Biochem. J. (1996) **319**, 851–860 (Printed in Great Britain)

D-3 phosphoinositide metabolism in cells treated with platelet-derived growth factor

Craig C. WHITEFORD*, Christie BEST†, Andrius KAZLAUSKAS†‡ and Emin T. ULUG*§

*Section of Virology and Oncology, Division of Biology, Kansas State University, Ackert Hall, Manhattan, KS 66506, U.S.A., and †National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206, U.S.A.

Despite extensive analysis of phosphoinositide 3-hydroxykinases (PI 3-kinases) at the molecular level, comparatively little is known about the mechanisms by which products of these enzymes exert their expected second-messenger functions. This study examines the metabolism of D-3 phosphoinositides in mouse Ph-N2 fibroblasts lacking the platelet-derived growth factor (PDGF) α-receptor. Treatment of these cultures with BB PDGF, but not AA PDGF, resulted in transient activation of PI 3-kinase activity measured *in vitro*. Treatment of *myo*-[^{3}H]inositol-labelled Ph-N2 cells with BB PDGF resulted in the rapid induction of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 and, to a smaller extent, PtdIns3P. The appearance of PtdIns(3,4,5)P_3 preceded that of PtdIns(3,4)P_2 and PtdIns3P after the addition of PDGF, suggesting that PtdIns(4,5)P_2 is the preferred substrate of the agonist-stimulated PI 3-kinase in intact cells. Treatment of both resting and PDGF-stimulated cells with the fungal metabolite wortmannin resulted in pronounced, selective effects on the levels of all D-3 phosphoinositides. Kinetic studies with this PI 3-kinase inhibitor revealed the presence of at least two independent routes for the biosynthesis of D-3 phosphoinositides in PDGF-treated cells.

INTRODUCTION

Since the identification of phosphoinositide 3-hydroxykinase (PI 3-kinase) activity physically associated with tyrosine kinase signalling complexes, considerable attention has focused on the functional roles of these enzymes as mediators of developmental and proliferative responses in eukaryotic cells [1–3]. Indeed, several lines of evidence indicate that PI 3-kinase activation is sufficient to drive quiescent cells into the cell division cycle [4–8]. It is suggested that D-3 phosphoinositide products of PI 3-kinases mediate these signalling responses, but the mechanisms of action and targets of these putative secondary messengers remain elusive.

Purified preparations of mammalian PI 3-kinases consist of a 110 kDa catalytic component that is present either in monomeric form or associated with an 85 kDa regulatory subunit [9]. Recent nucleic acid sequence data from several laboratories have established the existence of multiple PI 3-kinase isoforms in mammalian cells and tissues. The catalytic component of the tyrosine kinase-activated PI 3-kinase exhibits dual substrate specificity *in vitro*, and is capable of phosphorylating phosphoinositide substrates at the D-3 position of the inositol ring as well as a restricted repertoire of protein substrates (p85 and insulin receptor substrate 1) at serine residues [10–12]. p85 mediates binding of the PI 3-kinase catalytic subunit to tyrosine-phosphorylated signalling complexes [1,9]. However, one p110 homologue [13] as well as a recently cloned G-protein-regulated PI 3-kinase [14] fails to associate with p85 subunits, suggesting mechanisms of activation distinct from those elicited by tyrosine kinase signalling complexes. The identification of multiple PI 3-kinase isoforms reinforces the potential signalling properties of D-3 phosphoinositides during the transduction of cellular responses by diverse stimuli.

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesodermal origin and has provided a useful model system for investigating functional and mechanistic aspects of PI 3-kinase activation. PDGF consists of two disulphide-linked subunits arranged either as homodimers (AA, BB) or as AB heterodimers. Two structurally related receptors bind PDGF in an isoform-specific fashion: the PDGF α-receptor binds all isoforms, whereas the PDGF β-receptor binds only BB PDGF with high affinity [15]. PDGF α- and β-receptors are expressed to varying degrees in different cell types and induce overlapping, but also distinct, signals within cells [16–18]. Within 1 min of treatment of cells with PDGF, cytosolic PI 3-kinase isoforms containing p85α and p85β become physically associated with the PDGF receptor and phosphorylated [19–22]. Both phosphorylation of PI 3-kinase subunits by the receptor [23] and conformational changes resulting from interaction with the receptor [24–26] might contribute to the stimulation of PI 3-kinase activity in PDGF-treated cells.

In vitro, the PDGF-activated PI 3-kinase catalyses phosphorylation of PtdIns, PtdIns4P and PtdIns(4,5)P_2 at the D-3 position of the inositol ring to form PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively [27,28]. In intact cells, however, PtdIns(4,5)P_2 seems to be a preferred substrate of agonist-activated PI 3-kinases. Cellular levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are significantly elevated after addition of growth factors [29] or viral transformation [30–33]. [^{32}P]P$_i$ labelling studies have revealed that PtdIns(3,4,5)P_3 appears before PtdIns(3,4)P_2 in Swiss 3T3 cells stimulated with PDGF [34] and in neutrophils activated by the formyl peptide f-Met-Leu-Phe [35]. Analysis of radioactivity incorporated into individual phosphate groups of PtdIns(3,4,5)P_3 extracted from these cells confirmed that phosphorylation at D-4 and D-5 preceded phosphorylation at the D-3 position [34,35]. These results indicate that PtdIns(4,5)P_2 is a primary substrate of agonist-activated PI 3-kinases and that the product of this reaction, PtdIns(3,4,5)P_3,

Abbreviations used: DMEM, Dulbecco's modified essential medium; Gro*P*Ins, glycerophosphoinositol; PDGF, platelet-derived growth factor; PI 3-kinase, phosphoinositide 3-hydroxykinase.

‡ Present address: Schepens Eye Research Institute, Harvard Medical School, 20 Staniford Street, Boston, MA 02114, U.S.A.

§ To whom correspondence should be addressed.

is rapidly dephosphorylated to PtdIns(3,4)P_2. Presumably PtdIns(3,4)P_2 is then sequentially dephosphorylated to PtdIns3P and PtdIns, but this has not been demonstrated directly.

This study was initiated to define the metabolic route(s) of D-3 phosphoinositide synthesis following activation of PI 3-kinase by the PDGF β-receptor. Treatment of mouse cells lacking PDGF α-receptors with BB PDGF resulted in a rapid but transient stimulation of PI 3-kinase activity, measured in an assay *in vitro* and by formation of D-3 phosphoinositide products in intact cells. Metabolic labelling studies with *myo*-[^{3}H]inositol confirmed that PtdIns(4,5)P_2 is the primary substrate for the PI 3-kinase that is stimulated by the PDGF β-receptor. Treatment of both resting and PDGF-stimulated cultures with the PI 3-kinase inhibitor wortmannin resulted in specific changes in the turnover of cellular D-3 phosphoinositides, indicating that multiple pathways lead to formation of these products in intact cells.

EXPERIMENTAL

Materials

Recombinant human AA and BB PDGF were from Promega; [γ-^{32}P]ATP (5000 Ci/mmol) was from New England Nuclear; *myo*-[2-^{3}H]inositol (no. TRK-912; 80–120 mCi/mmol) was from Amersham; phospholipids, wortmannin and ammonium phosphate were from Sigma Chemical Co.; silica gel 60 chromatography plates were from EM Science (Gibbstown, NJ, U.S.A.); Bradford protein assay reagent was from Pierce (Rockford, IL, U.S.A.); and Uniscint BD scintillation fluid was from National Diagnostics (Atlanta, GA, U.S.A.). Antiserum to a C-terminal peptide of p85α was a gift of Dr. S. A. Courtneidge (Sugen Inc., Redwood City, CA, U.S.A.); Ph cells and 9235 antibody were provided by R. Seifert and D. Bowen-Pope (University of Washington, Seattle, WA, U.S.A.).

Cell cultures and constructs

Cultures used in this study were isolated from *Ph*/*Ph* mouse embryos in which the PDGF α-receptor gene had been deleted [36]. These cells express the PDGF β-receptor, but lack the PDGF α-receptor. Chimaeric PDGF receptors composed of extracellular, transmembrane and juxtamembrane domains of the human PDGF α-receptor fused to the kinase and C-terminal tail of the human PDGF β-receptor will be described in detail elsewhere (K. Demail, C. Best and A. Kazlauskas, unpublished work). The chimaeric construct was introduced into the pLXSN retroviral cloning vector and then transfected into the Ψ-2 packaging cell line [37]. Supernatants from the Ψ-2 cells were used to infect the PA317 packaging line, and virus-producing cells were selected in 0.5 mg/ml G-418. Virus from the PA317 cells was used to infect Ph cells, and mass populations of G-418-resistant PH-cWT cells were isolated. The Ph-N2 line was derived in a similar fashion by using an empty retroviral expression vector. Ph cells harbouring the chimaeric receptor, but not the Ph-N2 line, exhibited enhanced DNA synthesis when treated with AA PDGF and were capable of growth in serum-free medium supplemented with insulin, transferrin and AA PDGF (results not shown). Cell cultures were incubated in Dulbecco's modified essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin and streptomycin in a humidified, 10% CO_2 atmosphere at 37 °C.

For experimentation, nearly confluent 60 or 100 mm cultures were starved for serum by incubation in DMEM containing 5 μg/ml transferrin and 20 mM Hepes, pH 7.1, for 24–48 h before growth factor addition. Cells were stimulated by the addition of AA or BB PDGF to a final concentration of 40 ng/ml from stocks containing 5 μg/ml PDGF, 1 mg/ml BSA and 4 mM HCl.

Assays of protein kinase and PI 3-kinase activity

Protein kinase assays and Western immunoblotting were performed as described previously [5]. Sera that recognize the PDGF β-receptor and the external domain of the PDGF α-receptor have been described previously [38,39].

Immune complex PI 3-kinase activity was measured *in vitro* using conditions described previously [30]. Immunoprecipitates were prepared with 0.2 mg of cellular protein and antiserum to phosphotyrosine [30] or to a C-terminal peptide of p85α. PI 3-kinase assays were performed with total reaction volumes of 20 μl, containing 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 5 μg of lipid substrate and 10 μM [γ-^{32}P]ATP (5–10 μCi per reaction). Substrate for these assays contained a sonicated mixture of PtdIns, PtdIns$P$, PtdInsP_2 and PtdSer (1:1:1:3, w/w). After incubation for 10 min at 30 °C, reactions were terminated by addition of 180 μl of 1 M HCl and 400 μl of chloroform/methanol (1:1, v/v). Samples were mixed vigorously and phases were separated by brief centrifugation. The lower organic phase was washed with 400 μl of methanol/1 M HCl (1:1, v/v) and dried. Lipids were resuspended in 10 μl of chloroform and spotted on oxalate-impregnated, heat-activated silica chromatography plates. TLC used a solvent system containing chloroform/methanol/acetone/acetic acid/water (60:20:23:18:11, by vol.). ^{32}P-labelled phosphoinositides were identified by autoradiography, scraped from the plates and quantified by Čerenkov counting. When used for preparation of HPLC standards, individual ^{32}P-labelled D-3 phosphoinositides were scraped from the plate and deacylated as described below.

Metabolic labelling of cells with *myo*-[^{3}H]inositol

To optimize the incorporation of *myo*-[^{3}H]inositol into cellular phosphoinositides, subconfluent cultures in 60 mm dishes were rinsed with inositol-free DMEM and incubated for 8–18 h in inositol-free DMEM containing 5 μg/ml transferrin, 5 μg/ml insulin and 10% (v/v) dialysed fetal calf serum [dialysed against several changes of 150 mM NaCl/20 mM Hepes (pH 7.1) with a Spectra/Por 1000 Dalton MWCO membrane]. Cultures were then simultaneously starved for serum and metabolically labelled by replacement of this medium with 2 ml of inositol-free DMEM containing 5 μg/ml transferrin, 20 mM Hepes, pH 7.1, and 50 μCi of *myo*-[2-^{3}H]inositol. After 24 h, PDGF was added directly to the labelled cultures as described above. When used, wortmannin was added directly to the culture medium from a 500 × stock solution prepared in DMSO. For short-term timed experiments, cultures were treated with growth factor and/or inhibitor and placed in a shaking water bath at 37 °C.

myo-[^{3}H]Inositol-labelled cultures were harvested rapidly by removal of the culture medium and addition of 0.6 ml of 4.5% (v/v) ice-cold perchloric acid. After 15 min, cells were scraped from the dishes with rubber 'policemen', transferred to Microfuge tubes, and centrifuged at 16000 ***g*** for 10 min at 4 °C. The phosphoinositide-containing pellet was washed once with 100 mM EDTA by centrifugation at 4 °C and suspended in 50 μl of water. Lipids were deacylated by addition of 1 ml of methanol/40% methylamine/n-butanol (4:4:1, v/v) and incubation at 56 °C for 45 min [40]. After being dried under vacuum, the glycerophosphoinositide-containing pellets were suspended in 0.5 ml of water, clarified by centrifugation and extracted twice with butanol/ethyl ether/ethyl formate (20:4:1, v/v). These preparations were dried under vacuum and stored at −20 °C.

HPLC separation of glycerophosphoinositides

Glycerophosphoinositides were separated by anion-exchange HPLC on a Whatman Partisphere 5-SAX column [30]. The HPLC system consisted of two independent pumps [A pumped water; B pumped 1.25 M $(NH_4)_2HPO_4$ adjusted to pH 3.8 with H_3PO_4], a digital controller, a mixing device and a 1.0 ml injection loop. All solutions were filtered through a 0.22 μm membrane and degassed before use. Glycerophosphoinositide-containing samples were suspended in 0.5 ml of water, filtered and then injected as the chromatography system was pumping solution A at 1 ml/min. Immediately after injection, samples were subjected to the following linear elution gradient: 1 min to 2 % B, 19 min to 3 % B, 20 min to 9 % B, 44 min to 9 % B, 45 min to 27 % B, 53 min to 28 % B, 54 min to 100 % B, 58 min to 100 % B, 59 min to 0 % B. Fractions (0.4 ml) were collected directly into scintillation vials to which 1.2 ml of scintillation cocktail was added. the radioactive content of the samples was measured with a Beckman LS 6000 counter calibrated to correct for quenching. For quantification of weakly radioactive samples, count times were extended to 10 min to minimize error and background values (approx. 100 d.p.m. per fraction) were subtracted. Values for individual products were calculated as a ratio of radioactivity present in the PtdIns deacylation product, glycerophosphoinositol (Gro*P*Ins) to compensate for loss of sample during preparation.

Statistical analysis

Statistical significance was determined by Student's t test with the StatView statistics program (Brainpower, Calabasas, CA, U.S.A.). Correlation coefficients (r values) were obtained with Cricket Graph III 1.5.3 (Computer Associates International, Islandia, NY, U.S.A.).

RESULTS

PI 3-kinase activation in mouse fibroblasts lacking PDGF α-receptors

BB PDGF activates both α and β isoforms of the PDGF receptor and therefore studies to examine metabolic responses induced by PDGF β-receptor in mouse fibroblasts and other cells are confounded by the presence of endogenous PDGF α-receptors. To determine the contribution of the PDGF β-receptor to D-3 phosphoinositide metabolism in mouse fibroblasts, we have analysed PDGF activation of PI 3-kinase in Ph cells, a fibroblast line derived from mice lacking the PDGF α-receptor. These cells lack the PDGF α-receptor gene yet express PDGF β-receptors that trigger expected biological responses to BB PDGF [17,36]. To examine the activation of PI 3-kinase by the PDGF β-receptor, immune-complex PI 3-kinase assays were performed with lysates of Ph-N2 cells (Ph cells harbouring an empty expression vector) after treatment with AA or BB PDGF. Serum-deprived Ph-N2 cells were incubated for 5 min in the presence or absence of PDGF, lysed by the addition of Nonidet P40-containing buffer, and precipitated with antiserum to phosphotyrosine. PI 3-kinase activity in the immunoprecipitates was assayed by the addition of [γ-^{32}P]ATP in the presence of a mixed lipid substrate containing PtdIns, PtdIns4P and PtdIns(4,5)P_2. Products were separated by TLC and detected by autoradiography (Figure 1).

As illustrated in Figure 1(A) (lanes 1–3), antiserum to phosphotyrosine precipitated appreciable PI 3-kinase activity only from lysates of Ph-N2 cells that had been exposed to BB PDGF. Under the conditions of this assay, three radioactively labelled products were generated: PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. The identity of each was confirmed by anion-exchange HPLC after deacylation (see below). Čerenkov counting of products revealed that anti-phosphotyrosine precipitates derived from BB-PDGF-treated cultures contained approx. 50-fold more PI 3-kinase activity than those derived from either uninduced or AA-PDGF-treated cultures. Ph-N2 cells treated with AA PDGF exhibited low, baseline levels of PI 3-kinase activity similar to those in uninduced, quiescent Ph-N2 cells.

Lack of PI 3-kinase activation in Ph-N2 cultures treated with AA PDGF was not due to an inability of this growth factor to activate PDGF α-receptors. Substantial PI 3-kinase activation was observed in Ph-cWT cells after treatment with AA PDGF under identical assay conditions (Figure 1A, lane 5). These cells harbour a PDGF receptor chimaera composed of extracellular, transmembrane and juxtamembrane domains of the human PDGF α-receptor fused to the kinase and C-terminal tail of the human PDGF β-receptor. After treatment with AA PDGF, Ph-cWT cells exhibited levels of PI 3-kinase activity comparable to those observed in Ph-N2 cultures treated with BB PDGF (Figure 1A, lane 3). Uninduced cells expressing this receptor exhibited only baseline levels of PI 3-kinase activity (Figure 1A, lane 4). In this and other assays, treatment of Ph-cWT cells with BB PDGF (Figure 1A, lane 6) resulted in 15–20 % more PI 3-kinase activity than measured after treatment with AA PDGF, suggesting that the activities of the chimaeric and endogenous receptors were additive to only a small extent.

Western blot analysis (Figure 1B) was used to compare receptor levels in Ph-cWT and MG63 cells (a human osteosarcoma line containing approx. 10^5 receptors per cell). Whole-cell immunoblots prepared with antibody to the extracellular domain of the PDGF α-receptor revealed that the chimaeric receptor (Figure 1B, lanes 4–6) was expressed at about the same level as the PDGF α-receptor in MG63 cells (Figure 1B, lanes 7–9) and that Ph-N2 cells expressed no detectable PDGF α-receptors (Figure 1B, lanes 1–3).

To test activation of the chimaeric receptor by AA PDGF, resting or PDGF-stimulated Ph-N2 and Ph-cWT cells were lysed and immunoprecipitated with antibodies to the PDGF β-receptor C-terminus (which recognizes both the chimaeric receptor and the endogenous β-receptor) or antibodies directed against the PDGF α-receptor (which recognizes only the chimaeric receptor). Immunoprecipitates were subjected to kinase assays *in vitro*, and products were separated by SDS/PAGE and detected by autoradiography (Figure 1C). In all cases, BB PDGF activated the receptors, as indicated by the presence of phosphorylated receptor-associated proteins that were not observed in samples from unstimulated cells. In contrast, AA PDGF was able to activate only the chimaeric receptor (in Figure 1C, compare lane 2 with lanes 5 or 11). These studies demonstrate that Ph-N2 cells have no PDGF α-receptor and that AA PDGF selectively activates the chimaeric receptor. Furthermore Ph-N2 cells provide a unique system for the analysis of PDGF β-receptor signalling pathways in mouse fibroblasts, because BB PDGF treatment of these cells activates only the endogenous β-receptor.

Kinetics of PI 3-kinase activation in PDGF-treated Ph-N2 cells

It was reported previously that the activity of the PI 3-kinase isoform containing p85 is enhanced after treatment of cells with PDGF [19]. To determine the extent to which the PDGF β-receptor activates this PI 3-kinase isoform, Ph-N2 cells were treated with AA or BB PDGF and PI 3-kinase activity was measured in immunoprecipitates formed with antiserum to p85

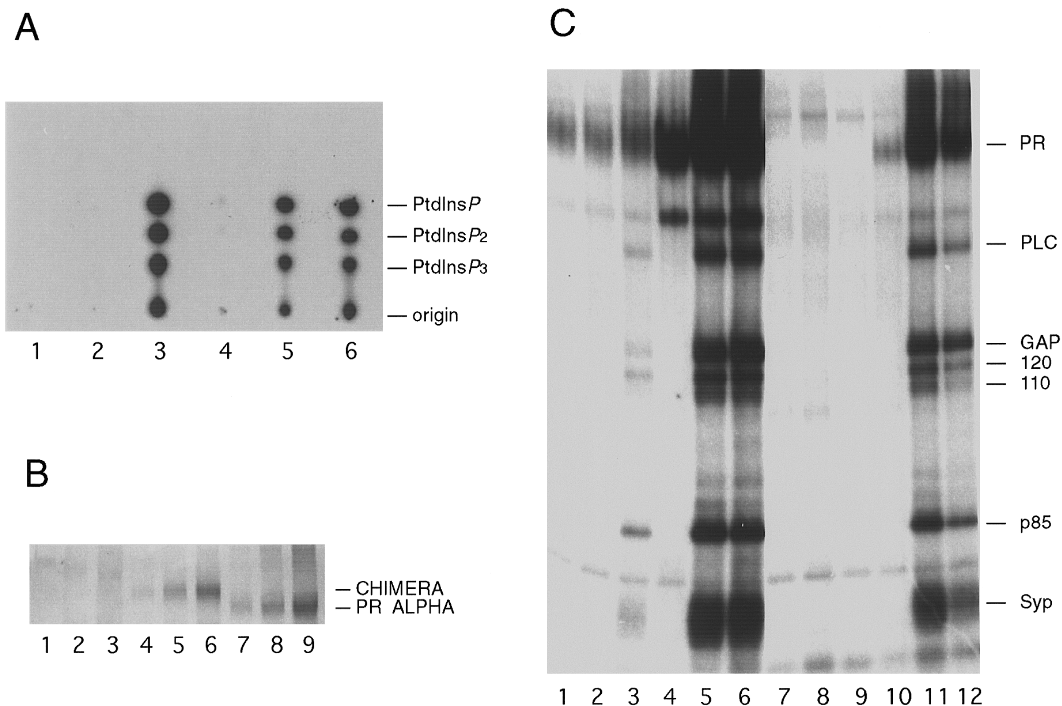


Figure 1 Activation of chimaeric and endogenous PDGF receptors in Ph cells

(**A**) Serum-starved Ph-N2 cells (lanes 1–3) and Ph-cWT cells (lanes 4–6) were incubated in the presence of AA PDGF (lanes 2 and 5) or BB PDGF (lanes 3 and 6), or untreated (lanes 1 and 4) for 5 min and harvested by the addition of lysis buffer. Immunoprecipitates were formed with antiserum to phosphotyrosine and assayed for PI 3-kinase activity by using a mixed lipid substrate. Products were separated by TLC and detected by autoradiography. (**B**) Whole cell lysates prepared from Ph-N2 cells (lanes 1–3), Ph-cWT cells (lanes 4–6) and MG63 cells (lanes 7–9) were resolved by SDS/PAGE, transferred to a poly(vinylidene difluoride) membrane and subjected to immunoblot analysis with antibody (9325) to the extracellular domain of the PDGF α-receptor. For each cell type, three different amounts of cell lysate were used, representing 1.2×10^4, 3.7×10^4 and 11.4×10^4 cells. As indicated, the chimaera migrated at a slightly higher molecular mass than the native PDGF α-receptor (PR alpha). (**C**) Quiescent cultures of Ph-N2 cells (lanes 1–3 and 7–9) and Ph-cWT cells (lanes 4–6 and 10–12) were stimulated with AA PDGF (lanes 2, 5, 8 and 11) or BB PDGF (lanes 3, 6, 9 and 12), or untreated (lanes 1, 4, 7 and 10) for 5 min. Lysates were prepared and immunoprecipitated with antibodies to the carboxyl tail of the PDGF β-receptor (lanes 1–6) or to the extracellular domain of the PDGF α-receptor (lanes 7–12). Kinase assays were performed *in vitro* and products were resolved by SDS/PAGE and identified by autoradiography. Migration positions of known PDGF receptor-associated proteins are indicated at the right. Abbreviations: PR, PDGF receptor; PLC, phospholipase C-gamma; GAP, GTPase-activating protein for ras; Syp, SH2-containing phosphotyrosine phosphatase.

(Figure 2, upper panel). Immunoprecipitates of p85 formed from lysates of BB PDGF-treated cultures (Figure 2, upper panel, lane 3) exhibited approx. 5-fold more PI 3-kinase activity than those from uninduced or AA PDGF-treated cultures. The low level of activity detected in precipitates derived from AA PDGF-treated cells (Figure 2, upper panel, lane 2) was not significantly distinct from the basal activity precipitated from lysates of uninduced cultures (Figure 2, upper panel, lane 1). These results indicate that an increase in the overall activity of this PI 3-kinase isoform occurs after activation of the PDGF β-receptor.

A time-course study was performed to define the kinetics by which the PI 3-kinase isoform containing p85 is activated after stimulation of the PDGF β-receptor. Immunoprecipitates of p85α were prepared from lysates of cells treated for various periods of time with BB PDGF, and PI 3-kinase assays were performed. After identification by autoradiography, PtdIns3*P* was scraped from the plates and quantified by Čerenkov counting (Figure 2, lower panel). A significant ($P \leqslant 0.005$) increase in PI 3-kinase activity associated with p85α was observed at all times after addition of BB PDGF to Ph-N2 cells. Maximal activation of PI 3-kinase was detected 2.5–5 min after addition of growth factor. By 15 min, PI 3-kinase activity had decreased to less than 40% of the maximally induced level. These results indicate that stimulation of the PDGF β-receptor results in a transient increase in activity of the PI 3-kinase isoform containing p85α.

Formation of PtdIns(3,4,5)P_3 precedes PtdIns(3,4)P_2 and PtdIns3*P* in PDGF-treated cells

To corroborate the analysis *in vitro* of PI 3-kinase activation by the PDGF β-receptor, D-3 phosphoinositide production was measured in Ph-N2 cells metabolically labelled with *myo*-[^{3}H]-inositol. For maximal incorporation of label into cellular phosphoinositides, nearly confluent cultures were starved for inositol and then simultaneously starved for serum and labelled with *myo*-[^{3}H]inositol. After 24 h, cultures were exposed to PDGF for 4 min and then rapidly extracted with perchloric acid. This extraction protocol was adopted to ensure the complete recovery of all membrane-associated compounds containing the labelled precursor. The lipid-containing fractions were then deacylated and analysed by anion-exchange HPLC to separate isomers within individual glycerophosphoinositide classes (see the Experimental section). Fractions were collected directly into scintillation vials and quantified by scintillation counting.

Representative HPLC profiles of deacylated phosphoinositides from uninduced and BB PDGF-treated cells are illustrated in Figure 3. Deacylation products of the most abundant cellular phosphoinositides were eluted consistently at expected times with this procedure: Gro*P*Ins eluted at fraction 16, Gro*P*Ins4*P* at fraction 53, and Gro*P*Ins(4,5)P_2 at fraction 113. To identify the elution patterns of D-3 phosphoinositide deacylation pro-

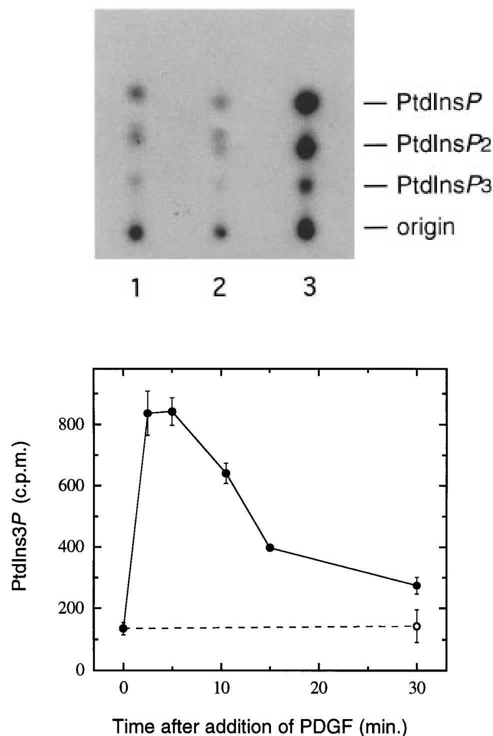


Figure 2 PI 3-kinase activation in Ph-N2 cells treated with BB PDGF

Upper panel: serum-starved Ph-N2 cultures were treated with AA PDGF (lane 2), BB PDGF (lane 3) or untreated (lane 1) for 5 min and then lysed by the addition of Nonidet P40-containing buffer. Immunoprecipitates were formed by using antisera to p85α and assayed for PI 3-kinase activity *in vitro*. PI 3-kinase products were separated by TLC and detected by autoradiography. Lower panel: duplicate cultures of serum-starved Ph-N2 cells were either untreated (○) or treated with BB PDGF (●) for various periods of time and then lysed by addition of Nonidet P40-containing buffer. Immunoprecipitates formed by using antisera to p85α were assayed for PI 3-kinase activity *in vitro* as in the upper panel. For quantification, PtdIns3P was scraped from the plates and analysed by Čerenkov counting. Each point represents the mean ± S.E.M. of replicate measurements.

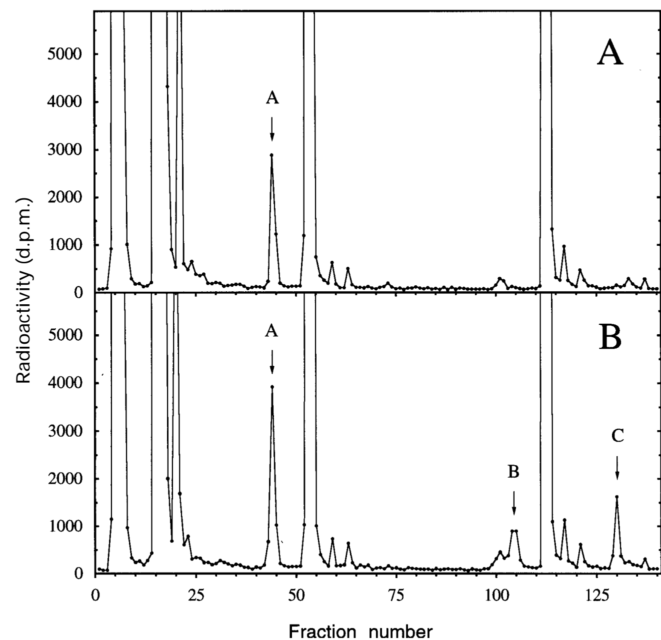


Figure 3 HPLC analysis of deacylated ³H-labelled phosphoinositides from PDGF-treated Ph-N2 cells

Quiescent *myo*-[^{3}H]inositol labelled Ph-N2 cells were untreated (**A**) or incubated in the presence of BB PDGF (**B**) for 4 min. Perchloric acid precipitates were deacylated and analysed by anion-exchange HPLC as described in the Experimental section. Deacylated products of cellular D-3 phosphoinositides were identified by co-migration with standards prepared *in vitro*: peak A, PtdIns3P; peak B, PtdIns(3,4)P_2; peak C, PtdIns(3,4,5)P_3. Samples analysed in each panel contained equivalent amounts of radioactivity (corresponding to approx. 2.8×10^6 d.p.m. of the PtdIns deacylation product Gro$P$Ins).

ducts, ^{32}P-labelled D-3 glycerophosphoinositide standards prepared *in vitro* were analysed in parallel HPLC runs. These were eluted at the following positions: Gro$P$Ins3P at fraction 44, Gro$P$Ins(3,4)P_2 at fraction 105, and Gro$P$Ins(3,4,5)P_3 at fraction 130.

When deacylated extracts of serum-deprived, *myo*-[^{3}H]inositol-labelled Ph-N2 cells were analysed by anion exchange HPLC, Gro$P$Ins3P (Figure 3A, peak A) was found to contain approx. 0.15 % of the radioactivity associated with Gro$P$Ins, the major labelled product in these preparations. Barely detectable levels of ^{3}H eluted at positions corresponding to the Gro$P$Ins(3,4)P_2 and Gro$P$Ins(3,4,5)P_3 standards, consistent with the finding that these products are not present in resting cells [29]. We note that a small peak of radioactivity (in fraction 101) was eluted consistently just before the Gro$P$Ins(3,4)P_2 standard. The identity of this compound is not known, but might represent a product tentatively identified by Auger et al. [29] as Gro$P$Ins(3,5)P_2.

HPLC analysis of deacylation products from PDGF-treated Ph-N2 cultures revealed clear induction of D-3 phosphoinositide synthesis. As illustrated in Figure 3(B), deacylation products of PtdIns(3,4)P_2 (peak B) and PtdIns(3,4,5)P_3 (peak C) were readily detected in extracts of cells treated for 4 min with BB PDGF. In addition, PDGF stimulated a small but reproducible increase (approx. 30 %) in the recovery of PtdIns3P deacylation product (peak A). No significant changes in cellular levels of PtdIns or PtdIns4P were detected after addition of BB PDGF in this or other experiments. However, treatment of serum-deprived cultures with BB PDGF did result in a 15–20 % decrease in the level of PtdIns(4,5)P_2, as observed previously [41]. These alterations in the levels of cellular phosphoinositides were not detected in Ph-N2 cells treated with AA PDGF but were readily detected in cells harbouring the PDGF receptor chimaera (results not shown).

Phosphate-labelling studies of Hawkins et al. [34] demonstrated that the formation of PtdIns(3,4,5)P_3 preceded PtdIns(3,4)P_2 in PDGF-treated Swiss 3T3 cells, suggesting that PtdIns(4,5)P_2 is the primary substrate for the PDGF-stimulated PI 3-kinase in intact cells. To test whether this pathway is activated by the PDGF β-receptor, *myo*-[^{3}H]inositol-labelled Ph-N2 cultures were treated for short periods of time with BB PDGF and rapidly harvested with perchloric acid. D-3 phosphoinositide deacylation products were then isolated by anion-exchange HPLC and quantified by liquid-scintillation counting. The results shown in Figure 4 are representative of three experiments in which PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 and PtdIns3P levels in Ph-N2 cells were measured at various times after the addition of PDGF.

These studies consistently revealed that increased levels of PtdIns(3,4,5)P_3 preceded the rise in PtdIns(3,4)P_2 levels. A significant increase ($P \leqslant 0.01$) in the level of PtdIns(3,4,5)P_3 was observed 30 s after treatment of Ph-N2 cells with BB PDGF (Figure 4A). PtdIns(3,4,5)P_3 levels continued to increase in a

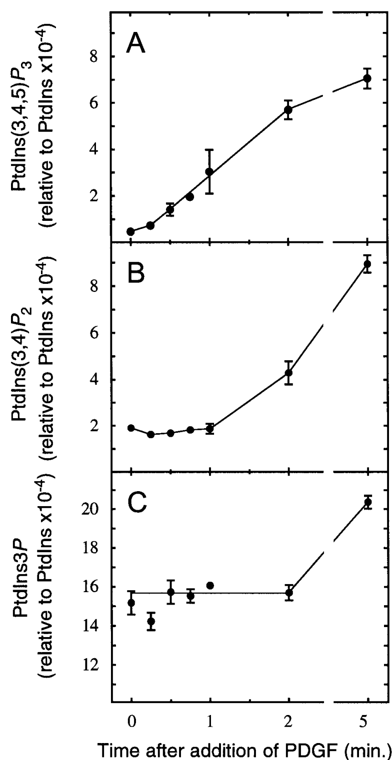


Figure 4 Time course of D-3 phosphoinositide accumulation in PDGF-treated Ph-N2 cells

Duplicate cultures of quiescent, *myo*-[^{3}H]inositol-labelled Ph-N2 cells were treated with BB PDGF for various time intervals and then rapidly harvested by the addition of perchloric acid. After deacylation, Gro*P*Ins(3,4,5)P_3 (**A**), Gro*P*Ins(3,4)P_2 (**B**) and Gro*P*Ins3*P* (**C**) were isolated by HPLC and quantified by scintillation counting. Levels of these products were adjusted for background radioactivity and normalized to the amount of Gro*P*Ins present in each sample. Each point represents the mean ± S.E.M. of replicate measurements.

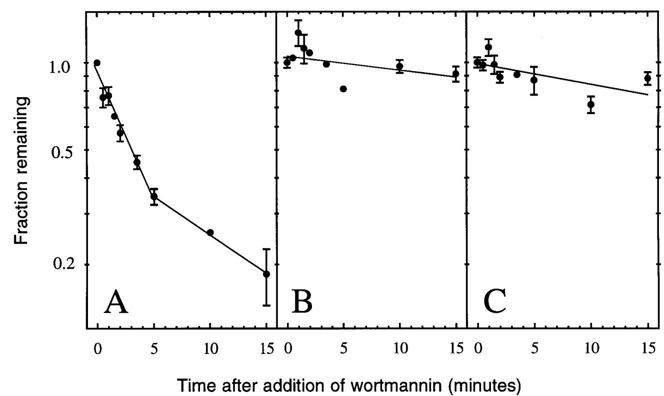


Figure 5 Effect of wortmannin on phosphoinositide levels in quiescent Ph-N2 cells

Replicate cultures of quiescent, *myo*-[^{3}H]inositol-labelled Ph-N2 cells were treated with 200 nM wortmannin for various periods of time and then rapidly harvested by the addition of perchloric acid. Amounts of Gro*P*Ins3*P* (**A**), Gro*P*Ins4*P* (**B**) and Gro*P*Ins(4,5)P_2 (**C**) in the deacylated extracts were quantified by scintillation counting after separation by HPLC. Levels of these products were adjusted for background radioactivity and normalized to the amount of Gro*P*Ins in each deacylated extract. Values (means ± S.E.M.) obtained after wortmannin treatment of cells are expressed as a ratio to those measured in the absence of inhibitor. In untreated cells, peaks corresponding to Gro*P*Ins3*P*, Gro*P*Ins4*P* and Gro*P*Ins(4,5)P_2 contained 0.15%, 4.91% and 4.92% respectively of the radioactivity associated with Gro*P*Ins. This figure contains results from two independent experiments and was repeated twice with similar results. In these experiments, levels of PtdIns3*P* in cultures treated with wortmannin were significantly distinct ($P < 0.05$) from untreated controls at all times assayed.

linear fashion until approx. 2 min after addition of growth factor. Maximum levels of PtdIns(3,4,5)P_3 were observed 2.5–4 min after addition of PDGF and then decreased steadily to approx. 20% of the maximally induced level by 10 min (results not shown).

Significantly elevated levels of PtdIns(3,4)P_2 were not detected until 2 min after addition of growth factor (Figure 4B). In this and other experiments, maximum levels of PtdIns(3,4)P_2 were observed 3–5 min after PDGF addition. In this particular experiment, deceptively high baseline levels of PtdIns(3,4)P_2 in resting cells were due to the presence of an unidentified product that eluted just before it. However, significant changes in the level of this contaminant were not observed after addition of PDGF in this or other experiments.

Significantly elevated levels of PtdIns3*P* were observed at later times after treatment of Ph-N2 cells with BB PDGF. In this experiment, a 30% increase in the level of PtdIns3*P* was detected 5 min after addition of growth factor (Figure 4C). In other experiments, increased levels of PtdIns3*P* were detected as early as 3–4 min after addition of PDGF, but in all cases this event was preceded by increased levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. These results are consistent with the suggestion [34] that PtdIns(3,4,5)P_3 is a precursor of PtdIns(3,4)P_2 in mouse cells treated with PDGF and that the latter is dephosphorylated to form PtdIns3*P*.

Wortmannin inhibits synthesis of PtdIns3*P* in quiescent Ph-N2 cells

Wortmannin is a potent inhibitor of certain PI 3-kinase isoforms *in vitro* and because of its cell-permeant properties it provides a useful tool for dissecting PI 3-kinase-mediated signalling pathways in intact cells [42]. It was recently reported that wortmannin treatment of unstimulated bovine adreno-reticulosa cells decreased the incorporation of [^{32}P]P_i into PtdIns3*P* during short labelling intervals [43]. Our pilot studies revealed that wortmannin treatment also resulted in decreased levels of PtdIns3*P* in serum-deprived *myo*-[^{3}H]inositol-labelled Ph-N2 cultures. The maximum decrease in the level of PtdIns3*P* was detected after exposure of Ph-N2 cells to 200 nM wortmannin for 10 min (results not shown). Collectively, these results suggested that in serum-deprived cultures PtdIns3*P* might be formed via a wortmannin-sensitive pathway distinct from that induced by PDGF. To characterize further the sensitivity of PtdIns3*P* formation to wortmannin, serum-deprived *myo*-[^{3}H]inositol-labelled Ph-N2 cultures were incubated with 200 nM wortmannin for various time intervals and then rapidly harvested by addition of perchloric acid. Cell extracts were deacylated, and Gro*P*Ins, Gro*P*Ins3*P*, Gro*P*Ins4*P* and Gro*P*Ins(4,5)P_2 were separated by anion-exchange HPLC and quantified by liquid-scintillation counting (Figure 5).

Treatment of resting Ph-N2 cultures with wortmannin resulted in a biphasic decrease in the level of PtdIns3*P* relative to PtdIns (Figure 5A). Incubation of Ph-N2 cells with wortmannin resulted initially in a rapid, exponential ($y = 0.944 \times 10^{-0.092x}$, $r = 0.985$) decline in the cellular level of PtdIns3*P*, with a 50% decrease occurring approx. 3 min after addition of the inhibitor. The rapid decline in PtdIns3*P* levels was observed until 5 min after treatment with wortmannin. From 5 to 15 min after treatment,

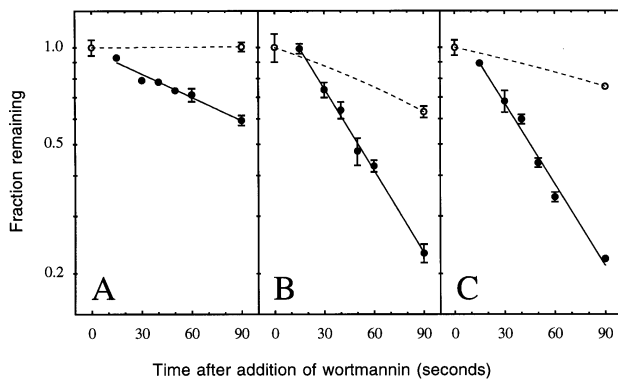


Figure 6 Effect of wortmannin on D-3 phosphoinositide levels in PDGF-treated Ph-N2 cells

Triplicate cultures of quiescent, *myo*-[^{3}H]inositol-labelled Ph-N2 cells were stimulated with BB PDGF for 3 min, treated with 200 nM wortmannin (●) or DMSO alone (○) for various periods of time and then rapidly harvested by the addition of perchloric acid. Deacylated cell extracts were analysed by HPLC and peak fractions corresponding to Gro*P*Ins3*P* (**A**), Gro*P*Ins(3,4)P_2 (**B**) and Gro*P*Ins(3,4,5)P_3 (**C**) were quantified by scintillation counting. Levels of these products were adjusted for background radioactivity and normalized to the amount of Gro*P*Ins present in the extracts. Values (means ± S.E.M.) obtained after wortmannin treatment of cells are expressed relative to those measured in the absence of inhibitor (i.e. 3 min after addition of growth factor). In the absence of wortmannin, peaks corresponding to Gro*P*Ins3*P*, Gro*P*Ins(3,4)P_2 and Gro*P*Ins(3,4,5)P_3 contained 0.177%, 0.039% and 0.143% respectively of the radioactivity associated with Gro*P*Ins. This experiment was performed twice with similar results.

PtdIns3*P* levels declined at a diminished rate ($y = 0.474 \times 10^{-0.027x}$, $r = 0.999$). Extended treatment of cells with wortmannin did not result in a further lowering of cellular PtdIns3*P* levels (results not shown).

In contrast to its dramatic effect on cellular PtdIns3*P* levels, wortmannin treatment of Ph-N2 cells had minimal effects on levels of PtdIns (results not shown), PtdIns4*P* (Figure 5B) or PtdIns(4,5)P_2 (Figure 5C). These results suggest that the inhibitory effect of wortmannin on phosphoinositide metabolism in resting cells is selective for synthesis of PtdIns3*P*.

Decay kinetics of D-3 phosphoinositides in PDGF-treated Ph-N2 cells

Recent studies have reported that wortmannin interferes with the incorporation of [^{32}P]P$_i$ into PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in cells treated with PDGF [43,44]. Our preliminary findings confirmed that pretreatment of *myo*-[^{3}H]inositol-labelled Ph-N2 cultures with wortmannin for 5 min completely prevented the PDGF-induced synthesis of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (results not shown). To further characterize the effect of wortmannin on this agonist-activated PI 3-kinase, we analysed the kinetics by which this inhibitor interferes with the accumulation of D-3 phosphoinositides in PDGF-treated cells. To test this, serum-starved *myo*-[^{3}H]inositol-labelled Ph-N2 cultures were treated with PDGF for 3 min, a time when levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 were maximal, and then treated with wortmannin for short time intervals. Perchloric acid was used to rapidly stop further metabolism of the lipids. After deacylation, phosphoinositide isomers in the extracts were quantified as described above.

As illustrated in Figure 6(A), treatment of PDGF-stimulated cells with wortmannin resulted in a rapid decline in cellular levels of PtdIns3*P*. The decrease in PtdIns3*P* levels was exponential ($y = 0.987 \times 10^{-0.002x}$, $r = 0.989$) and without a detectable lag period. Extrapolation of these data indicated that one-half of the initial PtdIns3*P* level would be present after approx. 150 s of treatment with wortmannin.

In contrast with the decline in PtdIns3*P* levels, the amounts of PtdIns(3,4)P_2 (Figure 6B) and PtdIns(3,4,5)P_3 (Figure 6C) diminished much more rapidly after the addition of wortmannin to PDGF-stimulated cells. PtdIns(3,4,5)P_3 levels declined exponentially ($y = 1.194 \times 10^{-0.008x}$, $r = 0.992$) after a short lag period estimated at 9.6 s (at $y = 1$). Treatment of cells with wortmannin resulted in a decline in PtdIns(3,4)P_2 levels at a rate ($y = 1.331 \times 10^{-0.008x}$, $r = 0.998$) very similar to that observed for the decline in PtdIns(3,4,5)P_3 levels. However, a somewhat extended lag period (calculated at 15.5 s) was observed before the levels of PtdIns(3,4)P_2 decreased. Levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 diminished with nearly identical kinetics, with a 50 % decrease occurring 37.6 s after the lag period in each case.

In contrast with its potent effects on D-3 phosphoinositide levels in PDGF-stimulated Ph-N2 cells, wortmannin had no significant effect on the levels of PtdIns, PtdIns4*P* or PtdIns(4,5)P_2 during the time scale analysed (results not shown). The inhibitory effects of wortmannin on phosphoinositide metabolism therefore seemed to be restricted to the formation of the D-3 phosphoinositide isomers.

DISCUSSION

Analysis of PI 3-kinase activation in cells lacking PDGF α-receptors

Studies to analyse responses of cells to PDGF or serum have been plagued by the pleiotropic nature of the signals that occur after addition of growth factor. For example, PDGF derived from non-primate sources consists primarily of the BB homodimer and elicits responses through both α and β isoforms of the PDGF receptor. To eliminate one component of the heterogeneous signalling responses after stimulation of mouse fibroblasts with PDGF, the studies reported here have utilized cells derived from mice that lack the PDGF α-receptor gene [36]. Accordingly, these cells lacked the PDGF α-receptor protein and failed to respond to AA PDGF [17,36]. Here we report that AA PDGF also failed to elicit a PI 3-kinase response in Ph-N2 cells, a derivative of the original Ph line infected with an empty retroviral vector. In contrast, treatment of Ph-N2 cells with BB PDGF resulted in enhanced PI 3-kinase activity, measured by the formation of PI 3-kinase products *in vivo* and by the presence of PI 3-kinase activity in immune complexes formed with antibodies to phosphotyrosine or to p85. Similar results were obtained with antisera prepared to the PDGF receptor (results not shown).

The Ph line isolated by Seifert et al. [36] will be valuable for analysis of functional domains within the PDGF β-receptor. As shown in Figure 1, chimaeric PDGF receptors composed of extracellular, transmembrane and juxtamembrane domains of the PDGF α-receptor and kinase and C-terminal domains of the PDGF β-receptor triggered activation of PI 3-kinase in response to AA PDGF. We are currently using Ph cells for the expression of additional PDGF receptor chimaeras that fail to bind PI 3-kinase and/or PLC-γ to assess the relative contributions of these signalling pathways in mediating cellular responses to PDGF.

BB-PDGF treatment of Ph-N2 cells transiently elevates PI 3-kinase activity

Results presented here indicate that activation of the PDGF β-receptor brought about enhanced activity of the PI 3-kinase

isoform containing p85α. This response was transient: maximal PI 3-kinase activation was detected 2.5–5 min after the addition of growth factor. By 15 min, PI 3-kinase activity had decreased to less than 40 % of the maximally induced level. Because PDGF treatment did not affect the amount of p85α protein present in these immune complexes (results not shown), we conclude that the elevated PI 3-kinase activity detected after the addition of growth factor was due to an increase in the specific activity of this PI 3-kinase isoform.

The rapid down-regulation of PI 3-kinase activity detected after PDGF treatment of cells is noteworthy. A decrease in PI 3-kinase activity might correlate with internalization and/or entry of the PDGF β-receptor into specific subcellular compartments [45]. It has been observed that only PDGF β-receptors that retain the ability to bind PI 3-kinase accumulate in the juxtanuclear region of cells in response to growth factor treatment [46]. Down-regulation of PI 3-kinase activity might also result from serine phosphorylation of p85 by p110. The PI 3-kinase catalytic subunit possesses an intrinsic serine kinase activity with specificity for p85 [10,11]. Serine phosphorylation of p85 was correlated with a decrease in PI 3-kinase activity. From the analysis of cellular D-3 phosphoinositide levels reported here, it is evident that maximal D-3 phosphoinositide synthesis was achieved within 5 min after addition of growth factor, a time at which maximal levels of PI 3-kinase activity could still be recovered from cells. It remains to be determined whether substrate depletion or internalization of this enzyme might trigger phosphorylation of p85 and/or down-regulation of PI 3-kinase activity.

Appearance of PtdIns(3,4,5)P_3 precedes PtdIns(3,4)P_2 in PDGF-treated Ph-N2 cells

Although the PDGF-stimulated PI 3-kinase can use PtdIns, PtdIns4P or PtdIns(4,5)P_2 as a substrate *in vitro*, results presented here confirm and extend previous findings that identified PtdIns(4,5)P_2 as the predominant substrate for this enzyme in intact cells [34]. As described in other systems [29], we observed a rapid induction of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in *myo*-[^{3}H]inositol-labelled Ph-N2 cells on addition of PDGF. Furthermore, significantly elevated levels of PtdIns(3,4,5)P_3 were detected before the appearance of PtdIns(3,4)P_2 at early times after growth factor addition. In addition, a significant increase in PtdIns3P levels was observed at later times after addition of PDGF. A similar pattern of D-3 phosphoinositide induction was observed in HepG2 cells harbouring PI 3-kinase add-back mutants of the PDGF receptor (results not shown).

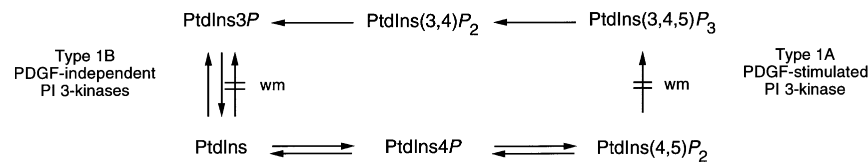
Results from kinetic studies with wortmannin (Figure 6) strongly support the notion that PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are generated from a common synthetic pathway in PDGF-treated cells. Cellular levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 diminished at nearly identical rates after treatment of PDGF-stimulated cultures with wortmannin, with half-life values for both compounds estimated at 37.6 s. Furthermore the decrease in PtdIns(3,4)P_2 levels was delayed by approx. 6 s relative to the decline in PtdIns(3,4,5)P_3 levels. The very similar turnover rates determined for these compounds indicate that other pathways, such as phosphorylation of PtdIns3P by PI 4-kinase [47,48], probably do not contribute significantly to formation of PtdIns(3,4)P_2 under these experimental conditions. Similarly, because the decrease in PtdIns(3,4)P_2 levels was delayed after wortmannin treatment relative to the drop in PtdIns(3,4,5)P_3, it would appear unlikely that both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are primary products of the same PI 3-kinase. Although these studies with intact cells cannot formally dismiss the possibility that the PI 3-kinase(s) forming these products exhibits differential sensitivity to wortmannin, the most straightforward interpretation of these results is that PtdIns(3,4,5)P_3 is the primary (if not the sole) product of the agonist-stimulated PI 3-kinase, and that it is rapidly dephosphorylated to form PtdIns(3,4)P_2. The rapid formation and turnover of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 might be expected of compounds with potential second-messenger properties.

Multiple pathways lead to formation of PtdIns3P in mouse fibroblasts

With the recent identification of new PI 3-kinases, it is becoming increasingly apparent that multiple enzymic routes might contribute to formation of D-3 phosphoinositides in mammalian cells. PtdIns3P is maintained at remarkably constant levels in resting and in actively growing/transformed cells. Constitutive activation of PI 3-kinase by polyomavirus middle T antigen [30] and activation of PI 3-kinase by PDGF (Figure 4) resulted in only modest increases in PtdIns3P levels, suggesting that PtdIns3P levels are maintained by metabolic pathways distinct from those stimulated by PDGF or other growth factors.

Evidence presented here (Figure 5) indicates that at least two pathways that maintain PtdIns3P levels in resting cells could be distinguished with regard to their sensitivity to wortmannin. PtdIns3P generated by a wortmannin-sensitive pathway had a half-life of approx. 3 min. It is unlikely that PtdIns3P generated by this pathway was a product of the enzyme stimulated by PDGF, because PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 were practically undetected in these cells under serum-free conditions. Furthermore the effect of this inhibitor on cellular PtdIns3P levels was immediate, whereas wortmannin treatment resulted in diminished levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 only after a significant lag period. This suggests that the enzyme(s) contributing to formation of this PtdIns3P pool is either more sensitive and/or accessible to wortmannin than is the PDGF-stimulated PI 3-kinase. Together these results suggest that a wortmannin-sensitive PtdIns 3-kinase contributes significantly to maintenance of PtdIns3P in resting cells. Although the enzyme(s) maintaining PtdIns3P levels in mouse fibroblasts has not been identified, it may be related to a recently cloned human PtdIns 3-kinase [13]. This enzyme is wortmannin-sensitive, fails to associate with p85, and phosphorylates PtdIns but not PtdIns4P or PtdIns(4,5)P_2 *in vitro*. A similar enzyme might be involved in activation of p70 S6 kinase by insulin, which recently was found to involve a wortmannin-sensitive pathway that functions independently of p85 [49].

A second pool of PtdIns3P in resting cells is distinguished by its relative insensitivity to wortmannin. Extrapolation of results in Figure 5 indicates that approx. 45–50 % of PtdIns3P in resting cells is comparatively resistant to the inhibitory effect of wortmannin. Approx. 15–20 % of PtdIns3P was completely resistant to treatment with wortmannin for 30 min (results not shown). This pool of PtdIns3P might arise from the action of a single PtdIns 3-kinase isoform that is weakly inhibited by wortmannin or, more probably, from two or more independent pathways that differ with regard to wortmannin sensitivity and/or turnover rates. Consistent with these results is the observation by Stephens et al. [43] that approx. 10–15 % of PtdIns3P synthesis in bovine adreno-reticulosa cells was resistant to wortmannin when unstimulated cells were treated with this inhibitor before labelling with [^{32}P]P$_i$. Furthermore a wortmannin-resistant PtdIns 3-kinase activity was partly purified from these cells, which could recognize PtdIns, but not PtdIns4P or PtdIns(4,5)P_2, as substrate [50]. This protein might be related to the yeast vesicular sorting proteins



Scheme 1 Routes of D-3 phosphoinositide biosynthesis in PDGF-treated Ph-N2 cells

PDGF-activated (Type 1A) and basal (Type 1B) PI 3-kinases contribute to synthesis of D-3 phosphoinositides in mouse fibroblasts treated with PDGF. PDGF induces a transient activation of the wortmannin-sensitive Type 1A enzyme that phosphorylates PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 is sequentially dephosphorylated to form PtdIns(3,4)P_2 and PtdIns3P. In addition, PtdIns3P is synthesized from PtdIns by two distinct Type 1B activities that differ in their sensitivity to wortmannin (wm).

that are resistant to wortmannin and exhibit substrate specificity for PtdIns.

Pathways of D-3 phosphoinositide metabolism in mouse fibroblasts treated with PDGF

The pathways that contribute to the formation of D-3 phosphoinositides in resting and PDGF-treated mouse fibroblasts are summarized in Scheme 1. As suggested originally by Stephens et al. [35], agonist-stimulated (Type 1A) PI 3-kinases phosphorylate PtdIns(4,5)P_2 to form PtdIns(3,4,5)P_3. This Type 1 enzyme is activated by growth factor receptors and viral oncogene products with intrinsic or associated tyrosine kinase activity [1]. A distinct PI 3-kinase that is activated by G-protein $\beta\gamma$ subunits seems to function in a similar fashion in myeloid and other cell types [50]. Phosphate labelling studies [34] and inositol labelling studies presented here implicate PtdIns(3,4,5)P_3 as the point of entry into the D-3 phosphoinositide cycle on activation by the PDGF receptor. PtdIns(3,4,5)P_3 is then sequentially dephosphorylated to form PtdIns(3,4)P_2, PtdIns3P and PtdIns. As discussed above, multiple 'basal' (Type 1B) PI 3-kinase activities also contribute to maintenance of PtdIns3P levels, in the presence and absence of agonist. These enzymes, referred to as PtdIns 3-kinases, catalyse phosphorylation of PtdIns to form PtdIns3P. At least two Type 1B activities have been detected that differ in regard to wortmannin sensitivity and/or turnover of their products. Although the number of agonists and enzymes that affect D-3 phosphoinositide metabolism is unknown, it would seem that all converge to produce a limited number of products with expected second-messenger function.

The mechanisms by which D-3 phosphoinositides signal cellular responses have remained elusive. Recent studies with wortmannin, growth factor-receptor mutants and dominantly acting PI 3-kinase components have implicated D-3 phosphoinositides in a number of cellular responses, including enhanced glucose uptake [49,51], membrane ruffling [44,52,53], activation of c-*fos* [7,54], activation of Akt kinase activity [55] and S6 kinase activation [8,56,57]. Consistent with these findings is our observation (E. T. Ulug, unpublished work) that entry of PDGF-stimulated Ph-N2 cells into the S-phase of the cell division cycle could be prevented by wortmannin, implicating the activation of PI 3-kinase as a primary mediator of the mitogenic response triggered by PDGF. However, as suggested previously [58], care must be taken in interpreting results from studies with inhibitors whose mode of action is not clearly understood. Results presented here demonstrate that wortmannin affects multiple pathways of D-3 phosphoinositide metabolism within a single cell population. Conclusive identification of potential effectors of PI 3-kinases will ultimately require precise identification of the D-3 phosphoinositide products that confer these biological responses.

We thank S. Courtneidge for the gift of antiserum to p85α, R. Seifert and D. Bowen-Pope for providing Ph cells and 9325 antiserum, and D. Rintoul and W. Odom for helpful comments during the course of this study. This research was supported by National Institutes of Health grants CA58291 (E.T.U.), CA55065 and GM48389 (A.K.), by a Grant-in Aid from the American Heart Association, KS Affiliate (to E.T.U.), and by funds from the Kansas Agricultural Experiment Station (to E.T.U). This manuscript is contribution no. 96-137J of the Kansas Agricultural Experiment Station.

REFERENCES

1 Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Cell **64**, 281–302
2 Kapeller, R. and Cantley, L. C. (1994) Bioessays **16**, 565–576
3 Stephens, L., Jackson, T. R. and Hawkins, P. T. (1993) Biochim. Biophys. Acta **1179**, 27–75
4 Coughlin, S. R., Escobedo, J. A. and Williams, L. T. (1989) Science **243**, 1191–1194
5 Valius, M. and Kazlauskas, A. (1993) Cell **73**, 321–334
6 Roche, S., Koegl, M. and Courtneidge, S. A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 9185–9189
7 Jhun, B. H., Rose, D. W., Seely, B. L., Rameh, L., Cantley, L., Saltiel, A. R. and Olefsky, J. M. (1994) Mol. Cell. Biol. **14**, 7466–7475
8 Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J. and Kahn, C. R. (1994) Mol. Cell. Biol. **14**, 4902–4911
9 Fry, M. J. and Waterfield, M. D. (1994) Phil. Trans. Roy. Soc. Lond. B **340**, 337–344
10 Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Vicendo, P., Yonezawa, K., Kasuga, M., Courtneidge, S. A. and Waterfield, M. D. (1994) EMBO J. **13**, 522–533
11 Carpenter, C. L., Auger, K. R., Duckworth, B. C., Hou, W. M., Schaffhausen, B. and Cantley, L. C. (1993) Mol. Cell. Biol. **13**, 1657–1665
12 Lam, K., Carpenter, C. L., Ruderman, N. B., Friel, J. C. and Kelly, K. L. (1994) J. Biol. Chem. **269**, 20648–20652
13 Volinia, S., Dhand, R., Vanhaesebroeck, B., MacDougall, L. K., Stein, R., Zvelebil, M. J., Domin, J., Panayotou, G. and Waterfield, M. D. (1995) EMBO J. **14**, 3339–3348
14 Stoyanov, B., Volinia, S., Hanck, T., Rubio, I., Loubtchenkov, M., Malek, D., Stoyanova, S., Vanhaesebroeck, B., Dhand, R., Nurnberg, B., Gierschik, P., Seedorf, K., Hsuan, J. J., Waterfield, M. D. and Wetzker, R. (1995) Science **269**, 690–693
15 Heldin, C.-H. (1995) Cell **80**, 213–223
16 Eriksson, A., Siegbahn, A., Westermark, B., Heldin, C.-H. and Claesson-Welsh, L. (1992) EMBO J. **11**, 543–550
17 Bazenet, C. E. and Kazlauskas, A. (1994) Oncogene **9**, 517–525
18 Heidaran, M. A., Beeler, J. F., Yu, J.-C., Ishibashi, T., LaRochelle, W. J., Pierce, J. H. and Aaronson, S. A. (1993) J. Biol. Chem. **268**, 9287–9295
19 Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J. and Waterfield, M. D. (1991) Cell **65**, 91–104
20 Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L. and Roberts, T. M. (1987) Cell **50**, 1021–1029
21 Escobedo, J. A., Kaplan, D. R., Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1991) Mol. Cell. Biol. **11**, 1125–1132
22 Susa, M., Keeler, M. and Varticovski, L. (1992) J. Biol. Chem. **267**, 22951–22956
23 Roche, S., Dhand, R., Waterfield, M. D. and Courtneidge, S. A. (1994) Biochem. J. **301**, 703–711
24 Shoelson, S. E., Sivaraja, M., Williams, K. P., Hu, P., Schlessinger, J. and Weiss, M. A. (1993) EMBO J. **12**, 795–802
25 Carpenter, C. L., Auger, K. R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S. and Cantley, L. C. (1993) J. Biol. Chem. **268**, 9478–9483

26 Klippel, A., Escobedo, J. A., Hirano, M. and Williams, L. T. (1994) Mol. Cell. Biol. **14**, 2675–2685
27 Whitman, M., Downes, C. P., Keeler, M., Keller, T. and Cantley, L. (1988) Nature (London) **332**, 644–646
28 Stephens, L., Hawkins, P. T. and Downes, C. P. (1989) Biochem. J. **259**, 267–276
29 Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P. and Cantley, L. C. (1989) Cell **57**, 167–175
30 Ulug, E. T., Hawkins, P. T., Hanley, M. R. and Courtneidge, S. A. (1990) J. Virol. **64**, 3895–3904
31 Serunian, L. A., Auger, K. R., Roberts, T. M. and Cantley, L. C. (1990) J. Virol. **64**, 4718–4725
32 Varticovski, L., Daley, G. Q., Jackson, P., Baltimore, D. and Cantley, L. C. (1991) Mol. Cell. Biol. **11**, 1107–1113
33 Ling, L. E., Druker, B. J., Cantley, L. C. and Roberts, T. M. (1992) J. Virol. **66**, 1702–1708
34 Hawkins, P. T., Jackson, T. R. and Stephens, L. R. (1992) Nature (London) **358**, 157–159
35 Stevens, L., Hughes, K. and Irvine, R. F. (1991) Nature (London) **351**, 33–39
36 Seifert, R. A., van Koppen, A. and Bowen-Pope, D. F. (1993) J. Biol. Chem. **268**, 4473–4480
37 Miller, A. D. and Rosman, G. L. (1989) Biotechniques **7**, 980–990
38 Valius, M., Bazenet, C. and Kazlauskas, A. (1993) Mol. Cell. Biol. **13**, 133–143
39 Kelley, J. D., Haldeman, B. A., Grant, F. J., Murray, M.J., Seifert, R. A., Bowen-Pope, D. F., Cooper, J. A. and Kazlauskas, A. (1991) J. Biol. Chem. **266**, 8987–8992
40 Downes, C. P., Hawkins, P. T. and Irvine, R. F. (1986) Biochem. J. **238**, 501–506
41 Berridge, M. J., Heslop, J. P., Irvine, R. F. and Brown, K. D. (1984) Biochem. J. **222**, 195–201
42 Ui, M., Okada, T., Hazeki, K. and Hazeki, O. (1995) Trends Biochem. Sci. **20**, 303–307
43 Stephens, L., Cooke, F. T., Walters, R., Jackson, T., Volinia, S., Gout, I., Waterfield, M. D. and Hawkins, P. T. (1994) Curr. Biol. **4**, 203–214

Received 12 February 1996/10 June 1996; accepted 9 July 1996

44 Wymann, M. and Arcaro, A. (1994) Biochem. J. **298**, 517–520
45 Kapeller, R., Chakrabarti, R., Cantley, L. C., Fay, F. and Corvera, S. (1993) Mol. Cell. Biol. **13**, 6052–6063
46 Joly, M., Kazlauskas, A., Fay, F. S. and Corvera, S. (1994) Science **263**, 684–687
47 Yamamoto, K., Graziani, A., Carpenter, C., Cantley, L. C. and Lapetina, E. G. (1990) J. Biol. Chem. **265**, 22086–22089
48 Graziani, A., Ling, L. E., Endemann, G., Carpenter, C. L. and Cantley, L. C. (1992) Biochem. J. **284**, 39–45
49 Hara, K., Yonezawa, K., Sakaue, H., Ando, A., Kotani, K., Kitamura, T., Kitamura, Y., Ueda, H., Stephens, L., Jackson, T. R., Hawkins, P., Dhand, R., Clark, A. E., Holman, G. F., Waterfield, M. D. and Kasuga, M. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 7415–7419
50 Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C. and Hawkins, P. T. (1994) Cell **77**, 83–93
51 Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) J. Biol. Chem. **269**, 3568–3573
52 Wennstrom, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L. and Stephens, L. (1994) Curr. Biol. **4**, 385–393
53 Kotani, K., Yonezawa, K., Hara, K., Ueda, H., Kitamura, Y., Sakaue, H., Ando, A., Chavanieu, A., Calas, B., Grigorescu, F., Nishiyama, M., Waterfield, M. D. and Kasuga, M. (1994) EMBO J. **13**, 2313–2321
54 Hu, Q., Klippel, A., Muslin, A. J., Fantl, W. J. and Williams, L. T. (1995) Science **268**, 100–102
55 Franke, T. F., Yang, S.-I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. and Tsichlis, P. N. (1995) Cell **81**, 727–736
56 Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A. and Blenis, J. (1994) Nature (London) **370**, 71–75
57 Hara, K., Yonezawa, K., Sakaue, H., Kotani, K., Kotani, K., Kojima, A., Waterfield, M. D. and Kasuga, M. (1995) Biochem. Biophys. Res. Commun. **208**, 735–741
58 Downward, J. (1995) Nature (London) **376**, 553–554