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# Inhibition of MG-63 Cell Proliferation and PDGF-Stimulated Cellular Processes by Inhibitors of Phosphatidylinositol 3-Kinase

**James E. Thomas,[1*] Murali Venugopalan,[2] Rachelle Galvin,[2] Yan Wang,[4,5] Gary M. Bokoch,[4,5] and Chris J. Vlahos[3]**

[1]Department of Cancer Research, Eli Lilly and Co., Indianapolis, Indiana
[2]Department of Endocrine Research, Eli Lilly and Co., Indianapolis, Indiana
[3]Department of Cardiovascular Research, Eli Lilly and Co., Indianapolis, Indiana
[4]Department of Immunology, Scripps Research Institute, La Jolla, California
[5]Department of Cell Biology, Scripps Research Institute, La Jolla, California

**Abstract** Studies on a platelet-derived growth factor (PDGF) responsive osteosarcoma cell line, MG-63, were initiated to determine the effects of phosphatidylinositol (PtdIns) 3-kinase inhibitors on serum-stimulated cell proliferation and PDGF-stimulated DNA replication, actin rearrangements, or PtdIns 3-kinase activity. In a dose-dependent manner, the fungal metabolite wortmannin and a quercetin derivative, LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), inhibited serum-stimulated MG-63 cell proliferation. The mitogenic effects of PDGF on MG-63 cells, as determined by incorporation of [$^3$H]-thymidine, were also substantially inhibited in the presence of 0.10 μM wortmannin or 10 μM LY294002. Furthermore, MG-63 cells stimulated by PDGF form distinct actin-rich, finger-like membrane projections which are completely inhibited by either 0.10 μM wortmannin or 10 μM LY294002. At these same concentrations, wortmannin and LY294002 were also effective at reducing levels of phosphatidylinositol 3-phosphate in PDGF-stimulated MG-63 cells. Treatment of these cells with increasing concentrations of wortmannin reduced the level of PDGF stimulated tyrosine phosphorylation of the PDGF receptor but did not significantly affect the amount of the PtdIns 3-kinase regulatory subunit, p85, associated with the receptor. Additionally, pretreatment of cells with 0.250 μM wortmannin followed by stimulation with PDGF resulted in a slightly reduced level of receptor autokinase activity; however, similar treatment with 50 μM LY294002 did not affect the level of autokinase activity. These results demonstrate the effects of two different PtdIns 3-kinase inhibitors on serum- and PDGF-stimulated MG-63 cell proliferation and PDGF-stimulated morphological changes and suggest a greater role for PtdIns 3-kinase in these processes. J. Cell. Biochem. 64:182–195. 

**Key words:** LY294002; wortmannin; signal transduction; tyrosine kinase; mitogen

Ligand induced activation of the PDGF receptor (PDGFR) results in receptor dimerization followed by autophosphorylation of the receptor on several tyrosine residues. The activated receptor complex associates with several signaling molecules, including phospholipase Cγ (PLCγ), nonreceptor tyrosine kinases from the Src family (Src, Fyn, and Yes), and the Ras GTPase activating protein GAP as well as phosphatidylinositol 3-kinase (PtdIns 3-kinase) [Cantley et al., 1991]. The individual functions of each of these signaling molecules have been clearly established; however, the roles they play in PDGF-stimulated cellular processes (e.g., mitosis, chemotaxis, actin rearrangements, receptor trafficking) have yet to be completely understood. PtdIns 3-kinase in part mediates several

Abbreviations used: EGF, epidermal growth factor; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; IGF-1, insulin-like growth factor-1; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PAS, protein A-Sepharose CL-4B; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PMSF, phenylmethylsulfonyl fluoride; PtdIns, phosphatidylinositol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPC, sphingosylphosphorylcholine; SS, serum-starved; TLC, thin-layer chromatography.

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*Correspondence to: James E. Thomas, Eli Lilly and Co., Department of Cancer Research, Mail Drop 0434, Indianapolis, IN 46285. E-mail: Thomas_James_E@Lilly.com

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of these responses to PDGF, including mitogenic responses [Fantl et al., 1992; Valius and Kazlauskas, 1993], receptor internalization [Joly et al., 1994], and changes in the actin organization [Wennstrom et al., 1994a,b; Wymann and Arcaro, 1994]. PtdIns 3-kinase activity is found co-localized with the PDGFR and associated microtubule-cytoskeletal structures [Kapellar et al., 1993]. Phosphorylated tyrosine residues on the PDGFR are the apparent docking sites for the SH2 domains of the p85 noncatalytic subunit of PtdIns 3-kinase [Kazlauskas and Cooper, 1990; Escobedo et al., 1991; Kashishian et al., 1992; Fantl et al., 1992; Hu et al., 1992; McGlade et al., 1992], while the 110 kDa catalytic subunit of PtdIns 3-kinase becomes activated following binding to a region between the p85 SH2 domains [Klippel et al., 1993, 1994; Holt et al., 1994]. The activated catalytic subunit subsequently processes the transfer of the terminal phosphate from ATP to either of two unique phosphoinositides, PtdIns 4-monophosphate or PtdIns 4,5-bisphosphate, yielding PtdIns 3,4-bisphosphate (PtdIns 3,4-$P_2$) and PtdIns 3,4,5-trisphosphate (PtdIns 3,4,5-$P_3$), respectively [Carpenter and Cantley, 1990].

Recently, potent inhibitors of PtdIns 3-kinase have been identified from natural sources [Yano et al., 1993; Okada et al., 1994; Matter et al., 1992]. Wortmannin, a microbial secondary metabolite, has been shown to be effective at inhibiting purified PtdIns 3-kinase with an $IC_{50}$ of 4.2 nM [Powis et al., 1994]. Another compound, which is a derivative of the bioflavinoid quercetin, LY294002, also inhibits purified bovine brain PtdIns 3-kinase, with an $IC_{50}$ of 1.4 μM [Vlahos et al., 1994]. These two inhibitors are structurally as well as mechanistically distinct [Vlahos, 1995]. Wortmannin acts in an irreversible, noncompetitive manner by covalently binding the ATP binding domain of PtdIns 3-kinase [Yano et al., 1993; Okada et al., 1994; Powis et al., 1994]. In contrast, LY294002 is a competitive, reversible inhibitor of the ATP binding site of PtdIns 3-kinase [Vlahos et al., 1994].

Treating cells with growth factors or mitogens, including PDGF, IGF-1, EGF, and sphingosylphosphorylcholine (SPC), results in distinct morphological changes which are attributed to rearrangement of the actin filaments and the formation of membrane ruffling. For example, ring-like structures have been observed on the dorsal side of human fibroblasts following stimulation with PDGF [Mellstrom et al., 1988]; however, in KB cells, IGF-1 and insulin stimulate darkly stained regions along the rim of the membrane, while treatment with EGF results in rod-like spiked structures and membrane ruffles [Kadowaki et al., 1986; Kotani et al., 1994]. Stimulation of Swiss 3T3 cells by the mitogen SPC has recently been shown to induce actin rearrangements resulting in the formation of unique actin microspikes at the plasma membrane [Seufferlein and Rozengurt, 1995].

Differences in the observed actin staining pattern may occur as a result of the signaling pathway which is activated by the mitogenic factor. Membrane ruffling occurs in EGF-stimulated KB cells apparently through a PKC-dependent pathway [Miyata et al., 1989], while insulin- and IGF-1–stimulated KB cells or PDGF-stimulated porcine endothelial cells utilize a PtdIns 3-kinase–dependent pathway [Kotani et al., 1994; Wennstrom et al., 1994a,b]. Changes in membrane morphology in PDGF-stimulated human foreskin fibroblasts may be the result of activating PtdIns 3-kinase [Wymann and Arcaro, 1994] but are inhibited by activators of PKC [Mellstrom et al., 1988]. Low concentrations of wortmannin disrupt neurite extension in PC12 cells [Kimura et al., 1994], suggesting a role for PtdIns 3-kinase in this process. SPC stimulation of actin microspikes is dependent upon phosphorylation of focal adhesion kinase (FAK) and paxillin through a $p21^{rho}$-dependent pathway [Seufferlein and Rozengurt, 1995]. Apparently, formation of microspikes in Swiss 3T3 cells is distinct from the membrane ruffling which is induced in these cells by several growth factors and activated rac1 or H-ras [Ridley et al., 1992].

To understand the role of PtdIns 3-kinase inhibitors on cell proliferation, DNA replication, and morphological alterations, the osteosarcoma cell line, MG-63, was treated with wortmannin and LY294002. Both compounds reduced serum-stimulated cell proliferation and inhibited PDGF-stimulated DNA replication at concentrations which also inhibited PtdIns 3-kinase activity in vivo. Furthermore, PDGF-stimulated MG-63 cells were shown to extend unique actin-rich, finger-like projections which were inhibited by both wortmannin and LY294002. These results compare two different PtdIns 3-kinase inhibitors and define their effects on cell-specific and distinct PDGF-stimulated processes.

## METHODS

### Materials

PDGF-BB and anti-PDGFR antibodies were purchased from Genzyme (Cambridge, MA). Rabbit antimouse IgG was obtained from Cappel (Durham, NC). Antiphosphotyrosine (4G10) and anti-p85 antibodies were products of UBI (Lake Placid, NY), and antiphosphotyrosine (Py20) was purchased from Transduction Laboratories (Lexington, KY). Protein A-Sepharose CL-4B was acquired from Pharmacia (Milwaukee, WI). Aprotinin, sodium orthovanadate, PMSF, and leupeptin were all purchased from Sigma (St. Louis, MO). [$\gamma$-$^{32}$P]-ATP (5,000 Ci mmol$^{-1}$), [$^{32}$P]-orthophosphate (8,500–9,120 Ci mmol$^{-1}$) were purchased from DuPont NEN (Boston, MA), and [methyl-$^{3}$H]-thymidine (79 Ci mmol$^{-1}$) was purchased from Amersham (Arlington Heights, IL). All tissue culture reagents were obtained from Gibco (Gaithersburg, MD). Ready Protein-plus scintillation cocktail was from Beckman (Fullerton, CA). PtdIns 3-kinase inhibitors wortmannin and LY294002 were synthesized at Eli Lilly and Co. All other reagents and materials were of the highest quality commercially available.

### Cell Proliferation Assays

MG-63 cells (CRL1427; ATCC, Rockville, MD) were grown in RPMI medium supplemented with 10% heat-inactivated FBS, 10 mM Hepes, and 2 mM glutamine (complete RPMI). Cells were seeded at 100,000 per 60 mm dish in complete RPMI. Twenty-four hours after the cells were seeded, the medium was removed and replaced with complete RPMI plus the described concentration of either wortmannin or LY294002 or an equal volume of solvent (DMSO). At this initial 24 h time point, a baseline cell number was established by trypsinizing and counting one plate of cells using a hemocytometer (improved Neubaurer type). The medium was changed every 24 h thereafter and replaced with fresh complete RPMI including inhibitors or solvent, and at this time one plate from each of the wortmannin, LY294002, and DMSO treatment groups was trypsinized and the cells counted.

### Thymidine Incorporation Assay

DNA replication was assayed by means of [$^{3}$H]-thymidine incorporation [Centrella et al., 1988]. MG-63 cells were seeded at a density of 100,000 cells/well into six-well plates in complete RPMI media. After 24 h, the cells were rinsed twice with PBS and re-fed with serum-free RPMI medium. Twenty-four hours later, this medium was replaced with the same serum-free RPMI medium supplemented with PDGF (10 ng/ml) and either 0.10 µM wortmannin, 10 µM LY294002, or an equivalent volume of DMSO. After 18 h the medium was replaced with serum-free RPMI medium supplemented with 2 µCi/ml [methyl-$^{3}$H]-thymidine, PDGF (10 ng/ml), and either 0.10 µM wortmannin, 10 µM LY294002, or an equivalent volume of DMSO. Six hours later, the medium was removed, the cell layer was washed three times with PBS, and the cells were trypsinized. The cells were pelleted by low speed centrifugation (1,000*g* for 10 min), washed twice with 1 ml PBS, and resuspended in lysis buffer (0.1 M NaOH, 0.1 M SDS). Following a 30 min incubation at 37°C, the solubilized cell lysates were cooled in an ice bath, and then an equal volume of ice-cold 10% trichloroacetic acid (TCA) was added to precipitate the DNA. The precipitate was spotted onto GF/A filters and then washed twice with ice-cold 5% TCA and once with ethanol. Following air-drying, the filters were placed in scintillation vials with 10 ml of cocktail. [$^{3}$H]-thymidine incorporation (DPM/well) was determined, and statistical analysis was by a one-way analysis of variance followed by a least significant difference multiple-comparison analysis (Tukey-Kramer). Data are presented as the mean of six samples and standard error of the mean with $P < 0.05$ considered significant.

### In Vivo Labeling

MG-63 cells in 10 cm petri dishes (80–90% confluent) were serum-starved for 18–24 h. The cells were then placed in phosphate-free medium supplemented with 0.5 mCi/plate [$^{32}$P]-orthophosphate for 2 h at 37°C. LY294002 (final concentration 10 µM) or wortmannin (final concentration 100 nM) in DMSO was added to the plates, which were then incubated an additional 10 min at 37°C. The cells were stimulated with 10 ng/ml PDGF-BB for 10 min and then washed twice with ice-cold PBS. Cells were harvested by scraping them into 750 µl of 1 N HCl:MeOH (1:1, v:v), and phospholipids were extracted as previously described [Serunian et al., 1991]. Phospholipids were resuspended in 60 µl $CHCl_3$ and separated by TLC on silica plates treated with 1.2% potassium oxalate as described previously [Vlahos and Matter, 1992]. Radioactive phosphatidylinositols

were extracted from the TLC plates, chemically deacylated with methylamine, and analyzed by anion-exchange HPLC using an on-line radiochemical detector as previously described [Vlahos and Matter, 1992].

### PDGFR Immunoprecipitations: Receptor Phosphorylation and Associated PtdIns 3-Kinase Assay

Subconfluent serum-starved (20–24 h) MG-63 cells were pretreated with either DMSO, wortmannin, or LY294002 in RPMI for 10 min at 37°C and 5% $CO_2$. The medium was removed, and the cells were subsequently treated with the same concentration of compound or solvent in the presence of 10 ng/ml PDGF-BB for 10 min at 37°C and 5% $CO_2$. The plates were then placed on ice and rinsed twice with cold PBS. Cells were lysed in ice-cold TNEN buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 1% Trasylol, 2.5 µM leupeptin, 1 mM sodium vanadate), and insoluble cell debris was removed by centrifugation at 2,000*g* for 10 min at 4°C [Seifert et al., 1989]. The supernatant was transferred to a fresh tube, and protein concentrations were determined. Subsequently, equal amounts of protein were precleared with 30 µl PAS for 15 min at 4°C on a rotator. The PAS was pelleted by centrifugation (2,000*g* for 1 min at 4°C) and the supernatant removed to a fresh tube. The supernatant was incubated with antibodies against PDGFR (5 µg/sample) for 1 h on ice. Immune complexes were absorbed to PAS conjugated with rabbit antimouse IgG for 1 h at 4°C on a rotator. The immune complexes were washed three times in TNEN buffer, resuspended in 2× Laemmli sample buffer, and fractionated by SDS-PAGE (7.5 or 10% polyacrylamide gel). Proteins were transferred to nitrocellulose and probed with either antiphosphotyrosine antibodies (Py20 and 4G10, both at 1:1,000 dilutions) or anti-p85 (1:1,000 dilution) in Western wash buffer (0.2% NP-40, 170 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing 3% BSA.

For PtdIns 3-kinase assays, the PAS-immune complex was washed successively with PBS; 0.5 M LiCl; 0.1 M Tris, pH 7.5; water; 0.1 M NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5 [Fukui et al., 1989]. The PAS-PDGFR immune complex was then resuspended in 50 µl of 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.5 mM EGTA. A 0.5 µl aliquot of 20 mg/ml phosphatidylinositol (Avanti-Polar Lipids, Alabaster, AL) dissolved in DMSO was then added to the reaction mixture and allowed to incubate at room temperature for 10 min. The reaction was initiated by the addition of 10 µCi per assay of [$\gamma$-$^{32}$P]-ATP, 10 µm ATP, and 20 mM MnCl and then incubated an additional 10 min at room temperature. The reaction was stopped by the addition of 1 N HCl, and lipid samples were dried in vacuo. Samples were resuspended in chloroform and spotted on silica gel plates, which were developed in chloroform-methanol-28% ammonium hydroxide-water (43:38:5:7).

### In Vitro Autokinase Activity

MG-63 cells, grown in 10 cm petri dishes to 80–90% confluence, were serum-starved for 18–24 h. The medium was withdrawn and the cells exposed to fresh medium containing solvent (DMSO) or compound (50 µM LY294002 or 0.25 µM wortmannin) for 10 min at 37°C. Cells were then stimulated with 10 ng/ml PDGF-BB. After a 10 min incubation at 37°C, the cells were washed twice with ice-cold PBS. Cells were lysed as described previously [Vlahos et al., 1993]. Protein concentrations in the lysate supernatants were normalized, and antiphosphotyrosine antibody (4G10, 5 µg) was incubated with the lysate supernatant for 1 h at 4°C. The antibody-antigen complexes were precipitated using immobilized PAS [Vlahos et al., 1993]. Immunopellets were washed twice with 0.1 M Tris-0.5 M LiCl (pH 7.5). During the second wash, 700 µl of the resuspended immunopellet was removed from use in the in vitro PtdIns 3-kinase assay (below). The remaining 300 µl of the resuspended immunopellet was washed with 0.2 M Tris-HCl buffer (pH 7.5) containing 5 mM $MnCl_2$. The in vitro autokinase reaction was performed as described previously [Vlahos et al., 1993]. The [$^{32}$P]-labeled proteins were separated by SDS-PAGE on an 8% gel (Novex, San Diego, CA). Gels were dried between cellophane sheets (Easy Breeze Gel Dryer; Hoefer Instrument Co., San Francisco, CA) and were analyzed by autoradiography.

### PtdIns 3-Kinase Activity in Antiphosphotyrosine Immunoprecipitates

The resuspended antiphosphotyrosine immunopellets (above) were split into two equal fractions of 350 µl. Each pellet was washed with 20 mM HEPES (pH 7.4) containing 10 mM $MgCl_2$ and then resuspended in 40 µl of the above buffer in the absence or presence of inhibitor (50 µM LY294002 or 0.25 µM wortmannin).

Thus, immunopellets derived from cells treated with either compound were washed and then assayed for PtdIns 3-kinase activity in the absence or presence of the compound. Phosphatidylinositol (10 µl of 2 mg/ml solution) was added to each sample, and the PtdIns 3-kinase reaction was then initiated by addition of 2 µl [$\gamma$-$^{32}$P]-ATP (32 µCi; 25 µM final concentration). Samples were incubated for 10 min at room temperature, after which time the reaction was quenched by addition of 80 µl 1 N HCl. Lipids were extracted and analyzed by TLC as described previously [Vlahos et al., 1993].

### Membrane Ruffling Experiments

MG-63 cells were grown in DMEM, 10% FCS, 1% Penn/Strep, 1% glutamine, and 10 mM Hepes until confluent. The cells were starved in serum-free media overnight and then pretreated with either wortmannin for 5 min at concentrations of 0.01, 0.10, or 1 µM or LY294002 for 10 min at concentrations of 1, 5, or 10 µM. The cells were then stimulated with PDGF (3 ng/ml) for 10 min in the presence or absence of inhibitors. Following stimulation, the cells were rinsed in 1× PBS for 1 min and fixed in 2% paraformaldehyde for 5–10 min, rinsed again in PBS, and allowed to soak in PBS for 15 min. The cells were permeabilized in 0.4% Triton X-100 in PBS for 15 min and stained with rhodamine phalloidin (2:100) in PBS for 30 min. Stained cells were then washed in PBS for 30 min and mounted with coverslips.

## RESULTS

### Effects of Wortmannin and LY294002 on MG-63 Cell Proliferation and PDGF-Stimulated DNA Synthesis

To determine the effects of PtdIns 3-kinase inhibitors on cell proliferation, MG-63 cells were treated with different concentrations of either wortmannin or LY294002. At most concentrations of wortmannin or LY294002, MG-63 cells continued to divide over 4 days; however, after 3 days, the effects of these compounds were sufficient to reduce cell proliferation compared to DMSO-treated cells (Fig. 1A,B). By the fourth day, the number of cells in 0.10 µM wortmannin- or 10 µM LY294002-treated plates was reduced by about 50% compared to the DMSO-treated cells. When MG-63 cells were treated with higher concentrations of the PtdIns 3-kinase inhibitors, the effects were toxic (data not shown).

Since MG-63 cells express relatively high levels of PDGF receptor [Seifert et al., 1989], it was of interest to determine whether PDGF-stimulated DNA replication was inhibited by either wortmannin or LY294002. Serum-starved MG-63 cells were stimulated with PDGF for 18 h in the presence of wortmannin, LY294002, or DMSO and then grown for an additional 6 h in PDGF-supplemented serum-free media with [$^3$H]-thymidine in the presence or absence of inhibitors. Under these conditions, 0.10 µM wortmannin (Fig. 1C) and 10 µM LY294002 (Fig. 1D) inhibited PDGF-stimulated DNA replication in MG-63 cells, although neither compound completely reduced DNA synthesis to levels observed in the unstimulated cells.

### Inhibition of PDGF-Stimulated PtdIns 3-kinase Activity

To determine whether PDGF-stimulated PtdIns 3-kinase activity was reduced in these cells at concentrations which inhibited DNA replication, MG-63 cells were labeled with [$^{32}$P]-orthophosphate and then stimulated with PDGF (10 ng/ml). HPLC analysis of cellular phospholipids showed a rapid rise in the levels of PtdIns 3,4-$P_2$ and PtdIns 3,4,5-$P_3$ in the PDGF- and DMSO-treated cells compared to unstimulated controls (Fig. 2). This increase in PtdIns 3,4-$P_2$ and PtdIns 3,4,5-$P_3$ was almost completely attenuated when MG-63 cells were pretreated with either 100 nM wortmannin or 10 µM LY294002 and then stimulated with PDGF in the presence of inhibitor (Fig. 2). Both compounds were specific for inhibiting PtdIns 3-kinase since levels of PtdIns 4-P and PtdIns 4,5-$P_2$ were not affected in cells treated with either wortmannin or LY294002.

The mechanism by which these compounds inhibit PtdIns 3-kinase was assessed by treating the cells with PDGF and inhibitors and then assaying for activity in the presence or absence of compound. When the antiphosphotyrosine immunopellets from the drug-treated cells were assayed in the absence of LY294002 (50 µM), more than 70% of the PtdIns 3-kinase activity was present compared to the PDGF-treated control in the absence of drug (Fig. 3). In contrast, only about 20% of the control activity was present in antiphosphotyrosine immunopellets from the wortmannin (250 nM) -treated cells (Fig. 3). When parallel samples were assayed in vitro in the presence of the same concentration of inhibitor, PtdIns 3-ki-

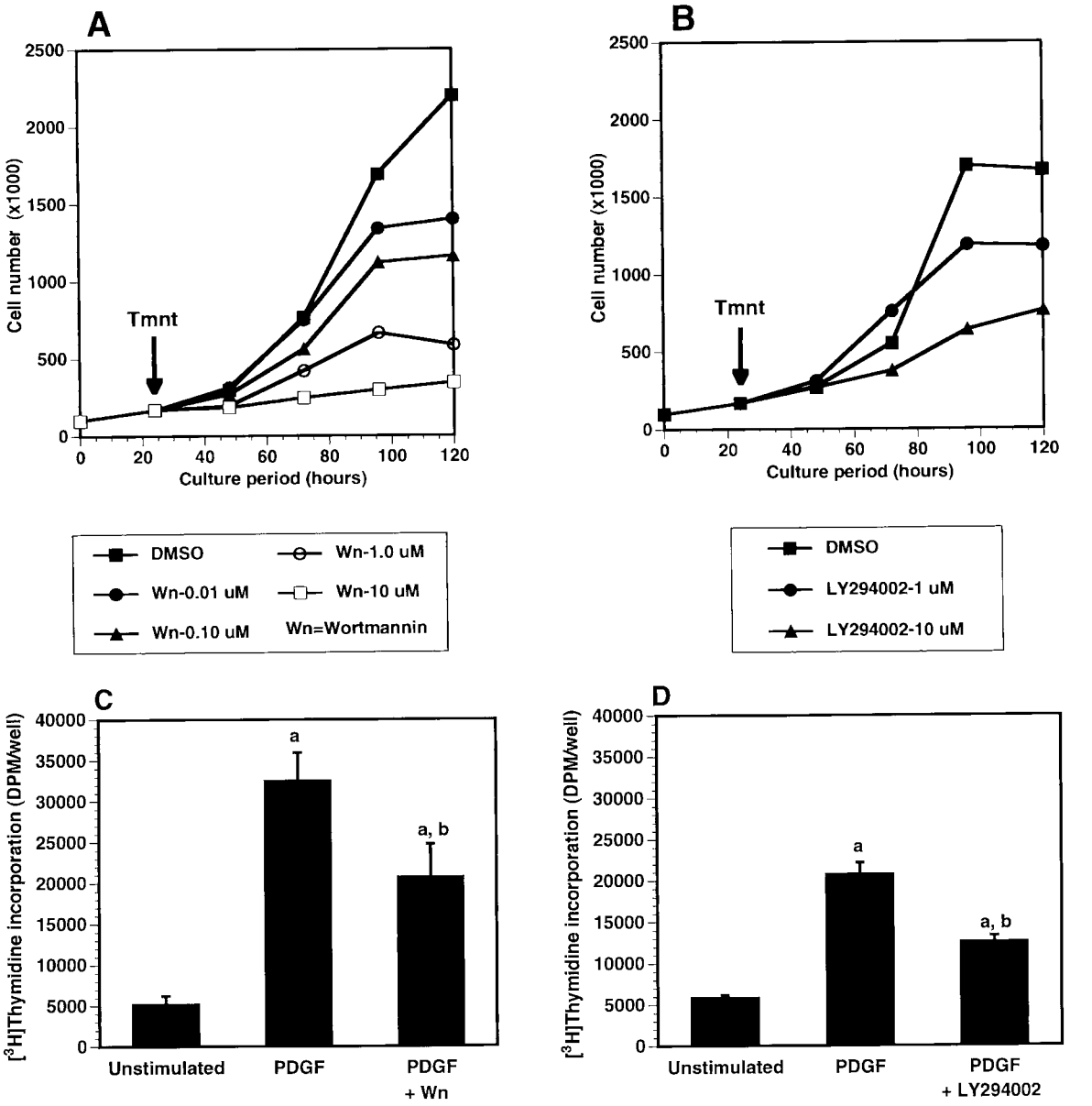


**Fig. 1.** Effects of wortmannin and LY294002 on cell proliferation and [$^3$H]-thymidine incorporation. Cell proliferation was determined for MG-63 cells grown in the presence of complete media and treated with DMSO or wortmannin **(A)** or complete media and DMSO or LY294002 **(B).** The results shown are a representative of three separate experiments. Serum-starved MG-63 cells were treated with PDGF and either DMSO, 0.10 μM wortmannin **(C)** or 10 μM LY294002 **(D)** in the presence of [$^3$H]-thymidine as described in Methods. Each bar represents the mean ± the standard error of the mean of six wells. $^aP < 0.05$, compared to unstimulated; $^bP < 0.05$, compared to PDGF stimulated cells. The results shown are representative of two different experiments.

nase activity was completely abolished (Fig. 3). Additionally, in a dose-dependent manner, wortmannin reduced the level of PtdIns 3-kinase activity associated with the PDGFR in PDGFR immunoprecipitates (Fig. 4, lanes 3–6). More than 65% of the PDGFR-associated PtdIns 3-kinase activity was inhibited at concentrations greater than or equal to 0.10 μM wortmannin. This inhibitory activity was specific for PtdIns 3-kinase, since HPLC analysis of the deacylated phosphatidylinositol phosphate showed reduced phosphorylation on the D3 site of the inositol ring (not shown). This decrease in PDGFR-associated PtdIns 3-kinase activity was not due to a decrease in PDGFR-associated p85 PtdIns 3-kinase regulatory subunit (see below

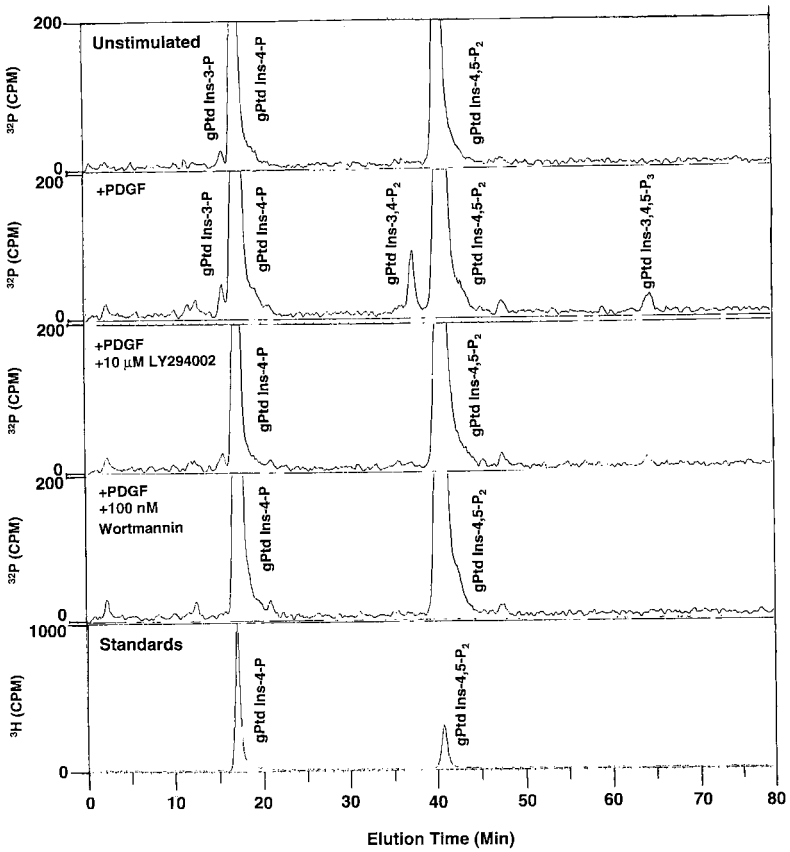


**Fig. 2.** Inhibition of PtdIns 3-kinase activity in PDGF-stimulated MG-63 cells. Phospholipids were extracted from [$^{32}$P]-orthophosphate-labeled MG-63 cells which were preincubated in either 10 μM LY294002, 100 nM wortmannin, or an equal volume of DMSO and then stimulated with 10 ng/ml PDGF. PtdIns-phosphates were extracted as described in Methods and subjected to anion exchange HPLC using an on-line radiochemical detector. Deacylated [$^{32}$P]-PtdIns 3,4-$P_2$ and [$^{32}$P]-PtdIns 3,4,5-$P_3$, synthesized using purified bovine brain PtdIns 3-kinase, were used as migration standards; in addition, deacylated [$^{3}$H]-PtdIns 4-P and [$^{3}$H]-PtdIns 4,5-$P_2$ were used as internal standards.

and Fig. 5B). These results indicated that the two inhibitors act by apparently different mechanisms. LY294002 acts in a competitive manner with respect to the substrate to bind PtdIns 3-kinase, while wortmannin is a covalent, irreversible inhibitor.

## Effects of Wortmannin and LY294002 on PDGF Receptor Phosphorylation and Autokinase Activity

Although both compounds have been shown to be relatively specific inhibitors of PtdIns 3-kinase, it was of interest to determine whether the reduced level of PtdIns 3-kinase activity might be due to an inhibitory effect on PDGFR autokinase activity. Hence, a reduced level of PDGFR tyrosine phosphorylation might result in less PtdIns 3-kinase regulatory subunit, p85, associating with the receptor. In PDGF-stimulated MG-63 cells, the level of tyrosine phosphorylation on the PDGFR decreased with increasing concentrations of wortmannin (Fig. 5A, lanes 3–5). At 0.10 μM and 1 μM wortmannin the level of tyrosine phosphorylation on the receptor was reduced by approximately 25 and 50%, respectively, compared to DMSO-treated controls. In contrast, there was no apparent decrease in tyrosine phosphorylation of the PDGFR in cells treated with either 1 or 10 μM LY294002 (Fig. 5A, lanes 6,7). These results were specific for PDGF stimulation of MG-63 cells since no other 190 kDa tyrosine phosphorylated protein could be immunoprecipitated from MG-63 cells with the PDGFR antibody used in these experiments following treatment with either insulin or EGF (data not shown). In addition, a subtle decrease in autokinase activity was also observed in PDGF-stimulated wortmannin (250 nM) treated cells (Fig. 6, lane 4, upper arrow) compared to PDGF-stimulated DMSO-treated controls (Fig. 6, lane 2), while no decrease was observed in the MG-63 cells

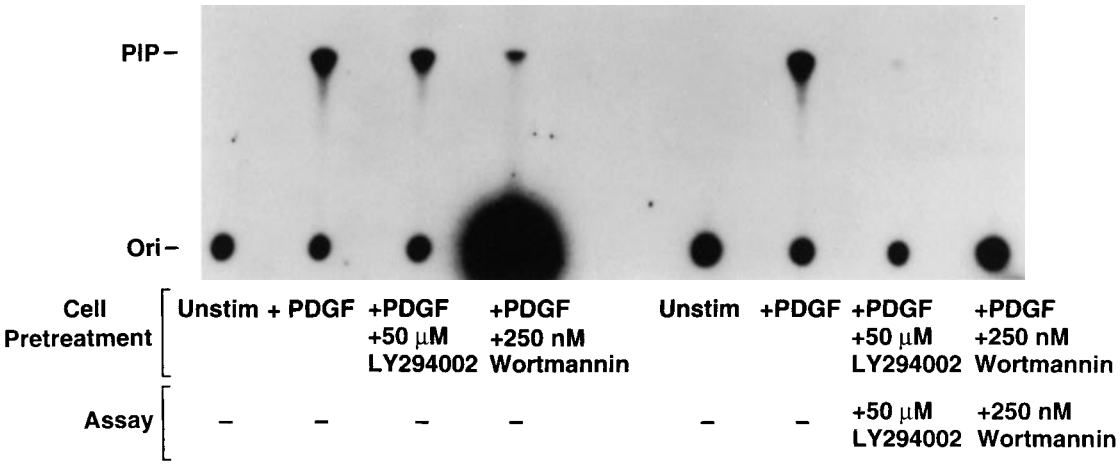


**Fig. 3.** Mechanistic analysis of PtdIns 3-kinase inhibitors. MG-63 cells were pretreated with compounds as described in Methods. Cells were left unstimulated or stimulated with PDGF-BB (10 ng/ml). PtdIns 3-kinase was immunoprecipitated from cell lysates with an antiphosphotyrosine antibody, and activity was assayed in the presence or absence of the described concentration of inhibitor.

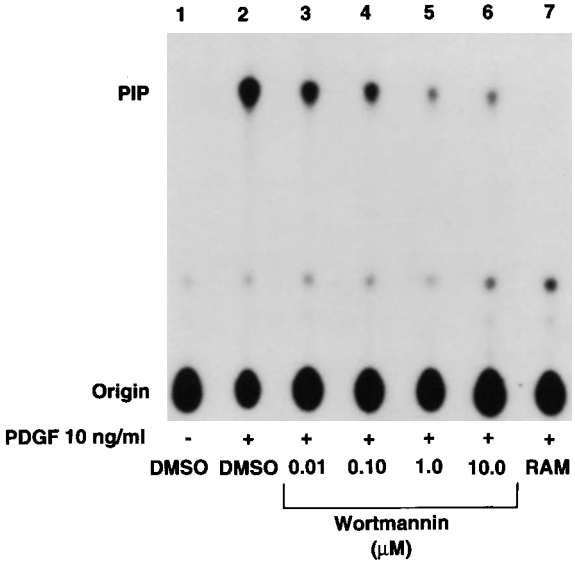


**Fig. 4.** Reduced level of PDGFR-associated PtdIns 3-kinase activity in wortmannin-treated cells. Serum-starved (24 h) MG-63 cells were pretreated with DMSO (*lanes 1,2*) or with the indicated concentration of wortmannin (*lanes 3–6*) in serum-free RPMI for 10 min and then treated for 10 min with RPMI supplemented with 10 ng/ml PDGF-BB and DMSO (lane 2) or the indicated concentration of wortmannin (lanes 3–6). PDGFR was immunoprecipitated from MG-63 cell lysates (300 μg) and subsequently assayed for associated PtdIns 3-kinase. Activity was reduced by more than 65% at 0.10 μM (n = 4 experiments) as determined by densitometer scans of autoradiography (Molecular Dynamics). *Lane 7:* PDGF-stimulated cells, rabbit anti-mouse immunoprecipitation.

stimulated with PDGF and treated with LY294002 (50 μM) (Fig. 6, lane 3).

Despite the decrease in PDGFR tyrosine phosphorylation in the PDGF-wortmannin-treated MG-63 cells, no significant decrease in p85 association with the PDGFR was observed compared to the PDGF and DMSO-treated controls (Fig. 5B, lanes 3–5). Additionally, there was no decrease in the level of p85 association with the receptor in PDGF-LY294002-treated cells compared to PDGF-stimulated and DMSO-treated cells (Fig. 5B, lanes 6,7). Interestingly, the level of p85 phosphorylation in the wortmannin-treated cells was not significantly affected in the autokinase reaction (Fig. 6, lanes 2–4, lower arrow). These results indicated that although wortmannin may inhibit other kinases, its effect on the PDGF receptor was not sufficient to reduce p85 association with the receptor and account for the observed decrease in PtdIns 3-kinase activity.

## PDGF-Induced Changes in Membrane Morphology are Inhibited by Wortmannin and LY294002

Previous work had shown that cells treated with PDGF underwent changes in their cytoskeletal architecture; therefore, it was of interest to determine how this growth factor might also affect MG-63 cell morphology. Within minutes of PDGF stimulation, rhodamine phalloidin staining revealed unique actin-rich, finger-like projections extending from the membrane of MG-63 cells creating a rough membrane surface (compare panels A and E in Fig. 7). To

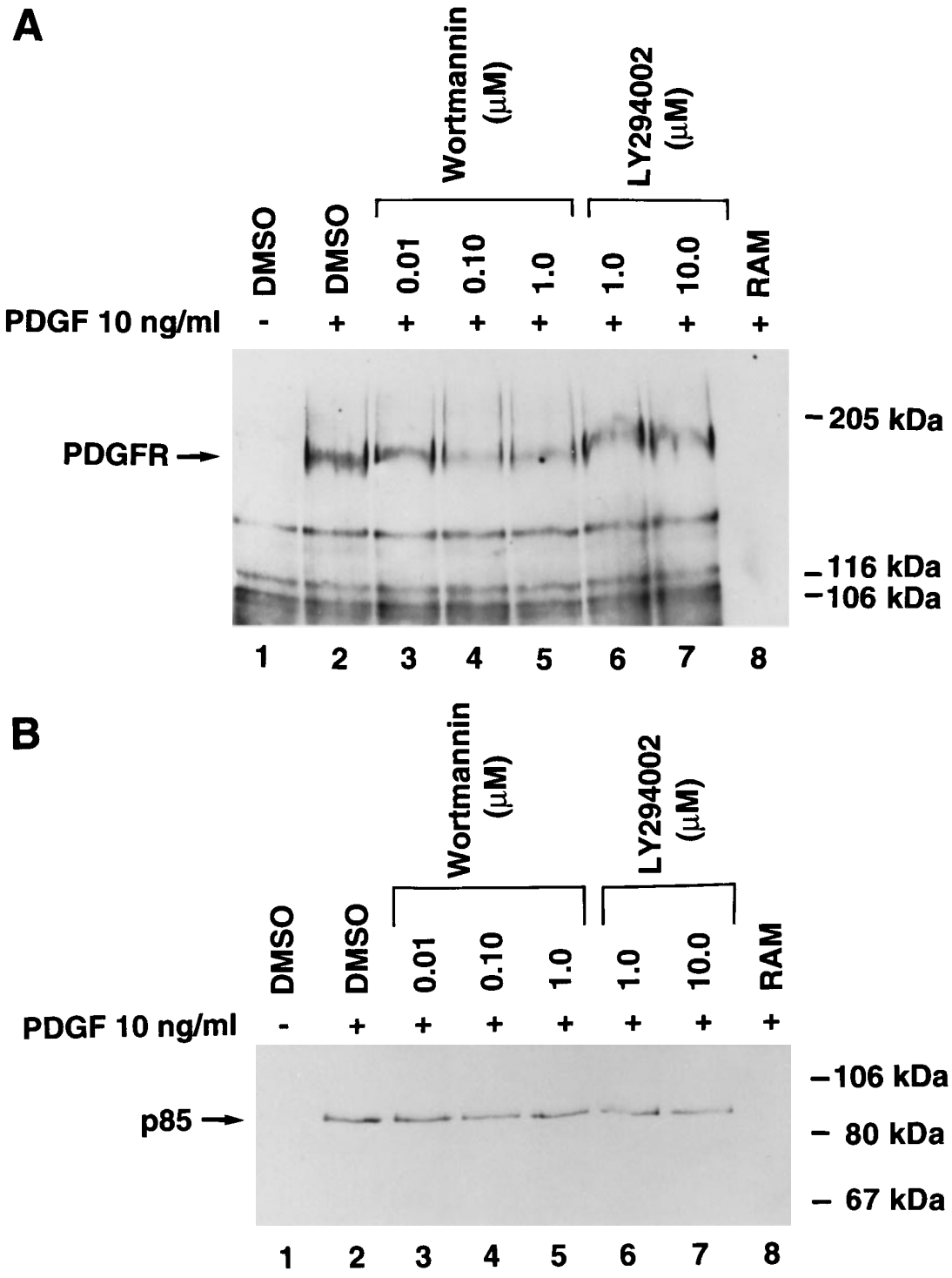


**Fig. 5.** Effects of wortmannin or LY294002 on PDGFR tyrosine phosphorylation and PDGFR-associated p85. MG-63 cells were treated with the indicated concentrations of wortmannin or LY294002 as described in Methods. *Lane 8:* Cells stimulated with PDGF and lysate immunoprecipitated with rabbit anti-mouse conjugated PAS (see Methods). **A:** Tyrosine phosphorylation of the PDGFR was identified in PDGFR immunoprecipitates and antiphosphotyrosine Western blots. The results shown are representative of seven different experiments for wortmannin and six different experiments for LY294002. **B:** The p85 subunit of PtdIns 3-kinase associated with the PDGFR immunoprecipitates was identified by probing Western blots with a polyclonal antibody. The results shown are representative of four different experiments. Levels of hybridization were determined by densitometer scans (Molecular Dynamics).

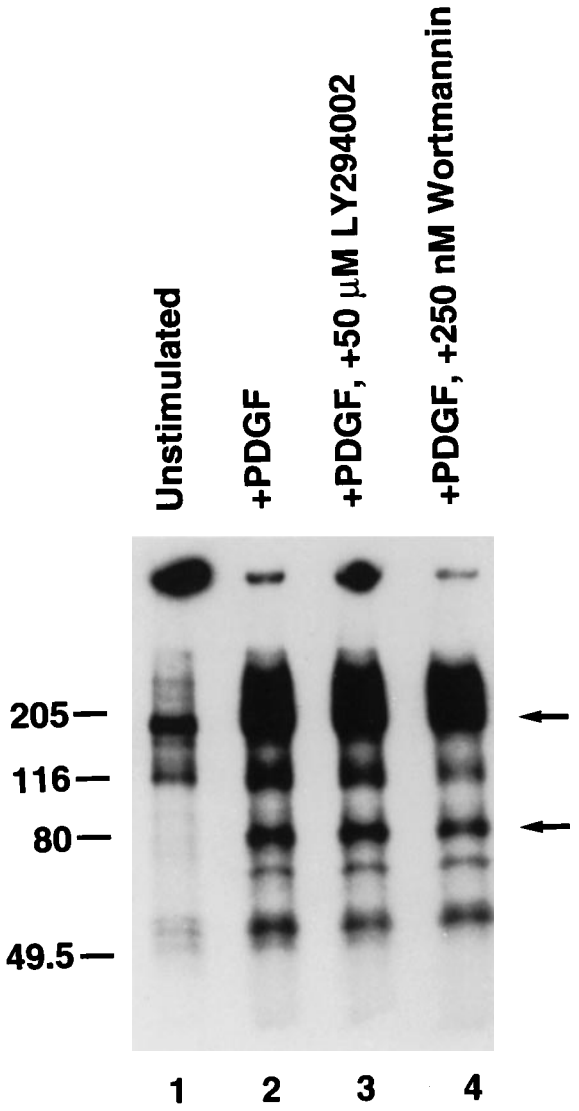


**Fig. 6.** PDGFR autokinase activity. MG-63 cells were pretreated with wortmannin, LY294002, or DMSO for 10 min and then treated with compounds in the presence of 10 ng/ml PDGF-BB. Tyrosine-phosphorylated proteins were immunoprecipitated from cell lysates as described in Methods and immune complexes subjected to an in vitro kinase reaction. Autophosphorylated 190 kDa PDGFR and p85 proteins are indicated by the upper and lower arrows, respectively. The results shown are representative of three different experiments.

determine whether these structures might be dependent upon PDGF activation of PtdIns 3-kinase, MG-63 cells were pretreated with different concentrations of either wortmannin or LY294002 and then stimulated with PDGF in the presence of inhibitors. Both wortmannin and LY294002 in a concentration-dependent manner significantly reduced the percentage of cells extending stainable PDGF-induced finger-like projections (Fig. 7B–D and F–H, respectively). At 0.01 μM wortmannin or 5 μM LY294002, the percentage of PDGF-stimulated cells which did not extend projections significantly increased, and at 0.10 μM wortmannin and 10 μM LY294002 this response to PDGF was nearly completed attenuated (Fig. 7; Table I). In contrast, lysophosphatidic acid–induced actin stress fibers were not affected by either of these compounds (data not shown).

**TABLE I. Effects of PtdIns 3-Kinase Inhibitors on MG-63 Cell Morphology**

| Condition | Percentage of Cells[a] With Projections (≥5) | Without projections |
|---|---|---|
| Serum-starved (SS) | 0 | 100 |
| SS + PDGF (3 ng/ml) | 86.6 | 13.4 |
| SS + PDGF + 0.01 μM wortmannin | 61.6 | 38.4 |
| SS + PDGF + 0.10 μM wortmannin | 0 | 100 |
| SS + PDGF + 1.0 μM wortmannin | 0 | 100 |
| SS + PDGF + 1 μM LY294002 | 81.3 | 18.7 |
| SS + PDGF + 5 μM LY294002 | 15.6 | 84.4 |
| SS + PDGF + 10 μM LY294002 | 0 | 100 |

[a]Ten fields counted for each condition.

## DISCUSSION

### Inhibition of MG-63 Cell Proliferation and DNA Synthesis by Inhibitors of PtdIns 3-Kinase

The antimitotic effects of wortmannin and LY294002 have not previously been assessed, yet these effects may be important when evaluating the relative role of both compounds in specific cellular processes. Both wortmannin and LY294002 reduced MG-63 cell proliferation in serum-supplemented media, although more than 3 days of exposure to these compounds was required before differences in cell numbers between control and treated groups were observed. Several reasons may explain why these inhibitors showed a protracted effect in the cell proliferation assay. One possibility is that mitogenic factors in serum either do not activate PtdIns 3-kinase or they concurrently activate PtdIns 3-kinase and other pathways. These other pathways may initially be unaffected by either inhibitor and the cells continue to proliferate for a time, although, eventually, the effect of wortmannin or LY294002 on PtdIns 3-kinase activity or other kinases is enough to disrupt cell division. A second possibility is that both

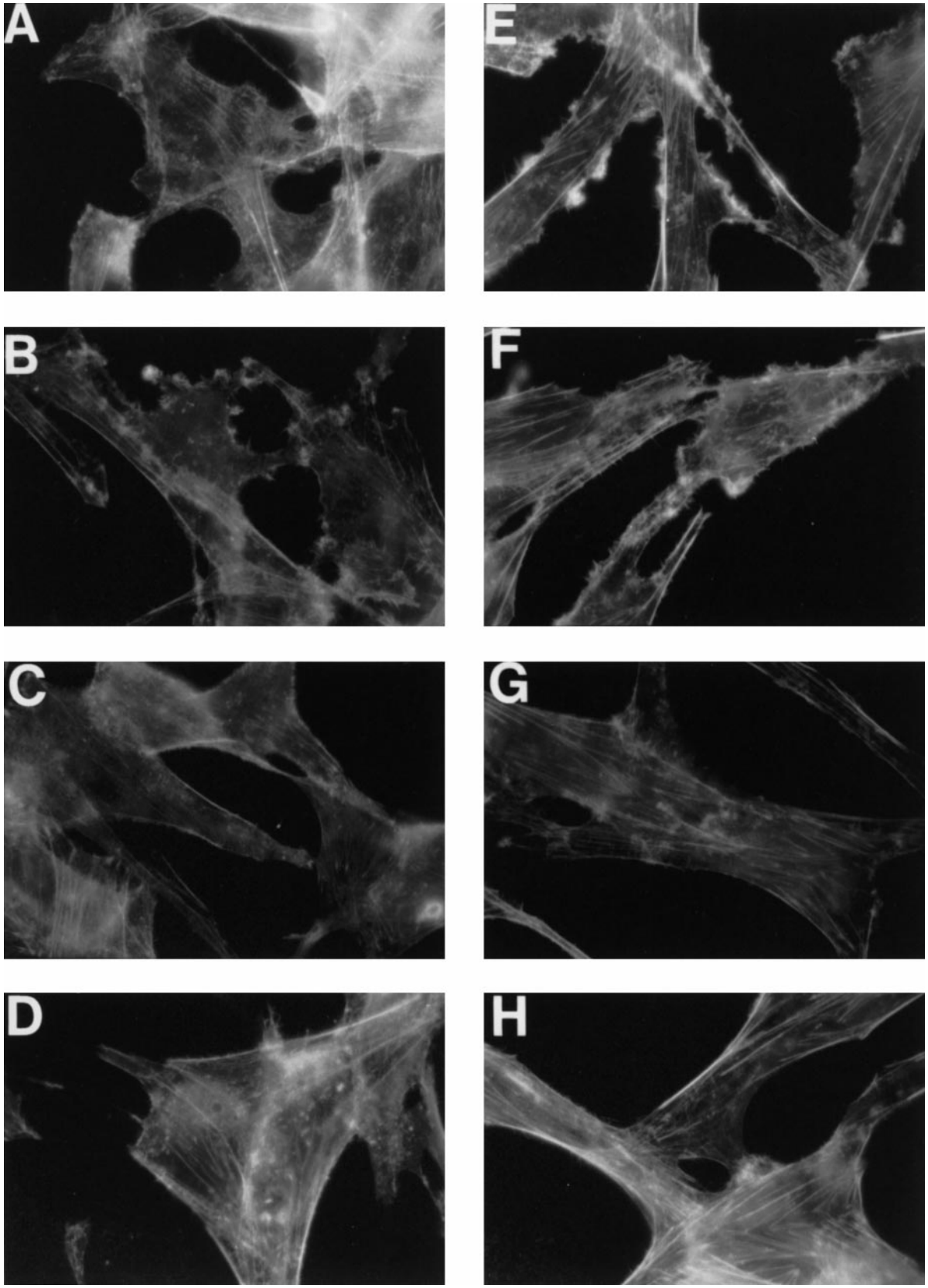


**Fig. 7.** Analysis of actin staining patterns observed in MG-63 cells stimulated with PDGF in the presence or absence of wortmannin or LY294002. Serum-starved MG-63 cells were stimulated with PDGF in the presence or absence of wortmannin **(B–D)** or LY294002 **(F–H).** The cells were fixed and stained with rhodamine phalloidin as described in Methods. **A:** Serum-starved MG-63 cells. **E:** Serum-starved cells stimulated with PDGF (3 ng/ml). Serum-starved cells stimulated with PDGF in the presence of 0.01 μM (B), 0.10 μM (C), or 1 μM (D) wortmannin or in the presence of 1 μM (F), 5 μM (G), or 10 μM (H) LY294002. Cells are shown at 100× magnification.

wortmannin and LY294002 are not active for long periods of time in serum-supplemented media. Kimura et al. [1994] have shown that in media wortmannin starts to lose effectiveness in less than 5 h. Therefore, in the cell proliferation assay, it is likely that wortmannin was degraded after only a few hours, possibly allowing some cells to survive and proliferate over the 3 day period. Little is known about the stability of LY294002 in serum-supplemented media. Cells grown in 1 or 10 μM LY294002 showed similar growth properties as those in 0.01 μM or 0.10 μM wortmannin, suggesting similar effectiveness. Furthermore, 10 μM LY294002 reduced cell proliferation by about 50% after 3 days, which is similar to its $IC_{50}$ against purified PtdIns 3-kinase enzyme, suggesting that the compound is relatively stable and available in vitro [Cheatham et al., 1994; Vlahos et al., 1995].

Both 0.10 μM wortmannin and 10 μM LY294002 reduced PDGF-stimulated DNA synthesis to nearly the same extent over a 24 h period of time. At a tenfold higher concentration, wortmannin inhibited the level of PDGF-stimulated DNA synthesis to about the same level shown in Figure 1C (data not shown). This result indicated that that portion of DNA synthesis dependent upon PDGF-stimulated PtdIns 3-kinase was nearly completely inhibited at both the 0.10 μM concentration of wortmannin and the 10 μM concentration of LY294002 shown in Figure 1C,D. Therefore, the observations in this paper that neither compound inhibited PDGF-stimulated DNA synthesis to the level of the unstimulated MG-63 cells (Fig. 1C,D) suggest that PDGF may be activating other mitogenic pathways in these cells, including Ras-GAP or PLCγ [Fantl et al., 1992; Valius and Kazlauskas, 1993]. Additional experiments are necessary to determine which different signaling pathways in these cells contribute to PDGF-stimulated DNA synthesis.

### PDGF-Stimulated PtdIns 3-Kinase Activity Inhibited by Wortmannin and LY294002

In PDGF-stimulated MG-63 cells, both wortmannin and LY294002 specifically inhibited PtdIns 3-kinase activity in both in vivo and in vitro kinase assays. Both compounds inhibited PtdIns 3-kinase activity in vivo at concentrations (0.10 μM wortmannin and 10.0 μM LY294002) which inhibited serum-stimulated cell proliferation by 50% and PDGF-stimulated DNA synthesis. Similarly, phosphatidylinositol trisphosphate production was inhibited in formyl peptide–stimulated guinea pig neutrophils by 0.05 μM wortmannin [Okada et al., 1994], although histamine secretion and PtdIns 3-kinase in RBL-2H3 cells was far more sensitive to wortmannin with an $IC_{50}$ of less than 10 nM [Yano et al., 1993]. LY294002 was effective at inhibiting proliferation of cultured rabbit aortic segments with an $IC_{50}$ of about 30 μM, a concentration higher than the $IC_{50}$ for purified PtdIns 3-kinase [Vlahos et al., 1994] but similar to results shown here. In addition, more than 65% of the PDGFR-associated PtdIns 3-kinase activity assayed in vitro was inhibited when PDGF-stimulated MG-63 cells were treated with 0.10 μM wortmannin. The similarity in the effective concentrations of wortmannin and LY294002 which inhibit cell proliferation or DNA replication and PtdIns 3-kinase activity suggests the involvement of PtdIns 3-kinase in serum- or PDGF-stimulated cellular processes.

The selective nature of wortmannin and LY294002 toward PtdIns 3-kinase has been demonstrated in other systems. Wortmannin will inhibit myosin light chain kinase with an $IC_{50}$ of 1.9 μM [Nakanishi et al., 1992], which is considerably higher than the $IC_{50}$ against purified PtdIns 3-kinase activity in vitro, 2–4 nM [Powis et al., 1994]. The level of PDGFR autokinase activity in PDGF-stimulated porcine endothelial cells treated with wortmannin (~0.10 μM) is apparently unchanged [Wennstrom et al., 1994a]. Similarly, we see only a slight decrease in phosphorylation on tyrosine residues in the PDGFR at 0.10 μM wortmannin (about 75% of PDGF-stimulated control levels) (Fig. 5A), but there is a further decrease at 1.0 μM wortmannin as well as a slightly lower level of PDGFR autokinase activity at 0.25 μM wortmannin. The concentrations (>100 nM) at which PDGFR activities are affected are similar to those that begin to inhibit other enzymatic activities; therefore, wortmannin or a metabolite of wortmannin may be acting on other kinases, including PtdIns 3-kinase. Since PDGFR-associated p85 levels in the wortmannin-treated PDGF-stimulated cells are unchanged, it is likely that wortmannin inhibited phosphorylation of different tyrosine residues on the receptor and not just the docking site for p85. In contrast, LY294002 appears to have no effect on PDGFR autokinase activity or association of p85 with the receptor. LY294002 is also not inhibitory to other kinases at the 10 μM concentration which reduced cell proliferation in se-

rum-stimulated cultures by 50% or significantly reduced PDGF-stimulated DNA replication [Vlahos et al., 1994].

### PDGF-Stimulated Projections Are Blocked by PtdIns 3-Kinase Inhibitors

MG-63 cells responded to PDGF by extending unique actin-rich, finger-like projections similar to those observed in SPC-stimulated Swiss 3T3 cells [Seufferlein and Rozengurt, 1995] and distinct from the ring-like structures observed in PDGF-stimulated human fibroblasts [Mellstrom et al., 1988] or the rod-like structures observed in EGF-stimulated KB cells [Kadowaki et al., 1986]. That the extension of these novel actin-rich projections is dependent upon PDGF stimulation of PtdIns 3-kinase is supported by results reported here. 1) Projections extended by MG-63 cells were sensitive to both PtdIns 3-kinase inhibitors in a concentration-dependent manner. This observation was confirmed by quantitative analysis which indicated that the percentage of cells with five or more finger-like extensions decreased with increasing concentrations of either wortmannin or LY294002 (Table 1). 2) PtdIns 3-kinase activity in vivo and in vitro is significantly reduced at 10 μM LY294002 or 0.10 μM wortmannin, concentrations which significantly inhibited these morphological changes in PDGF-stimulated MG-63 cells. 3) As other have reported, these concentrations do not inhibit the activity of other kinases [Nakanishi et al., 1992; Vlahos et al., 1994]. In contrast to the results with PDGF-stimulated MG-63 cells, extension of actin microspikes in SPC-stimulated Swiss 3T3 cells occurs apparently through a $p21^{rho}$-dependent pathway [Seufferlein and Rozengurt, 1995]. However, formation of ring-like structures in human fibroblasts seems to depend upon PtdIns 3-kinase, since mutation of p85 docking sites on the PDGF receptor or treating cells with wortmannin inhibits the formation of these rings [Wennstrom et al., 1994a,b; Wymann and Arcaro, 1994]. The results shown here and those demonstrated by others clearly show a complex relationship between morphological changes, growth factors, and cell types.

PtdIns 3-kinase appears to be a key enzyme in the signal transduction pathway elicited by PDGF. Mutants of the PDGFR that lack the PtdIns 3-kinase binding site fail to have associated PtdIns 3-kinase activity, and cells expressing these mutant receptors fail to show increased DNA synthesis, cell division, and receptor internalization. Using two structurally unrelated inhibitors of PtdIns 3-kinase, we present further evidence that PtdIns 3-kinase activity may be important for PDGF-dependent cellular processes, including DNA synthesis and membrane rearrangements. It is possible to treat cells with LY294002 and upon removal of the compound PtdIns 3-kinase activity is restored; however, pretreatment of the cells with wortmannin abolishes PtdIns 3-kinase activity, even when the immunoprecipitates are assayed in the absence of this compound. Although the exact role of PtdIns 3-kinase and its products in mitogenesis is still unknown, the use of specific inhibitors of PtdIns 3-kinase are valuable tools to elucidate specific signaling events that are dependent upon PtdIns 3-kinase.

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