Molecular Biology of the Cell
Vol. 6, 1145–1158, September 1995

# Wortmannin Blocks Lipid and Protein Kinase Activities Associated with PI 3-Kinase and Inhibits a Subset of Responses Induced by FcεR1 Cross-linking

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Submitted April 12, 1995; Accepted July 14, 1995
Monitoring Editor: Joseph Schlessinger

We have investigated the effects of wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), on antigen-mediated signaling in the RBL-2H3 mast cell model. In RBL-2H3 cells, the cross-linking of high affinity IgE receptors (FcεR1) activates at least two cytoplasmic protein tyrosine kinases, Lyn and Syk, and stimulates secretion, membrane ruffling, spreading, pinocytosis, and the formation of actin plaques implicated in increased cell-substrate adhesion. In addition, FcεR1 cross-linking activates PI 3-kinase. It was previously shown that wortmannin causes a dose-dependent inhibition of PI 3-kinase activity and also inhibits antigen-stimulated degranulation. We report that the antigen-induced synthesis of inositol(1,4,5)$P_3$ is also markedly inhibited by wortmannin. Consistent with evidence in other cell systems implicating phosphatidylinositol(3,4,5)$P_3$ in ruffling, pretreatment of RBL-2H3 cells with wortmannin inhibits membrane ruffling and fluid pinocytosis in response to FcεR1 cross-linking. However, wortmannin does not inhibit antigen-induced actin polymerization, receptor internalization, or the actin-dependent processes of spreading and adhesion plaque formation that follow antigen stimulation in adherent cells. Wortmannin also fails to inhibit either of the FcεR1-coupled tyrosine kinases, Lyn or Syk, or the activation of mitogen-activated protein kinase as measured by in vitro kinase assays. Strikingly, there is substantial in vitro serine/threonine kinase activity in immunoprecipitates prepared from FcεR1-activated cells using antisera to the p85 subunit of PI 3-kinase. This activity is inhibited by pretreatment of the cells with wortmannin or by the direct addition of wortmannin to the kinase assay, suggesting that PI 3-kinase itself is capable of acting as a protein kinase. We conclude that FcεR1 cross-linking activates both lipid and protein kinase activities of PI 3-kinase and that inhibiting these activities with wortmannin results in the selective block of a subset of FcεR1-mediated signaling responses.

## INTRODUCTION

Cross-linking the high affinity IgE receptors, FcεR1, on rat basophilic leukemia (RBL-2H3) cells initiates a signaling cascade that leads to release of granule contents, increased cell adhesion, membrane ruffling, and increased fluid-phase endocytosis (reviewed in Oliver *et al.*, 1989; Beaven and Metzger, 1993). Early signaling events associated with the receptor include activation of the tyrosine kinases Lyn (a member of the src family) and Syk (Eiseman and Bolen, 1992; Hutchcroft *et al.*, 1992), which in turn activate phospholipase Cγ (PLC)[1] (Park *et al.*, 1991; Li *et al.*, 1992). PLC cleaves

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[1] Abbreviations used: DNP, dinitrophenol; BSA, bovine serum albumin; FcεR1, the high affinity receptor for IgE; FITC, fluorescein isothiocyanate; Ins(1,4,5)$P_3$, inositol(1,4,5)trisphosphate; ITAM, immunoreceptor tyrosine activation motif; PAGE, polyacrylamide gel

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phosphatidylinositols, including phosphatidylinositol(4,5)-bisphosphate ($PtdIns(4,5)P_2$), which yields the second messengers $Ins(1,4,5)P_3$ and 1,2 diacylglycerol. Secretion is dependent upon increases in cytosolic calcium, resulting from the combination of release from $Ins(1,4,5)P_3$-sensitive calcium stores and the influx of extracellular calcium (Beaven *et al.*, 1984; Stump *et al.*, 1987).

In RBL-2H3 cells, FcεR1 cross-linking also activates a lipid kinase, phosphatidylinositol 3-kinase (PI 3-kinase) (Yano *et al.*, 1993). This enzyme is composed of two subunits, a regulatory 85-kDa and a catalytically active 110-kDa subunit. The 85-kDa subunit contains multiple protein-protein interaction motifs: two SH2 domains, one SH3 domain, a BCR homology domain, and two proline-rich SH3 binding domains (reviewed in Stephens *et al.*, 1993; Kapeller and Cantley, 1994). From these motifs, the 85-kDa subunit has been postulated to act as an "adaptor" protein coupling the 110-kDa catalytic subunit to activating signals. Activation of PI 3-kinase is common to receptor tyrosine kinases (Ullrich and Schlessinger, 1990), some G-protein–coupled receptors (Traynor-Kaplan *et al.*, 1989; Stephens *et al.*, 1994; Thomason *et al.*, 1994) and other members of the multi-chain immune system receptor family, including the T cell receptor, FcγRIII-A, and the mIg receptor on B cells (Thompson *et al.*, 1992; Ward *et al.*, 1992; Kanakaraj *et al.*, 1994; Ninomiya *et al.*, 1994). PI 3-kinase has been implicated in such diverse processes as cell growth control, intracellular trafficking, and cytoskeletal assembly (Kapeller and Cantley, 1994); however, mechanisms linking its products, the 3-phosphorylated phosphatidylinositols, to these responses are unknown. Recent evidence raised the novel possibility that PI 3-kinase may regulate cellular activities via an alternative role as a protein serine kinase. The 110-kDa subunit is capable of autophosphorylation and it phosphorylates the 85-kDa subunit on serine 608 (Dhand *et al.*, 1994); in addition, the complex has been shown to phosphorylate IRS-1 on serine following insulin stimulation in adipocytes (Lam *et al.*, 1994).

A useful tool in the analysis of PI 3-kinase activity in cells is the fungal toxin wortmannin. This sterol-like compound binds covalently to the 110-kDa catalytic subunit, probably via a mechanism involving nucleophilic substitution, and irreversibly inhibits kinase activity (Baggiolini *et al.*, 1987; Thelen *et al.*, 1994). Nanomolar concentrations of wortmannin specifically inhibit PI 3-kinase (Arcaro and Wymann, 1993; Yano *et al.*, 1993; Powis *et al.*, 1994). Thelen *et al.* (1994) showed that [$^3$H]-wortmannin at 5–20 nM binds covalently to

electrophoresis; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PI 3-kinase, phosphatidylinositol 3-kinase; PLC, phospholipase Cγ; PMA, phorbal myristate acetate; RBL-2H3, the 2H3-secreting subline of rat basophilic leukemia (RBL) cells.

a single protein species, the 110-kDa catalytic subunit of PI 3-kinase. Nakanishi *et al.* (1992) further demonstrated that micromolar concentrations of wortmannin are required to inhibit myosin light chain kinase ($IC_{50}$ of 1.9 μM) and that protein kinase C, cAMP-dependent protein kinase, cGMP-dependent protein kinase, and calmodulin-dependent protein kinase II are minimally affected by wortmannin concentrations as high as 10 μM. Confidence in the specificity of this pharmacological agent is strengthened by the work of Kotani *et al.* (1994), who showed that the effects of wortmannin upon insulin- or IGF-1–induced ruffling could be reproduced by microinjection of a mutant p85-kDa subunit of PI 3-kinase.

Previous studies demonstrated that PI 3-kinase is necessary for optimal secretion from RBL-2H3 cells (Yano *et al.*, 1993) and human basophils (Knol *et al.*, 1991). In the present study, wortmannin was used to investigate the role of PI 3-kinase in the regulation of additional signaling responses activated by FcεR1 cross-linking. We report that PI 3-kinase is critical for $Ins(1,4,5)P_3$ production, membrane ruffling, and pinocytosis. However, MAP kinase activation as well as antigen-induced spreading, assembly of actin plaques, and receptor internalization are unaffected by wortmannin treatment. Importantly, we show that PI 3-kinase may support functional responses in part by acting as a dual specificity enzyme in mast cells, because p85 immune complexes contain wortmannin-sensitive protein kinase activity.

## MATERIALS AND METHODS

### Cell Culture

RBL-2H3 cells were cultured on tissue culture grade plastic in minimal essential medium (MEM; Life Technologies, Grand Island, NY) with 15% fetal bovine serum (HyClone, Logan, UT) as described (Pfeiffer *et al.*, 1985; Wilson *et al.*, 1989). Experimental cells were incubated overnight with 1 μg/ml of monoclonal anti-dinitrophenol IgE (anti-DNP IgE; Liu *et al.*, 1980) to saturate the receptors and washed immediately before use with modified Hanks' buffered saline (Becker, 1972) containing 0.1% bovine serum albumin (Hanks'-BSA) to remove excess IgE. In experiments with adherent cultures, cells were plated on tissue grade plastic or on clean glass coverslips 1 to 3 days before use and were activated as adherent cells on the same surfaces. In experiments with suspension cultures, cells were plated onto suspension grade plastic and harvested by gentle scraping on the day of the experiment.

### Microscopy

To visualize actin by fluorescence microscopy, anti-DNP IgE-primed cells on glass coverslips were incubated for 15 min at 37°C in Hanks'-BSA buffer plus or minus wortmannin followed by activation with either 0.1 μg/ml DNP-BSA or 25 nM phorbal myristate acetate (PMA) for indicated times. Cells were fixed and stained as described in Pfeiffer and Oliver (1994) by a 30-min incubation with 2% paraformaldehyde plus 0.02% saponin and 4 U/ml of rhodamine phalloidin (Molecular Probes, Eugene, OR). The coverslips were washed in phosphate-buffered saline (PBS), mounted on slides, and photographed using a Zeiss Photomicroscope III equipped for epifluorescence microscopy. For electron microscopy,

monolayers of anti-DNP IgE-primed cells were incubated in Hanks'-BSA buffer with and without wortmannin for 15 min at 37°C. The cells were then activated by addition of DNP-BSA (0.1 $\mu$g/ml) or 25 nM PMA and incubated for 10 min at 37°C. The cells were fixed in 2% glutaraldehyde and processed as previously described (Pfeiffer *et al.*, 1985) for scanning electron microscopy.

### *Fluid-Phase Pinocytosis*

Suspension cultures of anti-DNP IgE–primed RBL-2H3 cells were harvested and resuspended in fresh MEM medium, 15% fetal bovine serum and incubated for 15 min at 37°C in the presence and absence of 10 nM wortmannin. FITC-dextran (1 mg/ml; Molecular Probes) and DNP-BSA (0.1 $\mu$g/ml) were added to cells, which were then incubated at 37°C for 0, 2, 5, or 10 min. Cells were washed twice with PBS and fixed by addition of 2% paraformaldeyde. Following 30 min of fixation at room temperature, the cells were washed twice and resuspended in 1 ml of PBS. Uptake of FITC-dextran was measured by mean channel detection of at least 10,000 cells using a Coulter EPICS Elite flow cytometer (Hialeah, FL), as described by Pfeiffer *et al.* (1985).

### *Internalization of Cross-linked Receptors*

Suspension cultures were primed for 1 h with FITC-conjugated anti-DNP IgE, harvested, and resuspended in fresh MEM ± wortmannin as above. Cells were then stimulated for indicated intervals with 0.1 $\mu$g/ml DNP-BSA, and reactions were stopped by the addition of 2% paraformaldehyde. Fixation was continued for 10 min at room temperature, followed by washing and incubation with and without anti-FITC antibody (a gift of Dr. Larry Sklar, UNM) that selectively quenches cell surface-associated FITC-anti-IgE. Samples were brought up to 0.5 ml in PBS, without washing, and mean fluorescence intensity of at least 10,000 cells was measured using a Coulter EPICS Elite flow cytometer. The rate of endocytosis was determined from the recovery of fluorescence in the anti-FITC–treated samples (Sklar *et al.*, 1981).

### *Quantitation of F-Actin*

IgE-primed cells were harvested from suspension culture dishes, resuspended in Hanks'-BSA at $10^6$/ml, and stirred in 12 × 75 mm polypropylene tubes at 37°C for 2–3 min before 25-$\mu$l pre-stimulus samples were collected. Cells were then stimulated while stirring with 0.1 $\mu$g/ml DNP-BSA and 25-$\mu$l samples were collected in triplicate at times indicated. Cell samples were added directly to 25 $\mu$l fixative solution (7.4% EM-grade formaldehyde in Hanks'-BSA minus calcium and magnesium) and fixation was continued for 10–20 min at room temperature. Cells were then simultaneously permeabilized and stained for F-actin by a 40- to 80-min incubation at room temperature with 3.7% formaldehyde, 0.1 mg/ml lysophosphatidylcholine, and 20 $\mu$M FITC-phalloidin (Sigma, St. Louis, MO). Samples were diluted with 3.7% formaldehyde in Hanks'-BSA, the FL1 emission was measured on a Becton Dickinson FACScan flow cytometer (Mountain View, CA) for 3000–5000 events per sample, and data were analyzed using Cyclops software.

### *Ins(1,4,5)$P_3$ Assays*

Ins(1,4,5)$P_3$ levels were determined using a radioreceptor assay modified from Challiss *et al.* (1988) as previously described (Deanin *et al.*, 1991b). For each measurement, 2 × $10^7$ primed cells were suspended in 0.75 ml of Hanks'-BSA medium and activated for specific intervals at 37°C with 0.1 $\mu$g/ml DNP-BSA. Reactions were stopped by the addition of an equal amount of 16% trichloroacetic acid. Samples were held on ice for 15 min and then spun in a microcentrifuge. The protein pellets were dissolved in 2 ml of 0.15 N NaOH for protein measurements, and the supernatant fractions, containing inositol phosphates, were extracted with $H_2O$-saturated ether to remove trichloroacetic acid. The pH of the extracts was adjusted to 6.5 with $NaHCO_3$ and EDTA was added to a final concentration of 5 mM. The amount of Ins(1,4,5)$P_3$ present in the extract was determined from its ability to compete with [$^3$H]Ins(1,4,5)$P_3$ (3000 cpm) for specific binding sites on bovine cerebellar membrane preparations. A standard curve was used to convert % inhibition of [$^3$H]Ins(1,4,5)$P_3$ binding to picomoles of Ins(1,4,5)$P_3$ in the extract.

### *Immune Complex Kinase Assays*

Anti-DNP IgE-primed RBL-2H3 cells were collected from suspension culture dishes, washed, and suspended to a concentration of 8 × $10^6$ cells/ml in Hanks'-BSA. Aliquots (0.5 ml) were dispensed into tubes containing DNP-BSA (0.1 $\mu$g/ml) and incubated at 37°C for various times. Reactions were terminated by transfer to an ice bath and the addition of 0.5 ml ice-cold PBS. Cells were collected by 5 s of centrifugation, the supernatants were discarded, and the cell pellets were lysed with 1.0 ml of ice-cold lysis buffer A (1% Brij in 50 mM Tris, 150 mM NaCl, 1 mM $NaVO_4$, pH 7.4, plus 1 $\mu$g antipain/ml and 1 $\mu$g leupeptin/ml); for MAP kinase assays, an alternative lysis buffer B was used (25 mM Tris, 140 mM NaCl, 1% NP-40, 10 mM NaF, 10 mM $NaP_2O_7$, 2 mM $NaVO_4$, 400 $\mu$M EDTA, 1 mM phenylmethylsulfonyl sulfate, and 2 $\mu$g/ml each of leupeptin, antipain, and aprotinin, pH 7.4). Insoluble material was removed by microcentrifugation and the supernatants were rocked for 1 h at 4°C with 30 $\mu$l of protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) that had been preincubated with one of the following: 1) anti-Lyn antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); 2) anti-Syk antibodies (provided by Dr. R. Geahlen, Purdue University); 3) antibodies to the p85 subunit of PI 3-kinase (anti-PI 3-K, lot 2; Transduction Labs, Lexington, KT); 4) antibodies to the $\beta$-subunit of the Fc$\epsilon$R1 receptor (provided by Dr. Juan Rivera, National Institutes of Health, Bethesda, MD); or 5) monoclonal antibody SC-14 (Santa Cruz Biotech) that recognizes both the ERK-1 and ERK-2 species of MAP kinase. Immunoprecipitates were washed three times with 1 ml of lysis buffer A containing 0.1% Brij 96, followed by two washes with lysis buffer without Brij 96. Exceptions were MAP kinase immunoprecipitates, where lysis buffer B was used for washes.

For protein kinase assays (except MAP kinase), immunoprecipitates were washed once with 200 $\mu$l of kinase buffer A (25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES], pH 7.5, containing 10 mM $MnCl_2$). Phosphorylation reactions were initiated by the addition of 40 $\mu$l of kinase buffer with 10 $\mu$Ci of [$\gamma^{32}$P]ATP (Amersham, Arlington Heights, IL; 3000 Ci/mmol) to the immune complexes and transfer to a 30°C heat block for 2 min (for Lyn and Syk) or for 5 min (for PI 3-kinase); in some cases 10 nM wortmannin was added directly to the kinase reaction. Precipitates were washed three times with 1 ml of ice-cold kinase buffer, followed by the addition of 40 $\mu$l of Laemmli sample buffer containing 5% 2-mercaptoethanol. Proteins were separated by SDS-PAGE using 10% linear or 5–15% gradient gels; $^{32}$P-phosphoproteins were identified by autoradiography of dried gels.

For MAP kinase assays, washed immunoprecipitates were resuspended in kinase buffer B (25 mM HEPES, 140 mM NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 400 uM EDTA, pH 7.8) containing 1.5 mg/ml myelin basic protein, and 225 $\mu$Ci/ml [$\gamma^{32}$P]ATP. Kinase reactions were carried out at 30°C for 20 min. Reactions were terminated by addition of Laemmli sample buffer, followed by immediate incubation at 98°C for 10 min, electrophoresis using 15% SDS-PAGE, and autoradiography of dried gels.

## RESULTS

In initial experiments, we confirmed the effects of wortmannin on Fc$\epsilon$R1-mediated responses, as previously reported by Kitani *et al.* (1992) and Yano *et al.* (1993). Using PtdIns as a substrate, we found that PI 3-kinase activity measured in anti-p85 immune com-

plex kinase assays is nearly doubled within 4 min of antigen stimulation. Wortmannin (10 nM) pretreatment blocked both basal PI 3-kinase activity and the increase in activity that accompanies FcϵR1 activation by ~80%. Similarly, pretreatment of cells with 10 nM wortmannin resulted in ~80% inhibition of $^3$H secretion in response to antigen. Like Kitani *et al.* (1992), we found that FcϵR1-activated increases in $[Ca^{2+}]_i$ were only modestly affected by wortmannin (approximately 20% reduction in the overall $[Ca^{2+}]_i$ response to antigen stimulation, with a peak value of approximately 680 nM and sustained $Ca^{2+}$ elevations above 300 nM).

### *Wortmannin Reduces Antigen-induced IP$_3$ Synthesis*

The synthesis of Ins(1,4,5)$P_3$ is required for $Ca^{2+}$ stores release and thus is a critical event in the FcϵR1-signaling pathway. We used a competitive radioligand receptor binding assay to specifically measure the 1,4,5 isomer of Ins$P_3$ present in cell lysates prepared from untreated and wortmannin-treated cells following FcϵR1 activation. As shown in Figure 1, wortmannin pretreatment causes up to 70% reduction in the antigen-induced increase in Ins(1,4,5)$P_3$ production when measured at 2 min post-stimulation and up to 50% reduction at 5 min post-stimulation. Ins(1,4,5)$P_3$-mediated $Ca^{2+}$ stores release is required for $Ca^{2+}$ influx mediated by the capacitative pathway (Fasolato *et al.*, 1993; Lee and Oliver, 1995). Thus, the reduced production of Ins(1,4,5)$P_3$ may contribute to the modest effects of wortmannin on $Ca^{2+}$ responses, by lowering both Ins(1,4,5)$P_3$-dependent $Ca^{2+}$ stores release and the closely coupled capacitative influx pathway.

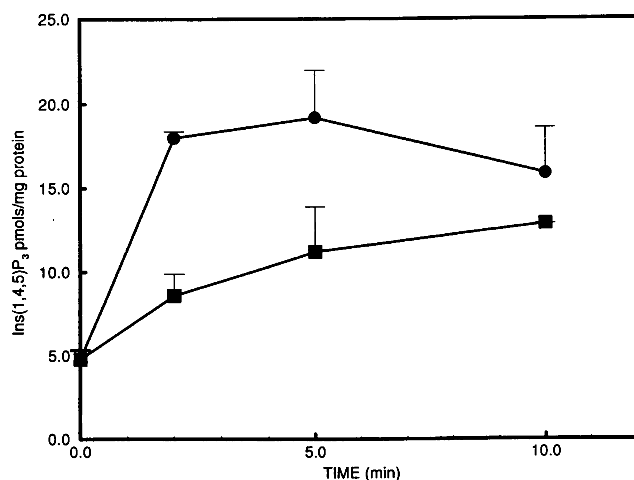


**Figure 1.** Antigen-induced Ins(1,4,5)$P_3$ production is inhibited by wortmannin. RBL-2H3 cells were primed with anti-DNP IgE. Control cells (●) or wortmannin-treated cells (■) were activated with DNP-BSA for indicated times. Ins(1,4,5)$P_3$ levels were measured in cell extracts by a radioreceptor assay as described in MATERIALS AND METHODS. Data are the averages of duplicates in a representative experiment.

### *Wortmannin Blocks Antigen-induced Membrane Ruffling, but not Spreading and Actin Plaque Assembly*

Previous work has demonstrated that FcϵR1 cross-linking stimulates the polymerization of actin (Pfeiffer *et al.*, 1985) and transforms the cell surface from a microvillous to lamellar architecture (illustrated in Figure 2, A and C). In adherent cells, FcϵR1 cross-linking also supports increased cell spreading and the formation of actin-rich adhesion structures, termed actin plaques (Pfeiffer and Oliver, 1994; illustrated in Figure 3B). Wortmannin pretreatment does not alter the normal microvillous surface of the unstimulated cell (Figure 2B). However, it prevents the membrane ruffling response to antigen (Figure 2D). To test the possibility that wortmannin renders the cell incapable of ruffling, both untreated and wortmannin-treated cells were stimulated with PMA, shown previously to mimic the antigen-induced ruffling response. Ruffling responses to PMA occur in both control and wortmannin-treated cells (Figure 2, compare E and F), although approximately 25% of the wortmannin-treated cells show a modified response to PMA that resulted in a "capped" appearance (arrow in Figure 2F) or in immature ruffles.

In contrast to the marked inhibition of FcϵR1-activated surface ruffling, wortmannin fails to alter the cytoskeletal responses associated with increased cell adhesion. Neither cell spreading (Figure 2D) nor the formation of actin plaques at the ventral surface (Figure 3C) in response to antigen are blocked by wortmannin. In addition, wortmannin treatment fails to inhibit the formation of actin stress fibers induced by PMA (Figure 3, D and E). Thus wortmannin selectively blocks a subset of actin-based responses linking FcϵR1 activation to membrane ruffling in RBL-2H3 cells.

### *Wortmannin Reduces Antigen-induced Pinocytosis*

Ridley (1994) has suggested that pinocytosis and membrane ruffling are tightly coupled membrane processes. We used FITC-dextran uptake as a marker for fluid-phase endocytosis (also referred to as *macropinocytosis*) and measured its accumulation in untreated and wortmannin-treated cells by flow cytometry after stimulus of IgE-primed RBL-2H3 cells with DNP-BSA. Results of a typical experiment are shown in Figure 4. As previously shown (Pfeiffer *et al.*, 1985; Wilson *et al.*, 1989), an increase in the rate of FITC-dextran uptake is a rapid and sustained response to FcϵR1 activation (compare open and closed circles). Wortmannin reduces the uptake of FITC-dextran in antigen-stimu-

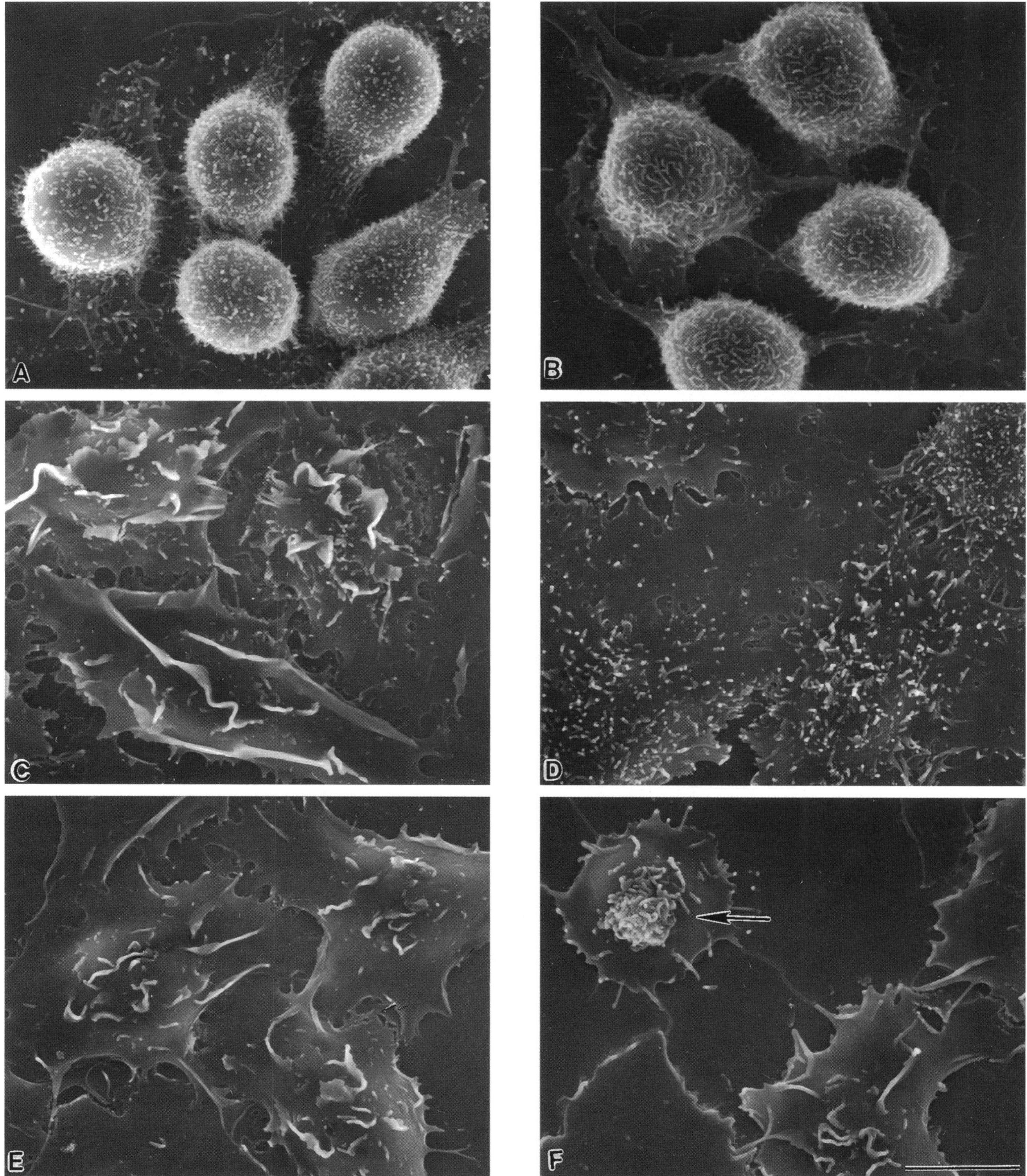


**Figure 2.** Wortmannin abolishes receptor-activated ruffling responses, but does not affect cell spreading (adhesion) responses. IgE-primed RBL-2H3 cells were cultured on glass coverslips. Cells shown in the right panels (B, D, and F) were treated with 10 nM wortmannin. (A and B) Cells were rinsed in Hanks'-BSA medium and held in buffer for 10 min at 37°C. (C and D) Cells were activated for 10 min at 37°C with DNP-BSA. (E and F) Cells were activated with PMA for 10 min at 37°C. Samples were fixed and processed for scanning electron microscopy. Bar, 10 $\mu$m.

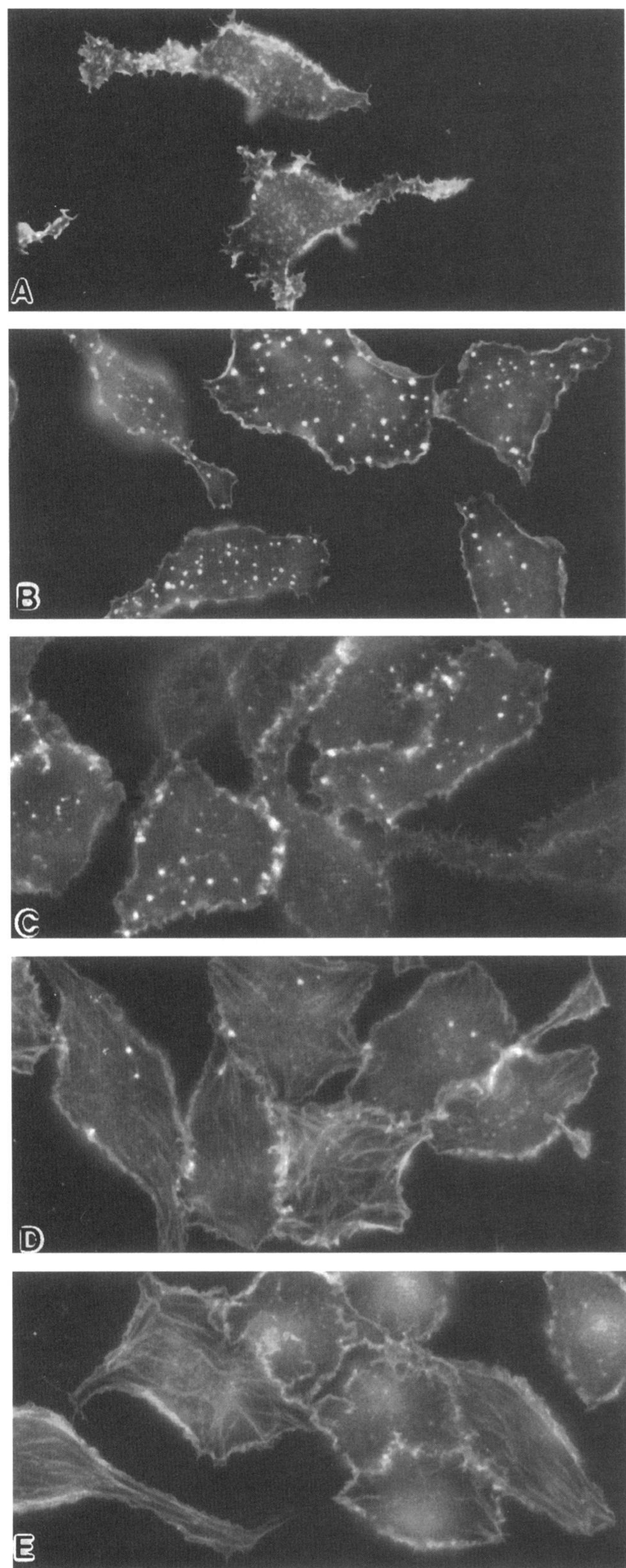


**Figure 3.** Wortmannin does not block antigen-induced actin plaque assembly or PMA-induced stress fiber assembly. RBL-2H3 cells were activated as in Figure 5, followed by fixation and staining

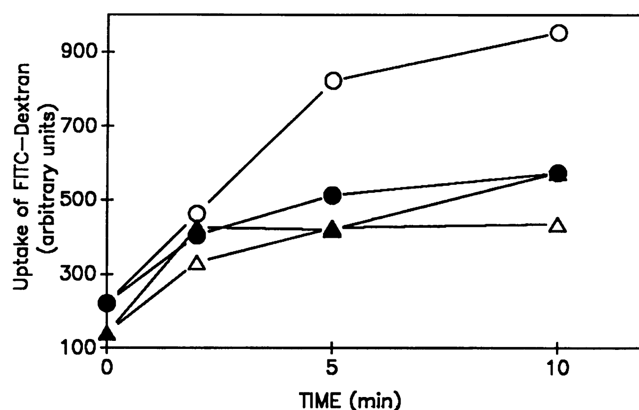


**Figure 4.** Antigen-induced pinocytosis is inhibited by wortmannin. FITC-dextran (1 mg/ml) was added to the following: control cells (●), wortmannin-treated cells (▲), control cells activated with DNP-BSA (○), and wortmannin-treated cells activated with DNP-BSA (△). RBL-2H3 cells were incubated for 0, 2, 5, or 10 min at 37°C, fixed with 2% paraformaldehyde, and internalized FITC-dextran was quantified by flow cytometry as described in MATERIALS AND METHODS. Results are representative of three separate experiments.

lated cells (open triangles) to levels comparable to unstimulated cells (solid circles).

### *Wortmannin Does Not Inhibit the Endocytosis of Cross-linked FcεR1 Receptors*

Previous work has shown that cross-linking of high affinity IgE receptors induces internalization via clathrin-coated vesicles (Pfeiffer *et al.*, 1985; Mao *et al.*, 1993). Because the bulk of fluid-phase endocytosis is expected to occur via a mechanism different from that of antigen-induced receptor internalization, we measured the specific internalization of cross-linked FcεR1 by flow cytometry. FcεR1 were primed with FITC-conjugated IgE and cross-linked with 0.1 μg/ml DNP-BSA. At indicated times, cells were fixed, washed, incubated with anti-FITC antibody that quenches cell surface-associated FITC, and their fluorescence intensity was measured by flow cytometry. In the absence of anti-FITC, cells show a high initial fluorescence intensity that decreases somewhat after 15 min of cross-linking. This small decrease is likely to reflect a

**Figure 3 cont.** for actin with rhodamine-phalloidin. Micrographs represent the following: (A) resting RBL-2H3 cells, which have low levels of F-actin; (B) RBL-2H3 cells following 10 min of activation with DNP-BSA; and (C) wortmannin-treated cells following 10 min of activation with DNP-BSA. Arrows point to specialized adhesion structures, termed actin plaques, that form in both control (B) and wortmannin-treated cells (C) following antigen stimulation. (E and F) Cells were activated with PMA, which results in stress fiber formation. Wortmannin treatment (F) does not affect this response to PMA.

combination of events: the slow dissociation of FITC-IgE from surface receptors; a small quenching of FITC-IgE fluorescence by DNP-BSA; and fluorescence resonance energy transfer between FITC-IgE molecules following their concentration in intracellular vesicles. Anti-FITC causes a large decrease in initial fluorescence intensity, due to the strong quenching fluorescence emission from surface-bound FITC-IgE. Antigen binding stimulates the progressive internalization of cross-linked receptors into vesicles that are inaccessible to anti-FITC. This is detected in cells treated after fixation with anti-FITC as a steady increase in fluorescence intensity with increasing times of incubation with antigen. As shown in Table 1, control and wortmannin-pretreated cells internalize the same proportion of surface FcεR1 receptors in 15 min under this protocol, indicating that PI 3-kinase does not play a significant role in the internalization of cross-linked receptors.

### *Wortmannin Does Not Inhibit Actin Polymerization Induced by FcεR1 Cross-linking*

To address the possibility that inhibition of membrane ruffling and pinocytosis was linked to a general lack of actin polymerization in wortmannin-treated cells, we measured total F-actin content by flow cytometry. In this method, cells are stimulated for designated times, then fixed, permeabilized, and incubated with FITC-phalloidin that specifically binds F-actin. In control cells stimulated with 0.1 μg/ml DNP-BSA, there is a rapid rise in F-actin that is typically followed by a transient drop before F-actin levels rise again to a plateau level at 4–5 min post-activation (Figure 5, open symbols). This transient drop in F-actin levels may be due to cortical actin disassembly that parallels an overall reorganization of the actin cytoskeleton in activated cells (Pfeiffer *et al.*, 1985). Also shown in Figure 5 (closed symbols), wortmannin

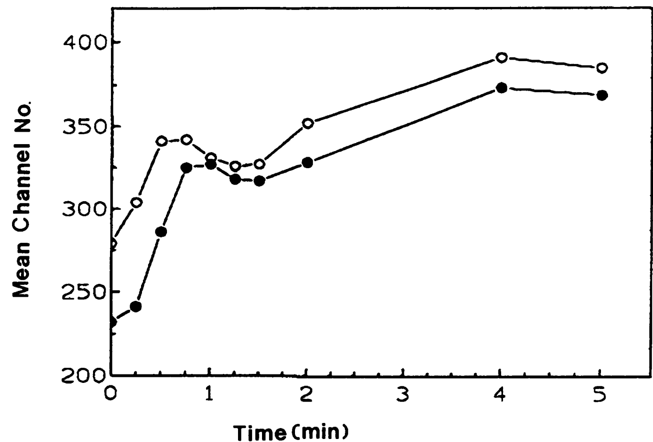


**Figure 5.** Wortmannin reduces the basal level of F-actin but does not inhibit antigen-stimulated actin polymerization. Control cells (○) or wortmannin-treated cells (●) were activated with DNP-BSA at 37°C as described in MATERIALS AND METHODS. At the indicated times after antigen addition, aliquots were removed, fixed, permeabilized, and incubated with FITC-phalloidin. F-actin content was quantified by flow cytometry and plotted as mean channel fluorescence. Results are representative of six separate experiments.

pretreatment results in a consistent decrease in the pre-stimulus levels of F-actin, as reflected by the lower mean at time zero; this represents a 21.1% (± 5.5) decrease in total F-actin. However, upon stimulation with DNP-BSA, wortmannin-treated cells showed a rapid rise in F-actin assembly. The rate and extent of this increase in total F-actin is not substantially different between control and wortmannin-treated cells.

**Table 1.** Endocytosis of cross-linked FcεR1 is not blocked by wortmannin

| | Control | Wortmannin |
|---|---|---|
| Autofluorescence | 0.5 | — |
| 0′ (unquenched) | 37.0 | 36.3 |
| 15′ (unquenched) | 28.3 | 27.0 |
| 0′ (quenched) | 7.4 | 7.7 |
| 5′ (quenched) | 15.2 | 14.3 |
| 15′ (quenched) | 18.6 | 18.0 |

RBL-2H3 cells were primed with FITC-conjugated anti-DNP IgE for 1 h at 37° C. Cells were activated with DNP-BSA for indicated times and fixed as described in MATERIALS AND METHODS. Endocytosis was measured by quenching of cell surface FITC fluorescence with anti-FITC antibodies and quantitation by flow cytometry. Data are presented as mean channel number for 10,000 events.

### *Wortmannin Does Not Inhibit the Antigen-induced Activation of Lyn, Syk, or MAP Kinases*

The activation of cytoplasmic tyrosine kinases, principally the Src-related kinase, Lyn, and the 72-kDa Syk kinase, are critical early responses to FcεR1 activation (Eiseman and Bolen, 1992; Hutchcroft *et al.*, 1992). Their importance is underscored by evidence from several independent laboratories that tyrosine kinase inhibitors block essentially all measurable responses to receptor activation (Deanin *et al.*, 1991a; Benhamou and Siraganian, 1992; Oliver *et al.*, 1994). Thus it was important to demonstrate that wortmannin is not mediating its effects via inhibition of these enzymes. Here, anti-kinase immune complexes were used as a source of active kinases for in vitro phosphorylation assays.

As reported previously (Oliver *et al.*, 1994), Lyn immunoprecipitates prepared from both resting and activated cells are capable of Lyn autophosphorylation (Figure 6A, lanes 1 and 2). Lyn autophosphorylation is not changed when immune complexes are prepared

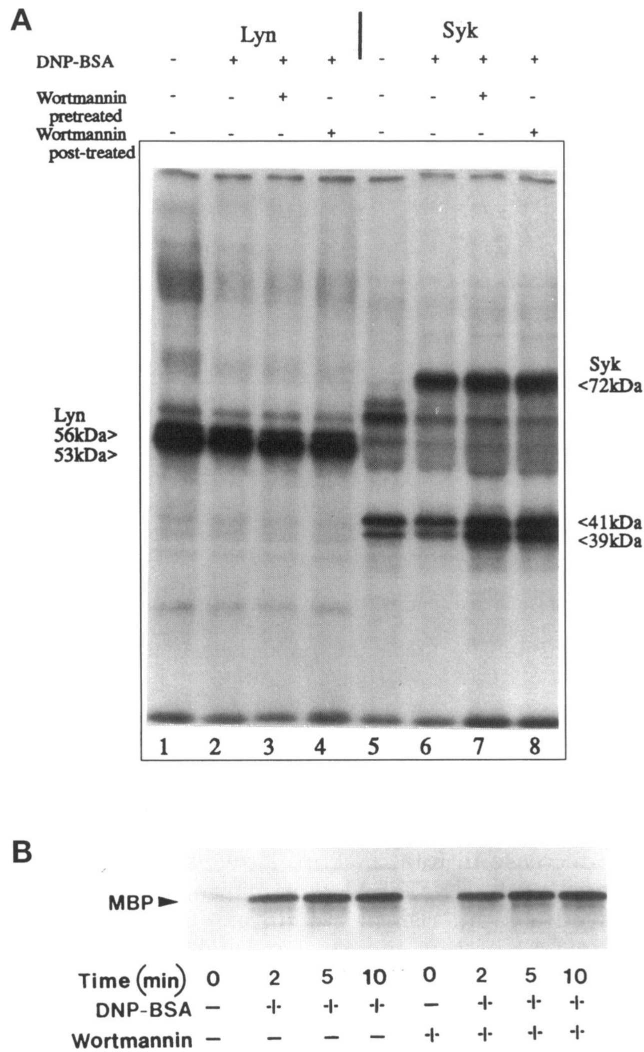


**Figure 6.** Wortmannin does not affect Lyn, Syk, or MAP kinases. IgE-primed RBL-2H3 cells were lysed with detergent and their supernatants were immunoprecipitated with anti-Lyn, anti-Syk, or anti Erk1/2 antibodies. Activation with 0.1 $\mu$g/ml DNP-BSA or 10 nM wortmannin pretreatment is indicated in top legends. (A) Kinase activities in Lyn (lanes 1–4) and Syk (lanes 5–8) immunoprecipitates are indicated by autophosphorylation as described in MATERIALS AND METHODS. At left, arrowheads point to the 53- and 56-kDa isoforms of Lyn. At right, arrowheads identify Syk and 31-kDa and 41-kDa unknown phosphoproteins. In lanes 4 and 8, wortmannin was added directly to the immune complex kinase reaction to establish insensitivity to the inhibitor. (B) MAP kinase activity in Erk 1/2 immunoprecipitates was measured using myelin basic protein as an exogenous substrate, as described in MATERIALS AND METHODS. Results are representative of three (A) or two (B) replicate assays.

from antigen-activated RBL-2H3 cells pretreated with wortmannin or when wortmannin is directly added to Lyn immune complexes (Figure 6A, compare lanes 3 and 4 with lane 2). Consistent with Yano *et al.* (1993), these results indicate that Lyn kinase is not a target for inactivation by wortmannin.

Immunoprecipitated Syk is only capable of autophosphorylation when immune complexes are prepared from antigen-activated cells (compare Figure 6A, lanes 5 and 6). The results in Figure 6A, lane 7 show that Fc$\epsilon$R1-mediated activation of Syk is unaffected by wortmannin pretreatment of cells before activation and cell lysis. Moreover, the direct addition of wortmannin to activated Syk immune complexes also fails to affect Syk autophosphorylation activity (Figure 6A, lane 7). The significance of the apparent wortmannin-induced increase in phosphorylation of two proteins at ~39 and 41 kDa that co-precipitate with Syk (Figure 6A, lanes 7 and 8) is unknown.

Recently, several groups have reported that Fc$\epsilon$R1 cross-linking activates MAP kinase in RBL-2H3 cells and have shown that nonselective protein tyrosine kinase inhibitors block this response (Santini and Beaven, 1993; Offerman *et al.*, 1994). The results in Figure 6B confirm that MAP kinase is activated by a time-dependent process in antigen-stimulated cells. They also show that antigen-induced MAP kinase activation is not inhibited by wortmannin. This identifies an additional pathway that is independent of PI 3-kinase activity.

### *Anti-p85 Immune Complexes Contain Antigen-activated Wortmannin-sensitive Serine/Threonine Kinase Activity*

The recent report (Dhand *et al.*, 1994) that PI 3-kinase is capable of autophosphorylation on serine residues prompted us to assay for protein kinase activity in anti-p85 immune complexes. Anti-p85 immune complexes were prepared from the following: 1) resting cells, 2) cells activated for 2 min with DNP-BSA, and 3) cells pretreated with wortmannin, followed by 2 min of activation with DNP-BSA and incubated with 10 $\mu$Ci [$^{32}$P]ATP in 10 mM $MnCl_2$-containing HEPES buffer. As shown in Figure 7, a small amount of protein kinase activity is present in p85 immune complexes isolated from resting cells (Figure 7, lane 1). Because phosphorylation of these proteins is unaffected by wortmannin (Figure 7, lanes 3 and 4) we attribute it to unidentified kinase(s) that coprecipitate with p85. Antigen stimulation results in intense phosphorylation of multiple proteins associated with the immune complex (Figure 7, lane 2), including a major band at ~105-kDa that most likely corresponds to the catalytic 110-kDa subunit of PI 3-kinase. Gradient (5–15%) gel analysis of similar experiments revealed that a minimum of 10 proteins are phosphorylated in p85 immune complexes isolated from activated RBL-2H3 cells (our unpublished result). This dramatic increase in protein kinase activity does not occur when cells are treated with wortmannin before antigen stimulation (Figure 7, lane 3) or when wortmannin is added directly to immune complexes prepared from cells acti-

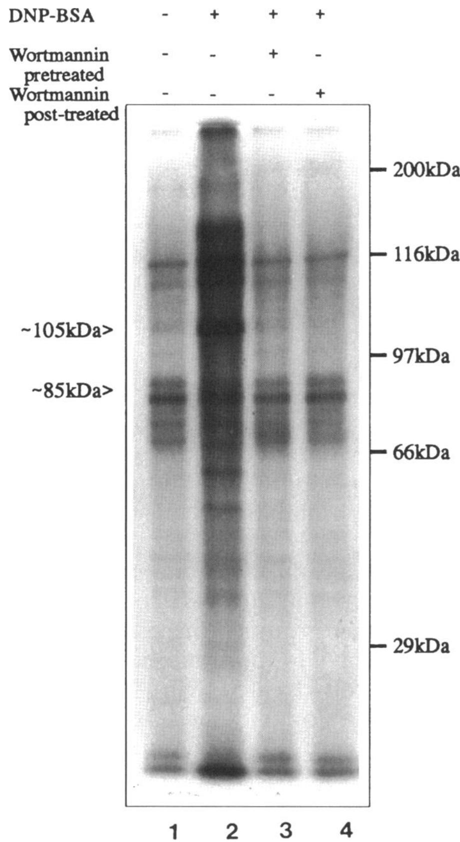


**Figure 7.** Anti-p85 immune complexes contain wortmannin-sensitive protein kinase activity. IgE-primed RBL-2H3 cells were lysed and their supernatants immunoprecipitated with anti-p85 antibodies. Activation with DNP-BSA in the absence or presence of wortmannin is indicated in top legend. The electrophoretic mobilities corresponding to proteins of ~85-kDa and ~110-kDa in lane 2 are marked with arrows.

vated in the absence of wortmannin (Figure 7, lane 4). The kinase activity found in p85 immune complexes is serine/threonine-specific, because it is completely removed by alkaline treatment (1 h at 60°C in 1 N KOH; our unpublished observation). Together, these results suggest that serine/threonine kinase activity associated with or intrinsic to PI 3-kinase is activated by FcεR1 cross-linking and renders it capable of phosphorylating a large number of associated proteins.

### *Protein Kinase Activity Associated with FcεR1 β Subunit Immune Complexes Increases upon Cross-linking and Is Inhibited in Wortmannin-treated Cells*

To determine whether PI 3-kinase associates with the FcεR1, in vitro kinase assays were performed using immunoprecipitates prepared with antibodies to the receptor β subunit. When anti-β immune complexes were incubated with [$^{32}$P]ATP in the absence of receptor activation, there is a low level of associated kinase activity (Figure 8, lane 1), resulting in the phosphorylation of 10–12 proteins, including species previously identified as the FcεR1 β and γ subunits and the tyrosine kinases Lyn and Syk (Hutchcroft *et al.*, 1992; Li *et al.*, 1992). Phosphorylation of these and other proteins is enhanced if receptors are precipitated from cells after 2 min of antigen stimulation (Figure 8, lane 2). When receptors are precipitated from cells pretreated with wortmannin before activation, there is a significant decrease in protein phosphorylation in the anti-β immune complex kinase assay. The most marked differences are in receptor-associated proteins, but levels of phosphorylation of the FcεR1 β and γ subunits that were shown previously to depend on Lyn-mediated tyrosine phosphorylation (Oliver *et al.*, 1994) are also reduced.

In similar unpublished experiments we added wortmannin directly to FcεR1 β immune complexes prepared from activated RBL-2H3 cells. Under these conditions, the inhibitor failed to modify the phosphorylation of receptor subunits or its associated pro-

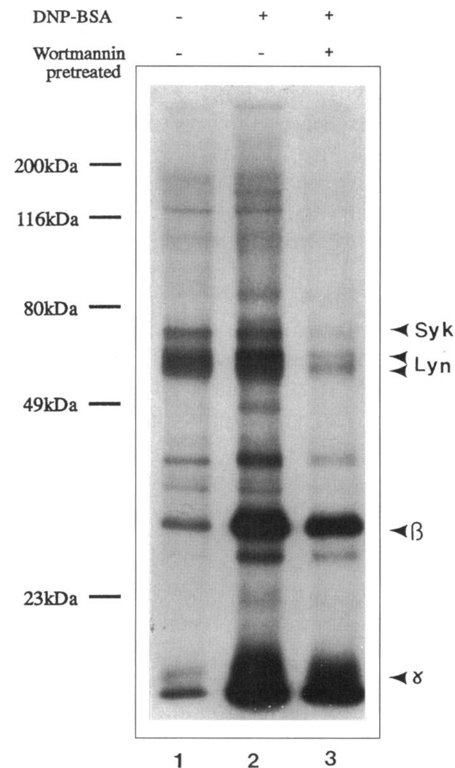


**Figure 8.** Pretreatment of RBL-2H3 cells with wortmannin modifies the pattern of in vitro protein phosphorylation in FcεR1 immune complexes. IgE-primed RBL-2H3 cells were lysed and their supernatants immunoprecipitated with anti-FcεR1-β subunit antibodies. Activation with DNP-BSA in the absence or presence of wortmannin is indicated in the top legend. Arrows identify the phosphorylated β and γ subunits of the FcεR1 receptor and Lyn and Syk tyrosine kinases, identified by their electrophoretic mobility.

teins. This does not exclude the possibility that PI3-kinase associates transiently with FcεR1 and thereby influences the stable association of other cytoplasmic kinases with the FcεR1 as implied by Figure 8.

## DISCUSSION

The data reported here show that treating RBL-2H3 cells with wortmannin inhibits both lipid and protein kinase activities of PI 3-kinase and reduces several key responses to FcεR1 cross-linking, including secretion, membrane ruffling, fluid-phase endocytosis, and Ins(1,4,5)$P_3$ production. In agreement with Kitani *et al.* (1992), the inhibition of receptor-mediated secretion does not appear to be directly related to reduction in $[Ca^{2+}]_i$, because the overall elevation of $[Ca^{2+}]$ is only mildly affected by wortmannin.

Wortmannin is the first reagent to dissociate the dorsal ruffling response to FcεR1 cross-linking from the spreading and actin plaque assembly responses that occur at the ventral surface. Indeed, the only previous evidence that these processes may be regulated independently comes from studies in PMA-treated cells, where cell spreading and ruffling proceed in the absence of actin plaque assembly (Pfeiffer and Oliver, 1994). Both dorsal and ventral responses rely on F-actin. Although basal F-actin levels are lower in wortmannin-treated than in control cells, we show here that the rate and extent of antigen-induced actin polymerization are similar between treated and untreated cells. Thus the block to ruffling in wortmannin-treated cells is not linked to a general lack of actin polymerization. The simplest explanation is that PI 3-kinase activity is specifically required for phosphorylation of a membrane-associated substrate that provides actin binding sites for ruffle formation. However, there is recent evidence that wortmannin blocks platelet-derived growth factor (PDGF)- and insulin-stimulated ruffling in fibroblasts but fails to inhibit ruffling induced by microinjection of recombinant V12rac1 protein (Nobes *et al.*, 1995). This suggests that PI 3-kinase is upstream of the ras-like GTPase, rac, in the pathway leading from growth factor receptor tyrosine kinases to ruffling. A similar mechanism may be evoked via the FcεR1 tyrosine kinase signaling cascade, because previous evidence showed that GTPγS elicits ruffling when introduced to permeabilized RBL-2H3 cells (Deanin *et al.*, 1991b). It remains to be established whether rac activation is dependent upon either the lipid kinase or protein kinase activity of this dual specificity enzyme.

It was previously demonstrated that cross-linked FcεR1 are endocytosed via clathrin-coated pits (Pfeiffer *et al.*, 1985). Unlike antigen-stimulated macropinocytosis, which is completely inhibited by wortmannin (Figure 4), we found that receptor endocytosis persists in wortmannin-treated cells (Table 1). This suggests that the antigen-stimulated, nonselective uptake of fluid phase markers like FITC-dextran occurs by a mechanism that is separate from the processes of selective endocytosis that occur via clathrin-coated vesicles. Site-directed mutagenesis of PI 3-kinase binding sites in PDGF receptors resulted in a block of receptor internalization (Joly *et al.*, 1994), leading to the hypothesis that PI 3-kinase, like its close relative VPS34 in yeast (Schu *et al.*, 1993), is required for protein sorting. However, similar studies of hPDGFRb by Mori *et al.* (1994) and of c-*kit* receptors by Yee *et al.* (1994) suggested that PI 3-kinase binding to cytoplasmic tails is not uniformly required for ligand-induced endocytosis. Our studies suggest that, similarly, PI 3-kinase activity is not a requirement for FcεR1 internalization. It does not exclude the possibility that subunits of PI 3-kinase, particularly the p85 adaptor protein, play a noncatalytic (ie., structural) role in endocytosis.

In the present study, antigen-induced Ins(1,4,5)$P_3$ synthesis was substantially reduced by 10 nM wortmannin. Two other groups (Bonser *et al.*, 1991; Nakanishi *et al.*, 1994) have found inhibition of Ins$P_3$ synthesis in cultured cells treated with wortmannin or its close structural analogue demethoxyviridin, although the concentrations of inhibitor required to effectively block Ins$P_3$ production were 10–1000 times that normally needed to inhibit PI 3-kinase. Our result was unexpected because inhibition of PI 3-kinase was anticipated to prevent phosphorylation of PtdIns(4,5)$P_2$ and thus potentially increase the amount of substrate for PLC-mediated Ins(1,4,5)$P_3$ synthesis. However, it is increasingly clear that the phosphoinositides are regulated by a complex interplay of phosphatases and kinases, some of which are tightly regulated. The newest member of receptor-regulated enzymes in this network is a PtdIns(4)P 5-kinase (Chong *et al.*, 1994). The 5-kinase is implicated in PDGF-stimulated calcium mobilization via a pathway that is dependent upon the ras-like GTPase rho, and presumably indirectly supports Ins(1,4,5)$P_3$ production by increasing the rate of PtdIns(4, 5)$P_2$ synthesis. Because there remains an unresolved connection between rho and PI 3-kinase (Kumagai *et al.*, 1993; Zhang *et al.*, 1993), it was conceivable that PI 3-kinase indirectly influences the production of new PtdIns(4, 5)$P_2$ via its association with rho or a closely related signaling molecule. This possibility has been ruled out by thin layer chromatography analysis of phospholipids isolated from $^{32}P$-labeled RBL-2H3 cells, which indicate that PtdIns(4,5)$P_2$ levels are not significantly reduced in wortmannin-treated cells (Barker and Caldwell, unpublished results).

There are at least two other potential mechanisms through which wortmannin could inhibit Ins(1,4,5)$P_3$ synthesis. First, because wortmannin is an irreversible inhibitor of PI 3-kinase that binds outside of the ATP-

binding site (Powis *et al.*, 1994), it is possible that wortmannin-modified PI 3-kinase may still be able to bind its preferred phospholipid substrate PtdIns(4, 5)$P_2$. In the absence of its ability to phosphorylate its substrate, the enzyme may stably associate with PtdIns(4, 5)$P_2$, preventing access by PLC. However, others (Yano *et al.*, 1993; A. Traynor-Kaplan, personal communication) have failed to detect a significant amount of PtdIns(3,4,5)$P_3$ in antigen-stimulated RBL-2H3 cells, as would be expected if PI 3-kinase successfully competed with PLC$\gamma$ for a significant portion of the PtdIns(4, 5)$P_2$ pool. Our own attempts to detect an accumulation of 3-phosphorylated phosphatidylinositols after Fc$\epsilon$R1 activation have been inconclusive. Another potential mechanism of action is suggested by the demonstration of wortmannin-sensitive protein kinase activity in p85 immune complexes (Figure 7). This raises the attractive possibility that PI 3-kinase may influence a subset of mast cell responses, including phosphoinositide metabolism and/or PLC activation, by acting as a serine/threonine protein kinase. It is clear from previous work (Li *et al.*, 1992) that Fc$\epsilon$R1 signaling is accompanied by the increased phosphorylation of receptor-associated proteins on serine and threonine as well as tyrosine. We speculate that signal transduction is determined by phosphorylation on all three amino acids.

This report makes the important observation that PI 3-kinase is a key component of some, but not all, of the signaling responses to antigen activation. Responses that are essentially unaffected in wortmannin-treated cells include the earliest events–activation of Lyn and Syk and $Ca^{2+}$ mobilization–and the later events marked by increases in cell adhesion and MAP kinase activation. This implies that there are several distinct branches of the tyrosine kinase signaling pathway initiated by the activated Fc$\epsilon$R1 and that PI 3-kinase is potentially a relay point at a early step of a major branch leading to ruffling and secretion. Alternatively, because PI 3-kinase is capable of both protein and lipid kinase activity following Fc$\epsilon$R1 cross-linking (Figure 7), it may phosphorylate multiple substrates that independently support such diverse processes as Ins(1,4,5)$P_3$ production, secretion, and ruffling.

How might PI 3-kinase be activated by Fc$\epsilon$R1 cross-linking? One possibility is that PI 3-kinase associates directly with Fc$\epsilon$R1 subunit cytoplasmic tails, as is the case for EGF and PDGF receptors. The complete structure of the heterotrimeric Fc$\epsilon$R1 was defined by Kinet and colleagues (Blank *et al.*, 1987). Analysis of the cytoplasmic tails of the Fc$\epsilon$R1 $\beta$ and $\gamma$ subunits revealed that each contains tyrosine activation motifs (Samelson and Klausner, 1992), now called ITAMs, which mediate coupling to soluble tyrosine kinases (Letourner and Klausner, 1991; Jouvin *et al.*, 1994; Wilson *et al.*, 1995). Although the Fc$\epsilon$R1 ITAMs do not fit the consensus sequence YXXM, previously shown to mediate the cooperative binding of PI 3-kinase's tandem SH2 domains (Cantley *et al.*, 1991; Kazlauskas, *et al.*, 1992; Felder *et al.*, 1993; Songyang *et al.*, 1993), it is known that PI 3-kinase can associate with similar ITAM motifs of the T cell receptor $\zeta$ chain, notably the $\zeta_A$ motif (Extley *et al.*, 1994), and with ITAMs in the Ig-$\alpha$ and Ig-$\beta$ chains (Clark *et al.*, 1992). In addition, recent results in the Cambier laboratory indicate that the p85 subunit of PI 3-kinase binds isolated ITAM motifs of both Fc$\epsilon$R1 $\gamma$ and $\beta$ (S. Johnson and J. Cambier, personal communication).

There are several alternative pathways for PI 3-kinase activation. We show (Figure 6) that the protein tyrosine kinases Lyn and Syk are not directly inhibited by wortmannin, suggesting that PI 3-kinase acts downstream or independently of these two important kinases. Nevertheless, PI 3-kinase may be activated indirectly via binding of p85 to the SH3 domains of other components of the Fc$\epsilon$R1 signaling pathway. For example, src family members Lyn and Fyn kinases were shown to interact with PI 3-kinase in B and T lymphocytes (Pleiman *et al.*, 1993; Prasad *et al.*, 1993; Karnitz *et al.*, 1994). This type of interaction could conceivably modify the ability of macromolecular assemblies to remain stably associated, as suggested by the reduced phosphorylation of Fc$\epsilon$R1-associated proteins when in vitro kinase assays are performed using receptor immune complexes prepared from wortmannin-treated cells (Figure 8).

We are also intrigued by the many parallels we have observed between Fc$\epsilon$R1 signalling and the pathways linked to receptors for insulin and IGF-1. The insulin/IGF-1 receptor uses IRS-1 as a docking platform for PI 3-kinase and other SH2-domain proteins in its tyrosine kinse signaling cascade (Keller and Lienhard, 1994). In this receptor system, PI 3-kinase also regulates membrane ruffling and a specialized form of regulated exocytosis, seen as the delivery of GLUT1- or GLUT4-containing vesicles to the plasma membrane (Clarke *et al.*, 1994; Hara *et al.*, 1994). In preliminary experiments, we have found that PI 3-kinase associates with a heavily phosphorylated 180-kDa protein in RBL-2H3 cells that is similar in size to IRS-1 and we speculate that this molecule may serve a similar function in mast cells.

In summary, the dual function protein and lipid kinase, PI 3-kinase, is activated by Fc$\epsilon$R1 cross-linking downstream of Lyn and Syk activation and mediates a subset of antigen-activated responses. Continued studies are focused on the mechanism by which PI 3-kinase is activated during the signaling cascade following cross-linking of Fc$\epsilon$R1.

## ACKNOWLEDGMENTS

This work was supported by an individual research allocation from American Cancer Society grant IRG192 to B.S.W., by American Cancer Society grant IM675 and National Institutes of Health grant GM-49814 to J.M.O., and by a grant from the Dedicated Health Research Funds of the University of New Mexico School of Medicine to K.K.C. A.H. was supported by National Institutes of Health grant RR-01315 (to L. Sklar). S.A.B. is a Howard Hughes predoctoral fellow. We thank Yehudit Platt, Terry Foutz, and Larry Seamer for technical assistance and Tim Graham for developing the MAP kinase assay. Dr. JeanClare Seagrave of the Lovelace Institutes, Albuquerque, NM, generously made available the PTI spectrofluorimeter. We also thank Drs. Robert Geahlen (Purdue University), Juan Rivera (National Institutes of Health), and Larry Sklar for the kind gifts of anti-Syk, anti-FcεR1-β subunit, and anti-FITC antibodies. The Flow Cytometry and Fluorescence Microscopy facilities are supported by the University of New Mexico Cancer Research and Treatment Center.

## REFERENCES

Arcaro, A., and Wymann, M.P. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-triphosphate in neutrophil responses. Biochem. J. *296*, 297–301.

Baggiolini, M., Dewald, B., Schnyder, J., Ruch, W., Cooper, P.H., and Payne, T.G. (1987). Inhibition of the phagocytosis-induced respiratory burst by the fungal metabolite wortmannin and some analogues. Exp. Cell Res. *169*, 408–418.

Beaven, M.A., and Metzger, H. (1993). Signal transduction by Fc receptors: the FcεR1 case. Immunol. Today *14*, 222–226.

Beaven, M.A., Roger, J., Moore, J.P., Hesketh, T.R., Smith, G.A., and Metcalfe, J.C. (1984). The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. J. Biol. Chem. *259*, 7129–7136.

Becker, E.L. (1972). The relationship of the chemotactic behavior of the complement-derived factors C3A, C5A and C567 and a bacterial chemotactic factor to their ability to activate the proesterase of rabbit polymorphonuclear leukocytes. J. Exp. Med. *135*, 376–387.

Benhamou, M., and Siraganian, R.P. (1992). Protein-tyrosine phosphorylation: an essential component of FcεR1 signalling. Immunol. Today *13*, 195–197.

Blank, U., Ra, C., Miller, L., White, K., Metzger, H., and Kinet, J.-P. (1987). Complete structure and expression in transfected cells of high affinity IgE receptor. Nature *337*, 187–189.

Bonser, R.W., Thompson, N.T., Randall, R.W., Tateson, J.E., Spacey, G.D., Hodson, H.F., and Garland, L.G. (1991). Demethoxyviridin and wortmannin block phospholipase C and D activation in the human neutrophil. Br. J. Pharmacol. *103*, 1237–1241.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. Cell *64*, 281–302.

Chaliss, R.A., Batty, I.Y., and Nahorski, S.R. (1988). Mass measurements of inositol(1,4,5)trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. Biochem. Biophys. Res. Commun. *157*, 684–691.

Chong, L.D., Traynor-Kaplan, A., Bokoch, G.M., and Schwartz, M.A. (1994). The small GTP-binding protein rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell *79*, 507–513.

Clark, M.R., Campbell, K.S., Kazlauskas, A., Johnson, S.A., Hertz, M., Potter, T.A., Pleiman, C., and Cambier, J.C. (1992). The B cell antigen receptor complex: association of Ig-α and Ig-β with distinct cytoplasmic effectors. Science *258*, 123–126.

Clarke, J.F., Young, P.W., Yonezawa, K., Kasuga, M., and Holman, G.D. (1994). Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. J. Biochem. *300*, 631–635.

Deanin, G.G., Martinez, A.M., Pfeiffer, J.R., Gardner, M.E., and Oliver, J.M. (1991a). Tyrosine kinase-dependent phosphatidylinositol turnover and functional responses in the FcεR1 signalling pathway. Biochem. Biophys. Res. Commun. *179*, 551–557.

Deanin, G.G., Pfeiffer, J.R., Cutts, J.L., Fore, M.L., and Oliver, J.M. (1991b). Isoprenoid pathway activity is required for IgE receptor-mediated, tyrosine kinase-coupled transmembrane signaling in permeabilized RBL-2H3 rat basophilic leukemia cells. Cell Regul. *2*, 627–640.

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). PI 3-kinase is a dual enzyme: autoregulation by an intrinsic protein-serine kinase activity. EMBO J. *13*, 522–533.

Eiseman, E., and Bolen, J.B. (1992). Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. Nature *355*, 78–80.

Extley, M., Varticovski, L., Peter, M., Sancho, J., and Terhorst, C. (1994). Association of phospatidylinositol 3-kinase with a specific sequence of the T cell receptor ζ chain is dependent on T cell activation. J. Biol. Chem. *269*, 15140–15146.

Fasolato, C., Hoth, J., and Penner, R. (1993). A GTP-dependent step in the activation mechanism of capacitative calcium influx. J. Biol. Chem. *268*, 20737–20740.

Felder, S., Zhou, M., Hu, P., Urena, J., Ullrich, A., Chaudhuri, M., White, M., Shoelson, S.E., and Schlessinger, J. (1993). SH2 domains exhibit high-affinity binding to tyrosine-phosphorylated peptides yet also exhibit rapid dissociation and exchange. Mol. Cell. Biol. *13*, 1449–1455.

Hara, K., *et al.* (1994). 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. Proc. Natl. Acad. Sci. USA *91*, 7415–7419.

Hutchcroft, J.E., Geahlen, R.L., Deanin, G.G., and Oliver, J.M. (1992). FcεRI-mediated tyrosine phosphorylation and activation of the 72-kDa protein tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. Proc. Natl. Acad. Sci. USA *89*, 9107–9111.

Joly, J., Kazlauskas, A., Fay, F.S., and Corvera, S. (1994). Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. Science *263*, 684–686.

Jouvin, M., Adamczewski, M., Nemerof, R., Letourneur, O., Valle, A., and Kinet, J.-P. (1994). Differential control of the tyrosine kinases lyn and syk by the two signaling chains of the high affinity immunoglobulin E receptor. J. Biol. Chem. *269*, 5918–5925.

Kanakaraj, P., Duckworth, B., Azzoni, L., Kamoun, M., Cantley, L.C., and Perussia, B. (1994). Phosphatidylinositol-3 kinase activation induced upon FcγRIIIA-ligand interaction. J. Exp. Med. *179*, 551.

Kapeller, R., and Cantley, L.C. (1994). Phosphatidylinositol 3-kinase. BioEssays *16*, 565–576.

Karnitz, L.M., Sutor, S.L., and Abraham, R.T. (1994). The Src-family kinase Fyn, regulates the activation of phosphatidylinositol 3-kinase in an interleukin 2-responsive T cell line. J. Exp. Med. *179*, 1799–1808.

Kazlauskas, A., Kashishian, A., Cooper, J.A., and Valius, M. (1992). GTPase-activating protein and phosphatidylinositol 3-kinase bind

to distinct regions of the platelet-derived growth factor receptor β subunit. Mol. Cell Biol. *12*, 2534–2544.

Keller, S.R., and Lienhard, G.E. (1994). Insulin signaling: the role of insulin receptor substrate 1. Trends Cell Biol. *4*, 115–119.

Kitani, S., Teshima, R., Morita, Y., Ito, K., Matsuda, Y., and Nonomura, Y. (1992). Inhibition of IgE-mediated histamine release by myosin light chain kinase inhibitors. Biochem. Biophys. Res. Commun. *183*, 48–54.

Knol, E.F., Koenderman, L., Mul, F.P.J., Verhoeven, A.J., and Roos, D. (1991). Differential activation of human basophils by anti-IgE and formyl-methionyl-leucyl-phenylalanine: indications for protein kinase C-dependent and -independent activation pathways. Eur. J. Immunol. *21*, 881–885.

Kotani, K., *et al.* (1994). Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling. EMBO J. *13*, 2313–2321.

Kumagai, N., Morii, N., Fujisawa, K., Nemoto, Y., and Narumiya, S. (1993). ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured Swiss 3T3 cells. J. Biol. Chem. *268*, 24535–24538.

Lam, K., Carpenter, C.L., Ruderman, N.B., Friel, J.C., and Kelly, K.L. (1994). The phosphatidylinositol 3-kinase serine kinase phosphorylates IRS-1. J. Biol. Chem. *269*, 20648–20652.

Lee, R., and Oliver, J.M. (1995). Two mechanisms of antigen-stimulated $Ca^{2+}$ influx in RBL-2H3 cells. Mol. Biol. Cell. *6*, 825–839.

Letourneur, F., and Klausner, R.D. (1991). T-cell and basophil activation through the cytoplasmic tail of T-cell-receptor ζ family proteins. Proc. Natl. Acad. Sci. USA *88*, 8905–8909.

Li, W., Deanin, G.G., Margolis, B., Schlessinger, J., and Oliver, J.M. (1992). FcεR1-mediated tyrosine phosphorylation of multiple proteins, including phospholipase Cγ1 and the receptor β-γ2 complex, in RBL-2H3 rat basophilic leukemia cells. Mol. Cell. Biol. *12*, 3176–3182.

Liu, F.T., Bohn, J.W., Ferry, E.L., Yamamoto, H., Molinaro, C.A., Sherman, L.A., Klinman, N.R., and Katz, D.H. (1980). Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation and characterization. J. Immunol. *24*, 2728–2736.

Mao, S.-Y., Pfeiffer, J.R., Oliver, J.M., and Metzger, H. (1993). Effects of subunit mutation on the localization to coated pits and internalization of cross-linked IgE-receptor complexes. J. Immunol. *151*, 2760–2774.

Mori, S., Ronnstrand, L., Claesson-Welsh, L., and Heldin, C.-H. (1994). A tyrosine residue in the juxtamembrane segment of the platelet-derived growth factor β-receptor is critical for ligand-mediated endocytosis. J. Biol. Chem. *269*, 4917–4921.

Nakanishi, S., Catt, K.J., and Balla, T. (1994). Inhibition of agonist-stimulated inositol 1,4,5-triphosphate production and calcium signaling by the myosin light chain kinase inhibitor, wortmannin. J. Biol. Chem. *269*, 6528–6535.

Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., and Matsuda, Y. (1992). Wortmannin, a microbial product inhibitor of myosin light chain kinase. J. Biol. Chem. *267*, 2157–2164.

Ninomiya, N., Hazeki, K., Fuku, Y., Seya, T., Okada, T., Hazeki, O., and Ui, M. (1994). Involvement of phosphatidylinositol 3-kinase in Fcγ receptor signaling. J. Biol. Chem. *269*, 22732–22737.

Nobes, C.D., Hawkins, P., Stephens, L., and Hall, A. (1995). Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J. Cell Sci. *108*, 225–233.

Offermans, S., Jones, S.V., Bombien, E., and Schultz, G. (1994). Stimulation of mitogen-activated protein kinase activity by different secretory stimuli in rat basophilic leukemia cells. J. Immunol. *152*, 250–261.

Oliver, J.M., Burg, D.L., Wilson, B.W., McLaughlin, J.L., and Geahlen, R.L. (1994). Role of the protein-tyrosine, syk, in signal transduction in mast cells: inhibition of FcεR1-mediated signaling by the syk-selective inhibitor, piceatannol. J. Biol. Chem. *269*, 29697–29703.

Oliver, J.M., Seagrave, J., Stump, R.F., Pfeiffer, J.R., and Deanin, G.G. (1989). Signal transduction and cellular response in RBL-2H3 mast cells. Prog. Allergy *42*, 185–245.

Park, D.J., Min, H.K., and Rhee, S.G. (1991). IgE-induced tyrosine phosphorylation of phospholipase C-γ1 in rat basophilic leukemia cells. J. Biol. Chem. *266*, 24237–24240.

Pfeiffer, J.R., Deanin, G.G., Seagrave, JC., Davis, B.H., and Oliver, J.M. (1985). Membrane and cytoskeletal changes associated with IgE-mediated serotonin release in rat basophilic leukemia cells. J. Cell Biol. *101*, 2145–2155.

Pfeiffer, J.R., and Oliver, J.M. (1994). Tyrosine kinase-dependent assembly of actin plaques linking FcεR1 cross-linking to increased cell-substrate adhesion in RBL-2H3 tumor mast cells. J. Immunol. *152*, 270–279.

Pleiman, C.M., Clark, M.R., Timson, L.K., Gauen, T., Wintz, S., Coggeshall, K.M., Johnson, G.L., Shaw, A., and Cambier, J.C. (1993). Mapping of sites on the Src family protein tyrosine kinases $p55^{blk}$, $p55^{fyn}$, and $p56^{lyn}$ which interact with the effector molecules phospholipase C-γ2, microtubule-associated protein kinase, GTPase-activating protein, and phosphatidylinositol 3-kinase. Mol. Cell. Biol. *13*, 5877–5887.

Powis, G., Bonjouklian, R., Berggren, M.M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W.F., Dodge, J., Grindey, G., and Vlahos, C.J. (1994). Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. Cancer Res. *54*, 2419–2423.

Prasad, K.V.S., Janssen, O., Kapeller, R., Raab, M., Cantley, L.C., and Rudd, C.E. (1993). Src-homology 3 domain of protein kinase p59*fyn* mediates binding to phosphatidylinositol 3-kinase in T cells. Proc. Natl. Acad. Sci. USA *90*, 7366–7370.

Ridley, A.J. (1994). Membrane ruffling and signal transduction. BioEssays, *16*, 321–327.

Samelson, L.E., and Klausner, R.D. (1992). Tyrosine kinases and tyrosine-based activation motifs. J. Biol. Chem. *267*, 24913–24916.

Santini, F., and Beavin, M.A. (1993). Tyrosine phosphorylation of a mitogen-activated protein kinase-like protein occurs at a late step in exocytosis. J. Biol. Chem. *268*, 22716–22722.

Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D., and Emr, S.D. (1993). Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. Science *260*, 88–91.

Sklar, L.A., Oades, Z.G., Jesaitis, A.J., Painter, R.G., and Cochrane, C.G. (1981). Fluoresceinated chemotactic peptide and a high affinity anti-fluorescein antibody as a probe of neutrophil stimulation. Proc. Natl. Acad. Sci. USA *78*, 7540–7544.

Songyang, Z., *et al.* (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767–778.

Stephens, L., Smrcka, A., Cooke, F.T., Jackson, T.R., Sternweis, P.C., and Hawkins, P.T. (1994). A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein βγ subunits. Cell *77*, 83–93.

Stephens, L.R., Jackson, T.R., and Hawkins, P.T. (1993). Agonist-stimulated synthesis of phosphatidylinositol (3,4,5)-trisphosphate: a new intracellular signalling system? Biochim. Biophys. Acta *1179*, 27–75.

Stump, R.F., Oliver, J.M., Cragoe, E.J., Jr., and Deanin, G.G. (1987). The control of mediator release from RBL-2H3 cells: roles for $Ca^{2+}$, $Na^{+}$, and protein kinase C. J. Immunol. *139*, 881–886.

Thelen, M., Wymann, M.P., and Langen, H. (1994). Wortmannin binds specifically to 1-phosphatidylinositol 3-kinase while inhibiting guanine nucleotide-binding protein-coupled receptor signaling in neutrophil leukocytes. Proc. Natl. Acad. Sci. USA *91*, 4960–4964.

Thomason, P.A., James, S.R., Casey, P.J., and Downes, C.P. (1994). A G-protein $\beta\gamma$-subunit-responsive phosphoinositide 3-kinase activity in human platelet cytosol. J. Biol. Chem. *269*, 16525–16528.

Thompson, P.A., Gutking, J.S., Robbins, K.C., Ledbetter, J.A., and Balen, J.B. (1992). Identification of distinct populations of PI-3 kinase activity following T cell activation. Oncogene *7*, 719–725.

Traynor-Kaplan, A.E., Thompson, B.L., Harris, A.L., Taylor, P., Omann, G.M., and Sklar, L.A. (1989). Transient increase in phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol trisphosphate during activation of human neutrophils. J. Biol. Chem. *264*, 15668–15673.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203–212.

Ward, S.G., Ley, S.C., MacPhee, C., and Cantrell, D.A. (1992). Regulation of D-3 phosphoinositides during T cell activation via the T cell antigen receptor/CD3 complex and CD2 antigen. Eur. J. Immunol. *22*, 45–49.

Wilson, B.S., Deanin, G.G., Standefer, J.C., Vanderjagt, D., and Oliver, J. (1989). Depletion of guanine nucleotides with mycophenolic acid suppresses IgE receptor-mediated degranulation in rat basophilic leukemia cells. J. Immunol. *143*, 259–265.

Wilson, B.S., Kapp, N., Lee, R.J., Pfeiffer, J.R., Martinez, A.M., Platt, Y., Letourneur, F., and Oliver, J.M. (1995). Distinct functions of the Fc$\epsilon$R1 $\gamma$ and $\beta$ subunits in the control of Fc$\epsilon$R1-mediated tyrosine kinase activation and signaling responses in RBL-2H3 cells. J. Biol. Chem. *270*, 4013–4022.

Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y., and Matsuda, Y. (1993). Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J. Biol. Chem. *268*, 25846–25856.

Yee, N.S., Hsiau, C.-W.M., Serve, H., Vosseler, K., and Besmer, P. (1994). Mechanism of down-regulation of c-kit receptor. J. Biol. Chem. *269*, 31991–31998.

Zhang, J., King, W.G., Dillon, S., Hall, A., Feig, L., and Rittenhouse, S.E. (1993). Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. J. Biol. Chem. *268*, 22251–22254.