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Wortmannin-Sensitive Phosphorylation, Translocation, and Activation of PLCγ1, but Not PLCγ2, in Antigen-stimulated RBL-2H3 Mast Cells

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In RBL-2H3 tumor mast cells, cross-linking the high affinity IgE receptor (FcεRI) with antigen activates cytosolic tyrosine kinases and stimulates Ins(1,4,5)P_3 production. Using immune complex phospholipase assays, we show that FcεRI cross-linking activates both PLCγ1 and PLCγ2. Activation is accompanied by the increased phosphorylation of both PLCγ isoforms on serine and tyrosine in antigen-treated cells. We also show that the two PLCγ isoforms have distinct subcellular localizations. PLCγ1 is primarily cytosolic in resting RBL-2H3 cells, with low levels of plasma membrane association. After antigen stimulation, PLCγ1 translocates to the plasma membrane where it associates preferentially with membrane ruffles. In contrast, PLCγ2 is concentrated in a perinuclear region near the Golgi and adjacent to the plasma membrane in resting cells and does not redistribute appreciably after FcεRI cross-linking. The activation of PLCγ1, but not of PLCγ2, is blocked by wortmannin, a PI 3-kinase inhibitor previously shown to block antigen-stimulated ruffling and to inhibit Ins(1,4,5)P_3 synthesis. In addition, wortmannin strongly inhibits the antigen-stimulated phosphorylation of both serine and tyrosine residues on PLCγ1 with little inhibition of PLCγ2 phosphorylation. Wortmannin also blocks the antigen-stimulated translocation of PLCγ1 to the plasma membrane. Our results implicate PI 3-kinase in the phosphorylation, translocation, and activation of PLCγ1. Although less abundant than PLCγ2, activated PLCγ1 may be responsible for the bulk of antigen-stimulated Ins(1,4,5)P_3 production in RBL-2H3 cells.

INTRODUCTION

In mast cells and basophils, cross-linking the high affinity cell surface receptors for IgE (FcεRI)[1] activates the tyrosine kinases Lyn and Syk (Eiseman and Bolen, 1992; Hutchcroft *et al.*, 1992) and initiates a signaling cascade that leads to the secretion of histamine and other granule constituents, to changes in adhesive properties, cell shape, and surface topography and to the de novo synthesis of lipid mediators and cytokines (reviewed in Beaven & Metzger, 1993; Oliver *et al.*, 1997). Tyrosine kinase activation by the FcεRI and related members of the multisubunit immunoreceptor family, which includes the T cell antigen receptor, the B cell antigen receptor, and several Fcγ receptors, depends on immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domains of individual receptor subunits (Cambier, 1995). The heterotrimeric ($\alpha\beta\gamma_2$) FcεRI of RBL-2H3 mast cells contains ITAM motifs in both the γ and β subunit cytoplasmic domains (Metzger, 1992). Recent studies indicate that cross-linking FcεRI activates Lyn, leading

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[1] Abbreviations used: FcεRI, high affinity IgE receptor; Ins(1,4,5)P_3, inositol 1,4,5 trisphosphate; PH, pleckstrin homology domain; PI 3-kinase, phosphatidylinositol 3-kinase; PLCγ, phospholipase γ; PtdIns(4,5)P_2, phosphatidylinositol (4,5) bisphosphate; RBL-2H3, rat basophilic leukemia cells; SH2, Src homology 2 domain; SH3, Src homology 3 domain.

to ITAM phosphorylation and Syk activation by its association with the γ subunit phospho-ITAM. Syk activation, resulting in the phosphorylation of multiple protein substrates, in turn initiates the signaling cascade (Li *et al.*, 1992; Oliver *et al.*, 1994; Wilson *et al.*, 1995; Rivera and Brugge, 1995).

Pharmacological studies have established that Syk-dependent tyrosine phosphorylation is required for the antigen-stimulated synthesis of inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3), presumably mediated by phospholipase Cγ (PLCγ) (Deanin *et al.*, 1991; Oliver *et al.*, 1994). It has been shown that RBL-2H3 cells contain two PLCγ isoforms, PLCγ1 and PLCγ2, with PLCγ2 being the more abundant species (S.G. Rhee, personal communication; also, below). PLCγ1 is phosphorylated on tyrosine and serine (Park *et al.*, 1991; Li *et al.*, 1992) and translocated to the membrane fraction (Atkinson *et al.*, 1992) after FcεRI cross-linking. Antigen-stimulated phosphorylation of PLCγ2 has not been reported previously, but Atkinson *et al.* (1993) reported its translocation to a particulate fraction after IgE receptor cross-linking.

The relative contributions of phosphorylation and redistribution to receptor-mediated PLCγ activation are not known in RBL-2H3 cells, and are incompletely understood in other systems. PLCγ1 and PLCγ2 are both monomeric enzymes that contain two pleckstrin homology (PH) domains, two SH2 domains and one SH3 domain (reviewed in Lee and Rhee, 1995). Nishibe *et al.* (1990) reported that PLCγ1 can be activated in vitro by incubation with epidermal growth factor (EGF) receptor preparations and ATP under conditions that also stimulate its tyrosine phosphorylation. However, PLCγ1 activated in vivo was only ~50% deactivated when PLCγ1 immunoprecipitates were incubated with tyrosine phosphatase, suggesting that several mechanisms contribute to maximal stimulation. Kim *et al.* (1991) showed that substituting phenylalanine for tyrosine 783 of PLCγ1 yielded a protein that could associate with tyrosine-phosphorylated sites in the platelet-derived growth factor (PDGF) receptor cytoplasmic domain and could become phosphorylated on other tyrosine sites, but was no longer activated by this interaction. These investigators also showed that mutations at Tyr1254 partially inhibited, and at Tyr771 enhanced, the PDGF-induced activation of PLCγ1. Several catalytically active PDGF and fibroblast growth factor receptors with cytoplasmic domain phenylalanine to tyrosine mutations that prevented their stable association with PLCγ also failed to stimulate tyrosine phosphorylation of PLCγ and Ins(1,4,5)P_3 production (Valilus *et al.*, 1993; Mohammadi *et al.*, 1992; Peters *et al.*, 1992). Additionally, a C-terminal mutant of the EGF receptor, which lacked a PLCγ binding site at Tyr 992, could phosphorylate PLCγ1 without stimulating PtdIns(4,5)P_2 hydrolysis (Vega *et al.*, 1992). Together, these studies suggest that a combination of the SH2 domain-mediated association of PLCγ1 with phosphotyrosine counterstructures and the tyrosine phosphorylation of PLCγ itself may be required for growth factor-mediated activation. Although the PH and SH3 domains of PLCγ isoforms have the potential to interact with membrane lipids and proteins and with cytoskeletal proteins (Pawson, 1995; Cohen *et al.*, 1995), the roles of these domains in PLCγ translocation and activation have not been addressed.

Recently, we (Barker *et al.*, 1995) confirmed the results of Yano *et al.* (1993) that FcεRI cross-linking activates a form of phosphatidylinositol 3-kinase (PI 3-kinase) that is composed of a p85 adaptor subunit and a 110-kDa catalytic subunit. This enzyme phosphorylates phosphatidylinositols in the 3 position of the inositol moiety (reviewed in Stephens *et al.*, 1993; Kapeller and Cantley, 1994). There is recent evidence that PI 3-kinase may regulate cellular activities via an additional role as a protein serine kinase (Dhand *et al.*, 1994; Lam *et al.*, 1994; Barker *et al.*, 1995). We demonstrated that wortmannin, at nM concentrations thought to specifically inhibit PI 3-kinase, blocks secretion, macropinocytosis, and ruffling in antigen-stimulated RBL-2H3 cells (Barker *et al.*, 1995). Despite the selective block of a subset of responses in antigen-stimulated RBL cells, wortmannin had no effect on the activation of Lyn, Syk or MAP kinases. From these results, we speculated that PI 3-kinase is located at a branch point in the FcεRI signaling cascade. Unexpectedly, we also demonstrated that wortmannin treatment results in a significant loss of antigen-stimulated Ins(1,4,5)P_3 production (Barker *et al.*, 1995). This novel finding prompted us to explore the potential role of PI 3-kinase in the FcεRI-signaling cascade leading to PLCγ activation. In this report, we directly measure enzymatic activity of PLCγ1 and PLCγ2 isoforms in immune complex phospholipase assays, examine their intracellular localization, and determine the effects of wortmannin on the activation and phosphorylation of both PLCγ isoforms.

MATERIALS AND METHODS

Material Sources

Wortmannin was purchased from Sigma (St. Louis, MO). Immunoprecipitating anti-PLCγ1 antibodies were obtained from Calbiochem, La Jolla, CA (immunizing antigen amino acids 113–132, near the amino terminus of bovine PLCγ1) or Santa Cruz Biotechnology, Santa Cruz, CA (immunizing antigen amino acids 1249–1262, near the carboxyl terminus of bovine PLCγ1). The Santa Cruz anti-PLCγ1 antibody was also used for immunofluorescence and immunoelectron microscopy. For immunoblotting, anti-denatured-PLCγ1 antibodies (immunizing antigen amino acids 82–100 from bovine PLCγ1) were purchased from Transduction Labs, Lexington, KY. The anti-PLCγ2 monoclonal antibody used for immunofluorescence and immunoelectron microscopy was a generous gift from Dr. S.G. Rhee (NIH). Immunoprecipitating anti-PLCγ2 antibodies (immu-

nizing antigen amino acids 1213–1232 from human PLCγ2) were also obtained from Santa Cruz. Phosphotyrosine was detected on Western blots with RC20-HRP antibody from Transduction. FITC-, HRP- and rhodamine-lissamine-conjugated secondary antibodies and the rabbit anti-mouse bridging antibody were from Jackson ImmunoResearch (West Grove, PA). Colloidal gold-labeled reagents were from Amersham (Arlington Heights, IL).

Cell Culture and Activation

RBL-2H3 cells were cultured on tissue culture flasks in minimal essential medium (MEM; Life Technologies, Gaithersburg, MD) supplemented with 15% fetal calf serum, penicillin-streptomycin, and L-glutamine. In some experiments, IgE receptors were primed by the addition of anti-DNP-IgE (1 μg/ml) for 12–20 h. Cells were then washed to remove excess IgE, incubated without or with 10 nM wortmannin for 15 min at 37°C, and activated by the addition of 1 μg/ml of the polyvalent antigen, DNP-BSA, at 37°C.

Western Blotting and Immune Complex Phospholipase Assays

Adherent, IgE-primed RBL-2H3 cells (1×10^7 cells for PLCγ2 experiments and 2×10^7 cells for PLCγ1) were activated for indicated times with DNP-BSA at 37°C. Culture dishes were transferred to a tray of ice, washed immediately with ice-cold PBS, and lysed with Buffer A (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 1 mM EGTA, 1 μg/ml aprotinin and leupeptin). Insoluble material was discarded after microcentrifugation (4 min at 13,000 × *g*, 4°C) and the supernatant rocked for 1 h with 30 μl Protein A/G Sepharose (Oncogene, Cambridge, MA) prebound to 1 μg anti-PLCγ1- or PLCγ2-specific antibodies.

For Western blotting, immune complexes were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine (anti-pY), or anti-PLCγ1 or γ2 antibodies, followed by HRP-labeled second antibodies. Blots were developed with Super-Signal ULTRA (Pierce, Rockford, IL) and detected by autoradiography. The relative amounts of the two isoforms were analyzed with a Molecular Dynamics PhosphorImager with ImageQuant software.

For phospholipase assays, the beads were washed once with reaction buffer (35 mM NaH_2PO_4, pH 6.8, 70 mM KCl, 0.8 mM EGTA, 0.8 mM $CaCl_2$) and assayed for phospholipase activity with a procedure adapted from Wahl *et al.* (1992). The substrate was prepared by drying a 100-μl aliquot of PtdIns(4,5)-P_2 (1 mg/ml; Boerhinger-Mannheim-USA, Indianapolis, IN) together with 30 μl of Ptd[^{3}H]Ins(4,5)P_2 (0.3 μCi; Dupont-New England Nuclear, Boston, MA), under a stream of nitrogen. The dried phospholipid was solubilized in 50 μl of 50 mM sodium phosphate, pH 6.8, 100 mM KCl with sonication, followed by adding 50 μl of 5% (80 mM) Triton X-100 and sonication to facilitate incorporation into Triton X-100 micelles. Excess wash buffer was removed from the immune complexes and 10 μl each of 5× reaction buffer and substrate solution added. The beads were incubated at 35°C for 20 min and reactions stopped by transfer to an ice bath with the addition of 100 μl of 1% (wt/vol) bovine serum albumin and 250 μl of 10% (wt/vol) TCA. Samples were centrifuged for 3 min in a swinging bucket microcentrifuge and release of [^{3}H]Ins(1,4,5)P_3 into the supernatant quantified by liquid scintillation counting.

In Vivo Phosphorylation of PLCγ1 and PLCγ2

In vivo phosphorylation of PLCγ species was measured in PLCγ immunoprecipitates from [^{32}P]orthophosphate-labeled, resting and antigen-activated RBL-2H3 cells. [^{32}P]-labeling was performed as described in Li *et al.* (1992). After 2 or 10 min activation with DNP-BSA, cells (4×10^7) were placed on a tray of ice, washed with ice cold phosphate-free MEM and lysed with 1 ml Lysis Buffer B (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% Brij-96, 1 mM sodium orthovanadate, 1 μg/ml leupeptin and antipain). Lysates were centrifuged and enzymes isolated from the supernatants by immunoprecipitation with anti-PLCγ1 or anti-PLCγ2 antibodies and Protein A/G Sepharose. Beads were washed once with lysis buffer containing 0.1% Brij-96 and three times with lysis buffer without detergent. Laemmli buffer (40 μl) was added after the final wash, samples were boiled for 5 min, and [^{32}P] incorporation into PLCγ isoforms analyzed by SDS-PAGE as follows. In some experiments, gels were fixed and stained for protein with Coomassie Blue. Dried gels were used for autoradiography, and phosphoproteins were identified as PLCγ1 or PLCγ2 based on electrophoretic mobility relative to Life Technologies "ladder" protein standards, as determined by immunoblotting similar PLCγ immunoprecipitates prepared from nonradioactive cell lysates. Phosphate incorporation was quantified by PhosphorImager analysis. In other experiments, wet gels were placed in a Millipore Semidry blotter, proteins were transferred to PVDF and dried membranes put to film. PLCγ bands were excised, digested to constituent amino acids with 6N HCL, and analyzed for phosphoamino acid content with a Hunter Thin Layer Peptide Mapping System (CBS Scientific Co, Del Mar, CA).

Immunolocalization of PLCγ Isoforms

For fluorescence microscopy, monolayers of RBL-2H3 cells on glass coverslips were activated for 10 min with DNP-BSA. To visualize F-actin, cells were labeled by 30 min incubation in 2% paraformaldehyde, 0.02% saponin and 4 U/ml of rhodamine phalloidin (Molecular Probes, Eugene, OR). For PLCγ localization, cells were fixed for 10 min with 2% paraformaldehyde, followed by 10 min permeabilization with 0.05% Triton X-100 in PBS. The coverslips were washed in PBS, and incubated sequentially with primary antibodies (1 μg/ml rabbit anti-PLCγ1 or 1 μg/ml monoclonal anti-PLCγ2), followed by FITC-conjugated secondary antibodies. Coverslips were mounted on slides and photographed with a Zeiss Photomicroscope III equipped for epifluorescence microscopy. For immunoelectron microscopy, cell suspensions were activated for 2 or 10 min with DNP-BSA. Reactions were stopped by 10 min incubation at room temperature with fixative (10% paraformaldehyde, 0.075% glutaraldehyde, 0.2% picric acid). Cells were collected by centrifugation, washed twice in PBS and held serially in 50% ETOH (ethyl alcohol) (10 min), 70% ETOH (10 min), 2% uranyl acetate in 70% ETOH (60 min), 75% ETOH (10 min), 2:1 LR White in 75% ETOH (10 min), 100% LR white, (4 × 20 min). Cell pellets were embedded in gelatin capsules in 10 ml LR White containing 20 μl accelerator, held on ice for 30 min, and allowed to harden for 2 days at room temperature. Thin sections were mounted on 150 mesh nickel, formvar, and carbon-coated grids. Grids with sections were held in distilled H_20 for 5 min at room temperature, and nonspecific protein-binding sites blocked for 15 min with 5% bovine calf serum, 0.5% BSA in TBS (20 mM Tris, 155 mM $NaCl_2$, 20 mM NaN_3, pH 7.6.). Samples were incubated overnight at room temperature with primary antibodies (anti-PLCγ1 at 0.1 μg/ml; anti-PLCγ2 at 1 μg/ml) in TBS supplemented with 1% serum, then washed through a series of 10 droplets of TBS, and incubated with 15 nm colloidal gold-conjugated Protein A or 30 nm colloidal gold-conjugated goat anti-mouse IgG in TBS, pH 8.2 (1:25; Amersham). They were again rinsed 10 times in TBS, pH 8.2, by the droplet method. Sections were postfixed with 2% glutaraldehyde, stained with uranyl acetate and lead citrate, and examined with an Hitachi 600 transmission electron microscope.

RESULTS

FcεRI Cross-linking Activates Both PLCγ1 and PLCγ2

FcεRI cross-linking induces Ins(1,4,5)IP_3 synthesis that reaches a maximum at around 2 min and persists over

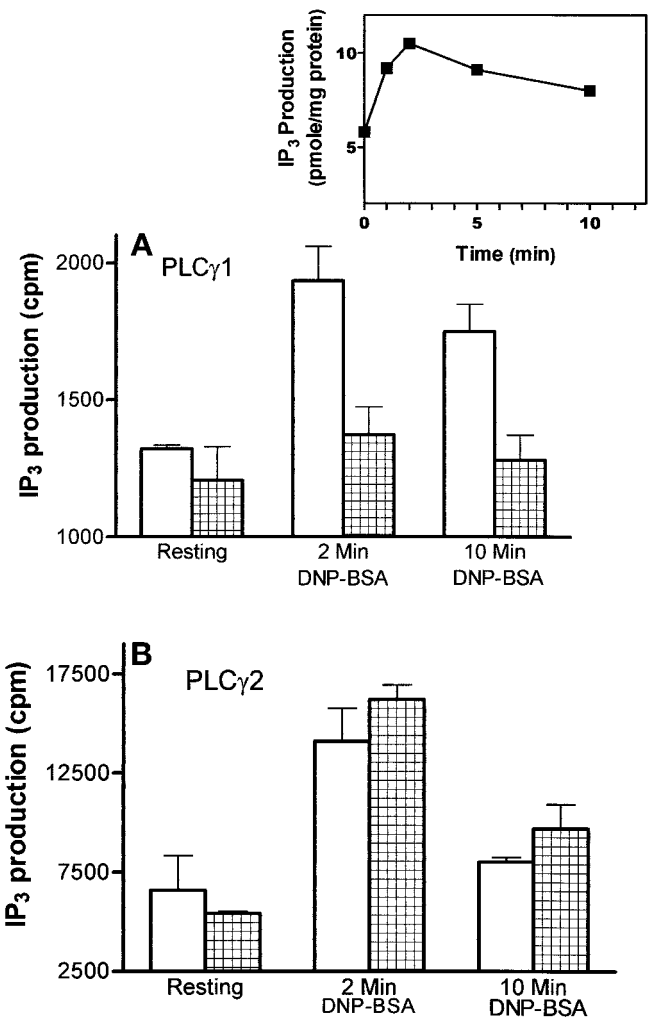

Figure 1. FcεRI cross-linking activates both isoforms of PLCγ and PLCγ1 activation is selectively blocked by wortmannin. PLC activity was measured in anti-PLCγ immune complexes prepared from resting and antigen-stimulated RBL-2H3 cells from the hydrolysis of [^{3}H]-PtdIns(4,5)P_2 in the Triton-based mixed micelle assay described in MATERIALS AND METHODS. Results show activation of PLCγ1(A) and PLCγ2 (B) at 2 and 10 min after addition of cross-linking reagent (1 μg/ml DNP-BSA) to control cells (open bars). PLCγ1 activation, but not that of PLCγ2, is inhibited in cells pretreated for 15 min with 10 nM wortmannin (hatched bars). Results are the average of duplicate samples in one of at least two similar experiments. Inset: The time course of antigen-induced Ins(1,4,5)IP_3 production in control cells, measured with the radioreceptor assay described in Deanin *et al.* (1991).

at least 10 min (inset, Figure 1). This time course should predict the time course of PLC activation and decay in RBL-2H3 cells. To test this, we directly measured the activity of the two PLCγ isoforms in immunoprecipitates from antigen-stimulated rat tumor mast cells, using methods modified from Wahl *et al.* (1992). As shown in Figure 1, FcεRI cross-linking of control cells (solid bars) causes a substantial increase in both PLCγ1 (A) and PLCγ2 (B) activities. We interpret the greater activity of PLCγ2 as a reflection of the greater abundance of PLCγ2 in RBL-2H3 cells. The increase in immune complex phospholipase activity is striking after 2 min of stimulation, when cytoplasmic Ins(1,4,5)IP_3 levels are highest (Inset). Consistent with the lower cytoplasmic Ins(1,4,5)IP_3 levels in cells treated with antigen for 10 min (Inset), the activities of both PLCγ isoforms are reduced, although PLCγ1 activity in particular is still well above basal levels in immune complexes prepared from cells that were exposed to antigen for 10 min.

Antigen-induced Activation of PLCγ1, but not PLCγ2, Is Inhibited by Wortmannin

We showed previously that 10 nM wortmannin inhibits the production of Ins(1,4,5)IP_3 by 50–70% in antigen-stimulated cells (Barker *et al.*, 1995). Therefore, we tested the effects of wortmannin on the activity of PLCγ isoforms in antigen-stimulated RBL-2H3 cells. As shown in Figure 1A (hatched bars), pretreatment of cells with 10 nM wortmannin, that irreversibly inhibits PI 3-kinase (Thelen *et al.*, 1994), effectively blocks antigen-induced activation of PLCγ1, as measured in the immune complex phospholipase assay. In contrast, the activation of PLCγ2 in response to FcεRI cross-linking is unaffected by wortmannin (Figure 1B). When wortmannin was added to PLCγ immune complexes together with substrate, there was no inhibition of phospholipase activity of either isotype (our unpublished results). Thus wortmannin inhibits antigen-stimulated Ins(1,4,5)P_3 production by selectively blocking a step upstream of PLCγ1 activation in the FcεRI signaling cascade.

Phosphorylation of PLCγ1, but not PLCγ2, Is Inhibited by Wortmannin

The phosphorylation states of PLCγ1 and PLCγ2 were determined in immunoprecipitates prepared from [^{32}P]-orthophosphate-labeled RBL-2H3 cells. Immunoprecipitates were separated by SDS-PAGE and transferred to PVDF before autoradiography. The results of these experiments are shown in Figure 2A. Both PLCγ isoforms have very low levels of phosphorylation in resting cells (lanes 1, 4). After 2 min cross-linking of anti-DNP IgE-primed receptors with DNP-BSA, both PLCγ1 (lane 2) and PLCγ2 (lane 5) are phosphorylated. The antigen-stimulated phosphorylation of PLCγ1 is barely detectable in wortmannin-treated cells (lane 3). In contrast, wortmannin does not affect the antigen-induced phosphorylation of PLCγ2 (lane 6). Similar results were obtained from analyses of immune complexes generated from cells exposed to antigen for 10 min (unpublished observations). Results shown here were obtained with anti-N terminal antibodies to precipitate PLCγ1; similar results were

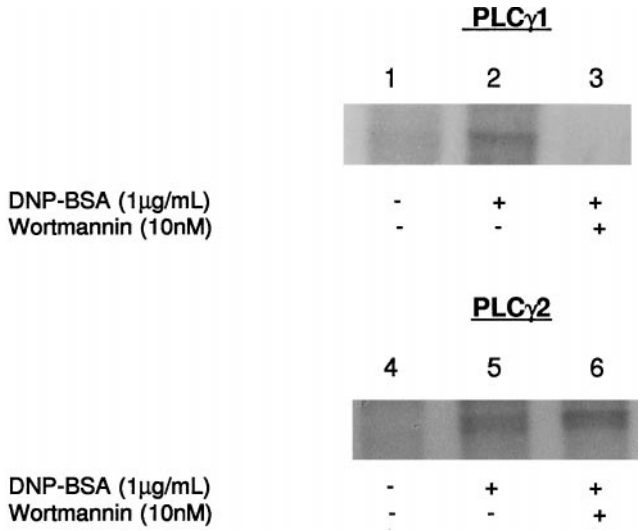

Figure 2. A). Wortmannin inhibits the antigen-stimulated phosphorylation of PLCγ1 but not PLCγ2. IgE-primed, [^{32}P]orthophosphate-labeled cells (4×10^7) were incubated for 15 min with or without 10 nM wortmannin, followed by 0 or 2 min with 1 μg/ml DNP-BSA. In lanes 1–3, PLCγ1 was immunoprecipitated from lysis supernatants, separated by SDS-PAGE and detected by autoradiography. PLCγ1 phosphorylation is not detected in resting cells (lane 1), although longer exposures to film can show low levels of phosphate incorporation (unpublished observation). FcεRI cross-linking causes the phosphorylation of PLCγ1 (lane 2). Wortmannin treatment blocks the phosphorylation of PLCγ1 (lane 3). Lanes 4 to 6 show that phosphorylation of PLCγ2 is weakly detectable in resting cells (lane 4), and increases in response to antigen (lane 5). Lane 6 shows that the increase in PLCγ2 phosphorylation is unaffected by the presence of wortmannin. Similar results were obtained in three experiments.

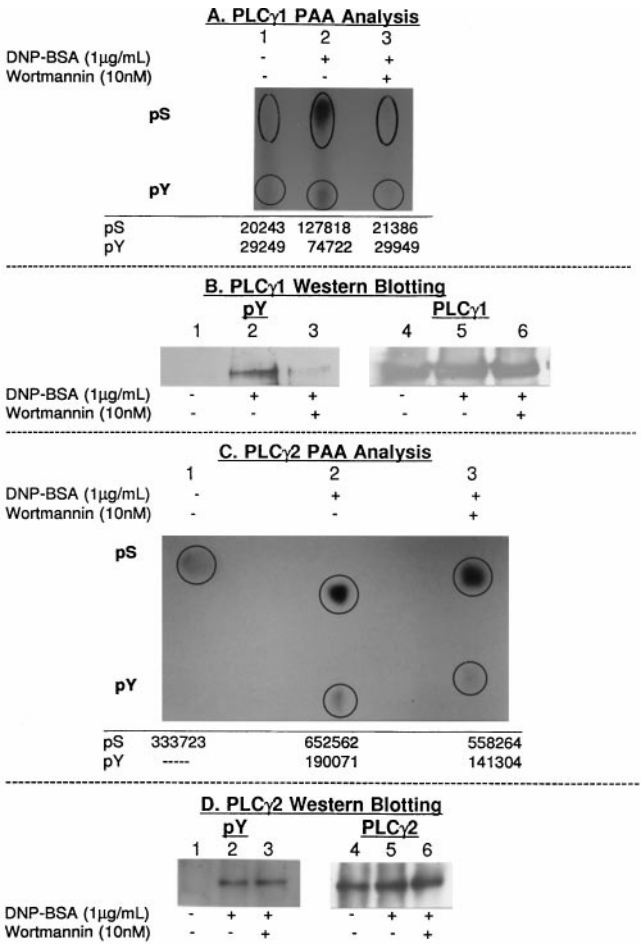

Figure 3. Phosphoamino acid analysis of PLCγ isoforms. Phosphate-labeled bands corresponding to PLCγ1 and PLCγ2 were excised from PVDF membranes after transfer from SDS-PAGE. Each band was digested to constituent amino acids and the entire sample was used for one-dimensional TLC analysis as described in MATERIALS AND METHODS. Mobilities of phosphoserine and phosphotyrosine were determined on the basis of authentic standards; neither isoform had detectable levels of phosphothreonine. The numbers under each lane represent the relative PhosphorImager units corresponding to phosphoserine and phosphotyrosine, adjusted for background. A) Phosphoamino acid content of PLCγ1 in resting cells (lane 1), after 2 min (lane 2) of cross-linking FcεRI with antigen and after 10 nM wortmannin treatment followed by 2 min with antigen (lane 3). B+D). Western blot analysis of PLCγ1(B) or PLCγ2(D) immunoprecipitated from resting and 2 min antigen-activated cells (with and without wortmannin pretreatment) probed with anti-pY (B+D; lanes 1–3), stripped and reprobed with anti-PLCγ1 antibodies (B; lanes 4–6) or anti-PLCγ2 (D; lanes 4–6). C) Phosphoamino acid content of PLCγ2 in resting cells (lane 4), in control cells after 2 min of antigen (lane 5) or in 10 nM wortmannin-treated cells after 2 min of antigen (lane 6).

seen with anti-C terminal antibodies to PLCγ1. The absence of phosphate-labeled bands in resting and wortmannin-treated cells is not a result of alterations in binding of antibodies to PLCγ1, as equal amounts of PLCγ1 are detected by immunoblotting methods in immunoprecipitates isolated under all three conditions (Figure 3B).

Because PLCγ1 was shown to be phosphorylated on both serine and tyrosine after FcεRI cross-linking (Li et al., 1992), the PLCγ bands were excised, acid hydrolysed, and analyzed by TLC to determine their phosphoamino acid content. The results, shown in Figure 3A, establish that wortmannin markedly reduces the incorporation of phosphate into both serine and tyrosine residues of PLCγ1 in antigen-stimulated cells. Similar results were obtained in two additional experiments and in cells activated for 10 min as well as for 2 min. To confirm these findings, we also used Western blotting methods to probe anti-PLCγ1 immunoprecipitates with anti-phosphotyrosine antibodies. Results in Figure 3B show neglible levels of phosphotyrosine in PLCγ1 isolated from resting cells (lane 1) and significant phosphotyrosine immunoreactivity in PLCγ1 after 2 min of antigen stimulation (lane 2). Wortmannin pretreatment substantially reduces, but does not completely abolish, tyrosine phosphorylation (Figure 3B, lane 3). The Western blots were stripped and reprobed with anti-PLCγ1 antibodies (Figure 3B, lanes 4–6), showing that equivalent amounts of

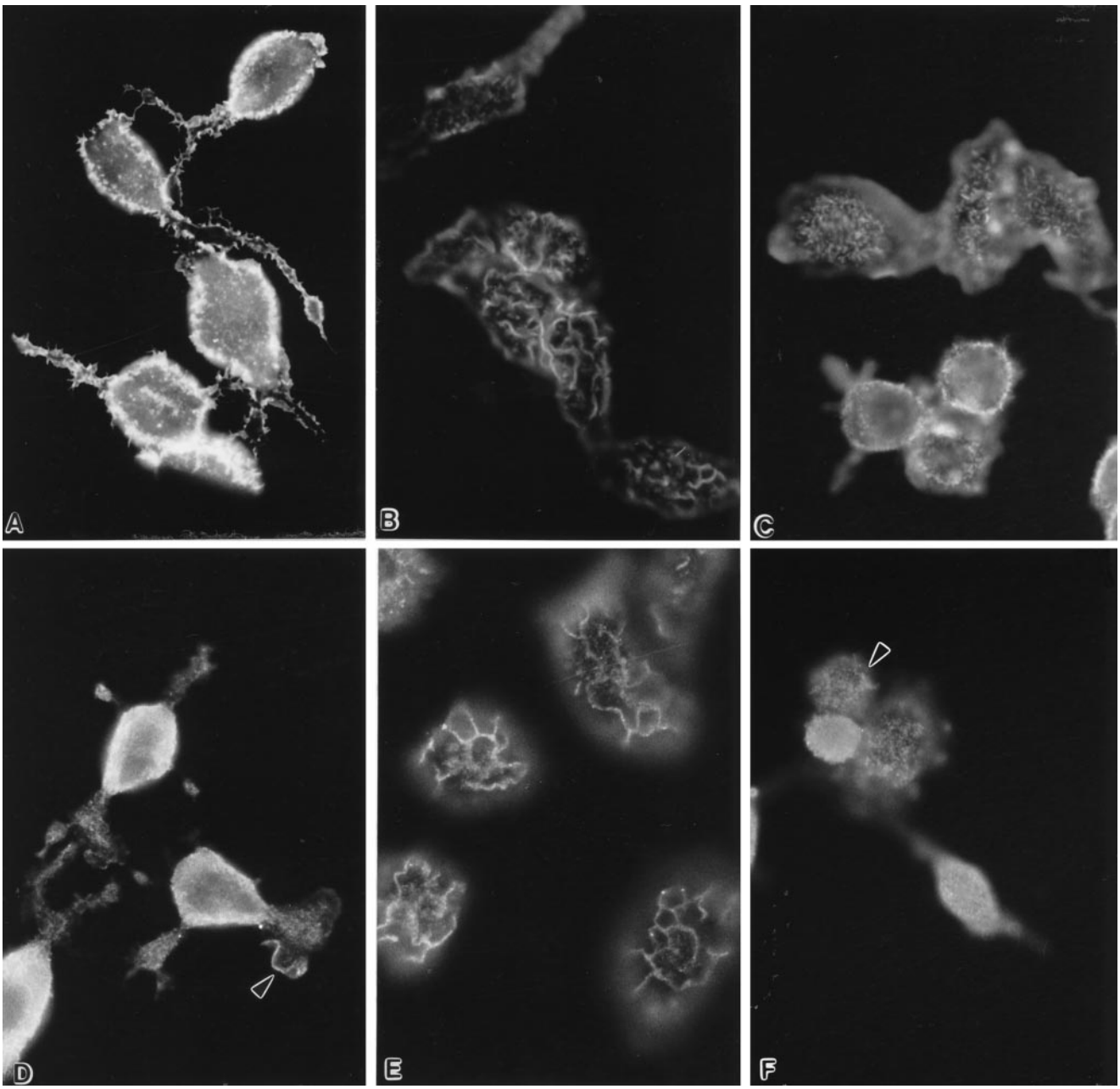

Figure 4. PLCγ1 translocates to actin-rich membrane ruffles in antigen-stimulated cells. Filamentous actin was labeled with rhodamine phalloidin in fixed and permeablized cells before and after FcεRI cross-linking. The actin stain shows the transformation from microvillous surface architecture in resting cells (A) to ruffles at 10 min of exposure to antigen (B); the majority of wortmannin-treated cells fail to make surface ruffles in response to antigen (C). PLCγ1 was localized by immunofluorescence microscopy in unstimulated RBL-2H3 cells (D) and antigen-stimulated (1 μg/ml DNP-BSA, 10 min) cells without (E) and with (F) wortmannin treatment. The anti-PLCγ1 stain shows a redistribution of enzyme from a generally diffuse distribution in resting cells to an association with membrane ruffles in antigen-stimulated cells. The presence of wortmannin inhibits membrane labeling of antigen-stimulated cells. The arrowheads in D and F point to occasional ruffles that form in resting or wortmannin-treated cells and label with PLCγ1 antibodies. Magnification 750×.

enzyme were immunoprecipitated from resting or antigen-stimulated cells.

In contrast, wortmannin had little or no effect on phosphorylation of PLCγ2. We conclude this based on no detectable differences when PLCγ2 immunoprecipitates were probed with anti-phosphotyrosine antibodies on Western blots (Figure 3D, lanes 1–3) and only slightly lower amounts (20–25%) of phospho-

serine and phosphotyrosine in two separate phosphoamino acid analyses of PLCγ2 from antigen-stimulated cells after wortmannin treatment (Figure 3C). As was the case for PLCγ1, the amount of immunoprecipitable PLCγ2 is the same in lysates of resting; antigen-activated; and wortmannin-treated, antigen-activated cells (Figure 3D, lanes 4–6).

Different Distribution of PLCγ1 and PLCγ2 in Antigen-stimulated RBL-2H3 Cells: Immunofluorescence Microscopy

Cross-linking the FcεRI on RBL-2H3 cells leads to cytoskeletal rearrangements, membrane ruffling, and increased cell adhesion and spreading (Pfeiffer *et al.*, 1984; Pfeiffer and Oliver, 1994). These changes in cell morphology are illustrated in cells stained with rhodamine phalloidin to visualize filamentous actin. Resting cells (Figure 4A) have rounded cell bodies with one or more processes and a microvillous surface. After antigen stimulation, the cells spread and have prominent membrane ruffles (Figure 4B). Antigen-stimulated membrane ruffling, but not spreading, is markedly inhibited in wortmannin-treated cells (Figure 4C).

Using isoform-specific antibodies and immunofluoresence microscopy, we found that the majority of PLCγ1 has a diffuse cytosolic distribution in resting cells, with some labeling of the plasma membrane that is most obvious at the tips of membrane processes (arrowhead, Figure 4D). The membrane association is specific, since it is not seen when antibody is pretreated with immunizing peptide (amino acids 1249–1262 near the carboxyl terminus of bovine PLCγ1; unpublished observation). In contrast, PLCγ1 in antigen-activated cells is strongly associated with membrane ruffles (Figure 4E). Membrane association of PLCγ1 is not apparent in antigen-stimulated cells treated with the PI 3-kinase inhibitor, wortmannin, with the exception of the rare cells that display an incomplete ruffling response (arrowhead, Figure 4F).

The γ2 isoform of PLC has a distinctly different distribution from PLCγ1 in RBL-2H3 cells. Immunofluorescence microscopy with a monoclonal antibody to PLCγ2 showed strong reactivity in the Golgi region and a patchy distribution along the plasma membrane (Figure 5A) of resting RBL-2H3 cells. Antigen stimulation induces cell spreading, which most likely accounts for the more dispersed appearance of the patches of membrane-associated anti-PLCγ2 reactivity (Figure 5B). However, the Golgi region still contains the highest concentration of PLCγ2. There is no detectable association of PLCγ2 with membrane ruffles at the dorsal surface of the cells. No difference in PLCγ2 labeling between antigen-stimulated control and wortmannin-treated cells was apparent at the level of immunofluorescence microscopy; in addition, there was no labeling above background levels in cells stained with FITC-conjugated anti-mouse IgG secondary alone (these, and other negative control illustrations below, omitted for space consideration).

Different Distributions of PLCγ1 and PLCγ2: Immunoelectron Microscopy

Because ruffles represent folds in membranes, there is potential to misinterpret brightly stained structures as targeted sites of membrane translocation. We therefore localized PLCγ1 at the ultrastructural level by anti-PLCγ1 immunogold-labeling of thin sections of LR-White-embedded RBL-2H3 cells and transmission electron microscopy. Typical micrographs of PLCγ1 distribution in resting and antigen-stimulated cells are shown in Figure 6. Gold labeling was essentially absent from identical samples treated with Protein A-15 nm gold alone. The majority of gold particles labeling PLCγ1 in resting cells are found in the cell interior. However, a subpopulation of gold particles bound to resting cells are membrane-associated, and these are predictably located on microvilli, rather than on smooth membrane regions (Figure 6A). Gold particles are also present in the cytoplasm and nucleus and at the membrane of antigen-activated cells. The micrographs in Figure 6B-D show that most of the membrane-associated particles in activated cells are associated with lamellae. The preferential labeling of PLCγ1 in surface projections (microvilli, ruffles) suggests that it targets to regions of high membrane curvature, which are rich in actin.

To verify that FcεRI cross-linking results in PLCγ1 recruitment to the plasma membrane, a series of micrographs from replicate experiments were blind-coded and scored for gold particles located within 60 nm of the plasma membrane, within the nucleus, and over the remainder of the cell. Because of the limited contrast of cytoplasmic organelles in LR White-embedded samples, no attempt was made to assign gold particles to intracellular organelles other than the nucleus.

In resting cells, approximately 6% of total gold particles identifying PLCγ1 were found within 60 nm of the plasma membrane (Figure 8A). The remaining gold particles were distributed between the nucleus (12%) and the cytoplasm (82%). Our observation of intranuclear PLCγ1 is consistent with earlier reports that detected PLCγ in nuclear fractions by immunoblotting methods (Marmiroli *et al.*, 1994; Martelli *et al.*, 1994).

FcεRI cross-linking of control cells for 2 min increased the proportion of membrane-associated gold particles to approximately 10% (Figure 8A). Almost 15% of gold particles were membrane-associated after 10 min exposure to antigen. We showed previously that the antigen-induced transition of surface topography from a microvillous to a lamellar architecture is

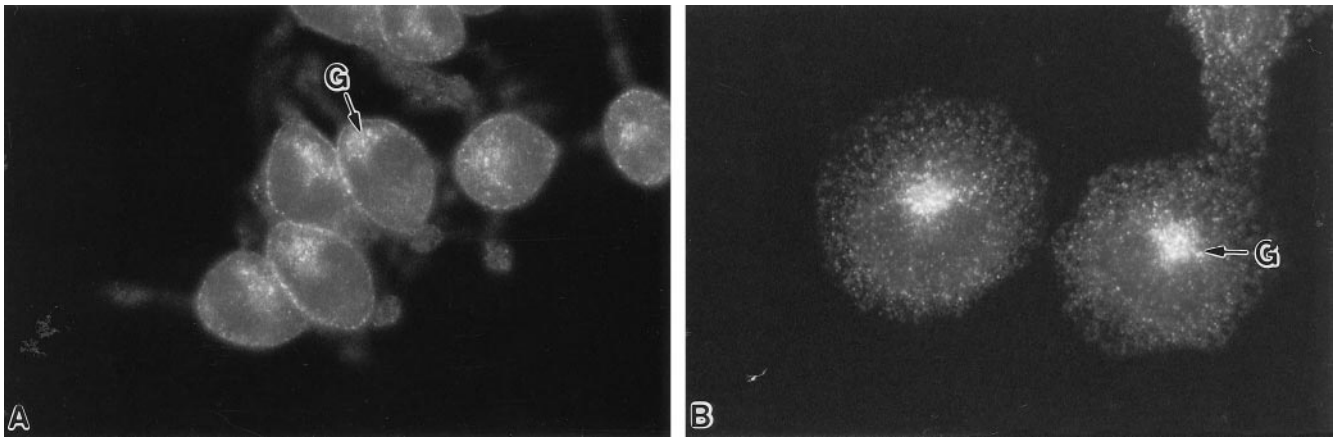

Figure 5. Immunoelectron microscopy of PLCγ1. Resting (A) and antigen-activated (B-D) (1 μg/ml DNP-BSA, 10 min) RBL-2H3 cells were embedded in LR White and thin sections labeled sequentially with anti-PLCγ1 and 15 nm Protein A-gold particles. Membrane-associated gold particles marking the location of PLCγ1 are circled in all panels to emphasize the concentration of membrane-associated PLCγ1 in membrane projections. Panels A and B show the presence of additional gold particles in the cell interior, including the nucleus. Micrographs are representative of results in three different experiments. Bar = 0.5 μm.

visible at 30 seconds, advanced after 2 min (when fringed lamellae are often visible), and complete by 5 to 10 min (Oliver *et al.*, 1997). It thus appears that PLCγ1 recruitment may accompany the ruffling response. In these experiments, we also observed that the proportion of gold particles in the nucleus did not change after antigen stimulation. Thus, PLCγ1 is recruited from the cytoplasmic pool to the plasma membrane in response to FcεRI cross-linking.

Results in Figure 8B show that the proportion of PLCγ1 at the plasma membrane of wortmannin-treated cells was slightly elevated at 2 min of antigen stimulation and had returned to basal levels by 10 min with antigen. These data implicate PI 3-kinase directly or indirectly in the process of antigen-induced PLCγ1 recruitment to the plasma membrane of RBL-2H3 cells.

In sharp contrast to the distribution of PLCγ1, the majority of gold particles localizing PLCγ2 were observed in close proximity to the Golgi stacks of both resting and activated cells (Figure 7C,D). Furthermore, the membrane-associated fraction of PLCγ2 failed to show a preferential localization to membrane ruffles in either resting (Figure 7A) or activated (Figure 7B) cells. Instead, PLCγ2 labeling was frequently just interior to the cortical actin network (arrow, Figure 7A). It was also noted in association with invaginations at the plasma membrane of activated cells (Figure 7E,F). Although clathrin coats are not visible in the LR White sections, our previous experience analyzing the membrane architecture of RBL-2H3 cells (Mao *et al.*, 1993) allows us to identify these structures as coated pits. Again, gold labeling was essentially absent from samples treated with Protein A 15 nm gold alone in combination with the rabbit anti-mouse bridge or with 30 nm goat anti-mouse colloidal gold.

The results of morphometric analyses showed little or no recruitment of PLCγ2 to the plasma membrane of activated cells (Figure 8C).

DISCUSSION

Antigen-induced PLCγ activation, leading to Ins(1,4,5)IP_3 production, is an early and important event in the FcεRI signaling cascade. The present study explores the relative contributions of PLCγ1 and PLCγ2 to total PLC activity in antigen-stimulated RBL-2H3 cells and begins to address mechanisms involved in activating these enzymes. The results of assays for phospholipase activity in isozyme-specific immunoprecipitates establish that FcεRI cross-linking activates both isoforms of PLCγ. Activation of both isoforms is associated with their increased phosphorylation on serine and tyrosine. The results of immunofluorescence and immunoelectron microscopic localization studies demonstrate that a small percentage of PLCγ1 associates with the plasma membrane of resting cells and, where present at the membrane, is primarily associated with membrane projections such as the leading edges of lamellae. After antigen stimulation, additional PLCγ1 is translocated to the plasma

Figure 6 (facing page). PLCγ2 distribution is not markedly altered after antigen stimulation. PLCγ2 was localized by immunofluorescence microscopy in unstimulated (A) and antigen-stimulated (B) (1 μg/ml DNP-BSA, 10 min) RBL-2H3 cell monolayers. PLCγ2 is distributed in small clusters associated with the Golgi region (G) and plasma membrane under both conditions. Magnification 750×.

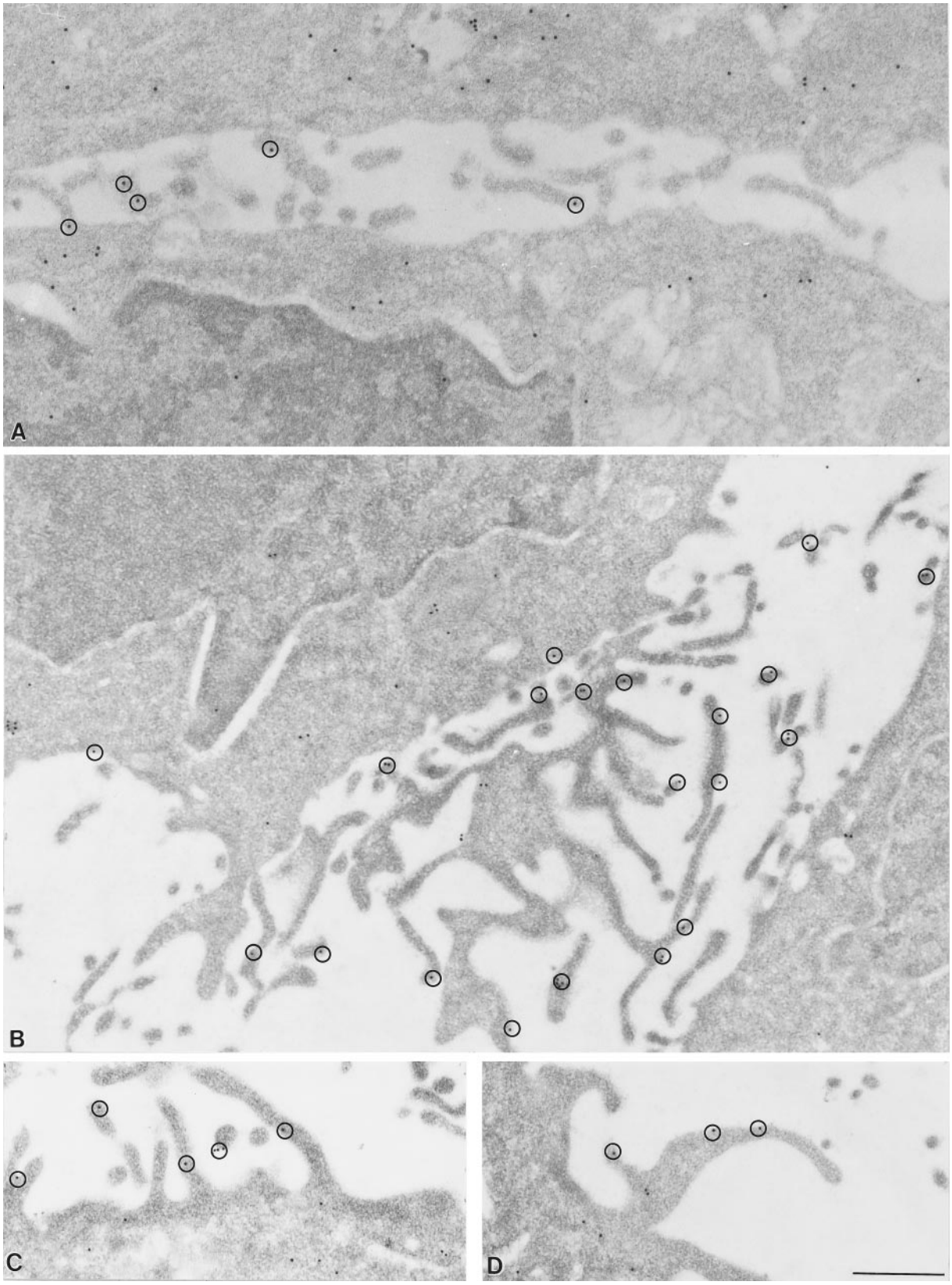

Figure 6.

membrane, where it associates with membrane ruffles. In contrast, the majority of PLCγ2 is concentrated in the Golgi region of both resting and activated cells, and the plasma membrane-associated portion of PLCγ2 does not increase appreciably after FcεRI cross-linking.

The topographical results add PLCγ1 to a growing list of cytoplasmic proteins that specifically target to specialized regions of the plasma membrane. Other examples include phospholipase A_2, Ras and Grb2 (Bar-Sagi *et al.*, 1988; Bar-Sagi *et al.*, 1993) that are found in microvilli and membrane ruffles of rat fibroblasts and the transmembrane protein, E-selectin, that is localized to the tips of microvilli in resting neutrophils (Erlandsen *et al.*, 1995). Bar-Sagi *et al.* (1993) also found that a truncated form of PLCγ1, containing only the SH3 domain, localizes to the stress fibers in fibroblasts. Since antigen-stimulated RBL-2H3 cells do not make stress fibers, but rather concentrate actin in surface projections, it is possible that association with the cytoskeleton is a primary means to recruit this PLCγ isoform to the plasma membrane. We note however that the other principal antigen-induced actin structures in RBL-2H3 cells, phosphotyrosine-containing adhesive structures known as actin plaques (Pfeiffer and Oliver, 1994), fail to label with anti-PLCγ1 antibodies. There is also precedent for the patchy distribution of the plasma membrane-associated component of PLCγ2. Wilson *et al.* (1994) reported a similar distribution for the heterotrimeric G protein, $Gi_{\alpha 2}$. Additionally, although FcεRI molecules are randomly distributed on the plasma membrane before activation, the cross-linked FcεRI redistributes away from membrane ruffles and projections and concentrates in clusters in the planar (flat) regions of the plasma membrane. These clusters are subsequently internalized through coated pits. Thus, the plasma membrane distributions of the PLCγ2 isoform and the cross-linked FcεRI partially overlap.

We established previously that nM wortmannin concentrations inhibit antigen-stimulated Ins(1,4,5)P_3 synthesis by 50 to 70% (Barker *et al.*, 1995). This result led us to hypothesize that PI 3-kinase may play a role in PLCγ activation. We report here that wortmannin blocks the activation of the PLCγ1 isoform, as measured in an in vitro phospholipase assay. This inhibition is associated with the inhibition of PLCγ1 phosphorylation and of PLCγ1 translocation to the plasma membrane. In contrast, wortmannin does not inhibit PLCγ2 activation and has little or no effect on PLCγ2 phosphorylation or distribution. These data suggest the possibility that, even though PLCγ2 is the more abundant enzyme, PLCγ1 may play a predominant role in mediating antigen-induced PtdIns(4,5)P_2 hydrolysis in RBL-2H3 cells. The results of immunolocalization studies, showing that a substantial proportion of PLCγ2 resides near the Golgi complex, may partially explain these results. Even though PLCγ2 is activated by FcεRI cross-linking, its ability to contribute to Ins(1,4,5)IP_3 production may be severely limited by its poor access to substrate, presumed to be most abundant at the plasma membrane.

On the basis of evidence that nM concentrations of wortmannin specifically inhibit PI 3-kinase (Thelen *et al.*, 1994; Wymann *et al.*, 1996), we hypothesize that PI 3-kinase contributes to the pathway leading to activation of PLCγ1. One likely mechanism involves a role for PI 3-kinase lipid products in PLCγ1 recruitment to the membrane. Both PLCγ isoforms have binding motifs, such as src (SH2, SH3) and pleckstrin homology (PH) domains, that are implicated in the interaction of other proteins with specific inositol phospholipids (Rameh *et al.*, 1995; Lemmon *et al.*, 1995; Hemmings, 1997). We suppose that distinct features within the hypervariable regions of the two PLCγ1 isoforms further defines the preferential targeting of the two isoforms to their predominant intracellular localizations. Once recruitment has occurred, interaction with PI 3-kinase lipid products has the potential to directly alter enzymatic activity. For example, D-3 phosphoinositides are known to activate protein kinase C types ϵ, η, and δ (Toker *et al.*, 1994). Lu *et al.* (1996) found basal PLCγ activity was enhanced approximately twofold in lipid micelle assays that incorporated PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. Thus, it is possible that PLCγ1 is recruited to the membrane and activated as a result of direct interaction with 3-phosphorylated phosphoinositides. Alternatively, PLCγ1 recruitment could occur indirectly via a membrane-associated platform/adaptor complex whose assembly is controlled by PI 3-kinase and its metabolites. Once at the membrane, PLCγ1 would be in close proximity to PtdIns(4,5)P_2 and its other substrates, PtdIns and PtdIns(4)P, as well as to membrane-associated tyrosine kinases. These possibilities–i.e., that D-3 phosphoinositides both recruit PLCγ1 to the membrane for activation by phosphorylation and directly enhance PLC activity–are not mutally exclusive. There is precedence for multiple roles for PI 3-kinase products in the activation of the serine kinase, c-Akt (also known as protein kinase B or PKB). PI 3-kinase lipid products

Figure 7 (facing page). Immunoelectron microscopy of PLCγ2. anti-PLCγ2 was localized in thin sections of LR White-embedded resting (A) and antigen-activated (B-F) RBL-2H3 cells by labeling with anti-PLCγ2 followed by 15 nm Protein A-gold with a rabbit anti-mouse bridge (B,C,E,F) or with 30 nm anti-mouse IgG-colloidal gold particles (A,D). 15 nm gold particles in B,C are circled for emphasis. Gold particles marking PLCγ2 associate with the plasma membrane and also prominently label vesicles in the Golgi region (G) in panels C,D. Occasional gold particles label Golgi stacks (arrows, D). Gold labeling was also found in association with coated pits (E,F). Micrographs are representative of results in 3 different experiments. Bar = 0.5 μm.

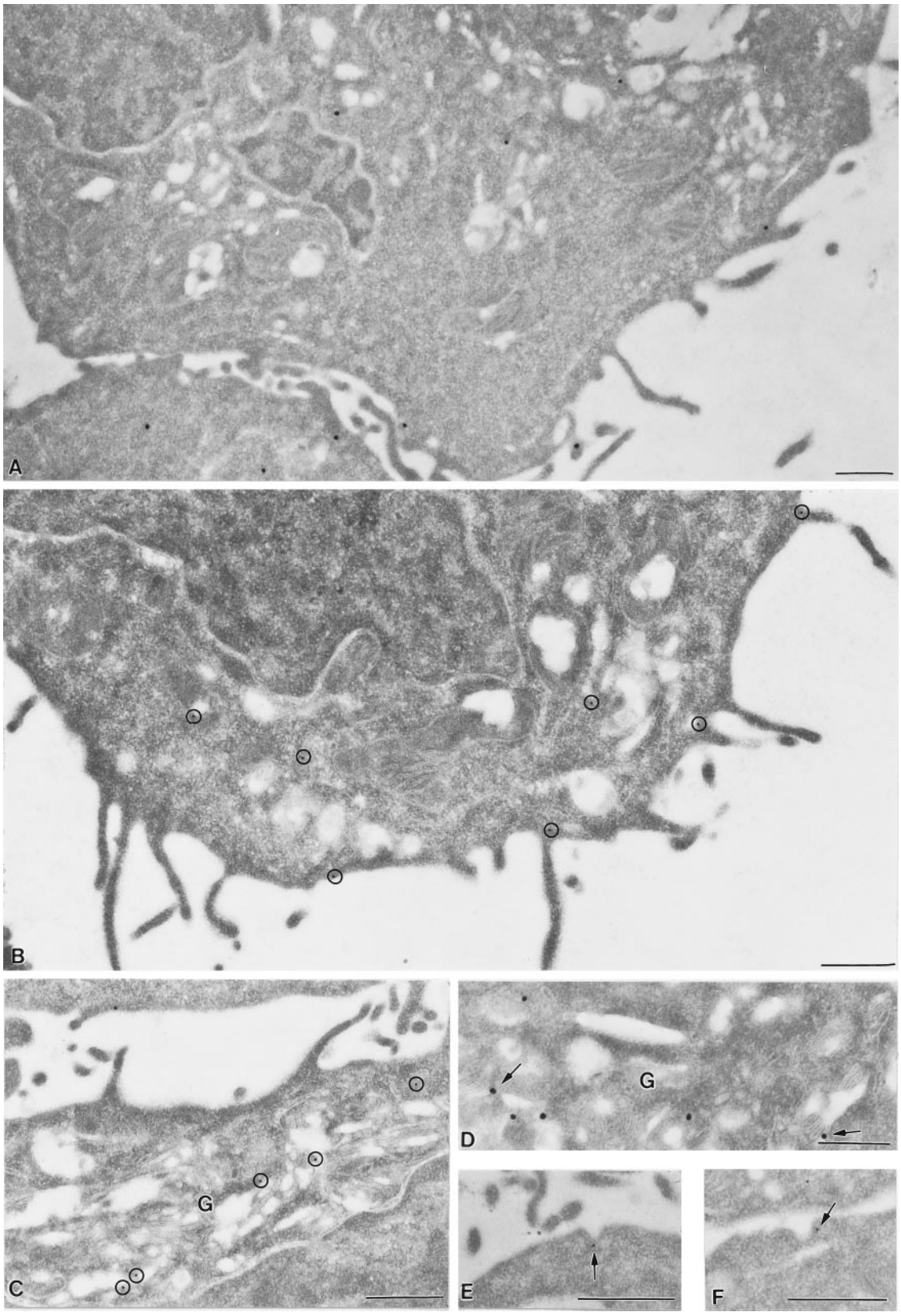

Figure 7.

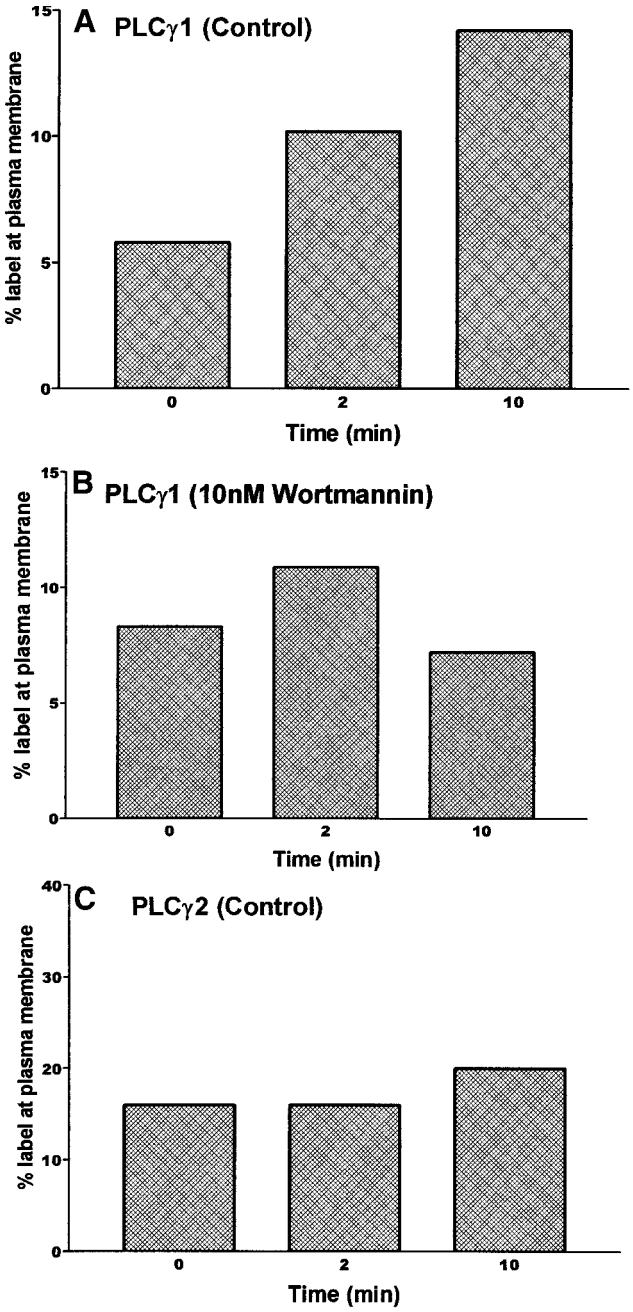

Figure 8. Morphometric measurements of gold-labeled PLCγ isoforms show wortmannin-sensitive translocation of PLCγ1, but not PLCγ2, to the plasma membrane. A) Percent of anti-PLCγ1 gold label within 60 nm of the plasma membrane of resting, control RBL-2H3 cells, followed by 2 or 10 min of antigen stimulation. B) Percent of anti-PLCγ1 gold label at the plasma membrane after 15 min wortmannin treatment, followed by 2 or 10 min of antigen stimulation. C) Percent of anti-PLCγ2 gold label at the plasma membrane of resting, control RBL-2H3 cells, followed by 2 or 10 min of antigen. Micrographs were blind-coded before counting; each experimental point represents the percent of total gold counts from replicate thin sections cut from blocks generated in 2 separate experiments. At least 1,000 gold particles were counted for each experimental condition.

bind and directly activate c-Akt (Franke *et al.*, 1997). In addition, PtdIns(3,4,5)P_3 activates PDK1, that phosphorylates c-Akt on threonine-308 and up-regulates Akt activity (Stokoe, *et al.*, 1997).

A large fraction of ^{32}P incorporated into PLCγ1 after IgE receptor stimulation is on serine (Li *et al.*, 1991; see also Figure 3). In earlier reports, Yamada *et al.* (1992) showed that several serine/threonine kinase inhibitors reduce the antigen-stimulated hydrolysis of total inositol phospholipids and tyrosine phosphorylation of PLCγ1 and we showed (Barker *et al.*, 1995) that nM concentrations of wortmannin block both serine and lipid kinase activities of PI 3-kinase. Thus it is also possible that the serine phosphorylation of PLCγ1 by PI 3-kinase may contribute to its maximal stimulation after receptor cross-linking. Alternatively PI 3-kinase may be upstream of another serine kinase, such as Akt (Burgering and Coffer, 1995; Bos, 1995), that in turn phosphorylates PLCγ1. The possibility that wortmannin directly inhibits another serine kinase, even at the low nM concentrations used in this study, also cannot be completely excluded. Previous reports of serine phosphorylation of PLCγ1 in other cell types implicated protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) in the *negative* regulation of PLCγ1 (reviewed in Rhee *et al.*, 1993). It follows that, if serine phosphorylation of PLCγ1 in RBL-2H3 cells is obligatory for maximal activation, then the target serine must be distinct from the PKC or PKA phosphorylation site at serine 1248.

Finally, we note the strong correlation between the ability of wortmannin treatment to inhibit both membrane ruffling and PLCγ1 activation. It is possible that activation of PLCγ1 precedes, or is dependent on, its assembly into macromolecular signaling complexes. These signaling complexes are likely to be associated with actin and other cytoskeletal elements that also participate in the formation of plasma membrane ruffles. Our current efforts are focused at defining which of these possible mechanisms underlies our observation that the FcεRI-mediated phosphorylation, translocation, and activation of PLCγ1 is blocked by wortmannin.

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