

# Mechanism of Tyrosine Phosphorylation and Activation of Phospholipase C- $\gamma$ 1

TYROSINE 783 PHOSPHORYLATION IS NOT SUFFICIENT FOR LIPASE ACTIVATION\*

Received for publication, May 7, 2004

Published, JBC Papers in Press, May 25, 2004, DOI 10.1074/jbc.M405116200

Fujio Sekiya $\ddagger$ , Benoit Poulin $\ddagger$ §, Yeun Ju Kim $\ddagger$ ¶, and Sue Goo Rhee $\ddagger$ ||

From the  $\ddagger$ Laboratory of Cell Signaling, NHLBI, National Institutes of Health, Bethesda, Maryland 20892 and the  $\ddagger$ Center for Cell Signaling Research and Division of Molecular Life Sciences, Ewha Womans University, Seoul 120-750, Korea

Phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1) is phosphorylated on three tyrosine residues: Tyr-771, Tyr-783, and Tyr-1253. With the use of antibodies specific for each of these phosphorylation sites, we have now determined the kinetics and magnitude of phosphorylation at each site. Phosphorylation of Tyr-783, which is essential for lipase activation, was observed in all stimulated cell types examined. The extent of phosphorylation of Tyr-1253 was ~50 to 70% of that of Tyr-783 in cells stimulated with platelet-derived growth factor (PDGF) or epidermal growth factor (EGF), but Tyr-1253 phosphorylation was not detected in B or T cell lines stimulated through B- and T-cell antigen receptors, respectively. Tyr-771 was phosphorylated only at a low level in all cells studied. In cells stimulated with PDGF, phosphorylation and dephosphorylation of Tyr-783 and of Tyr-1253 occurred with similar kinetics; the receptor kinase appeared to phosphorylate both sites, albeit with Tyr-783 favored over Tyr-1253, before the bound PLC- $\gamma$ 1 was released, and phosphorylation at the two sites occurred independently. PDGF and EGF induced similar levels of phosphorylation of Tyr-783 and of Tyr-1253 in a cell line that expressed receptors for both growth factors. However, only PDGF, not EGF, elicited substantial PLC activity, suggesting that Tyr-783 phosphorylation was not sufficient for enzyme activation. Finally, concurrent production of phosphatidylinositol 3,4,5-trisphosphate was found to contribute to the activation of phosphorylated PLC- $\gamma$ 1.

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>)<sup>1</sup> by phospholipase C (PLC) yields the intracellular messengers inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol and is an early signaling event triggered by many hormones, neurotrans-

mitters, and growth factors. The PLC family comprises a diverse group of isozymes. Mammalian cells thus express 12 different PLC gene products, which are grouped on the basis of their domain structure and mode of activation into five sub-families,  $\beta$  ( $\beta$ 1 to  $\beta$ 4),  $\gamma$  ( $\gamma$ 1 and  $\gamma$ 2),  $\delta$  ( $\delta$ 1 to  $\delta$ 4),  $\epsilon$ , and  $\zeta$  (1, 2). The activity of PLC isozymes is strictly controlled in cells; they are virtually inactive in resting cells but are able to generate their messenger products immediately in response to exposure of cells to various extracellular stimuli. Activation of the two  $\gamma$ -type isozymes, PLC- $\gamma$ 1 and PLC- $\gamma$ 2, requires their phosphorylation on tyrosine residues. PLC- $\gamma$ 1 is present in most cell types and at most developmental stages, and targeted deletion of its gene in mice results in early embryonic death, underlining the importance of this isozyme in cell proliferation and differentiation (1, 3, 4). In contrast, PLC- $\gamma$ 2 expression is restricted to hematopoietic cells, and deletion of the PLC- $\gamma$ 2 gene in mice results in impairment of immune function (5).

Activation of PLC- $\gamma$  by tyrosine phosphorylation is an important step in signaling by many stimuli that induce the activation of protein tyrosine kinases (PTKs). The intracellular domains of receptors for peptide growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor possess intrinsic PTK activity, and many of these receptor PTKs directly phosphorylate and activate PLC- $\gamma$ 1 (1, 4). A class of receptors present on hematopoietic cells that are collectively termed immunoreceptors also triggers the tyrosine phosphorylation of PLC- $\gamma$ 1 and PLC- $\gamma$ 2. Immunoreceptors comprise multiple polypeptide chains but do not possess intrinsic PTK activity; rather, in response to ligand binding, they recruit several distinct types of cytoplasmic PTKs and adapter proteins to form supramolecular complexes that activate PLC- $\gamma$  (1, 3, 6).

PLC- $\gamma$ 1 becomes phosphorylated on three tyrosine residues, Tyr-771, Tyr-783, and Tyr-1253 (amino acid numbering is based on the sequence of the rat protein), in cells stimulated with PDGF or EGF (7–9). The role of tyrosine phosphorylation of PLC- $\gamma$ 1 was investigated through substitution of Phe for Tyr at each of these three sites and expression of the mutant enzymes in NIH 3T3 cells. Substitution of Tyr-783 with Phe prevented the activation of PLC by PDGF, whereas substitution of Tyr-1253 or Tyr-771 resulted in partial or no inhibition, respectively (9). Further examination of the effects of Tyr  $\rightarrow$  Phe substitution by expression of the three mutant proteins in fibroblasts deficient in PLC- $\gamma$ 1 (Null TV-1 cells) revealed that Tyr-783 was essential for PDGF-dependent activation but that Tyr-771 and Tyr-1253 were dispensable.<sup>2</sup> The evaluation of the Y1253F mutant in Null TV-1 cells is likely more reliable than

\* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Interactions Cellulaires Neuroendocriniennes, UMR 6544, CNRS, Université de la Méditerranée, Institut Fédératif de Recherche Jean Roche, 13916 Marseille, France.

|| To whom correspondence should be addressed: Laboratory of Cell Signaling, NHLBI, National Institutes of Health, Bldg. 50, Rm. 3523, South Drive, MSC 8015, Bethesda, MD 20892. Tel.: 301-496-9646; Fax: 301-480-0357; E-mail: sgrhee@nih.gov.

<sup>1</sup> The abbreviations used are: PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PIP<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate; IP, inositol monophosphate; IP<sub>2</sub>, inositol bisphosphate; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PI3K, phosphoinositide 3-kinase; PTK, protein-tyrosine kinase; PDGF(R), platelet-derived growth factor (receptor); EGF(R), epidermal growth factor (receptor); DMEM, Dulbecco's modified Eagle's medium.

<sup>2</sup> B. Poulin, F. Sekiya, and S. G. Rhee, unpublished observation.

that in NIH 3T3 cells given that the latter contain endogenous wild-type PLC- $\gamma$ 1.

Activation of PLC- $\gamma$ 1 in cells is always accompanied by its tyrosine phosphorylation, and the extent of this phosphorylation is commonly regarded as a measure of its activation status. However, our unpublished observations have suggested that the lipase activity of PLC- $\gamma$ 1 measured in various stimulated cells does not correlate well with the extent of PLC- $\gamma$ 1 tyrosine phosphorylation as determined by immunoblot analysis with antibodies to tyrosine phosphate such as 4G10 or PY20. One possible explanation for these observations is that tyrosine phosphorylation alone might not be sufficient for the activation of PLC- $\gamma$ 1. Indeed, lipids such as phosphatidic acid (10), arachidonic acid (11, 12), and phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) (13–16) have been shown to augment the catalytic activity of PLC- $\gamma$ 1. Another possibility is that total tyrosine phosphorylation of PLC- $\gamma$ 1 measured with conventional antibodies to tyrosine phosphate may not closely reflect the extent of phosphorylation at the essential residue Tyr-783. Whether or not multiple phosphorylation, such as that on Tyr-783 and Tyr-1253, occurs in the same molecule or in different PLC- $\gamma$ 1 molecules also might be an important determinant of PLC function.

To address these questions, we have now measured the kinetics and extent of phosphorylation of each of the three tyrosine residues of PLC- $\gamma$ 1 with the use of phospho-specific antibodies raised against the three phosphorylation sites. We found that phosphorylation of PLC- $\gamma$ 1 on Tyr-783 is accompanied by phosphorylation on Tyr-1253 in growth factor-stimulated cells but not in B cells or T cells stimulated via their respective immunoreceptors. Our results also indicate that phosphorylation of PLC- $\gamma$ 1 on Tyr-783 *per se* is not sufficient to initiate phosphoinositide hydrolysis, with the lipase activity of the tyrosine-phosphorylated enzyme also being determined by other factors, including the production of PIP<sub>3</sub>.

#### MATERIALS AND METHODS

**Materials and Cell Lines**—PDGF-BB (human recombinant) and purified active tyrosine kinases (EGFR, ZAP-70, and Src) were obtained from Upstate Biotechnology. EGF (human recombinant) and LY294002 were from Calbiochem. Null-TV1 cells, which are derived from PLC- $\gamma$ 1<sup>-/-</sup> mouse embryonic fibroblasts (17), were kindly provided by G. Carpenter (Vanderbilt University School of Medicine, Nashville, TN). NIH 3T3/EGFR cells were as described previously (18). Null-TV1, A431, and NIH 3T3/EGFR cells were maintained under a humidified atmosphere of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium supplemented with penicillin-streptomycin and either 10% fetal bovine serum (Null TV-1 and A431) or 10% calf serum (NIH 3T3/EGFR). Ramos and Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin.

**Antibodies**—A mixture of mouse monoclonal antibodies to PLC- $\gamma$ 1 was previously described (19). Affinity-purified rabbit polyclonal antibodies specific for PLC- $\gamma$ 1 phosphorylated on Tyr-783 (anti-pY783) were obtained from Biosource/QCB. Antibodies to PLC- $\gamma$ 1 phosphorylated on Tyr-771 or on Tyr-1253 (anti-pY771 and anti-pY1253, respectively) were prepared by injection of rabbits with the phosphotyrosyl peptides EPD(pY)GALYEGC (corresponding to residues 768–777 of rat PLC- $\gamma$ 1 plus a terminal Cys residue for conjugation; pY, phosphorylated Tyr) or FEAR(pY)QQPFGC (residues 1249–1257 plus a linker Gly residue and a Cys residue), respectively, each conjugated with keyhole limpet hemocyanin and synthesized by BioSynthesis. Antisera were depleted of cross-reactive antibodies with nonantigen phosphorylated peptides (pY783 and pY1253 peptides for anti-pY771; pY771 and pY783 peptides for anti-pY1253) to prevent possible recognition of phosphorylated sites other than the target. Given that isolation of monospecific antibodies with the use of immobilized phosphopeptide antigens did not result in improvement of specificity, we used the antisera without further purification for our experiments. Mouse monoclonal antibody to Akt phosphorylated on Ser-473 was from Cell Signaling, goat F(ab')<sub>2</sub> fragments specific for human IgM were from Jackson ImmunoResearch, and the mouse monoclonal antibody OKT-3 to CD3 was kindly provided by L. Samelson (NCI, National Institutes of Health, Bethesda, MD).

**Expression of PLC- $\gamma$ 1 Mutants**—Mutants of rat PLC- $\gamma$ 1 in which the phosphorylation-site Tyr residues had been substituted with Phe were described previously (9). The coding sequences of these mutant proteins were transferred to the vector pSC11, and recombinant vaccinia viruses for expression in mammalian cells were prepared as described previously (20). Null TV-1 cells were exposed to the recombinant viruses for 3 h and then cultured in medium containing 1% fetal bovine serum for 24 h.

**Preparation of Cell Lysates and Immunoblot Analysis**—Adherent A431, Null TV-1, or NIH 3T3/EGFR cells grown in six-well culture plates to 50–80% confluence were incubated for 24 h in medium containing 1% serum before stimulation with growth factors. The serum-depleted cells were washed, incubated for 10 min at 37 °C with DMEM (1 ml per well) supplemented with 20 mM HEPES-NaOH (pH 7.4) (plus 1 mM sodium vanadate where indicated), and then stimulated as indicated. After incubation for the indicated times, the medium was removed, and lysis buffer I (20 mM HEPES-NaOH (pH 7.2), 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM sodium vanadate, 0.1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, leupeptin (5  $\mu$ g/ml), aprotinin (5  $\mu$ g/ml), 0.2 ml per well) was added to the cells, which were then incubated for 20 min on ice. The cell lysates were centrifuged and the resulting supernatants were subjected to SDS-PAGE on 6% gels (Novex). The separated proteins were transferred electrophoretically to a nitrocellulose membrane, which was then incubated with primary antibodies. Immune complexes were detected with alkaline phosphatase-conjugated secondary antibodies and the chemiluminescent reagent CDP-Star (Tropix). Images were captured and analyzed with a Kodak Image Station 440 equipped with a charge-coupled device camera.

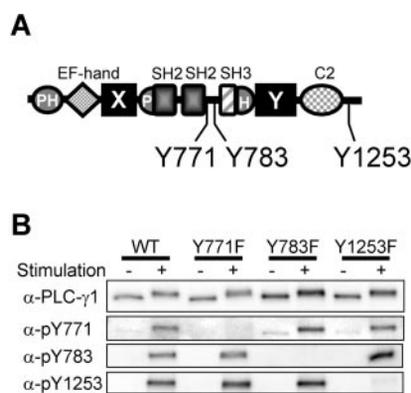
Ramos and Jurkat cells were incubated overnight in medium containing 0.5% serum, washed, suspended in RPMI 1640 supplemented with 20 mM HEPES-NaOH (pH 7.4) and 1 mM sodium vanadate, incubated for 10 min at 37 °C, and then stimulated with antibodies to their immunoreceptors. Stimulation was terminated by the addition of 0.25 volumes of ice-cold lysis buffer II (20 mM HEPES-NaOH (pH 7.5), 100 mM NaF, 5 mM sodium vanadate, 20 mM EDTA, 5% Triton X-100, 1.25% sodium deoxycholate, 0.5 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, leupeptin (25  $\mu$ g/ml), aprotinin (25  $\mu$ g/ml)) and incubation for 20 min on ice. After the removal of cell debris by centrifugation, the remaining supernatants were subjected to immunoblot analysis as described above. For stimulation with pervanadate, cells were equilibrated with 1 mM sodium vanadate for 10 min then stimulated with 2 mM H<sub>2</sub>O<sub>2</sub>.

**Immunoprecipitation**—Cell lysates were diluted 5-fold with lysis buffer I and then incubated at 4 °C first with appropriate antibodies for 1 h (with rocking) and then for an additional 2 h with 50  $\mu$ l of a 50% slurry of Protein G-Sepharose (Amersham Biosciences). The Protein G-Sepharose beads were then recovered by centrifugation, washed twice with lysis buffer I and once with water, suspended in SDS-PAGE sample buffer, and boiled. The eluted proteins were subjected to immunoblot analysis as described above.

**In Vitro Phosphorylation of PLC- $\gamma$ 1**—Purified rat PLC- $\gamma$ 1 (20  $\mu$ g/ml) was incubated for the indicated times at 30 °C in a solution containing 20 mM HEPES-NaOH (pH 7.0), 100 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 0.1 mM sodium vanadate, 1 mM ATP, bovine serum albumin (0.1 mg/ml), and either EGF receptor (EGFR, 0.5 unit/ml), ZAP-70 (1  $\mu$ g/ml), or Src (4  $\mu$ g/ml). The reaction was terminated by the addition of SDS-PAGE sample buffer, and the extent of PLC- $\gamma$ 1 phosphorylation was assessed by immunoblot analysis.

**Measurement of Inositol Phosphates**—Cells (in six-well plates) were transferred to *myo*-inositol-free DMEM containing 1% calf serum and incubated for 2 days in the presence of *myo*-[2-<sup>3</sup>H]inositol (1  $\mu$ Ci/ml, 25 Ci/mol; PerkinElmer Life Sciences). After washing with DMEM, the cells were incubated for 10 min in DMEM supplemented with 20 mM LiCl and 20 mM HEPES-NaOH (pH 7.4) (plus 1 mM sodium vanadate where indicated) and were then exposed to growth factors for various times. The medium was subsequently removed, and the cells were scraped into 1 ml of 5% perchloric acid on ice. The cell extracts were centrifuged to remove debris, and the resulting supernatants were neutralized with 2 M KOH before analysis of inositol phosphates by high-performance liquid chromatography on a Partisil SAX-10 (Whatman) ion-exchange column with a Flo-One on-line radiometric detector (Packard), as described previously (21).

**Measurement of PIP<sub>3</sub>**—Serum-depleted NIH 3T3/EGFR cells were washed with phosphate-free DMEM, incubated for 1 h in the same medium, and then labeled for 1 h with [<sup>32</sup>P]orthophosphate (0.5 mCi/ml; ICN) again in phosphate-free DMEM. After stimulation of the cells with



**FIG. 1. Characterization of antibodies specific for each phosphorylation site of PLC- $\gamma$ 1.** *A*, schematic representation of the domain organization of PLC- $\gamma$ 1 and the location of the three tyrosine phosphorylation sites. *B*, Null TV-1 cells were infected with recombinant vaccinia viruses encoding either wild-type (WT) PLC- $\gamma$ 1 or Tyr  $\rightarrow$  Phe mutants (Y771F, Y783F, and Y1253F) thereof. They were then deprived of serum overnight and stimulated (or not) for 10 min with a combination of PDGF (100 ng/ml) and pervanadate (2 mM H<sub>2</sub>O<sub>2</sub> in the presence of 1 mM sodium vanadate). Cell lysates were subjected to immunoblot analysis with anti-PLC- $\gamma$ 1 or with phospho-specific antibodies against PLC- $\gamma$ 1, as indicated.

EGF or PDGF, the medium was removed and the cells were scraped into 10% trichloroacetic acid on ice. The cell lysates were centrifuged, and phospholipids were extracted from the pellets as previously described (22). The extracted phospholipids were subjected to deacylation by treatment with methylamine (23), and the released fatty acids were removed by extraction with chloroform. The remaining aqueous phase containing glycerophosphoinositol phosphates was then analyzed by high-performance liquid chromatography on a Partisil SAX-10 column with a 70-min nonlinear gradient of 0 to 1 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>. The radioactivity associated with deacylated PIP<sub>3</sub> in the eluate was monitored with an on-line detector and was expressed as a percentage of the total radioactivity in the phospholipid fraction.

## RESULTS

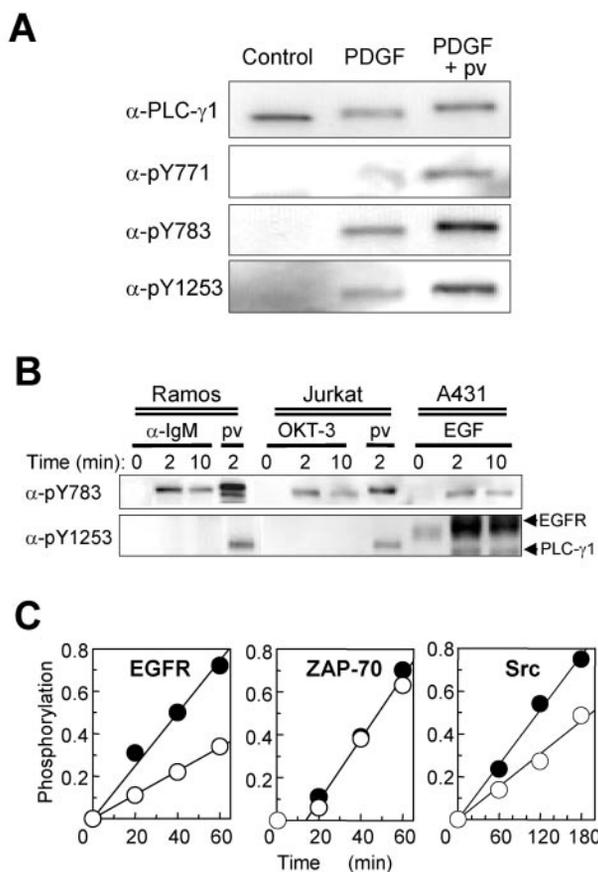
**Characterization of Antibodies Specific for Phosphorylated PLC- $\gamma$ 1**—Of the three phosphorylation sites of PLC- $\gamma$ 1, Tyr-771 and Tyr-783 are located between the X and Y catalytic domains and Tyr-1253 is localized near the COOH terminus (Fig. 1A). Polyclonal antibodies specific for PLC- $\gamma$ 1 phosphorylated on Tyr-783 (anti-pY783) are commercially available, and we have prepared anti-pY771 and anti-pY1253. The specificities of these various antibodies were tested with PLC- $\gamma$ 1-deficient fibroblasts (Null TV-1 cells) after their infection with vaccinia virus vectors for wild-type PLC- $\gamma$ 1 or for each of the Tyr  $\rightarrow$  Phe phosphorylation site mutants (Y771F, Y783F, and Y1253F). The cells were stimulated with a combination of PDGF and pervanadate to induce maximal phosphorylation of PLC- $\gamma$ 1; vanadate (VO<sub>4</sub><sup>3-</sup>) is a reversible inhibitor of a wide variety of phosphatases, but, in the presence of H<sub>2</sub>O<sub>2</sub>, it is converted to pervanadate (a general term for the various adducts formed between vanadate and H<sub>2</sub>O<sub>2</sub>), which irreversibly oxidizes the essential cysteine residue of protein tyrosine phosphatases (24). Lysates of the cells were then subjected directly to immunoblot analysis (Fig. 1B). Anti-pY783 and anti-pY1253 did not recognize PLC- $\gamma$ 1 before cell stimulation but yielded a single immunoreactive band at the position corresponding to the molecular size of PLC- $\gamma$ 1 after cell stimulation, indicating that they are specific for phosphorylated forms of PLC- $\gamma$ 1. Anti-pY771 exhibited a low level of reactivity with PLC- $\gamma$ 1 in nonstimulated cells but showed a much higher level of reactivity with a band of slightly lower mobility, corresponding to phosphorylated PLC- $\gamma$ 1, after cell stimulation.

The specificity of each of the three antibody preparations was high, as indicated by the fact that mutation of each of the target tyrosine residues prevented recognition of PLC- $\gamma$ 1 by the cor-

responding antibodies but not by the other two antibody preparations (Fig. 1B). The binding of these antibodies to PLC- $\gamma$ 1 thus requires not only phosphorylation of tyrosine but also the amino acid sequence surrounding each phosphorylated tyrosine residue. The detection of phosphorylated PLC- $\gamma$ 1 by anti-pY771 or anti-pY1253 was not affected by the inclusion of phosphotyrosine (0.1 mM) in the immunoblot reaction mixture, whereas that by anti-pY783 was partially inhibited (data not shown). The same treatment completely blocked the reactivity of tyrosylphosphorylated proteins with the anti-phosphotyrosine antibody 4G10. In addition, the reactivity of phosphorylated PLC- $\gamma$ 1 with each of the three antibody preparations was blocked by the respective phosphorylated peptide used as the antigen for antibody production but not by the other two phosphorylated peptides (data not shown). Anti-pY771 and anti-pY1253 sometimes recognized several cellular proteins other than PLC- $\gamma$ 1 in a phosphorylation-dependent manner, whereas anti-pY783 did not. In particular, phosphorylated EGFR was markedly reactive with anti-pY1253 (Figs. 2B and 4B) despite the fact that it does not contain an amino acid sequence similar to that surrounding Tyr-1253 of PLC- $\gamma$ 1.

**Phosphorylation of PLC- $\gamma$ 1 in Various Cell Types**—We next investigated the reactivity of the phospho-specific antibodies to PLC- $\gamma$ 1 with lysates of NIH 3T3/EGFR fibroblasts (NIH 3T3 cells that express exogenous EGFR in addition to endogenous PDGFR) that had been exposed to PDGF or to a combination of PDGF and pervanadate. Treatment of the cells with PDGF plus pervanadate induced a marked increase in PLC- $\gamma$ 1 reactivity with all the phospho-specific antibodies (Fig. 2A). Immunoblot analysis with anti-PLC- $\gamma$ 1 antibody revealed that the PLC- $\gamma$ 1 band of cells stimulated with PDGF plus pervanadate migrated more slowly than did that of nonstimulated cells or that of cells stimulated with PDGF alone and yet was as sharp as the band corresponding to nonphosphorylated PLC- $\gamma$ 1 in nonstimulated cells (Fig. 2A). Increasing the incubation time or the concentrations of PDGF and pervanadate did not increase further the blot intensities obtained with any of the three phospho-specific antibody preparations (data not shown). Treatment of immunoprecipitated PLC- $\gamma$ 1 with alkaline phosphatase abolished both the shift in the mobility of the PLC- $\gamma$ 1 band and its reactivity with the phospho-specific antibodies induced by treatment of the cells with PDGF and pervanadate (data not shown), indicating that the mobility shift was due to phosphorylation. Together, these results indicate that most PLC- $\gamma$ 1 molecules in the cells treated with PDGF and pervanadate are phosphorylated at all three tyrosine phosphorylation sites. In contrast, the broad immunoreactive band detected with antibodies to PLC- $\gamma$ 1 in cells stimulated with PDGF alone was suggestive of heterogeneity in the extent of phosphorylation (Fig. 2A). The mobility of this band was intermediate between those of the nonphosphorylated and fully phosphorylated PLC- $\gamma$ 1 bands; the extent of phosphorylation of Tyr-783 and Tyr-1253 was substantial but reduced compared with that apparent in the cells stimulated with PDGF plus pervanadate, and the phosphorylation of Tyr-771 was barely detectable.

We compared the phosphorylation profiles of PLC- $\gamma$ 1 in three different human cell types: Ramos B lymphoma cells stimulated by cross-linking of the B cell receptor, Jurkat T lymphoma cells stimulated by cross-linking of CD3, and A431 epidermoid carcinoma cells stimulated with EGF (Fig. 2B). The antigenic peptide (FEAR(pY1253)QQPF) used for the preparation of anti-pY1253 was based on the rat protein, which differs in this region by a single amino acid residue (underlined) from the human proteins (FESR(pY1254)QQPF). Although anti-pY1253 is able to recognize both the human and rodent proteins upon phosphorylation, reactivity of anti-pY1253 toward



**FIG. 2. Phosphorylation of PLC- $\gamma$ 1 monitored by immunoblot analysis with phospho-specific antibodies.** *A*, serum-deprived NIH 3T3/EGFR cells were stimulated (or not) in the presence of 1 mM sodium vanadate 10 min with PDGF (100 ng/ml) or PDGF plus pervanadate (pv). Cell lysates were then subjected to immunoblot analysis with the indicated antibodies. *B*, Ramos cells were stimulated with a saturating amount of goat anti-human IgM F(ab')<sub>2</sub> (10  $\mu$ g/ml) or with pervanadate. Jurkat cells were stimulated either with a saturating dose of the OKT-3 anti-CD3 antibody (5  $\mu$ g/ml) or with pervanadate. A431 cells were stimulated with EGF (20 ng/ml). Cell lysates were then subjected to immunoblot analysis with the indicated antibodies. The amounts of protein applied to the gel were adjusted so that samples from Ramos cells stimulated with anti-IgM for 2 min, from Jurkat cells stimulated with OKT-3 for 2 min, and from A431 cells stimulated with EGF for 2 min showed similar intensities on the blot with anti-pY783. The positions of PLC- $\gamma$ 1 and (cross-reactive) EGFR are indicated for the blot with anti-pY1253. *C*, purified rat PLC- $\gamma$ 1 was subjected for the indicated times to phosphorylation *in vitro* by either EGFR, ZAP-70, or Src and was then analyzed for the extent of phosphorylation on Tyr-783 (closed circles) and Tyr-1253 (open circles) by immunoblot analysis. Data are expressed as arbitrary units and are from a representative experiment. The lag period observed with ZAP-70 likely reflects the time required for activation of the kinase.

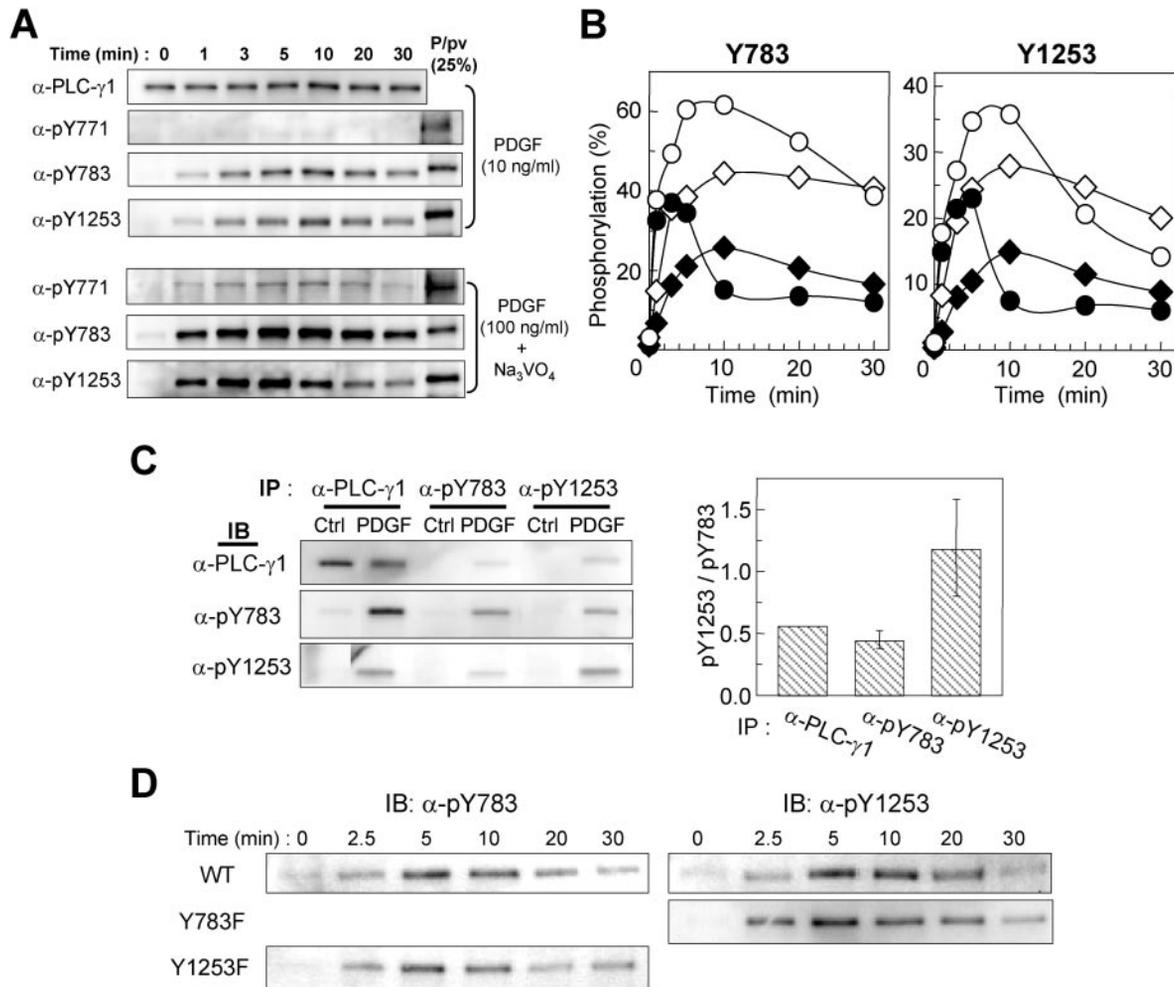
them may not be the same, which would make direct comparison of samples of different origins difficult. Phosphorylation of Tyr-783 was induced in all three cell lines by stimulation with their respective ligands, whereas phosphorylation of Tyr-1254 (equivalent to Tyr-1253 of rodent PLC- $\gamma$ 1) was readily observed in A431 cells but was virtually undetectable in Jurkat and Ramos cells (Fig. 2*B*). Phosphorylation of Tyr-771 was hardly detected in any of the three cell lines (data not shown). Stimulation of Ramos or Jurkat cells by pervanadate induced marked phosphorylation of PLC- $\gamma$ 1 on Tyr-1254 (Fig. 2*B*), indicating that the cells are capable of phosphorylating PLC- $\gamma$ 1 on this residue. Phosphorylation of Tyr-771 was also observed in all three cell lines stimulated by pervanadate (data not shown).

To determine whether the differences in the phosphorylation patterns of PLC- $\gamma$ 1 among these human cell lines were attributable to differences in the specificity of the responsible ki-

nases, we examined the phosphorylation of purified rat PLC- $\gamma$ 1 by EGFR, ZAP-70, and Src *in vitro* (Fig. 2*C*). ZAP-70, which belongs to the Syk family of PTKs, is thought to be the enzyme predominantly responsible for the phosphorylation of PLC- $\gamma$ 1 in Jurkat cells stimulated via the T cell receptor, and members of the Src family of PTKs are also implicated in the phosphorylation of PLC- $\gamma$ 1 in lymphocytes (25, 26). All three PTKs phosphorylated both Tyr-783 and Tyr-1253, whereas EGFR and Src catalyzed the phosphorylation of Tyr-783 faster than they did that of Tyr-1253, and ZAP-70 phosphorylated the two sites at similar rates. These results thus indicate that the lack of Tyr-1253 phosphorylation in the immune cells is not likely due to the specificity of kinases involved in PLC- $\gamma$ 1 phosphorylation.

**Mechanism of Growth Factor-induced PLC- $\gamma$ 1 Phosphorylation**—To gain insight into the mechanism of tyrosine phosphorylation of PLC- $\gamma$ 1, we measured the time course of phosphorylation at each site in NIH 3T3/EGFR cells stimulated with PDGF. As shown in Fig. 2*A*, PLC- $\gamma$ 1 is fully phosphorylated at all three sites in NIH 3T3/EGFR cells stimulated with PDGF plus pervanadate; we therefore defined 100% phosphorylation at a given position as the corresponding immunoblot intensity obtained with PLC- $\gamma$ 1 from these stimulated cells. The percentage of PLC- $\gamma$ 1 molecules phosphorylated on Tyr-783 and Tyr-1253 in cells stimulated with either a saturating (100 ng/ml) or a subsaturating (10 ng/ml) concentration of PDGF in the absence or presence of vanadate was calculated from the corresponding immunoblot intensities and plotted against time (Fig. 3, *A* and *B*). Treatment with vanadate alone did not induce any detectable phosphorylation of PLC- $\gamma$ 1. Under all conditions, the time courses of phosphorylation were similar for the two sites, and the extent of Tyr-783 phosphorylation was  $\sim$ 1.5- to 2-fold greater than that of Tyr-1253 phosphorylation. The higher concentration of PDGF (100 ng/ml) in the absence of vanadate elicited transient phosphorylation of both Tyr-783 and Tyr-1253; the extent of phosphorylation was maximal (40% for Tyr-783 and 25% for Tyr-1253) within 3–5 min and then decreased rapidly. Stimulation with PDGF at the lower concentration (10 ng/ml) in the absence of vanadate resulted in slower rates both of phosphorylation, which was maximal (25% for Tyr-783 and 15% for Tyr-1253) after  $\sim$ 10 min, and of dephosphorylation. Inclusion of vanadate enhanced phosphorylation at both sites, with maximal values of 60% for Tyr-783 and 35% for Tyr-1253 with PDGF at 100 ng/ml and of 45% for Tyr-783 and 30% for Tyr-1253 with PDGF at 10 ng/ml. It also markedly reduced the rate of decay of the phosphorylation at each site induced by PDGF at 100 ng/ml. Phosphorylation of PLC- $\gamma$ 1 on Tyr-771 was detectable only in cells stimulated with PDGF at 100 ng/ml in the presence of vanadate, but the maximal level achieved was  $<$ 5%.

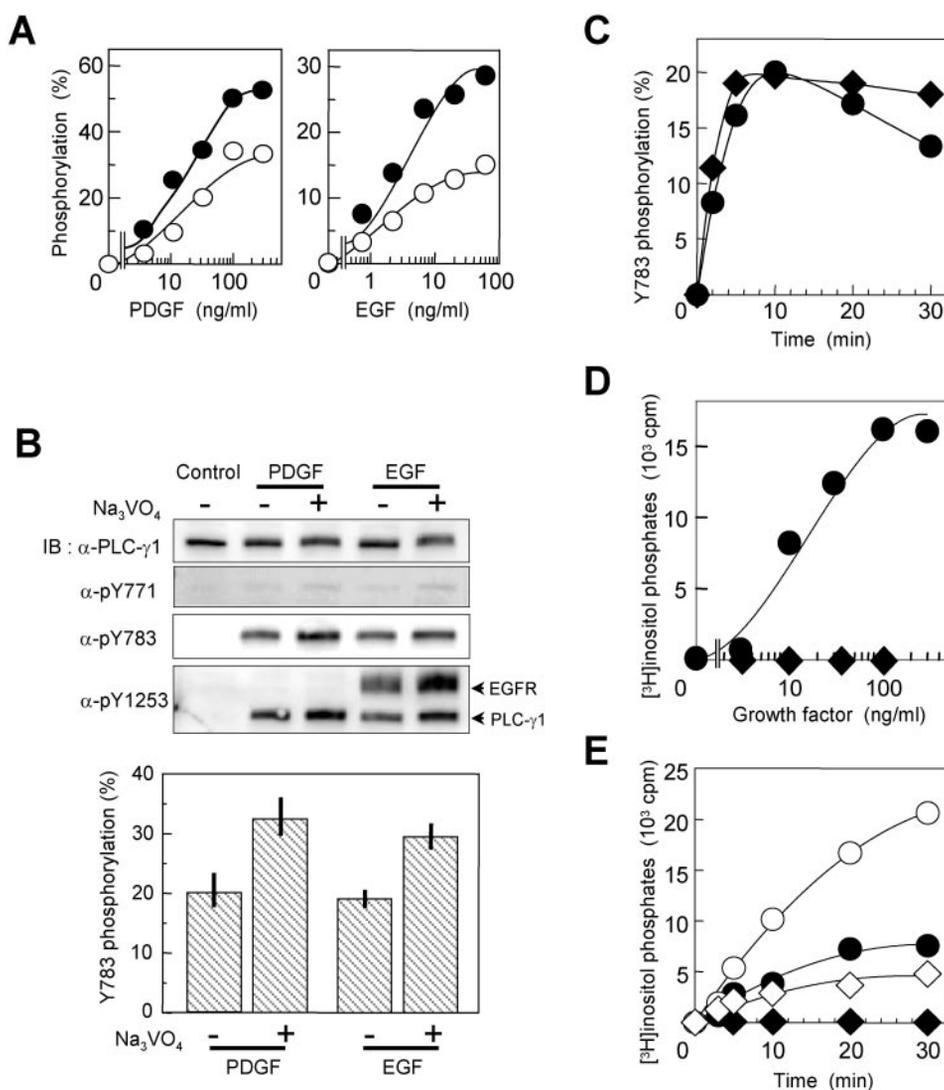
We also assessed the concordance of phosphorylation among sites. PLC- $\gamma$ 1 associates with receptor PTKs as a result of interaction between its NH<sub>2</sub>-terminal SH2 domain and a phosphorylated tyrosine residue of the receptor. We tested whether PLC- $\gamma$ 1 bound to PDGFR becomes phosphorylated on both Tyr-783 and Tyr-1253 before its dissociation from the receptor. In such a linked scenario, Tyr-1253 phosphorylation and Tyr-783 phosphorylation would thus occur together on the same bound PLC- $\gamma$ 1 molecule. In a random scenario, however, in which receptor-bound PLC- $\gamma$ 1 is phosphorylated on only one of these sites before its dissociation, phosphorylation of both residues of the same PLC- $\gamma$ 1 molecule would be expected to be less likely. We sought to distinguish between these scenarios by comparing the extent of Tyr-1253 phosphorylation in the pool of PLC- $\gamma$ 1 molecules phosphorylated on Tyr-783 with that in the total pool of PLC- $\gamma$ 1 molecules.



**FIG. 3. Mechanism of PDGF-induced PLC- $\gamma$ 1 phosphorylation.** A, NIH 3T3/EGFR cells were stimulated for various times either with PDGF (10 ng/ml) in the absence of sodium vanadate (*upper four blots*) or with PDGF (100 ng/ml) in the presence of 1 mM sodium vanadate (*lower three blots*). Cell lysates were then subjected to immunoblot analysis with the indicated antibodies. For comparison, cells were also stimulated with PDGF (100 ng/ml) plus sodium pervanadate (*right-most lanes, P/pv* (25%)), and the amount of lysate applied to the gels was 25% of that applied in the other lanes; the intensity of the PLC- $\gamma$ 1 bands in the *P/pv* lanes was taken as 25% to calculate the extent of phosphorylation at each site as shown in B. B, the extent of phosphorylation of PLC- $\gamma$ 1 on Tyr-783 and Tyr-1253 in experiments similar to that shown A was quantified by measurement of immunoblot intensities. Cells were thus stimulated with PDGF (100 ng/ml) in the absence (*closed circles*) or presence (*open circles*) of 1 mM sodium vanadate, or with PDGF (10 ng/ml) in the absence (*closed diamonds*) or presence (*open diamonds*) of 1 mM sodium vanadate. Data are means from four independent determinations, with the size of the symbols approximately representing  $\pm$  S.E. C, NIH 3T3/EGFR cells were left unstimulated (*Ctrl*) or stimulated for 5 min with PDGF (30 ng/ml) in the presence of 1 mM sodium vanadate. Cell lysates were then subjected to immunoprecipitation (*IP*) with the indicated antibodies, and the resulting precipitates were subjected to immunoblot analysis (*IB*) with the same antibodies (*left panel*). The ratio of the blot intensity obtained with anti-pY1253 to that obtained with anti-pY783 was measured for each of the various precipitates prepared from PDGF-stimulated cells and was then normalized relative to the value obtained for the precipitates prepared with the antibodies to PLC- $\gamma$ 1 (*right panel*); data are means  $\pm$  S.E. of four independent determinations. D, Null TV-1 cells were infected with vaccinia viruses for wild-type PLC- $\gamma$ 1 or for the Y783F or Y1253F mutants thereof and were then stimulated for the indicated periods with PDGF (100 ng/ml) in the absence of sodium vanadate. Cell lysates were then subjected to immunoblot analysis with the indicated antibodies.

NIH 3T3/EGFR cells were stimulated for 5 min with PDGF (30 ng/ml) in the presence of vanadate. In 3 independent experiments, this resulted in the average phosphorylation of  $\sim$ 35 and  $\sim$ 20% of PLC- $\gamma$ 1 molecules on Tyr-783 and Tyr-1253, respectively (see Fig. 4A). Cell lysates were then subjected to immunoprecipitation with either anti-PLC- $\gamma$ 1, anti-pY783, or anti-pY1253, and the resulting precipitates were each subjected to immunoblot analysis with these three antibodies (Fig. 3C). Both phospho-specific antibodies precipitated PLC- $\gamma$ 1 from lysates of stimulated cells but not from those of resting cells. The ratio of the blot intensity obtained with anti-pY1253 to that obtained with anti-pY783 was calculated for each of the precipitates obtained with anti-PLC- $\gamma$ 1, anti-pY783, and anti-pY1253. For the anti-PLC- $\gamma$ 1 precipitate, this pY1253/pY783 ratio was the same as the ratio ( $0.57 = 20\%/35\%$ ) obtained with the whole cell lysates. Under a linked scenario, all the PLC- $\gamma$ 1 molecules phosphorylated on Tyr-1253 are also phosphorylated

on Tyr-783. Therefore, the predicted pY1253/pY783 ratio for the anti-pY783 precipitate remains 0.57 (the same value as that for the whole population of PLC- $\gamma$ 1 molecules), whereas the predicted ratio for the anti-pY1253 precipitate is 1. However, if Tyr-1253 phosphorylation is randomly distributed, the predicted PLC- $\gamma$ 1 population would include three phosphorylated species: one bisphosphorylated on Tyr-783 and Tyr-1253 ( $0.35 \times 0.20 = 0.07$  or 7%), one monophosphorylated on Tyr-783 ( $35 - 7 = 28\%$ ), and one monophosphorylated on Tyr-1253 ( $20 - 7 = 13\%$ ). Therefore, the predicted pY1253/pY783 ratio for the anti-pY783 precipitate would be 0.2 ( $= 0.07/0.35$ ), and the predicted pY1253/pY783 ratio for the anti-pY1253 precipitate would be 2.86 ( $= 0.20/0.07$ ). The actual ratios obtained were  $0.45 \pm 0.07$  for the anti-pY783 precipitates and  $1.19 \pm 0.39$  for the anti-pY1253 precipitates (Fig. 3C), indicating that phosphorylation of Tyr-783 and Tyr-1253 occurs on the same molecules in most instances but that the kinase adds phospho-



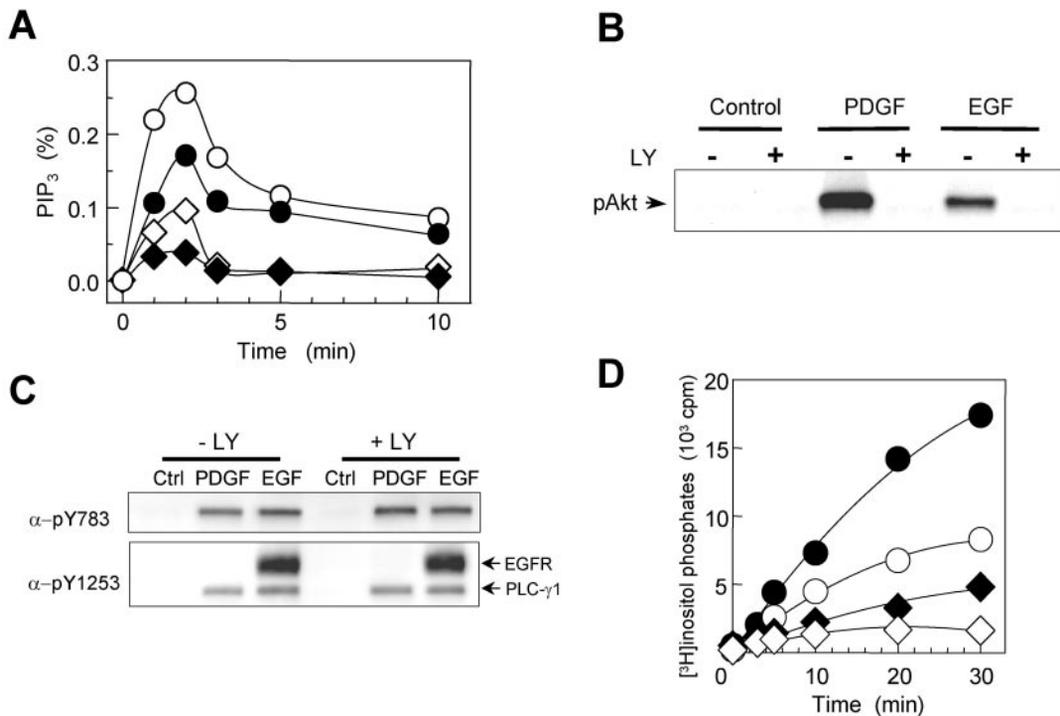
**FIG. 4. Tyrosine phosphorylation of PLC- $\gamma$ 1 and production of inositol phosphates in NIH 3T3/EGFR cells stimulated with PDGF or EGF.** *A*, serum-deprived cells were stimulated for 5 min with the indicated concentrations of PDGF or EGF in the presence of 1 mM sodium vanadate, after which the extent of phosphorylation of PLC- $\gamma$ 1 on Tyr-783 (closed circles) and on Tyr-1253 (open circles) was quantified as described in Fig. 3A. Data are means of three independent determinations. *B*, serum-deprived cells were left unstimulated or stimulated for 5 min with either PDGF (30 ng/ml) or EGF (20 ng/ml) in the absence or presence of 1 mM sodium vanadate, after which cell lysates were subjected to immunoblot analysis with the indicated antibodies (upper panel). The extent of Tyr-783 phosphorylation was quantified from immunoblot intensities (lower panel); data are means  $\pm$  S.E. of values from four independent determinations. *C*, time course of phosphorylation of PLC- $\gamma$ 1 on Tyr-783 in serum-deprived cells stimulated for 5 min with either PDGF (30 ng/ml) (circles) or EGF (20 ng/ml) (diamonds) in the presence of 1 mM sodium vanadate. *D*, cells that had been metabolically labeled with *myo*-[ $^3$ H]inositol were stimulated for 30 min with the indicated concentrations of PDGF (circles) or EGF (diamonds) in the absence of sodium vanadate, after which [ $^3$ H]inositol phosphates (IP, IP $_2$ , and IP $_3$ ) were measured. Data are representative of results from three similar experiments. *E*, cells that had been labeled with *myo*-[ $^3$ H]inositol as in *D* were stimulated for the indicated times with PDGF (30 ng/ml) (circles) or EGF (20 ng/ml) (diamonds) in the absence (closed symbols) or presence (open symbols) of 1 mM sodium vanadate, after which [ $^3$ H]inositol phosphates in cell extracts were measured. Data are representative of results from three similar experiments.

phate to Tyr-783 more frequently than it does to Tyr-1253. We also conducted the same experiments with PLC- $\gamma$ 1 phosphorylated in response to EGF stimulation to find essentially the same conclusion (not shown).

To characterize further the relation between the phosphorylation reactions at these two positions, we monitored the time course of PLC- $\gamma$ 1 phosphorylation in Null TV-1 cells expressing exogenous wild-type PLC- $\gamma$ 1 or the Y783F or Y1253F mutants after their stimulation with a saturating concentration (100 ng/ml) of PDGF in the absence of vanadate (Fig. 3D). Phosphorylation of both Tyr-783 and Tyr-1253 showed a transient increase similar to that observed for the endogenous enzyme in NIH 3T3/EGFR cells (Fig. 3A). The time courses were similar for the two sites, and the absence of phosphorylation at one site as a result of the Tyr  $\rightarrow$  Phe mutation did not affect the kinetics

of phosphorylation at the other site, suggesting that Tyr-783 and Tyr-1253 are phosphorylated independently.

**Relationship between Tyrosine Phosphorylation of PLC- $\gamma$ 1 and Lipase Activity**—We evaluated the relationship between the tyrosine phosphorylation of PLC- $\gamma$ 1 and phosphoinositide hydrolysis in NIH 3T3/EGFR cells. We measured phosphorylation of Tyr-783 and Tyr-1253 in cells stimulated for 5 min with various concentrations of PDGF or EGF (Fig. 4A). The maximal phosphorylation levels on both Tyr-783 and Tyr-1253 attained in the presence of saturating concentrations of PDGF were higher than those achieved with saturating concentrations of EGF. Similar to the situation with PDGF, the level of Tyr-783 phosphorylation was about twice that of Tyr-1253 phosphorylation in EGF-stimulated cells. The dose-response curves indicated that PDGF at 30 ng/ml and EGF at 20 ng/ml induced



**FIG. 5. Production of PIP<sub>3</sub>, activation of Akt, and formation of inositol phosphates in NIH 3T3/EGFR cells stimulated with PDGF or EGF.** *A*, serum-deprived cells that had been labeled with [<sup>32</sup>P]orthophosphate were stimulated for the indicated times with PDGF (30 ng/ml) (circles) or EGF (20 ng/ml) (diamonds) in the absence (closed symbols) or presence (open symbols) of 1 mM sodium vanadate, after which the amount of <sup>32</sup>P-labeled PIP<sub>3</sub> was measured. Data are expressed as a percentage of the total radioactivity associated with the phospholipid fraction and are means of three independent determinations. *B*, serum-deprived cells were incubated with 50  $\mu$ M LY294002 or vehicle for 10 min and then stimulated for 5 min with PDGF (30 ng/ml) or EGF (20 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies specific for Akt phosphorylated on Ser-473. *C*, cells were treated for 10 min with 50  $\mu$ M LY294002 or vehicle and then either left unstimulated (Ctrl) or stimulated for 10 min with PDGF (30 ng/ml) or EGF (20 ng/ml) in the presence of 1 mM sodium vanadate. Cell lysates were subjected to immunoblot analysis with anti-pY783 and anti-pY1253. *D*, cells that had been labeled with myo-[<sup>3</sup>H]inositol as in Fig. 4D were incubated for 10 min with 50  $\mu$ M LY294002 (open symbols) or vehicle (closed symbols) and then stimulated for the indicated times with PDGF (30 ng/ml) (circles) or EGF (20 ng/ml) (diamonds) in the presence of 1 mM sodium vanadate. The production of [<sup>3</sup>H]inositol phosphates was then analyzed.

similar levels of phosphorylation. This finding was confirmed by immunoblot analysis with the three phospho-specific antibodies of cells stimulated for 5 min with PDGF (30 ng/ml) or EGF (20 ng/ml) in the absence or presence of vanadate (Fig. 4B). As with PDGF, EGF-induced Tyr-771 phosphorylation was not detectable in the absence of vanadate and was barely detectable in its presence. The time courses of Tyr-783 phosphorylation induced by PDGF (30 ng/ml) or EGF (20 ng/ml) were similar, but a close inspection revealed some differences; in EGF-stimulated cells, phosphorylation took place more rapidly and dephosphorylation proceeded more slowly compared with those observed in PDGF-stimulated cells (Fig. 4C).

We next measured PIP<sub>2</sub>-hydrolyzing activity in NIH 3T3/EGFR cells after stimulation with PDGF or EGF. NIH 3T3/EGFR cells were thus metabolically labeled with myo-[<sup>3</sup>H]inositol and the production of [<sup>3</sup>H]inositol phosphates (the sum of IP, IP<sub>2</sub>, and IP<sub>3</sub>) in response to growth factor stimulation was determined. The incubation medium also included LiCl to block dephosphorylation of inositol monophosphates; phosphorylation of PLC- $\gamma$ 1 was not affected by the presence of LiCl (data not shown). We first measured PLC activity in cells stimulated with various concentrations of PDGF or EGF in the absence of vanadate. Despite the fact that EGF could induce substantial phosphorylation of PLC- $\gamma$ 1, no PLC activity was detected in cells exposed to the growth factor at any of the concentrations tested, whereas PDGF induced marked PIP<sub>2</sub> hydrolysis in a concentration-dependent manner (Fig. 4D). In cells stimulated with PDGF (30 ng/ml), the liberation of [<sup>3</sup>H]inositol phosphates increased with time, and this increase was faster in the presence of vanadate, concomitant with the extent of tyrosine phosphorylation (Fig. 4E). EGF (20 ng/ml), which induced compa-

able levels of phosphorylation on Tyr-783 and on Tyr-1253 as did PDGF (30 ng/ml), was unable to induce any detectable PIP<sub>2</sub> hydrolysis in the absence of vanadate. Although the addition of vanadate only modestly increased the phosphorylation (Fig. 4B), it had a pronounced stimulating effect on EGF-induced PLC activity to increase it to a detectable level. Although the extent of inositol phosphate production was still 20–25% of that induced by PDGF (30 ng/ml) in the presence of vanadate (Fig. 4E), however. It thus appears that phosphorylation of PLC- $\gamma$ 1 is, by itself, insufficient for the expression of enzymatic activity.

**PIP<sub>3</sub> Production in NIH 3T3/EGFR Cells Stimulated with PDGF or EGF**—PIP<sub>3</sub>, which is produced in response to cell stimulation with growth factors, enhances PLC- $\gamma$ 1 activity both *in vitro* and *in vivo* (13–16). We examined the time course of PIP<sub>3</sub> production in NIH 3T3/EGFR cells stimulated with PDGF (30 ng/ml) or EGF (20 ng/ml) in the absence or presence of vanadate (Fig. 5A). Each growth factor induced a rapid and transient increase in the amount of PIP<sub>3</sub>, which peaked at 1–2 min after the onset of stimulation. The effect of PDGF was greater than that of EGF, and the effects of both growth factors were enhanced by vanadate. Treatment of the cells with 50  $\mu$ M LY294002, an inhibitor of PI3K activity, virtually abolished PIP<sub>3</sub> production in response to either PDGF or EGF (data not shown).

The phosphorylation of Akt is commonly used as an indirect index of the activity of PI3K and the generation of PIP<sub>3</sub> (27). PDGF (30 ng/ml) induced a substantially larger increase in the phosphorylation of Akt than did EGF (20 ng/ml), but Akt phosphorylation at Ser-473 in response to either growth factor was completely blocked by prior incubation of the cells with LY294002 (Fig. 5B).

LY294002 also partially inhibited inositol phosphate production induced by PDGF or EGF (Fig. 5D), but it did not affect the phosphorylation of PLC- $\gamma$ 1 (Fig. 5C). These results indicate that PIP<sub>3</sub> is an important activator of phosphorylated PLC- $\gamma$ 1 and that the observed difference in PLC activity between cells stimulated with PDGF or with EGF is likely attributable in part to the difference in PIP<sub>3</sub> production elicited by these growth factors. However, given that ~50% of PLC activity persisted in stimulated cells treated with LY294002 at a concentration sufficient to fully block PIP<sub>3</sub> accumulation and Akt phosphorylation, PIP<sub>3</sub> may not be the only stimulatory factor for phosphorylated PLC- $\gamma$ 1.

#### DISCUSSION

The tyrosine phosphorylation and activation of PLC- $\gamma$ 1 are triggered by most growth factor receptors and immunoreceptors in response to ligand binding (1, 3, 4). Whereas activated growth factor receptors recruit and catalyze directly the phosphorylation of PLC- $\gamma$ 1 (28, 29), activated immunoreceptors appear to achieve this goal through the formation of supramolecular complexes that comprise the aggregated receptors, adapter proteins, and one member each of the Src, Syk, and Tec families of PTKs (3, 6). Although three tyrosine residues, Tyr-771, Tyr-783, and Tyr-1253, have been identified as phosphorylation sites of PLC- $\gamma$ 1 targeted by the receptors for PDGF and EGF, it has remained unknown whether immunoreceptors also elicit phosphorylation at all of these sites. In addition, given that phosphorylation of PLC- $\gamma$ 1 is generally monitored by immunoblot analysis with antibodies to phosphotyrosine, the extent and rate of phosphorylation of individual tyrosine residues have not been evaluated. We have now prepared and established the specificity of antibodies specific for individual tyrosine phosphorylation sites of PLC- $\gamma$ 1. Furthermore, with the use of these antibodies, we have analyzed the kinetics and stoichiometry of phosphorylation at each site and examined the relation of such phosphorylation to the lipase activity of PLC- $\gamma$ 1.

*Phosphorylation of PLC- $\gamma$ 1 on Tyr-1253 Depends on Receptor Type*—Our results have revealed that the mode of PLC- $\gamma$ 1 phosphorylation mediated by immunoreceptors differs from that mediated by growth factor receptors. Whereas PDGF and EGF each induced a substantial level of phosphorylation of PLC- $\gamma$ 1 on Tyr-1253 in addition to the marked extent of phosphorylation of Tyr-783, stimulation of the B cell receptor or T cell receptor resulted in measurable phosphorylation of only Tyr-783. Like EGFR, Src and ZAP-70 each phosphorylated both Tyr-783 and Tyr-1253 *in vitro*. The lack of Tyr-1253 phosphorylation apparent in Jurkat T cells and Ramos B cells is thus not likely attributable *per se* to the specificity of the kinases responsible for PLC- $\gamma$ 1 phosphorylation in these cells.

Phosphorylation of PLC- $\gamma$ 1 on Tyr-783 is essential for induction of phospholipase activity, whereas that of Tyr-1253 is dispensable for such activity (9). The role of Tyr-1253 phosphorylation might thus be related to something other than direct regulation of the catalytic function of PLC- $\gamma$ 1. In this regard, lipase-inactive PLC- $\gamma$ 1 mutants have been shown to possess biological activities (30–33). Lipase-inactive PLC- $\gamma$ 1 thus manifests mitogenic activity, probably by serving as a guanine nucleotide exchange factor for the nuclear GTPase PIKE (32). In addition, PLC- $\gamma$ 1 and PLC- $\gamma$ 2 play a lipase-independent role in agonist-induced Ca<sup>2+</sup> entry (33).

*Mechanism of Growth Factor-induced Phosphorylation of PLC- $\gamma$ 1*—Phosphorylation of PLC- $\gamma$ 1 is initiated on association of the enzyme with autophosphorylated receptors for PDGF or EGF (9, 34), and this association is mediated through recognition of phosphorylated tyrosine residues of the receptor PTKs by the NH<sub>2</sub>-terminal SH2 domain of PLC- $\gamma$ 1 (21, 35, 36). In

NIH 3T3/EGFR cells stimulated with PDGF, the level of phosphorylation of PLC- $\gamma$ 1 on Tyr-783 was 1.5- to 2-fold greater than that of Tyr-1253. Phosphorylation of Tyr-771 was barely detectable even in the presence of a protein tyrosine phosphatase inhibitor, sodium vanadate. A similar quantitative relation, pY783 > pY1253  $\gg$  pY771, was also observed in cells stimulated with EGF.

We investigated if phosphorylation of PLC- $\gamma$ 1 on Tyr-783 and on Tyr-1253 proceeded in a linked mechanism or in a random fashion in cells stimulated with PDGF or EGF by means of immunoprecipitation with the phospho-specific antibodies. With cell lysates containing partially phosphorylated PLC- $\gamma$ 1, we analyzed the distribution of pY783 and pY1253 residues among total PLC- $\gamma$ 1 molecules. All the experimentally determined pY783/pY1253 ratio values were similar to those predicted for a linked mechanism. If phosphorylation of the two sites occurred randomly, a far fewer amount of PLC- $\gamma$ 1 bisphosphorylated on Tyr-783 and Tyr-1253, as well as a significant amount of PLC- $\gamma$ 1 monophosphorylated on Tyr-1253, should have been found. It thus seems likely that a molecule of PLC- $\gamma$ 1 recruited by a receptor PTK is phosphorylated on both Tyr-783 and Tyr-1253 before being released from the receptor, albeit with phosphorylation of Tyr-783 occurring more frequently than that of Tyr-1253. Importantly, mutation of one site did not affect the phosphorylation of the other site, indicating that phosphorylation of Tyr-783 and Tyr-1253 is not a sequential process.

The kinetics of PDGF-induced phosphorylation of PLC- $\gamma$ 1 were similar for Tyr-783 and Tyr-1253 under various conditions, probably because the phosphorylation of PLC- $\gamma$ 1 by the associated receptor is not rate-limiting. Rather, prior events, such as the binding of PDGF to its receptor, the autophosphorylation of the receptor, and the binding of PLC- $\gamma$ 1 to the autophosphorylated receptor, might be relatively slow. Alternatively, the associated PTK might act at Tyr-783 and Tyr-1253 with similar rates.

The greater extent of phosphorylation of Tyr-783 compared with that of Tyr-1253 might result either from enhanced phosphorylation or reduced dephosphorylation of Tyr-783. Inhibition of protein tyrosine phosphatases by vanadate resulted in an increase in the levels of PDGF-induced phosphorylation of both Tyr-783 and Tyr-1253, but the pY783/pY1253 ratio remained largely unchanged. It is thus possible that the interaction of PLC- $\gamma$ 1 with a phosphatase results in the dephosphorylation of both sites by the phosphatase before it releases the PLC- $\gamma$ 1 molecule. Stimulation of cells with a saturating concentration of PDGF resulted in the rapid dephosphorylation of both pY783 and pY1253 after the peak level of phosphorylation was attained, suggesting that pronounced stimulation of the PDGFR also rapidly triggers a feedback mechanism, probably involving activation of phosphatase function.

*Requirement of Factors Other Than Tyr-783 Phosphorylation for PLC- $\gamma$ 1 Activation*—Although activated receptor PTKs phosphorylate both Tyr-783 and Tyr-1253 of PLC- $\gamma$ 1, only the phosphorylation of Tyr-783 is required for phospholipase activity. PLC activity is closely related to the extent of Tyr-783 phosphorylation in comparisons of various cell types stimulated with a specific growth factor. Our results have now shown, however, that PLC activity induced by EGF was much less than that induced by PDGF in NIH 3T3/EGFR cells, even though the extent and kinetics of phosphorylation of both Tyr-783 and Tyr-1253 elicited by the growth factors were not much different. Factors other than Tyr-783 phosphorylation thus appear to contribute to PLC- $\gamma$ 1 activation. EGF triggers PLC activation or Ca<sup>2+</sup> mobilization in various cell types (8, 37–39), and experiments with Null TV-1 cells have indicated that

PLC- $\gamma$ 1 is the isozyme responsible for the PLC activity (17). EGF alone failed to induce measurable PLC activity in NIH 3T3/EGFR cells, but EGF-induced production of inositol phosphates was detected in the additional presence of vanadate. These results suggest that the production of a factor necessary for PLC- $\gamma$ 1 activation is also enhanced by tyrosine phosphorylation.

We have now identified PIP<sub>3</sub> as an important regulator of PLC- $\gamma$ 1, which might explain, at least in part, the differences in PLC activity observed in NIH 3T3/EGFR cells stimulated with PDGF or EGF. Most, if not all, growth factor receptors that activate PLC- $\gamma$ 1 also elicit PIP<sub>3</sub> production. A role for PIP<sub>3</sub> in the activation of PLC- $\gamma$  isozymes has been demonstrated previously (13–16). The mechanism by which PIP<sub>3</sub> activates PLC- $\gamma$ 1 remains to be determined, however. We have previously suggested that PIP<sub>3</sub> activates PLC- $\gamma$ 1 by binding to its SH2 domains (14, 15). Falsca *et al.* (13) proposed that binding of PIP<sub>3</sub> to the NH<sub>2</sub>-terminal PH domain of PLC- $\gamma$ 1 is responsible for membrane targeting of the enzyme. Whereas yeast two-hybrid analysis did not support interaction of this PH domain with PIP<sub>3</sub> (40), molecular modeling did (41). The binding of PIP<sub>3</sub> and membrane targeting were studied with the isolated PH domain or SH2 domains, however, not with the intact PLC- $\gamma$ 1 molecule.

PIP<sub>3</sub> markedly activates nonphosphorylated PLC- $\gamma$ 1 *in vitro* (14). The production of PIP<sub>3</sub> alone, however, appears insufficient to activate PLC- $\gamma$ 1 in cells, with Tyr-783 phosphorylation being a prerequisite. Specific binding sites for various SH2 domain-containing proteins, including PI3K (Tyr-740 and Tyr-751) and PLC- $\gamma$ 1 (Tyr-1021), have been identified in  $\beta$ -PDGFR (42–44). Stimulation of HepG2 cells expressing a Y1021F mutant of this receptor with PDGF resulted in normal PIP<sub>3</sub> production but not in PLC- $\gamma$ 1 phosphorylation or PIP<sub>2</sub> hydrolysis (15, 44). The activation of PLC- $\gamma$ 1 by PIP<sub>3</sub> thus appears to require priming by phosphorylation of the enzyme on Tyr-783.

Unlike PDGFR, which activates PI3K by providing a tandem site (Tyr-740 and Tyr-751 for  $\beta$ -PDGFR) for the binding of the two SH2 domains of the p85 regulatory subunit of the enzyme (29, 44), EGFR does not possess a canonical p85 binding site and binding of p85 to the receptor is weak or nonexistent (45). Nevertheless, EGF-induced activation of PI3K has been described in various cell types. EGFR therefore likely recruits and activates PI3K indirectly, and different cell types appear to achieve this effect in different manners. Proteins such as ErbB3 and Gab1 have been shown to contribute to the EGF-induced activation of PI3K (46–48). Insufficiency of such mediators might thus be responsible in part for the low level of activation of PI3K by EGF in NIH 3T3/EGFR cells.

Treatment of NIH 3T3/EGFR cells with LY294002 at a concentration that completely blocked PIP<sub>3</sub> generation and Akt phosphorylation in response to PDGF resulted in only ~50% inhibition of PDGF-induced PLC activity. Furthermore, prevention of PI3K activation by mutation of Tyr-740 and Tyr-751 of  $\beta$ -PDGFR resulted in only modest inhibition of PDGF-dependent Ca<sup>2+</sup> mobilization in HepG2 cells (15). These results may suggest that Tyr-783-phosphorylated PLC- $\gamma$ 1 is partially active in the absence of PIP<sub>3</sub>, but this explanation can be excluded by the fact that EGF-induced tyrosine phosphorylation of PLC- $\gamma$ 1 alone does not lead to its activation in the absence of vanadate. It therefore seems most likely that the phosphorylated PLC requires other factors produced by PDGF for the activation. One possible candidate for such a factor is phosphatidic acid, a product of phospholipase D action. Phosphatidic acid activates PLC- $\gamma$ 1 *in vitro* (10), and phospholipase D activity is increased in most cells stimulated by growth factors. PDGF induces a larger increase in PLC activity than

does EGF in Swiss 3T3 cells and hippocampal stem cells; in the same cell types, PDGF induces activation of phospholipase D, but EGF does not (49, 50). Proteins such as Cbl might also contribute to the differential activation of PLC- $\gamma$ 1 by PDGF and EGF in NIH 3T3/EGFR cells. Cbl is thought to function as a negative regulator of growth factor signaling (51), and it inhibits PLC- $\gamma$ 1 activity in T cells (52). In mouse embryonic fibroblasts, EGF elicits tyrosine phosphorylation of Cbl as well as an association of the tyrosine-phosphorylated protein with PLC- $\gamma$ 1 that is mediated through interaction between the proline-rich region of Cbl and the SH3 domain of PLC- $\gamma$ 1; PDGF does not induce the association of Cbl and PLC- $\gamma$ 1 (53).

In conclusion, our results have revealed the phosphorylation and subsequent activation of PLC- $\gamma$ 1 to be complex and to vary greatly depending on cell type and stimulus. In addition to Tyr-783 phosphorylation, which is essential for the lipase activity of PLC- $\gamma$ 1, growth factors induce Tyr-1253 phosphorylation. The phosphorylation of Tyr-1253 is neither needed for lipase activity nor does it occur in lymphoid cells stimulated by immunoreceptors. Its role, which remains to be determined, therefore appears to be specific to growth factor-stimulated cells. Our results have also revealed that Tyr-783 phosphorylation is not sufficient for the lipase activity of PLC- $\gamma$ 1 and that concurrent activation of PI3K appears to be important for activation of the Tyr-783-phosphorylated enzyme.

## REFERENCES

- Rhee, S. G. (2001) *Annu. Rev. Biochem.* **70**, 281–312
- Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royle, J., Blayney, L. M., Swann, K., and Lai, F. A. (2002) *Development* **129**, 3533–3544
- Wilde, J. I., and Watson, S. P. (2001) *Cell Signal.* **13**, 691–701
- Carpenter, G., and Ji, Q. (1999) *Exp. Cell Res.* **253**, 15–24
- Wang, D., Peng, J., Wen, R., Marine, J. C., Sangster, M. Y., Parganas, E., Hoffmeyer, A., Jackson, C. W., Cleveland, J. L., Murray, P. J., and Ihle, J. N. (2000) *Immunity* **13**, 25–35
- Samelson, L. E. (2002) *Annu. Rev. Immunol.* **20**, 371–394
- Kim, J. W., Sim, S. S., Kim, U. H., Nishibe, S., Wahl, M. I., Carpenter, G., and Rhee, S. G. (1990) *J. Biol. Chem.* **265**, 3940–3943
- Wahl, M., and Carpenter, G. (1988) *J. Biol. Chem.* **263**, 7581–7590
- Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., and Rhee, S. G. (1991) *Cell* **65**, 435–441
- Jones, G. A., and Carpenter, G. (1993) *J. Biol. Chem.* **268**, 20845–20850
- Hwang, S. C., Jhon, D. Y., Bae, Y. S., Kim, J. H., and Rhee, S. G. (1996) *J. Biol. Chem.* **271**, 18342–18349
- Sekiya, F., Bae, Y. S., Jhon, D. Y., Hwang, S. C., and Rhee, S. G. (1999) *J. Biol. Chem.* **274**, 13900–13907
- Falasca, M., Logan, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A., and Schlessinger, J. (1998) *EMBO J.* **17**, 414–422
- Bae, Y. S., Cantley, L. G., Chen, C. S., Kim, S. R., Kwon, K. S., and Rhee, S. G. (1998) *J. Biol. Chem.* **273**, 4465–4469
- Rameh, L. E., Rhee, S. G., Spokes, K., Kazlaszkas, A., Cantley, L. C., and Cantley, L. G. (1998) *J. Biol. Chem.* **273**, 23750–23757
- Smith, A. J., Surviladze, Z., Gaudet, E. A., Backer, J. M., Mitchell, C. A., and Wilson, B. S. (2001) *J. Biol. Chem.* **276**, 17213–17220
- Ji, Q. S., Winnier, G. E., Niswender, K. D., Horstman, D., Wisdom, R., Magnuson, M. A., and Carpenter, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2999–3003
- Honegger, A. M., Dull, T. J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A., and Schlessinger, J. (1987) *Cell* **51**, 199–209
- Suh, P. G., Ryu, S. H., Choi, W. C., Lee, K. Y., and Rhee, S. G. (1988) *J. Biol. Chem.* **263**, 14497–14504
- Chakrabarti, S., Brechling, K., and Moss, B. (1985) *Mol. Cell. Biol.* **5**, 3403–3409
- Poulin, B., Sekiya, F., and Rhee, S. G. (2000) *J. Biol. Chem.* **275**, 6411–6416
- Zhang, L., and Buxton, I. L. (1998) *Methods Mol. Biol.* **105**, 47–63
- Serunian, L. A., Auger, K. R., and Cantley, L. C. (1991) *Methods Enzymol.* **198**, 78–87
- Huyer, G., Liu, S., Kelly, J., Moffat, J., Payette, P., Kennedy, B., Tsapralis, G., Gresser, M. J., and Ramachandran, C. (1997) *J. Biol. Chem.* **272**, 843–851
- Straus, D. B., and Weiss, A. (1992) *Cell* **70**, 585–593
- Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998) *Mol. Cell. Biol.* **18**, 1388–1399
- Hemmings, B. A. (1997) *Science* **275**, 628–630
- Pawson, T., and Nash, P. (2000) *Genes Dev.* **14**, 1027–1047
- Schlessinger, J. (2000) *Cell* **103**, 211–225
- Smith, M. R., Liu, Y. L., Matthews, N. T., Rhee, S. G., Sung, W. K., and Kung, H. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 6554–6558
- Huang, P. S., Davis, L., Huber, H., Goodhart, P. J., Wegryzn, R. E., Oliff, A., and Heimbrosk, D. C. (1995) *FEBS Lett.* **358**, 287–292
- Ye, K., Aghdasi, B., Luo, H. R., Moriarty, J. L., Wu, F. Y., Hong, J. J., Hurt, K. J., Bae, S. S., Suh, P. G., and Snyder, S. H. (2002) *Nature* **415**, 541–544
- Patterson, R. L., van Rossum, D. B., Ford, D. L., Hurt, K. J., Bae, S. S., Suh, P. G., Kurosaki, T., Snyder, S. H., and Gill, D. L. (2002) *Cell* **111**, 529–541

34. Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A., and Schlessinger, J. (1989) *Cell* **57**, 1101–1107
35. Ji, Q. S., Chattopadhyay, A., Vecchi, M., and Carpenter, G. (1999) *Mol. Cell. Biol.* **19**, 4961–4970
36. Chattopadhyay, A., Vecchi, M., Ji, Q., Mernaugh, R., and Carpenter, G. (1999) *J. Biol. Chem.* **274**, 26091–26097
37. Cunningham, T. W., Kuppuswamy, D., and Pike, L. J. (1989) *J. Biol. Chem.* **264**, 15351–15356
38. Yang, L. J., Rhee, S. G., and Williamson, J. R. (1994) *J. Biol. Chem.* **269**, 7156–7162
39. Haugh, J. M., Schooler, K., Wells, A., Wiley, H. S., and Lauffenburger, D. A. (1999) *J. Biol. Chem.* **274**, 8958–8965
40. Isakoff, S. J., Cardozo, T., Andreev, J., Li, Z., Ferguson, K. M., Abagyan, R., Lemmon, M. A., Aronheim, A., and Skolnik, E. Y. (1998) *EMBO J.* **17**, 5374–5387
41. Singh, S. M., and Murray, D. (2003) *Protein Sci.* **12**, 1934–1953
42. Larose, L., Gish, G., Shoelson, S., and Pawson, T. (1993) *Oncogene* **8**, 2493–2499
43. Kazlauskas, A., and Cooper, J. A. (1989) *Cell* **58**, 1121–1133
44. Valius, M., and Kazlauskas, A. (1993) *Cell* **73**, 321–334
45. Hu, P., Margolis, B., Skolnik, E. Y., Lammers, R., Ullrich, A., and Schlessinger, J. (1992) *Mol. Cell. Biol.* **12**, 981–990
46. Soltoff, S. P., Carraway, K. L., 3rd, Prigent, S. A., Gullick, W. G., and Cantley, L. C. (1994) *Mol. Cell. Biol.* **14**, 3550–3558
47. Rodrigues, G. A., Falasca, M., Zhang, Z., Ong, S. H., and Schlessinger, J. (2000) *Mol. Cell. Biol.* **20**, 1448–1459
48. Zhang, S. Q., Tsiaras, W. G., Araki, T., Wen, G., Minichiello, L., Klein, R., and Neel, B. G. (2002) *Mol. Cell. Biol.* **22**, 4062–4072
49. Yeo, E. J., and Exton, J. H. (1995) *J. Biol. Chem.* **270**, 3980–3988
50. Sung, J. Y., Lee, S. Y., Min, D. S., Eom, T. Y., Ahn, Y. S., Choi, M. U., Kwon, Y. K., and Chung, K. C. (2001) *J. Neurochem.* **78**, 1044–1053
51. Thien, C. B., and Langdon, W. Y. (2001) *Nat. Rev. Mol. Cell. Biol.* **2**, 294–307
52. Graham, L. J., DeBell, K. E., Veri, M., Stoica, B., Mostowski, H., Bonvini, E., and Rellahan, B. (2000) *FEBS Lett.* **470**, 273–280
53. Tvorogov, D., and Carpenter, G. (2002) *Exp. Cell Res.* **277**, 86–94

**Mechanism of Tyrosine Phosphorylation and Activation of Phospholipase C- $\gamma$ 1:  
TYROSINE 783 PHOSPHORYLATION IS NOT SUFFICIENT FOR LIPASE  
ACTIVATION**

Fujio Sekiya, Benoit Poulin, Yeun Ju Kim and Sue Goo Rhee

*J. Biol. Chem.* 2004, 279:32181-32190.

doi: 10.1074/jbc.M405116200 originally published online May 25, 2004

---

Access the most updated version of this article at doi: [10.1074/jbc.M405116200](https://doi.org/10.1074/jbc.M405116200)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 53 references, 30 of which can be accessed free at  
<http://www.jbc.org/content/279/31/32181.full.html#ref-list-1>