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Platelet-Derived Growth Factor Increases the In Vivo Activity of Phospholipase C-γ_1 and Phospholipase C-γ_2

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Upon binding to its cell surface receptor, platelet-derived growth factor (PDGF) causes the tyrosine phosphorylation of phospholipase C-γ_1 (PLC-γ_1) and stimulates the production of diacylglycerol and inositol 1,4,5-triphosphate. We showed that following stimulation by PDGF, rat-2 cells overexpressing PLC-γ_1 display an increase in the levels of both tyrosine-phosphorylated PLC-γ_1 and inositol phosphates compared with the parental rat-2 cells. This increased responsiveness to PDGF is a direct effect of PLC-γ_1 overexpression, as a cell line expressing similar levels of an enzymatically inactive point mutant of PLC-γ_1, PLC-$\gamma_1$335Q, did not show elevated inositol phosphate production in response to PDGF. Hematopoietic cells express PLC-γ_2, a PLC isoform that is closely related to PLC-γ_1. When rat-2 cells overexpressing PLC-γ_2 were treated with PDGF, an increase in both the tyrosine phosphorylation and the in vivo activity of PLC-γ_2 was observed. Aluminum fluoride (AlF_4^-), a universal activator of PLC linked to G-proteins, did not produce an increase in the levels of inositol phosphates in either of the overexpressing cell lines compared with parental rat-2 cells, demonstrating that PLC-γ isoforms respond specifically to a receptor with tyrosine kinase activity.

The binding of various ligands to their cell surface receptors rapidly induces the formation of two second messenger molecules derived from phosphatidylinositol (PI) 4,5-bisphosphate: diacylglycerol and inositol 1,4,5-triphosphate (IP_3) (2, 3). The production of these molecules is mediated by an activated PI-specific phospholipase C (PLC) (26). At present, PLC has been shown to be composed of several different isoforms, and cDNA clones encoding five different PLC isoforms, PLC-α, PLC-β_1, PLC-γ_1, PLC-γ_2, and PLC-δ_1, have been described (1, 7, 12, 24, 30, 31). All these isoforms have been shown to encode PI-specific PLC activity, with the notable exception of PLC-α, which also fails to share significant sequence similarity with any of the known PLCs (1). More recently, we have identified cDNA clones encoding four additional PLC isoforms. Based on their sequence similarity with the previously characterized PLCs, they reveal the existence of three classes of PLC and are referred to as PLC-β_2, PLC-β_3, PLC-δ_2, and PLC-δ_3 (16, 16a).

Presently, little is known about the regulation of these particular isoforms, but the complexity of the family may explain the diverse modes of regulation which have been observed (23). In fibroblasts, for example, the PI turnover induced by bombesin has been shown to be sensitive to pertussis toxin, an inhibitor of certain G-proteins. Platelet-derived growth factor (PDGF) stimulation of fibroblasts also induces PI turnover in these cells; however, this event is insensitive to pertussis toxin (8). These data have suggested that PDGF activates PLC via a pertussis toxin-insensitive G-protein or utilizes an alternative activation mechanism such as the direct phosphorylation of PLC-γ_1 by the PDGF receptor (17, 20, 34). In addition to PLCγ_1, several other cellular proteins known to be involved in the regulation of cell growth such as PI kinase (11), GTPase-activating protein (6, 11, 13, 21), and possibly Raf (22, 23) have been identified as direct substrates of the PDGF receptor, although at present it is not clear whether there is a modification in the activity of these proteins in response to tyrosine phosphorylation. By overexpressing PLC-γ_1 and PLC-γ_2 in rat-2 fibroblasts, we were able to demonstrate that the in vivo activity of PLC-γ_1 and PLC-γ_2 increases in response to PDGF treatment and that this increased activity is correlated with an increased serine and tyrosine phosphorylation of either PLC-γ_1 or PLC-γ_2.

MATERIALS AND METHODS

Cell lines. rat-2 cell lines were maintained in Dulbecco modified Eagle medium (DMEM) containing 50 units of penicillin and 50 μg of streptomycin per ml and supplemented with 10% fetal bovine serum in a humidified atmosphere with 10% CO_2. Cell lines overexpressing PLC-γ_1, PLC-$\gamma_1$335Q, or PLC-γ_2 were made by cotransfecting 1 μg of pSV2neo and 10 μg of the appropriate plasmid DNAs by the lipofection technique and selecting for colonies resistant to the antibiotic G418 (400 μg/ml).

Antibodies. PLC-γ_1 antibodies were raised to a peptide corresponding to bovine PLC-γ_1 residues 1255 to 1274, which was conjugated to keyhole limpet hemocyanin with glutaraldehyde and injected into rabbits as described previously (18). The PLC-γ_2 antibodies were raised by injecting rabbits with PLC-γ_2 produced in *Escherichia coli* under the control of the p_L promoter and purified from solubilized inclusion bodies by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electroelution. These antibodies are shown in Fig. 1 to cross-react with PLC-γ_1 to a very slight extent; however, any cross-reactivity with PLC-γ_1 can be readily distinguished because the two proteins migrate differently on SDS-polyacrylamide gels.

PLC overexpression analysis. For the determination of the relative amount of PLC present in transfected COS-1 cells or in the recombinant rat-2 cell lines, cells were incubated in 5 ml of methionine-free DMEM supplemented with 5% com-

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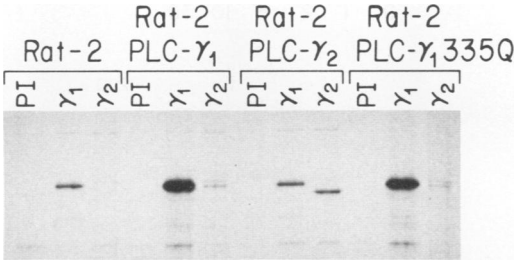

FIG. 1. Overexpression of PLC-γ_1, PLC-γ_2, and PLC-$\gamma_1$335Q in rat-2 cells. Parental rat-2, rat-2PLC-γ_1, rat-2PLC-γ_2, and rat-2PLC-$\gamma_1$335Q cells were labeled for 24 h with [^{35}S]methionine and immunoprecipitated with the indicated antibodies (PI, preimmune; γ_1, PLC-γ_1; γ_2, PLC-γ_2). Immunoprecipitates were separated by electrophoresis through an SDS-7.5% polyacrylamide gel and exposed to X-ray film for 10 h at −70°C.

plete DMEM, 10% dialyzed fetal bovine serum, and 200 μCi of [^{35}S]methionine (1,000 Ci/mmol; Dupont, NEN Research Products) per ml for 24 h at 37°C. Cells were lysed in NETN buffer (20 mM Tris [pH 8.1], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, 200 μM sodium orthovanadate, 10 mM PP_i) and centrifuged at 100,000 × *g* for 1 h. SDS was then added to a final concentration of 0.1%. Equal numbers of trichloroacetic acid-precipitable counts were immunoprecipitated with the indicated antibodies and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Western immunoblotting. Cells were grown to confluence and incubated for 48 h in medium containing 0.5% fetal bovine serum. Cells were then stimulated with 50 ng of PDGF-ββ (Amgen) per ml for 15 min and lysed in NETN, and equal amounts of protein were immunoprecipitated with the indicated PLC antibodies. The immunoprecipitates were separated by gel electrophoresis and immunoblotted with 1 μg of a murine monoclonal antiphosphotyrosine antibody (Upstate Biotechnology Inc.) per ml; this was followed by a 1-h incubation with 10 μCi of a ^{125}I-labeled goat anti-mouse antibody (Dupont) per ml.

Phosphoamino acid analysis. Cells were grown to confluence in 35-mm dishes and incubated for 48 h in medium containing 0.5% fetal bovine serum. The medium was removed and replaced with 3 ml of DMEM minus phosphate, 0.1% bovine serum albumin (BSA), and 2 mCi of $^{32}PO_4$ per ml. Cells were then stimulated with 50 ng of PDGF (Amgen) per ml for 10 min, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in NETN buffer plus 0.1% SDS, and equal amounts of protein were immunoprecipitated with the indicated PLC antibodies. The immunoprecipitates were separated by gel electrophoresis, and the $^{32}PO_4$-labeled PLC bands were excised from the gel and subjected to phosphoamino acid analysis as described previously (9).

In vitro mutagenesis, COS cell transfection, and in vitro PLC activity determination. In vitro mutagenesis was done as described previously (32). Plasmid DNA (10 μg) was transfected into four 100-mm dishes of COS-1 cells by the DEAE-dextran protocol with the addition of a chloroquine treatment as described previously (30). Cells were harvested or labeled 50 h after the addition of the DNA. COS-1 cells were homogenized, and the 100,000 × *g* supernatant was used for determining PLC activity with PI as a substrate as described previously (30).

[^{3}H]IP determination. For the measurement of inositol phosphates (IPs) (15), 5 × 10^5 cells were plated in 35-mm dishes and incubated for 48 h in 3 ml of inositol-free medium supplemented with 1.6% complete DMEM, 10% dialyzed fetal bovine serum, and [^{3}H]myoinositol (20 Ci/mmol; Amersham Corp.) at 10 μCi/ml. Cells were then rinsed twice in inositol-free medium and incubated a further 12 to 18 h in 2 ml of inositol-free medium supplemented with 10 μCi of [^{3}H]myoinositol per ml and 1% platelet-poor plasma. Cells were then rinsed twice with PBS and incubated for 1 h in DMEM plus 0.1% fatty acid-free BSA (Sigma Chemical Co.). LiCl (20 mM) was added for 10 min before the addition of PDGF (Amgen) (50 ng/ml) or AlF_4^- ($AlCl_3$ at 10 μM and NaF at 20 mM). Medium was removed, and the reaction was quenched by adding 0.5 ml of ice-cold 4% perchloric acid containing 1 mg of phytic acid carrier per ml. The mixture was then placed on ice for 10 min. Plates were scraped, and the precipitated protein was centrifuged (1,000 × *g*) for 5 min. The perchlorate was removed by adding 0.08 volume of ice-cold 50 mM EDTA (pH 7.0) and 1.5 volumes of freshly mixed ice-cold 1:1 (vol/vol) 1,1,2-trichlorotrifluoroethane: tri-*n*-octylamine to the supernatant. Samples were vortexed and centrifuged at 1,000 × *g* for 5 min at 4°C. A 400-μl sample of the upper phase was diluted with 10 ml of water and loaded onto a 0.6-ml (packed) Bio-Rad AG1-X8 200/400-mesh resin (formate form). The column was then washed with 10 ml of water followed by 10 ml of 0.06 M ammonium formate to elute the glycerol-PI. IP_1 was eluted with 10 ml of 0.18 M ammonium formate, IP_2 with 10 ml of 0.4 M ammonium formate–0.1 M formic acid, and IP_3 with 10 ml of 0.8 M ammonium formate–0.1 M formic acid. Radioactivity in the eluates was determined by scintillation counting.

RESULTS

PDGF induced tyrosine phosphorylation of PLC-γ_1 and PLC-γ_2 overexpressed in rat-2 cells. To study the in vivo regulation of PLC-γ_1, we established a PDGF-responsive rat-2 cell line which overexpresses PLC-γ_1. For this purpose, the PLC-γ_1 cDNA contained in the expression vector PMT was cotransfected with pSV2neo into rat-2 cells. G418-resistant colonies were isolated, and the relative level of PLC-γ_1 was determined by immunoprecipitation of [^{35}S]methionine-labeled proteins with PLC-γ_1-specific antibodies. One cell line, rat-2PLC-γ_1, was found to overexpress PLC-γ_1 approximately 5- to 10-fold compared with rat-2 cells (Fig. 1). This overexpression did not alter the morphology of the rat-2PLC-γ_1 cells, as they were indistinguishable from the parental cells (data not shown).

To determine whether the heterologous bovine PLC-γ_1 could be phosphorylated on tyrosine in response to PDGF treatment, as has been reported for the endogenous enzyme (17, 20, 34), lysates from unstimulated or PDGF-stimulated cells were immunoprecipitated with PLC-γ_1-specific antibodies, separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and incubated with a monoclonal antiphosphotyrosine antibody (Fig. 2). In response to PDGF stimulation, rat-2PLC-γ_1 cells had approximately three- to fourfold-higher levels of tyrosine-phosphorylated PLC-γ_1 than parental cells. An increased amount of a tyrosine-phosphorylated 180-kDa protein was also observed in the immunoprecipitates from the rat-2PLC-γ_1 cells compared with the parental rat-2 cells. This 180-kDa tyrosine-phosphorylated protein is likely to be the PDGF receptor, as it has been reported by several groups to coimmunoprecipitate with PLC-γ_1 (17, 20).

Recently, another PLC isoform closely related to PLC-γ_1,

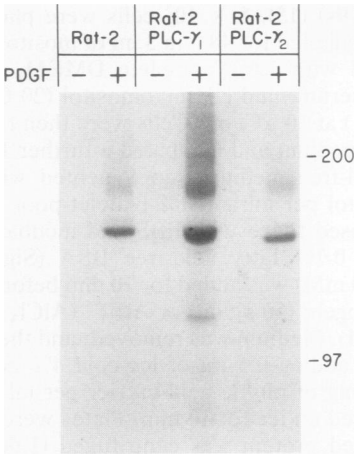

FIG. 2. PDGF-induced tyrosine phosphorylation of PLC-γ_1 and PLC-γ_2. Cells were treated with 50 nM PDGF for 5 min and immunoprecipitated with either PLC-γ_1 antibodies or PLC-γ_2 antibodies. Immunoprecipitates were electrophoresed through an SDS-7.5% polyacrylamide gel, transferred to nitrocellulose, and blotted with antiphosphotyrosine antibodies and ^{125}-labeled goat anti-mouse immunoglobulin G antibodies and then exposed to X-ray film at −70°C for 14 h. Relative molecular masses (in kilodaltons) are indicated on the right.

has been identified and termed PLC-γ_2 (7, 24). To date, this isoform has only been found in cells of the hematopoietic lineage, and we could not detect it in rat-2 fibroblasts (Fig. 1). We were interested in determining whether PLC-γ_2 could be activated by PDGF if expressed in the appropriate cell type. To test this possibility, we established a clonal cell line, rat-2PLC-γ_2, which expressed PLC-γ_2 at about the same level as the endogenous PLC-γ_1 (Fig. 1). To determine whether PLC-γ_2 was phosphorylated on tyrosine after stimulation of rat-2PLC-γ_2 cells with PDGF, immunoprecipitates of PLC-γ_2, and the endogenous PLC-γ_1 were immunoblotted and probed with antiphosphotyrosine antibodies. In response to PDGF stimulation, PLC-γ_1 and PLC-γ_2 were found to be phosphorylated on tyrosine to a similar extent (Fig. 2). The fact that PLC-γ_1 and PLC-γ_2 are expressed at similar levels indicates that the stoichiometry of phosphotyrosine in the two proteins is similar. A tyrosine-phosphorylated protein of 180 kDa which was noted to coprecipitate with PLC-γ_2 is again likely to be the PDGF receptor. We noted that PLC-γ_2 is rapidly phosphorylated on tyrosine, with maximal tyrosine phosphorylation occurring within 1 min of PDGF addition (Fig. 3). These tyrosine phosphorylation kinetics are similar to those reported previously for PLC-γ_1, which has been demonstrated to directly interact with the PDGF receptor (20, 34).

The PDGF-stimulated phosphorylation of PLC-γ_1 and PLC-γ_2 was also examined by metabolic labeling of rat-2PLC-γ_2 cells with $^{32}PO_4$. $^{32}PO_4$-labeled PLC-γ_1 or PLC-γ_2 was immunoprecipitated from control and PDGF-treated rat-2PLC-γ_2 cells, separated by gel electrophoresis, and visualized by autoradiography. The PDGF treatment caused an increase in the phosphorylation of both PLC-γ_1 and PLC-γ_2 (Fig. 4a). A phosphoamino acid analysis of the PLC-γ_1 or the PLC-γ_2 eluted from this gel indicated that PDGF treatment caused a large increase in phosphotyrosine and a smaller but significant increase in phosphoserine in both isoforms (Fig. 4b), confirming the antiphosphotyrosine immunoblotting analysis.

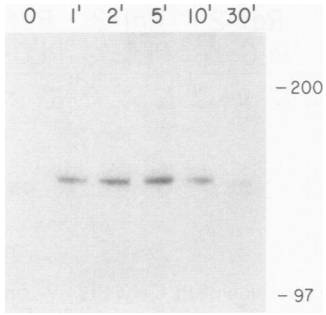

FIG. 3. Kinetics of PDGF-induced tyrosine phosphorylation of PLC-γ_2. rat-2PLC-γ_2 cells were stimulated with 50 nM PDGF for the indicated times (minutes). Cell lysates were immunoprecipitated with PLC-γ_2 antibodies, electrophoresed through an SDS-7.5% polyacrylamide gel, transferred to nitrocellulose, and blotted with antiphosphotyrosine antibodies and ^{125}I-labeled goat anti-mouse immunoglobulin G antibodies. Exposure time was 14 h at −70°C. Relative molecular masses (in kilodaltons) are indicated on the right.

PDGF activates PLC-γ_1 in whole cells. The PDGF-induced tyrosine phosphorylation of PLC-γ_1 in fibroblasts has been previously correlated with an increased production of IPs (30). Fibroblasts have been shown to contain several PLC isoforms, PLC-β_1, PLC-γ_1, and PLC-δ_1 (20). Recently, two additional isoforms, PLC-β_3 and PLC-δ_3, have been isolated from a fibroblast cDNA library, bringing the total number of PLCs expressed in fibroblasts to five (16). It is possible, therefore, that the PDGF-induced phosphoinositide breakdown could be due to the activation of any number of these isoforms. If, however, the PDGF-induced increase in phosphoinositide breakdown is mediated by PLC-γ_1, then after stimulation with PDGF, the rate of production of IPs in the rat-2PLC-γ_1 cell line should exceed that of PDGF-treated rat-2 cells. To test this, we labeled rat-2 cells and rat-2PLC-γ_1 cells with [^{3}H]myoinositol and determined the level of IPs as a function of time following PDGF treatment. The

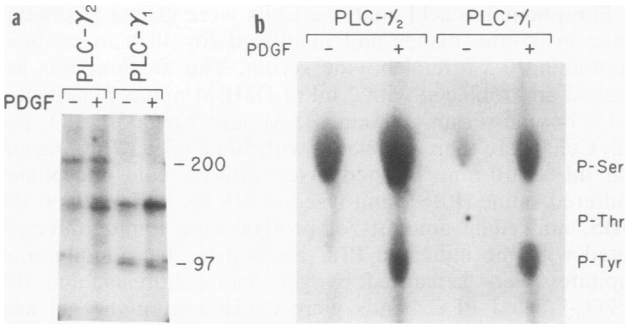

FIG. 4. PDGF-induced phosphorylation of PLC-γ_1 and PLC-γ_2. rat-2 cells expressing PLC-γ_2 were labeled with $^{32}PO_4$ as described in Materials and Methods and stimulated with 50 nM PDGF. $^{32}PO_4$-labeled PLC-γ_2 was immunoprecipitated from rat-2PLC-γ_2 cells (without and with PDGF treatment) with PLC-γ_2 antibodies. The endogenous $^{32}PO_4$-labelled PLC-γ_1 present in the resulting supernatant was immunoprecipitated with PLC-γ_1 antibodies. (a) Immunoprecipitates were separated by electrophoresis on an SDS-7.5% polyacrylamide gel and autoradiographed for 15 h. Relative molecular masses (in kilodaltons) are indicated on the right. (b) PLC-γ_1 and PLC-γ_2 were eluted from the SDS-polyacrylamide gel, subjected to phosphoamino acid analysis, and exposed to X-ray film at −70°C for 14 days. P-Ser, Phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

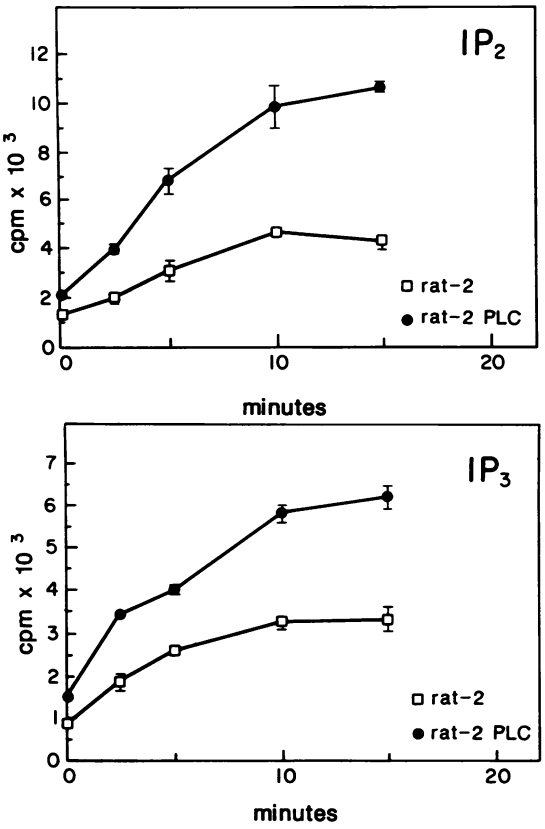

FIG. 5. Kinetics of PDGF-induced [^{3}H]IP$_2$ and [^{3}H]IP$_3$ accumulation in rat-2 and rat-2PLC-γ_1 cells. Cells were labeled with [^{3}H]myoinositol as described in Materials and Methods and stimulated with 50 nM PDGF for the indicated times. IP$_2$ and IP$_3$ were separated as described in the text, and the radioactivity of an aliquot was counted; error bars indicate the range of values obtained for duplicate samples.

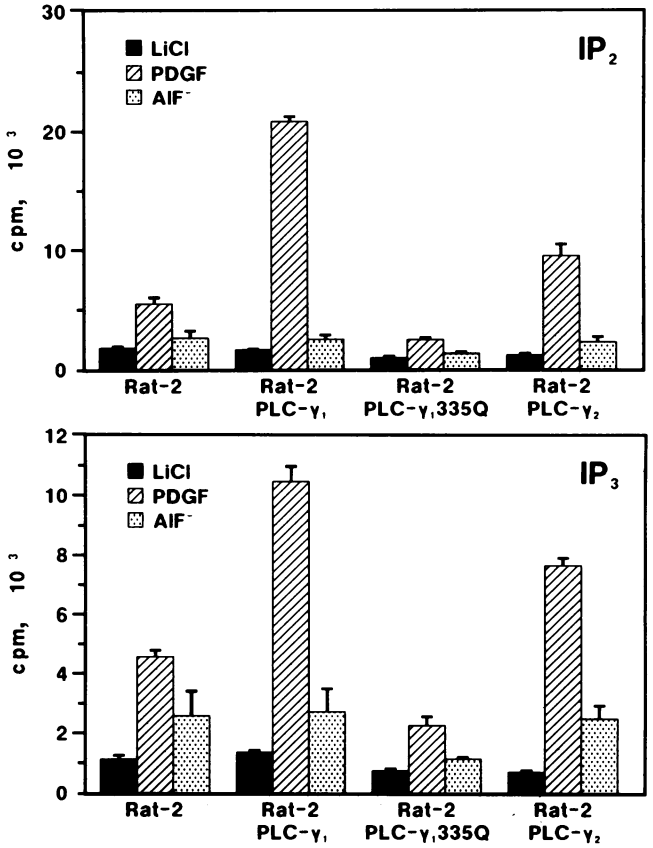

FIG. 6. PDGF- and AlF_4^--induced [^{3}H]IP accumulation in rat-2 and rat-2 cells expressing PLC-γ_1, PLC-$\gamma_1$335Q, or PLC-γ_2. Cells were labeled with [^{3}H]myoinositol as described in the text and stimulated with the indicated agonist, 50 nM PDGF or AlF_4^- (10 μM $AlCl_3$ and 20 mM NaF), for 10 min in the presence of LiCl. [^{3}H]IPs were extracted and separated, and the radioactivity in the eluates was determined by scintillation counting. Error bars indicate the range of values obtained for duplicate samples.

rat-2PLC-γ_1 cells produced both [^{3}H]IP$_2$ and [^{3}H]IP$_3$ at a greater rate and to a greater extent (about two- to threefold), than rat-2 cells (Fig. 5). This two- to threefold increase in IP production is closely correlated with the three- to fivefold increase in tyrosine-phosphorylated PLC-γ_1 shown in Fig. 2.

All activators of PLC, however, do not cause an increased production of IPs in the rat-2PLC-γ_1 cells compared with the parental rat-2 cells. AlF_4^- (aluminum fluoride), a universal activator of PLCs linked to G-proteins, caused a similar stimulation of [^{3}H]IP$_2$ and [^{3}H]IP$_3$ production in both rat-2 cells and rat-2PLC-γ_1 cells, in contrast to the results obtained with PDGF (Fig. 6). Therefore, the overexpression of PLC-γ_1 enhances the production of IPs in response to PDGF but not to AlF_4^-.

The in vivo activity of PLC-γ_2 is also increased in response to PDGF treatment. Since the in vivo activity of PLC-γ_1 was closely correlated with the amount of tyrosine-phosphorylated PLC-γ_1, we were interested to determine whether the tyrosine-phosphorylated PLC-γ_2 also caused an increase in the production of IPs. When rat-2PLC-γ_2 cells were labeled with [^{3}H]myoinositol, PDGF treatment induced a 2- and 1.7-fold increase in [^{3}H]IP$_2$ and [^{3}H]IP$_3$, respectively, in rat-2PLC-γ_2 cells compared with the PDGF-induced increase in [^{3}H]IP$_2$ and [^{3}H]IP$_3$ in rat-2 cells (Fig. 6). These results were confirmed by examining an independent cell line expressing PLC-γ_2, rat-2PLC-γ_2-20. rat-2PLC-γ_2-20 cells expressed about three- to fivefold more PLC-γ_2 than endogenous PLC-γ_1 (data not shown). Stimulation of these cells with PDGF resulted in the production of approximately threefold more [^{3}H]IP$_3$ than stimulation of the parental rat-2 cells (Fig. 7). The amount of tyrosine-phosphorylated PLC-γ_2 in the PLC-γ_2 cells was also noted to be slightly greater than that observed for the endogenous PLC-γ_1 (data not shown).

Although PDGF caused an increased IP release in the two cell lines expressing PLC-γ_2 compared with the parental rat-2 cells (or rat-2 cells transfected with pSV2neo [data not shown]), AlF_4^- caused a similar stimulation of IP production in rat-2 cells and in rat-2 cells expressing PLC-γ_2 (Fig. 6 and 7). The overexpression of PLC-γ_2, therefore, enhances the production of IPs in response to PDGF but not to AlF_4^-.

rat-2 cells overexpressing an enzymatically inactive PLC-γ_1 mutant fail to overrespond to PDGF. Although the increased PLC activity observed in rat-2PLC-γ_1 cells is likely to be due to the activation of the heterologous bovine PLC-γ_1, the increased production of IPs could be due to an indirect effect of the overexpression of PLC-γ_1. To test for this possibility, we constructed an enzymatically inactive PLC-γ_1 by changing a highly conserved histidine, residue 335, to a glutamine residue, and we refer to this mutant as PLC-$\gamma_1$335Q. COS

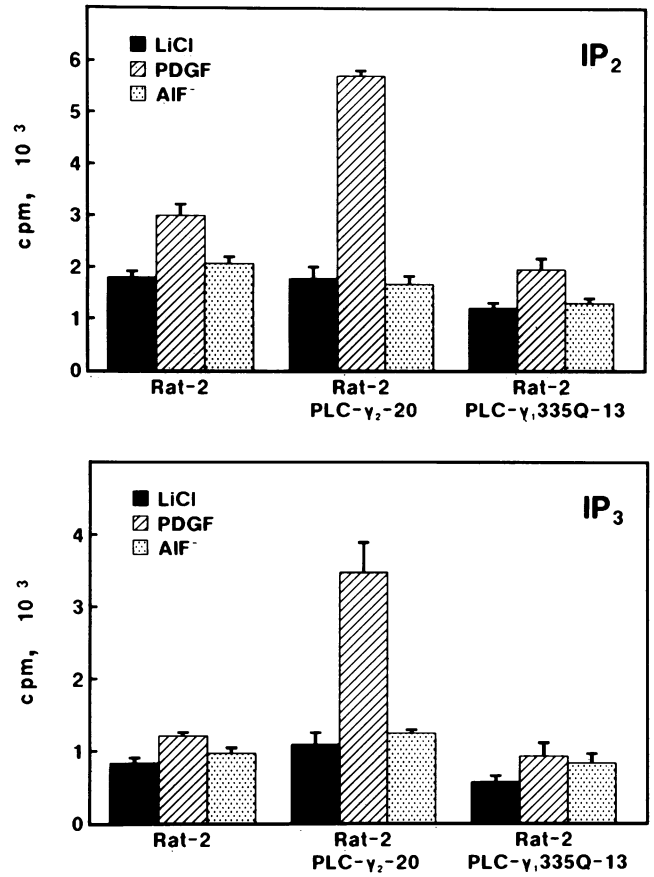

FIG. 7. PDGF- and AlF_4^--induced accumulation of IPs in rat-2, rat-2PLC-γ_2-20, and rat-2PLC-$\gamma_1$335Q-13 cell lines. Cells were labeled with [^{3}H]myoinositol as described in the text and treated with the indicated agonist, 50 nM PDGF or AlF_4^- (10 μM $AlCl_3$ and 20 mM NaF), as indicated for 10 min in the presence of 20 mM LiCl. The levels of IP_1, IP_2, and IP_3 were determined by scintillation counting. Error bars indicate the range of values obtained for duplicate samples.

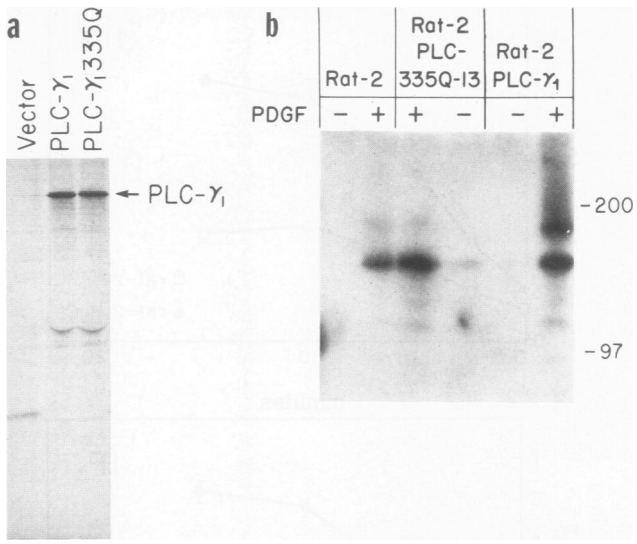

FIG. 8. Expression of PLC-$\gamma_1$335Q in COS-1 cells and tyrosine phosphorylation in rat-2 cells. (a) COS-1 cells were transfected with 10 μg of the indicated PLC plasmid DNA or vector. At 48 h posttransfection, the cells were labeled with [^{35}S]methionine for 24 h and immunoprecipitated with PLC-γ_1 antibodies. Immunoprecipitated proteins were analyzed by electrophoresis through an SDS-7.5% polyacrylamide gel and exposed to X-ray film for 12 h at −70°C. (b) Rat-2, rat-2PLC-γ_1, and rat-2PLC-$\gamma_1$335Q-13 cells were treated with 50 nM PDGF for 10 min. Control and treated cell lysates were immunoprecipitated with PLC-γ_1 antibodies, electrophoresed through an SDS-7.5% polyacrylamide gel, transferred to nitrocellulose, and blotted with antiphosphotyrosine antibodies.

cells were transfected with the pmt-2 vector alone, a construct of a cDNA for PLC-γ_1 subcloned into pmt-2 PLC (pmt-PLC-γ_1), or a construct of a mutant PLC-γ_1 in which histidine 335 was replaced by glutamine (pmt-PLC-$\gamma_1$335Q). Total cell extracts were prepared and assayed for hydrolysis of [^{3}H]IP (see Materials and Methods). PLC activity was 368 cpm/μg of protein for pmt, 3,200 cpm/μg for pmt-PLC-γ_1, and 276 cpm/μg for pmt-PLC-$\gamma_1$335Q. Thus, a 10-fold stimulation of activity is detectable in the cell lysates compared with vector-transfected cells. In contrast, however, when an expression plasmid encoding PLC-$\gamma_1$335Q was transfected into COS cells, no increase in PLC activity was detected compared with vector-transfected cells. Metabolic labeling of transfected COS cells with [^{35}S]methionine for 24 h followed by immunoprecipitation indicated that approximately equal amounts of PLC-γ_1 and PLC-$\gamma_1$335Q protein are produced and at levels at least 10-fold greater than observed in vector-transformed cells (Fig. 8a). Taken together, these data indicate that PLC-$\gamma_1$335Q PI hydrolysis activity must be reduced by at least 10-fold.

Studies on the in vivo activity of PLC-$\gamma_1$335Q were initiated by establishing a clonal cell line, rat-2PLC-$\gamma_1$335Q, overexpressing the mutant enzyme. The expression level of PLC-$\gamma_1$335Q in rat-2PLC-$\gamma_1$335Q cells was severalfold higher than the level of endogenous PLC-γ_1 and only about twofold lower than the level of PLC-γ_1 in the overproducing rat-2PLC-γ_1 cells (Fig. 1). The ability of PLC-$\gamma_1$335Q to become activated in whole cells was examined by labeling PLC-$\gamma_1$335Q cells with [^{3}H]myoinositol and then analyzing the [^{3}H]IPs released upon stimulation with PDGF. No increase in the responsiveness of PLC-$\gamma_1$335Q cells to PDGF was noted compared with their responsiveness to AlF_4^- (Fig. 6). A lack of increased responsiveness to PDGF-induced phosphoinositide turnover was also noted when an independent clonal cell line, PLC-$\gamma_1$335Q-13, which overexpresses PLC-$\gamma_1$335Q at a level three times the endogenous PLC-γ_1 level (data not shown), was tested for it's ability to respond to PDGF (Fig. 7).

Since tyrosine phosphorylation has been closely correlated with the activation of PLC-γ_1, we tested the ability of PLC-$\gamma_1$335Q to become phosphorylated on tyrosine in response to PDGF. Following stimulation with PDGF, PLC-$\gamma_1$335Q was immunoprecipitated from PLC-$\gamma_1$335Q-13 cell lysates with PLC-γ_1 antibodies, blotted, and then probed with phosphotyrosine antibodies. An approximately twofold increase in tyrosine-phosphorylated PLC-γ_1 was observed in the immunoprecipitates derived from the rat-2PLC-$\gamma_1$335Q cells compared with the parental rat-2 cells. The levels of tyrosine-phosphorylated PLC-$\gamma_1$335Q were similar to the levels of tyrosine-phosphorylated PLC-γ_1 found in PDGF-stimulated rat-2PLC-γ_1 cells (Fig. 8b), yet no increased responsiveness to PDGF was observed in the rat-2PLC-$\gamma_1$335Q cells. Therefore, although PLC-$\gamma_1$335Q becomes phosphorylated on tyrosine in response to PDGF treatment, it does not become activated. These results indicate that the

PDGF-induced increase in IP production in rat-2PLC-γ_1 cells is due to the activation of the PI-specific hydrolysis activity of the heterologous bovine PLC-γ_1 and not to some indirect effect caused by the overexpression of another functional domain(s) of the enzyme.

DISCUSSION

We showed that after PDGF stimulation of cells overexpressing PLC-γ_1, the levels of both tyrosine-phosphorylated PLC-γ_1, and IPs increased severalfold compared with those in parental cells, indicating that the in vivo activity of PLC-γ_1 is increased in response to PDGF treatment. The specificity of the response was demonstrated by showing that the overexpression of PLC-γ_1 does not alter the responsiveness to AlF_4^-. Although PLC-γ_1 was expressed at about 10 times the level of the endogenous enzyme, the production of IPs was increased only threefold compared with the level in the parental rat-2 cells. Interestingly, this threefold increase in IP production was noted to correlate with an approximately three- to fivefold increase in the level of tyrosine-phosphorylated PLC-γ_1. These data are consistent with the suggestion that the tyrosine-phosphorylated form of PLC-γ_1 is the active species in vivo (17, 20, 34).

We also showed that PDGF increases the serine and tyrosine phosphorylation of PLC-γ_2. Additionally, we noted that a tyrosine-phosphorylated 180-kDa polypeptide coimmunoprecipitates with PLC-γ_2 in response to PDGF treatment (Fig. 2). This 180-kDa polypeptide is likely to be the PDGF receptor as its presence has been previously detected in PLC-γ_1 immunoprecipitates (17, 20). Further support that the 180-kDa band is the PDGF receptor comes from COS cell studies which show that PLC-γ_2 coimmunoprecipitates with a 180-kDa band in response to PDGF treatment only when cells are transfected with both the PDGF receptor and PLC-γ_2 and not when cells are transfected with PLC-γ_2 alone (data not shown).

The PDGF-induced increase in PLC-γ_2 tyrosine phosphorylation was not a surprising result in light of the sequence similarity of these two isoforms. The most highly conserved region between the two isoforms is within the SH2 and SH3 domains, which are not necessary for catalytic activity and are likely to serve regulatory functions (4, 7, 25, 27).

Interestingly, two of the tyrosine residues of PLC-γ_1 which are rapidly phosphorylated by the epidermal growth factor receptor are located between the SH2 and SH3 regions, while two other more slowly phosphorylated sites are outside of these regions (14, 33). Of these four phosphorylation sites, only tyrosine 783 is conserved between the two PLC-γ isoforms. As it is one of the rapidly phosphorylated sites, it will be interesting to determine whether the phosphorylation of this residue is critical for the activation of PLC-γ. We presented evidence that the two enzymes appear to be phosphorylated on tyrosine with a similar stoichiometry, which argues that there must be additional nonconserved tyrosine phosphorylation sites on PLC-γ_2, but their lack of conservation may argue that they are not as important as tyrosine 783.

Concomitant with the increased phosphorylation of PLC-γ_2 we noted an increase in the in vivo activity of the enzyme. Expression of PLC-γ_2 at a level equivalent to that of the endogenous PLC-γ_1 resulted in a slight overresponsiveness to PDGF when the levels of IPs were measured. Expression of higher levels of PLC-γ_2 in an independent cell line resulted in a more marked overresponsiveness to PDGF, similar to the overresponsiveness observed in the cells overexpressing PLC-γ_1. As was noted for PLC-γ_1, the increase in IP production was correlated with an increase in tyrosine-phosphorylated PLC-γ_2.

Although our studies demonstrated the ability of the PDGF receptor to phosphorylate PLC-γ_2, it is an unlikely natural regulator of this enzyme because the expression of PLC-γ_2 is limited to cells of the hematopoietic lineage, which have not been reported to express the PDGF receptor. However, there are several candidate tyrosine kinases expressed in hematopoietic cells whose activation is linked to increased IP production.

Although our overexpression studies strongly suggested that the increased IP production is due to the hydrolytic activity of the overexpressed enzyme, an indirect effect of the overexpression of PLC-γ cannot be ruled out. Indeed, v-*crk* encodes a protein with significant sequence similarity to the putative regulatory region of PLC-γ, and v-*crk* is believed to mediate its transforming ability by indirectly increasing the activity of cellular tyrosine kinases which could in turn lead to increased PLC-γ activity (25). A similar result may be expected to occur when PLC-γ is overexpressed. However, the overexpression of an enzymatically inactive mutant failed to affect the responsiveness of these cells to PDGF when PI hydrolysis was measured. This enzymatically dead mutant appeared to interact with the PDGF receptor, as it was phosphorylated on tyrosine to a similar extent as the overexpressed PLC-γ_1. However, this interaction may not be identical to that observed with the wild-type enzyme. PLC-γ_1 has been observed to coimmunoprecipitate with several tyrosine-phosphorylated proteins in the range of 100 to 145 kDa (20, 34) (Fig. 8b), and when PLC-γ_1 was overexpressed, increased amounts of these proteins were noted in the immunoprecipitates. Similar results were obtained when the immunoprecipitates derived from the PLC-$\gamma_1$335Q-overexpressing cells were examined (Fig. 8b). These data indicate that the mutant enzyme is phosphorylated to a similar extent as the wild-type enzyme and is able to form complexes with several other tyrosine-phosphorylated polypeptides. Curiously, however, a corresponding amount of coimmunoprecipitating PDGF receptor was not detected in the immunoprecipitates, which may indicate that the interaction of the mutant enzyme with the PDGF receptor is not very stable, but additional experiments are necessary to verify this possibility. It remains possible that this mutant PLC is not properly activated in vivo and that this is the underlying cause of the lack of enzymatic activity. Whatever the mechanism, the overexpression studies using this mutant allowed us to eliminate the possibility that an indirect effect of the overexpression of PLC-γ_1 caused the increased IP production.

Following the submission of the manuscript, two reports appeared which also demonstrated that when PLC-γ_1 is overexpressed in fibroblasts, PDGF treatment induces an increased amount of IP production relative to parental cells (5, 19). Interestingly, however, both of these reports fail to observe an increased mitogenic responsiveness to PDGF, indicating that the activation of PLC-γ_1 is not a limiting factor in the PDGF induced mitogenic response.

During our studies, we and others (5; data not shown) noted that the transfection of PLC-γ_1 into NIH 3T3 cells fails to induce any morphological changes or focus formation. In apparent contradiction with these reports, however, the microinjection of 10,000 molecules of PLC-γ_1 into a 3T3 cell induces the cells to enter the S phase (29). From the amount of PLC-γ_1 present per milligram of protein in 3T3 cell lysates (28), we estimate that the endogenous levels of PLC-γ_1 in

3T3 cells are not likely to be much lower than about 30,000 molecules per cell. Therefore, it is interesting that the microinjection of only a fraction of the total endogenous PLC-γ_1 induces 3T3 cells to enter the S phase and that the microinjection of 150% of the endogenous PLC-γ_1 level results in cell lysis. Even if our estimate of the endogenous levels of PLC-γ_1 was 10-fold lower, 3,000 molecules per cell, these data would still be inconsistent with our observations as well as those of others. One possible explanation is that the microinjected PLC-γ_1 became irreversibly activated at some point in the purification process, or perhaps the microinjected PLC-γ_1 fails to become associated with negative regulatory elements. A clear understanding of the importance of tyrosine and/or serine phosphorylation in the activation of PLC-γ_1 awaits the overexpression of mutant PLC-γ_1 enzymes.

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REFERENCES

1. **Bennett, C. F., J. M. Balcarek, A. Varrichio, and S. T. Crooke.** 1988. Molecular cloning and complete amino-acid sequence of form-I phosphoinositide-specific phospholipase C. Nature (London) **334**:268–270.
2. **Berridge, M. J.** 1984. Inositol triphosphate and diacylglycerol as second messengers. Biochem. J. **220**:345–360.
3. **Berridge, M. J.** 1987. Inositol triphosphate and diacylglycerol: two interacting second messengers. Annu. Rev. Biochem. **56**: 159–193.
4. **Bristol, A., S. M. Hall, R. W. Kriz, M. L. Stahl, Y. S. Fan, M. G. Byers, R. L. Eddy, T. B. Shows, and J. L. Knopf.** 1988. Phospholipase C-148: chromosomal location and deletion mapping of functional domains. Cold Spring Harbor Symp. Quant. Biol. **53**:915–920.
5. **Cuadrado, A., and C. J. Molloy.** 1990. Overexpression of phospholipase C-γ in NIH 3T3 fibroblasts results in increased phosphatidylinositol hydrolysis in response to platelet-derived growth factor and basic fibroblast growth factor. Mol. Cell. Biol. **10**:6069–6072.
6. **Ellis, C., M. Moran, F. McCormick, and T. Pawson.** 1990. Phosphorylation of GAP and GAP associated proteins by transforming and mitogenic tyrosine kinases. Nature (London) **343**: 377–379.
7. **Emori, Y., Y. Homma, H. Sorimachi, H. Kawasaki, O. Nakanishi, K. Suzuki, and T. Takenawa.** 1989. A second type of rat phosphoinositide-specific phosphoinositide-hydrolyzing activity. J. Biol. Chem. **264**:21885–21890.
8. **Hoshijima, M., T. Ueda, Y. Hamamori, T. Ohmori, and Y. Takai.** 1988. Different sensitivity to phorbol esters and pertussis toxin of bombesin and platelet-derived growth factor-induced, phospholipase C-mediated hydrolysis of phosphoinositides in NIH/3T3 cells. Biochem. Biophys. Res. Commun. **152**:285–293.
9. **Kamps, M. P., and M. Sefton.** 1989. Acid and base hydrolysis of phosphoproteins bound to immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. Anal. Biochem. **176**:22–27.
10. **Kaplan, D. R., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** 1990. PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell **61**:125–133.
11. **Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M. White, L. Cantley, and T. M. Roberts.** 1987. Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell **50**:1021–1029.
12. **Katan, M., R. W. Kriz, N. Totty, R. Philp, E. Meldrum, R. A. Aldape, J. L. Knopf, and R. A. Parker.** 1988. Determination of the primary structure of PLC-154 demonstrates diversity of phosphoinositide-specific phospholipase C activities. Cell **54**: 171–177.
13. **Kazlauskas, A., C. Ellis, T. Pawson, and J. A. Cooper.** 1989. Binding of GAP to activated PDGF receptors. Science **247**: 1578–1581.
14. **Kim, J. W., S. S. Sim, U.-H. Kim, S. Nishibe, M. I. Wahl, G. Carpenter, and S. G. Rhee.** 1990. Tyrosine residues in bovine phospholipase C-γ phosphorylated by the epidermal growth factor receptor *in vitro*. J. Biol. Chem. **265**:3940–3943.
15. **Kirk, C. J., A. J. Morris, and B. Shears.** 1990. Inositol phosphate second messenger, p. 149–182. *In* K. Siddle and J. Hutton (ed.), Peptide hormone action: a practical approach. Oxford University Press, Oxford, England.
16. **Kriz, R., L.-L. Lin, L. Sultzman, C. Ellis, C.-H. Heldin, T. Pawson, and J. Knopf.** 1990. Phospholipase C isozymes: structural and functional similarities. Proto-oncogenes in cell development. CIBA Found. Symp. **150**:112–127.

16a. **Kriz, R., and J. Knopf.** Unpublished data.

17. **Kumjian, D. A., M. I. Wahl, S. G. Rhee, and T. O. Daniel.** 1989. Platelet-derived growth factor (PDGF) binding promotes physical association of PDGF receptor with phospholipase C. Proc. Natl. Acad. Sci. USA **86**:8232–8239.
18. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Levitzki, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces tyrosine phosphorylation of phospholipase C-11, a potential mechanism for EGF receptor signaling. Cell **57**:1101–1107.
19. **Margolis, B., A. Zilberstein, C. Francks, S. Felder, S. Kremer, A. Ullrich, S. G. Rhee, K. Skorecki, and J. Schlessinger.** 1990. Effect of phospholipase C-γ overexpression on PDGF-induced second messengers and mitogenesis. Science **248**:607–610.
20. **Meisenhelder, J., P. G. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor proteintyrosine kinases *in vivo* and *in vitro*. Cell **57**:1109–1122.
21. **Molloy, C. J., D. P. Bottaro, T. P. Fleming, M. S. Marshall, J. B. Gibbs, and S. A. Aaronson.** 1989. PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature (London) **34**:711–714.
22. **Morrison, D. M., D. K. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF β-receptor. Cell **58**:649–657.
23. **Morrison, D. K., D. K. Kaplan, U. Rapp, and T. M. Roberts.** 1988. Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. USA **85**:8855–8859.
24. **Ohta, S., A. Matsui, Y. Nazawa, and Y. Kagawa.** 1988. Complete cDNA encoding a putative phospholipase C from transformed human lymphocytes. FEBS Lett. **242**:31–35.
25. **Pawson, T.** 1988. Non-catalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene **3**:491–495.
26. **Rhee, S. G., P.-G. Suh, S.-H. Ryu, and S. Y. Lee.** 1989. Studies of inositol phospholipid-specific phospholipase C. Science **244**: 546–550.
27. **Sadowski, I., J. Stone, and T. Pawson.** 1986. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus p130$^{gag\text{-}fps}$. Mol. Cell. Biol. **6**:4396–4408.
28. **Smith, M. R., Y.-L. Liu, H. Kim, S. G. Rhee, and H.-F. Kung.** 1990. Inhibition of serum, and Ras stimulated DNA synthesis by antibodies to phospholipase C. Science **247**:1074–1077.
29. **Smith, M. R., S.-H. Ryu, P.-G. Suh, S. G. Rhee, and H.-F. Kung.** 1989. S-phase induction of transformation of quiescent NIH 3T3 cells by microinjection of phospholipase C. Proc. Natl. Acad. Sci. USA **86**:3659–3663.

30. **Stahl, M. L., C. R. Ferenz, K. L. Kelleher, R. W. Kriz, and J. L. Knopf.** 1988. Sequence similarity of phospholipase C with the non-catalytic region of *src*. Nature (London) **332**:269–272.
31. **Suh, P.-G., S. H. Ryu, K. H. Moon, H. W. Suh, and S. G. Rhee.** 1988. Cloning and sequence of multiple forms of phospholipase C. Cell **54**:161–169.
32. **Toole, J. J., D. D. Pittman, E. C. Orr, P. Murtha, L. C. Wasley, and R. J. Kaufman.** 1986. A large region (=95 kDa) of human factor VIII is dispensable for in vitro procoagulant activity. Proc. Natl. Acad. Sci. USA **83**:5939–5942.
33. **Wahl, M. I., S. Nishibe, J. W. Kim, H. Kim, S. G. Rhee, and G. Carpenter.** 1990. Identification of two epidermal growth factor-sensitive tyrosine phosphorylation sites of phospholipase C-γ in intact HSC-1 cells. J. Biol. Chem. **265**:3944–3948.
34. **Wahl, M. I., N. E. Olashaw, S. Nishibe, S. G. Rhee, W. J. Pledger, and G. Carpenter.** 1989. Platelet-derived growth factor induces rapid and sustained tyrosine phosphorylation of phospholipase C-γ in quiescent BALB/3T3 cells. Mol. Cell. Biol. **9**:2934–2943.