(S4-C) PDGF Stimulation of Inositol Phospholipid Hydrolysis Requires PLC-γ Phosphorylation on Tyrosine Residues 783 and 1254

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Introduction

The biological actions of PDGF and epidermal growth factor (EGF) are mediated through transmembrane cell-surface receptors that possess ligand-activated cytoplasmic tyrosine kinase activity (2, 5, 17, 21). One of the early cellular events induced by the binding of these growth factors to their respective receptors in the stimulation of PLC, which hydrolyzes inositol phospholipids and generates the second messengers, diacylglycerol and inositol phosphates (1, 9). The stimulation of PLC appears to require the intrinsic tyrosine kinase activity of the receptors because mutant PDGF and EGF receptors that lack tyrosine kinase activity bind the growth factors but fail to stimulate the hydrolysis of inositol phospholipids (3, 11, 14). However, the stimulation of PLC is not essential for PDGF-dependent mitogenesis (4). Both EGF and PDGF is not essential for PDGF-dependent mitogenesis (4). Both EGF and PDGF induce the association of one of the PLC isozymes, PLC-γ, with their respective receptors and stimulate the phosphorylation of PLC-γ on tyrosine residues by the receptor kinases and on serine residues by an unknown protein kinase (7, 10, 13, 15, 19, 20). Mammalian tissues contain at least four immunologically distinct pLC isoforms (16).

Purified EGF receptor phosphorylates tyrosine residues 771, 783, 1254, and to a lesser extent, tyrosine 472, in PLC-γ (6, 20). The major sites of phosphorylation by EGF and PDGF receptors *in vivo* or *in vitro* appeared to be identical (13, 15). Although the overal state of tyrosine phosphorylation in PLC-γ correlates well with growth factor-stimulated inositol phosphate formation, efforts to demonstrate enhanced activity after phosphorylation by purified EGF receptor have been unsuccessful. Furthermore, it is not known whether PLC-γ is the only isoform of PLC responsible for growth factor-induced inositol phospholipid hydrolysis. However, overexpression of PLC-γ in NIH3T3 cells was followed by increases in both tyrosine phosphorylation of PLC-γ and inositol phosphate formation in response to PDGF (12). To investigate the molecular mechanism underlying the stimulation of PLC by growth factor, we removed the sites of tyrosine phosphorylation on PLC-γ by substituting phenylalanine for tyrosine at these sites and characterized the properties of the point mutants.

Results

Expression of normal and mutant PLC-γ in NIH 3T3 cells

Tyrosine residues 771, 783, and 1254, which have been shown to be the main target for phosphorylation by purified EGF receptor kinase (6, 20), were individually changed to phenylalanine to produce mutants Y771F, Y783F, and Y1254F, respectively (where Y is the single-letter code for tyrosine and F is for phenylalanine). Oligonucleotide-directed mutagenesis was performed on a uracil-substituted vector (8). A double mutant, Y771-783F, in which residues 771 and 783 were simultaneously changed to phenylalanine was also generated. We inserted normal and mutant PLC-γ genes into a mammalian expression vector and cotransfected NIH 3T3 cells with the expression vector constructs and plasmid pSV_{neo}. Clones resistant to the antibiotic G418 were selected. To select clones overproducing PLC-γ, we measured the amount of PLC-γ in the lysates of the antibiotic-resistant cells by immunoblotting with antibodies to PLC-γ (anti-PLC-γ) and ^{125}I-labeled protein A. The extent of PLC-γ expression in the five cell lines that stably expressed the normal PLC-γ gene and each of the four mutant genes was varied and 4 to 11 times that in control cells transfected with pSV_{neo} only (data not shown).

We also assayed PLC activity in extract from the five cell lines using the exogenous substrate, [^{3}H]phosphatidylinositol 4,5-bisphosphate([^{3}H]PIP_2). Specfic activities of PLC in extracts from all of the cells transfected with PLC-γ constructs were 4 to 11 times that of PLC in control cells and were almost identical when corrected for the extent of PLC-γ expression (data not shown). Prior treatment of cells with PDGF did not affect the PLC activity measured in crude hemogenates (data not shown). These results confirm that PLC-γ is the most abundant PLC isozyme in NIH 3T3 cells (13) and suggest that neither phosphorylation of PLC-γ induced by PDGF nor mutation at residues 771, 783, or 1254 changes the catalytic activity of PLC-γ measured *in vitro*.

Effect of mutation on PLC-γ phosphorylation and its association with PDGF receptor

Stimulation of NIH 3T3 cells with PDGF causes the phosphorylation of PLC-γ on both tyrosine and serine residues, and antibodies to PLC-γ coprecipitate PDGF receptors from lysates of PDGF-stimulated cells but not from lysates of unstimulated cells (10, 13, 18, 19). We investigated these events with the transfected cells (Fig. 1). Cells were metabolically labeled with [^{32}P]orthophosphate, stimulated with PDGF, and lysed. The cell lysates were subjected to immunoprecipitation with anti-PLC-γ and approximately equal amounts of immunoprecipitated PLC-γ were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. As in the case of control cells, PDGF treatment enhanced both the phosphorylation of PLC-γ and promoted the association of PLC-γ with the autophosphorylated PDGF receptor in all of the PLC-γ-expressing cells except those expressing the Y771-783F enzyme (Fig. 1, upper panel). The phosphate content of PLC-γ was already high in cells expressing the Y771-783F enzyme before PDGF stimulation and further increased after addition of PDGF, but PDGF did not appear to promote association of the enzyme with the PDGF receptor. The extent of tyrosine phosphorylation of immunoprecipitated PLC-γ from unlabeled cells was also measured by immunoblotting with antibodies to phosphotyrosine (Fig. 1, lower panel). Compared to PLC-γ from cells transfected with the normal PLC-γ cDNA

construct, the phosphotyrosine content of the Y771F enzyme was slightly decreased and that of the Y783F and Y1254F enzymes were significantly decreased. No phosphotyrosine was detected in the Y771-783F enzyme. Phosphotyrosine was also not detected in PLC-γ and PDGF receptors from unstimulated cells. The antiphosphotyrosine antibodies revealed the presence of a small number of PDGF receptors associated with the Y771-783F enzyme from PDGF-stimulated cells, but as for the metabolic labeling experiment, this degree of association was comparable to that seen with cells transfected with pSV_{neo} alone. Near absence of PDGF receptors in the immunoprecipitate from Y771-783F cells was confirmed by the use of antibodies to PDGF receptor (data not shown). These results suggest that double mutations at residues 771 and 783 hamper the association of PLC-γ with the PDGF receptor, whereas single mutations do not.

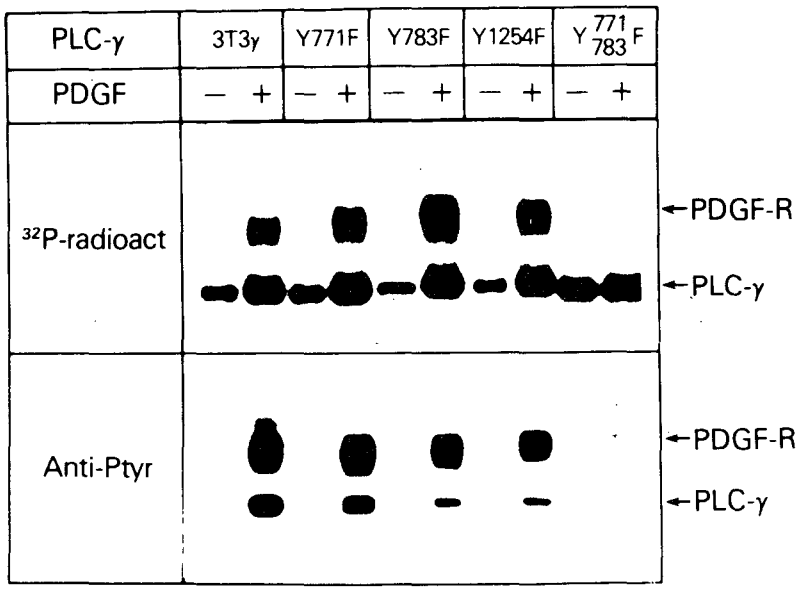

Fig. 1. Stimulation of phosphorylation of PLC-γ by PDGF and coimmunoprecipitation of PLC-γ and the PDGF receptor. (Upper panel) PLC-γ was immunoprecipitated from a 10-cm diameter dish of ^{32}P-labeled NIH 3T3 cells transfected with various PLC-γ expression vectors. Immunoprecipitated proteins were subjected to a 6% SDS-polyacrylamide gel. The volumes of samples from the different cell lines loaded onto the gel were adjusted so that each lane received equal amounts of PLC-γ. The gel was dried and exposed to Kodak XAR film at -70°C for 10 hr. (Lower Panel) Immunoprecipitates were prepared as in upper panel except that [^{32}P]orthophosphate was omitted. After the separation of immunoprecipitated protein on a SDS-polyacrylamide gel, proteins were transferred to a nitrocellulose sheet and immunoblotted with antibodies to phosphotyrosine (ICN Biomedicals, Inc., Costa Mesa, CA). Immune complexes were detected with ^{125}I-labeled protein A and autoradiography. 3T3γ refers to the cells transfected with the normal PLC-γ expression construct. The position of bands corresponding to the PDGF receptor (PDGF-R) and PLC-γ are indicated by arrows. Results are representative of four similar experiments.

Phosphopeptide maps

Two-dimensional analysis of phosphopeptides was undertaken to compare the peptide maps of PLC-γ from cells expressing normal and mutant enzymes (Fig. 2). The maps of PLC-γ from cells transfected with the normal PLC-γ gene closely resembled those previously described for PLC-γ from control NIH3T 3 cells (13, 15). Four peptides containing phosphoserine (peptides 1, 2, 3, and 4 in Fig. 2A) were prominent before stimulation with PDGF. PDGF treatment increased the phosphorylation of peptides 1 and 3 and resulted in the appearance of at least three new peptides containing phosphotyrosine (peptides a, b, and c in Fig. 2B). When the maps of PLC-γ from PDGF-treated cells expressing the mutant enzymes were compared with that of PLC-γ from PDGF-treated cells transfected with the normal PLC-γ gene, the following changes were apparent: (i) The intensity of peptide b in cells expressing Y771F was significantly reduced, but was still too strong to be attributed solely to endogenous PLC-γ present in the cells (Fig. 2C). (ii) The intensities of peptides a and b in cells expressing Y783F were reduced to levels which can be attributed to endogenous PLC-γ (Fig. 2D). (iii) In Y1254F-expressing cells, the intensity of peptide c decreased to the level of that seen with control cells (Fig. 2E).

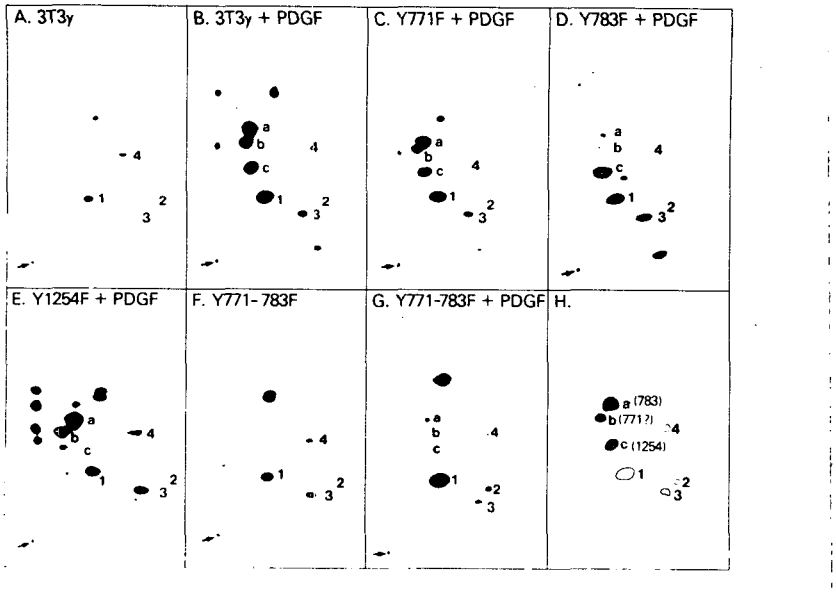

Fig. 2. Comparison of tryptic phosphopeptide maps of normal and mutant PLC-γ phosphorylated in NIH 3T3 cells in response to PDGF. Cell lysate from ^{32}P-labeled cells that had been treated in the absence or presence of PDGF were immunoprecipitated with anti-PLC-γ subjected to electrophoresis. Protein was eluted from the PLC-γ bands, digested with trypsin, and resolved in two dimensions on cellulose thin-layer plates by electrophoresis (horizontal dimension; anode on left) and chromatography (vertical dimension) (13). (A and B) Cells transfected with the normal PLC-γ gene incubated in the absence and presence of PDGF, respectively. (C to E) Cells expressing Y771F, Y783F, and Y1254F, respectively, incubated with PDGF. (F and G) Cells expressing Y771-783F

incubated in the absence and presence of PDGF, respectively. (H) Schematic representation of phosphopeptides in which filled and open spots represent phosphotyrosine-containing peptides and phosphoserine-containing peptides, respectively.

These results suggest that peptide a and c contain tyrosine 783 and tyrosine 1254, respectively. Upon treatment of cells expresing Y771-783F with PDGF, the intensity of phosphoserine peptide 1 increased significantly, whereas the increases in the intensities of phosphotyrosine peptides, a, b, and c were barely visible and could be attributed to the endogenous normal PLC-γ (Fig. 2, F and G). This means that tyrosine 1254 of the double mutant cannot be phosphorylated. In contrast, the phosphorylation of peptide 1 appears to require neither the association with nor tyrosine phosphorylation by the PDGF receptor

Effect of mutation on *in vivo* PLC-γ activity

We next investigated the effect of mutation on the activity of PLC-γ in intact cells as measured by inositol phosphate formation in response to PDGF (Fig. 3). As described previously (12) cells transfected with the normal PLC-γ gene produced five times the amount of inositol phosphates as control NIH 3T3 cells in response to PDGF. The amount of inositol phosphates formed by Y771F-expressing cells in response to PDGF was 50% more than that produced in cells transfected with the normal PLC-γ gene.

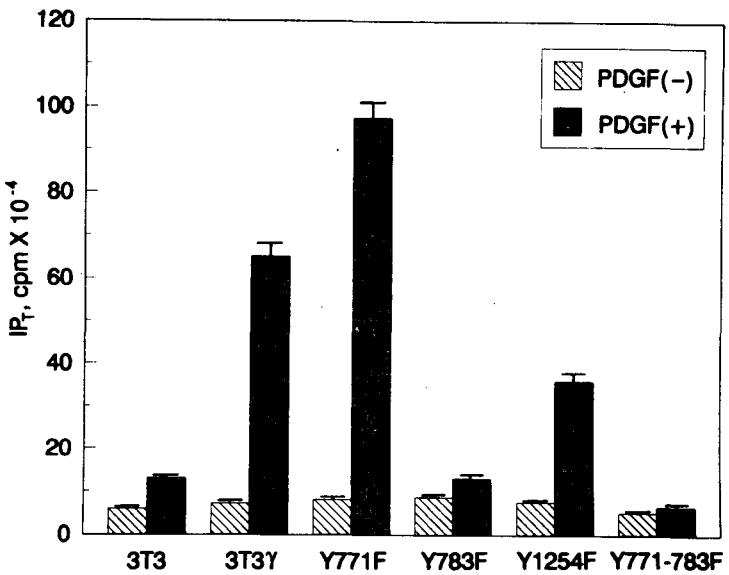

Fig. 3. Effect of mutation of PLC-γ on inositol phosphate generation. Cells were metabolically labeled with [^{3}H]myo-inositol for 60 hr and stimulated with 50 ng/ml PDGF (solid bars) or PDGF carrier buffer (hatched bars) at 37°C for 30 min. Total inositol phosphates formed during the stimulation were calculated per milligram of protein to correct for the differences in growth rate. 3T3 refers to control cells transfected with pSV_{neo} and 3T3γ to cells transfected

with the normal PLC-γ gene. Results shown are the means ± SD of values from five separate experiments. The normalized data obtained by taking the value for unstimulated 3T3 as 100 are listed (the first and second numbers in parenthesis are the values for unstimulated and stimulated cells, respectively): 3T3 (110, 215); 3T3γ (115, 1060); Y771F (135, 1600); Y783F (145, 215); Y1254 F (125, 580), and Y771-783F (86, 110).

In contrast, the response in Y783F-expressing cells was reduced to the level of that in control cells, suggesting that Y783F cannot be activated at all by PDGF. Total unresponsiveness to PDGF was also observed with cells expressing Y771-783F, even though the extent of PLC-γ expression in these cells is the highest among the transfected cells. It also appears that the double-mutant enzyme has an inhibitory effect on endogenous PLC as the amount of inositol phosphate formed in response to PDGF in cells expressing Y771-783F was 51% that formed in control cells. This inhibitory effect may be due to the mutant PLC-γ competing for PDGF receptors with endogenous PLC-γ, even though the binding affinity of the double mutant for PDGF receptors is not strong enough for the complex to survive immunoprecipitation procedures.

Although the levels of PLC-γ expression in cells transfected with the normal and Y1254F genes are similar, the response to PDGF in the latter cells is one-half that seen in the former. This indicates that phosphorylation of tyrosine 1254 is necessary for full activatoin of PLC-γ.

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