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Activation of Phospholipase Cγ2 by Tyrosine Phosphorylation

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ABSTRACT

Phospholipase Cγ2 (PLCγ2) has been implicated in collagen-induced signal transduction in platelets and antigen-dependent signaling in B-lymphocytes. It has been suggested that tyrosine kinases activate PLCγ2. We expressed the full-length cDNA for human PLCγ2 in bacteria and purified the recombinant enzyme. The recombinant enzyme was Ca^{2+}-dependent with optimal activity in the range of 1 to 10 μM Ca^{2+}. In vitro phosphorylation experiments with recombinant PLCγ2 and recombinant Lck, Fyn, and Lyn tyrosine kinases showed that phosphorylation of PLCγ2 led to activation of the recombinant enzyme. Using site-directed mutagenesis, we investigated the role of specific tyrosine residues in activation of PLCγ2. A mutant form of PLCγ2, in which all three tyrosines at positions 743, 753, and 759 in the SH2-SH3 linker region were replaced by phenylalanines, exhibited decreased Lck-induced phosphorylation and completely abolished the Lck-dependent activation of PLCγ2. Individual mutations of these tyrosine residues demonstrated that tyrosines 753 and 759, but not 743, were responsible for Lck-induced activation of PLCγ2. To confirm these results, we procured a phosphospecific antibody to a peptide containing phosphorylated tyrosines corresponding to residues 753 and 759. This antibody recognized phosphorylated wild-type PLCγ2 on Western blots but did not interact with unphosphorylated PLCγ2 or with PLCγ2 containing mutated tyrosine residues at 753 and 759. Using this antibody, we showed in intact platelets that collagen, a PLCγ2-dependent agonist, induces phosphorylation of PLCγ2 at Y753 and Y759. These studies demonstrate the importance of these two tyrosine residues in regulating the activity of PLCγ2.

Most of the regulatory interactions of PLCγ isozymes are mediated through receptor or nonreceptor tyrosine kinases. The stimulation of PLCγ1 has been linked to almost all polypeptide growth factor receptors having intrinsic tyrosine kinase activity (Kamat and Carpenter, 1997; Rhee and Bae, 1997). Upon stimulation, the cytoplasmic domains of growth factor receptors become autophosphorylated on tyrosine residues. This process creates phosphotyrosine binding sites for PLCγ1−SH2 domains, resulting in the interaction of PLCγ1 with the growth factor receptor and subsequent phosphorylation of the PLCγ itself. In vivo and in vitro tyrosine phosphorylation of PLCγ1 by purified epidermal growth factor or platelet-derived growth factor receptors occurs at analogous tyrosine residues at positions 771, 783, and 1254 (Kim et al., 1991). By substituting phenylalanine for tyrosine at these three sites and expressing the mutant PLCγ1 enzymes in NIH/3T3 cells, Kim and colleagues (1991) demonstrated the importance of these residues on the in situ functioning of PLCγ1. PLCγ1 activity can also be stimulated through the stimulation of a number of other receptors (e.g., T-cell antigen receptor, IgE receptor) which do not themselves possess tyrosine kinase activity but are associated with nonreceptor tyrosine kinases such as Src or Syk. It has been proposed that nonreceptor tyrosine kinases phosphorylate receptors or adapter proteins on tyrosine residues to generate a PLCγ binding site. Thus they have a role similar to that of the tyrosine receptor kinase's catalytic domain (Rhee and Bae, 1997).

PLCγ2 also is phosphorylated on tyrosine residues in response to growth factors and activation of nonreceptor tyrosine kinases. However, much less is known concerning the activation of PLCγ2, which is mainly, but not exclusively, found in hematopoietic cells. Platelet-derived growth factor increases the phosphorylation of PLCγ2 in rat-2 fibroblasts (Sultzman et al., 1991) and induces the expression of PLCγ2 in rabbit vascular smooth muscle cells (Homma et al., 1993). However, it is unclear whether growth factor signaling depends on PLCγ2-dependent activation to a major extent. Direct evidence for the importance of PLCγ2 in B-cell and platelet function comes from gene knockout studies in which the maturation of B but not T lymphocytes was found to be impaired (Hashimoto et al., 2000; Wang et al., 2000). In addition, signaling through appropriate receptors was found to be defective in both B lymphocytes and platelets.

In an attempt to delineate the mechanism of regulation of PLCγ2 activity, we expressed enzymatically active recombinant PLCγ2 in *Escherichia coli* and demonstrated its phos-

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ABBREVIATIONS: PLCγ2, phospholipase Cγ2; PCR, polymerase chain reaction; bp, base pair; DTT, dithiothreitol; PIP_2, phosphatidylinositol bisphosphate; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; IP_3, inositol 1,4,5-trisphosphate.

phorylation and activation by the recombinant Src-family kinases Lck, Lyn, and Fyn. We also identified the tyrosine residues in the SH2-SH3 linker region of PLCγ2 that are involved in the regulation of the enzyme activity of PLCγ2. We have shown that phosphorylation of these residues occurs in intact platelets when they are stimulated with collagen.

Materials and Methods

Materials. Human PLCγ2 cDNA containing pMT2 plasmid was a gift from Dr. Joseph Baldassare (Saint Louis University, Saint Louis, MO). Competent DH5α cells (subcloning efficiency) and competent BL21(DE3) cells were purchased from Invitrogen (Carlsbad, CA). The cloning vectors Bluescript KS±, pCAL-n, XL-10 Gold ultracompetent cells, and calmodulin affinity resin were from Stratagene (La Jolla, CA). Ready-To-Go T4 DNA ligase was from Amersham Biosciences Inc. (Piscataway, NJ). PIP_2 ammonium salt was from Sigma (St. Louis, MO), and [^{3}H]PIP_2 was obtained from PerkinElmer Life Sciences (Boston, MA). Monoclonal antibody YL 1/2 was from Harlan Bioproducts for Science (Indianapolis, IN). Anti-PLCγ2 antibody was a gift from Dr. Graham Carpenter (Vanderbilt University, Nashville, TN). Phosphatase-labeled secondary antibodies and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium membrane phosphatase substrate were from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Synthetic oligonucleotides were obtained from Genosys (Woodlands, TX). PCR products and plasmids were purified using QIAquick Gel Extraction Kit and QIAGEN Plasmid Kit from QIAGEN (Valencia, CA). Restriction enzymes and Wizard Plus Minipreps DNA Purification System were obtained from Promega (Madison, WI). GELCODE Blue staining reagent was from Pierce (Rockford, IL). All other reagents were purchased from Sigma unless otherwise indicated. Glutathione *S*-transferase–Lck and Fyn tyrosine kinases were gifts from Dr. Alexander Y. Tsygankov (Temple University, Philadelphia, PA). These kinases were produced in *Spodoptera frugiperda* cells as glutathione *S*-transferase fusion proteins and purified using glutathione agarose (Lehr et al., 1996). We also used Src-family kinases from commercial sources. Lck and Fyn were from Upstate Biotechnology (Lake Placid, NY) and Lyn was from Sigma.

Subcloning of PLCγ2 Coding Sequence to pCAL-n. The strategy for subcloning of human PLCγ2 into the bacterial expression vector pCal-n included PCR reactions at both ends of the cDNA molecule. These PCR reactions provided appropriate restriction sites for subcloning purposes. For the 5′ end PCR reaction, primer 1 (5′-GCTCTAGATC-TATGTCCACCACG GTCAAT-3′), primer 2 (5′-TTCGTCAAGCGGTC-3′), and template DNA in pMT2 were used, and the resultant product was digested with *Xba*I and *Eco*RV to generate a 222-bp fragment. Primer 1 (sense) contained *Xba*I and *Bgl*II sites (newly engineered into the noncoding region), and primer 2 (antisense) spanned a stretch beyond the internal *Eco*RV site that is found at position 291. This fragment, together with the 2716-bp *Eco*RV, *Sal*I fragment obtained from PLCγ2 cDNA, were subcloned into *Xba*I, *Sal*I digested Bluescript II KS±, generating a 5898-bp construct designated PLCγ2pBS1. This construct was propagated in DH5α cells. For the 3′ end PCR reaction, primer 3 (5′- GTCGCCAGCTGCGGCGGCGGCAA-3′), primer 4 (5′-CCCCAAGCTTCTAAAA TTCTTCTGAGTAAAACTTGCTGACTC-TCTTCTCTCTTAACCTCTTGTTGACTTTCTCCTGGTACAACTGGA-3′), and template DNA in pMT2 were used, and the resultant product was digested with *Pvu*II and Hind III to generate a 196-bp fragment. Primer 3 (sense) mutated AGGAGG arginine codons to CGGCGG arginine codons at positions 1204 and 1205. Tandem AGG-AGG arginine codons at the amino acid positions 1204 to 1205 were replaced by CGG-CGG arginine codons to allow the protein expression in a bacterial system (Bonekamp and Jensen, 1988). Primer 4 (antisense) contained a DNA sequence encoding a Glu-Glu-Phe epitope tag that is attached to the end of the coding sequence, as well as a Hind III restriction site after the stop codon and C-to-T point mutation to abolish the *Pvu*II site at the position 3699. This 196-bp fragment, together with the 692-bp *Sal*I, *Pvu*II fragment obtained from PLCγ2 cDNA in pMT2, were subcloned into *Sal*I, *Hin*dIII digested Bluescript II KS±, generating a 3848-bp construct designated PLCγ2pBS2. Digestion of PLCγ2pBS1 with *Bgl*II and *Sal*I and digestion of PLCγ2pBS2 with *Sal*I and Hind III yielded 3935-bp and 888-bp fragments, respectively. These two fragments were ligated into *Bam*HI and Hind III sites in the polycloning region of pCal-n vector downstream of and in frame with the calmodulin binding protein coding sequence, generating a 9592-bp construct, PLCγ2pCAL-n. The presence of the insert was verified with *Eco*RV digestion. This construct was used to produce a PLCγ2 fusion protein containing calmodulin binding peptide at its N-terminal end and the Glu-Glu-Phe tag at its C terminus. The codons for the C-terminal epitope Glu-Glu-Phe was attached to the 3′ end of the coding sequence to detect expressed protein with the commercially available monoclonal antibody YL 1/2 (Stammers et al., 1991).

Site-Directed Mutagenesis. Mutation of tyrosines Y743, Y753, and Y759 to phenylalanine was accomplished by overlapping PCR (Higuchi et al., 1988). After purification and digestion with *Sac*II and *Sal*I, the resultant mutant PCR product was substituted into the corresponding region of PLCγ2pCal-n. The constructs were designated PLCγ2pCal-n–Y743/753/759F, PLCγ2pCal-n–Y753/759F, PLCγ2pCal-n–Y743F, PLCγ2pCal-n–Y753F, and PLCγ2pCal-n–Y759F. All constructs were sequenced to confirm the mutations.

Expression and Purification of Human PLCγ2. The pCAL vector-based protein expression and purification system was developed by Zheng and colleagues (1997). When a cDNA is cloned in frame with the calmodulin binding peptide coding sequence, a calmodulin binding peptide fusion protein can be expressed, which can be rapidly purified by chromatography using commercially available calmodulin affinity resin. For bacterial expression of PLCγ2, PLCγ2pCAL-n–transformed *E. coli* BL21(DE3) strain was grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin at room temperature and induced with relatively low isopropyl thiogalactoside. Otherwise, PLCγ2 was found in inclusion bodies, which were easily purified, but PLCγ2 proved difficult to renature. Upon reaching an optical density at 550 nm of 0.8 to 1.0 A.U., the cells were induced with 0.1 mM isopropyl β-D-thiogalactoside and were harvested by centrifugation (30 min at 30,000*g*) after 6 h of induction time. The pellet was resuspended at a concentration of 10 mg/ml in binding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM DTT, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin A). The resuspended cells were lysed by sonication (5 × 20 s) while chilled on ice. The lysate was incubated with 1% Nonidet P-40 for 15 min at 4°C, and the cellular debris was formed into pellets by centrifugation (10 min at 10,000*g*). The supernatant was subjected to calmodulin affinity chromatography. Briefly, calmodulin affinity resin was equilibrated with binding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM DTT, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin A) and then incubated with the crude *E. coli* lysate at 4°C for 2 h. After binding, the beads were formed into pellets, and the unbound material was removed. The beads were washed three times with 100 volumes of binding buffer, and the fusion protein was eluted with 3 volumes of elution buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM DTT, 1 mM magnesium acetate, 1 mM imidazole, and 2 mM EGTA). When optimal induction conditions were used, BL21(DE3) cells having this vector expressed the PLCγ2 fusion protein at a level of approximately 10 to 20 μg/l soluble protein. Recombinant PLCγ2 was purified from crude extract to approximately 80% purity after one pass through the calmodulin affinity resin. The recombinant PLCγ2 retained its catalytic activity, and its specific activity was 60 nmol/min/mg as determined using PIP_2 as the substrate. In Western blots, the major band was recognized as PLCγ2 using two different antibodies: antibody YL 1/2 (against the glu-glu-phe epitope tag) (Harlan Bioproducts for Science), and anti-PLCγ2 antibody (donated by Dr. Graham Carpenter, Vanderbilt University, Nashville, TN) (data not shown).

Preparation of a Phosphospecific Antibody to a Phosphorylated Peptide Containing Residues Y753 and Y759. Phosphospecific antibodies were raised through a commercial contract with Research Genetics (Huntsville, AL). A 13-amino acid peptide (Asn-Ser-Leu-Tyr-Asp-Val-Ser-Arg-Met-Tyr-Val-Asp-Pro) was synthesized using multiple antigenic peptide resin technology (Tam, 1988). The corresponding phosphorylated peptide was prepared by synthesis of a new peptide using phosphorylated tyrosine residues. The resin-linked phosphopeptide was injected into New Zealand White rabbits. The initial injection was followed by two booster injections. Serum was taken, and the antibody was purified by two affinity procedures. The unphosphorylated peptide was used to absorb out antibodies that were not phosphospecific. The doubly phosphorylated peptide was used as an affinity reagent to isolate antibodies specific for doubly phosphorylated PLCγ2.

SDS-PAGE and Immunoblotting. SDS- PAGE was performed according to the procedures described by Laemmli (1970). The gels were either subjected to electrophoretic transfer for immunoblotting or were stained with reagent GELCODE Blue (Pierce) for visualization of the proteins. Electrophoresed SDS-polyacrylamide gels were electrophoretically transferred to Immobilon-P (Millipore Corporation, Bedford, MA). After blocking with 5% nonfat dry milk, blots were incubated with the primary antibody at 25°C for 1 h. Probing of antibody binding was performed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies. Detection was done by chemiluminescence using SuperSignal (Pierce) with a FujiFilm Las-1000 imaging system (FujiFilm Medical Systems, Stamford, CT). The digitized images were quantified with the use of Image Gauge software (version 3.4; FujiFilm Medical Systems). Alternatively in some experiments, phosphorylated bands were detected and analyzed using the Cyclone phosphoimaging system (PerkinElmer Life Sciences).

Assay of PLCγ2 Activity. The hydrolysis of Ptd[^{3}H]Ins-4,5-P_2 was measured in a reaction mixture (50 μl) that contained 35 mM NaH_2PO_4, pH 6.8, 70 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 0.6 mM $CaCl_2$ (1 μM final Ca^{2+} concentration), 5 μg/ml bovine serum albumin, 5 mM DTT, 200 μM Ptd[^{3}H]Ins-4,5-P_2 (25,000 dpm), 5 mM *n*-octyl glucoside, and the recombinant PLCγ2 purified from *E. coli*. An aliquot of PLCγ2 suspension (5 μl) was added to the substrate solution (45 μl), and the reaction mixture was incubated at 25°C for the various times. Reactions were stopped by transfer to an ice bath with the addition of 0.5 ml of chloroform/methanol/HCl (100:100:0.6) followed by 0.15 ml of 1 N HCl containing 5 mM EDTA. The aqueous and organic phases were separated by centrifugation, and a 200 μl portion of the upper aqueous phase was removed for liquid scintillation counting. Ca^{2+} dependence was measured in a reaction mixture in which free Ca^{2+} concentration was adjusted by varying the ratio of $CaCl_2$ to EDTA (see *Ca^{2+} Dependence of Recombinant PLCγ2 Activity)*.

In Vitro Kinase Assays. The reactions were performed in in vitro kinase buffer (50 mM MOPS, pH 7.4, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 mM DTT). The reactions were started by adding 25 μM of ATP (10 μCi of [^{32}P]ATP) to a mixture of Src-family kinase and PLCγ2, bringing the total volume to 50 μl with in vitro kinase buffer. The reactions were carried out at 24°C for the indicated periods of time, and the incorporation of [^{32}P]ATP was stopped on ice by adding 4× SDS-PAGE sample buffer. Samples were then analyzed with the use of SDS-PAGE and autoradiography. Autoradiograms were scanned, and the labeled bands were analyzed using the NIH image program (http://rsb.info.nih.gov/nih-image/). Gaussian fit was performed for the quantification of the labeled bands.

Ca^{2+} Dependence of Recombinant PLCγ2 Activity. Calcium is necessary for the activity of all mammalian PLC isozymes, and it interacts with several domains of the enzyme including catalytic domain, EF domains, and C2 domain (Katan, 1998). When PIP_2 is used as a substrate, low Ca^{2+} concentrations activate the enzyme, whereas high Ca^{2+} concentrations inhibit it, creating a peak of catalytic activity as a function of free calcium concentration. Therefore, the Ca^{2+} concentrations at which the peak occurs can be taken as strong evidence for the correct conformation of the enzyme. To make this measurement, we relied on the Mg^{2+}-EDTA buffer system described by Wolf (1973). This buffer system has the advantage of being pH-stable. For calibration of our buffers, we used the indicator dye BTC (Molecular Probes, Eugene, OR) and assumed an apparent K_d for Ca^{2+} of 7 μM. The Ca^{2+} dependence of the activity of recombinant PLCγ2 was determined (Fig. 1). We observed that PLCγ2 was stimulated by nanomolar concentrations of Ca^{2+}. The hydrolysis rate increased with increasing Ca^{2+} concentrations of up to approximately 1 to 10 μM and then decreased. The half-maximal stimulation of the enzyme was achieved at ~550 nM Ca^{2+} concentration. Thus, the calcium dependence of the purified recombinant PLCγ2 was found to be the same as those reported for PLCγ2 purified from platelets (Banno et al., 1990), PLCγ1 purified from bovine brain (Wahl et al., 1992; Koblan et al., 1995), and recombinant PLCγ1 expressed in bacteria (Koblan et al., 1995), the maximal activity being between 1 and 10 μM free Ca^{2+} concentrations. Note that this and all other assays were performed at 25°C because PLCγ2 was much more stable at this temperature. The addition of DTT to the assay buffer also helped to stabilize the enzyme.

Preparation of Human Platelets. Human blood was collected from informed healthy volunteers in acid/citrate/dextrose. Platelet-rich plasma was obtained by centrifugation at 180*g* for 15 min at ambient temperature and incubated with aspirin (1 mM) at 37°C for another 45 min. Platelets were isolated from the incubation medium by centrifugation (800*g* for 15 min, ambient temperature). The final buffer consisted of 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES, pH 7.4, 0.2% bovine serum albumin, and 20 μg/ml apyrase. The platelet count was adjusted to 2×10^8 cells/ml.

Results

The Phosphorylation of Recombinant PLCγ2 by Lck, Fyn, and Lyn Tyrosine Kinases. Stimulation of lymphocytes and platelets induces phosphorylation of PLCγ2 on tyrosine residues, and there is evidence to implicate Src-family kinases in these events (Ezumi, 1998; Briddon and Watson, 1999;

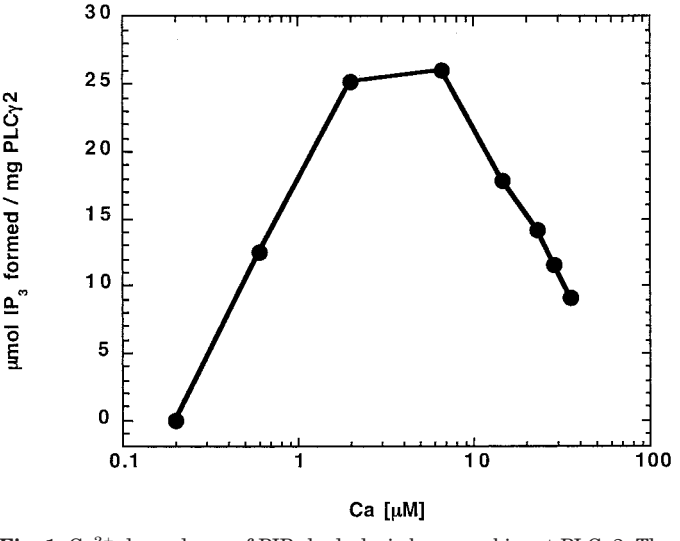

Fig. 1. Ca^{2+} dependence of PIP_2 hydrolysis by recombinant PLCγ2. The hydrolysis of PIP_2 was measured in a buffer containing varying amounts of free Ca^{2+}, which were adjusted by varying the ratio of Ca^{2+} to EDTA. The reaction was started with the addition of recombinant PLCγ2, incubated for 1 min at 37°C, then stopped and the radioactivity was determined as described under *Materials and Methods*. The amount of IP_3 formed was measured, and the data were plotted as the amount of IP_3 released versus log[free Ca^{2+}]. Each point represents a single determination and is representative of at least six determinations.

Wong, 1998; Liao et al., 1993). Thus, we determined the ability of the Src-family tyrosine kinases Lck, Fyn, and Lyn to phosphorylate recombinant PLCγ2 in vitro. Recombinant PLCγ2 was incubated with purified recombinant tyrosine kinases Lck, Fyn, and Lyn in the presence of [^{32}P]ATP. After in vitro kinase reaction, the proteins were separated by SDS-PAGE and subjected to autoradiography. All of the tyrosine kinases tested were able to phosphorylate recombinant PLCγ2 in a time-dependent fashion, with phosphorylation being complete in 15 to 30 min at 25°C (Fig. 2). In general, we performed all assays at this temperature because the enzyme was much more stable. It is important to determine whether the ability of tyrosine kinases to phosphorylate PLCγ2 was associated with increased PLCγ2 enzymatic activity. Nishibe and colleagues (1990) reported that the detergent Triton X-100 can be used to measure the regulation of PLCγ1. Thus, to demonstrate the phosphorylation-dependent increase in the catalytic activity of the enzyme, we modified the assay procedure to include Triton X-100. We found that although unphosphorylated PLCγ2 was inhibited, the Lck-phosphorylated enzyme was activated by increasing concentrations of Triton X-100 (Fig. 3). A concentration of 0.015% was found to be optimal in giving a difference between Lck-phosphorylated PLCγ2 and nonphosphorylated PLCγ2. This behavior is similar to that of PLCγ1 with the exception that the difference between phosphorylated and unphosphorylated PLCγ1 was optimal at a higher Triton X-100 concentration.

To determine whether PLCγ2 enzymatic activity was elevated in correlation with the tyrosine kinase induced phosphorylation, we preincubated recombinant Lck, Lyn, and Fyn kinases and ATP with PLCγ2 to phosphorylate PLCγ2 and then measured inositol-(1,4,5)trisphosphate production in the PLC assay. Lck stimulated wild-type PLCγ2 activity approximately 6-fold over the activity measured from unphosphorylated control PLCγ2 (Fig. 4). Both Fyn and Lyn were able to cause similar activation of PLCγ2.

Role of Tyrosine Residues in SH2-SH3 Linker Region in Phosphorylation and Activation of PLCg2. The major regulation sites of PLCγ1 by tyrosine phosphorylation in response to stimulation of different growth factor receptors are Y771 and Y783 in the SH2-SH3 linker region and Y1254 in the carboxyl terminal (Kim et al., 1991). Phosphorylation of Y783 is reported to be essential for growth factor receptor activation of PLCγ1 (Mohammadi et al., 1991). Determination of the possible tyrosine phosphorylation and activation sites in PLCγ2 is complicated by the fact that 58 tyrosine residues are present in the wild-type PLCγ2. It has been previously claimed that the sequences surrounding Y753 and Y759 in PLCγ2 are similar to those surrounding Y771 and

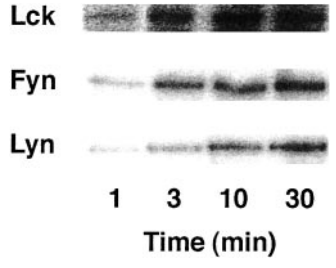

Fig. 2. Phosphorylation of recombinant PLCγ2 by recombinant Lck, Fyn, and Lyn tyrosine kinases. Reactions contained either Lck (4 mU/ml), Fyn (45 mU/ml), or Lyn (12 μg/ml) and recombinant PLCγ2 mixture in in vitro kinase buffer. Units given are those defined by Upstate Biotechnology Inc. The reactions were started by adding 25 μM of [^{32}P]ATP and were carried out at 25°C for the indicated periods of time. Phosphorylated bands were detected using Cyclone phosphoimaging system (PerkinElmer Life Sciences). These data are representative of more than three determinations.

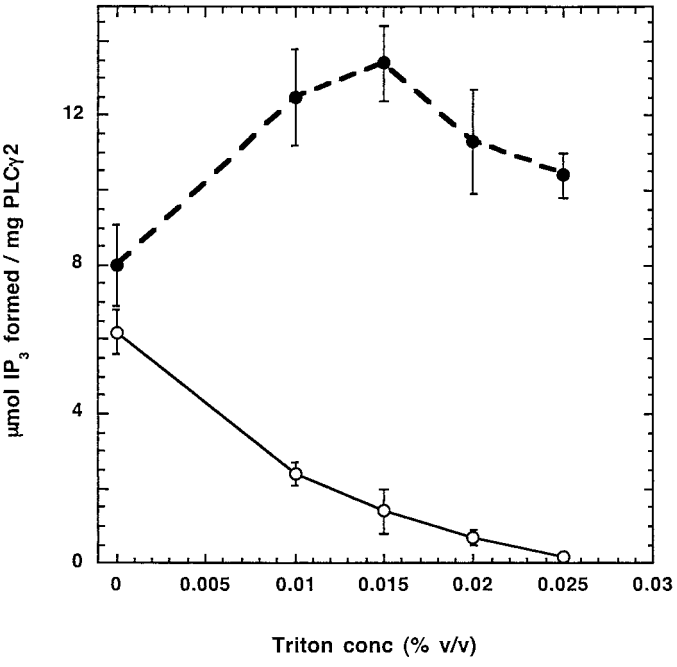

Fig. 3. The effect of Triton X-100 concentration on the activity of unphosphorylated and Lck-phosphorylated PLCγ2. Recombinant PLCγ2 was subjected to in vitro phosphorylation by Lck, in the presence (phosphorylated) or absence (unphosphorylated) of ATP and subsequently assayed for phospholipase activity. [^{3}H]IP_3 formation was quantified by scintillation counting. Each point is the mean of three determinations, and error bars indicate standard deviation. This experiment was repeated at least six times and was used to assess the quality of each batch of substrate.

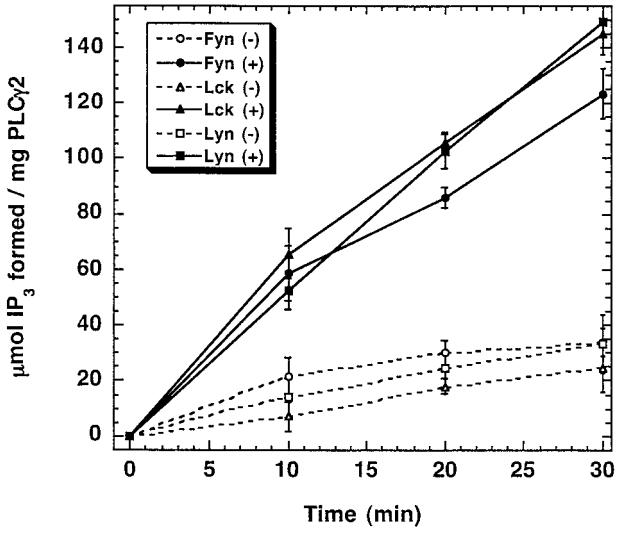

Fig. 4. Effect of Src-family tyrosine kinase phosphorylation on catalytic activity of recombinant PLCγ2. Recombinant PLCγ2 was subjected to in vitro phosphorylation by Lck, Fyn, or Lyn in the presence (+) or absence (−) of ATP and subsequently assayed for phospholipase activity as described under *Materials and Methods*. Triton X-100 (0.015%) was included in the assay. After the indicated periods of time, the assay reactions were stopped, and IP_3 formation was quantified by scintillation counting. Each point is the mean of three determinations, and error bars indicate standard deviation. The experiments are representative of three determinations with each kinase.

Y783 in PLCγ1 (Liao et al., 1993). However, we do not find a striking sequence homology in this region (Table 1).

Using site-directed mutagenesis, we prepared a mutant form of the PLCγ2 cDNA in which codons for all three tyrosines in the SH2-SH3 linker region (Y743, Y753, and Y759) were replaced by codons for phenylalanines. The mutant protein was expressed in *E. coli* and purified by calmodulin affinity chromatography. We measured the rate and level of phosphorylation of the mutant PLCγ2 by the Src-family tyrosine kinase Lck in vitro, and we compared the phosphorylation level to that of wild-type control. After in vitro kinase reaction, the proteins were separated by SDS-PAGE and subjected to autoradiography. Protein staining of these gels demonstrated that expression levels for wild-type and mutant PLCγ2 proteins were similar. Quantification of the corresponding bands in the phosphorylation experiment done with Lck demonstrated that phosphorylation of the mutant PLCγ2 lacking tyrosines 743, 753, and 759 was approximately 60% of that for wild-type PLCγ2 (Fig. 5). This finding indicated that one or more of the Lck-induced phosphorylation sites of human PLCγ2 are located in the SH2-SH3 linker region. The ability of Lck-induced phosphorylation of PLCγ2 to enhance the enzymatic activity the triple mutant PLCγ2 was determined. In contrast to wild-type enzyme, Lck-induced phosphorylation of the triple mutant PLCγ2 did not activate PLCγ2 (Fig. 6A). In view of the ability of Lck to effectively promote activation of wild-type but not mutant PLCγ2, it can be inferred that the observed stimulation of wild-type PLCγ2 activity is the result of phosphorylation of some or all of the tyrosines (Y743, Y753, and Y759) in the SH2-SH3 linker region. To determine the role of the individual tyrosine residues Y743, Y753, and Y759 on the Lck-induced activation, we expressed individual mutants Y743F, Y753F, and Y759F as well as the double mutant Y753/759F of PLCγ2 and purified the mutant proteins by calmodulin affinity chromatography. The expression level and activity of the mutants were assessed by SDS-PAGE and found to be similar to that of the wild type. Mutant Y743F was not significantly different from wild-type PLCγ2 in that Lck-induced phosphorylation was able to fully activate this mutant (Fig. 6A). On the other hand, Lck-induced activation of PLCγ2 was decreased significantly in the mutants Y753F and Y759F, and the mutation of both residues simultaneously (Y753/759F) resulted in complete inhibition of Lck-induced activation of PLCγ2 (Fig. 6, B and C). The results of site-directed mutagenesis studies suggest that tyrosine residues 753 and 759 but not 743 participate in the Lck-induced activation of PLCγ2.

To determine whether these mutations might cause global changes in the conformation of the mutant PLCγ2s, we measured the Ca^{2+} dependence of each mutant of PLCγ2 and found that they did not significantly differ from wild-type PLCγ2 (data not shown). We also determined the basal specific activity of three of the mutant PLCγ2s (Fig. 6D) and found no significant difference among any of the mutants and wild-type PLCγ2.

To confirm our identification of Y753 and Y759 as the residues involved in PLCγ2 activation, we used a phosphospecific antibody directed to the 13-amino acid peptide which contains these residues (Asn-Ser-Leu-Tyr(PO_4)-Asp-Val-Ser-Arg-Met-Tyr(PO_4)-Val-Asp-Pro). This antibody was able to recognized wild-type PLCγ2 that had been phosphorylated by Lck (Fig. 7A). However, it did not recognize unphosphorylated PLCγ2. In addition, neither Lck-phosphorylated Y753F/Y759F nor Y759F mutant PLCγ2 was recognized by the antibody (Fig. 7A). The phosphorylated mutant Y753F showed a faint reaction to this antibody (Fig. 7A). The probable explanation for the latter result is that antibodies to the monophosphorylated peptide were not completely eliminated during affinity purification. The ability of Lyn and Fyn to phosphorylate Y753 and Y759 was compared with that of Lck (Fig. 7B). Under conditions in which activation of the enzymatic activity of PLCγ2 was induced by Fyn and Lyn, we found similar levels of phosphorylation of the Y753/Y759 region of the enzyme. These results confirm the idea that all three Src-family kinases phosphorylate these two residues of PLCγ2 and shows that the antibody is specific for the doubly phosphorylated PLCγ2. Using this antibody, we determined whether the PLCγ2 in intact cells is phosphorylated on residues Y753 and Y759. Figure 8 shows the concentration response for collagen-induced specific phosphorylation of PLCγ2 in intact platelets. These data show that these residues are phosphorylated in cells and supports the concept that this phosphorylation regulates the activity of PLCγ2.

TABLE 1

Comparison of the sequences in the SH2-SH3 regions of PLCγ1 and PLCγ2

PLCγ1	GTAEPDYGALYEGRNPGFYVEAN
PLCγ2	ERDINSLYDVSRMYVDPSEI

Discussion

Phospholipase Cγ2 is an important enzyme in intracellular signaling (Katan, 1998). Its importance has been emphasized in recent knockout experiments in mice (Hashimoto et al., 2000; Wang et al., 2000). These mice had a reduction in mature B cells and a reduced response to B cell–receptor stimulation (Hashimoto et al., 2000). Fc receptor-γ signaling and collagen-induced platelet aggregation were also reduced (Wang et al., 2000). We have expressed catalytically active

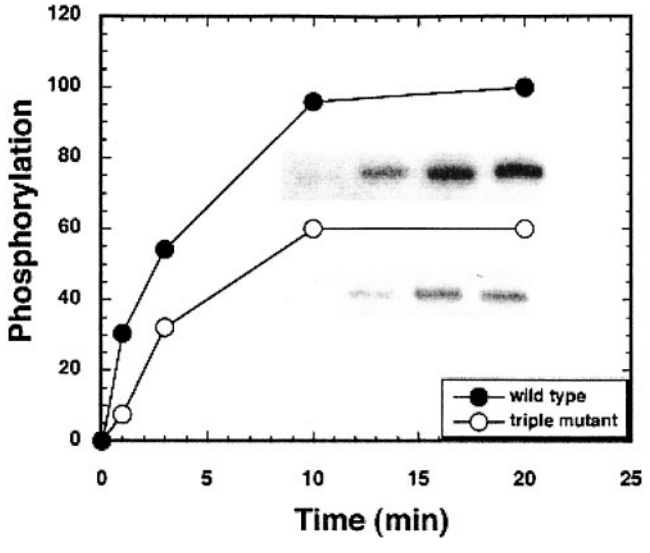

Fig. 5. Tyrosine phosphorylation of wild-type PLCγ2 and triple mutant (Y743/753/759F) PLCγ2 by Lck in vitro. Wild-type PLCγ2 and its mutant were expressed in BL21(DE3) cells and subjected to kinase assays by recombinant Lck tyrosine kinase. The bands on the resulting autoradiogram were quantified using densitometry and were plotted versus time. Below each curve, the insert shows the autoradiogram from which the data were derived. The result of an independent representative experiment from a total of three experiments is shown.

PLCγ2 in *E. coli* and have purified it and its mutants in quantities sufficient for biochemical characterization.

The phosphorylation of purified PLCγ2 by the Src-family tyrosine kinases Lck, Lyn, Hck, Fyn, and Src was studied in vitro previously (Liao et al., 1993). In this study (Liao et al., 1993), all five kinases tested phosphorylated PLCγ1 and PLCγ2 and provided evidence that both PLCγ isozymes theoretically could be phosphorylated in cells by any of the Src-family protein-tyrosine kinases in response to the activation of cell surface receptors (Liao et al., 1993). Immunoprecipitated Src-family kinases were used, and the impact of the phosphorylation of the enzyme on its function was not addressed. Our PLCγ2 phosphorylation results by Src-family kinases are in agreement with those of Liao and colleagues (1993). We could not find any specificity among Lck, Lyn, or Fyn in their ability to phosphorylate PLCγ2. All three tyrosine kinases phosphorylated PLCγ2 and were able to increase the enzymatic activity of purified recombinant PLCγ2. The enhancement of activity by Src-family kinases was detected using Triton X-100 in a mixed micellar assay. This assay was developed by Nishibe et al. (1990) and has been widely used. The exact basis for this effect is uncertain but Nishibe et al. (1990) suggested that it mimicked the role of profilin or glucosphingolipids in cells and selectively inhibited the PIP_2-hydrolyzing activity of unphosphorylated PLCγ1 compared with phosphorylated control. Zhou et al. (1999) proposed that Triton and Ca^{2+} both modified the accessibility of the substrate at the membrane interface.

The fact that the triple-mutant PLCγ2 is phosphorylated but fails to activate its enzymatic activity indicates that measure-

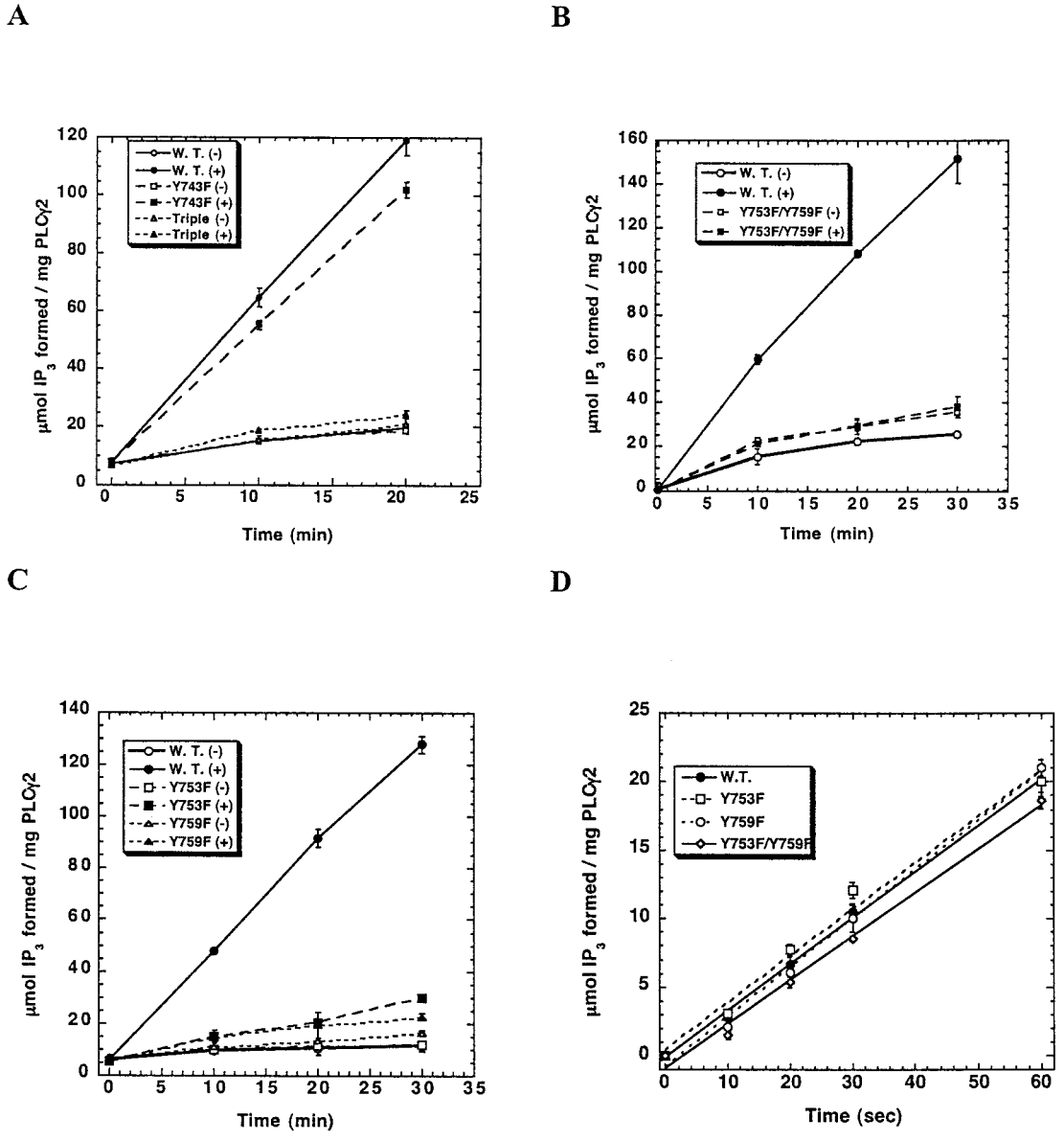

Fig. 6. The effect of mutations of all three tyrosines in the SH2-SH3 linker region (Y743/753/759F) on Lck-induced activation of PLCγ2. Wild-type PLCγ2 and indicated mutant forms were expressed, purified, and subjected to phosphorylation by recombinant Lck tyrosine kinase. The phosphorylated (closed symbols) and control enzymes (open symbols) were subsequently assayed for phospholipase activity in the presence of Triton X-100 (0.015%) as a function of time. The assay reactions were stopped, and IP_3 formation was quantified by scintillation counting. Each point is the mean of three determinations, and error bars represent standard deviation. This experiment is representative of two others. D, basal-specific activity of mutant PLCγ2s. The indicated mutants of PLCγ2 were assayed as a function of time. The data are expressed as specific activity.

ment of phosphorylation of PLCγ2 is not sufficient to determine that the enzyme has been activated. The nature of these non-activating phosphorylation events is not known. It is possible that in vitro phosphorylation occurs less specifically or may have some subtle regulatory function. PLCγ2 is phosphorylated to a similar extent in stimulated X-linked agammaglobulinemia B cells compared with normal B cells despite that there is a reduced Ca^{2+} response in the X-linked agammaglobulinemia cells (Fluckiger et al., 1998). These data suggest that there may be a "silent" phosphorylation of PLCγ2 in B cells.

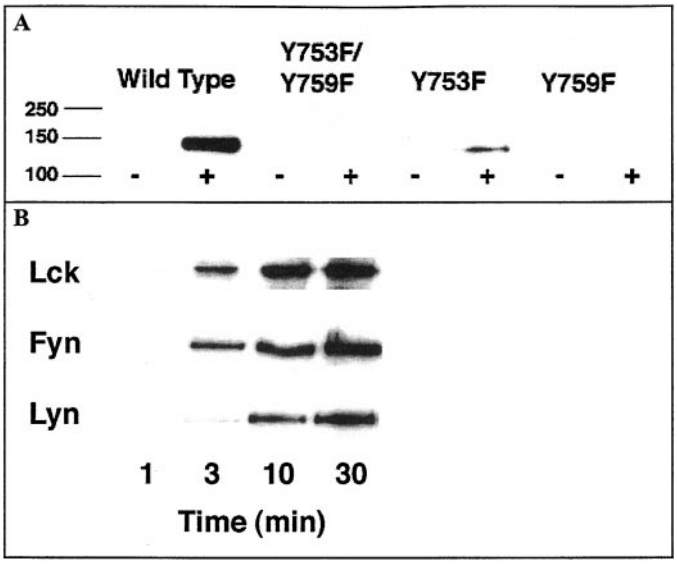

Fig. 7. Detection of phosphorylated PLCγ2 with a phosphospecific antibody. A, samples of recombinant wild-type, double-mutant (Y753F/Y759F), Y753F, or Y759F PLCγ2 isozymes were treated with either Lck alone (−) or Lck plus ATP (+) for 30 min at 25°C. Samples were analyzed by Western blotting using the phosphospecific antibody produced by Research Genetics (1:2000 dilution of a 0.9 mg/ml stock solution). B, wild-type PLCγ2 was phosphorylated by indicated tyrosine kinases for 30 min at 25°C and analyzed by Western blotting as in A.

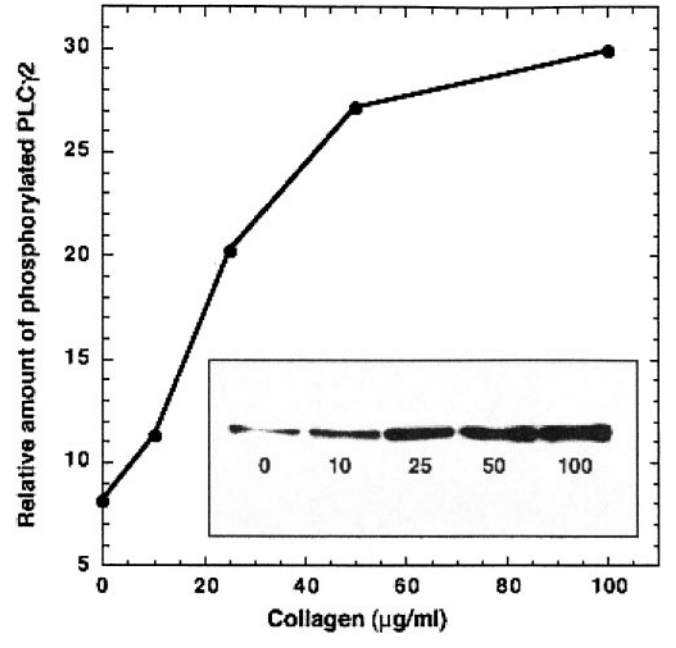

Fig. 8. Collagen-induced phosphorylation of PLCγ2 in intact human platelets. Washed platelets (1 ml) in suspension were treated with the indicated concentration of collagen for 2 min at 37°C with stirring in the presence of 1 mM SC 57101 to prevent aggregation, 20 mM creatine phosphate, and 25 U/ml creatine phosphokinase to prevent feedback by secreted ADP. Reactions were stopped by the addition of 1/10 volume of 6.6 N $HClO_4$. Proteins were solubilized and separated by SDS-PAGE to prepare for Western blotting. The inset shows the Western blot, and the graph shows the quantification of that blot using Image Gauge software (version 3.4; FujiFilm Medical Systems).

The physiological significance of the phosphorylation of PLCγ2 by the Src-family kinases used in this study is unclear. Our hope in initiating these studies was that we could find a single tyrosine kinase that would be key in the activation of PLCγ2. Ting et al. (1995) overexpressed Lck in natural killer cells and found enhanced phosphorylation of PLCγ2. Lck could not be replaced by overexpression of either Fyn or Src or by inactive Lck. Tyrosine phosphorylation of raft-targeted PLCγ1 was recently shown to require Lck but did not require Zap-70 or the interaction with the adapters Lat and Slp76 (Veri et al., 2001). Watson's group implicated both Lyn and Fyn in the signaling pathways that lead to PLCγ2 activation in platelets (Briddon and Watson, 1999; Quek et al., 2000). Recently Watanabe et al. (2001) showed that Btk phosphorylates Y753 and Y753 as well as Y1197 and Y1217. Rodriguez et al. (2001) showed that Btk can phosphorylate PLCγ2 in vitro, whereas Syk cannot. These authors also showed that several Src-family members, including Src, Fyn, and Lck, were able to phosphorylate PLCγ2.

The importance of the SH region with respect to basal and stimulated enzymatic activity of PLCγ1 has been well established. Homma and Takenawa (1992) reported that the addition of a bacterially expressed protein corresponding to the SH2-SH2-SH3 domains of PLCγ1 or PLCγ2 decreased the activity of PLC isoforms, suggesting an interaction of these domains with the conserved X and Y catalytic domains. Recently, Horstman and colleagues (1999) reported that the SH3 domain and at least one of the SH2 domains were needed for maximal attenuation of basal activity of PLCγ1. Therefore, theoretically the activation of the enzyme can occur by modulating the structure of the SH region and altering its inhibitory influence on the catalytic domain. All of the mechanisms that are known to activate PLCγ1 modulate the SH region. The binding of phosphatidylinositol-3,4,5-trisphosphate to SH2 domains (Bae et al., 1998) and ligation of SH2 domains with a phosphotyrosine-containing peptide (Koblan et al., 1995) are shown to increase PLCγ1 activity in vitro. Tyrosine phosphorylation (Koblan et al., 1995) at sites close to the SH2 and SH3 domains also activates PLCγ1.

Therefore, during our investigation of the mechanism of Lck-induced activation of PLCγ2, we substituted all three tyrosines in the SH2-SH3 linker region (Y743, Y753, and Y759) for phenylalanines. Phenylalanine substitution at all three sites (triple mutant) decreased the Lck-induced phosphorylation by 40%. To show that our mutants did not have major alterations in conformation, we measured the Ca^{2+} dependence of several of these mutants and found them to be the same as wild type. We also found no difference in the basal activity of the mutants compared with wild type.

In the activation studies, Lck-induced activation of PLCγ2 was abolished in both triple mutant and Y753F/Y759F double-mutant PLCγ2, whereas it was not affected in the individual mutant Y743F. It is possible that Lck does not phosphorylate tyrosine 743 or, alternatively, it is phosphorylated but this phosphorylation has no effect on the catalytic activity of the enzyme. Lck-induced activation was decreased in

individual mutants Y753F and Y759F, indicating that they were both phosphorylated by Lck and they both participated in the activation process. These results are in contrast with studies on PLCγ1, in which only one phosphorylation seemed to be required (Kim et al., 1991). However, our results are supported by the recent studies of Rodriguez et al. (2001). Thus, site-directed mutagenesis studies provide direct evidence that Lck-induced activation of PLCγ2 is mediated by phosphorylation on tyrosines 753 and 759 in the SH2-SH3 linker region. These results demonstrate the importance of the SH region for the regulation of enzymatic activity of both enzymes. We used a phosphospecific antibody to distinguish between unphosphorylated and Lck-phosphorylated PLCγ2. The antibody was specific for dually phosphorylated PLCγ2. This result supports the conclusion that these two residues are important in regulating PLCγ2. We also showed that collagen-induced activation of human platelets results in the phosphorylation of these residues, providing evidence that phosphorylation plays a role in vivo. The reason for the relatively high levels of basal phosphorylation of PLCγ2 in this experiment is unclear. However, it is often seen that there is an idling state in signaling system which may in part account for this observation. Recent results from two groups support our results in that both Y753 and Y759 have an important role in regulating the activity of PLCy2 (Rodriguez et al., 2001; Watanabe et al., 2001). In addition, it is now clear that phosphorylation is not the only factor regulating the activity of the γ-isoforms of PLC.

In conclusion, we provided the first evidence that tyrosine phosphorylation of PLCγ2 can activate this enzyme. However, phosphorylation alone is not sufficient to imply an activation of PLCγ2, because the mutant forms of PLCγ2 could be phosphorylated without activating enzyme activity. Thus Src-family kinases can phosphorylate PLCγ2 on apparently nonessential tyrosine, and activation of PLCγ2 requires phosphorylation at two tyrosines in the SH2-SH3 linker region of PLCγ2. Our results can be extended to intact cells in which we have shown that agonist-dependent activation of PLCγ2 results from its phosphorylation on Y753 and Y759.

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