src-Homology 2 (SH2) domain ligation as an allosteric regulator: modulation of phosphoinositide-specific phospholipase C γ 1 structure and activity

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Phosphoinositide-specific phospholipase C γ 1 (PI-PLC γ 1) catalyses the hydrolysis of PtdIns(4,5) P_2 to generate the second messengers diacylglycerol and Ins(1,4,5) P_3 . PI-PLC γ 1, an src-homology 2/3 (SH2/SH3)-domain-containing enzyme, is activated in response to growth-factor-induced tyrosine phosphorylation, and, *in vivo*, is translocated from the cytosol to the particulate cell fraction. Here we report the bacterial expression of rat brain PI-PLC γ 1 under the control of the T7 promoter. Production of the active enzyme in amounts suitable for structure–function analysis depended on coupling the translation of PLC γ 1 to the expression of the phage- ϕ 10 coat protein. Purification of the enzyme was facilitated by the presence of a three-amino-acid C-terminal antibody epitope tag (Glu-Glu-Phe) engineered into the cloned PLC γ 1. Examination of the specific activity, pH–rate profile, [Ca $^{2+}$]-dependence and substrate specificity of bacterially expressed PLC γ indicated that it had

kinetic properties similar to those of PLC γ isolated from bovine brain. The substrate specificity was dependent on [Ca $^{2+}$]: at low [Ca $^{2+}$] (1–10 μ M) PtdIns(4,5) P_2 was a better substrate than PtdIns. Addition of phosphotyrosine-containing peptides (12-mers) with the cognate sequence of the high-affinity binding site for PLC γ 1 on the activated epidermal-growth-factor (EGF) receptor (Tyr-992) increased enzyme activity (up to 85%) *in vitro*. Cognate non-phosphorylated peptides had no effect on activity. When c.d. spectroscopy was used to monitor the effect of added phosphotyrosine-containing peptide on the structure of recombinant PLC γ 1, significant spectral shifts, indicative of a conformational change, were observed upon complexation with the EGF-receptor phosphotyrosine-containing 12-residue peptide (Tyr*-992). How SH2 domains from PLC γ 1 can mediate structural rearrangements and modulate enzymic activity on their ligation by growth-factor receptors is discussed.

INTRODUCTION

Phosphoinositide-specific phospholipase C (PI-PLC) catalyses the hydrolysis of membrane PtdIns(4,5) P_2 , producing the intracellular second-messenger signalling molecules Ins(1,4,5) P_3 , which induces the release of intracellular Ca $^{2+}$ stores, and 1,2-diacylglycerol, which activates protein kinase C [see reviews by Majerus (1992) and Rhee and Choi (1992)]. Myriad PLC isoenzymes have been purified and their corresponding cDNAs cloned (Ryu et al., 1987b; Suh et al., 1988a; Emori et al., 1989). On the basis of their primary-sequence similarity, the sundry PLC forms can be classified as follows: phospholipase C β (PLC β) (β_1 , β_2 , β_3 , *Drosophila* norpA, *Drosophila* PLC-21), PLC γ (γ_1 , γ_2) and PLC δ (δ_1 , δ_2 , δ_3) (Rhee and Choi, 1992). Alignment of sequences from all groups reveals that highly conserved residues are clustered into two distinct regions (one of $\sim$ 170 amino acids and the other of $\sim$ 260 amino acids), designated the X and Y regions respectively (Majerus, 1992; Rhee and Choi, 1992). PLC γ 1 also contains src-homology (SH2 and SH3) domains that mediate the interaction between the enzyme and receptors with tyrosine kinase activity, such as the epidermal-growth-factor (EGF) receptor (Stahl et al., 1988; Rotin et al., 1992).

The PLC isoenzymes are activated by different mechanisms in response to stimulation of specific cell-surface receptors. Activation of PLC β is achieved by guanine-nucleotide-binding proteins of the G $_q$ class (Simon et al., 1991; Smrcka et al., 1991; Taylor et al., 1991). Although coupling of PLC δ , which has recently been expressed as a recombinant fusion protein (Ellis et

al., 1993), to specific receptors or downstream effectors has not been reported, this isoenzyme is believed to be associated with mechanisms that regulate the tone of vascular smooth muscle (Kato et al., 1992). PLC γ is the only isoenzyme that is phosphorylated by activated tyrosine kinase growth-factor receptors (Kim et al., 1991; Mohammadi et al., 1992; Rotin et al., 1992). Following growth-factor stimulation, cytosolic PLC γ is rapidly and extensively phosphorylated *in vivo* (50–70% of the PLC γ molecules are modified within 5 min). This phosphorylation apparently induces the relocation of PLC γ from the cytosol to the plasma membrane, where presumably it is better able to interact with its phospholipid substrates (Kim et al., 1991; Vega et al., 1992). *In vitro* studies utilizing enzyme that had been immunoprecipitated from cells suggest that the catalytic activity of the phosphorylated form of PLC γ 1 is increased compared with that of the unphosphorylated form, although this effect depends upon assay conditions (Kim et al., 1991; Wahl et al., 1992). Moreover, such altered PLC γ 1 activity may correlate with some disease states. For example, an increase in the concentration of PLC γ 1 has been documented in cells derived from primary human breast carcinomas, which also overexpress the EGF receptor (Arteaga et al., 1991). Inhibition of PLC γ 1 activity, particularly of the activated form, may therefore be of therapeutic value in the treatment of breast cancer.

Most biochemical studies have used PLC γ 1 that has been immunoprecipitated from cell lysates, because the existing method for purifying the enzyme from mammalian tissue yields low quantities of enzyme. For enzymological and structural

Abbreviations used: PLC β (etc.), phosphoinositide-specific phospholipase C β ; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; SH2, src-homology region 2; SH3, src-homology region 3; DTT, dithiothreitol.

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studies, a ready source of pure enzyme is essential, and thus our first goal was to devise a method for producing large quantities of homogeneous enzyme. Here, the bacterial expression, purification, and characterization of rat brain PLC γ 1 is detailed. The enzyme purified from *Escherichia coli* was fully active and functionally identical with the one isolated from bovine brain with respect to its [Ca²⁺]-dependence and substrate specificity. The effect of phosphotyrosine-containing peptides [identical in sequence with regions of the EGF and fibroblast-growth-factor (FGF) receptors] on PLC γ 1 structure and activity was also evaluated.

EXPERIMENTAL

Materials

Competent DH5 α cells (subcloning efficiency) were obtained from GIBCO/BRL (Gaithersburg, MD, U.S.A.). Competent BL21(DE3) cells were purchased from Novagen (Madison, WI, U.S.A.). The general cloning vector, pBSII(S/K)+, was from Stratagene (La Jolla, CA, U.S.A.). Purified EGF receptor was purchased from Promega (Madison, WI, U.S.A.), while purified p60^{src} kinase and its peptide substrate, Raytide, were from Oncogene Science (Uniondale, NY, U.S.A.). PtdIns was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.), PtdIns(4,5)P₂ ammonium salt was from Boehringer Mannheim, and [³H]PtdIns and [³H]PtdIns(4,5)P₂ were obtained from du Pont-NEN (Boston, MA, U.S.A.). [γ -³²P]ATP was obtained from Amersham International (Chicago, IL, U.S.A.). Isoenzyme-specific anti-PLC $\beta/\gamma/\delta$ antibodies were purchased from UBI (Lake Placid, NY, U.S.A.). Phosphotyrosine-containing peptides were synthesized by Research Genetics (Huntsville, AL, U.S.A.). The antibody YL1/2 (Kilmartin et al., 1982) was obtained from Harlan Bioproducts for Science (Indianapolis, IN, U.S.A.).

Synthetic deoxyribonucleotides were obtained from Midland Certified Reagent Co. (Midland, TX, U.S.A.). The sequences of the oligonucleotides are (5' to 3'):

01

CCC-GGG-CAT-ATG-GAT-CCA-TTG-GAG-GAT-GAT-TAA-ATG-GCG-GGC-GCC-GCG-TCC

02

CTG-CTT-CCG-GAG-CCA-CCT-CTC

03

TC-GCC-ATT-CGT-CCT-GAG-GGC

04

GG-GCC-CAA-GCT-TCT-AGA-CTA-AAA-TTC-TTC-GAG-GCG-GTT-GTC-TCC-ATT-GAC-CCG-AGT-TCG-TCG

05

G-ATG-ATG-GAG-CGG-CGG-AAG-AAG-ATC-G

06

C-GAT-CTT-CTT-CCG-CCG-CTC-CAT-CAT-C

Subcloning of the PLC γ 1 coding sequence

PCR mutagenesis was carried out according to literature protocol (Higuchi, 1990). pT5T was constructed as described by Eisenberg et al. (1990). DNA sequencing was performed using the dideoxy chain-termination method of Sanger et al. (1977). Standard

DNA manipulations were carried out as described by Sambrook et al. (1989). Rat brain PLC γ 1 (clone 2-2568), as a *Bam*HI fragment, was obtained from S. G. Rhee (Suh et al., 1988b). From this plasmid, a *Bam*HI–*Hind*III fragment containing the coding sequence of PLC γ 1 was subcloned into pBSII(S/K)+, generating pPLC1. The rest of the manipulations were accomplished using PCR to generate the appropriate DNA fragment. The 5' end of the PLC γ gene was altered to include a new *Bam*HI site and a sequence that would eventually translationally couple the expression of PLC γ 1 to the phage- ϕ 10 coat-protein gene of the pT5T vector (Eisenberg et al., 1990). Primers 01 and 02 were used to generate a 508-bp fragment (from template DNA pPLC1) containing a 5' *Bam*HI site and 3' *Eco*RI site. This new restriction fragment was substituted for the analogous fragment in pPLC1 to produce pPLC2. To restructure the 3' end so as to add DNA sequence encoding the epitope tag Glu-Glu-Phe (Kilmartin et al., 1982), and to change the tandem AGG-AGG codons to CGG-CGG at amino acid positions 944–945 and 1279–1280, we used PCR-mediated mutagenesis with template pPLC2 and with primers (03 and 05) to generate one mutant fragment and primers (04 and 06) to generate the second mutant fragment. Note that tandem AGG codons are associated with poor protein expression in *Escherichia coli*, possibly because they can cause the ribosome to frameshift during translation (Bonekamp and Jensen, 1988). Using the isolated mutant fragments from each of these PCRs together as template, the final PCR was carried out using primers (03, 06) which yielded, after restriction-enzyme digestion, a *Sph*I–*Hind*III fragment which was used to replace the analogous fragment in pPLC2. The resulting plasmid, pPLC3, contained the coding sequence of the PLC γ 1 gene on a *Bam*HI–*Hind*III fragment with both the sequence encoding the Glu-Glu-Phe tag at the 3' end as well as the changed AGG codons. Finally, the *Bam*HI–*Hind*III fragment from pPLC3 was transferred into the *Bam*HI–*Hind*III site of pT5T, generating pT5T-PLC4. This construct was used to produce PLC γ 1 (containing the Glu-Glu-Phe epitope tag at the C-terminus) translationally coupled to the expression of phage- ϕ 10 protein in the pT5T vector.

Expression and purification of PLC γ 1

All purification steps were carried out at 4 °C. Bovine brain PLC γ was purified by modification of a previously described protocol (Ryu et al., 1987a) in which a three-stage chromatographic run was performed: DEAE-cellulose, followed by reverse-phase chromatography on a Bio-Gel phenyl 5-PW (Bio-Rad) column, and final purification on a Mono-Q column (Pharmacia). For bacterial expression of PLC γ 1, the plasmid pT5T-PLC4 was transformed into *E. coli* BL21 (DE3) and grown in Luria–Bertani medium containing 100 μ g/ml ampicillin and 12.5 μ g/ml tetracycline at 20 °C. When the cells reached early exponential phase (A_{550} = 0.6–0.8), isopropyl thio-galactoside was added to a final concentration of 0.5 mM, and the cells were grown for an additional 6 h. PLC γ was purified as described below. After harvesting the cells by centrifugation (20 min at 9000 g), the pellet was resuspended in buffer A [50 mM Tris/HCl (pH 8.0)/2 mM MgCl₂/10 mM CaCl₂/1 mM EGTA/5 mM dithiothreitol (DTT)/5 mM streptomycin sulphate/1 mM phenylmethanesulphonyl fluoride/leupeptin (2 μ g/ml)/antipain (2 μ g/ml)/aprotinin (10 μ g/ml)] (approx. 5 g wet packed cells/10 ml of buffer). The resuspended cells were lysed by sonication and the cellular debris pelleted by centrifugation (30 min at 30 000 g). An affinity column (2 ml) consisting of monoclonal antibody YL1/2 immobilized on CNBr-activated Sepharose (4 mg of antibody/ml of resin) was prepared

and equilibrated with buffer A (see below). After loading the supernatant on to the affinity column (0.5 ml/min) and washing with standard buffer (100 ml), PLC γ 1 was eluted with three 5 ml aliquots of buffer A containing 5 mM Asp-Phe dipeptide (Sigma). The column was regenerated by washing with PBS containing 2 M NaCl and then stored in PBS containing 0.02% (w/v) NaN₃. PLC γ 1 was obtained at > 80% purity after the immunoaffinity-column step. To purify PLC γ 1 further, enzyme eluted from the YL1/2 column was fractionated by h.p.l.c. using a MonoQ HR10-10 column (Pharmacia). Enzyme was applied to the column equilibrated in buffer A at a flow rate of 1 ml/min. PLC γ was eluted by running linear gradients of 0–30% buffer B (buffer A containing 1 M KCl) over 40 min, 30–50% buffer B over 50 min and 50–100% buffer B over 70 min. PLC γ 1 was eluted at approx. 25–30% buffer B. After the anion-exchange-chromatography step the enzyme was greater than 95% pure and typically in a 0.4–0.5% yield based on the total starting soluble *E. coli* protein.

Assay of purified PLC γ activity

The activity of the purified PLC γ was assayed at 30 °C. Reactions were never allowed to proceed to more than 10% completion on the basis of limiting substrate. A typical activity assay mixture contained the following (in a total volume of 200 μ l): 50 mM Na-Hepes, pH 7.0, 2.4 mM sodium deoxycholate, 3 mM CaCl₂, 1 mM EGTA, 0.1 mM DTT with PtdIns (1–1000 μ M) and 0.02 μ Ci of [³H]PtdIns as substrate. The phospholipid components were dried under a gentle stream of nitrogen and resuspended in assay buffer. The substrate mixture was then vortex-mixed and sonicated (10 s with probe sonicator) to disperse the lipid and to form micelles. For the measurement of Ca²⁺-dependent activities, small unilamellar vesicles of [³H]PtdIns, and [³H]PtdIns(4,5)P₂ were prepared as described by Ryu et al. (1987a). These reaction mixtures contained PtdIns or PtdIns(4,5)P₂ (125 μ M) in a buffer containing 50 mM Na-Hepes, pH 7.0, 0.1 mM DTT, and indicated concentrations of free Ca²⁺ (which were adjusted by varying the ratio of Ca²⁺ to EGTA). The hydrolysis of [³H]PtdIns(4,5)P₂ was measured in an assay mixture which contained 50 mM Na-Hepes, pH 7.0, 100 mM KCl, 0.8 mM EGTA, 0.8 mM CaCl₂, [³H]PtdIns(4,5)P₂, and Triton X-100 at the indicated concentrations. Some experiments included 5 mM β -N-octyl glucopyranoside or 2.4 mM deoxycholate in the reaction mixture [as described previously by Wahl et al. (1992)]. After thermally pre-equilibrating the assay mixture in the absence of enzyme, the reaction was initiated by adding PLC γ 1.

Assays to determine the effect of phosphotyrosine-containing peptides on activity were conducted as described above, except that the enzyme was preincubated (30 min) in the presence of the peptide prior to its addition to the assay mixture containing substrate. Aliquots (200 μ l) of the reaction were withdrawn at timed intervals and terminated by addition of 0.25 vol. of 1 M HCl containing 5 mM EGTA and then transferred to an ice bath. The quenched-reaction mixtures were loaded on to a Q-Sepharose column (1 ml) equilibrated with 20 ml of 10 mM NH₄H₂PO₄, pH 3.5. The product was eluted with 3 ml of 10 mM NH₄H₂PO₄, pH 3.5, and collected in a scintillation vial, mixed with 10 ml of scintillation fluid, and counted for radioactivity in a Beckman LS3801 liquid-scintillation counter. Control experiments utilizing a more conventional extraction protocol (Ryu et al., 1987a; Wahl et al., 1992) were conducted, and validated results of the assay are presented here. The Q-Sepharose separation assay was developed for these studies on account of its shorter processing time and higher throughput than the con-

ventional extraction assay. All experiments were conducted in triplicate on separate occasions and with similar results. Each Figure contains data from a single representative experiment.

C.d. spectroscopy

C.d. spectra (far-u.v., 260–184 nm) were recorded at 30 °C with a JASCO J720 spectropolarimeter, which was calibrated with aq. 0.1% D-10-camphorsulphonic acid ($|\lambda_{192.5}/\lambda_{290.5}|$ ratio was 2.01) (Chen and Yang, 1977). The spectra for PLC γ 1 were recorded at a protein concentration of 0.53 mg/ml, using a 0.01 cm thermal-jacketed cylindrical quartz cuvette (Hellma). The following settings were used: wavelength range, 260–184 nm; bandwidth, 1 nm; step resolution, 0.5 nm; scan speed, 10 millidegrees/min. Each spectrum represents an average of three scans with the baseline subtracted. C.d. studies were conducted in 5 mM Na-Hepes (pH 7.0)/10 mM KCl/0.8 mM CaCl₂. The peptides (12-mers) were prepared in the same buffer and added to PLC γ 1 at a concentration of 2 μ g/ml for a final peptide/protein molar ratio of 1.5:1. The recorded spectra are presented in terms of mean residue ellipticity, based on the protein concentration. The protein concentration was based on an absorption coefficient (ϵ_{280} 173550 M⁻¹·cm⁻¹) determined by the tyrosine and tryptophan composition of the protein. Secondary-structure analysis was performed using the program of Sreerama and Woody (1993).

RESULTS

Cloning, expression, and purification of PLC γ 1

The amino acid sequence derived from the rat brain cDNA encoding full-length PLC γ 1 is 97% identical with that derived from human PLC γ 1 cDNA clones (Burgess et al., 1990). Soluble rat brain PLC γ 1 was produced in *E. coli* under control of the T7 promoter by translationally coupling its expression to that of phage- ϕ 10 coat protein in the pT5T vector (Eisenberg et al., 1990). Lower growth temperatures (20 °C instead of 37 °C) resulted in significantly decreased amounts (from 100% to ~40%) of insoluble PLC γ 1. Attempts to express PLC γ 1 as a fusion protein (of either maltose binding protein or glutathione S-transferase) led to protein that was either inactive or insoluble (located in inclusion bodies). Replacement of the tandem AGG-AGG codons at amino acid positions 944–945 and 1279–1280 by CGG-CGG codons enhanced protein expression (yield increased approx. 20-fold) (Bonekamp and Jensen, 1988). The codons for the C-terminal epitope Glu-Glu-Phe were genetically appended on to the 3' end of the coding sequence to facilitate purification of expressed PLC γ 1 with the monoclonal antibody YL 1/2 (Stammers et al., 1991). This epitope tag has previously been utilized for the immunoaffinity purification of human-immunodeficiency-virus reverse transcriptase (Stammers et al., 1991), *ras* GTPase-activating protein (Skinner et al., 1991), and farnesyl: protein transferase (Omer et al., 1993).

Bovine brain PLC γ was purified by a modification of a previously published protocol (Ryu et al., 1987a; see the Experimental section). Since in our hands the enzyme was unstable through the early isolation steps, we developed a quicker and more streamlined procedure. The purification scheme described in the Experimental section consists of just three chromatography steps (two of which are h.p.l.c.-based) compared with the six-step sequence described by others (Ryu et al., 1987a). By comparison, isolation of pure PLC γ 1 expressed in *E. coli* was simple. Due to the presence of the C-terminal epitope tag, the enzyme could be bound specifically to an immunoaffinity column. After elution from this column, a final anion-exchange-chromatography step

Figure 1 SDS/PAGE of *E. coli*-expressed PLC γ 1 and bovine brain PLC γ 1

PLC γ purified as described was subjected to SDS/7.5%-PAGE and stained with Coomassie Blue. Lane 1, PLC γ 1 isolated from bovine brain; lane 2, rat PLC γ 1 expressed in, and purified from, *E. coli*.

Table 1 Kinetic parameters for recombinant PLC γ 1

Assays utilizing phosphatidylinositol as a substrate were conducted in the presence of 50 mM Hepes (pH 7.0)/2.4 mM sodium deoxycholate/3 mM CaCl₂/1 mM EGTA/0.1 mM DTT with PtdIns (125 μ M) and 0.02 μ Ci of [³H]PtdIns. PtdIns(4,5) P_2 hydrolysis was assayed utilizing small unilamellar vesicles in a buffer containing 50 mM Hepes, pH 7.0, 10 μ M CaCl₂, 2 mM EGTA, 0.1 mM DTT, 125 μ M PtdIns(4,5) P_2 and 0.02 μ Ci of [³H]PtdIns(4,5) P_2 .

PLC γ 1 source	Substrate ...	Activity (μ mol/min per mg)	
		PtdIns	Ptd-Ins(4,5) P_2
Bovine brain		11.4	0.73
<i>E. coli</i>		13.8	0.60

yielded enzyme of greater than 95% purity. PLC γ 1 has a predicted molecular mass (148 400 Da) that is in good agreement with that (145 000) determined by SDS/PAGE (Figure 1) and by immunoblotting with isoenzyme-specific antibodies (results not shown).

Characterization of PLC γ 1

The apparent $V_{\max}$ of both PLC γ 1 expressed in *E. coli* and PLC γ 1 isolated from bovine brain was measured using either PtdIns or PtdIns(4,5) P_2 as substrate (Table 1). Under the conditions of the assay, the apparent $V_{\max}$ values for these substrates were comparable for the two PLC γ 1 preparations and in reasonable agreement with those previously determined (Ryu et al., 1987a). Likewise, the dependence of catalytic activity on pH of the bacterially expressed material was the same as for bovine brain PLC γ 1 and displayed a pH optimum at pH 5.0, in agreement with previous reports (Ryu et al., 1987a). Given the characteristic [Ca²⁺] requirements for all mammalian PLC isoenzymes studied to date (Suh et al., 1988a; Wahl et al., 1992), the effect of [Ca²⁺] on PtdIns and PtdIns(4,5) P_2 hydrolysis was next investigated for the recombinant enzyme. The enzyme-catalysed dependence of rates of hydrolysis upon [Ca²⁺] was measured using small unilamellar vesicles as substrate. The

Figure 2 Ca²⁺-dependence of phosphoinositide hydrolysis by PLC γ

Small unilamellar vesicles derived from [³H]PtdIns (a) and [³H]PtdIns(4,5) P_2 (b) were used as substrates. The reaction mixtures contained PLC γ purified from *E. coli* (○) or PLC γ isolated from bovine brain (■), 125 μ M PtdIns or PtdIns(4,5) P_2 in a buffer containing 50 mM Hepes, pH 7.0, 0.1 mM DTT and increasing amounts of free Ca²⁺, which were adjusted by varying the ratio of Ca²⁺ to EGTA (Ryu et al., 1987a).

results in Figure 2 show that, with PtdIns as substrate, the reaction velocity increases with increasing [Ca²⁺] up to 10 mM (the highest concentration tested), while PtdIns(4,5) P_2 hydrolysis rates reached a maximum at 1–10 μ M Ca²⁺ and were inhibited at higher concentrations (at and above 100 μ M Ca²⁺). Thus bacterially expressed PLC γ 1 activity shows not only the same pH- and [Ca²⁺]-dependences, but also the same specific activity and substrate specificity, as previously reported for PLC γ 1 from bovine brain (Ryu et al., 1987a; Wahl et al., 1992).

Effect of phosphotyrosine-containing peptides on the catalytic activity of PLC γ 1

Recent studies from a number of laboratories have demonstrated that SH2 domains mediate interactions between auto-phosphorylated growth-factor receptors, such as EGF receptor, and signal-transducing molecules, such as PLC γ [reviewed by Koch et al. (1991) and Pawson and Gish (1992)]. In the present study, synthetic phosphotyrosine-containing peptides (12-mers) were incubated with purified PLC γ 1 to determine the effect of PLC γ -SH2-domain ligation upon enzymic activity. The phosphotyrosine-containing peptides employed in this study (Table 2) are expected to bind to the PLC γ -SH2 domain because their sequences are the same as those surrounding high-affinity binding sites from the EGF receptor (at Tyr*-992 [Tyr* indicates phosphotyrosine]) (Pawson and Gish, 1992) and from the FGF receptor (at Tyr*-766) (Mohammadi et al., 1992). A peptide corresponding to the sequence from the human platelet-derived

Table 2 Phosphotyrosine-containing peptides utilized

Peptide	Sequence	Basis of sequence
Tyr*-992	DDVVDAD ^Y *LIP	EGF receptor [Tyr(P)-992]
Tyr-992	DDVVDAD ^E YLIP	EGF receptor (Tyr-992)
Tyr*-766	VALTSNQ ^{EY} *LDL	FGF receptor [Tyr(P)-766]
Tyr-766	VALTSNQ ^E YLDL	FGF receptor (Tyr-766)
Tyr*-751	MSKDESVD ^Y *VPM	PDGF- β receptor [Tyr(P)-751]

Figure 3 Phosphotyrosine-containing peptides affect the catalytic activity of recombinant PLC γ 1

PLC γ 1 was incubated with peptides (10 μ M final concn.) and added to reaction mixtures containing [3 H]PtdIns(4,5) P_2 (100, 200, 400 and 800 μ M) and Triton X-100 at substrate mol fraction of 0.33, 1.0 μ M free Ca^{2+} and incubated. The reaction velocity was determined and the data plotted in double-reciprocal form for each peptide. Control peptides (see the text) were plotted with open symbols, Tyr-992 ($\square$), Tyr-766 ($\circ$), Tyr*-751 ($\triangle$), while phosphotyrosine-containing peptides were plotted with closed symbols, Tyr*-992 ($\bullet$) and Tyr*-766 ($\blacklozenge$). A control reaction containing no added peptide had a reaction velocity identical with that of the control peptide samples (results not shown).

growth factor (PDGF) β -receptor at Tyr*-751, to which the p85 subunit of phosphatidylinositol 3'-kinase binds (Fantl et al., 1992), was used as a control ligand. PLC γ 1 enzymic activity (V_{max}) was increased approx. 50% (compared with the activity measured in the presence of unphosphorylated cognate control peptides) in the presence of either Tyr*-992 peptide (10 μ M) from the EGF receptor or Tyr*-766 peptide from the FGF receptor (10 μ M) (Figure 3). However, there was no appreciable change in the specificity constant (V_{max}/K_m), because the increase in the value of V_{max} was compensated for by an increase in the value of K_m [PtdIns(4,5) P_2]. The effect on enzyme activity was dependent upon the concentration of phosphotyrosine-containing peptide in the assay (e.g. 85% activation at 100 μ M Tyr*-992). Enzyme activity was unaffected in samples with either the cognate peptides containing no phosphotyrosine (Tyr-992 and Tyr-766) or the non-specific phosphotyrosine-containing peptide Tyr*-751 from the PDGF μ -receptor (Figure 3). Reactions without peptide showed kinetics that were the same as the control peptide samples (results not shown). Phosphotyrosine-containing peptides utilized in this study compete for PLC γ 1 binding to the activated EGF receptor in the same concentration range (1 μ M) where enzymic activity is increased.

Figure 4 Conformational change of PLC γ 1 upon binding phosphotyrosine-containing peptides

Far-u.v. c.d. spectra (260–184 nm) of PLC γ 1 (0.53 mg/ml) in 5 mM Na-Hepes (pH 7.0)/10 mM KCl/0.8 mM CaCl $_2$. The protein spectra were collected either alone (—), in the presence of the phosphotyrosine-containing peptide Tyr*-992 (derived from the EGF receptor) (---), or in the presence of the control unphosphorylated Tyr-992 peptide (·····). The protein to peptide molar ratio was 1:1.5.

Table 3 Secondary-structure prediction by c.d. analysis

The percentages assessed from the far-u.v. c.d. spectra were obtained with the program described by Sreerama and Woody (1993) utilizing either the H/J (Hennessy and Johnson, 1981) or K/S (Kabsch and Sander, 1983) basis set. Root-mean-square residual errors were in the range 0.001–0.003.

Sample	Analysis	Proportion in each form (%)			
		α -Helix	β -Sheet	β -Turn	Random
PLC γ 1	H/J	43.6 $\pm$ 0.9	14.0 $\pm$ 0.8	14.3 $\pm$ 0.9	27.2 $\pm$ 0.9
	K/S	42.3 $\pm$ 1.0	15.1 $\pm$ 0.8	18.1 $\pm$ 0.8	23.4 $\pm$ 0.8
PLC γ 1 + Tyr-992	H/J	43.0 $\pm$ 0.9	16.7 $\pm$ 0.7	14.1 $\pm$ 0.9	25.2 $\pm$ 0.9
	K/S	42.6 $\pm$ 0.9	17.0 $\pm$ 0.8	18.4 $\pm$ 0.9	21.4 $\pm$ 1.0
PLC γ 1 + Tyr*-992	H/J	52.0 $\pm$ 1.0	11.4 $\pm$ 1.0	13.0 $\pm$ 1.0	23.6 $\pm$ 0.9
	K/S	50.9 $\pm$ 0.9	12.1 $\pm$ 1.1	18.7 $\pm$ 0.8	18.3 $\pm$ 1.1

Phosphotyrosine-containing peptides affect the conformation of PLC γ 1

In order to obtain information about the general secondary-structural elements (α -helices, β -strands, turns) of PLC γ 1, the far-u.v. c.d. spectra (Figure 4) were analysed by the SELCON program (Table 3). The c.d. spectra of PLC γ 1 in the presence of the phosphotyrosine-containing peptide Tyr*-992 from the EGF receptor and the non-phosphorylated control peptide Tyr-992 were compared to test the possibility of a structural change upon complex formation. The effects of phosphotyrosine-containing-peptide addition upon the far-u.v. c.d. spectra are dramatic, with large changes evident in both the 190 nm and 215–220 nm regions (Figure 4). No spectral changes were observed when the non-phosphorylated Tyr-992 peptide was present. Examination of the peptides in the absence of PLC γ 1 demonstrated that no c.d. spectral differences exist between the Tyr-992 and Tyr*-992 peptides (12 residues each), and neither appeared to have any secondary structure when free in solution. The shifts in the c.d. spectra are consistent with a large structural reorganization in PLC γ 1 upon phosphotyrosine-containing-peptide binding. The

close agreement in the secondary-structural analysis using two different basis sets [H/J (Hennessy and Johnson, 1981); K/S (Kabsch and Sander, 1983)] that is presented in Table 3 is an indication of the reliability of the data. Unliganded PLC γ 1 contains both β -sheet and α -helical regions, and upon SH2-domain ligation, there is calculated to be an increase in the α -helical content of the protein (8%) as well as a concomitant decrease in the β -sheet and random-coil content.

DISCUSSION

Expression of a rat brain cDNA in *E. coli* has yielded catalytically active PLC γ 1. The purified enzyme is identical with PLC γ 1 isolated from bovine brain with respect to substrate specificity [PtdIns, PtdIns(4,5) P_2], pH-rate profile, and [Ca $^{2+}$] requirements. Production of functional recombinant PLC γ 1 that is easily purified will greatly facilitate additional cell-biological, enzymological and biophysical studies [such as the role of profilin and profilin-bound PtdIns P_2 in intracellular signal transduction (Goldschmidt-Clermont et al., 1991)].

The phosphorylation of specific serine and tyrosine residues on PLC γ 1 and the role which phosphorylation plays in activation *in vivo* has been well studied [Rhee and Choi (1992) and references cited therein]. Some *in vitro* studies have shown that purified PLC γ 1 is activated after phosphorylation by the EGF receptor (Nishibe et al., 1990; Wahl et al., 1992), while other studies indicate that phosphorylated and unphosphorylated PLC γ 1 have similar activities (Kim et al., 1991). In preliminary experiments, we have evaluated the effects of phosphorylation on recombinant PLC γ 1 catalytic activity. The PLC γ 1 expressed in *E. coli* is not phosphorylated, since anti-PLC γ 1 immunoprecipitates of lysates from bacteria grown in ortho[32 P]phosphate-containing media did not reveal radiolabelled PLC γ 1 (results not shown). PLC γ 1 was a substrate for tyrosine phosphorylation by purified EGF receptor, A431 vesicles containing EGF receptor, or p60 src kinase; however, the stoichiometry of *in vitro* phosphorylation was small (5 mol%), and no detectable activity changes were measured (K. S. Koblan, M. D. Schaber, G. Edwards, J. B. Gibbs and D. L. Pompliano, unpublished observations).

The interactions between activated growth-factor receptors and effector proteins are mediated, at least in part, by SH2 and SH3 domains. While it is clear that at least one of the SH2 domains in PLC γ can bind to specific phosphotyrosine sequences in activated growth-factor receptors such as the EGF receptor, the precise effect of this protein-protein interaction remains elusive. In the present study, phosphotyrosine-containing peptides derived from sequences corresponding to known high-affinity binding sites for PLC γ 1 from the EGF and FGF receptors were tested to determine the effect of ligating PLC γ 1-SH2 domains on PLC γ 1 catalytic activity. Peptides corresponding to known PLC γ 1 binding sites (Tyr*-992 and Tyr*-766) increased the rate of PtdIns(4,5) P_2 hydrolysis approx. 50 and 85% when tested at 10 μ M and 100 μ M respectively. Unphosphorylated control peptides Tyr-992 and Tyr-766 and the phosphotyrosine-containing peptide Tyr*-751 derived from the PDGF- β receptor did not alter the catalytic activity (when tested at 10 or 100 μ M). Thus the activation was dependent on both phosphotyrosine and a relevant peptide sequence, and therefore was not a general peptide effect. Cantley and co-workers have reported a similar allosteric effect whereby phosphotyrosine-containing peptides activate the phosphatidylinositol 3'-kinase 2-3-fold *in vitro* (the peptides utilized were tested at 1-100 μ M, a concentration range similar to that used in the present study) (Carpenter et al., 1993). It therefore appears that regulation of PLC γ 1 enzyme activity

may involve more than simple phosphorylation of specific tyrosine residues.

C.d. spectroscopy was used to determine whether structural changes are induced upon ligation of the PLC γ 1 SH2 domain(s) with a peptide corresponding to a major autophosphorylation site on the EGF receptor. Significant spectral changes, indicative of secondary-structural reorganization (i.e. conformational change), were observed upon complex formation with the phosphorylated peptide (Tyr*-992). Since the same peptide also activated PLC γ 1 activity, a protein conformational change could play a role in regulating PLC γ 1 activity. In the activity measurements the non-phosphorylated control peptide Tyr-992 had no effect under identical conditions, and thus the conformational change does not seem to be a general peptide effect. The N-terminal SH2 domain of the p85 subunit of the intracellular signalling enzyme phosphatidylinositol 3'-kinase undergoes a significant structural change upon binding to an autophosphorylation site of the PDGF receptor as determined by c.d. spectroscopy (Panayotou et al., 1992). The N-terminal SH2 domain of p85, but not of Ras GTPase-activating protein (GAP), also undergoes a Ca $^{2+}$ -dependent conformational change (Mahadevan et al., 1994). When considering PLC γ 1 regulation there is no reason why SH2-mediated interactions must be *intermolecular*: possibly an SH2 domain interacts *intramolecularly* with PLC γ 1 phosphotyrosine residues to regulate the activity of the enzyme. For example, the PLC γ 1 sequence surrounding Tyr*-783 is (Y-PO $_4$)VEA, which closely resembles a motif [(Y-PO $_4$)VEV] that is known experimentally to bind to the N-terminal SH2 domain of PLC γ 1 (Songyang et al., 1993). Indeed, the activation of p60 src is thought to occur by changes in conformation which depend upon phosphotyrosine ligation by its own SH2 domains (Cooper and Howell, 1993). Whether or not the structural changes of SH2-domain-containing proteins upon phosphotyrosine binding will be a general phenomenon must await additional investigation. These effects with SH2 domains also beg the question of SH3-domain-ligation effects on protein structure and enzyme activity. The levels of regulation for PLC γ 1 may be manifold: SH2- or SH3-domain ligation by activated growth factor receptors may affect both conformation and activity.

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