THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 19, Issue of May 8, pp. 11650–11659, 1998
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Catalytic Domain of Phosphoinositide-specific Phospholipase C (PLC)

MUTATIONAL ANALYSIS OF RESIDUES WITHIN THE ACTIVE SITE AND HYDROPHOBIC RIDGE OF PLCδ1*

(Received for publication, November 20, 1997, and in revised form, February 18, 1998)

Moira V. Ellis‡, Stephen R. James§¶, Olga Perisic‖, C. Peter Downes§, Roger L. Williams‖, and Matilda Katan‡**

From the ‡Cancer Research Campaign Centre for Cell and Molecular Biology, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, the ‖Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, and the §Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, United Kingdom

Structural studies of phospholipase C δ1 (PLCδ1) in complexes with the inositol-lipid headgroup and calcium identified residues within the catalytic domain that could be involved in substrate recognition, calcium binding, and catalysis. In addition, the structure of the PLCδ1 catalytic domain revealed a cluster of hydrophobic residues at the rim of the active site opening (hydrophobic ridge). To assess a role of each of these residues, we have expressed, purified, and characterized enzymes with the point mutations of putative active site residues (His^{311}, Asn^{312}, Glu^{341}, Asp^{343}, His^{356}, Glu^{390}, Lys^{438}, Lys^{440}, Ser^{522}, Arg^{549}, and Tyr^{551}) and residues from the hydrophobic ridge (Leu^{320}, Phe^{360}, and Trp^{555}). The replacements of most active site residues by alanine resulted in a great reduction (1,000–200,000-fold) of PLC activity analyzed in an inositol lipid/sodium cholate mixed micelle assay. Measurements of the enzyme activity toward phosphatidylinositol, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate (PIP_2) identified Ser^{522}, Lys^{438}, and Arg^{549} as important for preferential hydrolysis of polyphosphoinositides, whereas replacement of Lys^{440} selectively affected only hydrolysis of PIP_2. When PLC activity was analyzed at different calcium concentrations, substitutions of Asn^{312}, Glu^{390}, Glu^{341}, and Asp^{343} resulted in a shift toward higher calcium concentrations required for PIP_2 hydrolysis, suggesting that all these residues contribute toward Ca^{2+} binding. Mutational analysis also confirmed the importance of His^{311} (~20,000-fold reduction) and His^{356} (~6,000-fold reduction) for the catalysis. Mutations within the hydrophobic ridge, which had little effect on PIP_2 hydrolysis in the mixed-micelles, resulted in an enzyme that was less dependent on the surface pressure when analyzed in a monolayer. This systematic mutational analysis provides further insights into the structural basis for the substrate specificity, requirement for Ca^{2+} ion, catalysis, and surface pressure/activity dependence, with general implications for eukaryotic phosphoinositide-specific PLCs.

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2)[1] to the second messengers inositol 1,4,5-trisphosphate and diacylglycerol by phosphoinositide-specific phospholipase C (PI-PLC) is one of the earliest key events in the regulation of various cell functions by a number of extracellular signaling molecules (1–3). Three families of mammalian PI-PLCs with 10 different isozymes (PLCβ1–β4, PLCγ1–γ2, and PLCδ1–δ4) have been characterized; PI-PLC molecules from other eukaryotes have properties shared with mammalian isozymes from one of the main families. Regulation of PI-PLCs has been extensively studied and reveals several distinct mechanisms that link multiple isozymes to various receptors (1). Among those mechanisms, the activation of mammalian PLCβ by G protein subunits and PLCγ isozymes by tyrosine kinase-linked receptors are best understood.

Studies of the catalytic properties of PI-PLC revealed that, unlike regulatory mechanisms, all eukaryotic enzymes have some common characteristics (4). The catalytic activity is strictly dependent on calcium as a cofactor and increases with a rise of calcium concentrations within the physiological range (0.01–10 μM). Phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), and PIP_2 are hydrolyzed with a preference for PIP_2 and PIP, but the enzymes are unable to hydrolyze 3-phosphoinositides. There is a high stereospecificity for the D-*myo*-inositol configuration of the headgroup but not for the configuration of the C-2 position of the diacylglycerol moiety (5). Although glycerophosphorylinositol phosphates can be hydrolyzed by PI-PLC (6), the presence of at least short lipid side chains is required for the efficient catalysis (7). Kinetic studies of some PLCβ, PLCγ, and PLCδ isozymes have also demonstrated that the membrane associated isozymes can catalyze several cycles of PIP_2 hydrolysis functioning in a processive mode of catalysis (8–10), and, when analyzed in monolayer assays, show activity dependence on the monolayer surface pressure (11–14).

Critical to further understanding of PI-PLCs catalytic functions has been the determination of PLCδ1 crystal structure (15, 16). This revealed a four-domain organization of the enzyme consisting of a pleckstrin homology (PH) domain, an EF-hand domain, a catalytic domain, and a C2 domain. An alignment of PI-PLC sequences has suggested that PLCβ, PLCγ, and PLCδ isozymes have all four domains found in PLCδ1 (17). Although PLCδ1 shares sequence similarity with

* This work was supported by grants from the Cancer Research Campaign (to M. K.), the Medical Research Council/DTI/ZENECA LINK Program (to R. L. W.), and the British Heart Foundation (to R. L. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Current address: Pharmacia and Upjohn AB, 112 87 Stockholm, Sweden.

** To whom correspondence should be addressed. Tel.: 44-171-352-8133; Fax: 44-171-352-3299; E-mail: matilda@icr.ac.uk.

[1] The abbreviations used are: PIP_2, phosphatidylinositol 4,5-bisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PLC, phospholipase C; PI-PLC, phosphoinositide-specific phospholipase C; PCR, polymerase chain reaction; PH, pleckstrin homology; PC, phosphatidylcholine; PS, phosphatidylserine; N, newton(s); IP_3, inositol 1,4,5-trisphosphate.

This paper is available on line at http://www.jbc.org

TABLE I
Oligonucleotides used to generate PLCδ1 mutants

Primers with mutated nucleotides (underlined) and the wild type primers were used to introduce mutations into PLCδ1 by the two-stage PCR-based overlap extension method. Only forward primer sequences are shown for each mutant oligonucleotide.

	Primer sequence
Mutant	
$Asp^{343} \rightarrow Ala$	TG GAG CTC GCC TGC TGG G
$Asp^{343} \rightarrow Arg$	GC TTG GAG CTC CGC TGC TGG G
$Glu^{341} \rightarrow Ala$	TGC CGG TGC TTG GCG CTC GAC TGC TGG
$Glu^{341} \rightarrow His$	TGC CGG TGC TTG CAC CTC GAC TGC TGG GAG
$Glu^{341} \rightarrow Gln$	TGC CGG TGC TTG CAG CTC GAC TGC TGG CAG
$Glu^{390} \rightarrow Ala$	TCC CTG GCG AAC CAC TGT AG
$Glu^{390} \rightarrow His$	TCC CTG CAC AAC CAC TGT AG
$Glu^{390} \rightarrow Lys$	TCC CTG AAG AAC CAC TGT AG
$Glu^{390} \rightarrow Gln$	TCC CTG CAG AAC CAC TGT AG
$Phe^{360} \rightarrow Ala$	CAC GGC TAC ACT GCT ACC TCT AAG ATA
$His^{311} \rightarrow Ala$	TTA GTG TCT TCT TCC GCC AAC ACC TAC CTG
$His^{356} \rightarrow Ala$	CC ATC ATC TAC GCC GGC TAC
$Lys^{438} \rightarrow Ala$	TC CTG TTG GCA GGG AAG AAG
$Lys^{440} \rightarrow Ala$	TG AAA GCC AAG CTG G
$Leu^{320} \rightarrow Ala$	CTG GAA GAC CAG GCC ACA GGG CCC AGC
$Asn^{312} \rightarrow Ala$	CT TCT TCC CAC GCC ACC TAC CTG CTG
$Arg^{549} \rightarrow Ala$	G AGC TGT CTG AGC GCG ATC TAC CCG GCT
$Ser^{552} \rightarrow Ala$	TAT GAG ATG GCT GCC TTC TCT GAG AGC
$Trp^{555} \rightarrow Ala$	TAC CCG GCT GGG GCG AGA ACA GAT TCC
$Tyr^{551} \rightarrow Ala$	CTG AGC AGG ATC GCC CCG GCT GGG TGG
Wild type	
1. Forward (691–710)	GAG AGG CTA GTG ACG TTT C
2. Reverse (1613–1594)	CCA TTT CCT GAT TCT TGC AG
3. Reverse (2226–2210)	GCT GGT CTC CAT TCT TAG

other PI-PLC throughout its sequence, the regions with the highest sequence similarity are contained within the catalytic domain. The residues within the conserved region X and the most conserved part of the Y region (4) form two halves of the catalytic α/β barrel (15). Based on structural studies of complexes with the PIP_2 headgroup and the catalytic calcium, the importance of individual residues within the catalytic domain for the substrate binding, catalysis, and membrane interactions has been suggested (15). Furthermore, the crystal structure of the complex with cyclic inositol phosphate (18) and kinetic studies (6) have suggested a reaction mechanism for PI-PLC catalysis. The data support general acid/base catalysis in a sequential mechanism with cyclic inositol phosphate as a reaction intermediate.

In this study, the data obtained from structural studies of PLCδ1 complexes with the ligands have been used as a framework for a structure/function analysis. The residues that constitute putative active site and several hydrophobic residues in the vicinity of the active site opening have been subjected to site-directed mutagenesis. The impact of individual replacements on the PLC activity was analyzed under different conditions to establish further structural requirements for substrate recognition, calcium binding, catalytic steps, and surface pressure dependence.

EXPERIMENTAL PROCEDURES

Materials

Oligonucleotides for mutagenesis and sequencing were supplied by Oswel DNA Services and also made in-house using an Applied Biosystems DNA synthesizer (model 394). PCR reagents and the ABI PRISM dye terminator cycle sequencing ready reaction kit for automated sequencing, using an ABI 377 sequencer, were from Perkin-Elmer. Wizard PCR preps were from Promega and mini-prep kits from Qiagen. PGEX-2T vector, glutathione-Sepharose 4B, and Mono Q column (PC1.6/5) were from Amersham Pharmacia Biotech. Human thrombin, sodium salts of soybean PI, bovine brain PIP and PIP_2, as well as dioleyl phosphatidylcholine (PC) and Folch extract of bovine brain (for purification of PIP_2 for the monolayer assay) were purchased from Sigma. Pig brain phosphatidylserine (PS) was from Doosan Serdary Research Laboratories. Phosphatidyl([^{3}H])inositol ([^{3}H]PI) and Phosphatidyl [^{3}H]inositol 4,5-bisphosphate ([^{3}H]PIP_2) were obtained from NEN Life Science Products. Phosphatidylinositol 4-phosphate (inositol-2-^{3}H) ([^{3}H]PIP) was obtained from American Radiolabeled Chemicals Inc. [^{33}P]ATP (used for synthesis of [^{33}P]PIP_2 with partially purified PIP kinase from rat brain) was from Amersham Pharmacia Biotech.

Methods

Plasmid Construction and Site-directed Mutagenesis—The 2.2-kilobase pair *Bam*HI/*Sma*I fragment from rat brain PLCδ1 cDNA (19) was subcloned into PGEX-2T vector, which encodes the enzyme as a glutathione *S*-transferase fusion protein. A *Bam*HI site had been engineered at the 5′ end of the cDNA to enable cloning into the PGEX-2T vector; an internal *Bam*HI site had first been mutated by PCR without changing the amino acid sequence. A mutation has also been introduced by PCR at residue 60, an internal thrombin cleavage recognition site, from arginine to lysine ($Arg^{60} \rightarrow Lys$) resulting in higher yields of the glutathione *S*-transferase fusion protein and its cleavage by thrombin only at the engineered cleavage site. This mutation did not interfere with any other function of the enzyme, and this mutant will be referred to as the control PLCδ1.

Mutations were introduced into PLCδ1 $Arg^{60} \rightarrow Lys$ by the two-stage PCR-based overlap extension method (20). The control PLCδ1 $Arg^{60} \rightarrow Lys$ was used as the template for the first stage PCR reaction; gene fragments with overlapping complementary ends from the first round were paired to provide templates for the second round using wild type external primers. One mutant, $His^{311} \rightarrow Ala$, prepared by M13-based site-directed mutagenesis has been described previously (21). Mutants $Arg^{549} \rightarrow Ala$, $Ser^{522} \rightarrow Ala$, $Trp^{555} \rightarrow Ala$, and $Tyr^{551} \rightarrow Ala$ were made using wild type primers 1 and 3; all others, excluding $Asp^{343} \rightarrow Arg$ and $Glu^{390} \rightarrow Lys$, were made using wild type primers 1 and 2. Oligonucleotides used in these procedures are summarized in Table I.

Mutant fragments $Asp^{343} \rightarrow Ala$, $Glu^{341} \rightarrow Ala$, $Glu^{341} \rightarrow His$, $Glu^{341} \rightarrow Gln$, $Glu^{390} \rightarrow Ala$, $Glu^{390} \rightarrow His$, $Glu^{390} \rightarrow Gln$, $Phe^{360} \rightarrow Ala$, $His^{356} \rightarrow Ala$, $Arg^{438} \rightarrow Ala$, $Lys^{440} \rightarrow Ala$, $Leu^{320} \rightarrow Ala$, and $Asn^{312} \rightarrow Ala$ were digested with *Acc*I/*Bsm*I and subcloned individually into *Acc*I/*Bsm*I sites of PLCδ1 $Arg^{60} \rightarrow Lys$. Mutant fragments $Arg^{549} \rightarrow Ala$, $Ser^{522} \rightarrow Ala$, $Trp^{555} \rightarrow Ala$, and $Tyr^{551} \rightarrow Ala$ were digested with *Acc*I/*Kpn*I and subcloned individually into the *Acc*I/*Kpn*I sites of PLCδ1 $Arg^{60} \rightarrow Lys$. All mutations were verified by sequencing, and each fragment was completely sequenced to confirm that no unwanted mutations had been created.

Expression and Purification of Recombinant Proteins—Expression

and purification of recombinant PLCδ1 protein lacking the first 57 amino acids residues has been described previously (19, 21).The same procedures were used to obtain PLCδ1 $Arg^{60} \rightarrow Lys$ and mutant enzymes. Briefly, glutathione *S*-transferase fusion protein was isolated from the *Escherichia coli* extract by affinity chromatography on glutathione-Sepharose. The PLCδ1 protein was separated from glutathione *S*-transferase and removed from the affinity matrix by thrombin cleavage. Soluble PLCδ1 was further purified from minor contaminants by chromatography on a Mono Q column (PC 1.6/5) using a SMART system (Amersham Pharmacia Biotech). Determination of protein concentration was according to Bradford (22), using bovine serum albumin as a standard, and aliquots of purified protein (5–10 mg/ml) stored at −20 °C. Electrophoresis in SDS-acrylamide gels, performed according to Laemmli (23), showed that purity of all PLCδ1 proteins was >90%. In most cases, the protein yield was about 10 mg/liter of starting bacterial culture.

PLC Activity in Mixed Micelles—The assay of hydrolysis of PIP_2 and PI was based on methods described previously (19, 21, 24, 25). The standard reaction mixture for PIP_2 hydrolysis contained 50 mM Tris-HCl, pH 6.8, 100 mM NaCl, 0.5% sodium cholate, 5 mM 2-mercaptoethanol, 0.4 mg/ml bovine serum albumin, 220 μM PIP_2 (0.025 μCi), and CaHEDTA buffer for the final concentration of free calcium of 50 μM. Incubation was at 37 °C for 10 min. In this assay, 1 unit of PLC activity corresponds to hydrolysis of 1 μmol of PIP_2/min. The same assay conditions were used to monitor hydrolysis of PIP and PI. In addition, PI hydrolysis was also analyzed in sodium deoxycholate mixed micelles (0.05% sodium deoxycholate) in the presence of 1 mM calcium. The calcium dependence of PIP_2, PIP, and PI hydrolysis was analyzed using a range of calcium buffers as described previously (24).

For kinetic analysis of control and mutant enzymes, initial velocities were measured at PIP_2 concentrations of 0.055, 0.110, 0.220, 0.440, and 0.660 mM, with incubation times of 0, 2.5, 5, 10, and 20 min. Apparent K_m and V_{max} values were determined by plotting results as the double reciprocal Lineweaver-Burk plot.

PLC Activity in Monolayers—Monolayer assay of the activity of the control and mutant PLCδ1 enzymes were performed as described previously for β-isoforms of PLC (13, 14, 26). The composition of the monolayers was 70% PC, 27% PS, 3% PIP_2 by molarity, which were spread over a buffer comprising 10 mM Hepes, pH 7.2, 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 1 mM $MgCl_2$, and 1 μM free Ca^{2+} ions. Enzyme (200 ng) was added to the subphase, via an injection port in the Teflon trough, 5 min after the monolayer was spread to allow the surface pressure to stabilize. PIP_2 hydrolysis was assayed for 15 min, during which time the monolayer radioactivity was assayed continuously as described previously. Induction times were determined by computer-aided integration of the trace recording of changes in radioactivity in the monolayer, and the extent of the reaction was determined by sampling the radioactivity remaining in the monolayer and in 1 ml of subphase buffer at the end of the reaction.

RESULTS

Residues Selected for Site-directed Mutagenesis—Based on the structure of PLCδ1 complexes with inositol phosphates and/or calcium analogues (15, 18), the following residues have been implicated in interactions with the ligands: His^{311}, Asn^{312}, Glu^{341}, Asp^{343}, His^{356}, Glu^{390}, Lys^{438}, Lys^{440}, Ser^{522}, Arg^{549}, and Tyr^{551} (Fig. 1*A*). These putative active site residues are present within a broad, solvent accessible depression on the C-terminal end of the catalytic α/β-barrel. Comparison of 23 sequences of PI-PLC from mammalian sources and other organisms such as slime mold, yeast, and plants demonstrated that these residues are well conserved among eukaryotic enzymes (Fig. 2). Residues corresponding to His^{311}, Asn^{312}, Glu^{341}, Asp^{343}, His^{356}, Ser^{522}, and Arg^{549} in PLCδ1 are invariant in all PI-PLCs. Other active site residues in PLCδ1 (Glu^{390}, Lys^{438}, Lys^{440}, and Tyr^{551}) are replaced conservatively in only a few sequences. Thus, Glu^{390} is replaced by aspartic acid only in PLCδ4, Lys^{438} by serine in plant PI-PLCs, Lys^{440} by histidine in the enzyme from *Dictyostelium discoideum*, and Tyr^{551} by phenylalanine in *Arabidopsis thaliana*. This strict conservation of residues that could comprise the PI-PLC active site suggests that substrate recognition and mechanism of catalysis are likely to be common to all eukaryotic enzymes.

Structural studies of PLCδ1 (15) also revealed a cluster of hydrophobic residues around the active site opening (Fig. 1*B*). This hydrophobic ridge, located at one end of the active site opening, consists of a residue from helix α5 and residues from three loops connecting β1 with α1, β2 with α2, and β7 and α6. The hydrophobic residues include Leu^{320} in the loop β1/α1, Tyr^{358} and Phe^{360} in the β2/α2 loop, Trp^{555} in the β7/α6 loop, and Leu^{529} from helix α5. Comparison of PI-PLC sequences (Fig. 2) have shown that positions equivalent to PLCδ1 Leu^{320} are occupied by hydrophobic residues in all PI-PLCs. There is at least one hydrophobic residue in the loop β2/α2 at positions corresponding to PLCδ1 Tyr^{358} and Phe^{360}; Phe^{360} is better conserved with a hydrophobic residue absent in only two (bovine γ1 and human γ2) sequences. Conservation of hydrophobic residues equivalent to Trp^{555} is limited to only a few sequences. However, in most other sequences the position corresponding to residue 557, also present in the β7/α6 loop, is occupied by hydrophobic residues and could also contribute to a hydrophobic ridge as described for PLCδ1.

All PLCδ1 active site residues and Leu^{320}, Phe^{360}, and Trp^{555} within the hydrophobic ridge were individually replaced, first by alanine and, in the case of Glu^{341}, Glu^{390}, and Asp^{343}, by other residues as well (Table II). All changes have been introduced into the full-length PLCδ1 using PCR-based site-directed mutagenesis. The control enzyme and PLCδ1 with the point mutations were expressed as fusion proteins, subjected to thrombin cleavage, and further purified and concentrated on a Mono Q column. Expression levels of different point mutants were comparable to the control (~10 mg/liter of bacterial culture) with the exception of Ser^{522} that was reduced 5–10-fold.

Effects of Replacements of the Active Site Residues on Activity of PLCδ1—PLC activity of the enzymes with single point mutations of the active site residues was compared with the activity of the control enzyme using a sodium cholate/PIP_2 mixed micelle assay. In previous studies using this assay system (19, 21, 27), it was demonstrated that the deletion of the PH domain (containing the high affinity, non-catalytic PIP_2-binding site) had no effect on the rate of PIP_2 hydrolysis and that the remainder of the enzyme could directly bind and hydrolyze the substrate presented in this way.

Data obtained from analysis of the active site residue replacements by alanine are summarized in Table II and Fig. 3. Specific activity of the control enzyme at a calcium concentration of 50 μM (control conditions) was about 1,000 units/mg, consistent with our previous measurements (19). The $Glu^{341} \rightarrow$ Ala mutation caused the greatest reduction of specific activity to 0.004 unit/mg, *i.e.* over 200,000-fold. The $His^{311} \rightarrow$ Ala, $Asn^{312} \rightarrow$ Ala, and $Ser^{522} \rightarrow$ Ala mutations resulted in reduction of specific activity to 0.03–0.09 unit/mg (10,000–35,000-fold). A reduction of 600–6,000-fold (specific activity 0.1–2 units/mg) was observed for the mutants $Asp^{343} \rightarrow$ Ala, $His^{356} \rightarrow$ Ala, $Glu^{390} \rightarrow$ Ala, $Arg^{549} \rightarrow$ Ala, and $Tyr^{551} \rightarrow$ Ala. Replacements of Lys^{438} and Lys^{440} resulted in only 30-fold and 5-fold reductions, respectively.

The impact of the point mutations on activity of PLCδ1 was also analyzed using PI and conditions adjusted for maximum hydrolysis of this substrate (1 mM calcium, sodium deoxycholate mixed micelles) (Fig. 3). The -fold reductions of PI hydrolysis calculated for replacements of His^{311}, Asn^{312}, Glu^{341}, Asp^{343}, His^{356}, Glu^{390}, and Tyr^{551} to Ala were comparable with the data obtained for hydrolysis of PIP_2 (all differences were less than 3-fold). In contrast, the specific activities of $Lys^{438} \rightarrow$ Ala, $Lys^{440} \rightarrow$ Ala, $Ser^{522} \rightarrow$ Ala, and $Arg^{549} \rightarrow$ Ala toward PI were not reduced to the levels measured with PIP_2 as a substrate with the ratio PIP_2/PI between 5- and 60-fold. Substrate specificity of these mutants toward PIP_2, PIP, and PI were studied further (see below).

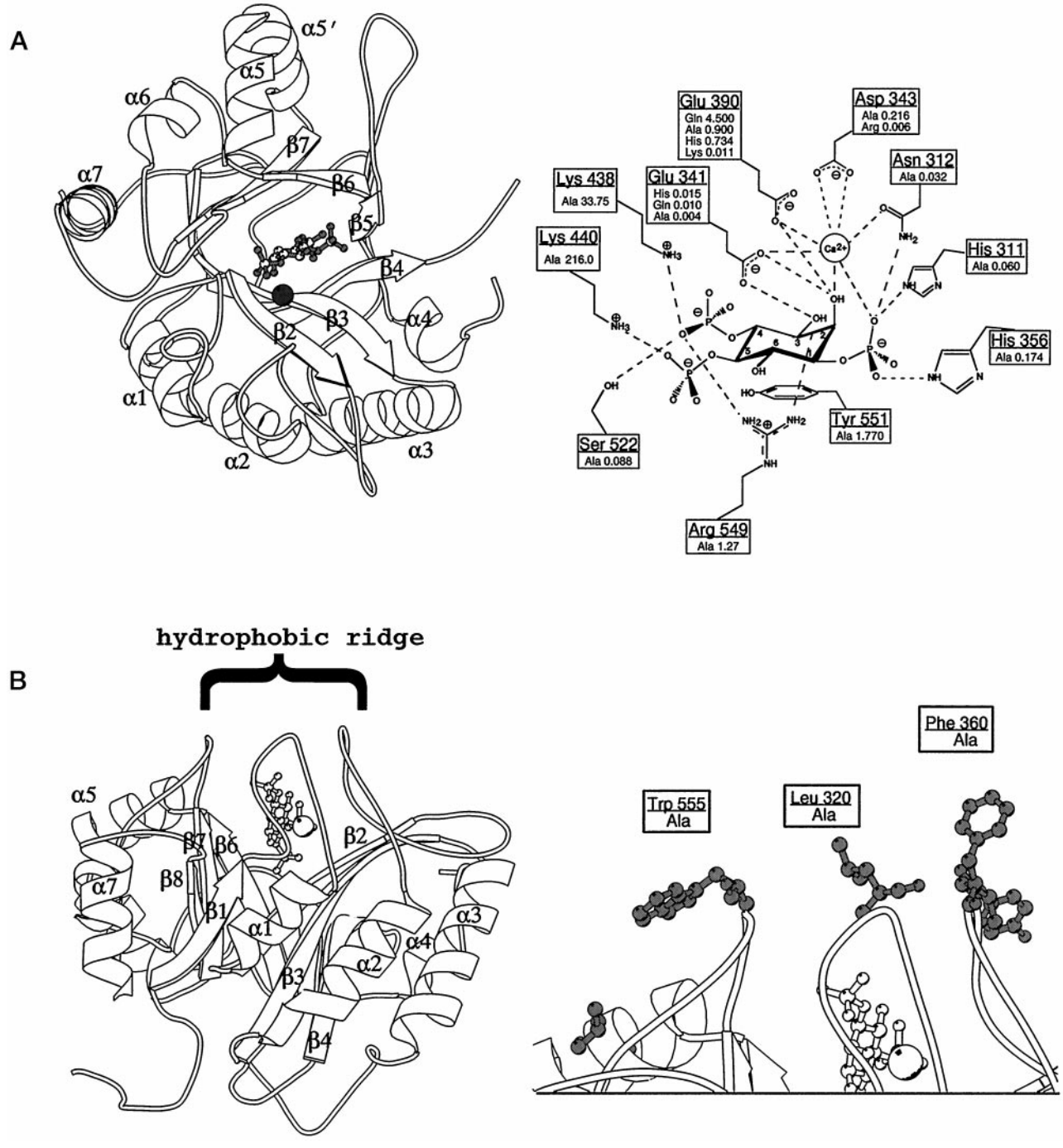

FIG. 1. **Schematic representation of the catalytic domain of phosphoinositide-specific phospholipase C.** A ribbon representation of the PLCδ1 catalytic domain/IP_3 complex as reported by Essen *et al.* (15). *A*, *left panel*, view looking down into the active site of the domain with the bound IP_3 substrate analogue shown in ball-and-stick representation and the calcium co-factor for the reaction is shown as a large sphere; *right panel*, schematic representation for the interactions between the protein and the substrate analogue. Beneath each residue interacting with the substrate analogue are listed the mutants that were constructed and the specific activities of the mutants toward PIP_2 (μmol/min/mg). *B*, *left panel*, view of the catalytic domain roughly 90° from the view shown in *A*. The loops forming the hydrophobic ridge at the rim of the active site opening are indicated. *Right panel*, expanded view of the hydrophobic ridge with hydrophobic side chains shown in ball-and-stick representation. The residues that were mutated are indicated.

In addition to alanine replacements, Glu^{341} was also mutated into glutamine or histidine, Asp^{343} into arginine, and Glu^{390} into glutamine, histidine, or lysine (Table II). The impact of the histidine and glutamine replacements on PLC activity was similar to the effects of alanine mutations except for the $Glu^{390} \rightarrow$ Gln mutant, which had a slightly higher residual activity. In the case of the $Asp^{343} \rightarrow$ Arg and $Glu^{390} \rightarrow$ Lys mutations, where negative charges have been replaced by positively charged residues, activity was reduced an additional 30- or 80-fold, respectively.

Since a relatively large number of mutations have been made and many replacements greatly reduced the enzyme activity, a detailed kinetic analysis was performed only with the control PLCδ1, $Glu^{390} \rightarrow$ Ala, $Arg^{549} \rightarrow$ Ala, $His^{356} \rightarrow$ Ala, and $His^{311} \rightarrow$ Ala. Based on structural studies (15), these selected resides are likely to perform different functions. The main differences for all tested mutants were in the V_{max} values. The K_m values determined for the $Glu^{390} \rightarrow$ Ala (83 ± 15 μM), $His^{356} \rightarrow$ Ala

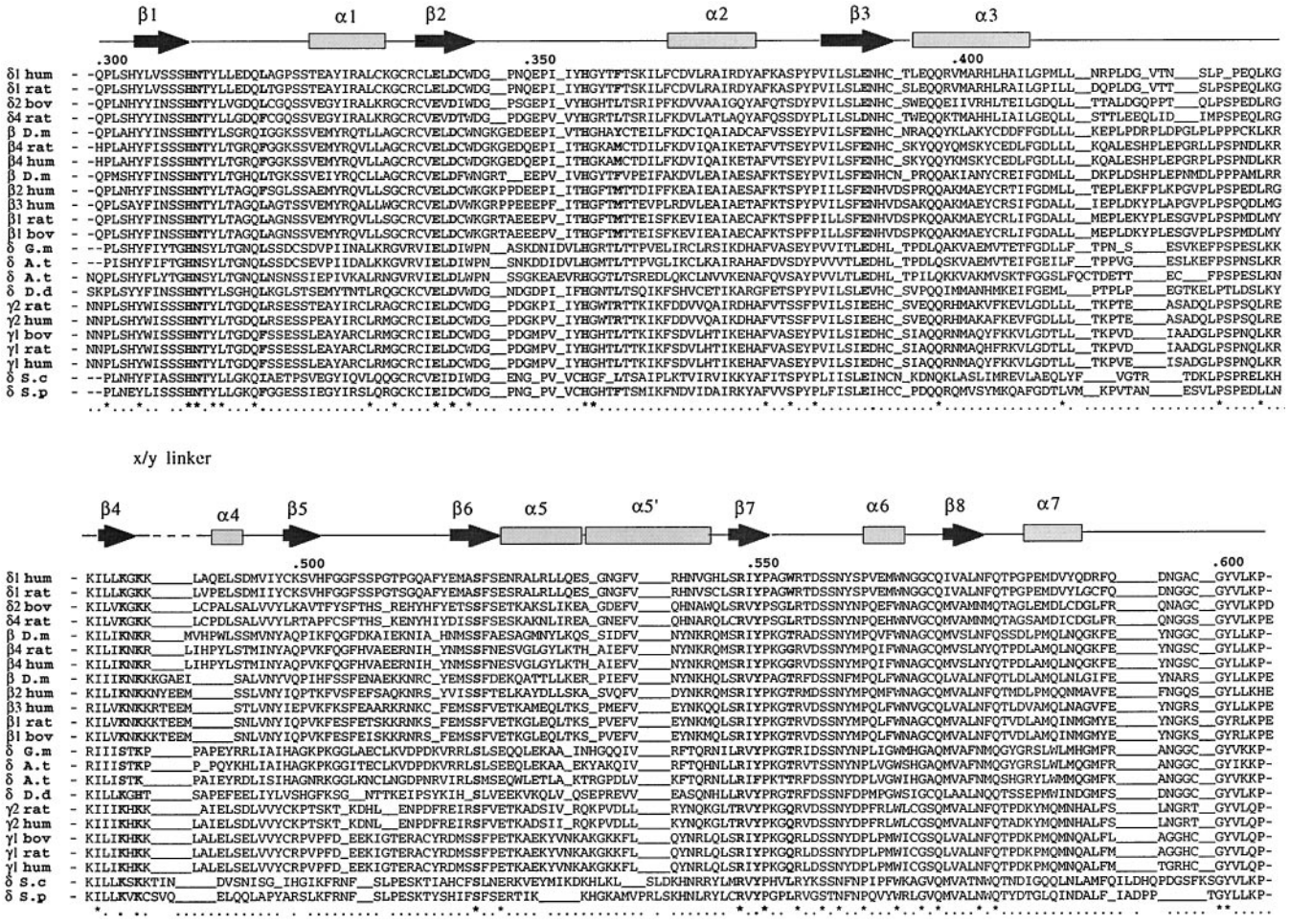

FIG. 2. **An alignment of the catalytic domain amino acid sequence from different PI-PLC isozymes.** The catalytic domain sequences incorporating residues from the X and Y regions of sequence similarity have been compared; the linker region (X/Y linker) was omitted. The following sequences, described in Ref. 17, were compared: human PLCδ1 (*δ1 hum*; residues 299–442 and 489–606), rat PLCδ1 (*δ1 rat*; residues 299–442 and 489–606), bovine PLCδ2 (*δ2 bov*; residues 293–437 and 487–603), rat PLCδ4 (*δ4 rat*; residues 293–437 and 500–616), *Drosophila melanogaster* norpA (β-like) PLC (*β D.m*; residues 322–471 and 546–633), rat PLCβ4 (*β4 rat*; residues 316–465 and 562–679), human retinal PLCβ4 (*β4 hum*; residues 163–312 and 408–525), *D. melanogaster* (β-like) plc-21 (*β D.m*; residues 321–468 and 596–712), human PLCβ2 (*β2 hum*; residues 315–465 and 539–655), human PLCβ3 (*β3 hum*; residues 320–470 and 587–703), rat PLCβ1 (*β1 rat*; residues 319–469 and 537–653), bovine PLCβ1 (*β1 bov*; residues 319–469 and 537–653), soybean (*Glycine max*) δ-like PLC (*δ G.m*; residues 109–252 and 330–447), *A. thaliana* δ-like PLC 2 (*δ A.t*; residues 106–249 and 313–430), *A. thaliana* δ-like PLC 1 (*δ A.t*; residues 108–250 and 290–407), *D. discoideum* δ-like PLC (*δ D.d*; residues 325–467 and 536–650), rat PLCγ2 (*γ2 rat*; residues 315–458 and 927–1041), human PLCγ2 (*γ2 hum*; residues 315–458 and 927–1041), bovine PLCγ1 (*γ1 bov*; residues 323–466 and 950–1067), rat PLCγ1 (*γ1 rat*; residues 323–466 and 950–1067), human PLCγ1 (*γ1 hum*; residues 323–466 and 950–1067), *Saccharomyces cerevisiae* δ-like PLC (*δ S.c*; residues 383–522 and 584–707), and *Schizosaccharomyces pombe* δ-like PLC (*δ S.p*; residues 444–586 and 635–750). All-against-all comparisons of the sequences were performed using the Darwin suite of programs running on the Computational Biochemistry Research Group Server at Eidgenossische Technische Hochschule, Zurich (http://cbrg.inf.ethz.ch/welcome.html). Residues in PLCδ1 selected for mutagenesis are shown in *bold*. Invariant residues in all sequences are indicated by an *asterisk*. Secondary structure elements and residue numbers of PLCδ1 are also shown.

(110 ± 21 μM), and His^{311} → Ala (74 ± 14 μM) were similar to the control (83 ± 17 μM), whereas the value determined for Arg^{549} → Ala was somewhat higher (125 ± 21 μM).

Mutations Affecting Substrate Specificity—Among mutations that differentially affected hydrolysis of PIP_2 and PI, Lys^{438}, Arg^{549}, and Ser^{522} have been implicated in interactions with the 4-phosphoryl group and Lys^{440} in interaction with the 5-phosphoryl group of the inositol ring. To distinguish whether the reduction in PIP_2 hydrolysis resulted from a loss of interactions with the 4- or 5-phosphate, we compared the PLC activity of these mutants toward PI, PIP, and PIP_2, prepared as sodium cholate mixed micelles, in the presence of 50 μM calcium (Fig. 4). Activity of the Lys^{440} → Ala mutant was reduced (~5-fold) only with PIP_2 as a substrate. The specific activity of this mutant was similar to the specific activity of the control enzyme toward PIP (Table II), consistent with Lys^{440} interaction with the 5-phosphoryl group. The Lys^{438} → Ala mutation resulted in a reduction of both PIP_2 and PIP hydrolysis (15–20-fold) with very little effect on PI hydrolysis. The specific activity of this mutant was comparable with the activity of the control enzyme using PI as a substrate. The mutation Arg^{549} → Ala had an effect on hydrolysis not only of PIP_2 and PIP but also PI. Both the hydrolysis of PIP_2 and PIP were greatly reduced relative to hydrolysis of PI. This ratio (PIP_2/PI and PIP/PI) for Arg^{549} → Ala was the most pronounced (about 50-fold) among the analyzed mutants. The effect of this mutation on PI hydrolysis could be due to an additional interaction with the 2-hydroxyl of the inositol (Fig. 1*A*) that would affect hydrolysis of all inositol-lipid substrates. In the case of Ser^{522} → Ala replacement, the effect on PI hydrolysis was a reduction of about 1,000-fold with a further decrease in specific activity (5–10-fold) toward PIP and PIP_2.

Calcium Dependence of Substrate Hydrolysis—It has been shown previously that activity of PLCδ1 (as well as other PI-PLC enzymes) has different calcium dependence curves when analyzed with PIP_2 and PI as a substrate presented as sodium

TABLE II
Phospholipase C activity of control and mutant PLCδ1

Control PLCδ1 and different mutants were expressed in bacteria and purified to homogeneity. For calculation of specific activity, the activity of the control and mutant PLCδ1 were analysed using PIP_2 as a substrate in the presence of 50 μM calcium (control conditions). Specific activities of control protein towards PIP_2, PIP, and PI were determined at 50 μM calcium. The specific activity towards PIP_2 was also determined in the presence of 3 mM EGTA. The data are averages of two measurements that did not differ more than 20%.

PLCδ1	Activity
	units/mg
Control PLCδ1	
PIP_2, 50 μM calcium	1080.0
PI, 50 μM calcium	21.6
PIP, 50 μM calcium	360.0
PIP_2, 3 mM EGTA	0.059
Mutations in the active site	
PIP_2, 50 μM calcium	
$His^{311} \rightarrow Ala$	0.060
$Asn^{312} \rightarrow Ala$	0.032
$Glu^{341} \rightarrow Ala$	0.004
$Glu^{341} \rightarrow Gln$	0.010
$Glu^{341} \rightarrow His$	0.015
$Asp^{343} \rightarrow Ala$	0.216
$Asp^{343} \rightarrow Arg$	0.006
$His^{356} \rightarrow Ala$	0.174
$Glu^{390} \rightarrow Ala$	0.900
$Glu^{390} \rightarrow Gln$	4.50
$Glu^{390} \rightarrow His$	0.734
$Glu^{390} \rightarrow Lys$	0.011
$Lys^{438} \rightarrow Ala$	33.75
$Lys^{440} \rightarrow Ala$	216.0
$Ser^{522} \rightarrow Ala$	0.088
$Arg^{549} \rightarrow Ala$	1.27
$Tyr^{551} \rightarrow Ala$	1.770
Mutations in the hydrophobic ridge	
PIP_2, 50 μM calcium	
$Leu^{320} \rightarrow Ala$	284.2
$Phe^{360} \rightarrow Ala$	276.9
$Trp^{555} \rightarrow Ala$	302.1

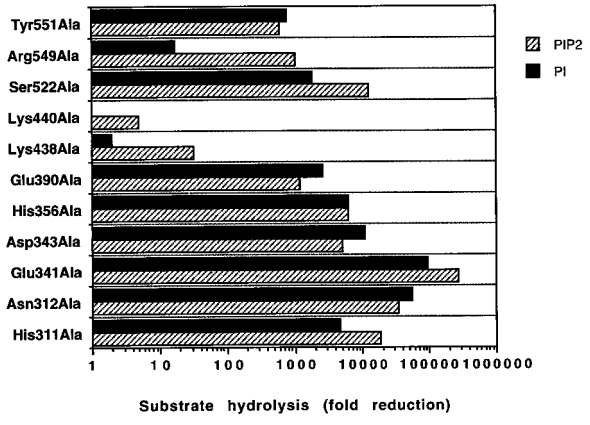

FIG. 3. **Effect of replacement of active site residues by alanine on substrate hydrolysis.** Purified proteins were analyzed for PIP_2 (*light hatched bars*) and PI (*black bars*) hydrolysis under optimal conditions for the substrates. The data are averages of two measurements that did not differ more than 20% and are expressed as -fold reduction of control PLCδ1 activity.

cholate or sodium deoxycholate micelles (19). The specific activity of the control PLCδ1 used in this study toward PIP_2 was ~300 units/mg at 0.5 μM calcium, ~1,000 units/mg at 50 μM calcium, and ~140 units/mg at 1 mM calcium. The specific activity toward PI in a similar assay system increased within the tested range of calcium concentrations (0.5 μM to 10 mM) and was ~7 units/mg at 0.5 μM calcium, ~18 units/mg at 50 μM calcium, and ~25 units/mg at 1 mM calcium. Although simpler relationship of calcium dependence was observed with PI than with PIP_2 as a substrate, the reduced activity of the mutants made the measurements difficult with this less efficiently hydrolyzed substrate. Therefore, the activity of the control and mutant enzymes with the point mutations of the active site residues was compared at different calcium concentrations (0.5 μM to 10 mM) using PIP_2 as a substrate. The control (Fig. 5) and $His^{356} \rightarrow Ala$, $Lys^{438} \rightarrow Ala$, $Lys^{440} \rightarrow Ala$, $Ser^{522} \rightarrow Ala$, $Arg^{549} \rightarrow Ala$, and $Tyr^{551} \rightarrow Ala$ mutants (data not shown) had a similar calcium dependence of PIP_2 hydrolysis with the maximum at about 50 μM calcium and decreasing at higher calcium concentrations. Mutations of Asn^{312}, Glu^{341}, Asp^{343}, and Glu^{390}, however, resulted in changes of PLCδ1 calcium dependence (Fig. 5). Some differences have been also observed for the $His^{311} \rightarrow Ala$ mutant, which shows less inhibition of activity at high calcium concentrations than the control enzyme. The activity of the $Asp^{343} \rightarrow Ala$ and $Asn^{312} \rightarrow Ala$ mutants at 1 mM calcium was comparable to their activities at concentrations of 50 μM, whereas for $Glu^{341} \rightarrow Ala$ and $Glu^{390} \rightarrow Ala$ the peak of the activity was shifted to 1 mM calcium. A similar shift was observed for the $Asp^{343} \rightarrow Arg$ and $Glu^{390} \rightarrow Lys$ mutants (data not shown). Residues Asn^{312}, Glu^{341}, Asp^{343}, and Glu^{390} have been implicated in calcium binding (Fig. 1*A*), and this difference in PLC dependence of calcium concentrations further supports their proposed role.

Analysis of the control PLCδ1 has shown that the enzyme prepared and analyzed in the presence of 3 mM EGTA had a specific activity reduced to about 0.06 unit/mg (Table II). Under these conditions, it is still possible that some calcium remained bound to PLCδ1 and that the complete removal of the metal could have an even more pronounced effect. Based on these observations, it is expected that mutation of residues essential for the calcium binding would result in a great reduction of the enzyme activity. All mutations of Asn^{312}, Glu^{341}, Asp^{343}, and Glu^{390} residues (in particular, $Glu^{341} \rightarrow Ala$, $Asp^{343} \rightarrow Arg$, and $Glu^{390} \rightarrow Lys$) had a large impact on the activity (1,000–200,000-fold) (Table II). It is however difficult to assess the relative contribution of each of these residues, since a role of some residues may not be limited to calcium binding (see "Discussion").

Mutations within the Hydrophobic Ridge—Analyses of the $Leu^{320} \rightarrow Ala$, $Phe^{360} \rightarrow Ala$, and $Trp^{555} \rightarrow Ala$ mutants in PIP_2/sodium cholate mixed micelles at 50 μM calcium have shown that these mutants, in comparison with the mutants in the active site, had little effect; they resulted in a small reduction in PIP_2 hydrolysis (Table II). Similar data have been obtained using the substrate/dodecyl maltoside mixed micelles, and measurements of K_m values in this assay revealed that enzymes with mutations in the hydrophobic ridge behaved similarly to the control enzyme, having largely unaltered interfacial K_m values (data not shown).

For further studies of these mutants, the possibility that the hydrophobic ridge could penetrate into a phospholipid membrane was examined by measuring PIP_2 hydrolysis in monolayers at different surface pressures. Fig. 6*A* shows that increasing monolayer surface pressure in PC/PS/PIP_2 monolayers (70:27:3 by molarity) was accompanied by a decline in PIP_2 hydrolysis catalyzed by the control PLCδ1. The decrease in rate of hydrolysis was linear with about a 5-fold difference between activity at the surface pressure of 10 mN/m and 35 mN/m. Analysis of the mutants in this system, in contrast to the mixed micelle assay, revealed an increase in activity compared with the control. Furthermore, as illustrated for the $Phe^{360} \rightarrow Ala$ mutant (Fig. 6*B*), activity was less dependent on increasing surface pressure. The surface pressure/activity relationship was also analyzed by measurements of the induction time τ between addition of the enzyme to the subphase and

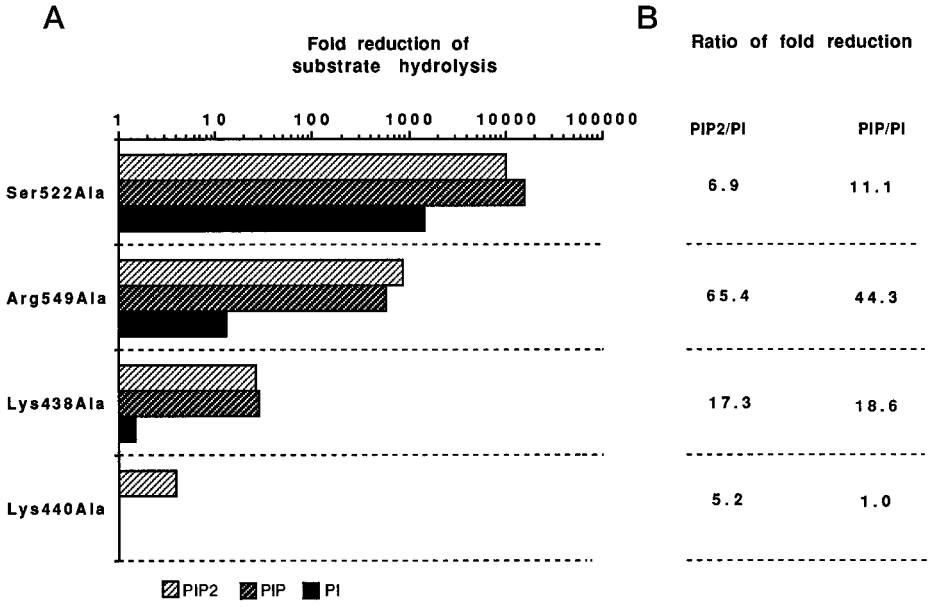

FIG. 4. **Mutations that selectively affect hydrolysis of PIP_2 and PIP compared with hydrolysis of PI.** Activity of control and mutant PLCδ1 was determined using PIP_2 (*light hatched bars*), PIP (*dark hatched bars*), or PI (*black bars*) as a substrate at 50 μM calcium. The data are expressed as -fold reduction compared with the control PLCδ1 (*A*). Ratio of -fold reduction of PIP_2 and PI hydrolysis (*PIP2/PI*) and PIP and PI hydrolysis (*PIP/PI*) is also shown for each mutant (*B*).

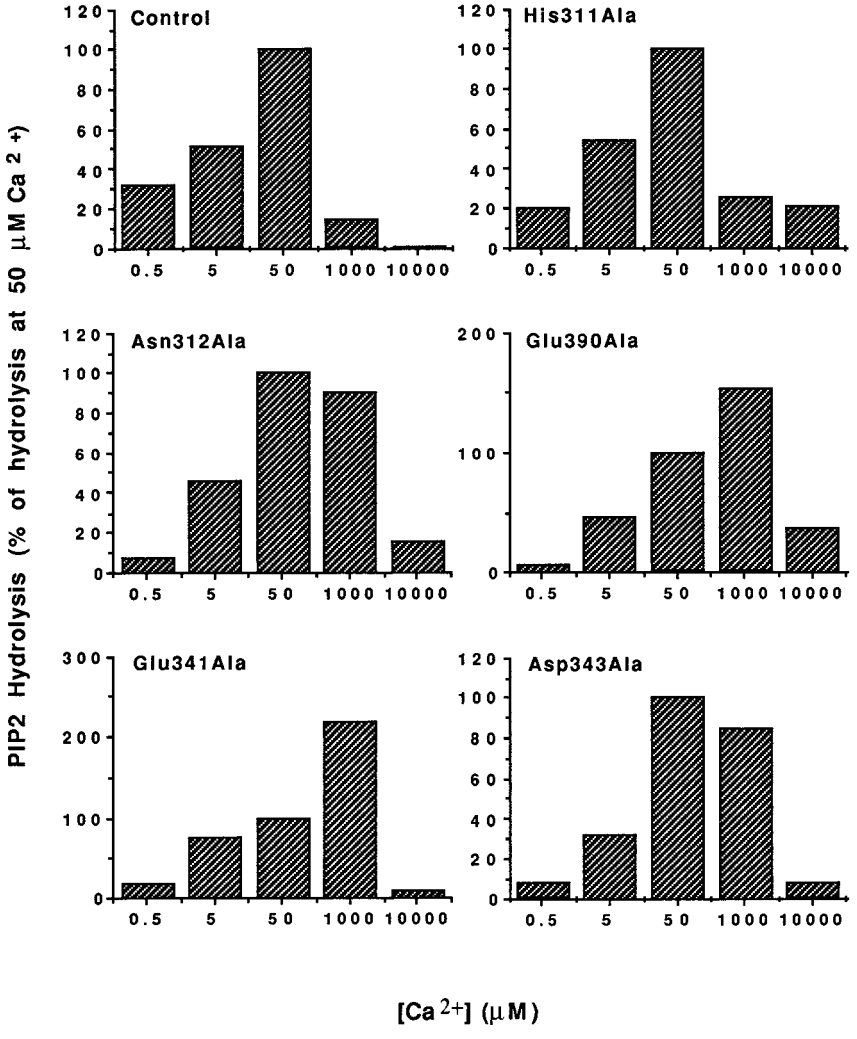

FIG. 5. **PLCδ1 mutations showing changes in Ca^{2+} requirement for PIP_2 hydrolysis.** Preparations of purified proteins were diluted to similar PLC activity as determined under control conditions and then analyzed at different calcium concentration for PIP_2 hydrolysis. Activity of each mutant is expressed as percent of hydrolysis at 50 μM calcium.

onset of catalysis. The induction time measured for the control PLCδ1 was greater at higher monolayer pressures (Fig. 6*C*). It has been suggested that in this assay system the penetration rate (k_p) would be inversely proportional to the τ time (13). Therefore, the data for the control PLCδ1 would suggest that increasing surface pressure reduces the penetration rate into the monolayer. When PLCδ1 enzymes with the point mutations within the hydrophobic ridge were compared with the control, it was found that the τ time of $Trp^{555} \rightarrow Ala$ and $Leu^{320} \rightarrow Ala$ mutants was less dependent on monolayer pressure and that the $Phe^{360} \rightarrow Ala$ mutant was independent of the surface pressure (Fig. 6*C*).

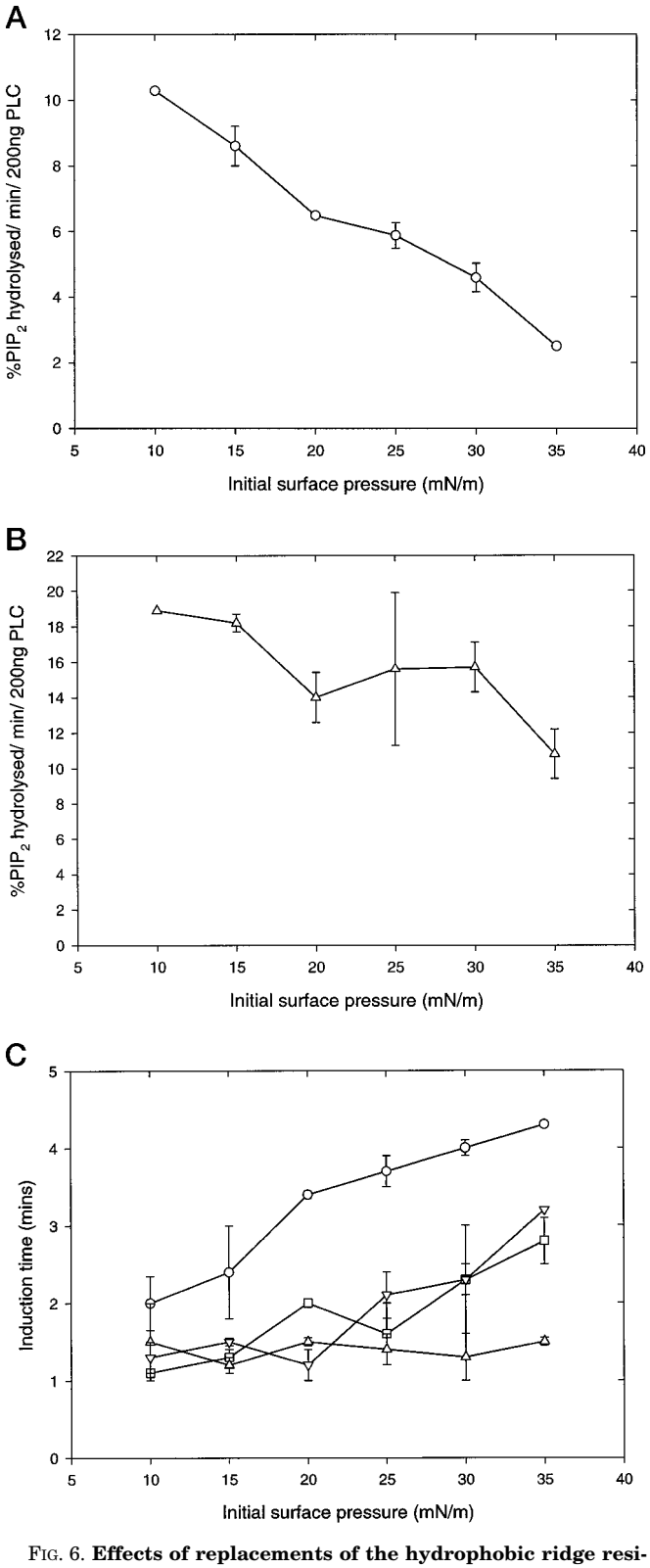

FIG. 6. **Effects of replacements of the hydrophobic ridge residues on PLCδ1 activity in a monolayer assay.** *A*, pressure-activity relationship of the control PLCδ1 in mixed phospholipid monolayers. PC/PS/PIP_2 monolayers containing ^{33}P-labeled PIP_2 were spread as described previously (13, 26) and 200 ng of enzyme added after 5 min. Reaction times were 15 min, and the rate of PIP_2 hydrolysis was determined from linear portions of continuous trace recordings of radioactivity in the monolayer. Data points are the mean ± range of duplicate monolayers, except 10 and 35 mN/m, which were taken from single experiments. *B*, the Phe^{360} → Ala mutant was analyzed as described in *A*. *C*, induction times for PIP_2 hydrolysis in PC/PS/PIP_2 mixed monolayers by the control and three hydrophobic ridge point mutants of PLCδ1. Induction times (τ) were derived by computer-aided integration of trace recordings of ^{33}P-labeled PIP_2 hydrolysis by the different enzymes at all surface pressures examined, as described previously (13). Each data point is the mean (± range or S.D.) of at least two determinations of τ from each assay performed. *Circles*, control PLCδ1; *inverted triangles*, Trp^{555} → Ala; *squares*, Leu^{320} → Ala; *triangles*, Phe^{360} → Ala.

DISCUSSION

In this study, we describe the first systematic analysis of amino acid replacements within the PLCδ1 catalytic domain based on the crystal structure of this enzyme. The data provide further evidence for a relationship between the individual residues and the catalytic properties of PI-PLC enzymes (including substrate specificity, requirement for Ca^{2+} ion, and surface pressure/activity dependence) with implications for all eukaryotic PI-PLCs.

It is generally accepted that agonist stimulation of PI-PLC activity can result in preferential hydrolysis of polyphosphoinositides and, in particular, PIP_2 (see, *e.g.*, Ref. 28). Although presentation of PIP_2 in cells could contribute toward a preference for this inositol lipid, it has been shown that the substrate selectivity is, to at least some extent, a property of PI-PLC enzymes (4). Characterization of PI-PLC enzymes *in vitro* revealed that all eukaryotic enzymes, unlike bacterial PI-PLC, can hydrolyze PIP_2 and PIP. Generally, a preference $PIP_2 > PIP \gg PI$ has been observed. However, some differences between PI-PLC families have been found, such as lower ratio of PIP_2/PI hydrolysis for PLCγ than for PLCβ and PLCδ (29). The degree of preference for polyphosphoinositides described for the same isozyme also varies considerably depending on the conditions and substrate presentation used in different assays *in vitro* (30–33). Some of these differences could be due to composition and concentration of detergents and metal ions affecting directly hydrolysis by the enzyme and also substrate presentation of different inositol-lipids. Within a multidomain structure of eukaryotic PI-PLCs, some of the domains could interact non-catalyticaly with the inositol lipids and (depending on the assay conditions) also influence the rate of substrate hydrolysis (8, 10). In this study, we used mixed micelles of each inositol-lipid substrate with sodium cholate and found that the PLCδ1 specific activity with PIP_2 as a substrate was about 3-fold higher than with PIP and about 50-fold higher than with PI (Table II). The ratio of substrate hydrolysis in this assay was not affected by a deletion of the PH domain, eliminating a contribution of this high affinity non-catalytic PIP_2 binding site. The structural studies of the PLCδ catalytic domain (15, 18) implicated Ser^{522}, Lys^{438}, and Arg^{549} in interactions with the 4-phosphate and suggested their importance for preference of both PIP and PIP_2 over PI. The only residue that could interact with the 5-phosphate, Lys^{440}, emerged as a candidate residue that could provide specificity for PIP_2. Replacements of each of these residues by alanine resulted in selective changes of PIP_2, PIP, and PI hydrolysis consistent with their predicted functions (Fig. 4). Among these mutations, the greatest impact on preference for polyphosphoinositides was the Arg^{549} → Ala replacement. However, replacements of some residues, most notably Ser^{522}, not only selectively reduced hydrolysis of polyphosphoinositides, but also significantly reduced hydrolysis of PI suggesting their role in some other functions of the enzyme or their importance for the protein stability. Replacement of Tyr^{551}, implicated in hydrophobic interactions with the sugar ring of inositol, equally reduced hydrolysis of PIP_2 and PI (Table II and Fig. 3). Several of the residues implicated in substrate binding and selectivity have been analyzed previously. A study of the conserved region rich in basic residues in PLCβ2 (residues 457–464) identified Lys^{461} and Lys^{463}, corresponding to Lys^{438} and Lys^{440} in PLCδ1, as important for PIP_2

hydrolysis (34). It is, however, difficult to compare those data and this study further, since a different assay system has been used and PLCβ2 was analyzed only with PIP_2 as a substrate. Replacements of four arginine residues within the Y region of conserved sequences in human PLCδ1 identified Arg^{549} as selectively important for PIP_2 compared with PI hydrolysis (30, 35). The replacement of this residue with glycine, as with our $Arg^{549} \rightarrow$ Ala mutant, also somewhat reduced PI hydrolysis, whereas $Arg^{549} \rightarrow$ His mutation only affected PIP_2 hydrolysis (30).

Kinetic analysis of PI and PIP_2 hydrolysis by PI-PLC enzymes, in the assay systems where preference for PIP_2 was clearly observed, revealed only small differences in the apparent K_m values (24, 31–33, 36). Consistent with these observations, the main effect of Arg^{549} replacements, which selectively reduced hydrolysis of polyphosphoinositides, was on V_{max} (Ref. 30 and this report). It is therefore possible that additional interactions with the 4- and 5-phosphoryl groups of the inositol ring in PIP_2 have little effect on the affinity for this substrate. However, these interactions could greatly reduce flexibility of inositol headgroup movements within the active site, resulting in higher hydrolytic efficiency. Recent structural studies using an inositol monophosphate support this possibility (18).

Structural studies and kinetic analysis of both eukaryotic and bacterial enzymes have outlined a common mechanism of substrate hydrolysis: general acid/base catalysis with formation of cyclic inositol in a phosphotransfer step, followed by its conversion to an acyclic inositol in a phosphohydrolysis step (5, 37). However, a distinct characteristic of substrate hydrolysis by all eukaryotic enzymes is dependence on Ca^{2+} as a cofactor. Structural studies of PLCδ1 have suggested that the principal function of the Ca^{2+} is to lower the pK_a of the 2-hydroxyl group of the inositol moiety so as to facilitate its deprotonation and subsequent nucleophilic attack on the 1-phosphate. A second role of the metal could be to stabilize the negatively charged transition state (18). Although all eukaryotic enzymes require Ca^{2+}, some differences in calcium dependence have been observed among different PI-PLCs and among inositol-lipid substrates (24, 29, 32, 33, 36). Generally, maximum hydrolysis of PIP_2 was achieved at 5–50 μM calcium and further increase in calcium concentrations (1–10 mM) had an inhibitory effect. Crystallographic studies of complexes of PLCδ1 with Ca^{2+} and PIP_2 headgroup (IP_3) revealed complex interactions with the catalytic Ca^{2+} involving several negatively charged residues in the active site (Glu^{390}, Glu^{341}, and Asp^{343}), Asn^{312}, and 2-hydroxyl group of IP_3 (15, 18). Replacement of these residues by alanine resulted in a great reduction of the enzyme activity (1,000–200,000-fold) (Table II). Furthermore, calcium dependence of these mutants was shifted toward higher calcium concentrations (Fig. 4) showing the importance of all these residues in coordination of calcium. In previous studies, based on sequence alignments, only one of these residues (Glu^{341} in PLCδ1) had been analyzed. The replacement of Glu^{341} in PLCδ1 by glycine resulted in a loss of the enzyme activity; the calcium dependence of this mutant, however, was not analyzed (35). The same replacement has been found in the p130 protein, containing X and Y regions found in PI-PLCs with the closest similarity to PLCδ1, but without detectable PI-PLC activity (38).

The acidic residues that coordinate the catalytic Ca^{2+} in eukaryotic PI-PLCs are not present in the enzyme from *Bacillus cereus*. Instead, basic residues (Arg^{69} and Lys^{115}) are present in the positions equivalent to Asp^{343} and Glu^{390} within a similar α/β barrel structure (37, 39). It has been suggested that these basic residues, like Ca^{2+} in eukaryotic enzymes, facilitate nucleophilic attack and stabilize the resulting transition state (37, 39). A recent mutational analysis of Arg^{69} is consistent with this proposal (40). In our attempts to generate a PLCδ1 mutant that is calcium-independent like the bacterial enzyme, mutations $Glu^{390} \rightarrow$ Lys and $Asp^{343} \rightarrow$ Arg (Table II) as well as the double mutant (data not shown) have been made. However, these mutations did not reduce calcium dependence, possibly due to small structural differences between the active sites of eukaryotic and the bacterial enzymes, which could still be too complex to allow this conversion. Another reason for the inability to convert PLCδ1 into a calcium-independent enzyme by these mutations could be that the role of calcium is not restricted to a positive charge. The structure of the enzyme in complexes with intermediate analogues suggests that the Ca^{2+} makes additional ligations with the transition state that may sterically accelerate catalysis (18).

An effort has also been made to clarify the identity of residues important for the phosphotransfer and phosphohydrolysis steps. It has been suggested that in PI-PLC from *B. cereus* His^{32} and His^{82} act as general base/acid catalysts (37, 39). A residue in eukaryotic enzymes that could have an equivalent role to His^{82} is likely to be the conserved histidine corresponding to His^{356} in PLCδ1. A replacement of this residue by alanine resulted in a great reduction of the enzyme activity (Table I). Similar observations have been reported previously for PLCδ1 Leu^{356} mutant (35) and a replacement of the corresponding His^{380} in PLCγ1 to phenylalanine (41). However, three candidate residues in PLCδ1, His^{311}, Glu^{390}, and Glu^{341}, have been considered for the role of His^{32} from the bacterial enzyme, which is located within hydrogen bonding distance of the 2-OH group of the inositol and could deprotonate the hydroxyl in a step leading to the formation of the cyclic intermediate (15, 37). Mutational analysis in this (Table II) and previous studies (21, 35, 41) identified PLCδ1 His^{311}, and the corresponding His^{335} in PLCγ1, as an important catalytic residue. In the structural studies, using inositol phosphates that mimic the binding of substrates and the reaction intermediates, His^{311} appears to be too distant and unfavorably oriented for hydrogen-bonding with the 2-hydroxyl group of any of the studied inositol phosphates (18). The structural data are more consistent with the notion that His^{311} is essential for the stabilization of a pentavalent transition state. Alternative candidates for the general acid/base catalyst, Glu^{341} and Glu^{390}, are not only the calcium ligands but also form hydrogen bonds to the 2-OH group of the inositol. A proton transfer between either of the glutamate residues and the 2-hydroxyl group would be feasible even in the presence of the positively charged calcium, because any change in the partial charge of the carboxyl group would be compensated by an opposing charge at the 2-hydroxyl. An example for a glutamate residue acting both as a metal ligand and as a nucleophile is Glu^{70} in inositol monophosphatase. In addition to structural data supporting these roles of Glu^{70} (42, 43), it has been shown that the mutation of this residue to glutamine decreased k_{cat} dramatically while leaving metal binding unaffected (44). This is consistent with the prediction that the $Glu^{70} \rightarrow$ Gln mutant would be able to coordinate the metal but would not function as a nucleophile. The mutational analysis of PLCδ1 (Table II) has shown about a 100-fold greater impact of the $Glu^{341} \rightarrow$ Ala replacement compared with the $Glu^{390} \rightarrow$ Ala mutation. To analyze the function of these two glutamate residues further, replacements to glutamine were also made; this mutation would eliminate the function of a general acid/base catalyst. Since the $Glu^{390} \rightarrow$ Gln mutation resulted in significant residual activity compared with the $Gln^{390} \rightarrow$ Ala mutant, the Glu^{341} residue would be more likely to act as the general base of the first step of the reaction.

Studies of eukaryotic (8–10) and bacterial (45) PI-PLC enzymes suggested that interactions with the membrane involve interactions additional to the substrate binding in the active site. Although some of these sites in eukaryotic enzymes are clearly outside the catalytic domain (*e.g.* the PH domain of PLCδ1), the hydrophobic ridge at the rim of the active site opening could provide a non-catalytic membrane interaction site for this domain (17, 46, 47). Experiments where dependence of PLC activity on surface pressure was examined in lipid monolayers have shown a decrease of PLCβ, PLCγ, and PLCδ activity as the pressure increases. The surface pressure/activity relationship observed for PLCδ was less complex than for PLCβ and PLCγ, where changes of the pressure within a small range resulted in a dramatic reduction of the activity (11). A more linear relationship for PLCδ observed in this study (Fig. 6*A*) is in agreement with the the data reported previously (11, 12). One possible interpretation offered for the surface pressure dependence was that a part of the PLC molecule (estimated to be ~1 nm^2 in PLCδ1) inserts into monolayers in a work-requiring step prior to activation (12). The surface area of the PLCδ1 hydrophobic ridge is roughly consistent with the area that could penetrate into the membrane. The possibility that the residues present in the hydrophobic ridge could underlie this activity/surface pressure relationship was supported by the observation that the replacements of these residues by alanine-generated enzymes that were less dependent on the surface pressure (Fig. 6, *B* and *C*). The greatest change is caused by the replacement of Phe^{360}, the hydrophobic residue most exposed in this area. These data (Fig. 6*C*) have also shown that at high surface pressure (30–35 mN/m), when the packing of phospholipids is believed to be comparable to that in biological membranes, the enzymes with the reduced hydrophobic surface had shorter induction times than the control, suggesting a negative rather than positive contribution of the hydrophobic ridge to the rate of substrate hydrolysis. Thus, overcoming such a negative influence potentially could play a part in the activation of PI-PLC's *in vivo*.

Acknowledgment—We thank Damian Counsell for help with the sequence alignments.

REFERENCES

1. Rhee, S. G., and Bae, Y., S. (1997) *J. Biol. Chem.* **272,** 15045–15048
2. Lee, S. B., and Rhee, S. G. (1995) *Curr. Opin. Cell Biol.* **7,** 183–189
3. Cockcroft, S., and Thomas, G. M. H. (1992) *Biochem. J.* **288,** 1–14
4. Rhee, S. G., and Choi, K. D. (1992) *J. Biol. Chem.* **267,** 12393–12396
5. Bruzik, K. S., and Tsai, M.-D. (1994) *Bioorg. Med. Chem.* **2,** 49–72
6. Wu, Y., Perisic, O., Williams, R. L., Katan, M., and Roberts, M. F. (1997) *Biochemistry* **37,** 11223–11233
7. Rebecchi, M. J., Eberhardt, R., Delaney, T., Ali, S., and Bittman, R. (1993) *J. Biol. Chem.* **268,** 1735–1741
8. James, S. R., Paterson, A., Harden, T. K., and Downes, C. P. (1995) *J. Biol. Chem.* **270,** 11872–11881
9. Wahl, M. I., Jones, G. A., Nishibe, S., Rhee, S. G., and Carpenter, G. (1992) *J. Biol. Chem.* **267,** 10447–10456
10. Cifuentes, M. E., Honkanen, L., and Rebecchi, M. J. (1993) *J. Biol. Chem.* **268,** 11586–11593
11. Boguslavsky, V., Rebecchi, M., Morris, A. J., Jhon, D.-Y., Rhee, S. G., and McLaughlin, S. (1994) *Biochemistry* **33,** 3032–3037
12. Rebecchi, M., Boguslavsky, V., Boguslavsky, L., and McLaughlin, S. (1992) *Biochemistry* **31,** 12748–12753
13. James, S. R., Paterson, A., Harden, K., Demel, R. A., and Downes, C. P. (1997) *Biochemistry* **36,** 848–855
14. James, S., Demel, R. A., and Downes, C. P. (1994) *Biochem. J.* **298,** 499–506
15. Essen, L.-O., Perisic, O., Cheung, R., Katan, M., and Williams, R. L. (1996) *Nature* **380,** 595–602
16. Ferguson, K., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1995) *Cell* **83,** 1037–1048
17. Williams, R. L., and Katan, M. (1996) *Structure* **4,** 1387–1394
18. Essen, L.-O., Perisic, O., Katan, M., Wu, Y., Roberts, M. F., and Williams, R. L. (1997) *Biochemistry* **36,** 1704–1718
19. Ellis, M. V., Carne, A., and Katan, M. (1993) *Eur. J. Biochem.* **213,** 339–347
20. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 51–59
21. Ellis, M. V., U., S., and Katan, M. (1995) *Biochem. J.* **307,** 69–75
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685
24. Katan, M., and Parker, P. J. (1987) *Eur. J. Biochem.* **168,** 413–418
25. Katan, M., Kriz, R. W., Totty, N., Philip, R., Meldrum, E., Aldape, R. A., Knopf, J. L., and Parker, P. J. (1988) *Cell* **54,** 171–177
26. James, S. R., Smith, S., Paterson, A., Harden, T. K., and Downes, C. P. (1996) *Biochem. J.* **314,** 917–921
27. Paterson, H. F., Savopoulos, J. W., Perisic, O., Cheung, R., Ellis, M., Williams, R. L., and Katan, M. (1995) *Biochem. J.* **312,** 661–666
28. Hughes, A. R., and Putney, J. W., Jr. (1989) *J. Biol. Chem.* **264,** 9400–9407
29. Ryu, S. H., Suh, P.-G., Lee, K.-Y., and Rhee, S. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6649–6653
30. Wang, L.-P., Lim, C., Kuan, Y.-S., Chen, C.-L., Chen, H.-F., and King, K. (1996) *J. Biol. Chem.* **271,** 24505–24516
31. Rebecchi, M., and Rosen, O. M. (1987) *J. Biol. Chem.* **262,** 12526–12532
32. Fukui, T., Lutz, R. J., and Lowenstein, J. M. (1988) *J. Biol. Chem.* **263,** 17730–17737
33. Ginger, R. S., and Parker, P. J. (1992) *Eur. J. Biochem.* **210,** 155–160
34. Simões, A. P., Camps, M., Schnabel, P., and Gierschik, P. (1995) *FEBS Lett.* **365,** 155–158
35. Cheng, H.-F., Jiang, M.-J., Chen, C.-L., Liu, S.-M., Wong, L.-P., Lomasney, J. W., and King, K. (1995) *J. Biol. Chem.* **270,** 5495–5505
36. Meldrum, E., Katan, M., and Parker, P. J. (1989) *Eur. J. Biochem.* **182,** 673–677
37. Heinz, D. W., Essen, L.-O., and Williams, R. L. (1998) *J. Mol. Biol.* **275,** 635–650
38. Kanematsu, T., Misumi, Y., Watanabe, Y., Ozaki, S., Koga, T., Iwanaga, S., Ikehara, Y., and Hirata, M. (1996) *Biochem. J.* **313,** 319–325
39. Heinz, D. W., Ryan, M., Bullock, T. L., and Griffith, O. H. (1995) *EMBO J.* **14,** 3855–3863
40. Hondal, R. J., Riddle, S. R., Kravchuk, A. V., Zhao, Z., Liao, H., Bruzik, K. S., and Tsai, M.-D. (1997) *Biochemistry* **36,** 6633–6642
41. Smith, M. R., Liu, Y.-L., Matthews, N. T., Rhee, S. G., Sung, W. K., and Kung, H.-F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6554–6558
42. Bone, R., Frank, L., Springer, J. P., and Atack, J. R. (1994) *Biochemistry* **33,** 9468–9476
43. Bone, R., Frank, L., Springer, J. P., Pollack, S. J., Osborne, S., Atack, J. R., Knowles, M. R., McAllister, G., Ragan, C. I., Broughton, H. B., Baker, R., and Fletcher, S. R. (1994) *Biochemistry* **33,** 9460–9467
44. Pollack, S. J., Knowles, M. R., Atack, J. R., Broughton, H. B., Ragan, C. I., Osborne, S., and McAllister, G. (1993) *Eur. J. Biochem.* **217,** 281–287
45. Volwerk, J. J., Filthuth, E., Griffith, O. H., and Jain, M. K. (1994) *Biochemistry* **33,** 3464–3474
46. Katan, M., and Williams, L. R. (1997) *Cell Dev. Biol.* **8,** 287–296
47. James, S. R., and Downes, C. P. (1997) *Cell. Signalling* **9,** 329–336