Reassembly of Phospholipase C-β2 from Separated Domains

ANALYSIS OF BASAL AND G PROTEIN-STIMULATED ACTIVITIES*

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Phosphatidylinositol-specific phospholipase C-βs (PLCs) are the only PLC isoforms that are regulated by G protein subunits. To further understand the regulation of PLC-β2 by G proteins and the functional roles of PLC-β2 structural domains, we tested whether the separately expressed amino and carboxyl halves of PLC-β2 could associate to form catalytically active enzymes as two polypeptides, and we explored how the complexes thus formed would be regulated by G protein βγ subunits (Gβγ). We expressed cDNA constructs encoding PLC-β2 fragments of different lengths in COS-7 cells and demonstrated by coimmunoprecipitation that the coexpressed fragments could assemble and functionally reconstitute an active PLC-β2. The pleckstrin homology domain of PLC-β2 was required for its targeting to the membrane and for substrate hydrolysis. Reconstituted enzymes that contained the linker region that joins the two catalytic domains were as active or more active than the wild-type PLC-β2. When the linker region was removed, basal PLC-β2 enzymatic activity was increased further, suggesting that the linker region exerts an inhibitory effect on basal PLC-β2 activity. The reconstituted enzymes, like wild-type PLC-β2, were activated by Gβγ when the C-terminal region was present in these constructs, they were also activated by Goαγ, Gβγ and Goα, activated these PLC-β2 constructs equally in the presence or absence of the linker region. We conclude that the linker region is an inhibitory element in PLC-β2 and that Gβγ and Goα do not stimulate PLC-β2 through easing the inhibition of enzymatic activity by the linker region.

Phosphatidylinositol-specific phospholipase Cs (PLCs) are enzymes that catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 releases Ca2+ from intracellular stores, and diacylglycerol activates protein kinase C for review see Refs. 1, 2. PLCs are also more sensitive to Ca2+ than the other isoforms and, unlike PLC-γs and PLC-βs, are activated by Ca2+ alone (8). All the ten mammalian PLC isoforms identified to date are modular proteins. As shown in Fig. 1, the PLCs contain a pleckstrin homology (PH) domain, four EF-hand motifs, a catalytic domain (composed of X and Y regions separated by a linker region) and a C2 domain. PLC-βs have an additional 400-residue C-terminal region, which is required for activation by Goα (9, 10) and may also contribute to membrane localization (11).

Among the PLC isoforms, only members of the PLC-β family (PLC-β1–3) are activated by Gβγ. Part of the Gβγ-binding site on PLC-β2 is located in the Y region as shown by cross-linking (12) and copurification (13). Gβγ can also bind to the isolated PH domains from PLC-β2 (14). Indirect evidence suggests that this interaction may lead to activation of PLC-β2 (15). Despite these progresses, the mechanism whereby Gβγ activates PLC-β2 is still unclear. It seems unlikely that PLC-β2 activation by Gβγ involves membrane translocation of PLC-β2 to the plasma membrane, because Gβγ does not significantly alter the binding affinity of PLC-β2 to phospholipid vesicles (16–18).

The goal of this study was to further understand the mechanism of substrate hydrolysis of PLC-β2, its regulation by G protein subunits, and the functional contribution of some of the PLC-β2 domains to enzyme function. Although some PLC domains are homologous to known domains in other proteins, and highly homologous domains can be found among the various PLC isoforms, these domains may have different functions in the different isoforms. For example, PH domains are found in many proteins, but only some of them can bind WD-repeat-containing proteins (such as Gβγ) (for review see Ref. 19). In addition, the PH domains of various PLC isoforms show very different affinities to phospholipids (14, 20, 21). It is therefore necessary to test individual proteins to find out which role(s) a particular domain plays in a particular context.

The roles of other specific PLC domains in basal and ligand-regulated PLC catalytic activities have not yet been clearly identified. For example, the role of the linker region between X and Y regions of the catalytic domain is less well understood. In the crystal structure of the PLC-δ1 molecule, the X and Y regions are tightly associated to form a triose phosphate isomerase barrel-like structure (22). Although the X and Y

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The abbreviations used are: PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; PH, pleckstrin homology; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
regions are well conserved among the PLC isozymes, the linker region possesses little similarity among the PLC isozymes. For example, PLC-γs have a long linker region that contains two SH2 domains, one SH3 domain, and an additional PH domain, whereas the linker regions in PLC-β and PLC-δ are less than 100 residues long and contain no obvious structural domains within them. In the crystal structure of PLC-δ1, the linker region shows a disordered structure (22). The linker region is not essential for PLC catalytic activity. Coexpression of the N- and C-terminal fragments of PLC-γ lacking the linker region produces a catalytically active complex with an activity substantially higher than the holoenzyme (23). Trypsin digestion of PLC-δ1 cleaves the enzyme at the linker region and generates two associated fragments that retain catalytic activity (24). Proteolysis at or near the linker region of a truncated form of PLC-β2 after it had folded into an active enzyme suggested that the linker region served as an inhibitory element (25). In this study, the linker region was cleaved but not removed and the exact site of trypic or V8 protease cleavage was not determined. Because the authors used a truncated form of PLC-β2 that was not stimulated by Goq, it was not possible to determine the effect of proteolysis of the enzyme in or near its linker region on Goq-dependent PLC activity.

It is usually straightforward to analyze the contributions of domains at the N or C termini of a protein, because truncated forms of the enzyme can be made, and these truncated proteins are often active. In addition to analyzing the role of the N-terminal PH domain and the C terminus, we were particularly interested in the linker region. Because it is often difficult to study the function of internal domains due to misfolding of proteins with internal deletions, we attempted to reconstitute PLC-β2 from two separate fragments, each containing one of the two catalytic X and Y regions. We tested whether or not the N- and C-terminal halves of PLC-β2 could associate to form catalytically active enzymes when expressed as two separate polypeptides and whether reconstituted PLC-β2 could still be activated by Gβγ and Goq. Using PLC fragments of different lengths, we examined the functional contribution of the PH domain, the linker region and the C-terminal region to basal activity and Gα- and Gβγ-mediated PLC-β2 activation. Answers to these questions will expand our understanding of the mechanism of substrate hydrolysis by PLC-β2 and its regulation by G proteins.

EXPERIMENTAL PROCEDURES

cDNA Constructs—Plasmids containing cDNA sequences encoding various fragments of human PLC-β2 were constructed by polymerase chain reaction. Full-length wild-type PLC-β2 in pMT2 vector (a gift from M. Simon of the California Institute of Technology, Pasadena, CA) was used as template. The primer at the 5′-end included a HindIII site, a Kozak sequence (GGCCGCC), and a start codon. The primer at the 3′-end included an EcoRI site and a stop codon. To add a FLAG or hemagglutinin (HA) epitope tag to a construct, one of the two primers contained the sequence encoding the epitope. The polymerase chain reaction products were digested with HindIII and EcoRI and cloned into an HindIII/EcoRI-cut pcDNA3 vector. All the sequences were confirmed by DNA sequencing.

Cell Culture and Transfection—COS-7 cells were maintained in complete growth medium (Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin). Cells in 6-well plates (for immunoprecipitation) or 12-well plates (for PLC activity assay) were transfected using LipofectAMINE (Life Technologies). Prior to transfection, cells were transfected to Opti-MEM I medium (Life Technologies) for 1 h. The medium was replaced with 1 ml (6-well plates) or 500 μl (12-well plates) of complete growth medium and added to each well. The medium was replaced with complete growth medium the next day.

35S Metabolic Labeling and Immunoprecipitation—Forty-eight hours after transfection, cells on 6-well plates were starved for 2 h in 2 ml of starvation medium (RPMI 1640 without glutamine, methionine, and cysteine (Sigma) supplemented with 10% dialyzed, heat-inactivated fetal bovine serum and 2 mM l-glutamine). The cells were then metabolically labeled in 1 ml of starvation medium containing 150 μCi of [35S]-Express Protein Labeling Mix (NEN) for 4 h. The cells were rinsed with PBS and lysed in 1 ml of lysis buffer (50 mM HEPES-Na (pH 7.5), 6 mM MgCl2, 1 mM EDTA, 75 mM sucrose, 3 mM benzamidine, 1% (v/v) Triton X-100, and 1 mM dithiorthreitol) at 4 °C for 30 min. The cell lysates were preclarified with 30 μl of protein G-agarose (Roche Molecular Biochemicals) or 50 μl of protein A-Sepharose (Sigma) slurry (50% (v/v) in PBS) for 30 min. After a 10-min centrifugation, the supernatants were mixed with 2 μl of M2 anti-FLAG antibody (Sigma) or anti-FLAG antibody 12CA5 (Babco) at 4 °C overnight. The samples were centrifuged at 15,000 × g for 15 min. The supernatants were then mixed with 30 μl of protein G-agarose or 50 μl of protein A-Sepharose slurry for 1.5 h. The resins were washed twice at 4 °C for 15 min each with 1 ml of lysis buffer containing 150 mM NaCl and once at room temperature for 15 min with 1 ml of PBS. 25 μl of 3 × sample buffer (187.5 mM Tris-Cl (pH 6.8), 6% SDS, 30% glycerol, 0.003% bromphenol blue) was added to each of the final pellets, and 20 μl was loaded onto an SDS-PAGE gel. The gel was Coomassie Blue-stained, destained, treated with EN3HANCE (NEN), dried, and used for autoradiography with intensifying screens at ~80 °C.

Inositol Phosphate Production in COS-7 Cells—PLC activity was analyzed as production of inositol phosphates (26, 27). Twenty-four hours after transfection, the medium was replaced with 1 ml of inositol-free DMEM supplemented with 5% fetal bovine serum. Two hours later, the medium was again replaced with the same medium containing 2 μM of myo-[3H]inositol. After 15 min, 10 μl of 1 M LiCl was added to each well (the final LiCl concentration was 10 mM). No difference in the uptake/incorporation of myo-[3H]inositol was found in cells incubated with LiCl-containing medium for 1 h and 24 h. Forty-eight hours after transfection, the cells were washed with 1 ml of PBS and extracted twice for 30 min each with 500 μl of 20 mM formic acid. The extracts were combined and neutralized to pH 7.5 with a solution containing 7.5 mM HEPES and 150 mM KOH. The neutralized extracts were loaded onto 0.5 ml AG1-X8 (Bio-Rad) anion exchange columns. Prior to use, the columns were washed with 2 ml of 1 M NaOH and 2 ml of 1 M formic acid and equilibrated with H2O to neutrality. After the extracts were loaded onto the column, the columns were washed with 5 ml of H2O and 5 ml of 5 mM Borax and 60 mM sodium formate. The inositol phosphates were eluted with 3 μl of 0.9 M ammonium formate and 0.1 M formic acid. The eluent was counted in a γ-counter for 2 min.

Subcellular Fractionation—COS-7 cells cultured in 6-well plates were transfected and metabolically labeled with [35S]-Express Protein Labeling Mix as described above. The cells were washed with PBS and detached by incubation in 500 μl of Trypsin-EDTA solution (1×) (Sigma) at 37 °C for 1 min. After mixing with 2 ml of DMEM/5% fetal bovine serum, the cells were collected by centrifugation at 500 × g at 4 °C for 5 min. After being washed with 3 ml of a buffer identical to the lysis buffer used in immunoprecipitation but containing no Triton X-100, the cells were resuspended in 600 μl of the same buffer and went through freeze-and-thaw in ethanol/dry ice three times (3 min per period). The broken cells were then passed through a 23-gauge (or smaller) needle ten times to shear DNA and centrifuged at 100,000 × g in a Beckman SW55 rotor at 4 °C for 30 min. The supernatant was the soluble fraction. The pellet was resuspended in 600 μl of lysis buffer containing 1% Triton X-100 and incubated at 4 °C for 30 min. The supernatant, after a 5-min centrifugation at 15,000 × g was the particulate fraction. Both the soluble and the particulate fractions were later used in immunoprecipitation.

Western Blot Analysis—Forty-eight hours after transfection, cells in 6-well plates were washed with 2 ml of PBS and harvested in 1 ml of lysis buffer. The cells were lysed at 4 °C for 30 min. After a 10-min centrifugation at 15,000 × g, an aliquot (10 μl) of the supernatant was loaded on an SDS-PAGE mini-gel, and the resolved proteins were wet-electroblotted to a nitrocellulose membrane and probed with specific primary and peroxidase-conjugated secondary antibodies using a chemiluminescence kit according to the manufacturer’s instructions (NEN).

RESULTS

Design of PLC-β2 Plasmids—We constructed several mammalian expression plasmids encoding various fragments of
PLC-β2, as shown in Fig. 1. To further understand the mechanism of substrate hydrolysis of PLC-β2 and its regulation by G protein subunits, we used these constructs to determine whether the amino and carboxyl halves of PLC-β2 could associate when expressed as two polypeptides and, if they could, how the complexes thus formed would be regulated by Gβγ and Goα. These constructs were designed to allow us to test the role of the PH domain, to compare the activity and G protein regulation of enzyme with the linker region either attached to the C-terminal fragment or completely removed, and to compare the activity of reconstituted enzyme with and without the C-terminal domain required for activation by Gβγ and Goα. To all these fragments (except construct A), a FLAG or an HA epitope tag-encoding sequence was attached at one end (see Fig. 1). Construct A had both a FLAG tag at the N terminus and an HA tag at the C terminus to compare the results of immunoprecipitation through the FLAG and HA tags.

Assembly of PLC-β2 Fragments Expressed in COS-7 Cells—Antibodies directed against the epitope tags were used to (co)immunoprecipitate metabolically labeled PLC-β2 fragments that had been expressed in COS-7 cells. The representative autoradiograph in Fig. 2A shows that most of the fragments were robustly expressed. Only A and E fragments were expressed at significantly lower levels (about one-tenth to one-fifth) when compared with their corresponding PH domain-containing fragments (A’ fragment and wild-type, respectively).

PLC-β2 has an endogenous proteolytic site that cleaves off the C-terminal region necessary for Goα activation (10). The wild-type enzyme expressed in COS-7 cells was partially cleaved at this site giving rise to a fragment of about the same size as A’ fragment. The A’ fragment was further cleaved to a polypeptide of approximately the same size as the B’ fragment. Similarly, A and E fragments had shorter polypeptides of the same size as the B fragment, suggesting that there is another proteolytic site near the C terminus of the X region. The remaining proteolytic or background bands were not identified but represented only a small fraction of the total protein.

Lanes 8–11 of Fig. 2A show that the B’ fragment could bind C, C’, D, and D’ fragments (C’ and D’ fragments lacked the linker region, whereas C and D fragments included this region). The expression level of the B’ fragment was higher when the C-terminal fragments were coexpressed, suggesting that they stabilized the B’ fragment. Immunoprecipitation through the FLAG tag on the B’ fragment was able to coimmunoprecipitate the C, C’, D, and D’ fragments, indicating that the B’ fragment was able to form complexes with each. The B’ fragment coimmunoprecipitated approximately equal amounts of C and C’ fragments. The numbers of methionines in these fragments were: 15 in B’, 17 in C, 16 in C’, 27 in D, and 26 in D’. The D and D’ fragments were cleaved at or near the site described by Park et al. (10) to generate C and C’ fragments, which also bound to the B’ fragment. About 90% of D fragment and 75% of D’ fragment were cleaved. The implication of this cleavage for interpretation of activity measurements will be described below. The B’ fragment, which lacked the PH domain, could also coimmunoprecipitate a C-terminal fragment (C, C’, D, and D’ fragments), but the capacity was lower when compared with the B’ fragment (Fig. 2B). Therefore, removal of the PH domain reduced but did not block assembly. Immunoprecipitation and coimmunoprecipitation could also be performed through the HA epitope tag on A, C, C’, D, and D’ fragments, but the efficiency was lower. For this reason, we performed the coimmunoprecipitation in all our other experiments through the FLAG epitope tag.

We also examined whether the PLC-β2 fragments could form complexes after they had been synthesized. When the B’ fragment and C, C’, D, or D’ fragments were expressed in COS-7 cells separately and later mixed after cell lysis, none of the C, C’, D, nor D’ fragments were coimmunoprecipitated by the B’
Reassembly and Regulation of PLC-β₂

Fig. 3. Activity of PLC-β₂ fragments and reconstituted PLC-β₂ constructs. A, activity of single PLC-β₂ fragments. COS-7 cells in each duplicate well in 12-well plates were transfected with 625 ng/ml of each DNA. In all cases, vector DNA was added to give a total DNA concentration of 2.5 μg/ml. Black bars, no Gᵦ₁₋₂, shaded bars, in the presence of Gᵦ₁₋₂ WT, wild-type. A representative experiment analyzed in duplicate is shown. The error bars indicate the ranges of duplicate determinations. Each construct was tested at least three times. B, activity of two cotransfected PLC-β₂ fragments. Experimental conditions were identical to those in A of this figure except that the concentration of each PLC-β₂ DNA was 125 ng/ml. Wild-type PLC-β₂ and the A’ fragment were used as positive controls.

Fig. 4. Subcellular distribution of PLC-β₂ fragments. Lysates of COS-7 cells transfected with various PLC-β₂ constructs and metabolically labeled were resolved into soluble and particulate subcellular fractions by ultracentrifugation. PLC-β₂ fragments were immunoprecipitated from these fractions. S, soluble fraction; P, particulate fraction. The error bars indicate standard deviations of three independent experiments. See details under “Experimental Procedures.”

Roles of the PH Domain for the Enzymatic Activity and Subcellular Distribution of PLC-β₂ Constructs—We next tested the catalytic activity of the PLC-β₂ fragments measured as production of inositol phosphates. Full-length, wild-type PLC-β₂ was used as control. As shown previously in this laboratory (26, 27), inositol phosphate production increased when COS-7 cells were transfected with PLC-β₂ itself (Fig. 3A). Coexpression of Gᵦ₁₋₂ caused a pronounced rise in PLC activity. PLC-β₂ truncated at the C terminus (A’ fragment) had basal and Gᵦ₁₋₂-stimulated activity equal to the full-length enzyme. Even though the wild-type enzyme was substantially cleaved, if the activity of the wild-type enzyme was much higher than A’ fragment, the mixture should still show higher activity than the A’ fragment. These results were consistent with previous reports (9, 10, 28). However, when the PH domain was removed from the full-length or truncated enzyme (E fragment and A fragment, respectively), both were inactive. Fig. 3A also shows that individual fragments containing only one of the two catalytic regions (B, B’, C, C’, D, and D’) had no PLC activity whether or not the PH domain was present. Fig. 3B illustrates that basal and Gᵦ₁₋₂-stimulated PLC-β₂ activity could be reconstituted from two fragments each containing one of the catalytic domains only if the PH domain was present. The characteristics of the reconstituted activity will be discussed below. These results indicate that the PH domain was required for PLC-β₂ to hydrolyze its substrate in COS-7 cells.

Because the fragments and reconstituted complexes lacking the PH domain (i.e. E, A, and complexes formed with the B fragment) were expressed at levels significantly lower than those containing the PH domain (the wild-type enzyme, A’, and complexes formed with the B’ fragment) (Fig. 2), it was possible that their lower catalytic activity was simply a result of lower expression. To test this possibility, we compared the expression and PLC activity of B + C’ at a higher DNA dose (625 ng of DNA/ml at transfection) with those of B’ + C transfected with one-tenth of this DNA dose (62.5 ng/ml). We chose this pair, because, in contrast to the wild-type enzyme and the A’ fragment, at the lower DNA dose B’ + C’ were expressed well and had a basal activity substantially higher than the blank. Despite a higher expression level of the B fragment (due to higher DNA dosage) compared with the B’ fragment and a similar amount of immunoprecipitated C’, B + C’ showed no enzymatic activity. Similar results were also observed for other fragments (data not shown). Although this experiment did not completely exclude the possibility that the absence of PLC activity of E and A was in part due to low expression, our results indicated that removal of the PH domain abolished the function of PLC-β₂ in COS-7 cells.

Removal of the PH domain also altered the subcellular distribution of the expressed proteins (Fig. 4). When the PH domain was present (wild-type, A’, and B’ fragments), ~30% of the enzyme was found in the particulate fraction. Constructs lacking the PH domain (E, A, and B fragments) were found almost exclusively in the soluble fraction. Therefore, the accessibility to a membrane-associated substrate may account for the observed loss of PLC-β₂ activity in constructs lacking the PH domain. However, using these experimental approaches in transfected cells, we could not distinguish an intrinsic loss of catalytic activity in truncated fragments from effects secondary to alterations in subcellular localization.

Effects of Go₁ on Gᵦ₁₋₂ Activation of Wild Type and Reconstituted PLC-β₂—Gᵦ₁₋₂ needs to dissociate from Go₁ to interact with its effectors. Therefore, excess Go₁ should block the Gᵦ₁₋₂ activation of PLC-β₂ by scavenging free Gᵦ₁₋₂ to form heterotrimers (26, 27). This is an important control, because it shows that Gᵦ₁₋₂ is activating PLC-β₂ with characteristics expected for a heterotrimeric G protein. Fig. 5 shows that, although Go₁ itself did not exhibit any significant effect in any group, it
Reassembly and Regulation of PLC-β2

Our study is the first to reconstitute active enzymes with PLC-β fragments. In this study, we have characterized several constructs encoding various fragments of human PLC-β2. We reassembled PLC-β2 from enzyme fragments each containing one of the two catalytic regions (Fig. 2) and found that the PH domain was required for both enzymatic activity (Figs. 3) and membrane targeting of PLC-β2 (Fig. 4). These reassembled enzymes were still subject to regulation by G protein subunits (Figs. 5 and 6). We identified the X-Y linker region as an inhibitory element in the intact enzyme. However, changes at the linker region did not affect the regulation of PLC-β2 by G protein subunits (Figs. 3, 5, and 6).

The Roles of the PH Domain—We found that, although the targeting of PLC-β2 fragments lacking the PH domain to the membrane may be impaired (Fig. 4), these fragments were still assembled (Fig. 2). Moreover, the PH domain was essential for PLC-β2 to hydrolyze its substrates in COS-7 cells (Fig. 3). We could not distinguish whether removal of the PH domain in PLC-β2 prevented the enzyme from getting access to its substrates in the plasma membrane, caused a loss in enzymatic activity, or both. A PLC-δ construct in which the PH domain was replaced by glutathione S-transferase has full enzymatic activity (32), suggesting that the major role of the PH domain in PLC-δ1 is to ensure membrane localization. The PH domain of PLC-δ1 binds to the PIP2 polar headgroup with an affinity

![Image](http://www.jbc.org/)

**FIG. 5.** Regulation of PLC-β2 fragments by Gβγ2 and Gαq.

COS-7 cells in duplicate wells in 12-well plates were transfected with 125 ng/ml of each PLC-β2 DNA, but the concentrations of Gβγ2, Gγ2, and Gαq DNA were kept at 625 ng/ml each. In all cases, vector DNA was added to give a total DNA concentration of 2.5 μg/ml. A representative experiment out of four independent experiments analyzed in duplicate is shown. The error bars indicate the ranges of duplicate determinations.

**FIG. 6.** Activation of PLC-β2 fragments by Gαq. A, activation of PLC-β2 fragments containing the PH domain. COS-7 cells in duplicate wells in 12-well plates were transfected with 125 ng/ml of each PLC-β2 DNA. The concentration of Gαq DNA was 625 ng/ml. In all cases, vector DNA was added to give a total DNA concentration of 1.5 μg/ml. The counts of the blank group (vector DNA only) with or without Gαq were subtracted from those of the other groups under the same conditions. The error bars indicate standard deviations of three independent experiments. Black bars, no Gαq; shaded bars, in the presence of Gαq. B, absence of activation of PLC-β2 fragments lacking the PH domain. The error bars indicate the ranges of duplicate determinations in the same experiment, which was repeated twice with similar results.

(E, B, B’+C’, and B’+D’). These fragments or reconstituted proteins showed no increase in PLC activity, providing further evidence that removal of the PH domain abolished the function of PLC-β2 (Fig. 6B).
and specificity comparable to the native enzyme (20) and is proposed as the anchor localizing the enzyme to the plasma membrane in the "tether-and-fix" model based on the crystal structure of PLC-δ1 (22). The PH domain of PLC-γ1 binds to PIP₃ strongly and specifically and targets the enzyme to the membrane in response to growth factor stimulation (21). In contrast, PLC-β₁, PLC-β₂, and their PH domains bind to phospholipid membrane surfaces with lower affinities, and the binding is PIP₂ concentration-independent (14). We observed that PLC-β₂ fragments lacking the PH domain were not found in the particulate fraction, whereas constructs containing the PH domain were partially targeted to the particulate fraction (Fig. 4). Therefore, this domain is also involved in membrane targeting of PLC-β₂. Besides binding to some inositol phosphates, PH domains identified in some proteins bind to proteins containing WD-repeats (33). An example is the strong interaction between β-adrenergic receptor kinase and Gβγ (34). The isolated PH domain of PLC-β₂ binds to Gβγ with an affinity comparable to that of the full-length PLC-β₂ (14), but the significance of this interaction for the enzyme's regulation by Gβγ needs to be further tested.

The Roles of the Linker Region—The X and Y regions form the catalytic domain of PLC. In the present study, we demonstrated by communoprecipitation experiments that, when COS-7 cells were cotransfected with two plasmids each containing the DNA sequence encoding one of the two catalytic regions, the two in vivo coexpressed fragments associated tightly with each other (Fig. 2). In contrast, fragments that were separately expressed and then combined could not bind to each other, suggesting that the association occurs during translation.

The reassembled enzymes possessed catalytic activity similar to or higher than that of the wild-type PLC-β₂ (Figs. 3B and 5). The most dramatic elevation of the basal catalytic activity was found in the two combinations lacking the linker region, B' + C' and B' + D'. These results suggest that the linker region, when present in the intact enzyme, inhibits basal PLC-β₂ activity. When the linker region was attached to the C-terminal fragment containing the Y domain, but (in contrast to wild-type PLC-β₂) was not linked to the X domain, thereby allowing for more flexibility, the basal activity almost doubled (Figs. 3B and 5), compare the wild-type PLC-β₂ with B' + D'). However, the linker region may still interfere with PIP₂ hydrolysis, because complete removal of the linker region resulted in even greater increase in the basal activity.

The long C-terminal region was not essential for the basal or Gβγ-stimulated activity of PLC-β₂ (Fig. 3A). However, the highest basal activity of all was given by B' + D' that lacked the linker region but retained the long C-terminal domain. As was shown in Fig. 2, the C-terminal domain did not lead to the formation of more reassembled PLC-β₂. Therefore, we conclude that the presence of the C-terminal domain allows those complexes that do reassemble to acquire a more active conformation.

We found that even when the linker region was cleaved or completely removed, PLC-β₂ was activated by Gβγ₂, and this activation was completely blocked by Goq (Fig. 5). These PLC-β₂ fragments were also subject to activation by Goq. Consistent with previous findings (9, 10), activation by Goq was contingent upon the presence of the C-terminal region (Fig. 6). In addition, all PLC-β₂ fragments showed similar increment in PLC-β₂ activity upon activation by Gβγ₂ or Goq. Therefore, it is highly unlikely that the G protein α₁ and Gβγ subunits regulate PLC-β₂ by direct effects on the linker region.

Conclusions—Our experiments show that the PH domain is required for the basal as well as the Goq- and Gβγ-stimulated PLC-β₂ activity in a heterogeneous cell expression system. Like PLC-γ₁, functional PLC-β₂ can be reconstituted from two coexpressed enzyme fragments, each containing one of the two catalytic regions. The linker region is an inhibitory element in PLC-β₂, but cleavage or removal of the linker region does not affect the G protein-mediated regulation of PLC-β₂. Therefore, Gβγ and Goq appear to activate PLC-β₂ by mechanisms other than easing the inhibition of PLC-β₂ activity by the linker region, thereby providing evidence for a regulatory pathway for PLC-β₂-involving mechanisms distinct from other PLC isoforms.

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