Activation of Phospholipase C-ε by Heterotrimeric G Protein βγ-Subunits*

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PLC-ε was identified recently as a phosphoinositide-hydrolyzing phospholipase C (PLC) containing catalytic and regulatory domains (X, Y, and C2) common to all PLC isozymes as well as unique CDC25- and Ras-associating domains. Novel regulation of this PLC isozyme by the Ras oncoprotein and α-subunits (Gα12) of heterotrimeric G proteins was illustrated. Sequence analyses of PLC-ε revealed previously unrecognized PH and EF-hand domains in the amino terminus. The known interaction of Gβγ subunits with the PH domains of other proteins led us to examine the capacity of Gβγ to activate PLC-ε. Co-expression of Gβ1γ2 with PLC-ε in COS-7 cells resulted in marked stimulation of phospholipase C activity. Gβ2 and Gβ3, in combination with Gα11, Gα2, Gα3, or Gγ12 also activated PLC-ε to levels similar to those observed with Gβγ-containing dimers of these Gγ-subunits. Gββ2 in combination with the same Gγ-subunits was less active, and Gβγ-containing dimers were essentially inactive. Gβγ-promoted activation of PLC-ε was blocked by cotransfection with either of two Gβγ-interacting proteins, Gα11, or the carboxyl terminus of G protein receptor kinase 2. Pharmacological inhibition of PI3-kinase-γ had no effect on Gβ1γ2-promoted activation of PLC-ε. Similarly, activation of Ras in the action of Gβγ is unlikely, because a mutation in the second RA domain of PLC-ε that blocks Ras activation of PLC failed to alter the stimulatory activity of Gβ1γ2. Taken together, these results reveal the presence of additional functional domains in PLC-ε and add a new level of complexity in the regulation of this novel enzyme by heterotrimeric G proteins.

PLC-ε-catalyzed hydrolysis of polyphosphoinositides is a necessary target cell response in the physiological action of many hormones, neurotransmitters, growth factors, and other extracellular stimuli (1). Three classes of PLC isozymes have been considered historically to underlie these signaling responses (2). A panoply of seven transmembrane-spanning receptors activate isozymes of the PLC-β class through release of α-subunits of the Gq family of heterotrimeric G proteins (3–6). Certain PLC-β isozymes also are activated by Gβγ (7–9), which has been proposed largely to originate from activated Gi family G proteins. PLC-γ isozymes are activated by protein phosphorylation following from activation of tyrosine kinase receptors or from receptors linked to tyrosine kinases (10–12). Although there is evidence for Ca2+ - and transglutaminase II-promoted regulation of PLC-δ (13, 14), the importance of hormonal regulation of this PLC isozyme remains unclear (15).

The recent identification of PLC-ε revealed a fourth class of PLC isozymes (16–19). PLC-ε contains both a CDC25 homology domain at its amino terminus and a pair of RA domains at the carboxyl terminus, suggesting direct involvement of this PLC isozyme in signaling promoted by Ras superfamily GTPases. Indeed, GTPase-deficient, constitutively active, mutants of Ras promote increases in inositol phosphate accumulation in cells cotransfected with PLC-ε (17). Moreover, this PLC isozyme may function at a nexus between Ras GTPase signaling and signaling through heterotrimeric G proteins because Gα12, but not Gαq, also activates PLC-ε in cotransfection experiments (18).

We illustrate here an additional layer in the architecture of PLC-ε-mediated signaling. A previously unrecognized PH domain followed by an EF-hand domain exists in the amino terminus of PLC-ε. In light of previous observations that Gβγ interacts with PH domains in other signaling proteins, we speculated that Gβγ might act as an efficacious activator of PLC-ε and found that PLC-ε catalytic activity was stimulated by co-expression with various Gβγ dimers. This activity was not dependent on activation of PI3-kinase or on an intact RA domain. These results add further complexity to the signaling pathways that may converge on PLC-ε.

EXPERIMENTAL PROCEDURES

Materials—Rat PLC-ε (17), Ras, Gα12, and Gα13 (20), and Gβ and Gγ expression vectors (21) were as previously described. The expression vector for the carboxyl-terminal domain of GRK2 was generously provided by Dr. Robert J. Lefkowitz, Duke University. Wortmannin and LY294002 were purchased from Biomol (Plymouth Meeting, PA). Inositol-free DMEM was purchased from ICN Biomedicals (Costa Mesa, CA). All other reagents were from sources previously noted (22, 23).

Computational Tools—PLC-ε protein sequences were analyzed for structural relationships using the three-dimensional-PSSM method (24) as implemented on the ICRF Fold Recognition Server. Two-dimensional -PSSM analyses were performed using the following data

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‡ The abbreviations used are: PLC, phospholipase C; PH, pleckstrin homology; RA, Ras-binding domain; GRK, G protein receptor kinase; PI3-kinase, phosphoinositide 3-kinase; PSSM, position-specific scoring matrix; DMEM, Dulbecco’s modified Eagle’s medium; aa, amino acid(s); PDB, Protein Data Bank; GTPγS, guanosine 5’-3-O-(thio)triphosphate.

‡‡ Found on the Web at www.bmm.icnet.uk/∼3dpssm.
Identification of Pleckstrin Homology and EF-hand Domains within PLC-ε. PLC-ε contains the core catalytic domains (X, Y, and C2) common to all PLC isoforms (Fig. 1A) as well as CDC25 homology and RA domains unique to PLC-ε and specifying upstream and downstream interactions with Ras superfamily GTPases (16–18). To identify additional functional modules in PLC-ε, the three-dimensional-PSSM method of Kelley and colleagues (24) was applied to the polypeptide sequences that separate known structural domains within rat and human PLC-ε and PLC210, the PLC-ε orthologue of Caenorhabditis elegans (25). The top two predictions for structural motifs between the CDC25 and X domains of rat PLC-ε (aa 829–1373) and human PLC-ε (aa 832–1392) were the PH domain (PDB code 1mai) and third and fourth EF-hands of rat PLC-ε (PDB code 1qas) (26). The top prediction for the analogous region (aa 334–910) of PLC210 was the rat PLC-ε EF-hand domain; the PLC-ε PH domain match was tenth on the list (the other eight predictions were EF-hands of other proteins including tropolin-C, calmodulin, and aequorin). The scoring matrix method was repeated with the isolated NH2- and COOH-terminal halves of this region of the three PLC-ε isoforms. Significant three-dimensional-PSSM E-values were obtained for the PH domain assignment (rat PLC-ε, E = 0.01, 95% certainty; human PLC-ε, E = 0.01, 95% certainty; PLC210, E = 0.18; 80% certainty) and the EF-hand assignment (rat PLC-ε (aa 1001–1372), E = 0.01, 95% certainty; human PLC-ε (aa 1001–1392), E = 0.14, 80% certainty; PLC210 (aa 501–893), E = 0.01, 95% certainty). Multiple sequence alignments of the predicted PLC-ε/PLC210 PH and EF-hand domains are presented in Fig. 1, B

RESULTS

Identification of Pleckstrin Homology and EF-hand Domains within PLC-ε. PLC-ε contains the core catalytic domains (X, Y, and C2) common to all PLC isoforms. The term molten-helix refers to a highly mobile extension of the PH domain COOH-terminal α-helix (28) required for GRK2 binding to Gβγ (29). Conserved positions are denoted by black boxes. Predicted (based on PSI-Pred) α-helices are highlighted in red and predicted β-sheets in blue. C, multiple sequence alignment of the EF-hands of rat PLC-ε-1, rat and human PLC-ε, and C. elegans PLC210. Known boundaries of rat PLC-ε-1 α-helices (26) are overlined. The color scheme for amino acids is the same as described for panel B.
and C, respectively. These alignments include known boundaries of secondary structure elements (α-helices and β-strands) within the rat PLC-α1 PH and EF-hand-3/4 regions (26) as well as secondary structure predictions of the corresponding PLC-ε/PLC210 regions as derived from the PSI-Pred algorithm (27). Overall concordance was high between the predicted secondary structures and known structural elements within both domains. However, differences were observed in the predicted α-helical character within rat and human PLC-ε corresponding to β-strands β4 and β5 of the PLC-α1 PH domain (Fig. 1B) and in a short stretch of predicted β-strand within rat and human PLC-ε corresponding to α-helix E4′ of the fourth PLC-α1 EF-hand (Fig. 1C). The predicted loops between β-strand pairs β3/β4 and β5/β6 in the PLC-ε/PLC210 PH domains are longer than their counterparts within PLC-α1; in the case of the β3/β4 intervening region, additional secondary structure elements are predicted by the PSI-Pred algorithm. The intervening sequence between the PH and EF-hand domains is also much longer in PLC-ε/PLC210 proteins (e.g. 274 amino acids in human PLC-ε) than in other PLC isoforms (10 – 30 amino acids; Fig. 1A). This intervening polypeptide may fold to form additional EF-hands currently unidentifiable by primary sequence searches or structure threading algorithms, because all other PLC isoforms encode four EF-hand α-helical pairs carboxy-terminal to the PH domain.

An additional feature of the putative PH domain within PLC-ε/PLC210 proteins is a predicted extension of the carboxyl-terminal α-helix well beyond the α1 helix of PLC-α1 (aa 115–128; Fig. 1B). This α-helical extension is rich in arginine and lysine residues and thus reminiscent of the highly mobile, positively charged "molten helix" observed in the NMR structure of the human GRK2 PH domain (PDB code 1bah; Fig. 1B) and critical for GRK2 binding to G-protein βγ dimers (28, 29).

Activation of PLC-ε by Gβγ–PH domains serve as recognition motifs for phosphoinositides or proteins such as Gβγ. The presence of a PH domain in PLC-ε suggested that, in addition to regulation by Gα12 and Ras, PLC-ε also is regulated by Gβγ.

Fig. 2. Gβγ dimers stimulate PLC-ε. COS-7 cells were co-transfected with varying amounts of each indicated DNA (supplemented to 700 ng of total DNA with empty vector), and [3H]inositol phosphate accumulation was measured 36 h later as described under "Experimental Procedures." A, increasing amounts of PLC-ε DNA were transfected in combination with 200 ng of Gα12, Gα13, or Gβγ DNA. B, PLC-ε (300 ng) was co-transfected with 200 ng of Gβγ and 200 ng of Gα12 (Q229L) or Gα13 (Q228L). C, PLC-ε (300 ng) was transfected with 200 ng of Gβγ and 200 ng of the indicated Gγ isoform. Data shown are the mean ± S.D. for triplicate samples in one experiment. These results are representative of three or more experiments in each case.

Fig. 3. Inhibition of Gβγ-stimulated PLC-ε activity. A, PLC-ε (300 ng) was co-transfected with 200 ng of Gβγ or GαQ/L and either 200 ng of empty vector, GRK2 carboxyl-terminal domain (GRK2-ct), or Gα13. Dotted lines represent the inositol phosphate accumulation in the presence of either Gβγ or PLC-ε alone. B, cells were transfected with 300 ng-plasmid DNA encoding PLC-ε and 200 ng Gβγ or GαQ/L. Either 100 nM wortmannin or 20 μM LY294002 was added in combination with LiCl, and [3H]inositol phosphate accumulation was measured as described. Data shown are the mean ± S.D. for triplicate samples in one experiment. These results are representative of three or more experiments in each case.

4 M. R. Wing, D. P. Siderovski, and T. K. Harden, unpublished observations.
Therefore, COS-7 cells were transfected with DNA expression vectors encoding rat PLC-ε and various G protein subunits, and PLC activity was quantitated 24–36 h later by the addition of 10 mM LiCl to inhibit inositol phosphate phosphatase and thus allow accumulation of [3H]inositol phosphate products of PLC-catalyzed [3H]phosphoinositide hydrolysis. Transfection of COS-7 cells with PLC-ε alone resulted in negligible increases in inositol phosphate accumulation (Fig. 2A). However, cotransfection of PLC-ε with Gβ1γ2 produced marked increases in [3H]inositol phosphate accumulation to levels similar to those observed with Gα12 or Gα13 (Fig. 2A). Cotransfection of maximally effective concentrations of Gβ1γ2 and Gα12 (or Gα13) with PLC-ε produced no greater activity than with cotransfection of PLC-ε with each subunit alone (data not shown). Similarly, co-expression of Gβ1γ2 together with GTPase-deficient, constitutively active mutants of Gα12(Q229L) or Gα13(Q226L) resulted in activity similar to that observed with the GTPase-deficient mutant alone (Fig. 2B).

The capacity of other Gβ- and Gγ-subunits to activate PLC-ε was tested. Gβ5, in combination with Gγ13, Gγ26, or Gγ11, all activated PLC-ε (Fig. 2C). The stimulated activities observed with Gβ5 and Gβ6 in combination with Gγ13 were similar to activities observed with Gβ1-containing dimers (Fig. 2D). Gβ5γ2 (Fig. 2D) and Gβ5γγ13, Gβγ26, and Gβ6γ11 (data not shown) were clearly less active than Gβ1γ2. Moreover, Gβ5, which is an atypical Gβ exhibiting only 50% identity with Gβ1-Gα, was essentially inactive in combination with Gγ2 (Fig. 2D) or Gγ13, Gγ26, or Gγ11 (data not shown); this finding is consistent with the prevailing hypothesis that the physiologically relevant partners for Gβ5 are the GGL-domain-containing RGS proteins, rather than conventional Gγ-subunits (30).

Two separate experiments were employed to illustrate that free Gβγ is likely necessary for activation of PLC-ε. First, whereas Gα11 alone had no effect on PLC-ε activity (data not shown), coexpression of Gβ1γ2 with Gα11 resulted in loss of capacity of Gβ1γ2 to activate the enzyme (Fig. 3A). Similarly, the GRK2 carbon terminus, which was previously demonstrated to bind to Gβγ (31, 32), also reduced the capacity of Gβ1γ2 to activate PLC-ε upon coexpression in COS-7 cells (Fig. 3A). Activation of PI3-kinase-γ by Gβγ is not likely involved in activation of PLC-ε, because neither wortmannin nor LY294002, which are known inhibitors of PI3-kinase-γ (33, 34), inhibited Gβ1γ2-stimulated [3H]inositol phosphate accumulation (Fig. 3B).

Ras stimulation of PLC-ε is dependent on intact RA domains (17). Therefore, a PLC-ε construct with two point mutations (K2150E and K2152E) in the RA domain nearest the COOH terminus (17) was used to determine whether the effects of Gβγ on the enzymatic activity of PLC-ε are indirect and due to activation of Ras. Cotransfection of COS-7 cells with G12V-activated H-Ras and wild-type PLC-ε but not with mutant PLC-ε resulted in marked increases in inositol phosphate accumulation (Fig. 4, inset). In contrast, coexpression of Gβ1γ2 with either wild-type PLC-ε or PLC-ε with mutated RA domain resulted in similar levels of Gβγ-stimulated activity (Fig. 4). Thus, the action of Gβγ on PLC-ε is apparently a separate event from the activation of this isozyme by Ras.

**DISCUSSION**

Identification of a PH domain and EF-hand domain in rat, human, and *C. elegans* PLC-ε orthologues and demonstration of Gβγ-mediated activation of rat PLC-ε suggest an additional mechanism for the regulation of this PLC isozyme. The recently established dual activation of PLC-ε by Ras and by the G12 family of heterotrimeric G protein α-subunits now must be expanded to include potential input from all G protein-coupled receptors through release of Gβγ.

PLC-ε initially was reported as a PLC isozyme that possesses the TIM-barrel X and Y domains and C2 domains conserved in the PLC-β, -γ, and -δ isozyme families. However, PLC-ε is notably unique by virtue of a large amino-terminal region that contains a CDC25 homology domain and by a carboxyl-terminal region consisting largely of two RA domains. Our illustration here of a PH domain and EF-hand domain interspersed in the large amino terminus between the CDC25 homology domain and X domain adds an additional commonality in structure with the other PLC isozyme families.

Ras apparently activates PLC-ε, in part, by promoting its translocation to the plasma membrane (17). Consequently, it will be important to resolve whether Gβγ activates PLC-ε by recruitment of the isozyme to the plasma membrane, by direct stimulation of catalytic activity, or by both mechanisms. Activation of PLC-ε by Gβγ apparently is independent of PI3-kinase-γ or the Ras signaling pathways. Our working hypothesis is that activation by both Gβγ and Gα12/Gα13 is direct and requires independent domains in the enzyme. However, lack of additivity in activation by Gβ1γ2 and GTPase mutants of Gα12 and Gα13 may preclude involvement of interacting or mutually exclusive domains in the activation by Gβγ and Goα-subunits. Neither GTPγS-bound Ras nor Gβ1γ2-activated purified PLC-ε when mixed with [3H]PtdIns(4,5)P2-containing vesicles under conditions in which marked activation of PLC-β2 by Gβ1γ2 was observed (17). Our data illustrate robust Gβγ-promoted phosphoinositide hydrolysis by PLC-ε, but whether Gβγ modulates the catalytic activity of the PLC-ε CDC25 homology domain, which serves as a guanine nucleotide exchange factor for Ras GTPases (18, 19), also will be important to establish.

The presence of a PH domain in PLC-ε together with the well established interactions of Gβγ with PH domains of GRK2 and GRK3 (15, 35, 36), Bruton’s tyrosine kinase, IRS-1, and other proteins (37, 38) prompted the testing of Gβγ as a potential activator of PLC-ε. Although our studies revealed a marked stimulation of PLC-ε by Gβγ, they did not address the direct role of the PH domain in this activation. Isozymes of the PLC-β, PLC-γ, and PLC-δ families all contain PH domains (15). However, only PLC-β2 and PLC-β3 are robustly activated by Gβγ, whereas PLC-β1, PLC-β4, and members of the PLC-γ and PLC-δ families, all of which possess PH domains, are not. That the PH domain is involved in activation of PLC-β2 by Gβγ is suggested by the observation that a chimeric PLC-δ containing the PH domain of PLC-β2 is activated by Gβγ (35). Similarly,
sequence in PLC-β3 carboxyl-terminal to the PH domain was shown by Barr et al. (36) to be important for interaction with and activation by Gβγ. This region exhibits similarity to the basic residues in the extended PH domain of GRK2, i.e. the molten helix, involved in interaction with Gβγ (28, 29). Other studies have illustrated the importance of sequence in the Y-domain of PLC-β2 in Gβγ-promoted activation (39), and therefore the precise structural determinants for activation of PLC-β isoforms by Gβγ remain to be resolved.

In summary, observation of activation of PLC-ε by Gβγ extends the possibilities for activation of this unique inositol lipid-hydrolyzing effector protein to a broad range of heptahelical receptors for extracellular stimuli. This ubiquitously expressed multifunctional protein may provide a major point of integration between heterotrimeric G protein and Ras GTPase signaling pathways.

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