RhoA Activates Purified Phospholipase C-ε by a Guanine Nucleotide-dependent Mechanism*

Phospholipase C-ε (PLC-ε) is a recently identified PLC isozyme activated by subunits of heterotrimeric G proteins (Gα12, Gα13, and Gβγ) as well as by the low molecular weight GTPases, Rho and Rac. To define the enzymatic activity and substrate specificity of PLC-ε as well as its potential direct activation by Rho family GTPases, a major fragment of PLC-ε encompassing the catalytic core (EF-hand repeats through the tandem Ras-associating domains; −118 kDa) was purified to near homogeneity and assayed after reconstitution under various conditions. Similar to the enzymatic profiles of previously purified PLC-β isozymes, the purified fragment of PLC-ε maximally hydrolyzed phosphatidylinositol 4-phosphate at a rate of −10 μmol/mg of protein/min, exhibited phospholipase activity dependent on the concentration of free calcium, and favored phosphatidylinositol 4,5-bisphosphate as substrate relative to other phosphoinositides. Furthermore, in mixed detergent phospholipid micelles, RhoA stimulated the phospholipase activity of the PLC-ε fragment in both a concentration-dependent and guanosine 5′-O-(3-thiotriphosphate)-dependent manner. This activation was abolished by the deletion of a unique ~65 amino acid-insert within the catalytic core of PLC-ε. Although Rac1 activated purified PLC-β2 in a guanine nucleotide-dependent manner, Rac1 failed to promote guanine nucleotide-dependent activation of purified PLC-ε. These results indicate that PLC-ε is a direct downstream effector for RhoA and that RhoA-dependent activation of PLC-ε depends on a unique insert within the catalytic core of the phospholipase.

Phospholipase C (PLC) isozymes respond to a variety of extracellular signaling molecules to stimulate hydrolysis of PtdIns(4,5)P2 into the second messengers, inositol1,4,5-trisphosphate and diacylglycerol (1). The generation of these two second messengers triggers the release of intracellular Ca2+ and stimulates protein kinase C isozymes, respectively. Five different isoforms of PLC have been identified, each with distinct mechanisms of activation. The PLC-β isozymes are directly activated by heterotrimeric G protein α subunits of the Gα family (2–4) and by Gβγ subunits (5–7). More recently, these isoforms were shown to be activated by the small GTPase Rac (8–11). Activation of receptor and cytosolic tyrosine kinases promotes activation of PLC-γ via phosphorylation and translocation (12–14). The mechanisms by which extracellular stimuli regulate PLC-δ (15, 16) and the recently identified PLC-ζ (17, 18) are less understood, although both are believed to require the mobilization of Ca2+.

The fifth family member of PLC isoforms, PLC-ε, initially was identified in Caenorhabditis elegans as a Ras-binding protein (19). Mammalian PLC-ε subsequently was identified as a phospholipase C isoform containing multiple Ras interaction domains (20–22) including a CDC25 homology domain in the amino terminus and tandem Ras-associating (RA) domains at the carboxyl terminus. Coexpression of GTPase-deficient, constitutively active Ras mutants with PLC-ε results in accumulation of inositol phosphates in COS-7 cells (22). Transient coexpression with Gα12, Gα13 (21, 23), or Gβγ (23) also promotes phospholipase activity of PLC-ε. More recently, a screen of Rho family GTPases revealed a marked activation of PLC-ε when coexpressed with RhoA, RhoB, or RhoC but not with Rac or Cdc42 (24). This activation depended on a unique ~65 residue-insert within the Y box of the catalytic TIM barrel of PLC-ε, and co-precipitation studies indicated an interaction between PLC-ε and RhoA. Although these results suggest that PLC-ε is a direct, downstream effector of Rho GTPases, other interpretations are also consistent with the data.

To provide incontrovertible evidence for direct activation of PLC-ε by GTP-bound Rho GTPases, we overexpressed and purified a large fragment of the phospholipase (PLC-ε EF-RA2; ~118 kDa) and present here the enzymatic characterization of this fragment reconstituted with phospholipids and GTPases. Relative to previously purified PLC isoforms, purified PLC-ε EF-RA2 exhibited similar specific phospholipase activity and phosphoinositide substrate preferences. Moreover, our analyses illustrate direct, guanine nucleotide-dependent activation of PLC-ε by RhoA and further implicate the catalytic core of PLC-ε as the effector region necessary for RhoA-stimulated phospholipase activity.

**EXPERIMENTAL PROCEDURES**

Materials—The open reading frame of rat PLC-ε was a kind gift from Grant Kelley (State University of New York, Syracuse, NY). A PLC-ε-specific antibody was generated by BIOSOURCE against a predicted exposed sequence (amino acids 1523–1540; LKAHQL) directly C-terminal to the X box of the catalytic core. 3H-Labeled phosphoinositide substrates were prepared from [3H]inositol-
PLC-β domain, and tandem RA domains. Human PLC-β2 was amplified from full-length PLC-β2 PH-C2 and PLC-β2 PH-C2 was a fragment of full-length PLC-β2 that encompasses amino acid residues 1–801 and was used to transfect 20 μg of each PLC-ε vector construct per dish according to the manufacturer's protocol. The overexpressed recombinant PLC isozymes were purified using anti-FLAG M2 affinity gel according to the manufacturer's protocol (Sigma). Briefly, ~36 h after transfection, growth medium was removed from the cells, and the dishes were rinsed with phosphate-buffered saline (10 mM phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4). After aspiration of the phosphate-buffered saline, 1 ml of lysis buffer was added per dish, and the dishes were incubated for 15 min with shaking. Cells were scraped from dishes, and the lysate was centrifuged at 12,000 × g for 10 min. The resultant supernatant was subjected to a single gravity flow passage over a 1-ml bed volume of anti-FLAG M2 affinity gel and washed with 10 bed volumes of Tris-buffered saline (50 mM Tris, pH 7.5, 150 mM NaCl) to remove unbound proteins. Recombinant protein was then collected in six 500-μl elutions with 100 μg/ml FLAG peptide in Tris-buffered saline.

Purification of Small GTPases—The coding sequences of human monomeric GTPases were amplified by PCR and ligated into BamHI/XhoI-digested pFastBacHT, Baculovirus encoding the Rho family GTPases was produced using the Bac-to-Bac system. Recombinant proteins were expressed in HighFive insect cells at a density of ~2.0 × 10^6 cells/ml and loaded onto a 5-ml HighTrap metal cholate column (Amersham Biosciences) charged with Ni^2+. Purification steps included a 10-column volume buffer A wash and a 10-column volume 3% buffer B wash (buffer A + 1 mM imidazole). Recombinant protein was eluted with 10 column volumes of 40% buffer B collected in 5-ml fractions. Fractions containing the recombinant protein were dialyzed overnight against buffer A in the presence of His<sub>6</sub>-tagged tobacco etch virus protease to remove the His<sub>6</sub> tag from PLC-ε EF-RA2. The cleaved protein was subjected to a second passage over a Ni<sup>2+</sup>-charged metal cholate column and eluted with 5 mM imidazole as a final purification step. Fractions containing the purified protein were pooled and concentrated using a 50,000 MWCO PES centrifugal filtering device (Vivascience). The final concentration of the recombinant protein was determined by comparing a Bradford assay to bovine serum albumin standards. Purification of avian PLC-β was described previously (26). PLC-β2 PH-C2 is a fragment of full-length human PLC-β2 that encompasses amino acid residues 1–801 and was purified as described previously (11).

Purification of FLAG-tagged PLC-ε Constructs—Construction of mammalian expression vectors for PLC-ε EF-Ct and PLC-ε EF-CAY was described previously (24). PLC-ε EF-Ct spans amino acids 1198–2281, and PLC-ε EF-CAY spans amino acids 1198–2281 but lacks 62 amino acids (residues 1667–1728) that occur as an ~70-amino acid insert that is conserved in all orthologues of PLC-ε. COS-7 cells were seeded on 150-mm dishes at a density of 5.0 × 10<sup>5</sup> cells per dish and maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C in an atmosphere of 90% air, 10% CO<sub>2</sub>. Sixty microliters of FuGENE 6 transfection reagent (Roche Applied Science) was used to transfect 20 μg of each PLC-ε vector construct per dish according to the manufacturer's protocol. The overexpressed recombinant PLC isozymes were purified using anti-FLAG M2 affinity gel according to the manufacturer's protocol (Sigma). Briefly, ~36 h after transfection, growth medium was removed from the cells, and the dishes were rinsed with phosphate-buffered saline (10 mM phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4). After aspiration of the phosphate-buffered saline, 1 ml of lysis buffer was added per dish, and the dishes were incubated for 15 min with shaking. Cells were scraped from dishes, and the lysate was centrifuged at 12,000 × g for 10 min. The resultant supernatant was subjected to a single gravity flow passage over a 1-ml bed volume of anti-FLAG M2 affinity gel and washed with 10 bed volumes of Tris-buffered saline (50 mM Tris, pH 7.5, 150 mM NaCl) to remove unbound proteins. Recombinant protein was then collected in six 500-μl elutions with 100 μg/ml FLAG peptide in Tris-buffered saline.

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phospholipid vesicles combined with purified GTPases in cholate-containing buffers prior to the addition of PLC-ε. Assays were performed interchangeably using either [3H]PtdIns(4)P or [3H]PtdIns(4,5)P2 as substrate as indicated in the figure legends.

Maximum enzymatic activity was determined as previously described (4) with minor modification. Briefly, PLC isozymes were incubated in final buffer conditions including 10 mM HEPES, pH 7.4, 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 5.8 mM MgSO4, 0.5% cholate, 100 μM GDP or 10 μM GTP-S, 0.16 mg/ml fatty acid-free bovine serum albumin, 100 μM PtdIns(4)P (Avanti Polar Lipids), and ~10,000 cpm/assay [3H]PtdIns(4,5)P2 in a final volume of 60 μL. Assays were at 30 °C for 10 min and were terminated by the addition of 200 μL of 10% trichloroacetic acid and 100 μL of 10 mg/ml fatty acid-free bovine serum albumin. Centrifugation of the reaction mixture separated aqueous [3H]Ins(1,4,5)P3 from precipitated [3H]PtdIns(4)P, and soluble [3H]Ins(1,4,5)P3 was quantified by liquid scintillation counting.

Most experiments to determine enzyme characteristics and potential regulation by small GTPases utilized phosphatidylethanolamine (PE)- and PtdIns(4,5)P2-containing lipid vesicles in assays that were previously described (27) with minor modifications. Briefly, recombinant proteins were presented in lipid vesicles containing 50 μM PtdIns(4,5)P2, 500 μM PE (Avanti Polar Lipids), and ~10,000 cpm of [3H]PtdIns(4,5)P2 per assay. Final buffer conditions were 20 mM HEPES, pH 7.4, 70 mM KCl, 3 mM EGTA, 2 mM dithiothreitol, 0.16 mg/ml fatty acid-free bovine serum albumin, and 1 μM free Ca2+ in a final volume of 60 μL. The concentration of free calcium was calculated using WinMAXC software (Stanford University). All reactions were performed at 25 °C for 10 min after the addition of the indicated PLC enzyme. Experiments designed to determine the substrate selectivity of PLC-ε EF-RA2 utilized 1 nmol each of PtdIns, PtdIns(4)P, and PtdIns(4,5)P2, along with 100 μM GDP or 10 μM GTP-S under assay conditions for 20 min at 25 °C prior to the addition of PLC. Assays were initiated by the addition of PLC enzyme and incubated for 10 min at 25 °C. Reactions were terminated by the addition of 200 μL of 10% trichloroacetic acid and 100 μL of 10 mg/ml fatty acid-free bovine serum albumin. [3H]Insitol(1,4,5)P3 triphosphate was quantified by liquid scintillation counting of the soluble fraction after centrifugation of the reaction mixture.

RESULTS

Purification of PLC-ε EF-RA2—A fragment of PLC-ε (Fig. 1A) encompassing approximately half the EF-hand repeats through the second RA domain (amino acids 1258–2215) was purified after overexpression from a recombinant baculovirus in HighFive insect cells as described under “Experimental Procedures.” The soluble fraction of the enzyme bound efficiently to a metal chelate column as a first purification step. After tobacco etch virus protease cleavage of the affinity tag, a second passage over a metal chelate column yielded a nearly homogeneous preparation of the recombinant protein (Fig. 1B). The purified PLC-ε fragment migrated as a species of ~118 kDa on SDS-PAGE as seen by Coomassie staining and immunoblot analysis with a PLC-ε-specific antibody. Contaminating proteins observed by Coomassie staining were not immunoreactive, suggesting that the impurities were not breakdown products of the PLC-ε fragment. The typical yield of purified PLC-ε was 0.5–1.0 mg/liter baculovirus-infected insect cells. Multiple attempts to purify full-length PLC-ε after insect cell expression have yielded insufficient amounts of purified protein.

PLC Activity of PLC-ε EF-RA2—The purified PLC-ε fragment exhibited phospholipase C activity in assays utilizing a detergent and lipid mixture containing 50 μM [3H]PtdIns(4)P as substrate (Fig. 1C). Enzymatic activity depended on the
were performed utilizing PE- and PtdIns(4,5)P2-containing vesicles either in the absence of detergent when examining PLC-ε activity alone or in the presence of 0.05% sodium cholate when reconstituting PLC-ε with vesicles containing purified small GTPases. Phosphoinositide hydrolysis was linear for at least 10 min under the conditions of these assays (Fig. 2A).

Similar to other PLC isozymes, enzymatic activity of PLC-ε EF-RA2 depended on the concentration of free calcium with an EC50 value of ~65 ± 11 nM (n = 3), and maximum activity was observed at a concentration of ~1 μM free Ca2+ (Fig. 2B).

Kinetic analyses of PLC-ε EF-RA2 were performed utilizing PE- and PtdIns(4,5)P2-containing vesicles presented at various concentrations in the bulk medium (see "Experimental Procedures"). These experiments and Hofstee analyses revealed a Vmax of ~6.5 μmol/min/mg of protein and a Km value for PtdIns(4,5)P2 of ~6.0 μM (n = 3) (Fig. 2C). The phospholipid substrate selectivity of PLC-ε EF-RA2 was determined using PE- and PtdIns-containing vesicles composed of an equal ratio of PtdIns:PE (1:1). The recombinant PLC-ε fragment hydrolyzed phosphoinositides with a rank order of selectivity of PtdIns(4,5)P2 > PtdIns(4)P > PtdIns (Fig. 2D).

**Activation of Recombinant PLC-ε by RhoA**—Previous studies indicate that the Rho family members, RhoA, RhoB, and RhoC, induce stimulation of phospholipase activity when transiently coexpressed with PLC-ε in COS-7 cells (24). To determine whether the stimulation of PLC-ε by Rho family GTPases is direct, RhoA was expressed from a recombinant baculovirus in insect cells, purified to near homogeneity from detergent-extracted membranes, and reconstituted in substrate-containing phospholipid vesicles. As illustrated in the time course assay in Fig. 2A, recombinant RhoA (300 nM) stimulated phospholipase activity of PLC-ε by 2.5-fold in the presence of GTPγS but had no effect in the presence of GDP. To further assess this phenomenon, RhoA was reconstituted over a wide range of concentrations in mixed detergent phospholipid micelles, and PtdIns(4,5)P2 hydrolysis was quantified in the presence of the purified PLC-ε fragment (Fig. 3). The amount of recombinant PLC-ε used (~2 ng) for reconstitution with the GTPases was adjusted to achieve a basal activity of 5–10 pmol/min. RhoA caused a 2–3-fold stimulation of PLC-ε in all experiments. Stimulation was GTPγS-dependent, and half-maximal activation was observed at a concentration of RhoA of 58 ± 18 nM (n = 4). The calcium dependence for activation of PLC-ε was not changed in the presence of GTPγS-activated RhoA (Fig. 2B). GTPγS-dependent activation of PLC-ε was not observed with non-lipidated RhoA purified from bacteria, suggesting that C-terminal isoprenylation of RhoA may be required for activation (data not shown).

The selectivity for activation of PLC-ε by Rho family GTPases was examined by assessing the potential capacity of purified Rac1 to activate PLC-ε following purification of the lipidated form of the GTPase from baculovirus-infected insect cells. At relatively high concentrations (300 nM), Rac1 caused an increase in PLC-ε activity. However, no guanine nucleotide-dependence was observed (Fig. 4). Lower concentrations of Rac1 also had no effect on PLC-ε in the absence or presence of GTPγS (data not shown), and the increased activity observed at higher GTPase concentrations may result from contaminating proteins that accompany the purification. Although Rac1 had no effect on PLC-ε activity, this Rho family GTPase caused an ~2-fold GTPγS-dependent stimulation of PLC-β2 PH-C2 (Fig. 4), which is consistent with previously published data (8, 10, 11).

**Activity of the Unique Y Box Insert of PLC-ε**—Previous transient expression studies in COS-7 cells indicate that a unique amino acid insert within the Y box of the catalytic TIM barrel of PLC-ε present in other PLC isozymes is required for activation of PLC-ε by Rho, Gaqα, and Gαq (24). To further determine the importance of the Y region insert in PLC-ε, two FLAG-tagged fragments of PLC-ε were purified after overex-
pression in COS-7 cells (Fig. 5, inset). Both constructs of PLC-ε spanned the EF-hand repeats through the C terminus with one fragment lacking 62 amino acids within the Y box (Fig. 5A).

Analyses of the purified PLC-ε fragments in the absence of RhoA indicated that removal of the insert within the Y box decreased basal enzymatic activity of the enzyme ~20-fold. Therefore, when reconstituted with RhoA-containing vesicles, the added amount of purified PLC-ε fragment was normalized to give a basal lipase activity of ~5 pmol/min with each construct. Under these conditions, 300 nM purified RhoA caused an ~2-fold stimulation of the PLC-ε EF-Ct fragment, and this activation was entirely GTPγS-dependent. In contrast, no GTPγS-dependent activation by RhoA was observed with the PLC-ε fragment lacking the 62-amino acid Y box insert (Fig. 5B). These data further support in vivo data suggesting that the Y insert region is necessary for RhoA-dependent activation of PLC-ε.

**DISCUSSION**

The recent identification of PLC-ε as a downstream effector of Ras and Rho adds a new level of complexity to phospholipid signaling involving monomeric GTPases. In this study, we illustrate the direct, GTP-dependent stimulation of PLC-ε in phospholipid vesicles reconstituted with purified RhoA. These data also extend previous in vivo studies in which phosphoinositide hydrolysis by PLC-ε promoted by Rho depended on the presence of a unique ~65-amino-acid sequence within the Y box of the catalytic TIM barrel of PLC-ε. PLC-ε lacking this region retained activation by Gβγ or H-Ras in coexpression experiments in COS-7 cells; however, Rho-, Go12-, and Go13-dependent activation was not observed (24).

A subfamily of RhoGEFs (i.e. p115RhoGEF, LARG, PDZ-RhoGEF) activated by Go12 and Go13 provides a direct link between stimulation of G-protein-coupled receptors and the activation of Rho GTPases (30–33). Because deletion of the unique insert within the Y box of PLC-ε causes loss of responsiveness to Rho, Go12, and Go13, we hypothesize that activation of PLC-ε by Go12 and Go13 occurs indirectly by promoting Rho activation. This is supported by the fact that C3 toxin, which ADP-ribosylates and inactivates Rho, prevented activation of PLC-ε by Rho, Go12, or Go13 and prevented agonist activation of a lysophosphatidic acid receptor but did not inhibit the activation of PLC-β isozymes by Go6. Direct activation of PLC-ε by Rho does not rule out the possibility of direct activation of PLC-ε by Go12 or Go13. However, our combined in vivo and in vitro data suggest that Go12 and Go13 mostly likely activate PLC-ε indirectly via a subfamily of RhoGEFs typified by p115RhoGEF.

Although the complete set of determinants within PLC-ε required for interaction with Rho have yet to be identified, our current results combined with previous in vivo data (24) have unambiguously identified a region within the catalytic core of PLC-ε that is necessary for the direct stimulation of the phospholipase activity of PLC-ε by GTP-bound Rho. Sequence analyses of the catalytic regions of PLC-ε have not revealed obvious regions of homology to other known Rho-binding domains (34).

In a similar vein, the PH domain of PLC-β2 binds GTP-activated Rac (11). However, the current experiments directly illustrate that the PH domain of PLC-ε is not required for acti-

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2 M. Hains, M. Wing, and T. K. Harden, unpublished data.
activation by RhoA. These results suggest that eventual structural determination of the interface will define a novel mode of effector modulation by Rho GTPases.

The mechanisms whereby Rho family GTPases activate PLC isozymes remain a point of conjecture. Our unpublished studies suggest that prenylation of RhoA is required for stimulation of PLC-ε because soluble bacterial RhoA failed to activate PLC-ε. GTP-dependent binding of the lipidated GTPase to PLC isozymes may promote phospholipase activity by causing translocation of the lipase to the phospholipid bilayer. However, a more active role of GTPases may involve conformational changes in PLC isozymes that enhance phospholipase activity. Perhaps the strongest case for GTPase-stimulated phospholipase activity occurs with the Rho-promoted activation of PLC-ε studied here, whereby binding of Rho within the catalytic core of PLC-ε is readily envisioned to induce conformational changes favorable for lipase activity. Cooperative mechanisms of Rho-dependent activation of PLC-ε are also possible, whereby Rho induces the lipase activity of PLC-ε through both spatial and conformational regulation of the enzyme.

The addition of PLC-ε as a direct effector in Rho-dependent signaling expands the role of Ras superfamily proteins in phospholipid signaling and metabolism. Historically, Rho family GTPases have been studied as regulators of actin dynamics, gene transcription, cell cycle progression, and cell adhesion (34). More recently, Rho has been shown to increase PtdIns(4,5)P₂ levels in cells through activation of phosphatidylinositol 4-phosphate 5-kinase (35, 36) and to negatively regulate diacylglycerol kinase activity (37). Rho may potentiate inositol lipid signaling by increasing PtdIns(4,5)P₂ substrate levels and increasing PKC activity by inhibition of phosphorylation of diacylglycerol. PLC-ε potentially is involved in several signal transduction pathways involving multiple Ras family GTPases. The data reported here show PLC-ε is an effector for GTP-bound RhoA, and future experiments will more completely define the binding regions within PLC-ε and the physiological role played by PLC-ε-dependent pathways in Rho-activated signaling.

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RhoA Activates Purified Phospholipase C-δ by a Guanine Nucleotide-dependent Mechanism
Jason P. Seifert, Michele R. Wing, Jason T. Snyder, Svetlana Gershburg, John Sondek and T. Kendall Harden

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