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

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Phospholipase C-ϵ (PLC-ϵ) is a recently identified PLC isoform activated by subunits of heterotrimeric G proteins ($G\alpha_{12}$, $G\alpha_{13}$, and $G\beta\gamma$) as well as by the low molecular weight GTPases, Rho and Ras. 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)[1] isozymes respond to a variety of extracellular signaling molecules to stimulate hydrolysis of PtdIns(4,5)P_2 into the second messengers, inositol(1,4,5) trisphosphate and diacylglycerol (1). The generation of these two second messengers triggers the release of intracellular Ca^{2+} 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 G_q family (2–4) and by Gβγ subunits (5–7). More recently, these isozymes 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 Ca^{2+}.

The fifth family member of PLC isozymes, PLC-ϵ, initially was identified in *Caenorhabditis elegans* as a Ras-binding protein (19). Mammalian PLC-ϵ subsequently was identified as a phospholipase C isozyme 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 $G\alpha_{12}$, $G\alpha_{13}$ (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 isozymes, 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; LKAHQTPVDILKQKAHQL) directly C-terminal to the X box of the catalytic core. ^{3}H-Labeled phosphoinositide substrates were prepared from [^{3}H]inositol-

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[1] The abbreviations used are: PLC, phospholipase C; RA, Ras-associating; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; PH, pleckstrin homology; GEF, guanine nucleotide exchange factor; EF, elongation factor; TIM, triose-phosphate isomerase.

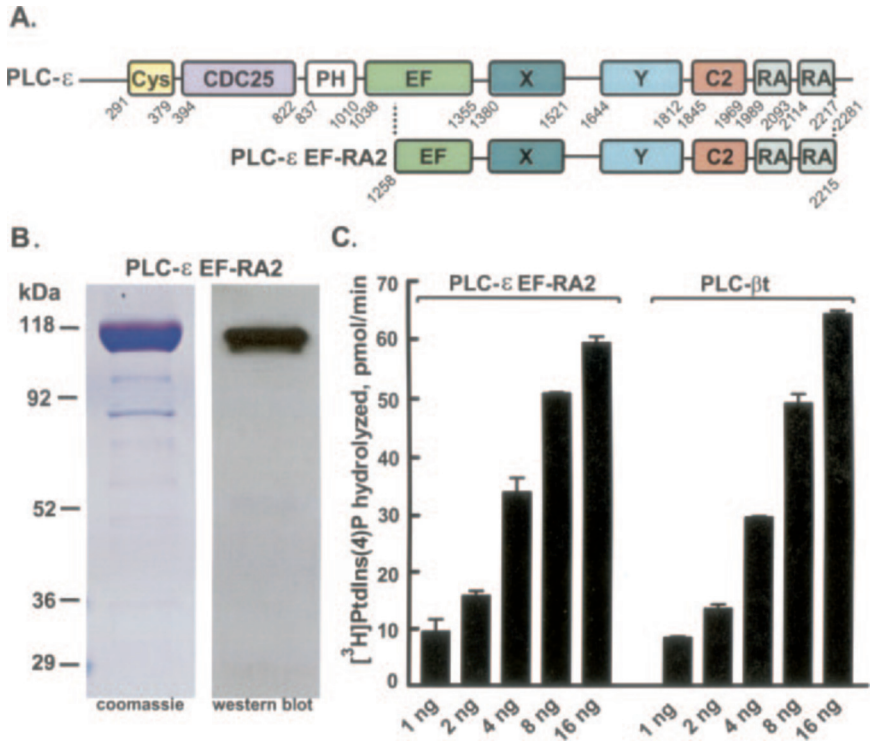

FIG. 1. **Purification and functional activity of PLC-ϵ EF-RA2.** *A*, schematic representation of full-length PLC-ϵ (2281 total amino acids, *top*) and PLC-ϵ EF-RA2 (residues 1258–2215, *bottom*). PLC-ϵ EF-RA2 lacks the amino-terminal cysteine-rich domain (*Cys*), CDC25 domain, and PH domain but retains approximately half the EF-hand repeats as well as the catalytic TIM barrel (*X* and *Y regions*), C2 domain, and tandem RA domains. *B*, PLC-ϵ EF-RA2 was purified as described under "Experimental Procedures," and final samples of recombinant protein were analyzed by SDS-PAGE using a 4–15% polyacrylamide gradient. Gels were either Coomassie-stained (*left*) or transferred to nitrocellulose and immunoblotted with an antibody specific to the X-Y linker region of PLC-ϵ (*right*). *C*, the specific activity of the purified PLC-ϵ fragment was measured using [^{3}H]PtdIns(4)P as substrate in the presence of 0.5% sodium cholate for 10 min at 30 °C (*left*) and compared with the specific activity of purified avian PLC-βt (*right*).

labeled turkey erythrocytes as described previously (4). His_6-tagged tobacco etch virus protease was purified as described previously (25).

Purification of PLC-ϵ EF-RA2—A fragment of PLC-ϵ encompassing approximately one-half of the EF-hand repeats through the second RA domain (amino acids 1258–2215) was amplified from full-length PLC-ϵ by PCR and subcloned into NcoI/XhoI-digested pFastBacHT, which incorporates a His_6 tag and a tobacco etch virus protease site at the amino terminus of the expressed enzyme. Baculovirus encoding PLC-ϵ EF-RA2 was generated using the Bac-to-Bac method (Invitrogen). HighFive insect cells at a density of ~2.0×10^6 cells/ml were infected with virus encoding PLC-ϵ EF-RA2 at a multiplicity of infection of 1.0. After 48 h of incubation at 27 °C, cells were harvested by low speed centrifugation and resuspended in 100 ml of buffer A (20 mM HEPES, pH 8.0, 300 mM NaCl, 1 mM $CaCl_2$, and 10% glycerol) with two EDTA-free complete protease inhibitor tablets (Roche Applied Science). Cells were lysed using an EmulsiFlex C5 cell homogenizer (Avestin), and the lysate was centrifuged at 100,000 × *g* for 45 min. The clarified supernatant was diluted to a final volume of 150 ml and loaded onto a 5-ml HighTrap metal chelate 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 M 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_6-tagged tobacco etch virus protease to remove the His_6 tag from PLC-ϵ EF-RA2. The cleaved protein was subjected to a second passage over a Ni^{2+}-charged metal chelate 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-βt 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-CΔY was described previously (24). PLC-ϵ EF-Ct spans amino acids 1198–2281, and PLC-ϵ EF-CΔY 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 5.0×10^6 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_2. 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.

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 after infection with baculovirus at a multiplicity of infection of 1.0. The cells were harvested after 48 h at 27 °C by low speed centrifugation and resuspended in 50 ml of buffer A (20 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 5% glycerol) with one EDTA-free complete protease inhibitor tablet. Membranes of virus-infected cells were harvested by high speed centrifugation at 100,000 × *g* of the cell lysate for 1 h. Lipidated, membrane-bound GTPases were solubilized by detergent extraction of the harvested membranes in buffer A containing 1% purified sodium cholate and one EDTA-free complete protease inhibitor tablet for 1 h at 4 °C. Solubilized membrane proteins were recovered by centrifugation of the extracted membranes at 100,000 × *g* for 1 h. The clarified supernatant was diluted with buffer A to achieve a final detergent concentration of 0.3% and was subsequently loaded onto a HighTrap metal chelate column charged with Ni^{2+}. Recombinant protein was collected over an imidazole gradient running from 0 to 1 M over 20 column volumes. Fractions containing the recombinant GTPase were pooled and concentrated using 10,000 MWCO PES centrifugal filtering device. The concentrations of the individual GTPases were determined by quantifying the binding of [^{35}S]GTPγS under the same buffer conditions used for reconstitution of the GTPases in phospholipid vesicles described below.

Phospholipase C Assay—Two different procedures were utilized for measuring phospholipase activity of PLC enzymes. Initial experiments measuring maximum enzymatic activity were carried out in the presence of a detergent substrate mixture. The second assay employed

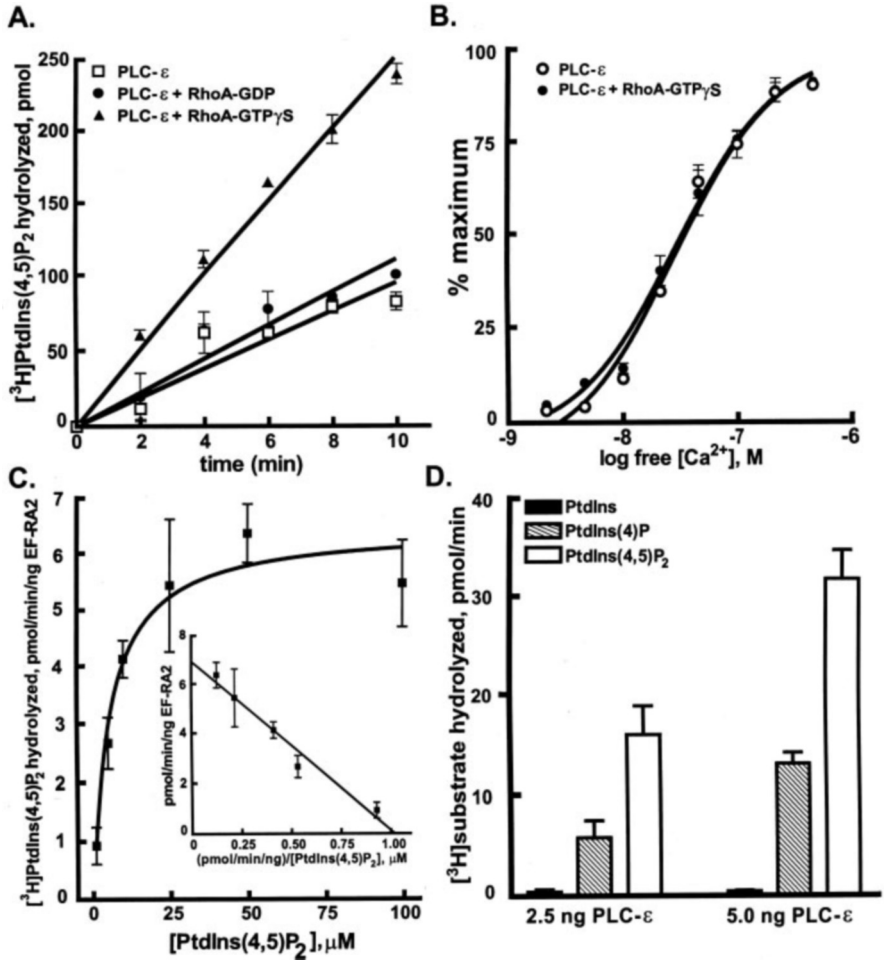

FIG. 2. **Characterization of the phospholipase activity of PLC-ϵ EF-RA2.** *A*, time course of PtdIns(4,5)P_2 hydrolysis in the presence of PLC-ϵ EF-RA2 (10 ng) was tested either in the absence (*open squares*) or presence (*closed circles*) of 300 nM inactive (GDP-bound) RhoA or in the presence of activated (GTPγS-bound) RhoA (*closed triangles*). *B*, PLC-ϵ EF-RA2 was incubated with phospholipid vesicles containing [^{3}H]PtdIns(4,5)P_2 substrate under varying calcium concentrations in the absence of RhoA (*open circles*) or presence of 300 nM GTPγS-bound RhoA (*closed circles*). *C*, the lipase activity of PLC-ϵ EF-RA2 (2 ng) was assayed with increasing concentrations of PtdIns(4,5)P_2 substrate. The data shown are the mean ± S.E. of three separate experiments, and the *inset* is a Hofstee plot of the data. *D*, the substrate selectivity of PLC-ϵ EF-RA2 was tested by presentation of either [^{3}H]PtdIns (*black bars*), [^{3}H]PtdIns(4)P (*dashed bars*), or [^{3}H]PtdIns(4,5)P_2 (*open bars*) with equal amounts of each cold substrate per assay as described under "Experimental Procedures." Enzyme activity was tested with two different concentrations of the purified enzyme.

phospholipid vesicles combined with purified GTPases in cholate-containing buffers prior to the addition of PLC-ϵ. Assays were performed interchangeably using either [^{3}H]PtdIns(4)P or [^{3}H]PtdIns(4,5)P_2 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 $MgSO_4$, 0.5% cholate, 100 μM free calcium, 0.16 mg/ml fatty acid-free bovine serum albumin, 100 μM PtdIns(4)P (Avanti Polar Lipids), and ~10,000 cpm/assay [^{3}H]PtdIns(4)P 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 [^{3}H]Ins(1,4)P_2 from precipitated [^{3}H]PtdIns(4)P, and soluble [^{3}H]Ins(1,4)P_2 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)P_2-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)P_2, 500 μM PE (Avanti Polar Lipids), and ~10,000 cpm of [^{3}H]PtdIns(4,5)P_2 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 Ca^{2+} 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)P_2, along with 30 nmol of PE per assay. Only the ^{3}H-labeled phosphoinositide substrate was changed for each condition.

For experiments determining the effects of small GTPases on phospholipase activity, 10 μl of purified G protein was reconstituted in phospholipid vesicles, resulting in a final cholate concentration of 0.05%. The small GTPases were loaded with nucleotide by incubation with 10 μM GDP or 10 μM GTPγS 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. [^{3}H]Inositol(1,4,5) trisphosphate 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. 1*A*) 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. 1*B*). 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 [^{3}H]PtdIns(4)P as substrate (Fig. 1*C*). Enzymatic activity depended on the

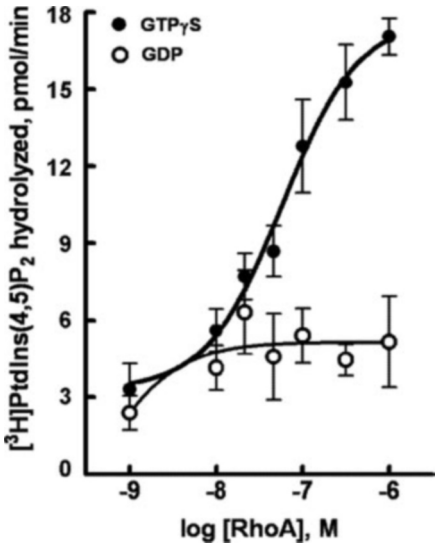

FIG. 3. **Activation of PLC-ϵ by RhoA.** Increasing concentrations of recombinant RhoA purified from detergent-extracted HighFive insect cell membranes were reconstituted in phospholipid vesicles as described under "Experimental Procedures." Assays included PLC-ϵ EF-RA2 in mixed detergent phospholipid micelles containing [^{3}H]PtdIns(4,5)P_2. After a 20-min preincubation of RhoA with either 10 μM GDP (*open circles*) or 10 μM GTPγS (*closed circles*), PLC-ϵ EF-RA2 (1–2 ng) was added and the assay was performed for 10 min at 25 °C. Basal activity of PLC-ϵ EF-RA2 in the absence of RhoA (5–15 pmol/min) was subtracted to give the final values. The results presented are the mean ± S.E. of four individual experiments.

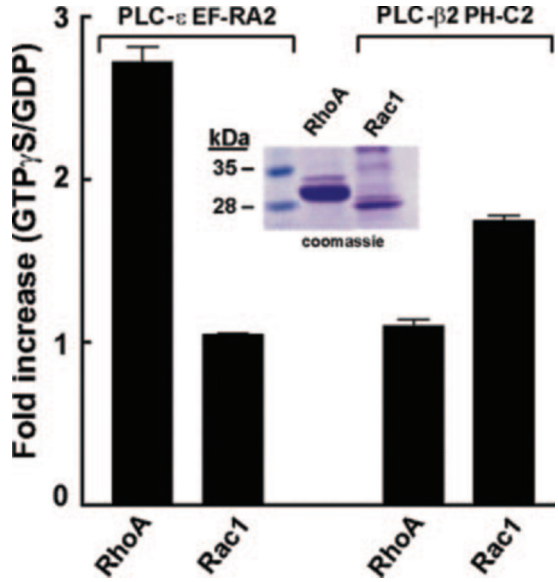

FIG. 4. **Stimulation of PLC isozymes by small GTPases.** RhoA and Rac1 were purified from detergent-extracted HighFive insect cell membranes after expression from recombinant baculoviruses as described under "Experimental Procedures." *Inset*, purified proteins were resolved by SDS-PAGE, and gels were stained with Coomassie Blue. Purified PLC-ϵ EF-RA2 (2 ng) or PLC-β2 PH-C2 (2 ng) was incubated with mixed detergent phospholipid micelles reconstituted with 300 nM monomeric GTPase in the presence of 10 μM GDP or 10 μM GTPγS. Data are presented as -fold increase of activity observed in the presence of GTPγS after subtraction of the basal PLC activity.

amount of purified phospholipase and was linear over a range of concentrations. The observed enzymatic activity of ~10 μmol/min/mg of protein was similar to that of the widely studied purified avian PLC-βt (4, 6, 28, 29). Subsequent assays were performed utilizing PE- and PtdIns(4,5)P_2-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. 2*A*). Similar to other PLC isozymes, enzymatic activity of PLC-ϵ EF-RA2 depended on the concentration of free calcium with an EC_{50} value of ~65 ± 11 nM (n = 3), and maximum activity was observed at a concentration of ~1 μM free Ca^{2+} (Fig. 2*B*). Kinetic analyses of PLC-ϵ EF-RA2 were performed utilizing PE- and PtdIns(4,5)P_2-containing vesicles presented at various concentrations in the bulk medium (see "Experimental Procedures"). These experiments and Hofstee analyses revealed a V_{max} of ~6.5 μmol/min/mg of protein and a K_m value for PtdIns(4,5)P_2 of ~6.0 μM (n = 3) (Fig. 2*C*). The phospholipid substrate selectivity of PLC-ϵ EF-RA2 was determined using PE- and PtdIns-containing vesicles composed of an equal ratio of PtdIns:PtdIns(4)P:PtdIns(4,5)P_2. The recombinant PLC-ϵ fragment hydrolyzed phosphoinositides with a rank order of selectivity of PtdIns(4,5)P_2 > PtdIns(4)P > PtdIns (Fig. 2*D*).

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. 2*A*, 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)P_2 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. 2*B*). 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 not present in other PLC isozymes is required for activation of PLC-ϵ by Rho, $G\alpha_{12}$, and $G\alpha_{13}$ (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-

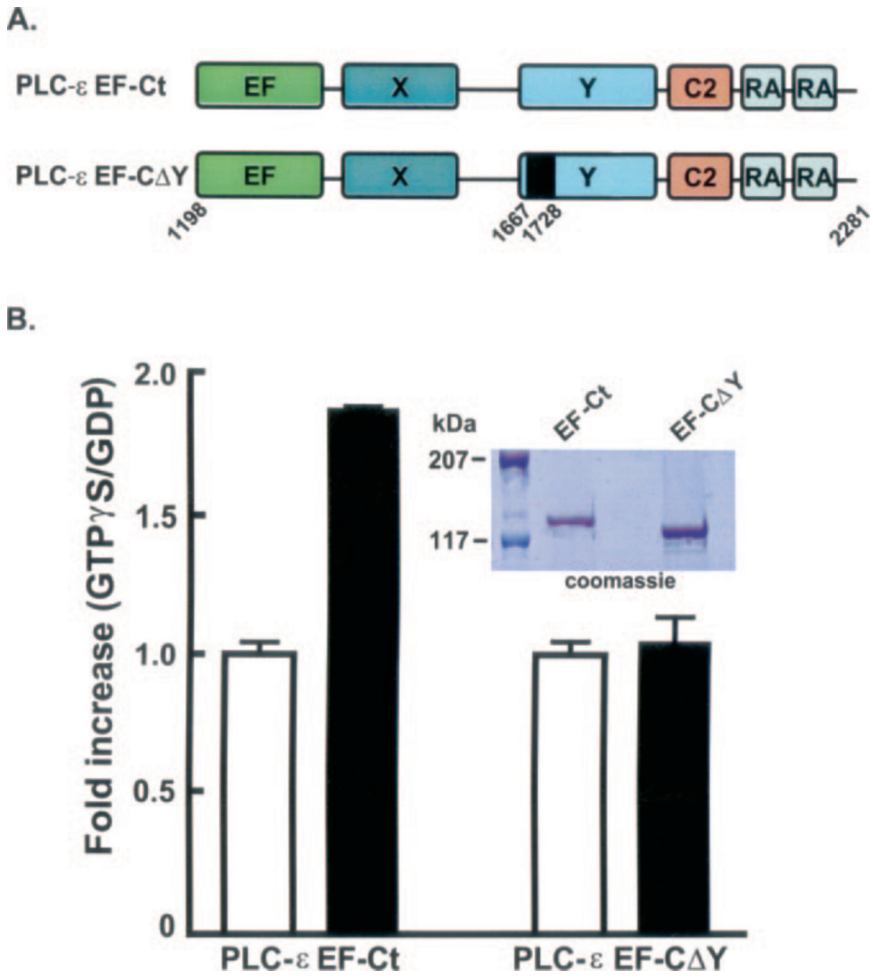

FIG. 5. **Loss of Rho-dependent regulation in a PLC-ϵ fragment lacking a conserved insert within the Y box.** *A*, domain architecture of two PLC-ϵ fragments that span the EF-hand repeats through the carboxyl terminus. Both constructs span amino acids 1198–2281; however, PLC-ϵ EF-CΔY lacks residues 1667–1728 within the Y box (*dark region*). *B*, both PLC-ϵ fragments were purified as described under "Experimental Procedures" and analyzed by SDS-PAGE using a 4–15% polyacrylamide gradient (*inset*). PLC-ϵ EF-Ct (1 ng) and PLC-ϵ EF-CΔY (20 ng) were incubated with detergent lipid vesicles reconstituted with 300 nM RhoA in the presence of 10 μM GDP (*open bars*) or 10 μM GTPγS (*solid bars*) using [^{3}H]PtdIns(4,5)P_2 as substrate. The data are presented as the -fold increase in phospholipase activity in the presence of GTPγS-bound RhoA over GDP-bound RhoA after subtraction of basal PLC activity.

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. 5*A*).

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. 5*B*). 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-, $G\alpha_{12}$-, and $G\alpha_{13}$-dependent activation was not observed (24).

A subfamily of RhoGEFs (*i.e.* p115RhoGEF, LARG, PDZ-RhoGEF) activated by $G\alpha_{12}$ and $G\alpha_{13}$ 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, $G\alpha_{12}$, and $G\alpha_{13}$, we hypothesize that activation of PLC-ϵ by $G\alpha_{12}$ and $G\alpha_{13}$ 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, $G\alpha_{12}$, or $G\alpha_{13}$ and prevented agonist activation of a lysophosphatidic acid receptor but did not inhibit the activation of PLC-β isozymes by $G\alpha_q$.[2] Direct activation of PLC-ϵ by Rho does not rule out the possibility of direct activation of PLC-ϵ by $G\alpha_{12}$ or $G\alpha_{13}$. However, our combined *in vivo* and *in vitro* data suggest that $G\alpha_{12}$ and $G\alpha_{13}$ 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-

[2] M. Hains, M. Wing, and T. K. Harden, unpublished data.

vation 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)P_2 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)P_2 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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