A Novel Bifunctional Phospholipase C That Is Regulated by Gα_{12} and Stimulates the Ras/Mitogen-activated Protein Kinase Pathway*

Isabel Lopez‡, Eric C. Mak‡, Jirong Ding‡, Heidi E. Hamm§, and Jon W. Lomasney‡§

From the ‡Veterans Affairs Chicago Health Care System-Lakeside Division and the §Department of Pathology and Feinberg Cardiovascular Research Institute, Northwestern University Medical School, Chicago, Illinois 60611 and the ¶Institute of Neuroscience and Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, 60611

Three families of phospholipase C (PI-PLCβ, γ, and δ) are known to catalyze the hydrolysis of polyphosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate the second messengers inositol 1,4,5 trisphosphate and diacylglycerol, leading to a cascade of intracellular responses that result in cell growth, cell differentiation, and gene expression. Here we describe the founding member of a novel, structurally distinct fourth family of PI-PLC. PLCɛ not only contains conserved catalytic (X and Y) and regulatory domains (C2) common to other eukaryotic PLCs, but also contains two Ras-associating (RA) domains and a Ras guanine nucleotide exchange factor (RasGEF) motif. PLCɛ hydrolyzes PIP₂, and this activity is stimulated selectively by a constitutively active form of the heterotrimeric G protein Gα_{12}. PLCɛ and a mutant (H1144L) incapable of hydrolyzing phosphoinositides promote formation of GTP-Ras. Thus PLCɛ is a RasGEF. PLCɛ, the mutant H1144L, and the isolated GEF domain activate the mitogen-activated protein kinase pathway in a manner dependent on Ras but independent of PIP₂ hydrolysis. Our findings demonstrate that PLCɛ is a novel bifunctional enzyme that is regulated by the heterotrimeric G protein Gα_{12} and activates the small G protein Ras/mitogen-activated protein kinase signaling pathway.

Many hormones, neurotransmitters, and growth factors elicit intracellular responses by activating a family of inositol phospholipid-specific phospholipase C (PLC)1 enzymes (1, 2).

* This work was supported by a Merit Review Grant from the Department of Veterans Affairs (to J. W. L.), by National Institute of Health Grants HL03836 (to I. L.) and HL55591 and HL03961 (to J. W. L.), by the Robert H. Lurie Comprehensive Cancer Center’s American Cancer Society Institutional Research Grant IRS-93-037-06 (to I. L.), and by American Heart Association Grant-in-Aid 9951330Z (to I. L.), and by American Cancer Society Institutional Research Grant IRG-93-037-06 (to I. L.) and HL55591 and HL03961 (to J. W. L.), by National Institute of Health Grants HL03836 (to I. L.) and HL55591 and HL03961 (to J. W. L.), by the Robert H. Lurie Comprehensive Cancer Center’s American Cancer Society Institutional Research Grant IRS-93-037-06 (to I. L.), and by American Heart Association Grant-in-Aid 9951330Z (to I. L.) and HL55591 and HL03961 (to J. W. L.).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF170071.

To whom correspondence should be addressed: Dept. of Pathology and Feinberg Cardiovascular Research Institute, Tarry Bldg, 12-703, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, IL 60611. Tel.: 312-503-0450; Fax: 312-503-0137; E-mail: j-lomasney@northwestern.edu.

1 The abbreviations used are: PLC, phospholipase C; PI-PLC, PI-PLC; PIP₂, phosphatidylinositol 4,5-bisphosphate; IF₃, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; MAP, mitogen-activated protein; RasGEF, Ras guanine nucleotide exchange factor; RA, Ras-associating; RBD, Ras-binding domain; PI, phosphatidylinositol; EST, expressed sequence tag; PCR, polymerase chain reaction; HA, hemagglutinin; GST, glutathione S-transferase.

There are three established families of PLC termed β (~150 kDa), γ (~145 kDa), and δ (~85 kDa) (3). All three families of the PI-PLC family are able to recognize phosphatidylinositol (PI), phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate (PIP₂) and to carry out the Ca²⁺-dependent hydrolysis of these inositol phospholipids. It is presumed that the primary substrate for hydrolysis is PIP₂, which yields the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ releases intracellular Ca²⁺ from the endoplasmic reticulum via interaction with a specific receptor located on the surface of the endoplasmic reticulum. DAG, as well as increased intracellular Ca²⁺, activate protein kinase C leading to a cascade of intracellular events including regulation of cellular growth, smooth muscle contraction, and cardiac hypertrophy (2).

The mode of regulation differs considerably for members of the different isoform families. The β isoforms are regulated by large heterotrimeric G proteins. After activation by agonists such as epinephrine, α₁ adrenergic receptors are able to couple to Gα subunits from the G_{αq} family and stimulate hydrolysis of phosphatidylinositol lipids via PLC β isoforms. For some β isoforms, G_{αq} alone is sufficient for activation, whereas for others the coordinated action of both G_{αq} and βY is necessary. The γ isoforms of PLC contain SH2 and SH3 domains; hence, they are activated by both receptor and nonreceptor tyrosine kinases. Until recently, the mode of regulation of the δ class was unknown. Work from our laboratory and that of others has determined that this class is regulated in vitro by lipid ligands and ionized free calcium (regulation by calcium via C2 domain is described by Lomasney et al. (4–7).

Activation of G protein-coupled receptors modulates various aspects of cellular growth and proliferation, processes that are primarily controlled by small Ras-related G proteins and their downstream effector, the mitogen-activated protein (MAP) kinases (8, 9). There appear to be multiple mechanisms involving heterotrimeric Gα and βγ subunits by which G protein-coupled receptors regulate small monomeric G protein function (10). Gßγ subunits can induce phosphorylation of the Shc adapter protein leading to association with the Grb2 docking protein and eventual stimulation of Ras guanine nucleotide exchange activity (11). Gα₅ and Gα₁₅ subunits can lead to activation of MAP kinase via a protein kinase C-dependent pathway (12). Recently, a novel molecule, p115 RhoGEF, has been identified that serves as a direct link between the heterotrimeric Gα subunit Gα₁₅ and the small G protein Rho (13, 14). Gα₁₅ stimulates the nucleotide exchange activity of p115 RhoGEF for Rho. p115 RhoGEF also serves as a GTPase-activating protein for Gα₁₃ and Gα₁₂. This is the first example of a protein that is

2 J. W. Lomasney and K. King, submitted for publication.
able to directly link large and small G protein pathways. In this report we identify a novel fourth class of PI-PLC that we designate PLCe and demonstrate that PLCe interacts with large and small G proteins, although it is very different from p115 Rhos6F.

**Experimental Procedures**

Cloning of hPLCe cDNA—A computer search of the human GenBankTM expressed sequence tag (EST) data base was conducted using three relatively short amino acid sequences from the conserved X and Y domains of the mammalian PLCs. An EST clone, z585912.1, showed high sequence identity and contained a putative open reading frame. Screening of a human placental cDNA library with the EST cDNA probe yielded a larger (3.0 kilobases) but still incomplete cDNA fragment. The full-length PLCe cDNA was generated by 5′ rapid amplification of cDNA ends utilizing the Marathon cDNA amplification kit (CLONTECH). The cDNA was reverse transcribed from poly(A) RNA obtained from human heart (CLONTECH), using the cDNA synthesis primer provided with the kit. The PCR amplification was carried out using adaptor primer 1 (5′-CCATCCTTAATAGCTCACTGATTGGCGG-3′) and a gene specific reverse primer, 5′-TCACCGCTGTCGCAACACACAACTCACA-3′. The first round PCR was performed according to the recommendations of the manufacturer of 94 °C for 1 min, five cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by 25 cycles at 94 °C for 5 s and 68 °C for 4 min. The second round of PCR was performed under the same conditions using a 1:200 dilution of the first round PCR amplification mixture as the template, adaptor primer 2 (5′-ACTACCATAGGCT-CGACCCGGG-3′) and a nested gene-specific primer (5′-AGGACGGGCGAAGAAGTTGTTGATGCTCC-3′). Southern blot analysis using an end-labeled internal oligonucleotide (5′-TGCACAGACCTCTCGACGTATCCC-3′) primers from the PCR primers confirmed that the PCR product contained the expected sequence. The PCR product was subcloned into the PCR2.1 TA cloning vector (Invitrogen) according to the recommendations of the manufacturer. Plasmids were isolated by hybridizing filter lifts with the same labeled oligonucleotide that was described for the Southern blots. Clones containing the expected size DNA fragment were then sequenced along both strands using the Tgy d terminator method at the University of Georgia Molecular Genetics Facility. Department of Genetics, using Applied Biosystems 373 and 377 automatic sequencers. The entire hPLCe cDNA was obtained from heart cDNA by PCR using the following primers: 5′-ATGGTTCAGAGAAGGATGCGCAAC-3′ (sense) and 5′-TACCTATGCGATATCCAC-3′ (antisense). The PCR product was subcloned in-frame into pBluescript KS+. The sequence of the inserted DNA was determined as previously mentioned.

Creation of the Phosphodiesterase-deficient Mutant hPLCeH1144L—

The phosphodiesterase-deficient mutant of hPLCe, PLCeH1144L, was generated by changing the histidine residue at position 1144 to leucine using the QuickChange Kit (Stratagene). Briefly, the fragment of PLCe containing the mutation was amplified using Pfu Turbo Polymerase (Stratagene) using sense (5′-GCTTGACGGCCTCAGG-3′) and an antisense (5′-CTACCCCTACGGTAAGTATAAAAGCTGATGACGTGTTT-3′) primers from the PCR primers confirmed that the PCR product contained the expected sequence. The PCR product was subcloned into the PCR2.1 TA cloning vector (Invitrogen) according to the recommendations of the manufacturer. The recombinant plasmid was propagated in E. coli and purified using Qiagen kits. TSA201 cells were cultured in complete Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing glucose, high glucose, 10% fetal bovine serum (Life Technologies, Inc.), and 50 mg/ml gentamicin (Life Technologies, Inc.) at 37 °C in a humidified 5% CO2 incubator. The cells were transfected with Myc-hPLCe or pcDNA3 empty vector using Lipofectamine (Life Technologies, Inc.) according to manufacturer’s instructions with slight modifications. Cells were harvested 48 h after transfection.

Immunoblotting—Samples were subjected to 6% or 15% SDS-polyacrylamide gel electrophoresis and were electrophoretically transferred to nitrocellulose as described previously (15). hPLCe was detected by Western blot analysis using a primary anti-Myc tag antibody (Invitrogen). Goat IgG subunits were detected using specific anti-Go antibodies. Alkaline phosphatase or horseradish peroxidase-conjugated secondary antibody (IG) was used for detection.

**PLC Activity in TSA201 Cells**—TSA201 cells were transfected with either pcDNA3 vector (control), hPLCe, hPLCeH1144L, Gαs*, Gα*α*, Gα*γ*, or Ras (2 mg/35-mm plate) alone or in combination using Lipofectamine (Life Technologies, Inc.). To maintain uniform distribution of transfected cells the configuration was altered by adding the desired combination when necessary. The cells were labeled with [3H]inositol-sodium orthophosphate (PerkinElmer Life Sciences) for 24 h and harvested 48 h after transfection. The amount of inositol phosphates (inositol 1-phosphate, inositol 1,4-bisphosphate, and IP₃) was determined using anion exchange chromatography (16). The percentage of PI hydrolyzed is expressed as the total inositol phosphates formed relative to the amount of [3H]inositol-sodium orthophosphate pool. The data are presented as mean ± SE.

**Cell Free Assays**—TSA201 cells were transfected with either vector (control) or hPLCe cDNA (12 mg DNA/100-mm plate). Cells were harvested 48 h after transfection and homogenized in ice-cold lysis buffer (50 mM Tris, pH 7.5, 30 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 1 mM aprotinin, and 1 mM pepstatin). Cytosolic and particulate fractions were isolated as described previously (15). Cytosolic and membrane fractions were then assayed for PLC activity using a vesicle assay previously described with slight modification (17). Substrate was provided as mixed phospholipid vesicles containing phosphatidylinositol-4,5-bisphosphate, PI(4,5)P₂ (28 µM), and phosphatidylethanolamine (280 µM) in a ratio of 1:10 with 25,000-35,000 cpm of [3H]IP₃/assay. PLC assays were performed at 37 °C for varying time periods in a mixture (70 µl) containing 50 mM HEPES, pH 7.3, 3 mM EGTA, 0.2 mM EDTA, 1 mM MgCl₂, 20 mM NaCl, 30 mM KCl, 4 mM dithiothreitol, 0.1 mM mg/acetate buffered bovine albumin, 1.6 mM sodium deoxycholate, and 1 mM CaCl₂. The reactions were started by addition of the transfected cell membranes and terminated by adding 350 µl of chloroform/methanol/concentrated HCl (500:500:3, v/v/v). The samples were vortexed, and 100 µl of 1 M HCl containing 5 mM EGTA were added. Phases were separated by centrifugation and assayed for radioactivity by liquid scintillation counting.

**MAP Kinase Assay**—TSA201 cells were co-transfected (12 mg DNA/100 mm plate) with vector, hPLCe, hPLCeH1144L, or RasGRF2 in combination with HA-tagged MAP kinase (2 mg). 24 h after transfection the cells were serum starved over night. Cells were lysed 48 h after transfection as described previously with slight modifications (18).
Briefly, cells were washed twice with ice-cold phosphate-buffered saline (Life Technologies, Inc.) and lysed by addition of one volume 10× MAP kinase lysis buffer (5.5% Triton X-100, 0.2 mM phenylmethanesulfonyl fluoride, 0.7 mg/ml pepstatin A, 10 mg/ml leupeptin, 2 mg/ml aprotinin, 20 mM sodium orthovanadate, and 20 mM sodium pyrophosphate) in phosphate-buffered saline. The cells were ultrasonicated for 10 min at 4°C and centrifuged at 4°C for 15 min at 10,000 × g. Activated MAP kinase was immunoprecipitated by incubating the cells for 2 h with anti-HA antibodies (Upstate Biotechnology Inc., 0.4 mg/ml). At the end of the incubation period precleared protein A beads (50% slurry) were added, and the samples were rotated 2 h at 4°C. The ability of MAP kinase to phosphorylate myelin basic protein was measured by autoradiography. One volume of SDS containing sample buffer was added to the samples and boiled for 5 min. Following the manufacturer's protocol (Upstate Biotechnology Inc.), samples were separated on a 7.5% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose at room temperature for 1 or 2 h at 50 V, respectively, using a semidry transfer cell (Bio-Rad). The membranes were probed with anti-HA antibodies, anti-FLAG M2 antibody (Sigma) was used to detect FLAG-tagged RasGRF2, and anti-Myc antibody (InVitrogen) was used to detect hPLC and the H1144L mutant. The horseradish peroxidase-coupled goat antimouse antibody (1:2,000, Sigma) was used as the secondary antibody. The blots were developed by ECL (Amersham Pharmacia Biotech).

**Results and Discussion**

**Isolation of the cDNA Encoding Human PLCε, Determination of Expression in Human Tissues, and Identification of Structural Features**—X and Y domains are regions of ~170 and ~260 amino acids, respectively, which share 20% to 40% amino acid identity among all PLC isoforms. The X and Y domains are necessary for phosphodiesterase activity of PLC. We selected three relatively short amino acid sequences from these conserved X and Y domains to use in a BLAST search. The Basic Local Alignment Search Tool (BLAST) is an extremely powerful tool that allows one to simultaneously search multiple nucleotide sequence data bases. Using the BLAST server at the National Center for Biotechnology Information, we were able to identify an EST clone that partially encoded for a novel PLC isoform. Using the EST cDNA as a probe, a human placental cDNA library was screened yielding a larger but still incomplete cDNA. A full-length cDNA clone was ultimately generated with 5′ rapid amplification of cDNA ends PCR using human heart mRNA as template that we termed PLCε. The full-length cDNA (Fig. 1A) possesses an open reading frame of 6,055 bases encoding a 1,994-amino acid protein with a calculated molecular mass of 230,000, making this the largest PLC isoform to date. The next largest would be a member of the β family at ~1,300 amino acids and ~150 kDa. Hybridization of human multiple tissue Northern blots with PLCε CDNA revealed a ~7.5-kilobase message corresponding to PLCε expressed in a wide variety of tissues including brain, lung, kidney, testis, and colon with highest expression detected in the heart (Fig. 2). An additional transcript of larger size (~9.5 kilobases) could be observed in most tissues, suggesting the possibility of an alternatively spliced form of PLCε or differentially polyadenylated. Results from Southern blotting indicate that isoforms likely do not exist (data not shown).

Structural analysis reveals that PLCε contains the conserved catalytic X and Y domains, thus identifying this isoform conclusively as a PI-PLC (Fig. 1B). Like members of the three other PLC families, PLCε also contains the regulatory C2 domain. Unlike other eukaryotic PLCs, PLCε appears not to contain a pleckstrin homology domain. A phylogenetic comparison of all known mammalian PLC isoforms (Fig. 1C) demonstrates that PLCε shares little homology with other PLC families (β, γ, and δ) and therefore constitutes a distinct family of PLCs. PLCε is most similar to PLC210, a largely uncharacterized isoform from the nematode Caenorhabditis elegans (20). PLC210 was first identified by open reading frame prediction of genomic sequences generated from the C. elegans genome sequencing project. Although there are significant similarities, human PLCε also differs considerably from PLC210. PLCε is considerably larger than PLC210 (~200 amino acids) and differs extensively in the primary structure of the C terminus and portions of the N terminus. We predict that the C terminus of PLCε will mediate protein-protein interactions that are completely different from those of PLC210. Interestingly, both PLCε and PLC210 share structural domains that are not present in any other PLC, further suggesting the existence of a novel PLC family. Both PLCs contain a Ras binding motif denoted as the RA domain (21). Although the functional role of RA domains is presently unknown, a recent report has demonstrated that PLC210 binds to Ras via this domain, suggesting a possible role of this isoform in Ras signaling (22). A number of other RA domain-containing proteins such as Ral-GDS and RGL are also known to bind to Ras (21). PLC210 has two RA domains at the C terminus (amino acids 1688–1792 and 1813–1916), suggesting that it might interact with the effector region of Ras (Fig. 1B). PLCε also contains domains in the N terminus, which suggests that it may interact with small G proteins of the Ras superfamily. Very significant homology (p < 0.00001) is found with aimless RasGEF from Dictyostelium, CDC25 RasGEF from yeast (both Candida albicans and Saccharomyces cerevisiae), human RasGEF homolog Sos1, human RasGEF H-GRF55, and son-of-sevenless RasGEF from mouse. The area of homology is encompassed by the RasGEF catalytic domain signature (G/A/P/C(V/P/Y)X4/L/MF/Y)XD(N/D/L)/V/M) PROSITE121 (22). No other eukaryotic PLC contains these Ras binding motifs. This strongly suggests that PLCε activates Ras signal transduction pathways.

**Expression of PLCε in TSA201 Cells and Characterization of Polyphosphoinositide Hydrolysis**—Western blot analysis of cell lysates from TSA201 cells (a clone of human embryonic kidney 293 cells stably expressing simian virus 40 large T antigen) revealed the presence of a single protein of approximately 230 kDa from cells transfected with PLCε CDNA inserted into the mammalian expression vector pCDNA3 but not from control cells (Fig. 3A) (23). The observed apparent molecular mass of 230 kDa is consistent with the predicted molecular mass of PLCε. Extensive PLCε immunoreactivity resides in the partic-
ulate fraction of transfected TSA201 cells (data not shown) similar to eukaryotic PLCβ isoforms but unlike PLCγ and PLCδ isozymes that are primarily localized in cytosolic fractions (24, 25).

PLCe has PI-PLC activity, measured by its ability to hydrolyze exogenous PIP$_2$, a selective substrate of PI-PLCs. As can be seen in Fig. 3B, 10 µg of plasma membranes obtained from PLCe transfected TSA201 cells had a 2–3-fold (61–114 pmol PIP$_2$ hydrolyzed) greater PLC activity over a 15-min incubation period than membranes of cells transfected with control vector (20–46 pmol of PIP$_2$ hydrolyzed).

The Heterotrimeric G Protein Ga$_{12}$ Selectively Stimulates PLCe-mediated Hydrolysis of Polyphosphoinositides—As depicted in the phylogenetic tree (Fig. 1C), the closest mammalian homolog of PLCs is PLCβ, an effector for heterotrimeric G protein Ga and β subunits. Currently, there are no identifica-
A Novel Phospholipase C that Interacts with Ga₁₂ and Ras

**Fig. 2. Tissue distribution of hPLCe mRNA.** Multiple human tissue Northern blots were analyzed as described under “Experimental Procedures.” Each lane contains approximately 2 mg of poly(A)⁺ RNA. Blots were hybridized with the full-length cDNA or β-actin probe. The hybridized membrane was exposed to x-ray film for 24 h. **kb**, kilobases.

**Fig. 3. Expression of PLCε in TSA201 cells reveals a PI-PLC.** A. Western blot analysis reveals a band of approximately 230 kDa corresponding to Myc-tagged PLCε (arrow) only in cells lysates (50 mg) obtained from TSA201 cells transfected with PLCε, whereas none is found in lysates (50 mg) obtained from TSA201 cells transfected with control (vector). B. membranes (10 mg) isolated from TSA201 cells expressing PLCε (filled circles) exhibit time dependent increases in InaP formation when reconstituted with mixed phospholipid vesicles containing [³²P]PIP₂, as described under “Experimental Procedures.” Data represent the averages ± S.E. of duplicate samples. Similar results were obtained in three independent experiments. Where *error bars* are not shown, the range was less than the size of the point. **MWt.**, molecular mass.

**A**

| Control | myc-PLC ε |
|---------|-----------|
| 205     |           |
| 250     |           |
| 275     |           |

**B**

[Graph showing time-dependent increases in PIP₂ hydrolysis.]
signaling, the ability of PLC to activate Ras was determined by measuring phosphorylation of MAP kinase. To determine whether PLC could activate Ras and the MAP kinase pathway independent of PI hydrolysis, the ability of an X domain phosphodiesterase deficient mutant of PLC (Fig. 4A) to activate Ras was examined. X and Y domains are regions of 170 to 260 amino acids, respectively, which share 60% to 40% amino acid identity among all eukaryotic PI-PLCs. The X and Y domains are necessary for phosphodiesterase activity and make up the catalytic core of the enzyme. Bacterial PLCs contain only the X domain. We have previously demonstrated through extensive site-directed mutagenesis of PLC that the X domain is responsible for the catalytic hydrolysis of polyphosphoinositides, whereas the Y domain is responsible for substrate binding (33). Mutation of amino acid residues Arg338, Glu341, and His356 in the X domain of PLC induces cleavage-defective enzymes (33). These residues are absolutely conserved in all eukaryotic PLCs including PLC. A cleavage-defective PLC was created by mutating the conserved histidine at position 1144 to leucine. This residue is analogous to the histidine at position 356 of PLC ε. PLC ε 1144L is expressed normally as determined by Western blot using anti-Myc antibodies (Fig. 5B) but does not hydrolyze substrate (data not shown). Thus, any effects on Ras mediated by PLC ε 1144L would be due to the Ras binding domains of PLC ε rather than due to indirect effects of PI hydrolysis and the production of the second messengers DAG and IP3.

To determine whether PLC ε and PLC ε 1144L could indeed activate the MAP kinase pathway, subtraction (31.9 versus 29.3 relative units, respectively) in T9A201 cells, as was the level of HA-MAP kinase. Therefore, levels of expression did not account for the differences in activity (Fig. 5B). The products of PI hydrolysis IP3 and DAG could activate the MAP kinase pathway (Fig. 5A). Expression of PLC H1144L also stimulates phosphorylation of MAP kinase, indicating that PI hydrolysis is not necessary for activation of the Ras effector MAP kinase. In fact, PI hydrolysis seems to inhibit the activation of MAP kinase, thereby act as a negative feedback loop. For example, stimulation of protein kinase C by DAG might lead to phosphorylation of PLC ε 1144L and RasGRF2 transfected cells, and MAP kinase activity was assayed as described under "Experimental Procedures." The data are the means ± S.E. of two to six experiments using separate transfections. B, Western blot analysis of cell lysates (100 mg), using HA antibody 12CA5 for MAP kinase, anti-Myc for PLC, or anti-FLAG antibody for RasGRF2 as described under "Experimental Procedures." Antibodies were used at a dilution of 1:1000. This figure is a representative of three to five different experiments repeated with similar results.
of PLCε and inhibition of the RasGEF activity. The known RasGEF, RasGRF2, gave the most robust stimulation (123 pmol/min/mg) yet was comparable with the stimulation by H1144L and PLCε, demonstrating that PLCε is a fairly robust activator of MAP kinase.

The role of the Ras-associating or RA domains is presently unknown. Proteins such as RalGDS that contain both Ras-GEF and RA domains are assumed to serve as links between different Ras family members. Although RA domains are known to bind to the effector loop of activated members of the Ras superfamily, the RA domain seems to not be necessary for regulation of GDP/GTP exchange by some proteins. For example, activated Ras stimulates RGL (for RalGDS-like), which exchanges GDP for GTP on Ral \textit{in vivo}. However, the RA domain of RGL is not necessary for GDP/GTP exchange on Ral \textit{in vivo}. It is presumed that the role of the RA domain in RGL activation of Ral is to mediate redistribution of RGL to the membranes where Ral is located. To differentiate the role of the RasGEF domain from that of the RA domain of PLCε in stimulating MAP kinase, a Myc-tagged construct PLCε-GEF was made of the N-terminal 600 amino acids of PLCε. This construct contains the entire RasGEF domain but lacks the X and Y phosphodiesterase domains and both of the C-terminal RA domains. PLCε-GEF was able to stimulate MAP kinase to a level comparable with the wild type holoenzyme (Fig. 6), suggesting that the RasGEF domain is sufficient for activation of the Ras/MAP kinase pathway by PLCε and that the C-terminal RA domains are not necessary. This result is consistent with the previously described findings for RGL (34).

It is difficult to predict the function of the RA domains in

![FIG. 6. The GEF domain of PLCε is sufficient to activate MAP kinase. A, cell lysates (∼3–5 mg of total protein) were acquired from control, PLCε, PLCε-GEF transfected cells, and MAP kinase activity assayed as described under “Experimental Procedures.” The data are the means ± S.E. of three experiments using separate transfections. B, Western blot analysis of cell lysates (100 mg), using HA antibody 12CA5 for MAP kinase, and anti-Myc for PLCε and PLCε-GEF as described under “Experimental Procedures.” Antibodies were used at a dilution of 1:1000. This figure is a representative of three different experiments with similar results.](Image)

By guest on July 23, 2018

![FIG. 7. Activated Ras inhibits PLCε phosphodiesterase activity \textit{in vivo}. A, PI hydrolysis (InsP formation) is assayed in whole cells after transfection in TSA201 cells. Each value represents the means ± S.E. of two experiments each carried out in duplicate. B, Western blot demonstrating expression of v-Ras and PLCε. This figure is a representative of two different experiments with similar results.](Image)

![FIG. 8. Effect of PLCε and H1144L on Ras activation. A, cells were transfected with either vector (control), PLCε, PLCε H1144L, or RasGRF2 in combination with Ha-Ras. Cell lysates were prepared, and the amount of protein was determined. GTP-Ras was isolated by binding to GST-RBD and visualized by Western blot Analysis as described under “Experimental Procedures.” Approximately equal amounts of protein were loaded per lane. This figure is a representative of three to five different experiments with similar results. B, Western blot analysis of cells lysates (100 mg), using anti-Myc, anti-HA, anti-FLAG, or anti-Ras antibodies. Antibodies were used in dilutions of 1:1000.](Image)
PLCε from these studies. They do not seem to be involved with membrane association, because the PLCε-GEF construct is highly targeted to membranes (data not shown). The domains might be closely associated with the phosphodiesterase catalytic domains (X and Y) because when one or more of the RA domains are truncated, the resulting enzyme has very little phosphodiesterase activity, suggesting that these domains are necessary for proper folding of the enzyme (data not shown). This is similar to truncation of the ζ1 isoform of PLC, where deletion of even a few C-terminal residues leads to inactivation of phosphodiesterase activity.3 Because of the potential interaction of the RA domains with the X and Y domains, the ability of activated Ras to regulate the phosphodiesterase activity of PLCε was assessed by co-transfection. As can be seen from Fig. 7A, v-Ras does not stimulate but may inhibit PLCε in vivo. Transfection of cells with PLCε alone increased PI hydrolysis 3.5-fold, whereas co-transfection with PLCε and v-Ras lead to an increase of only 2-fold. Expression levels of v-Ras and PLCε did not vary substantially among different conditions. Actually, PLCε expression was slightly greater when co-transfected with v-Ras. Overall, these results suggest a potential role for PLCε in regulating Ras activation and thus activation of the MAP kinase pathway in a manner dependent upon the RasGEF domain and independent of PI hydrolysis.

PLCε Activates Ras—More direct evidence of the ability of PLCε to activate Ras comes from experiments in which GTP-Ras is trapped using the Ras effector Raf1. The minimal RBD of Raf1 (amino acids 51–131) binds very tightly and specifically to the GTP-bound form of Ras (K_d 20 nm), whereas the affinity for RasGDP is 3 orders of magnitude lower (35). To determine whether PLCε could indeed act as an exchange factor for Ras, TSA201 cells were transiently co-transfected with either pcDNA3 (control), PLCε, PLCε H1144L, or RasGRF2 (as a GEF control for activation of Ras) and Ha-Ras. The cells were harvested after 48 h and RasGTP was identified by precipitation with GST-RAβ and immunoblotting using anti-Ras antibody. As such, PLCε may mediate the effects of G protein-coupled receptors, especially those coupled with G12,13 through two divergent pathways involving phosphatidylinositol hydrolysis as well as direct activation of the Ras/MAP kinase pathway. Thus, this new member of the PLC family may play a vital role in transducing signals from the plasma membrane to the nucleus through multiple pathways to modulate cytoskeletal changes, cell growth, and mitogenesis.

Acknowledgments—We thank Tatiana A. Vovo-Yasenetskaya for kindly providing the constitutively active Gα12ζ, Gα13ζ, Gα12G13ζ, Gα12, Gα13, and Gα12ζ and David Manning for providing antibodies against Gα12ζ, Gα13ζ, and Gα12ζ. We also thank Dr. Alan Wofman and Johannes L. Bos for kindly providing GST-Raf1 minimal Ras-Binding Domain (GST-RB), Mike Moran for providing RasGRF2, and Mark Marshall for v-Ras G12V and Ha-Ras. We also thank John Campion for technical assistance.

REFERENCES

1. Rhee, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 15045–15048
2. Berri, M. J., and Irvine, R. F. (1989) Nature 341, 197–205
3. Rhee, S. G., Suh, P. G., Ryu, S. H., and Lee, S. Y. (1989) Science 244, 546–550
4. Lomasney, J. W., Cheng, H. F., Wang, I. P., Kuan, Y. S., Liu, S. M., Fesik, S. W., and King, K. (1996) J. Biol. Chem. 271, 25316–25326
5. Bromann, P. A., Boetticher, E. E., and Lomasney, J. W. (1997) J. Biol. Chem. 272, 16240–16246
6. Yagisawa, H., Sakuma, K., Paterson, H. F., Cheung, R., Allen, V., Hirata, H., Watanabe, Y., Hira, M., Williams, R. L., and Katan, M. (1998) J. Biol. Chem. 273, 417–424
7. Kim, Y. H., Park, T. J., Lee, Y. H., Baek, K. J., Suh, P. G., Ryu, S. H., and Kim, K. T. (1999) J. Biol. Chem. 274, 26127–26134
8. Bokoch, G. M. (1996) FASEB J. 10, 1290–1295
9. Gutkind, J. S. (1998) Oncogene 17, 1331–1342
10. Post, G. R., and Brown, J. H. (1996) FASEB J. 10, 741–749
11. Luttrell, L. M., Della Rocca, G. J., van Biesen, T., Luttrell, D. K., and Leftkowit, R. J. (1997) J. Biol. Chem. 272, 4637–4644
12. van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Koch, W. J., and Leftkowit, R. J. (1996) J. Biol. Chem. 271, 1366–1373
13. Hart, M. J., Jiang, X. J., Koza, T., Roscoe, W., Singer, D. W., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Science 280, 2112–2114
14. Koza, T., Jiang, X. J., Hart, M. J., Sternweis, P. M., Singer, D. W., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) Science 280, 2109–2111
15. Lopez, I., Arnold, R. S., and Lambeth, J. D. (1998) J. Biol. Chem. 273, 12846–12852
16. Martin, T. F. (1998) J. Biol. Chem. 273, 14816–14822
17. Kim, M. J., Min, D. S., Ryu, S. H., and Suh, P. G. (1998) J. Biol. Chem. 273, 3618–3624
18. Zhu, Y. L., Ai, Y., Gilevrist, A., Labadia, M. E., Sha’afi, R. I., and Huang, C. K. (1998) Blood 87, 5287–5296
19. de Rooji, J., and Bos, J. L. (1997) Oncogene 14, 623–625
20. Shihabohge, M., Kariya, K., Liao, Y., Hu, C. D., Watarai, Y., Goshima, M., Shimna, F., and Kataoka, T. (1997) J. Biol. Chem. 272, 6218–6222
21. Ponting, C. P., and Benjamin, D. R. (1996) Trends Biochem. Sci. 21, 422–425
22. Boguski, M. S., and McCormick, F. (1995) Nature 366, 643–654
23. Margolskee, R. F., McHendry-Rinde, B., and Horn, R. (1995) BioTechniques 19, 906–911
24. Kim, G. C., Park, D., and Rhee, S. G. (1996) J. Biol. Chem. 271, 21187–21192
25. Ryu, S. H., Suh, P. G., Cho, K. S., Lee, K. Y., and Rhee, S. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6649–6653
26. Wu, D., Jiang, H., Katz, A., and Simon, M. I. (1993) J. Biol. Chem. 268, 3704–3709
27. Strathmann, M. P., and Simon, M. I. (1993) Proc Natl Acad Sci U. S. A. 90, 5582–5586
28. Xu, N., Bradley, L., Ambdukar, I., and Gutkind, J. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6741–6745
29. Vovo-Yasenetskaya, T. A., Faure, M. P., Ahr, N. G., and Bourne, H. R. (1996) J. Biol. Chem. 271, 21081–21087
30. Collins, L. M., Minden, A., Karin, M., and Brown, J. H. (1996) J. Biol. Chem. 271, 17349–17353
31. Wadsworth, S. J., Gebauer, G., van Rossum, G. D., and Dhanelashkar, N. (1997) J. Biol. Chem. 272, 28829–28832
32. Lowy, D. R., and Willumsen, B. M. (1993) Ann. Rev. Biochem. 62, 851–891
33. Cheng, H., Jiang, M., Chen, C., Liu, S., Wong, L., Lomasney, J. W., and King, K. (1995) J. Biol. Chem. 270, 1–11
34. Murai, H., Ide, M., Kishida, S., Ishida, O., Okazaiki-Kishida, M., Matsuura, Y., and Kikuchi, A. (1997) J. Biol. Chem. 272, 10483–10490
35. Herrmann, C., Martin, G. A., and Wittinghofer, A. (1995) J. Biol. Chem. 270, 2901–2905

2 K. King and J. W. Lomasney, personal communication
