Phosphoinositide-specific phospholipase C (PI-PLC) plays a pivotal role in regulation of intracellular signal transduction from various receptor molecules. More than 10 members of human PI-PLC isoforms have been identified and classified into three classes \( \beta, \gamma, \) and \( \delta \), which are regulated by distinct mechanisms. Here we report identification of a novel class of human PI-PLC, named PLC\( \varepsilon \), which is characterized by the presence of a Ras-associating domain at its C terminus and a CDC25-like domain at its N terminus. The Ras-associating domain of PLC\( \varepsilon \) specifically binds to the GTP-bound forms of Ha-Ras and Rap1A. The dissociation constant for Ha-Ras in a GTP-dependent manner is estimated to be approximately 40 nM, comparable with those of other Ras effectors. Co-expression of an activated Ha-Ras mutant with PLC\( \varepsilon \) induces its translocation from the cytosol to the plasma membrane. Upon stimulation with epidermal growth factor, similar translocation of ectopically expressed PLC\( \varepsilon \) is observed, which is inhibited by co-expression of dominant-negative Ha-Ras. Furthermore, using a liposome-based reconstitution assay, it is shown that the phosphatidylinositol 4,5-bisphosphate-hydrolyzing activity of PLC\( \varepsilon \) is stimulated in vitro by Ha-Ras in a GTP-dependent manner. These results indicate that Ras directly regulates phosphoinositide breakdown through membrane targeting of PLC\( \varepsilon \).

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) by phosphoinositide-specific phospholipase C (PI-PLC) is a key event initiating intracellular signal transduction from various receptor molecules at the plasma membrane (1). This reaction yields two intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, which induce activation of protein kinase C and mobilization of Ca\(^{2+}\) from intracellular stores, respectively. Concurrently, the reduction in the PIP\(_2\) concentration is likely an important signal because activities of various actin-binding proteins and pleckstrin homology (PH) domain-containing proteins are modulated through interaction with PIP\(_2\) (2). PIP\(_2\) concentration at the membrane has also been shown to be important in maintenance of the cell shape through regulation of the actin cytoskeleton (3). More than 10 members of human PI-PLC isoforms have been identified and classified into three classes, \( \beta, \gamma, \) and \( \delta \), exemplified by 150-kDa PLC\( \beta1 \), 145-kDa PLC\( \gamma1 \), and 85-kDa PLC\( \delta1 \), respectively (1). All the PI-PLC isoforms contain two regions of high sequence homology, designated X and Y, which constitute the PLC catalytic domain. In addition, other accessory modules, a PH domain, an EF-hand domain, and a C2 domain, are also shared by all the mammalian PLC isoforms reported so far (see Fig. 1A). The three classes of PI-PLCs are linked to receptors by distinct mechanisms (1): PLC\( \beta \) isozymes are activated by the \( \alpha \) subunits of the G\(^\text{q} \) subfamily of heterotrimeric G proteins as well as by the G\( \beta \gamma \) dimers, whereas PLC\( \gamma \) isozymes are activated by both receptor-type and nonreceptor-type protein tyrosine kinases through tyrosine phosphorylation.

Ras proto-oncoproteins are small GTP-binding proteins that function as molecular switches by cycling between the active GTP-bound state and the inactive GDP-bound state (4). They regulate a variety of biological responses including proliferation and differentiation of mammalian cells. In mammalian cells, membrane-anchored GTP-bound Ras interacts directly with the serine/threonine kinase Raf-1, leading to activation of the mitogen-activated protein kinase cascade (5). In addition, recent searches have identified a number of candidate Ras effectors other than Raf-1 and its isoforms B-Raf and A-Raf in mammalian cells (6). All these effector proteins exhibit a GTP-dependent interaction with Ras, for which the intact effector region (residues 32–40 of mammalian Ras) is required (4–6). This interaction, when made with post-translationally modified Ras, induces translocation of the effectors from the cytosol to the plasma membrane, where their substrates or activators are localized. The Ras-dependent membrane translocation has been shown to be prerequisite to activation of the Ras effectors, Raf-1 (7), phosphoinositide 3-kinase (8), and Raf guanine nucleotide dissociation stimulator (RalGDS) (9). Some of the candidate effectors of Ras, including RalGDS and AF-6/Afadin (10, 11), have been shown to possess homologous motifs of about 100 amino acids in their Ras-associating (RA) regions (12). Recently, the RA domain of RalGDS was reported to share a
similar tertiary structure, the ubiquitin superfold, with the Ras-binding domain of Raf-1, although they exhibit no apparent homology in their amino acid sequences (13, 14).

Rap1A, another member of the Ras family small GTP-binding proteins, possesses an identical effector region with that of Ras and associates with almost all effector molecules of Ras (15). Although its physiological functions remain to be investigated, the findings that Rap1A can activate the Ras effector B-Raf and may cooperate with Rap1 in regulation of Ras-effector-mediating responses in some cell types (16, 17) suggest that Rap1A may regulate the activity of other Ras effectors as well. Recently, Rap1A has been implicated in integrin-mediated cell adhesion signaling following stimulation of cell surface molecules, such as the granulocyte colony-stimulating factor (18), CD3(19), and the T cell receptor (20).

In this report, we have identified a novel class of human PI-PLC, named PLC*e, which is characterized by the presence of an RA domain, as a human homolog of Caenorhabditis elegans PLC210 (21). We demonstrate that PLC*e is regulated through direct association with Rap1A, adding another member to the growing list of Ras effectors.

EXPERIMENTAL PROCEDURES

Cloning of PLC*e cDNA—The BLAST search (22) of GenBankTM entries identified a human EST clone zb59f12, which had a striking homology to the C-terminal segment of C. elegans PLC210 (see Fig. 1A). To isolate cDNAs coding for the upstream sequences, we performed 5' RACE (23) by using a cDNA library, synthesized from human fetal brain mRNA (Invitrogen, San Diego, CA) by Marathon cDNA amplification procedure, as a template according to the manufacturer's instruction (CLONTECH, Palo Alto, CA). The nucleotide sequences of the 5'-RACE-amplified cDNAs were confirmed by isolating and sequencing multiple clones.

In vitro Binding and Two-hybrid Assays—The post-translationally modified forms of human Ha-Ras and Rap1A were purified as described before (24–26). Residues 2094–2303 of PLC*e, encompassing the RA domain, were expressed in Escherichia coli as a maltose-binding protein (MBP) fusion by using the pMAL-c plasmid. The resulting MBP-PLC*e was examined for in vitro association with Ha-Ras and Rap1A, which had been loaded with GTP*S or GDP*S, as described before (24–26). Interaction of PLC*e (2094–2303) with various effector region mutants of Ha-Ras was examined by the yeast two-hybrid assay employing pGAD-PLC*e (2094–2303) and pGBT-Ha-RasG12V carrying the mutations as described (27). The β-galactosidase activity was measured by a filter assay as described (28).

Adenylyl Cyclase Inhibition Assay—Measurements of Saccharomyces cerevisiae adenylyl cyclase activity dependent on GTP*S-loaded Ha-Ras and its inhibition by purified MBP-PLC*e (2094–2303) were carried out as described before (29).

Assay of PI-PLC Activity—Full-length PLC*e was expressed with a FLAG epitope tag in Spodoptera frugiperda Sf9 cells by using a baculovirus vector pVL1393 (PharMingen, San Diego, CA). FLAG-PLC*e was affinity purified with resin conjugated with anti-FLAG monoclonal antibody M2 (Sigma). The PI-PLC activities were measured essentially as described (21). Briefly, the fusion proteins were incubated in 50-μl reaction mixtures containing 50 mM Mes, pH 6.8, 10 μM Ca2+/EGTA, 100 mM NaCl, 0.2 mg/ml bovine serum albumin, 0.1 mM diithiothreitol, 90 μM [3H]PIP2 (20,000 cpm), and 80 μM phosphatidylcholine at 30 °C for 30 min. [3H]Insitol 1,4,5-trisphosphate produced was extracted and quantitated by liquid scintillation counting. Various EGTA/Ca2+ buffers giving different concentrations of free Ca2+ were prepared as described (30). GFP-PLC1 (provided by Dr. Tadami Takenawa, University of Tokyo, Tokyo, Japan) was used for expression of the full-length rat PLC1 as a glutathione S-transferase fusion in E. coli. For a liposome-based reconstitution assay, liposomes were made similarly as described (8) by sonication of dried lipids in buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM fatty acid-free bovine serum albumin (Sigma), 7 mM EDTA, and 5 mM MgCl2. The final lipid concentration was 640 μg phosphatidylethanolamine, 600 μg phosphatidyserine, 280 μg phosphatidylcholine, 60 μg sphingomyelin and 160 μg [3H]PI (5,000 cpm/μmol). Post-translationally modified Ha-Ras preloaded with either GTP*S or GDP*S was added to the liposomes at a final concentration of 35 mM and mixed at 4 °C for 90 min. Liposomes were collected by centrifugation at 100,000 × g for 2 h and resuspended in the same volume of the buffer. The PIP2-hydrolyzing activity of PLC*e was measured as described above by incubating 0.1 pmol of purified FLAG-PLC*e with the liposomes in a total volume of 50 μl.

Fluorescence Microscopy—Full-length PLC*e was expressed in COS-7 cells as an enhanced green fluorescent protein (EGFP) fusion. COS-7 cells transfected with pFLAG-CMV-PLC*e were observed under a confocal laser microscope (MRC-1024; Bio-Rad).

RESULTS

Cloning of Human PLC*e—Nucleotide sequence determination of the human EST clone zb59f12 revealed a striking homology to the C-terminal region of C. elegans PLC210 including the Y, C2, and RA domains (Fig. 1, A and B), suggesting that this may represent a human homolog of PLC210. We isolated cDNAs coding for the upstream sequences by the 5'-RACE procedure using a human fetal brain cDNA library as a template. A putative initiator ATG was identified in the 5'-RACE-amplified cDNAs that matched the Kozak consensus sequence (31) and preceded by in-frame stop codons. A composite cDNA was reconstructed by joining the 5'-RACE-amplified clones and the EST clone zb59f12 and shown to encode the full-length protein with the size of 2,303 amino acid residues. Comparison of the deduced amino acid sequence with GenBankTM entries indicated that this protein possessed the X, Y, and C2 domains, which were sandwiched between an N-terminal CDC25-like domain and a C-terminal RA domain (Fig. 1, A and B). The CDC25-like domain is homologous to a family of guanine nucleotide exchange proteins for Ras, represented by S. cerevisiae CDC25. All of these structural elements are conserved with PLC210 except for a difference in the number of the RA domains, confirming that we identified a human homolog of PLC210. As observed with PLC210, this protein lacks a PH domain and an EF-hand domain, both of which are invariably present in all the known human PLC isoforms (Fig. 1A). The observations led us to propose that this protein defines a novel class of human PI-PLC, designated PLC*e. The PLC*e mRNAs were 7.6 kb in size and detected in various fetal and adult human tissues, such as brain, muscle, and lung (data not shown). The PI-PLC activity was also found in the supernatant with an anti-HA monoclonal antibody. Purified PLC*e specifically hydrolyzed PIp2, with a specific activity of 1.35 μmol/min/mg protein, which was comparable with that of PLC1. 304 nmol/min/mg protein, obtained under the same assay condition. Like most of other PI-PLC isoforms, the PIp2-hydrolyzing activity of PLC*e exhibited a dependence on the addition of Ca2+. This indicates that PLC*e is a Ca2+-dependent enzyme.

The observations described above suggest that PLC*e participates in signal transduction in some human tissues as well as the C. elegans PLC210, although the physiological roles of the PLC*e are not yet elucidated.
Ca\textsuperscript{2+} concentration, and the maximal activity was obtained around 10 μM Ca\textsuperscript{2+} (Fig. 1C).

**Specific Binding of PLC\textsubscript{e} to Ha-Ras and Rap1A—** MBP-PLC\textsubscript{e} (2094–2303), encompassing the RA domain, was examined for association with Ha-Ras and Rap1A, which had been loaded with GTP\textsubscript{gS} and GDP\textsubscript{bS}. A dose-dependent association was observed with the GTP-bound forms of Ha-Ras and Rap1A but not with their GDP-bound forms (Fig. 2A). The association with Ras was further analyzed quantitatively by the yeast adenylyl cyclase inhibition assay (29). A dose-dependent inhibition of Ha-Ras-dependent adenylyl cyclase activity was observed by purified PLC\textsubscript{e} (2094–2303) added into the reaction mixture (Fig. 2B). MBP-PLC\textsubscript{e} (2094–2303) had no effect on the Mn\textsuperscript{2+}-dependent adenylyl cyclase activity, indicating that this protein exerted its effect by interacting with Ras but not with adenylyl cyclase (data not shown). We carried out this experiment in the presence of various concentrations of Ha-Ras and MBP-PLC\textsubscript{e} (2094–2303) to prove the competitive nature of the inhibition as described (29). At each point of Ha-Ras concentration in the presence of MBP-PLC\textsubscript{e} (2094–2303), we obtained the free Ha-Ras concentration available for adenylyl cyclase activation as that required for giving the same adenylyl cyclase activity in the absence of MBP-PLC\textsubscript{e} (2094–2303). A difference between the original and the free concentrations of Ha-Ras was regarded as that bound to MBP-PLC\textsubscript{e} (2094–2303), and a reciprocal of this value was plotted against a reciprocal of the free Ha-Ras concentration (Fig. 2C). This gave a series of straight lines for each value of MBP-PLC\textsubscript{e} (2094–2303), which converged on the horizontal axis. The data indicated that MBP-PLC\textsubscript{e} bound directly to Ha-Ras and competitively sequestered it from adenylyl cyclase. The $K_{d}$ value for Ha-Ras was calculated from the point of intersection with the horizontal axis and determined to be approximately 40 nM, which is comparable with those of other Ras effectors (27). The data also indicated that the maximal Ha-Ras binding capacity of PLC\textsubscript{e} (2094–2303), calculated from the point of intersection with the vertical axis (29), was about 0.5 pmol of Ha-Ras bound to 1 pmol of MBP-PLC\textsubscript{e} (2094–2303).

The interaction of the PLC\textsubscript{e} RA domain with Ha-Ras was also confirmed by the yeast two-hybrid assay (Table I). The
mutations affecting Asn-26, Tyr-32, and Thr-35 specifically abolished the interaction of Ha-Ras with PLCε (2094–2303). Comparison with our past results obtained with other Ras effectors (21, 27) indicated that the binding specificity of PLCε was indistinguishable from those of PLCε210 and Schizosaccharomyces pombe Rap1A by the yeast two-hybrid assay. +, positive interaction; −, no interaction. The data on PLCε210, Byr2, Raf-1, Rap1A, Rap1GDS, and AF-6 are taken from Refs. 21, 27, 38, 39, and 40.

mutating the effector region mutants with PLCε and other effectors

| Ha-Ras mutants | PLCε | PLCε210 | Byr2 | Raf-1 | Rap1GDS | AF-6 |
|----------------|------|---------|------|-------|---------|------|
| G12V           | +    | +       | +    | +     | +       | +    |
| G12V, N26G     | +    | +       | +    | +     | +       | +    |
| G12V, V29A     | +    | +       | +    | +     | +       | +    |
| G12V, Y32F     | +    | +       | +    | +     | +       | +    |
| G12V, P34G     | +    | +       | +    | +     | +       | +    |
| G12V, T35S     | +    | +       | +    | +     | +       | +    |
| G12V, E37G     | +    | +       | +    | +     | +       | +    |
| G12V, D35N     | +    | +       | +    | +     | +       | +    |
| G12V, D33F     | +    | +       | +    | +     | +       | +    |
| ND, not determined.

extract of COS-7 cells co-expressing them (Fig. 2D), demonstrating their in vivo association.

Ras Induces Membrane Translocation of PLCε and Stimulates Its PIP2-hydrolyzing Activity—To examine whether the association with Ras has a function to recruit PLCε to the plasma membrane, we co-expressed FLAG-tagged PLCε with Ha-RasG12V in serum-starved COS-7 cells and examined its distribution in the membrane and cytosolic fractions. PLCε was predominantly localized in the cytosolic fraction when expressed alone (Fig. 3A). However, co-expression with Ha-RasG12V caused a gross increment of the amount of PLCε in the membrane fraction. In contrast, co-expression with Ha-RasG12V,Y32F failed to affect the distribution of PLCε. Next, we took advantage of the GFP fusion and examined the intracellular localization of PLCε under a confocal laser microscope. GFP-PLCε was evenly distributed in the cytosol when expressed alone in the serum-starved COS-7 cells (Fig. 3B, top panels). However, GFP-PLCε became enriched at the plasma membrane when co-expressed with Ha-RasG12V (Fig. 3B, middle panels). Again, Ha-RasG12V,Y32F was found to be incompetent (Fig. 3B, bottom panels). Similar results were obtained by using NIH3T3 cells (data not shown). These data taken to-
together indicate that Ras associates with PLCe and induces its translocation from the cytosol to the plasma membrane.

We further investigated whether the PIP2-hydrolyzing activity of PLCe is regulated through interaction with Ras. Because simple mixing of GTP-bound Ha-Ras with purified PLCe had no effect on its activity (data not shown), we examined the effect of the Ras-induced membrane translocation, which was in principle expected to cause an increase of the PLCe activity by bringing it to the proximity of its substrate PIP2. To this end, we employed an in vitro reconstitution assay, in which post-translationally modified Ha-Ras and 3H]PIP2 were incorporated into liposomes and incubated with purified PLCe. As shown in Fig. 3C, GTPγS-loaded Ha-Ras stimulated the PIP2-hydrolyzing activity of PLCe by approximately 2.4-fold. In contrast, no stimulation was observed with the GDPβS-loaded form. This result suggests that the Ras-dependent membrane translocation stimulates the PLCe activity.

Epidermal Growth Factor Stimulates Translocation of PLCe through Ras and Rap1A—To further examine whether the Ras-dependent translocation of PLCe could be induced by a physiological stimulus, COS-7 cells expressing PLCe were stimulated with EGF and analyzed for the distribution of PLCe by immunoblotting. EGF induced transient membrane translocation of PLCe, starting from 5 min after the stimulation and terminating at 40 min (Fig. 4A, left panels). The EGF-stimulated membrane translocation was efficiently blocked by co-expression of the dominant-negative Ha-Ras mutant Ha-RasS17N (Fig. 4A, right panels). Further, translocation of GFP-PLCe to the plasma membrane upon EGF stimulation was observed (Fig. 4B). In addition, an enrichment of GFP-PLCe in the perinuclear region was also noticed (Fig. 4B). We speculated that this perinuclear enrichment was mediated by Rap1A because Rap1A was reported to be activated upon EGF stimulation (32) and because Rap1A was found to be associated with PLCe (Fig. 2A). In fact, we observed co-localization of GFP-PLC with Rap1AG12V in this region (Fig. 4C). Moreover, following EGF stimulation, GFP-PLCe was translocated to the plasma membrane in cells overexpressing wild type Ha-Ras, whereas in wild type Rap1A-overexpressing cells, GFP-PLCe was translocated to the perinuclear region (Fig. 4D). Taken together, EGF may direct translocation of PLCe to different subcellular regions through Ras or Rap1A.

DISCUSSION

We have discovered yet another class of PI-PLC, PLCe, which is characterized by the presence of the CDC25-like domain and the RA domain as well as by the absence of PH and EF-hand domains. As predicted from the close homology at the X, Y, and C2 domains with the known PLC isoforms, PLCe exhibited a Ca2+-dependent PI-PLC activity with the maximal activity observed at 10 μM Ca2+. PLCe possessed the RA domain, which exhibited a specific binding activity to GTP-bound Ha-Ras with an affinity comparable with those of other Ras-effector proteins (27). This binding was abolished by specific mutations in the effector region, which constitutes a major interface for interaction with Ras effectors (4). Further, it was shown that Ras could induce translocation of PLCe to the plasma membrane and stimulate its PIP2-hydrolyzing activity in the liposome reconstitution assay. These observations indicate that PLCe functions as a direct downstream effector of Ras. Considering that full-length PLCe expressed in Sf9 cells by itself possessed a higher specific activity than PLC6 and could not be activated in vitro by mixing with Ras, the association with Ras is likely to induce activation of PLCe through its recruitment to the plasma membrane, where its substrate PIP2 exists. This mechanism is similar to those observed for other Ras effectors, phosphoinositide 3-kinase (8) and RaIGDS (9).

However, we cannot exclude the possibility that Ras has another role of directly activating PLCe as observed for Raf-1 (7).

The physiological function of PLCe remains to be elucidated. The observed membrane recruitment of PLCe by EGF treatment suggests that PLCe may be involved in signal transduction from growth factor receptors. In mammalian cells, an increase in PI-PLC activity upon treatment with growth factors has been largely attributed to the activation of PLCγ (1). However, the observation that the rate of phosphoinositide turnover in Ras-transformed NIH3T3 cells was three times that in untransformed cells implied a persistent stimulation of PI-PLC in these cells (33). Further, injection of anti-PI-PLC antibody has been shown to inhibit Ras-induced mitogenesis (34). These observations are consistent with the possibility that an as yet unidentified species of PI-PLC regulated by Ras may play a role in mammalian cell proliferation, although they might be explained by accessory events accompanying the Ras-induced

![Figure 4](http://www.jbc.org/)
transformation such as autocrine stimulation of receptors coupled to the known PI-PLCs. Our present findings suggest that they might be accounted for by PLCε.

PLCε binds not only to Ras but also to Rap1A as shown in Fig. 2A. Furthermore, Rap1A may mediate translocation of PLCε to the perinuclear region in response to EGF (Fig. 4). Considering that Rap1A (35, 36) and protein kinase C (37) were reported to be localized at the Golgi apparatus, PLCε downstream of Rap1A, may have some role in the regulation of Golgi functions mediated by protein kinase C. Further analysis of the function of PLCε may reveal its unique role in some cellular phenomena.

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Regulation of a Novel Human Phospholipase C, PLCε, through Membrane Targeting by Ras
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