Interaction of Elongation Factor-1α and Pleckstrin Homology Domain of Phospholipase C-γ1 with Activating Its Activity*

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Jong-Soo Chang, a,b,c,‡ Heon Seok, a Deuk Kwon, b Do Sik Min, c Bong-Hyun Ahn, b Young Han Lee, d, i Ju-Won Suh, e Jong-Woo Kim, e Shintaro Iwashita, f Akira Omori, f
Sachiyu Ichinose, a Osamu Numata, g Jeong-Kon Seo, a Yong-Seok Oh, a and Pann-Ghill Suh a

From the aDepartment of Life Science, College of Natural Science, Daejin University, Kyonggi-do 487-711, Korea, bDepartment of Immunology, College of Medicine, Keimyung University, Taegu 700-712, Korea, cDepartment of Physiology, College of Medicine, The Catholic University of Korea, Seoul 137-710, Korea, dDepartment of Biochemistry, College of Medicine, Yeungnam University, Taegu 705-717, Korea, eDepartment of Biological Science, College of Natural Science, Myongji University, Kyonggi-do 449-728, Korea, fMitsubishi Kasei Institute of Life Sciences, Machida-shi, Tokyo 194, Japan, gInstitute of Biological Science, University of Tsukuba, Ibaraki 305-8572, Japan, and hIn2Gen, Cancer Research Institute, Seoul National University, College of Medicine, Seoul 110-789, Korea, and iDepartment of Life Science, Pohang University of Science and Technology, Kyungbuk 790-784, Korea

The pleckstrin homology (PH) domain is a small motif for membrane targeting in the signaling molecules. Phospholipase C (PLC)-γ1 has two putative PH domains, an NH2-terminal and a split PH domain. Here we report studies on the interaction of the PH domain of PLC-γ1 with translational elongation factor (EF)-1α, which has been shown to be a phosphatidylinositol 4-kinase activator. By pull-down of cell extract with the glutathione S-transferase (GST) fusion proteins with various domains of PLC-γ1 followed by peptide sequence analysis, we identified EF-1α as a binding partner of a split PH domain of PLC-γ1. Analysis by site-directed mutagenesis of the PH domain revealed that the β2-sheet of a split PH domain is critical for the interaction with EF-1α. Moreover, Dot-blot assay shows that a split PH domain specifically binds to phosphoinositides including phosphatidylinositol 4-phosphate and phosphatidylinositol 4, 5-bisphosphate (PIP2). So the PH domain of PLC-γ1 binds to both EF-1α and PIP2. The binding affinity of EF-1α to the GST-PH domain fusion protein increased in the presence of PIP2, although PIP2 does not bind to EF-1α directly. This suggests that EF-1α may control the binding affinity between the PH domain and PIP2. PLC-γ1 is substantially activated in the presence of EF-1α with a bell-shaped curve in relation to the molar ratio between them, whereas a double point mutant PLC-γ1 (Y509A/F510A) that lost its binding affinity to EF-1α shows basal level activity. Taken together, our data show that EF-1α plays a direct role in phosphoinositide metabolism of cellular signaling by regulating PLC-γ1 activity via a split PH domain.

Regulation of phosphoinositide metabolism by PLC-γ1 is important for cell proliferation, differentiation, and migration.

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‡ To whom correspondence should be addressed. Tel.: 82-31-539-1853; Fax: 82-31-539-1850; E-mail: jchang@road.daejin.ac.kr.

1 The abbreviations used are: PLC, phospholipase C; EF, elongation factor; PH, pleckstrin homology; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4, 5-bisphosphate; PIP3, phosphatidylinositol 3, 4, 5-trisphosphate; GST, glutathione S-transferase; HRP, horseradish peroxidase; PE, phosphatidylethanolamine; IP3, inositol 1,4,5-trisphosphate; SH, Src homology; TBT, Tris-buffered Tween 20; n, NH2-terminal portion; c, COOH-terminal portion; t, T. pyriformis.

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probably true that EF-1α, as a PI-4 kinase activator, has a pivotal role in regulating phospholipid metabolism. There is, however, no report on the roles of EF-1α as a PI-4 kinase activator in mammalian cells, so our present data are the first demonstration of the roles of eukaryotic EF-1α in mammalian phosphoinositide metabolism. In addition to the involvement of EF-1α protein translation (28, 29), EF-1α phosphoinositide metabolism. In addition to the involvement of EF-1α domains. A split PH domain consists of NH2-terminal portion (nPH2) and COOH-terminal portion (cPH2).

**FIG. 1.** Isolation of EF-1α as a split PH domain of PLC-γ1 binding protein. A, PLC-γ1 has two putative PH domains (PH1 and split PH2) in addition to the SH2n, SH2c, SH3, and catalytic X and Y domains. A split PH domain consists of NH2-terminal portion (nPH2) and COOH-terminal portion (cPH2). B, three GST fusion proteins of GSTPH1, GSTnPH2, and GSTcPH2 incubated with (+) or without (−) NIH 3T3 cell lysates. The bound proteins were isolated by pull-down and subjected to 10% SDS-PAGE. A prominent protein with 48 kDa (indicated by an open arrowhead) was detected from GSTnPH2 fusion protein purified by Coomassie Brilliant Blue staining. C, various GST fusion proteins incubated with NIH 3T3 cell lysates. The bound proteins were resolved on 10% SDS-PAGE followed by immunoblotting using anti-EF-1α monoclonal antibody. WCL indicates the whole cell lysates used for each pull-down experiment.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Anti-EF-1α monoclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-GST antibody and HRP-conjugated donkey anti-goat antibody were from Amersham Biosciences and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively. All the phospholipids including phosphatidylycholines (PC), PI, PIP, and PIP2, were from Sigma. Lysylendopeptidase AP-1 was obtained from Wako Pure Chemical (Osaka, Japan).

In Vitro Binding Assay Using GST Fusion Protein—All the GST fusion proteins were engineered by PCR using rat EF-1α CDNA (33) and rat PLC-γ1 CDNA (34) as templates. Briefly, PCR was carried out between a 5′ primer with a EcoRI recognition site and a 3′ primer with a XhoI recognition site at the 5′ extension, corresponding to the individual PH domains, respectively. PCR products were ligated into the pGEX-5X-1 vector (Amersham Biosciences). Point mutant PH domains of PLC-γ1 were also engineered by PCR. All of the DNA constructs were confirmed by DNA sequencing. Expression and purification of fusion proteins using glutathione-Sepharose 4B (Amerham Biosciences) were performed per the manufacturer’s specifications. GST fusion proteins used in this study were as follows: GSTPH1 (coding residue amino acids 25–145 of PLC-γ1), GSTnPH2 (amino acids 477–547 of PLC-γ1), GSTcPH2 (amino acids 850–979 of PLC-γ1), GSTPH2 (amino acids 850–979 of PLC-γ1), GSTPHGAP (amino acids 461–612 of p120 kDa rasGTPase activating protein), GSTSH2n (amino acids 550–667 of PLC-γ1), GSTSH2c (amino acids 668–735 of PLC-γ1), GSTSH3 (amino acids 791–836 of PLC-γ1), and GSTEF-1α (whole amino acid sequence of rat EF-1α). Proteins bound to GST fusion proteins were washed extensively with Nonidet P-40 buffer (20 mM Tris pH 7.5, 1% Nonidet P-40, 300 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate), resolved in 10% SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The membrane-bound proteins were detected with the ECL detection system using anti-EF-1α monoclonal antibody and HRP-conjugated goat anti-mouse antibody.

**Determination of the Partial Amino Acid Sequence—**GST-PH-bound proteins were separated by 10% SDS-PAGE and stained with Coomasie Brilliant Blue R-250. The prominent band was excised and digested with lysylendopeptidase AP-1 for 14 h, and the resulting peptides were separated by reverse-phase high pressure liquid chromatography C18 column chromatography as described previously (35). Amino acids from the NH2 terminus of the peptides were analyzed by a pulse-labeled phase protein sequencer (PE-Biosystems, model 492 cLC).

**PLC-γ1 Activity Assay—**PLC-γ1 activity was measured as described previously (36). Briefly, substrate was prepared as sonicated vesicles of 75 mM [3H]IP3 (9,000–10,000 cpm/assay, PerkinElmer Life Sciences) and 750 mM PE in 50 mM HEPES buffer (pH 7.0) containing 2 mM ATP, 1 mM MgCl2, 0.5 mM CaCl2. Reactions were performed for 20 min at 30 °C. The reaction was terminated by the addition of 1 ml of chloroform/methanol/1 M HCl (50:50:0.5) and 0.45 ml of 1 N HC1. The mixtures were vortexed and centrifuged for 10 min at 2,000 rpm. The aqueous phase containing [3H]IP3 was collected and subjected to a scintillation counter. The effect of EF-1α was examined by adding the indicated amount of EF-1α to the PLC-γ1 assay mixture. Tetrahymanina pyriformis EF-1α was homogenously purified by the method described before (37). Wild type PLC-γ1 and its mutant form (Y509A/F510A) were homogenously prepared as described previously (38).

**Far Western Blot Analysis—**Purified EF-1α (0.2 μg/lane) was resolved in 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Nonspecific binding to the membrane was blocked by adding 2% skim milk in Tris-buffered Tween 20 (TBT) for 1 h at room temperature. The membranes were then incubated with GST, GSTnPH2, or GSTnPH2 proteins (0.5 μg/ml) for 1 h in blocking buffer for 14 h at 4 °C. After washing in TBT buffer, the membranes were incubated with anti-GST antibody for 2 h at room temperature. After washing the membrane with TBT buffer, bound proteins were detected by successive incubation with HRP-conjugated anti-goat antibody as a secondary antibody using the ECL detection system.

**Dot-blot Analysis—**The ability of the proteins to bind different phospholipids was examined using Dot-blot analysis (39). Briefly, chloroform-solubilized phospholipids (3 μg of each) were spotted onto nitrocellulose membrane (PROTRAN, Schleicher & Schuell), and then the membrane was dried at room temperature for 1 h. The following steps were exactly the same method as for Far Western blotting. The membrane was blocked with 2% non-fat skim milk in TBT buffer for 1 h. The membranes were then incubated with purified EF-1α, GST, GST-EF-1α,
Interaction of PLC-\(\gamma\)-1-Ph Domain and EF-1\(\alpha\)

A Split PH Domain of PLC-\(\gamma\)-1 Directly Binds to EF-1\(\alpha\)—PLC-\(\gamma\)-1 has two putative PH domains in the molecule. To search for proteins specifically binding to the PH domains of PLC-\(\gamma\)-1, we prepared three kinds of GST-PH domain fusion proteins in *Escherichia coli* (GST-PH\(_5\), GST-PH\(_{1}\), and GSTcPH\(_2\)). These purified GST-PH fusion proteins were incubated with lysate of NIH 3T3 cells, respectively (Fig. 1, A and B). Among them, GST-PH\(_2\) fusion proteins specifically pulled down a prominent protein with a molecular size of 48 kDa. To identify the protein, the band was cut from the gel and subjected to a protein sequencer after lysozyme treatment. We obtained two peptide sequences, P1 and P2. P1 is YYVTIDAPGHRDFIK, and P2 is TGHLIYK. When these sequences were searched by the NCBI data base of SWISS-PROT, they were found to match 58 species of EF-1\(\alpha\) sequence reported. P1 and P2 correspond to the 105–120th and 24–30th amino acids of human EF-1\(\alpha\), respectively. Then we confirmed the band with 48 kDa as EF-1\(\alpha\) with Western immunoblotting using anti-EF-1\(\alpha\) monoclonal antibody (Fig. 1C). To further clarify the binding region of PLC-\(\gamma\)-1 to EF-1\(\alpha\), we examined binding capacity using several other GST fusion proteins including GST-PH\(_{5}\), GSTcPH\(_2\), GST-SH2, GST-SH3, GST-PH-GAP, and PH-GAP. As shown in Fig. 1C, only GST-PH\(_2\) associates with EF-1\(\alpha\), as judged by Western immunoblotting. We next tested whether the binding is direct or not; Far Western blotting using purified protozoan T. pyriformis EF-1\(\alpha\) (tEF-1\(\alpha\)) was used for this. Since EF-1\(\alpha\) is highly conserved and has very similar biochemical properties among different species in eukaryotes (28), we used tEF-1\(\alpha\) due to its success with purification steps with high purity (37). The result of Far Western blotting clearly showed a direct binding between GST-PH\(_2\) and tEF-1\(\alpha\) (Fig. 2, A and B). Moreover, a double point mutation in GST-PH\(_2\) fusion protein (Y509A/F510A) lost its binding affinity to tEF-1\(\alpha\) (Fig. 3, A and B). To confirm the interaction between the PH domain and EF-1\(\alpha\) in vivo, immunoprecipitation was carried out to detect a PLC-\(\gamma\)-1/EF-1\(\alpha\) complex in COS-7 cells. The immunoprecipitates of EF-1\(\alpha\) isolated by anti-EF-1\(\alpha\) antibody included PLC-\(\gamma\)-1, detected by Western immunoblot with anti-PLC-\(\gamma\)-1 antibody or vice versa (Fig. 2C). Also, the yeast two-hybrid assay was introduced to show in vivo interaction between EF-1\(\alpha\) and the nPH\(_2\) domain of PLC-\(\gamma\)-1 (Fig. 2D). The mutant nPH\(_2\) domain did not bind to EF-1\(\alpha\) in either yeast two-hybrid assay. These results clearly demonstrate that the nPH\(_2\) domain of PLC-\(\gamma\)-1 directly binds to EF-1\(\alpha\).

\(\beta\)-Sheet of nPH\(_2\) Domain Is Critical for Binding to EF-1\(\alpha\)—Fine mapping of the EF-1\(\alpha\) binding site was carried out within the nPH\(_2\) domain of PLC-\(\gamma\)-1. Since aromatic residue has a potential for protein-protein interaction via hydrophobic interaction, we substituted aromatic residues including tyrosine and phenylalanine for alanine within the P2 region (YYVTIDAPGHRDFIK: P1: Y509A/F510A, respectively). The result of Far Western blotting clearly showed a direct binding between GST-PH\(_2\) and tEF-1\(\alpha\) (Fig. 2, A and B). Moreover, a double point mutation in GST-PH\(_2\) fusion protein (Y509A/F510A) lost its binding affinity to tEF-1\(\alpha\) (Fig. 3, A and B). To confirm the interaction between the PH domain and EF-1\(\alpha\) in vivo, immunoprecipitation was carried out to detect a PLC-\(\gamma\)-1/EF-1\(\alpha\) complex in COS-7 cells. The immunoprecipitates of EF-1\(\alpha\) isolated by anti-EF-1\(\alpha\) antibody included PLC-\(\gamma\)-1, detected by Western immunoblot with anti-PLC-\(\gamma\)-1 antibody or vice versa (Fig. 2C). Also, the yeast two-hybrid assay was introduced to show in vivo interaction between EF-1\(\alpha\) and the nPH\(_2\) domain of PLC-\(\gamma\)-1 (Fig. 2D). The mutant nPH\(_2\) domain did not bind to EF-1\(\alpha\) in either yeast two-hybrid assay. These results clearly demonstrate that the nPH\(_2\) domain of PLC-\(\gamma\)-1 directly binds to EF-1\(\alpha\).
and another double point mutant Y506A/P507A of the mutant Y509A/F510A completely abolishes the interaction, PIP2/GSTnPH2, whereas GSTEF-1 glutathione-Sepharose beads. Bound proteins were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Bound EF-1α was detected by immunoblotting using anti-EF-1α antibody. WCL, the whole cell lysates used for each pull-down experiment.

FIG. 3. Mapping of the EF-1α binding site within the nPH2 domain of PLC-γ1. A, schematic representation of the elements of the secondary structure. Amino acid sequences of the nPH2 domain (corresponding to the β-sheets 1–3) of PLC-γ1 are depicted with the single-letter codes. Positions of the mutated amino acids are shown under the wild type amino acids. B, in vitro binding assay with various mutant GSTnPH2 fusion proteins. NIH 3T3 cell lysates (300 μg) were incubated with 2–5 μg each of various mutant GST fusion proteins immobilized onto glutathione-Sepharose beads. Bound proteins were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Bound EF-1α was detected by immunoblotting using anti-EF-1α antibody. WCL, the whole cell lysates used for each pull-down experiment.

FIG. 4. Dot-blot assay of GST fusion proteins with phospholipids. Each chloroform-solubilized phospholipid (3 μg) was spotted on nitrocellulose membrane for Dot-blot assay. The filter was incubated with 0.5 μg/ml GST or GST fusion proteins for 14 h at 4 °C. The phospholipid-bound proteins were detected by the ECL detection system as described under “Experimental Procedures.”

nPH2 Domain of PLC-γ1 Specifically Binds to PIP and PIP2—To explore the binding region of PLC-γ1 to phosphoinositides, we used GSTnPH2, mutant GSTnPH2-Y509A/F510A, GSTEF-1α, and GST proteins for dot-blotting (lipid-protein blotting). Different lipids (each 3 μg) including PE, PIP, and PIP2 were spotted onto nitrocellulose membrane, and the membrane was blotted as described under “Experimental Procedures.” As shown in Fig. 4, a double point mutant of GSTnPH2-Y509A/F510A without binding affinity for EF-1α binds to PIP and PIP2 with a similar capacity as wild type GSTnPH2, whereas GSTEF-1α and GST as a control do not show any binding affinity to phospholipids. Also, purified tEF-1α did not show any binding affinity to phospholipids (data not shown). These results suggest that the nPH2 domain of PLC-γ1 has different binding sites for EF-1α and phospholipid. It is noteworthy that the nPH2 region serves as a substrate PIP2-binding site, whereas the NH2-terminal PH domain (PH2) of PLC-γ1 has been reported to interact with PIP2 for membrane-targeted translocation (25).

nPH2 Potentiates the Binding Affinity of PH Domain to EF-1α—Since both substrate PIP2 and EF-1α bind to the nPH2 domain of PLC-γ1, we investigated whether they compete with each other for binding to the PH domain. However, we found that the association between the nPH2 domain of PLC-γ1 and EF-1α significantly increased in the presence of PIP2 but not in either PE or PIP up to its concentration of 100 μg/ml phospholipid (Fig. 5). Complex formation of GSTnPH2/EF-1α increased in a PIP2 dose-dependent manner.

EF-1α Activates PLC-γ1 Activity—To examine whether the complex formation of both proteins affects PLC-γ1 enzymatic activity, we measured its catalytic activity. Since we confirmed that tEF-1α specifically associates with PLC-γ1 (Fig. 2, A and B), we used tEF-1α for its effect on PLC-γ1 activity. After preincubation of the purified tEF-1α with either PLC-γ1 or
phorylation of PLC-

domains of PLC-

activity even in the presence of EF-1

of GST

nPH2 proteins (highly induce the production of IP3 with weak tyrosine phos-

identify a responsible molecule(s) for regulation of PLC-

mutant PLC-

drolyzing activity was measured. As shown in Fig. 6, EF-1

by immunoblotting with anti-EF-1

by relative image density (Quantity One, Bio-Rad), which was normal-

functional roles of PLC-

example, some ligands strongly stimulate tyrosine phosphoryl-

CaM molecules are contaminated in EF-1

EF-1

to the autophosphorylated receptor, and

another possibility is that component(s) such as Ca2+

CaM might sequester Ca2+

Although extensive studies on the role of the PH domains were done in PLC-β (17), -δ (41, 42), and -γ1 (25) and PI-4 kinase (39), those of a split PH domain of PLC-γ1 had not been examined. By pull-down experiments with GST-nPH2 using a detergent lysate of NIH 3T3 cells, EF-1α was identified by peptide sequence analysis. The association between the nPH2 domain and EF-1α is highly specific. Since EF-1α has been reported to be an activating protein of PI-4 kinase (26, 27), it is meaningful that the association might play a critical role for PLC-γ1 in cellular signaling.

The addition of PIP2, a PLC-γ1 substrate, to the incubation mixture of the GST-nPH2 domain fusion protein and cell extracts containing EF-1α, the complex formation of GST-nPH2-

EF-1α, was dramatically increased in a PIP2 concentration-de-

dependent manner. On this point, it is interesting that the stable complex between PLC-γ1 and its substrate PIP2 was detected by Dot-blot analysis (Fig. 4). Therefore, we can speculate that the PH domain of PLC-γ1 associates with PIP2 first, and the PH domain/PIP2 complex formation induces the conforma-
tional change to allow EF-1α to bind PLC-γ1. EF-1α binding to

PLC-γ1 might facilitate the hydrolysis of PIP2 by PLC-γ1. In this context, the role of PLC-γ1-bound EF-1α is a possible regulator for PIP2 hydrolysis.

The activation of PLC-γ1 activity by EF-1α showed a bell-shaped curve (Fig. 6). The maximum activity was at around a 1:2 molar ratio, whereas the activity decreased to basal level at higher than 1:8 molar ratios. There might be several reasons to explain the bell-shaped curve. One is that EF-1α has a very basic isoelectric point and is easily aggregated at high density (43, 44). Another possibility is that component(s) such as Ca2+/CaM molecules are contaminated in EF-1α preparation. Although the preparation of tEF-1α is highly pure, the contamination of Ca2+/CaM molecules or other components could not be completely excluded. Generally, EF-1α preparation contains Ca2+/CaM molecules to some extent (40, 45). In this regard, Ca2+/CaM might sequester Ca2+ supplements for maximal PLC-γ1 activity at high doses of EF-1α addition.

EF-1α promotes the production of PIP and PIP2 by the activation of PI-4 kinase, and eventually this newly produced PIP2 hydrolysis is also accelerated by EF-1α via PLC-γ1 activation. EF-1α activates both PI-4 kinase (26, 27) and PLC-γ1, which
can bind to PIP and PIP$_2$. However, they regulate the level of PIP and PIP$_2$ in a different manner. The former regulates the level of PIP and PIP$_2$ by phosphorylation of PI at the D-4 position of the inositol ring, whereas the latter regulates the phospholipid level by hydrolyzing PIP$_2$ to IP$_3$ and diacetylgerol. Therefore, EF-1α has the potential to induce a rapid PI turnover in a cell.

In vivo, the complex of PLC-γ1-EF-1α was detected by immunoprecipitation not only from quiescent cells but also by the different growth factor- or platelet-derived growth factor-stimulated cells, and so far no significant difference was observed between them. However, using green fluorescent protein fusion proteins, serum, and lysosomopatic acid increased their complex formation around the cell membrane.

Although we need more detailed analysis for the PLC-γ1 activation mechanism, our results show a direct interaction between PLC-γ1 and EF-1α that elucidates the phospholipid metabolisms induced by PLC-γ1 in cellular signaling.

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Interaction of Elongation Factor-1α and Pleckstrin Homology Domain of Phospholipase C-γ1 with Activating Its Activity  
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