THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 22, Issue of May 31, pp. 19697–19702, 2002

Printed in U.S.A.

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

Received for publication, November 26, 2001, and in revised form, February 7, 2002
Published, JBC Papers in Press, March 8, 2002, DOI 10.1074/jbc.M111206200

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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 NH_2-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 (PIP_2). So the PH domain of PLC-γ1 binds to both EF-1α and PIP_2. The binding affinity of EF-1α to the GST·PH domain fusion protein increased in the presence of PIP_2, although PIP_2 does not bind to EF-1α directly. This suggests that EF-1α may control the binding affinity between the PH domain and PIP_2. 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[1] is important for cell proliferation, differentiation, and migration. Many extracellular signals stimulate the hydrolysis of PIP_2 by the activation of PLC-γ1, which produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. Both second messengers regulate the release of Ca^{2+} from intracellular stores and activate protein kinase C, respectively (1, 2). On the roles of PLC-γ1 in cell growth and differentiation, recent findings demonstrate that overexpression of PLC-γ1 induces malignant transformation in nude mice (3), and targeted deletion of PLC-γ1 results in embryonic lethality in mice (4).

For protein-protein interactions, PLC-γ1 has two Src homology (SH) 2 domains to bind to tyrosine-phosphorylated proteins including several growth factor receptors (5–8). Although a large variety of proteins are identified as interacting counterparts of the SH2-SH2-SH3 domain of PLC-γ1, the activation mechanism of PLC-γ1 remains obscure.

The PH domain is a 120-amino acid residue stretch that has been identified in over 100 proteins (9–12). The PH domain binds with high specificity and affinity to phosphoinositides including PIP, PIP_2, and IP_3 (13–15). The PH domain of signaling molecules is involved in targeted translocation of molecules to cell membranes (13, 16, 17). Also, the PH domain mediates protein-protein interaction as well as protein-lipid interaction including the βγ-subunit of heteromeric G-protein (18, 19), protein kinase C (20), actin (21), and BAP-135 (22). By analyzing the tertiary structure, the PH domain is an antiparallel β-sheet consisting of seven strands (23, 24).

PLC-γ1 has two putative PH domains: one is located in the amino-terminal 150-amino acid residue, and the other is split by the SH2-SH2-SH3 domain (see Fig. 1*A*). Upon growth factor stimulation, the NH_2-terminal PH domain of PLC-γ1 is targeted to the plasma membrane and binds to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) but not to PIP_2 (25). In an effort to identify the PH domain ligands and to understand the phosphoinositide regulation mechanism of PLC-γ1, we used the GST·PH fusion protein system. We found that a split half of the PH domain of PLC-γ1 directly binds to EF-1α, which is known for PI-4 kinase activating protein in plants (26, 27).

Since the first step of PIP_2 biosynthesis is the phosphorylation of PI by PI-4 kinase to produce PI-4-P, which is phosphorylated by PI-4-P-5 kinase (PI-4-P kinase) to produce PIP_2, it is

* This work was supported by Grant 2000-015-DP0317 from the Korea Research Foundation (to J.-S. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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[1] The abbreviations used are: PLC, phospholipase C; EF, elongation factor; PH, pleckstrin homology; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4, 5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; PE, phosphatidylethanolamine; IP_3, inositol 1,4,5-trisphosphate; SH, Src homology; TBT, Tris-buffered Tween 20; n, NH_2-terminal portion; c, COOH-terminal portion; t, *T. pyriformis*.

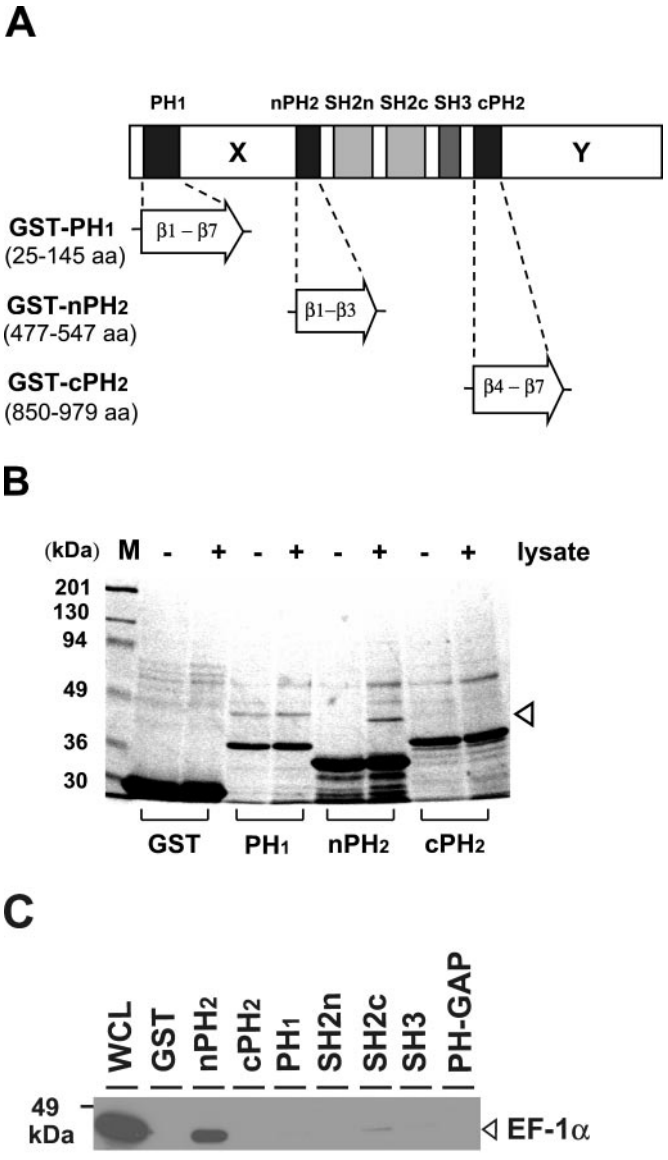

FIG. 1. **Isolation of EF-1α as a split PH domain of PLC-γ1 binding protein.** *A*, PLC-γ1 has two putative PH domains (PH_1 and split PH_2) in addition to the SH2n, SH2c, SH3, and catalytic X and Y domains. A split PH domain consists of NH_2-terminal portion (nPH_2) and COOH-terminal portion (cPH_2). *B*, three GST fusion proteins of GST·PH_1, GST·nPH_2, and GST·cPH_2 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 GST·nPH_2 fusion protein followed 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.

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 protein translation (28, 29), EF-1α is involved in cytoskeletal rearrangement (30). Furthermore, overexpression of EF-1α correlates with metastasis (31) and leads to increased susceptibility to oncogenic transformation (32). Here we describe that EF-1α directly binds to the PH domain to activate PLC-γ1.

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 phosphatidylethanolamine (PE), PIP, and PIP_2 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 *Eco*RI recognition site and a 3′ primer with a *Xho*I 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 (Amersham Biosciences) were performed per the manufacturer's specifications. GST fusion proteins used in this study were as follows: GST·PH_1 (coding residue amino acids 25–145 of PLC-γ1), GST·nPH_2 (amino acids 477–547 of PLC-γ1), GST·cPH_2 (amino acids 850–979 of PLC-γ1), GST·PHGAP (amino acids 461–612 of p120 kDa rasGTPase activating protein), GST·SH2n (amino acids 550–667 of PLC-γ1), GST·SH2c (amino acids 668–735 of PLC-γ1), GST·SH3 (amino acids 791–836 of PLC-γ1), and GST·EF-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-Cl, pH 7.5, 1% Nonidet P-40, 300 mM NaCl, 2 mM $MgCl_2$, 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 Coomassie 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 C_8 column chromatography as described previously (35). Amino acids from the NH_2 terminus of the peptides were analyzed by a pulse-liquid 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 [^{3}H]PIP_2 (9,000–10,000 cpm/assay, PerkinElmer Life Sciences) and 750 mM PE in 50 mM HEPES buffer (pH 7.0) containing 2 mM $CaCl_2$. Reactions were performed for 20 min at 30 °C in a 60-μl final volume and terminated by the addition of 1 ml of chloroform/methanol/HCl (50:50:0.3) and 0.45 ml of 1 N HCl. The mixtures were vortexed and centrifuged for 10 min at 2,000 rpm. The aqueous phase containing [^{3}H]IP_3 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. *Tetrahymena pyriformis* EF-1α was homogeneously purified by the method described before (37). Wild type PLC-γ1 and its mutant form (Y509A/F510A) were homogeneously 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, GST·nPH_2, or GST·nPH_2·Y509A/F510A mutant proteins (0.5 μg/ml) in blocking buffer for 14 h at 4 °C. After washes 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 second 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 are 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,

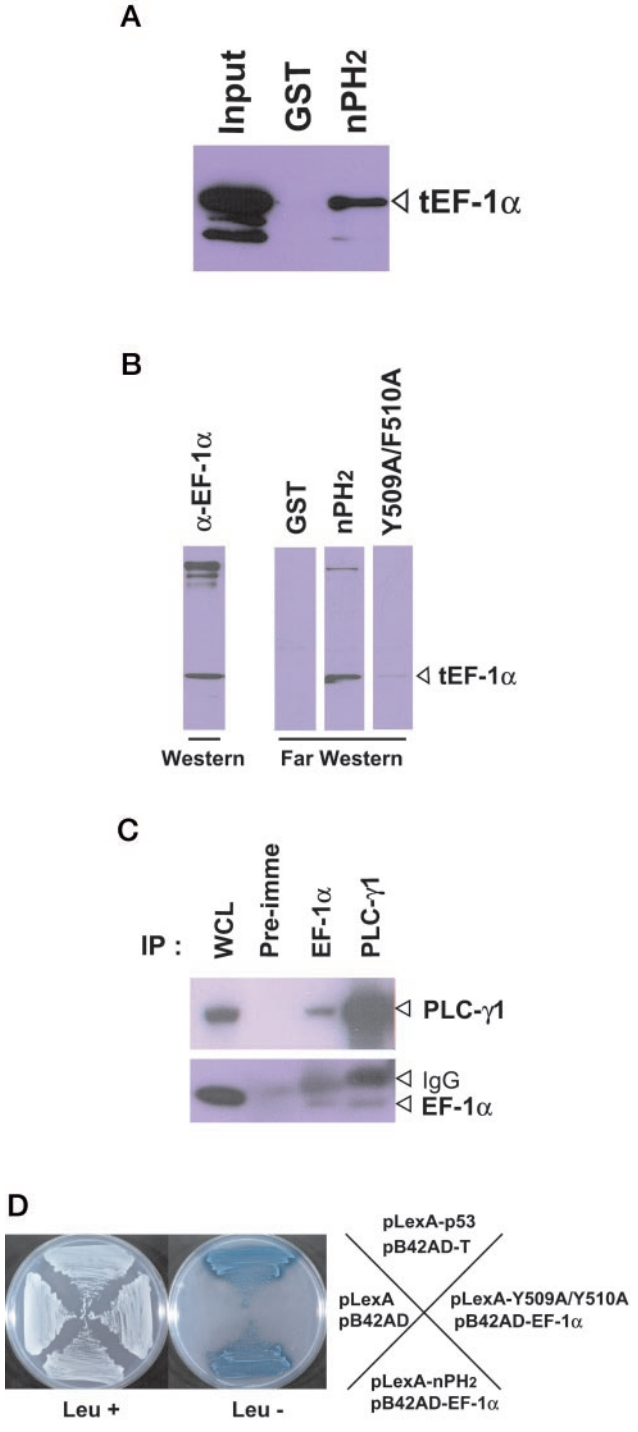

FIG. 2. **nPH_2 domain of PLC-γ1 directly binds to EF-1α.** *A*, GST·nPH_2 fusion protein was incubated with purified *Tetrahymena* EF-1α (tEF-1α) in Nonidet P-40 buffer containing 1% bovine serum albumin. The bound tEF-1α was subjected to immunoblotting with anti-EF-1α antibody. *B*, purified tEF-1α (0.2 μg/lane) was subjected to 10% SDS-PAGE. The protein was then transferred to a nylon membrane and probed with either anti-EF-1α antibody (*left*, Western blot) or purified GST, GST·nPH_2 and GST·nPH_2·Y509A/F510A proteins (*right*, Far Western blot), respectively. *Left panel*, the filter was probed with HRP-conjugated goat anti-mouse antibody. *Right panel*, the filter was incubated with anti-GST antibody followed by HRP-conjugated donkey anti-goat antibody. *C*, immunoprecipitation (*IP*) analysis. COS-7 cell lysate was immunoprecipitated using anti-EF-1α antibody and anti-PLC-γ1 antibody (F-7), and the immunoprecipitates were subjected to immunoblotting with anti-PLC-γ1 antibody (*upper*) or anti-EF-1α antibody (*lower*). *WCL*, the whole cell lysates used for each pull-down experiment. *D*, yeast two-hybrid assay. cDNAs from GST·nPH_2 and GST·nPH_2·Y509A/F510A, respectively, were inserted into the *Eco*RI/*Xho*I site of the pGilda LexA vector (CLONTECH). A full-length EF-1α cDNA was inserted into the *Eco*RI/*Xho*I site of the pB42AD (activation domain) vector (CLONTECH). The yeast two-hybrid assay was carried out according to the manufacturer's specifications.

GST·nPH_2, GST·mutant (Y509A/F510A) nPH_2 proteins (0.5 μg/ml), respectively, in blocking buffer for 14 h at 4 °C. After washes with TBT buffer, the membranes were incubated with anti-EF-1α monoclonal antibody and anti-GST antibody for 2 h at room temperature, respectively. After washing the membrane again with TBT buffer extensively, bound proteins were detected by successive incubation with HRP-conjugated anti-mouse for EF-1α and anti-goat antibody as second antibody for GST fusion proteins using the ECL detection system, respectively.

RESULTS

A Split PH Domain of PLC-γ1 Directly Binds to EF-1α—PLC-γ1 has two putative PH domains in the molecule. To search for proteins that specifically bind to the PH domains of PLC-γ1, we prepared three kinds of GST·PH domain fusion proteins in *Escherichia coli* (GST·PH_1, GST·nPH_2, and GST·cPH_2). These purified GST·PH fusion proteins were incubated with lysate of NIH 3T3 cells, respectively (Fig. 1, *A* and *B*). Among them, GST·nPH_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 lysylendopeptidase digestion. We obtained two peptide sequences, P1 and P2. P1 is YYVTIIDAPGHRDFIK, and P2 is TGHLIYK. When these sequences were searched by the NCBI data base of SWISS-PLOT, they were found to match 58 species of EF-1α sequence reported. P1 and P2 correspond to the 105–120th and 24–30th amino acids of human EF-1α, respectively. Then we confirmed the band with 48 kDa as EF-1α with Western immunoblotting using anti-EF-1α monoclonal antibody (Fig. 1*C*). To further clarify the binding region of PLC-γ1 to EF-1α, we examined binding capacity using several other GST fusion proteins including GST·PH_1, GST·cPH_2, GST·SH2, GST·SH3, GST·PH·GAP, and PH·GAP. As shown in Fig. 1*C*, only GST·nPH_2 associates with EF-1α, as judged by Western immunoblotting. We next tested whether the binding is direct or not; Far Western blotting using purified protozoan *T. pyriformis* EF-1α (tEF-1α) was used for this. Since EF-1α is highly conserved and has very similar biochemical properties among different species in eukaryotes (28), we used tEF-1α due to its success with purification steps with high purity (37). The result of Far Western blotting clearly showed a direct binding between GST·nPH_2 and tEF-1α (Fig. 2, *A* and *B*). Moreover, a double point mutation in GST·nPH_2 fusion protein (Y509A/F510A) lost its binding affinity to tEF-1α (Fig. 3, *A* and *B*). To confirm the interaction between the PH domain and EF-1α *in vivo*, immunoprecipitation was carried out to detect a PLC-γ1·EF-1α complex in COS-7 cells. The immunoprecipitates of EF-1α isolated by anti-EF-1α antibody included PLC-γ1, detected by Western immunoblot by anti-PLC-γ1 antibody or vice versa (Fig. 2*C*). Also, the yeast two-hybrid assay was introduced to show *in vivo* interaction between EF-1α and the nPH_2 domain of PLC-γ1 (Fig. 2*D*). The mutant nPH_2 domain did not bind to EF-1α in either yeast two-hybrid assay. These results clearly demonstrate that the nPH_2 domain of PLC-γ1 directly binds to EF-1α.

β2-Sheet of nPH_2 Domain Is Critical for Binding to EF-1α—Fine mapping of the EF-1α binding site was carried out within the nPH_2 domain of PLC-γ1. 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 β2- and β3-sheet (Fig. 3*A*). Several mutants of the nPH_2 domain induced by site-directed mutagenesis were expressed as GST fusion proteins, mixed with lysates of NIH 3T3 cells, pulled down, and sub-

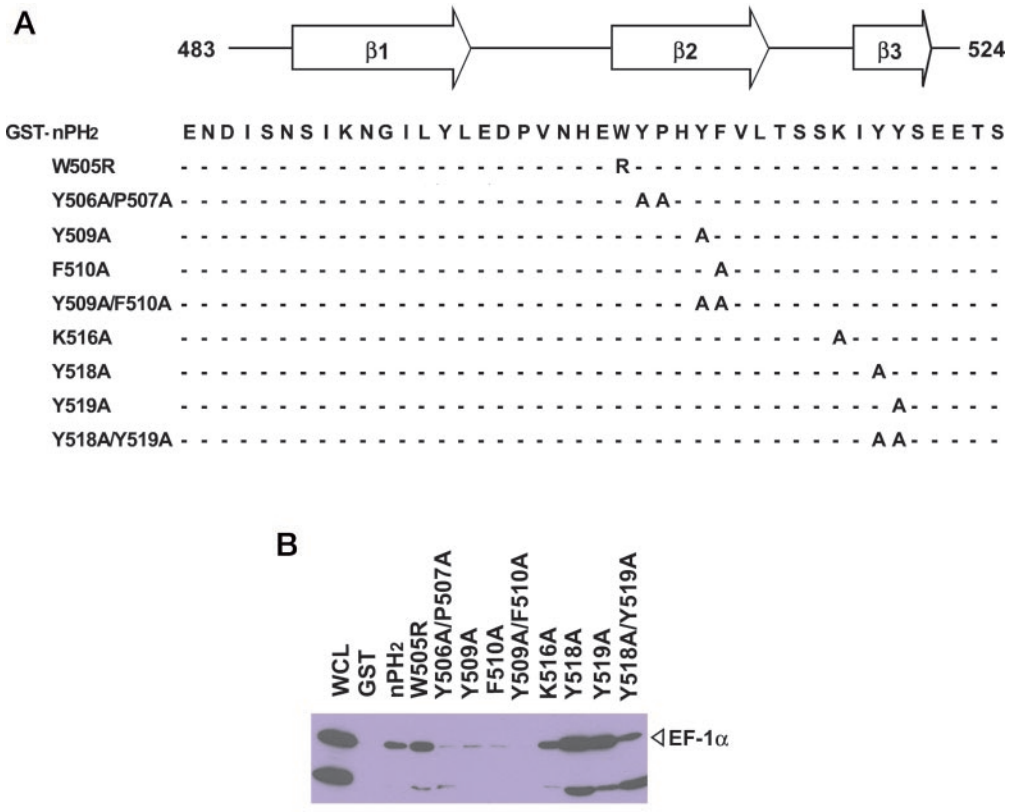

FIG. 3. **Mapping of the EF-1α binding site within the nPH_2 domain of PLC-γ1.** *A*, schematic representation of the elements of the secondary structure. Amino acid sequences of the nPH_2 domain (corresponding to the β-sheets 1–3) of PLC-γ1 are depicted with the single-letter codes. Positions of the mutated amino acids are shown *under* the wild type amino acids. *B*, *in vitro* binding assay with various mutant GST·nPH_2 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.

jected to immunoblotting with anti-EF-1α antibody. The results demonstrate that amino acid residues Tyr-509 and Phe-510 of the β2-sheet play critical roles in the interaction between EF-1α and the PH domain of PLC-γ1 (Fig. 3*B*). A double point mutant Y509A/F510A completely abolishes the interaction, and another double point mutant Y506A/P507A of the β2-sheet of PLC-γ1 also shows reduced binding affinity to EF-1α. However, the truncated mutant GST·$tnPH_2$ (amino acids 495–547) that lacks a portion of the β1-sheet has an equal binding capacity to that of nPH_2 (amino acids 477–547) (data not shown). These results indicate that the association between EF-1α and the nPH_2 domain of PLC-γ1 is due to hydrophobic interaction via the β2-sheet of PLC-γ1.

nPH_2 Domain of PLC-γ1 Specifically Binds to PIP and PIP_2—To explore the binding region of PLC-γ1 to phosphoinositides, we used GST·nPH_2, mutant GST·nPH_2·Y509A/F510A, GST·EF-1α, and GST proteins for dot-blotting (lipid-protein blotting). Different lipids (each 3 μg) including PE, PIP, and PIP_2 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 GST·nPH_2·Y509A/F510A without binding affinity for EF-1α binds to PIP and PIP_2 with a similar capacity as wild type GST·nPH_2, whereas GST·EF-1α and GST as a control do not show any binding capacity to phospholipids. Also, purified tEF-1α did not show any binding affinity to phospholipids (data not shown). These results suggest that the nPH_2 domain of PLC-γ1 has different binding sites for EF-1α and phospholipid. It is noteworthy that the nPH_2 region serves as a substrate PIP_2-binding site, whereas the NH_2-terminal PH domain (PH_1) of PLC-γ1 has been reported to interact with PIP_3 for membrane-targeted translocation (25).

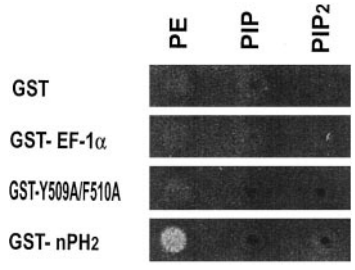

FIG. 4. **Dot-blot assay of GST-fusion proteins with phospholipids.** Each chloroform-solubilized phospholipid (3 μg) was spotted on nitrocellulose membrane for Dot-blot assay. The filter was incubated with 0.5 μg/ml GST or GST fusion proteins for 14 h at 4 °C. The phospholipid-bound proteins were detected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

PIP_2 Potentiates the Binding Affinity of PH Domain to EF-1α—Since both substrate PIP_2 and EF-1α bind to the nPH_2 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 nPH_2 domain of PLC-γ1 and EF-1α significantly increased in the presence of PIP_2 but not in either PE or PIP up to its concentration of 100 μg/ml phospholipid (Fig. 5). Complex formation of GST·nPH_2·EF-1α increased in a PIP_2 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

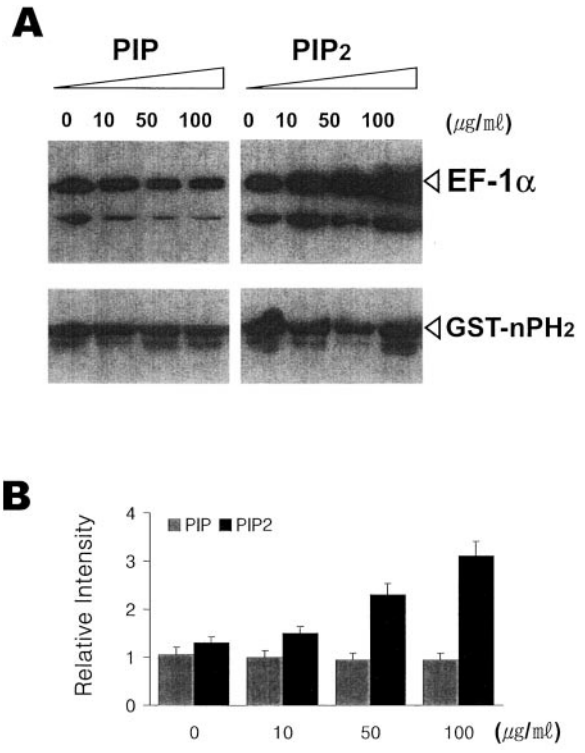

FIG. 5. **PIP_2 increases binding activity of GST·nPH_2 domain to EF-1α.** *A*, GST·nPH_2 (4 μg)-coupled beads incubated with NIH 3T3 cell lysate in the presence of 0, 10, 50, or 100 μg/ml sonicated phospholipid vesicles. Each resulting bead was divided into two equal portions for measuring the bound EF-1α (*upper*) and for normalization by amounts of GST·nPH_2 proteins (*lower*), respectively. Both proteins were accessed by immunoblotting with anti-EF-1α antibody or by anti-GST antibody followed by the second antibody. *B*, amounts of bound EF-1α expressed by relative image density (Quantity One, Bio-Rad), which was normalized by GST·nPH_2. The experiments were carried out three times with similar results.

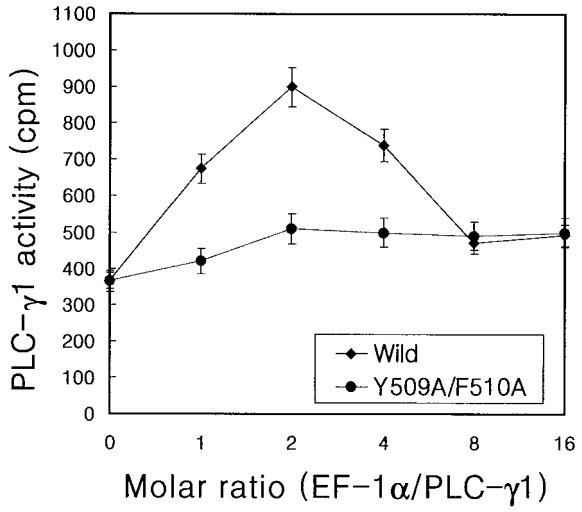

FIG. 6. **Modulation of PLC-γ1 activity in the presence of EF-1α.** PIP_2 hydrolyzing activity of PLC-γ1 and its Y509A/F510A mutant form was measured in an assay mixture containing various amounts of EF-1α as indicated by the molar ratio to PLC-γ1. PLC-γ1 activity is expressed as the radioactivity of amounts of [^{3}H]IP_3. Data represent the average of duplicate determination (mean ± range) from one of the two experiments with similar results.

mutant PLC-γ1 (Y509A/F510A) at 4 °C for 1 h, [^{3}H]PIP_2 hydrolyzing activity was measured. As shown in Fig. 6, EF-1α activates wild type PLC-γ1 activity in a bell-shaped manner, whereas mutant PLC-γ1 (Y509A/F510A) shows basal level activity even in the presence of EF-1α. The activity of wild type PLC-γ1 is accelerated about 3-fold under the condition of 1:2 molar ratio (PLC-γ1 to EF-1α).

DISCUSSION

Many reports have described the activation mechanisms and functional roles of PLC-γ1 in cellular signaling. Generally, growth factor stimulation leads to the binding of the SH2 domains of PLC-γ1 to the autophosphorylated receptor, and then PLC-γ1 is subsequently activated by tyrosine phosphorylation of Tyr-783 followed by PIP_2 hydrolysis to IP_3 and diacylglycerol. However, the degree of tyrosine phosphorylation of PLC-γ1 does not correlate well with its enzyme activity. For example, some ligands strongly stimulate tyrosine phosphorylation of PLC-γ1 with low IP_3 production (6), and some ligands highly induce the production of IP_3 with weak tyrosine phosphorylation of PLC-γ1 (7). There might be alternative controlling mechanism(s) of PLC-γ1 activity in cellular signaling. To identify a responsible molecule(s) for regulation of PLC-γ1, we have searched for binding proteins to PLC-γ1 and found EF-1α, a PI-4 kinase activator.

We showed that an NH_2-terminal split PH domain of PLC-γ1 specifically binds to EF-1α and that PIP_2, a substrate of PLC-γ1, increases its association with EF-1α. It is noteworthy that the strict region of PLC-γ1 plays a role for protein-protein interaction other than the SH2 and SH3 domains in PLC-γ1. 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·nPH_2 using a detergent lysate of NIH 3T3 cells, EF-1α was identified by peptide sequence analysis. The association between the nPH_2 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 PIP_2, a PLC-γ1 substrate, to the incubation mixture of the GST·nPH_2 domain fusion protein and cell extracts containing EF-1α, the complex formation of GST·nPH_2·EF-1α, was dramatically increased in a PIP_2 concentration-dependent manner. On this point, it is interesting that the stable complex between PLC-γ1 and its substrate PIP_2 was detected by Dot-blot analysis (Fig. 4). Therefore, we can speculate that the PH domain of PLC-γ1 associates with PIP_2 first, and the PH domain/PIP_2 complex formation induces the conformational change to allow EF-1α to bind PLC-γ1. EF-1α binding to PLC-γ1 might facilitate the hydrolysis of PIP_2 by PLC-γ1. In this context, the role of PLC-γ1-bound EF-1α is a possible regulator for PIP_2 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 Ca^{2+}/CaM molecules are contaminated in EF-1α preparation. Although the preparation of tEF-1α is highly pure, the contamination of Ca^{2+}/CaM molecules or other components could not be completely excluded. Generally, EF-1α preparation contains Ca^{2+}/CaM molecules to some extent (40, 45). In this regard, Ca^{2+}/CaM might sequester Ca^{2+} supplements for maximal PLC-γ1 activity at high doses of EF-1α addition.

EF-1α promotes the production of PIP and PIP_2 by the activation of PI-4 kinase, and eventually this newly produced PIP_2 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 diacylglycerol. 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 from epidermal 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 lysophosphatidic acid increased their complex formation around the cell membrane.[2] 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.

Acknowledgment—We thank Dr. Takuji Shirasawa (Tokyo Metropolitan Institute of Gerontology) for providing rat EF-1α cDNA.

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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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J. Biol. Chem. 2002, 277:19697-19702.
doi: 10.1074/jbc.M111206200 originally published online March 8, 2002
