Pleckstrin Homology Domains of Phospholipase C-γ1 Directly Interact with β-Tubulin for Activation of Phospholipase C-γ1 and Reciprocal Modulation of β-Tubulin Function in Microtubule Assembly*

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Phosphoinositide-specific phospholipase C-γ1 (PLC-γ1) has two pleckstrin homology (PH) domains, an N-terminal domain and a split PH domain. Here we show that pull down of NIH3T3 cell extracts with PLC-γ1 PH domain-glutathione S-transferase fusion proteins, followed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, identified β-tubulin as a binding protein of both PLC-γ1 PH domains. Tubulin is a main component of microtubules and mitotic spindle fibers, which are composed of α- and β-tubulin heterodimers in all eukaryotic cells. PLC-γ1 and β-tubulin colocalized in the perinuclear region in COS-7 cells and cotranslocated to the plasma membrane upon agonist stimulation. Membrane-targeted translocation of depolymerized tubulin by agonist stimulation was also supported by immunoprecipitation analyses. The phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolyzing activity of PLC-γ1 was substantially increased in the presence of purified tubulin in vitro, whereas the activity was not promoted by bovine serum albumin, suggesting that β-tubulin activates PLC-γ1. Furthermore, indirect immunofluorescent microscopy showed that PLC-γ1 was highly concentrated in mitotic spindle fibers, suggesting that PLC-γ1 is involved in spindle fiber formation. The effect of PLC-γ1 in microtubule formation was assessed by overexpression and silencing PLC-γ1 in COS-7 cells, which resulted in altered microtubule dynamics in vivo. Cells overexpressing PLC-γ1 showed higher microtubule densities than controls, whereas PLC-γ1 silencing with small interfering RNAs led to decreased microtubule network densities as compared with control cells. Taken together, our results suggest that PLC-γ1 and β-tubulin transmodulate each other, i.e. that PLC-γ1 modulates microtubule assembly by β-tubulin, and β-tubulin promotes PLC-γ1 activity.

PLC-γ1 is an important signaling molecule for cell proliferation and differentiation. Activated PLC-γ1 hydrolyzes PIP2 to produce inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. These second messengers regulate the release of Ca2+ from intracellular stores and activate protein kinase C, respectively (1, 2). PLC-γ1 has two Src homology (SH) 2 domains and an SH3 domain, both responsible for protein-protein interactions, and two pleckstrin homology (PH) domains, responsible for protein-protein and protein-lipid interactions. Of these, one PH domain is located in the 150 N-terminal amino acid residues, whereas the other is split by the SH2-SH2-SH3 domain (3). PH domains bind with high specificity and affinity to phosphoinositides such as phosphatidylinositol phosphate, PIP3, phosphatidylinositol 1,4,5-trisphosphate, and IP3 (4, 5), and the PH domains of signaling molecules are often involved in targeted translocation of molecules to cell membranes (6, 7). PH domains can also specifically bind cellular signaling proteins, such as the βγ-subunit of the heteromeric G-protein (8) and protein kinase C (9). Although numerous studies have investigated protein-protein interactions via the SH domain of PLC-γ1, the PH domain-mediated signaling of PLC-γ1 has not yet been elucidated. Here we sought to identify proteins that specifically associate with the PLC-γ1 PH domains in cells, and we found that both PH domains of PLC-γ1 specifically bind to β-tubulin.

Heterodimers of α- and β-tubulin are essential cytoskeletal components of the microtubules in all eukaryotes. Microtubules regulate cell division, cell shape, and cell motility via cycles of tubulin polymerization and depolymerization called microtubule instability (10, 11). The mitotic spindle, which is a dynamic array of microtubules, is responsible for chromosome segregation into daughter cells during mitosis. Therefore, numerous studies have sought to identify proteins controlling

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1 The abbreviations used are: PLC-γ1, phospholipase C-γ1; PH, pleckstrin homology; GST, glutathione S-transferase; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PIP2, phosphatidylinositol 4,5-bisphosphate; BSA, bovine serum albumin; siRNA, small interfering RNA; IP3, inositol 1,4,5-trisphosphate; SH, Src homology; HRP, horseradish peroxidase; FBS, fetal bovine serum; PVDF, polyvinylidene difluoride; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; GFP, green fluorescent protein; mAb, monoclonal antibody; MES, 4-morpholineethanesulfonic acid; EF, elongation factor; GEF, guanine nucleotide exchange factor; GIF, S-3-O-thio triphosphate.
microtubule instability. Two major groups of these proteins have been identified. The microtubule destabilizers, capable of depolymerizing microtubules into α- and β-tubulin heterodimers, include XKCM1 (12), XKIF2 (13), stathmin/Op18 (14), and katanin (15). The microtubule stabilizers, which polymerize tubulin heterodimers into microtubules, include Ran (16), CRM-P-2 (17), Tau (18), and microtubule-associated protein (MAP) (19). Recently, a third group has been identified as regulating microtubule assembly. This group includes Hsp90 (20), Gβγ subunit (21), G-protein coupled receptor kinase-2 (22), and pyruvate kinase (23).

Because PLC-γ1 plays pivotal roles in the cellular signaling pathways responsible for triggering the production of second messengers including Ca2+ and IP3, and because overexpression of PLC-γ1 results in cellular transformation (24), it is critical to identify the proteins that regulate activity of PLC-γ1. Here we sought to identify proteins that specifically interact with the PH domains of PLC-γ1, and we showed for the first time that β-tubulin can bind both PH domains of PLC-γ1. In addition, we determined that PLC-γ1 interacts with β-tubulin transmodeulate each other in vivo.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Monoclonal anti-β-tubulin and anti-α-tubulin were purchased from Chemicon (Temecula, CA) and Sigma, and polyclonal anti-β-tubulin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-FLAG M5, polyclonal anti-PLC-γ1, and highly purified tubulin from bovine brain were obtained from Sigma. Fluorescein-conjugated Affinipure goat anti-rabbit IgG and rhodamine-conjugated Affinipure goat anti-mouse IgG were from Jackson ImmunoResearch (West Grove, PA).

**Gel Electrophoresis with GST Fusion Proteins**—By using rat PLC-γ1 cDNA (25) as the template, GST constructs for fusion proteins were generated by PCR as described previously (26). GST fusion proteins were expressed in Escherichia coli, and the lysates were incubated with glutathione-Sepharose beads, then washed extensively with Igepal buffer (20 mM Tris-Cl, pH 7.5, 1% Igepal CA-630, 300 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), resolved by 10% SDS-PAGE, and transferred onto a PVDF membrane. The membrane-bound proteins were detected with an ECL detection system (Amersham Biosciences) using monoclonal anti-FLAG and HRP-conjugated goat anti-mouse antibodies.

**DNA Construction and Expression**—PCR-amplified mouse cDNAs encoding tubulin isotypes (Mθ, Mθ; Mβ, Mγ, and Mδ, kindly provided by Dr. Sally Lewis, New York University) (27) were ligated into the EcoRI/Sall restriction sites of the N-terminal epitope tagging vector, pFLAG-CMV-2 (Sigma). When expressed, all α- and β-tubulin cDNAs encoded an N-terminal 9-amino acid FLAG epitope tag. Similarly, the rat cDNA encoding PLC-γ1 was inserted into the HindIII/Xbal sites of the pFLAG-CMV-2 vector for mammalian expression. For localization studies in COS-7 cells, we constructed vectors in which the enhanced green fluorescent protein (GFP) was fused with the PH domains of PLC-γ1. cDNAs encoding the PH domains of PLC-γ1 were subcloned into the EcoRI/Xbal sites of pEGFP-C2 (Clontech). Expression of GFP-PH-1, (amino acids 25–145 of PLC-γ1) and GFP-PH-2, (amino acids 477–547 of PLC-γ1) was assessed by immunofluorescence microscopy and immunoblotting using an anti-GFP antibody (Zymed Laboratories Inc.). All constructs were expressed in Escherichia coli, and the lysate supernatants were precleared by incubation with anti-FLAG M2 antibodies for 2 h at room temperature. The membranes were then incubated with the GST, GST-PH-1, GST-PH-2, or GST-SH3 fusion proteins (0.5 μg/ml) in blocking buffer for 14 h at 4 °C. After washing in TBS buffer, the membranes were incubated with anti-GST antibody for 2 h at room temperature. The blots were then washed and bound proteins were detected by incubation with a secondary HRP-conjugated anti-goat antibody and visualized with an ECL detection system.

**Expression and Purification of PLC-γ1**—The cDNAs for the wild type PLC-γ1 were introduced into the HindIII/Xbal sites of the pFLAG-CMV-2 vector. The recombinant PLC-γ1 proteins contained a 9-amino acid FLAG epitope at their N termini. COS-7 cells grown on 100-mm culture dishes were transfected with 5 μg of recombinant plasmid and 10 μl of Lipofectamine (Invitrogen) according to the manufacturer's specifications. Two days after transfection, cells were harvested, and the recombinant PLC-γ1 proteins were recovered using a pFLAG-CMV-2 purification kit (Sigma).

**Immunoprecipitation and Immunoblotting**—Cells transfected with plasmids encoding various tubulin isotypes (Mθ, Mθ; Mβ, Mγ, and Mδ) and one of the following: 2 μg of the pFLAG-CMV-2-PLC-γ1 vector, small interfering RNA (siRNA) for PLC-γ1, or empty pFLAG-CMV-2 vector. The siRNAs for human PLC-γ1 were purchased from Dharmacon Inc. (Lafayette, CO); the SMARTpool siRNAs consisted of combinations of GGAAAGACGACGTGTTGTT, CCAACACGCTTAAGGAGAA, GAAGTGACACGTGGTCA, and GACGACGTCCCTTTAGAAGA. Following transfection, the cells were grown and doubly transfected with Dharmacon's modified Eagle's medium with 0.5% FBS. For agonist stimulation, the cells were serum-starved for at least 30 h and then stimulated with either 20% FBS (Invitrogen) or 0.2 μg/ml EGF (Sigma) for 10–20 min. The cells were fixed at 37 °C for 10 min in 4% paraformaldehyde and then incubated with affinity-purified monoclonal anti-FLAG or polyclonal anti-PLC-γ1 antibodies for 2 h at room temperature. Anti-GFP antibody and visualized with an ECL detection system.

**Immunofluorescent Microscopy**—COS-7 cells were seeded on glass coverslips in 6-well plates and transfected with 5 μg of recombinant plasmid and one of the following: 2 μg of the pFLAG-CMV-2-PLC-γ1 vector, small interfering RNA (siRNA) for PLC-γ1, or empty pFLAG-CMV-2 vector. The siRNAs for human PLC-γ1 were purchased from Dharmacon Inc. (Lafayette, CO); the SMARTpool siRNAs consisted of combinations of GGAAAGACGACGTGTTGTT, CCAACACGCTTAAGGAGAA, GAAGTGACACGTGGTCA, and GACGACGTCCCTTTAGAAGA. Following transfection, the cells were grown and doubly transfected with Dharmacon's modified Eagle's medium with 0.5% FBS. For agonist stimulation, the cells were serum-starved for at least 30 h and then stimulated with either 20% FBS (Invitrogen) or 0.2 μg/ml EGF (Sigma) for 10–20 min. The cells were fixed at 37 °C for 10 min in 4% paraformaldehyde and then incubated with affinity-purified monoclonal anti-FLAG or polyclonal anti-PLC-γ1 antibodies for 1 h at room temperature in a humidity chamber. Following complete washing with phosphate-buffered saline, the cells were incubated with fluorescein-conjugated goat anti-rabbit IgG or rhodamine-conjugated Affinipure goat anti-mouse IgG. Immunostained cells were observed with a fluorescent microscope (Nikon Eclipse E600 Epifluorescence Microscope), and the images were captured with a digital image microscope camera.

**Mass Spectrometry**—Gel slices corresponding to the appropriate protein bands were excised and destained before being digested with 50% acetonitrile in 25 mM NH4HCO3. The gel slices were then incubated overnight with trypsin (Promega) in 25 mM NH4HCO3 at 37 °C. The resulting peptides were eluted with matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid, and 50% acetonitrile) and applied to the MALDI target plate. Peptide molecular weights were measured on a MALDI-TOF mass spectrometer (Voyager-DE STR, Applied Biosystems, Inc.). Peptide mass maps were searched against theoretically derived maps from proteins found in...
Interaction of PLC-γ1 PH Domains and β-Tubulin

The PH Domains of PLC-γ1 Directly Bind to β-Tubulin—To identify new PLC-γ1-associated proteins, the two PH domains of PLC-γ1 were fused to GST and used for pull-down assays. GSH-Sepharose-coupled GST-PH₁ and GST-nPH₂ proteins were incubated with NIH3T3 cell lysates (Fig. 1A), using the technique that had been used previously to isolate eukaryotic translational elongation factor (EF)-1α as a PH domain-associated protein (26). Because the GST-cPH₂ fusion protein did not show any visible protein band on our SDS-polyacrylamide gel (26), we used the GST-PH₁ and GST-nPH₂ fusion proteins in this experiment. As shown in Fig. 1A, the GST-nPH₂ fusion protein specifically pulled down a prominent 48-kDa protein band that had been identified previously as EF-1α (26). In addition, both the GST-PH₁ and GST-nPH₂ fusion proteins specifically pulled down a 55-kDa protein that was not pulled down by the control GST fusion protein. This band was excised from the gel and analyzed by MALDI-TOF-mass spectrometry.

Table 1 shows the peptide sequences and mass data derived from a trypsin digest of the 55-kDa protein. When the peptide mass maps were searched against the NCBI protein data base, the 55-kDa protein was identified as mouse β-tubulin. This identification was confirmed by Western blotting with monoclonal anti-β-tubulin (Fig. 1B). We further confirmed that the interaction between PLC-γ1 and β-tubulin occurs in vivo by

![Diagram](image-url)

FIG. 1. β-Tubulin binds both PH domains of PLC-γ1. A, GST-PH domain fusion proteins were incubated with (+) or without (−) NIH3T3 cell lysates, and the bound proteins were resolved by 10% SDS-PAGE. GST-PH₁ and GST-nPH₂ fusion proteins pulled down a 55-kDa protein on the gel as shown by Coomassie Brilliant Blue staining (indicated by the closed arrowhead). WCL indicates the whole cell lysates used for each pull-down experiment. B, the bound proteins were resolved by 10% SDS-PAGE followed by Western blotting with a monoclonal anti-β-tubulin antibody (mAb TU-20). C, immunoprecipitation analysis of the in vivo interaction between β-tubulin and PLC-γ1. PLC-γ1 from COS-7 cell lysates was coimmunoprecipitated with β-tubulin (upper panel) and β-tubulin was coimmunoprecipitated with PLC-γ1 (lower panel). mAb TU-20 and mAb F-7 were used for immunoprecipitation of β-tubulin and PLC-γ1, respectively. D, far Western blot hybridization revealed a direct interaction between PLC-γ1 and β-tubulin. Purified tubulin from bovine brain (0.2 μg per lane) was resolved on a 10% SDS-PAGE. The protein was then transferred to a nylon membrane and probed with either anti-β-tubulin antibody (left) or purified GST, GST-PH₁, GST-nPH₂, and GST-SH3 fusion proteins, respectively. The filters were then probed with either HRP-conjugated goat anti-mouse antibody (left) or anti-GST antibody followed by HRP-conjugated donkey anti-goat antibody. E, sedimentation analysis revealed the interaction of microtubules and PLC-γ1. Supernatants (S15) and precipitates (P15) were separated at 15,000 × g, resolved by 10% SDS-PAGE, and visualized by Coomassie Brilliant Blue staining (upper panel). PLC-γ1 and tubulin were co-polymerized in polymerizing-depolymerizing cycles. PLC-γ1 in the supernatant (sup) or pellet (ppt) of 100,000 × g fraction at each step was detected by Western blotting using anti-PLC-γ1 antibody (lower).
The translocation of PLC-γ1 in COS-7 cells. Generally, agonist treatment results in cells. Overlays of the fluorescent microscopy images showed with tubulin in the supernatant and pellet fractions (Fig. 1 treated with 20% FBS or EGF for 10 min, both PLC- agonist stimulation, we performed double immunostaining and 

| Peptide sequence | Start-end | Monoisotopic mass (M+H+) |
|------------------|-----------|-------------------------|
| FWEVSDSHEGDTPTGTYHGSDLGLER | 11–37 | 3115.42 | 3115.37 |
| SGPFGQFPRDPVFPGGSGAGNNWAK | 69–94 | 2797.34 | 2797.30 |
| GHTYEGAEVLSVLDVVR | 95–112 | 1957.97 | 1957.97 |
| LTPPTYGDNLHILVATMSGVTTCCLR | 206–232 | 2707.33 | 2707.29 |
| FPGQLNADER | 233–242 | 1129.59 | 1129.62 |
| LAVMMYPPPR | 244–253 | 1142.63 | 1142.65 |
| LHFMPGFQAPLTSR | 254–267 | 1619.83 | 1619.84 |
| YLTVAAVFR | 301–309 | 1038.59 | 1038.61 |
| NSSYFVEWIPNVK | 328–341 | 1856.93 | 1856.94 |
| MSATFIGNSTAIQELFK | 354–370 | 1695.83 | 1695.83 |
| ISEQTMAMP | 372–381 | 1228.59 | 1228.61 |

Amino acid residues derived from the 55-kDa protein.

Position of the amino acid residue in the deduced peptide sequence of mouse β-tubulin.

Interactions of PLC-γ1 PH Domains with β-Tubulin

Table I

| Tryptic peptides of β-tubulin identified by MALDI-TOF |
|------------------------------------------------------|
| Peptide sequence | Start-end | Monoisotopic mass (M+H+) |
|------------------|-----------|-------------------------|
| FWEVSDSHEGDTPTGTYHGSDLGLER | 11–37 | 3115.42 | 3115.37 |
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Amino acid residues derived from the 55-kDa protein.

Position of the amino acid residue in the deduced peptide sequence of mouse β-tubulin.
Western blotting, whereas the other domains (GST-SH2N, -SH2C or -SH3) did not. Most interestingly, H9251-tubulin did not bind to the PLC-H92531 PH domains in our in vitro binding assay (Fig. 4C) or immunoprecipitation (Fig. 5A) experiments.

Serum and EGF Enhances PLC-γ1-β-Tubulin Complex Formation in COS-7 Cells—Although the exact mechanism for PLC-γ1 activation is not yet clearly understood, it has been reported that stimulation with growth factors including EGF and PDGF resulted in PLC-γ1 activation in various cell lines (30, 31). Generally, as described above, agonist stimulation results in translocation of PLC-γ1 from the cytosol to the plasma membrane, where tyrosine phosphorylation of PLC-γ1 by its receptors activates hydrolysis of PIP2. Alternatively, PLC-γ1 could be activated by PLC-γ1-activating proteins, such as Tau (32), AHNAK (33, 34), and EF-1α (26). Hence, it is critical to examine whether the level of complex formation is modulated by agonist treatment. To examine the effects of agonist stimulation on the interaction between β-tubulin and PLC-γ1 in vivo, FLAG-Mβ-tubulin isotypes were transiently expressed in COS-7 cells. The cells were then stimulated with the agonist, and coimmunoprecipitation of PLC-γ1 with the FLAG-Mβ-tubulin isotypes was assessed. As shown in Fig. 5A, 20% FBS treatment augmented the amount of FLAG-Mβ2-tubulin coimmunoprecipitated with the anti-PLC-γ1 antibody.

β-tubulin and a polyclonal anti-PLC-γ1 antibody followed by rhodamine- and fluorescein isothiocyanate-labeled secondary antibody staining, respectively. B and C, COS-7 cells treated with either 20% FBS (B) or 0.2 µg/ml EGF (C) for 10 min were stained with anti-β-tubulin and anti-PLC-γ1 antibodies as described above. D, double immunofluorescence of mitotic COS-7 cells, prepared as in A.

Fig. 2. Colocalization of PLC-γ1 and β-tubulin in COS-7 cells. A, double immunostaining of β-tubulin and PLC-γ1. Serum-deprived quiescent COS-7 cells were fixed and stained with a monoclonal anti-
In this experiment, all β isoforms of mouse tubulin showed similar results (data not shown). Following EGF treatment, Mβ1, Mβ2, Mβ3, and Mβ6 all showed time-dependent increases in coimmunoprecipitation with PLC-γ1 (Fig. 5B). These results are consistent with the subcellular localization of both proteins (Fig. 2, B and C, and Fig. 3A) and suggest that Mβ-tubulin but not Mα-tubulin might promote PLC-γ1 activity. Furthermore, when we examined the effects of taxol, which accelerates microtubule formation by β-tubulin heterodimers and inhibits microtubule depolymerization, the amount of complex formation between β-tubulin and PLC-γ1 was dramatically decreased in comparison to that in control cells, suggesting that taxol inhibits translocation of tubulin to the plasma membrane (data not shown).

β-Tubulin Activates PLC-γ1 Activity—To examine whether the complex formation affects PLC-γ1 activity, we measured the PIP2 hydrolyzing activity of PLC-γ1 in the presence of purified bovine tubulin. The purified bovine α- and β-tubulin heterodimers were pre-incubated with PLC-γ1 at 4 °C for 1 h, and then [3H]PIP2 hydrolyzing activity was measured. As shown in Fig. 6, bovine tubulin substantially promoted PLC-γ1 activity, whereas purified BSA (control) did not. We performed activity assays with 10 and 100 ng of PLC-γ1 (Fig. 6), and we found that both concentrations of PLC-γ1 showed about 1.6-fold increases in activity in the presence of the tubulin heterodimers.

PLC-γ1 Modulates Microtubule Assembly in COS-7 Cells—Because purified tubulin promotes PLC-γ1 activity via protein-protein interactions, we next examined the effects of PLC-γ1 on microtubule assembly by overexpression and silencing of PLC-γ1 in COS-7 cells. PLC-γ1 cDNAs or siRNAs for overexpression or silencing were transiently transfected into COS-7 cells, which were then examined by double immunostaining with anti-PLC-γ1 and anti-β-tubulin antibodies. The expression levels of PLC-γ1 in cDNA- or siRNA-transfected COS-7 cells were examined by Western blotting with the anti-PLC-γ1 antibody (Fig. 7A). The PLC-γ1-overexpressing cells were found to express ~5-fold the amount of PLC-γ1 as compared with control cells, whereas the PLC-γ1-silenced cells expressed 0.5-fold the normal levels. As shown in Fig. 7B, cells overexpressing PLC-γ1 showed a higher intensity of microtubule staining than did control cells, suggesting that PLC-γ1 affects microtubule assembly. In contrast, PLC-γ1 silencing resulted in a diffuse microtubule network as compared with control cells (Fig. 7C). Thus, in the PLC-γ1-altered COS-7 cells, microtubule assembly appeared to be proportional to the amount of PLC-γ1.

Furthermore, quantitative Western immunoblotting of the polymerized tubulin pool and depolymerized tubulin pool in PLC-γ1-altered cells showed that PLC-γ1 regulates tubulin assembly in vivo (Fig. 7D). When we compared monomeric (or heterodimeric) and polymeric tubulin levels in PLC-γ1-altered cells by Western immunoblotting, overexpression of PLC-γ1 resulted in more than 67% polymeric tubulin, and silencing of PLC-γ1 resulted in less than 47% polymeric tubulin. In vector-transfected control cells, the level of polymeric tubulin was...
Similar results were obtained with three different immunoprecipitation experiments. Previously, the N-terminal half of αtubulin isotypes (Mα1 and Mβ2-tubulin). Cells were either unstimulated or treated with 20% FBS for 10 min prior to harvest. PLC-γ1 immunoprecipitates were subsequently subjected to immunoblotting using anti-FLAG (upper panel) and anti-PLC-γ1 (lower panel) antibodies. EGF enhances the amount of PLC-γ1-β-tubulin complex in vivo. After treatment with EGF, the amount of FLAG-β-tubulin (BT) isotypes in PLC-γ1 immunoprecipitates (IP) increased with time as indicated. Similar results were obtained with three different immunoprecipitation experiments. WCL, whole cell lysates.

FIG. 5. Serum and EGF facilitate the interaction of PLC-γ1 with β-tubulin in vivo. A, PLC-γ1 was immunoprecipitated from COS-7 cell lysates transiently overexpressing FLAG-tagged mouse tubulin isotypes (Mα1- and Mβ2-tubulin). Cells were either unstimulated or treated with 20% FBS for 10 min prior to harvest. PLC-γ1 immunoprecipitates were subsequently subjected to immunoblotting using anti-FLAG (upper panel) and anti-PLC-γ1 (lower panel) antibodies. B, EGF enhances the amount of PLC-γ1-β-tubulin complex in vivo. After treatment with EGF, the amount of FLAG-β-tubulin (BT) isotypes in PLC-γ1 immunoprecipitates (IP) increased with time as indicated. Similar results were obtained with three different immunoprecipitation experiments. WCL, whole cell lysates.

Fig. 6. Purified tubulin promotes PLC-γ1 activity. The PIP$_2$-hydrolyzing activity of PLC-γ1 was measured in the presence of tubulin heterodimers. PLC-γ1 and tubulin used in this experiment were purified from COS-7 cells (FLAG-PLC-γ1) and bovine brain extracts (Sigma), respectively. PLC-γ1 activity is expressed as the radioactivity of \textsuperscript{[3]H}IP$_3$. The data represent the average of duplicate determinations (mean ± S.D.) from three experiments with similar results.

~55% (Fig. 7D). These biochemical data indicate that the level of PLC-γ1 is important for microtubule assembly in COS-7 cells. Taken together with our observations of colocalization of PLC-γ1 with the mitotic spindle during cell division (Fig. 2D), these results suggest that PLC-γ1 might promote tubulin polymerization within the cell.

DISCUSSION

Our present study demonstrates that the PH domains of PLC-γ1 interact with β-tubulin in vivo. The PH domains of PLC-γ1 have been shown to facilitate membrane targeting via interactions with phosphoinositide (7) and activate enzymatic activity via protein-protein interactions (26). In this paper, we focused on the roles of PH domains of PLC-γ1 by examination of protein-protein interactions. Previously, the N-terminal half of the split PH domain (nPH$_2$) of PLC-γ1 was shown to specifically bind EF-1α (26) and the βγ-subunit of the small G-protein (Gβγ) (35). This makes β-tubulin the third protein known to associate with the nPH$_2$ domain of PLC-γ1. Most interestingly, all the PLC-γ1 PH domain binding proteins, including EF-1α, Gβγ, and β-tubulin (in this paper), are known as guanine nucleotide-binding proteins; the SH3 domain of PLC-γ1 has been shown to act as a guanine nucleotide exchange factor (GEF) for PIKE (36) and dynamin-1 (37). Although we have not yet explored the functional roles of PLC-γ1 as a GEF for such proteins, these results suggest that PLC-γ1 might be involved in GTP/GDP exchanges with a guanine nucleotide-binding protein in cellular signaling. In addition, we found that PLC-γ1 is reciprocally activated by tubulin heterodimers following agonist stimulation.

Microtubules are composed of α- and β-tubulin heterodimers and are involved in a variety of cellular functions, including spindle fiber formation during mitosis and maintenance/alteration of cell shape. β-Tubulin reversibly binds to GTP to polymerize into a microtubule; its GTPase activity allows the tubulin heterodimer to depolymerize. During the cell cycle, tubulin polymerization and depolymerization are tightly regulated (11, 38). There are six different isotypes of β-tubulin in mammalian cells. We expressed four of these (Mα1, Mβ1, Mβ2, and Mβ3) in COS-7 cells with N-terminal FLAG tags; the transfected cells expressed each isotype with efficient microtubule formation, except in the case of Mβ3, which was unable to form microtubules. As most previous papers have described C-terminal epitope-tagged expression of β-tubulin, this is the first report that N-terminal epitope-tagged β-tubulin could be efficiently polymerized into microtubules in vivo.

In quiescent COS-7 cells, PLC-γ1 and β-tubulin colocalized in the perinuclear region. After treatment of cells with 20% FBS, both proteins clearly translocated to the plasma membrane, indicating that 20% serum stimulation resulted in tubulin depolymerization and translocation to the plasma membrane (Fig. 3, A and B). This membrane translocation of PLC-γ1 was robustly mediated by both the PH$_1$ and nPH$_2$ domains (Fig. 3C). Previous reports (7, 39) had investigated PH$_1$ domain-mediated PLC-γ1 translocation in various cell lines. Here we showed for the first time that a truncated portion of the nPH$_2$ domain of PLC-γ1 was also sufficient to mediate membrane translocation of PLC-γ1 (Fig. 3C). Furthermore, the nPH$_2$ domain predominantly binds to phosphatidylinositol 3-phosphate on an Echelon filter. It also binds to PIP$_2$ (26), although with a negative electrostatic profile (40). Therefore, the nPH$_2$ domain of PLC-γ1 is independently involved in protein-protein and protein-phosphoinositide interactions.

Colocalization of PLC-γ1 and β-tubulin after agonist stimulation was strongly supported by our immunoprecipitation experiments using COS-7 cells before and after 20% serum or EGF stimulation. In our experiments, 20% FBS was much more potent than EGF (0.2 μg/ml) in stimulating PLC-γ1-β-tubulin complex translocation to the plasma membrane and in coimmunoprecipitation experiments in COS-7 cells, suggesting that serum contains unknown agonist(s) for COS-7 cell stimulation, in addition to EGF. Consistent with tubulin translocation, carbachol stimulation resulted in microtubule depolymerization, tubulin translocation to the membrane, and colocalization of tubulin with membrane Gβ in SK-N-SH cells (41).

Based on the above results, we speculated on the biological relevance of the PLC-γ1-β-tubulin interaction. There are two nonexclusive possibilities: (i) β-tubulin regulates PLC-γ1 activity; and (ii) PLC-γ1 affects microtubule assembly. To address

\[ S.-K. Kim and J.-S. Chang, unpublished results. \]
the first possibility, we examined the PIP$_2$ hydrolyzing activity of PLC-$\gamma$1 in the presence or absence of $\beta$-tubulin. In the presence of tubulin (Fig. 6), the PIP$_2$ hydrolyzing activity of PLC-$\gamma$1 was increased about 1.6-fold under the conditions of a 1:2 molar ratio (PLC-$\gamma$1 to $\beta$-tubulin). $\beta$-Tubulin is expected to activate PLC-$\gamma$1, based on our immunoprecipitation experiments using COS-7 cells before and after agonist stimulation and the observation that the PLC-$\gamma$1-$\beta$-tubulin complex gradually increased in EGF-treated cells. Moreover, when we treated COS-7 cells with taxol, a microtubule stabilizer, the amount of $\beta$-tubulin was greatly reduced in the immunocomplexes precipitated by anti-PLC-$\gamma$1 (data not shown), suggesting that taxol inhibits $\beta$-tubulin translocation to the membrane. These results suggest that tubulin heterodimers, rather than assembled microtubules, promote PLC-$\gamma$1 activity in the plasma membrane. Moreover, when we examined in vivo PLC-$\gamma$1 activity by measuring the amount of IP$_3$ after treatment with 20% serum or EGF, the enzyme activity was increased about 3-fold (data not shown), which is consistent with previous reports (30, 31) and indicates that depolymerized tubulin is required for PLC-$\gamma$1 activation in cells.

Although we did not directly investigate the mechanism for PLC-$\gamma$1 activation, we speculate that $\beta$-tubulin may modulate the phosphoinositide-binding affinity of PLC-$\gamma$1 in the membrane, because $\beta$-tubulin directly binds to PIP$_2$ (42). In this scenario, a plausible possibility for the PLC-$\gamma$1 activation mechanism can be deduced from that of the actin-binding protein, CapG (43, 44). Overexpression of CapG enhances PDGF-stimulated PLC-$\gamma$1 activity either by CapG-dependent transport of PIP$_2$ to PLC-$\gamma$1 or CapG-induced modulation of the $V_{\text{max}}$.
or $K_m$ values of PLC-γ1 activity through direct protein-protein interactions (43). Most interestingly, our previous results revealed that overexpression of a truncated mutant molecule consisting of the nPH2-SH2-SH3-cPH2 domain of PLC-γ1 completely blocked PDGF-induced IP$_3$ generation in NIH3T3 cells, suggesting that the split PH domain dominantly occupied the β-tubulin pool of PI-P$_2$ rather than endogenous PLC-γ1 in the membrane (45). In the case of PLC-β1, it has been suggested that membrane-localized tubulin regulates the PI-P$_2$ hydrolyzing activity of PLC-β1 (42). At low concentrations (nanomolar), tubulin activates PLC-β1 via Ga activation, whereas at higher concentrations (micromolar), tubulin inhibits its PLC-β1 activity by blocking the Ga by tubulin-PI-P$_2$ complex and rendering the enzyme inaccessible for receptor-activated Ga (42, 46). In contrast, receptor-activated Ga is not required for PLC-γ1 activation, which is probably mediated by tyrosine phosphorylation by receptor-tyrosine kinase, including EGF receptor and PDGF receptor (30, 31). Thus, we suggest that PH domain-bound tubulin likely facilitates transportation of the PI-P$_2$ pool to membrane-localized PLC-γ1. In this way, the PLC-γ1 PH domains play a critical role in PI-P$_2$ hydrolysis.

The next question in terms of the physiological relevance of the PLC-γ1-β-tubulin interaction is whether PLC-γ1 affects microtubule assembly. When we performed double immunostaining using anti-PLC-γ1 and anti-β-tubulin antibodies in COS-7 cells, we found that PLC-γ1 was highly concentrated in the mitotic spindle fiber (Fig. 2D). This image evokes the possibility that PLC-γ1 might be involved in tubulin polymerization. To explore the possibility, microtubule formation in vivo was assessed by overexpression and depletion of PLC-γ1 in COS-7 cells. Fig. 7, B–D, shows that PLC-γ1 has a positive role in tubulin polymerization. Generally, microtubules are polymerized by β-tubulin-GTP complexes and depolymerized by β-tubulin-GDP complexes. In this context, the PLC-γ1 may function as a GEF in microtubule assembly, as described above.

Because PLC-γ1-SH3 showed GEF activity for PIKE (36) and dynamin-1 (37), we examined the GEF activity of GST-PLC-γ1-SH3 for tubulin. Addition of the GST control (36, 37), whereas that for PIKE showed GEF activity for PIKE (36) and dynamin-1 (37), whereas that for PIKE was 5-fold higher than that of the GST control (36, 37), whereas that for β-tubulin was only 0.3-fold higher. This may suggest that the SH3 domain has a weaker GEF activity for β-tubulin than for PIKE or dynamin-1. Although further work will be necessary to examine these apparent differences, this may indicate that the SH3 domain of PLC-γ1 requires the involvement of other proteins for full activity.

In summary, our present results demonstrate that transmodulation of PLC-γ1 and β-tubulin activities via protein-protein interactions regulates cell growth and differentiation. β-Tubulin is constitutively associated with PLC-γ1 in the cytosol, and agonist stimulation results in translocation of both proteins to the membrane, where PLC-γ1 is activated by depolymerized β-tubulin. In addition, PLC-γ1 promotes tubulin polymerization in vivo, as demonstrated by altered polymerization in response to PLC-γ1 level changes in COS-7 cells.

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