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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 (PIP_2) 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[1] is an important signaling molecule for cell proliferation and differentiation. Activated PLC-γ1 hydrolyzes PIP_2 to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. These second messengers regulate the release of Ca^{2+} 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, PIP_2, phosphatidylinositol 1,4,5-trisphosphate, and IP_3 (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; PIP_2, phosphatidylinositol 4,5-bisphosphate; BSA, bovine serum albumin; siRNA, small interfering RNA; IP_3, 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; GTPγS, guanosine 5′-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), CRMP-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 Ca^{2+} and IP_3, 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 and β-tubulin transmodulate 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).

In Vitro Binding Assay 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 $MgCl_2$, 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 then transferred to polyvinylidene difluoride (PVDF) membranes. 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 various tubulin isotypes (Mβ1, Mβ2, Mβ3, Mβ6, and Mα1, kindly provided by Dr. Sally Lewis, New York University) (27) were ligated into the EcoRI/SalI 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/XbaI 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/XbaI sites of pEGFP-C2 (Clontech). Expression of GFP-PH_1 (amino acids 25–145 of PLC-γ1) and GFP-nPH_2 (amino acids 477–547 of PLC-γ1) was assessed by immunofluorescent microscopy and immunoblotting using an anti-GFP antibody (Zymed Laboratories Inc.). All constructs were prepared using the plasmid maxi kit (Qiagen, Santa Clarita, CA) and confirmed by DNA sequencing of the ligation sites. COS-7 cells were transfected with the various constructs using the Lipofectamine reagent (Invitrogen). Forty eight hours after transfection, the cells were either harvested for immunoblotting/immunoprecipitation or fixed for indirect immunofluorescent microscopy.

Immunoprecipitation and Immunoblotting—Cells transfected with plasmids encoding the various FLAG-tagged mouse tubulin constructs were washed twice with phosphate-buffered saline and lysed with radioimmunoprecipitation assay buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). For agonist stimulation, cells were serum-starved for 30 h and then stimulated with either 20% fetal bovine serum (FBS) or 0.2 μg/ml epidermal growth factor (EGF) for 10–20 min. The lysate supernatants were precleared by incubation with anti-FLAG M2 affinity gel (Sigma) for 30 min. Precleared cell lysates were then incubated for 2 h with anti-FLAG antibodies conjugated with 50 μl of a 50% slurry of anti-FLAG M2 affinity gel. The immune complexes were collected by centrifugation, washed three times with ice-cold radioimmunoprecipitation assay buffer, then resolved by 10% SDS-PAGE, and blotted to a PVDF membrane. The blot was probed with either anti-PLC-γ1 or anti-FLAG antibodies, and the immunoreactive bands were visualized by ECL detection using HRP-conjugated goat anti-mouse IgG.

Far Western Blot Analysis—Purified bovine tubulin (0.2 μg per lane) was resolved by 10% SDS-PAGE and transferred onto a PVDF membrane. Nonspecific binding was blocked by incubation of membranes in 2% skim milk in Tris-buffered Tween 20 (TBT) for 1 h at room temperature. The membranes were then incubated with the GST, GST-PH_1, GST-nPH_2, or GST-SH3 fusion proteins (0.5 μg/ml) 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. The membranes were then washed in TBT buffer, 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 was introduced into the HindIII/XbaI 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).

Sedimentation Experiments—Purified tubulin (10 μM) was incubated with 0.5 μM of PLC-γ1 or BSA (control) in tubulin polymerizing buffer (0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 2.5 M glycerol and 0.1 mM GTP, pH 6.5) for 30 min at 37 °C. The samples were then centrifuged at 15,000 × *g* for 1 h at room temperature. The supernatant and pellet fractions were resolved by 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue for visualization. A separate fractionation was performed; the resulting supernatant was mixed with 20% glycerol and 1 mM GTP, and the sample was incubated at 37 °C for polymerization for 30 min and then centrifuged at 100,000 × *g* for 45 min. The pellet was resuspended in ice-cold tubulin polymerizing buffer, homogenized, incubated on ice for depolymerization, and centrifuged at 100,000 × *g*. These polymerization/depolymerization cycles were repeated two times, and the supernatants and pellets from each step were resolved by 10% SDS-PAGE and transferred to PVDF membranes for Western immunoblotting using an anti-PLC-γ1 antibody.

Immunofluorescent Microscopy—COS-7 cells were seeded on glass coverslips in 6-well plates and transfected with 5 μl of Lipofectamine 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 GGAAGAAGCAGCTGTGGTT, CCAACCAGCTTAAGAGGAA, GAAGTGAACATGTGGATCA, and GAGCAGTGCCTTTGAAGAA. Following transfection, the cells were grown for 2 days in Dulbecco'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 Affinipure 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 crushed and destained by washing with 50% acetonitrile in 25 mM NH_4HCO_3. The gel slices were then incubated overnight with trypsin (Promega) in 25 mM NH_4HCO_3 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

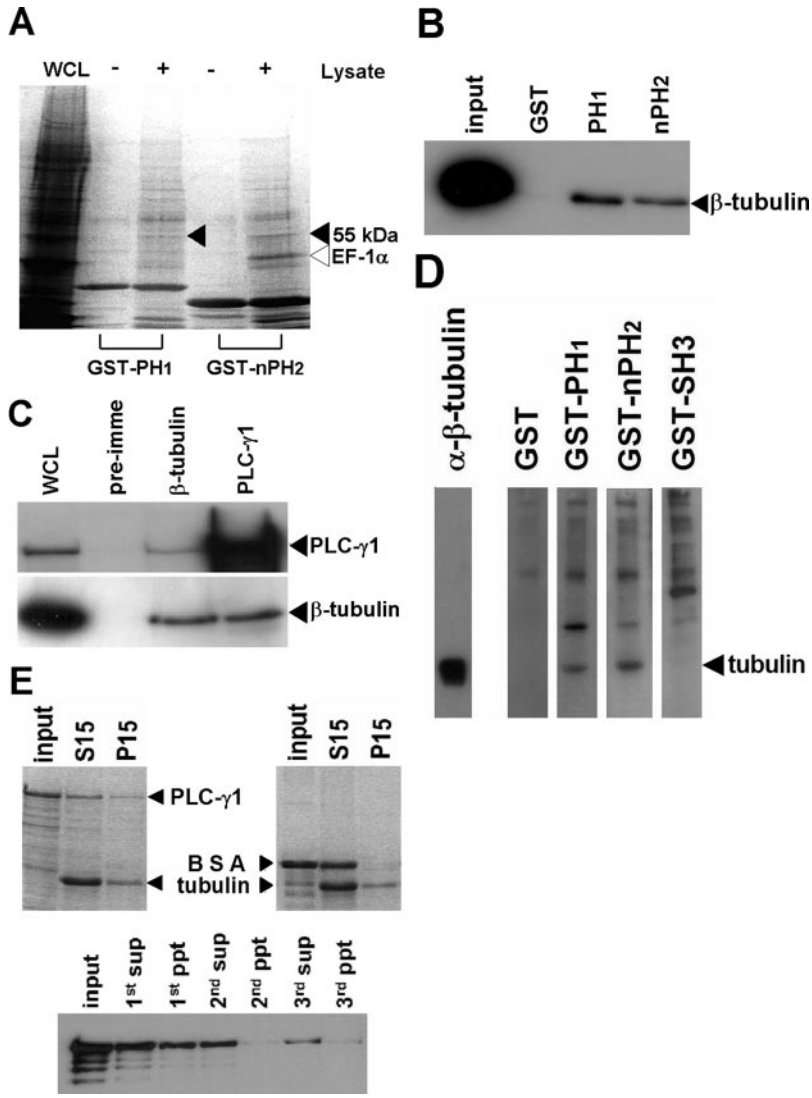

FIG. 1. **β-Tubulin binds both PH domains of PLC-γ1.** *A,* GST-PH domain fusion proteins were incubated with (+) or without (−) NIH3T3 cell lysates, and the bound proteins were resolved by 10% SDS-PAGE. GST-PH_1 and GST-nPH_2 fusion proteins pulled down a 55-kDa protein on the gel as shown by Coomassie 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-PH_1, GST-nPH_2, 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 nonredundant protein data base (NCBI) using the ProFound online program (www.proteometrics.com).

Fractionation of Monomeric and Polymeric Tubulin—Polymerized (polymeric) and depolymerized (monomeric) tubulins were differentially fractionated from COS-7 cells transfected with vector alone, pFLAG-CMV-2-PLC-γ1, and siRNA for PLC-γ1 according to procedures described previously (28). Briefly, the transfected cells on a 6-well plate were washed two times with phosphate-buffered saline and incubated with 0.3 ml of tubulin polymerizing buffer plus 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM $MgSO_4$, and 0.1% Triton X-100 for 20 min at 37 °C. The supernatant from a 6-well plate was removed carefully as monomeric tubulin fractions. Polymeric tubulin was extracted from the remaining Triton X-100-insoluble fraction by extracting in SDS lysis buffer (25 mM Tris, pH 7.4, 0.4 M NaCl, 0.5% SDS) for 10 min at 37 °C. Aliquots from each fraction were resolved by 10% SDS-PAGE and Western immunoblotted with anti-tubulin and anti-PLC-γ1 antibodies.

PLC-γ1 Activity Assay—PLC-γ1 activity was measured as described previously (29). Briefly, the substrate was prepared as sonicated vesicles of 75 mM PIP_2, 75 mM [^{3}H]PIP_2 (9,000–10,000 cpm/assay), and 750 mM phosphatidylethanolamine in 50 mM HEPES buffer, pH 7.0. Reactions were performed for 20 min at 30 °C in a 100-μl final volume containing 10 or 100 ng of PLC-γ1 and 2 mM Ca^{2+}, and terminated by addition of 1 ml of chloroform/methanol/HCl (50:50:0.5) and 400 μl 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 scintillation counting. The effect of β-tubulin was examined by adding the indicated amounts of α- and β-tubulin to the PLC-γ1 assay mixture.

RESULTS

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-PH_1 and GST-nPH_2 proteins were incubated with NIH3T3 cell lysates (Fig. 1*A*), 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-cPH_2 fusion protein did not show any visible protein band on our SDS-polyacrylamide gel (26), we used the GST-PH_1 and GST-nPH_2 fusion proteins in this experiment. As shown in Fig. 1*A*, the GST-nPH_2 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-PH_1 and GST-nPH_2 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 I 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. 1*B*). We further confirmed that the interaction between PLC-γ1 and β-tubulin occurs *in vivo* by

TABLE I
Tryptic peptides of β-tubulin identified by MALDI-TOF

Peptide sequence[a]	Start-end[b]	Monoisotopic mass ([M + H$^+$])	
		Theoretical	Experimental
FWEVISDEHGIDPTGTYHGDSDLQLER	11–37	3115.42	3115.37
SGPFGQIFRPDNFVFGQSGAGNNWAK	69–94	2797.34	2797.30
GHYTEGAELVDSVLDVVR	95–112	1957.97	1957.97
LTTPTYGDLNHLVSATMSGVTTCLR	208–232	2707.33	2707.29
FPGQLNADLR	233–242	1129.59	1129.62
LAVNMVPFPR	244–253	1142.63	1142.65
LHFFMPGFAPLTSR	254–267	1619.83	1619.84
YLTVAAVFR	301–309	1038.59	1038.61
NSSYFVEWIPNNVK	328–341	1695.83	1695.83
MSATFIGNSTAIQELFK	354–370	1856.93	1856.94
ISEQFTAMFR	372–381	1228.59	1228.61

[a] Amino acid residues derived from the 55-kDa protein.
[b] Position of the amino acid residue in the deduced peptide sequence of mouse β-tubulin.

reciprocal immunoprecipitations: anti-β-tubulin immunoprecipitated PLC-γ1 and vice versa (Fig. 1*C* and see Fig. 4*B*). To examine whether the binding is direct or not, we performed far Western blot analysis using purified tubulin. The result shows that GST-PH_1 and GST-nPH_2 directly bind to β-tubulin, whereas GST alone or GST-SH3 does not (Fig. 1*D*). Finally, we performed a sedimentation experiment to verify the interaction between PLC-γ1 and β-tubulin. Because self-assembled tubulin microtubules can be pelleted by centrifugation, we incubated purified tubulin (α- and β-tubulin heterodimers) and allowed them to polymerize into microtubules in the presence of PLC-γ1 or BSA (control). As shown in Fig. 1*E*, PLC-γ1 coprecipitated with polymerized tubulin, whereas BSA did not, indicating that PLC-γ1 binds to microtubules and tubulin heterodimers *in vivo*. Moreover, when further polymerization and depolymerization cycles were performed with the polymerized tubulin precipitates, PLC-γ1 was found to consistently coexist with tubulin in the supernatant and pellet fractions (Fig. 1*E*).

Colocalization of PLC-γ1 and β-Tubulin—To examine the subcellular localizations of PLC-γ1 and β-tubulin in COS-7 cells, we performed double immunostaining by using anti-PLC-γ1 and anti-β-tubulin antibodies. As shown in Fig. 2*A*, PLC-γ1 and β-tubulin were localized in the cytoplasm of resting cells. Overlays of the fluorescent microscopy images showed that both proteins dominantly colocalized in the perinuclear region of the COS-7 cells. Generally, agonist treatment results in translocation of PLC-γ1 from the cytosol to the plasma membrane, where it binds to receptor tyrosine kinase (30, 31). To examine the localizations of PLC-γ1 and β-tubulin following agonist stimulation, we performed double immunostaining with anti-PLC-γ1 and anti-β-tubulin antibodies. In cells treated with 20% FBS or EGF for 10 min, both PLC-γ1 and β-tubulin were localized in the plasma membrane (Fig. 2, *B* and *C*).

Furthermore, in mitotic cells, PLC-γ1 was highly concentrated in the mitotic spindle with enrichment toward the spindle pole, which is composed of pure α- and β-tubulin heterodimers (Fig. 2*D*). Our observation that PLC-γ1 colocalizes with the mitotic spindle in the absence of mitosis-inducing drugs suggests that PLC-γ1 might be involved in spindle formation in mitotic COS-7 cells (see below).

Membrane-targeted Translocation of PLC-γ1 and β-Tubulin in COS-7 Cells—Because treatment of COS-7 cells with 20% FBS resulted in membrane localization of PLC-γ1 and β-tubulin, we explored how PLC-γ1 and β-tubulin might translocate from the cytosol to the plasma membrane. Anti-β-tubulin antibody staining revealed that COS-7 cells showed remarkable microtubule depolymerization after treatment with 20% FBS (Fig. 3*A*), indicating that depolymerized β-tubulin translocates together with PLC-γ1 to the membrane. To examine the tubulin polymerization and depolymerization cycles caused by agonist stimulation, we surveyed the subcellular localization of both proteins over time. We found that most of the microtubules depolymerized into tubulin heterodimers within 15–20 min of agonist stimulation and then gradually reformed into microtubules over time, with a portion of them having membrane localization (Fig. 3*B*). Next, we examined whether the PH domains of PLC-γ1 were responsible for the membrane-targeted translocation. We expressed the PH_1 and nPH_2 domains as green fluorescent protein fusions (GFP-PH_1 and GFP-nPH_2) in COS-7 cells with or without stimulation by 20% serum for 20 min. As shown in Fig. 3*C*, the PH_1 and nPH_2 domains were both found to localize in the membrane following treatment with 20% FBS. This result indicates that the PH domains likely act as a tether for membrane localization of PLC-γ1. Taken together, these data show that agonist stimulation results in tubulin and PLC-γ1 translocation from the cytosol to the plasma membrane in COS-7 cells.

Expression of Various Mβ-tubulin Isotypes—Mouse cells have been shown to express at least six different β-tubulin isotypes (Mβ1 to Mβ6) in a tissue-restricted manner, so we next sought to determine which isotype of Mβ-tubulin specifically binds to the PH domains of PLC-γ1. We constructed and expressed FLAG-tagged Mβ1-, Mβ2-, Mβ3,- and Mβ6-tubulin isotypes. First, we determined whether the transfected Mβ-tubulin isotypes were capable of tubulin assembly into microtubules. We transiently transfected each recombinant DNA into COS-7 cells and examined the cells with anti-FLAG monoclonal antibody staining. As shown in Fig. 4*A*, three of the four transfected isotypes, Mβ1, Mβ2, and Mβ6, were expressed and efficiently assembled into microtubules. In the case of the Mβ3 isotype, the protein was expressed, but it was unable to assemble into microtubules, suggesting that the Mβ3 isotype likely exists as monomer or heterodimer with α-tubulin isotypes. When cells expressing FLAG-Mβ2-tubulin were treated with 20% FBS, the FLAG-Mβ2-tubulin translocated from the cytosol to the plasma membrane together with PLC-γ1 (data not shown) in a manner similar to that of endogenous tubulin (Fig. 2*B*). Furthermore, FLAG-Mβ2-tubulin was coimmunoprecipitated with FLAG-PLC-γ1 by anti-PLC-γ1 and vice versa in transiently transfected COS-7 cell lysates (Fig. 4*B*). Mα1-tubulin was also expressed in this system and shown to assemble into microtubules (Fig. 4*A*). Together, these data indicate that the ectopically expressed tubulin isotypes behaved in a manner similar to the endogenous proteins and were therefore an acceptable assay system. To examine the binding specificity of the PHs to these isotypes, we compared the binding reactions of GST fusion proteins including GST-PH_1, -nPH_2, -cPH_2, -SH2, and -SH3. As shown in Fig. 4*C*, GST-PH_1 and GST-nPH_2 associated with all tested Mβ-tubulin isotypes, as shown by

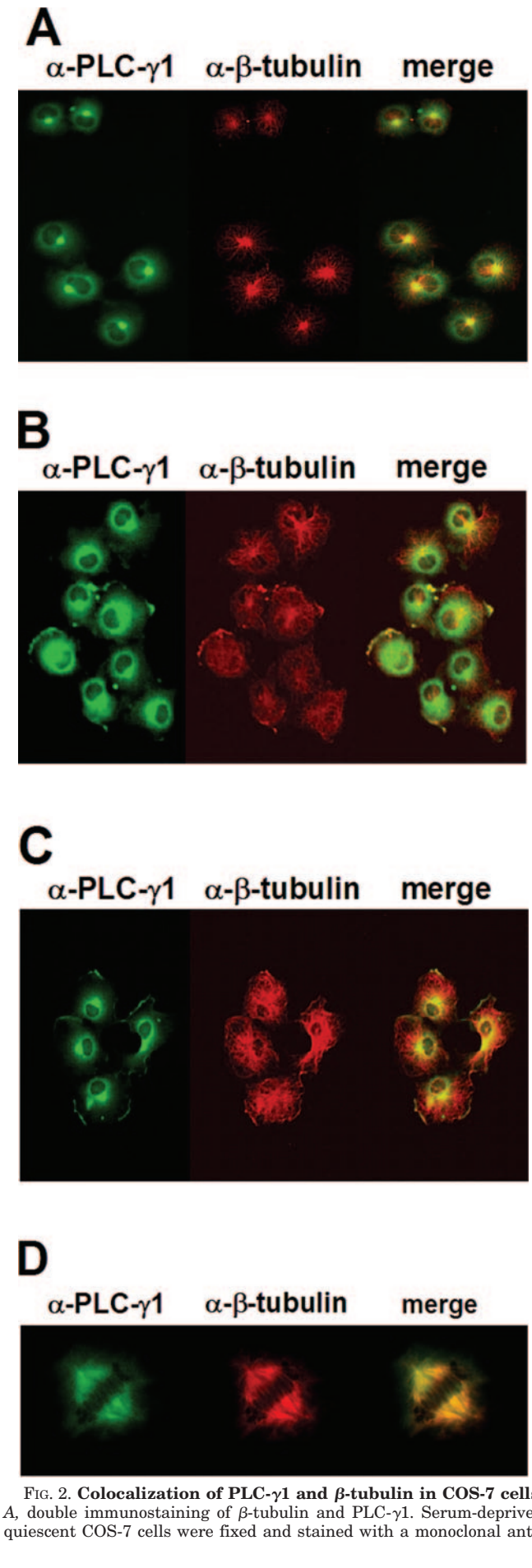

FIG. 2. **Colocalization of PLC-γ1 and β-tubulin in COS-7 cells.** *A,* double immunostaining of β-tubulin and PLC-γ1. Serum-deprived quiescent COS-7 cells were fixed and stained with a monoclonal anti-β-tubulin and a polyclonal anti-PLC-γ1 antibody followed by rhodamine- and fluorescein isothiocyanate-labeled 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*.

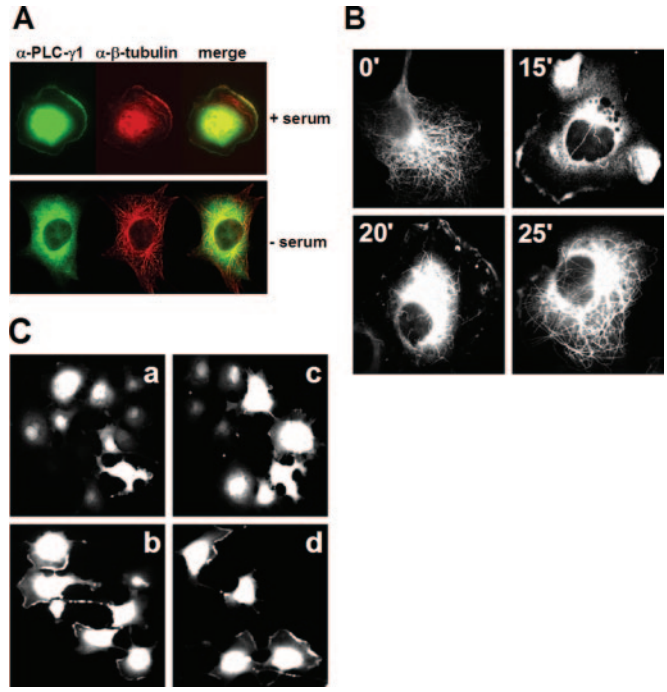

FIG. 3. **Translocation of β-tubulin and PLC-γ1 following agonist stimulation.** *A,* membrane-targeted translocation of β-tubulin and PLC-γ1 was visualized by immunofluorescence. COS-7 cells stimulated with (*upper panel*) or without (*lower panel*) 20% FBS for 20 min were double immunostained with anti-PLC-γ1 and anti-β-tubulin antibodies. *B,* to examine the tubulin depolymerization and translocation upon agonist stimulation, subcellular localization of β-tubulin after 20% FBS stimulation was examined at the indicated time intervals. *C,* GFP-PH_1 (amino acids 25–145) (*a* and *b*) and GFP-nPH_2 (amino acids 477–547) (*c* and *d*) were expressed in COS-7 cells, and both GFP-PH_1 (*b*) and GFP-nPH_2 (*d*) proteins translocated to the plasma membrane following 20% FBS treatment for 20 min.

Western blotting, whereas the other domains (GST-SH2N, -SH2C or -SH3) did not. Most interestingly, α-tubulin did not bind to the PLC-γ1 PH domains of in our *in vitro* binding assay (Fig. 4*C*) or immunoprecipitation (Fig. 5*A*) 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 PIP_2. 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. 5*A*, 20% FBS treatment augmented the amount of FLAG-Mβ2-tubulin coimmunoprecipitated with the anti-PLC-γ1 antibody.

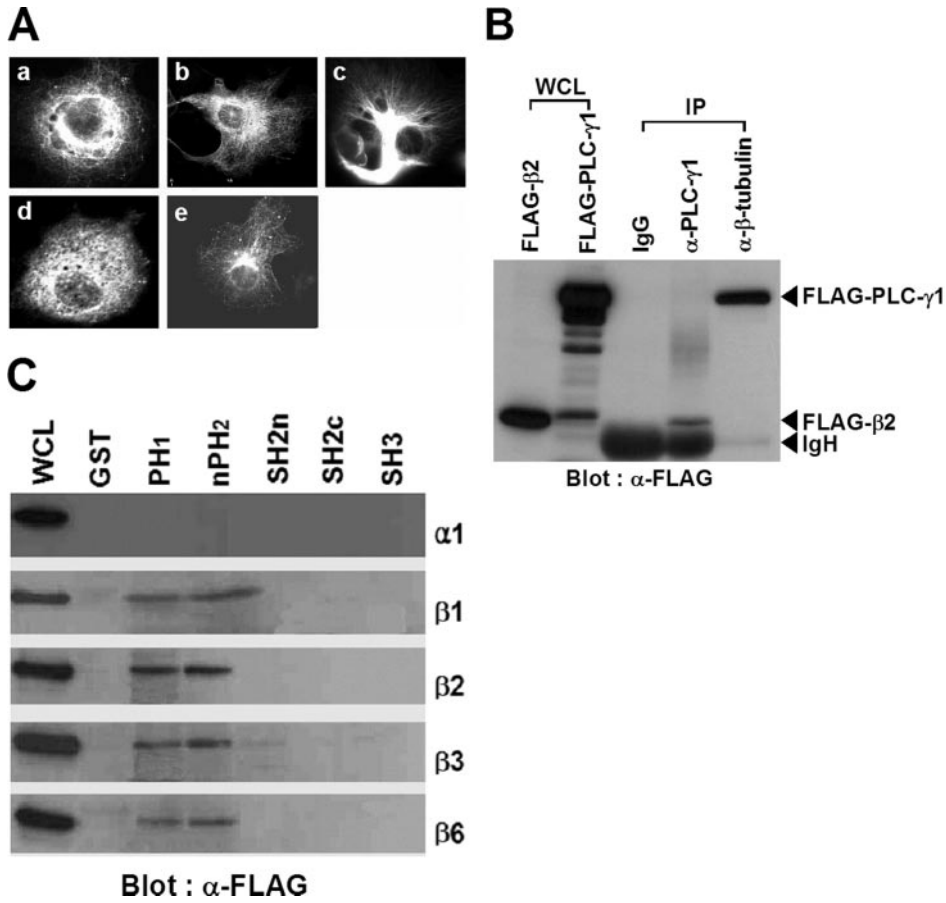

FIG. 4. **Immunofluorescence of transfected FLAG-Mβ-tubulin isotypes in COS-7 cells.** *A,* cells transfected with N-terminal FLAG-tagged mouse α- and β-tubulin isotypes were visualized by immunofluorescence microscopy. cDNAs encoding FLAG-Mα1 (*a*), -Mβ1 (*b*), -Mβ2 (*c*), -Mβ3 (*d*), and -Mβ6 (*e*) were transiently transfected into COS-7 cells. After 48 h, the cells were fixed and stained with anti-FLAG antibodies. *B,* FLAG-Mβ2-tubulin coimmunoprecipitates with FLAG-PLC-γ1 in COS-7 cell lysates. COS-7 cells transfected with FLAG-tagged mouse β2-tubulin and rat PLC-γ1 were used for immunoprecipitations (*IP*) of FLAG-Mβ2-tubulin and FLAG-PLC-γ1 using their antibodies. The membrane was probed with a monoclonal anti-FLAG antibody followed by HRP-conjugated anti-mouse IgG. *C,* to examine the domain specificity of β-tubulin association, various GST fusion proteins were incubated with COS-7 cell lysates containing each FLAG-Mβ tubulin protein. The bound proteins were resolved by 10% SDS-PAGE followed by immunoblotting with an anti-FLAG antibody. *WCL,* whole cell lysates.

In this experiment, all β isotypes 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. 5*B*). These results are consistent with the subcellular localizations of both proteins (Fig. 2, *B* and *C,* and Fig. 3*A*) 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 α- and 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 PIP_2 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 [^{3}H]PIP_2 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 for overexpression or siRNAs for 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. 7*A*). 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. 7*B*, 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. 7*C*). 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. 7*D*). 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

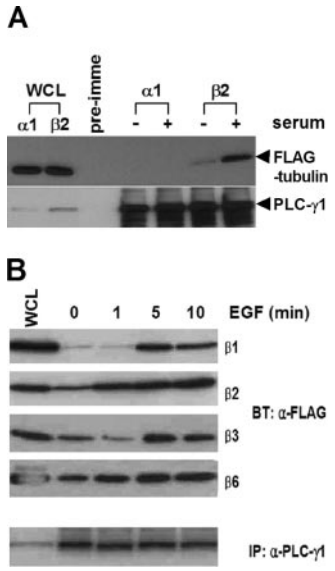

FIG. 5. **Serum and EGF facilitate the interaction of PLC-γ1 with β-tubulin *in vivo*.** *A,* PLC-γ1 was immunoprecipitated from COS-7 cell lysates transiently overexpressing FLAG-tagged mouse tubulin isotypes (Mα1- and Mβ2-tubulin). Cells were either unstimulated (−) or treated with 20% FBS (+) for 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.

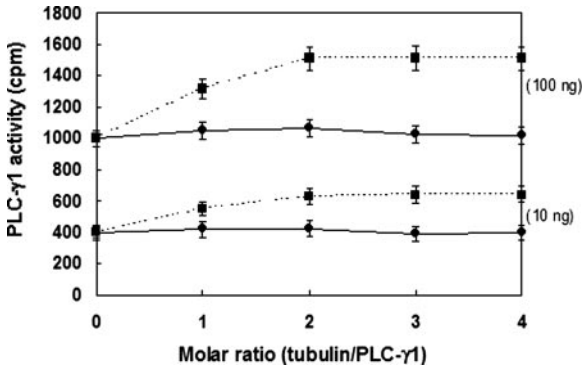

FIG. 6. **Purified tubulin promotes PLC-γ1 activity.** The PIP_2 hydrolyzing activity of PLC-γ1 was measured in the presence of tubulin heterodimers. PLC-γ1 and tubulin used in this experiment were purified from COS-7 cells (FLAG-PLC-γ1) and bovine brain extracts (Sigma), respectively. PLC-γ1 activity is expressed as the radioactivity of $[^3H]IP_3$. The data represent the average of duplicate determinations (mean ± S.D.) from three experiments with similar results.

~55% (Fig. 7*D*). 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. 2*D*), 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 Mβ6) 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. 3*C*). 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. 3*C*). Furthermore, the nPH_2 domain predominantly binds to phosphatidylinositol 3-phosphate on an Echelon filter.[2] 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 relevancy 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

[2] S.-K. Kim and J.-S. Chang, unpublished results.

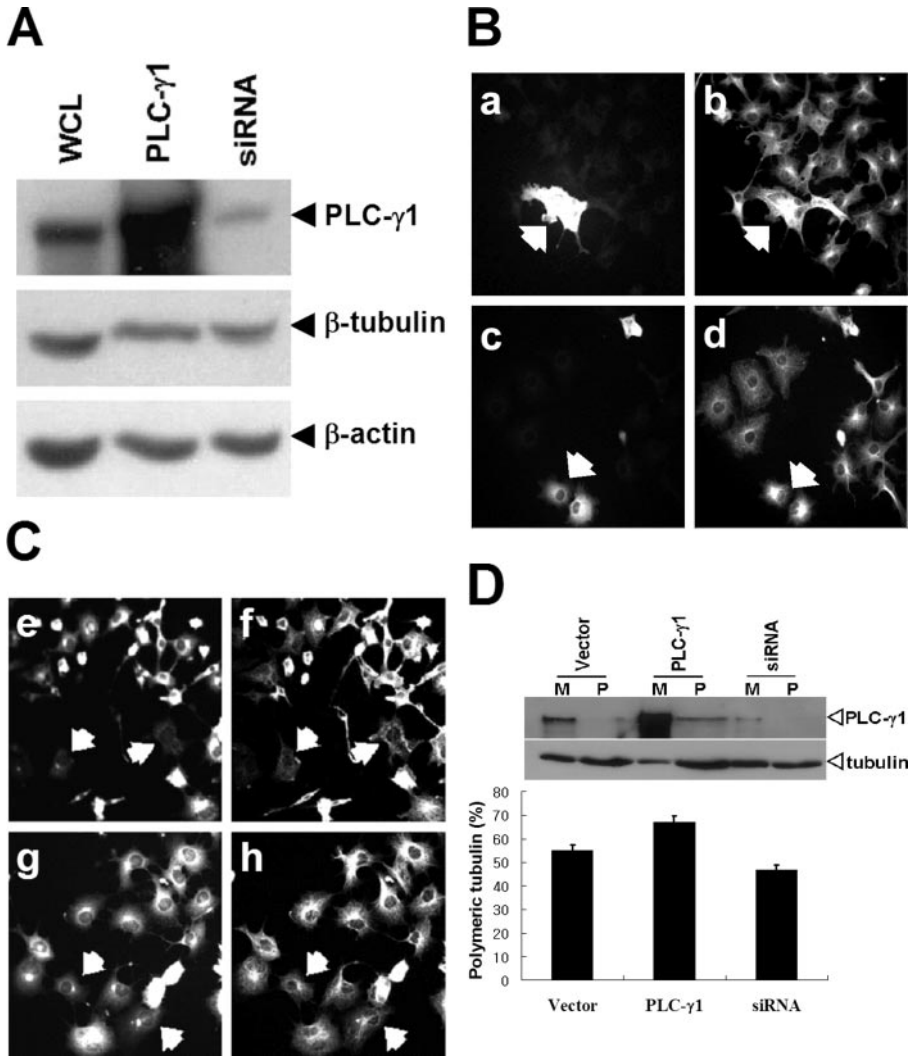

FIG. 7. **PLC-γ1 modulates microtubule assembly *in vivo*.** *A,* the amounts of PLC-γ1 in COS-7 cell overexpressing PLC-γ1 and silencing PLC-γ1 or wild type (endogenous) were compared. Cells were transfected with FLAG-PLC-γ1, siRNA for PLC-γ1, or FLAG vector alone in 6-well culture plates. Fifty μg of protein per well was resolved by 10% SDS-PAGE, and Western blotting was performed using anti-PLC-γ1 antibody (*upper panel*). For normalization, the amount of β-tubulin (*middle panel*) and β-actin (*lower panel*) was measured using anti-β-tubulin and anti-β-actin antibodies, respectively. *WCL,* whole cell lysates. *B* and *C,* double immunofluorescence of PLC-γ1 and β-tubulin was examined in cells overexpressing PLC-γ1 (*B*) and silencing PLC-γ1 (*C*). Cells overexpressing PLC-γ1 at high (*a* and *b*) and moderate (*c* and *d*) levels or silencing PLC-γ1 to low (*e* and *f*) or moderate (*g* and *h*) levels were stained with anti-PLC-γ1 (*a, c, e,* and *g*) and anti-β-tubulin antibodies (*b, d, f,* and *h*), respectively. *D,* quantitative Western immunoblotting shows that PLC-γ1 regulates tubulin assembly. After transfection of the cells with pFLAG-CMV-2 vector, pFLAG-CMV-2-PLC-γ1, or siRNA for PLC-γ1, depolymerized (monomeric) tubulin was extracted in 0.1% Triton X-100 extraction buffer plus protease inhibitors at 37 °C for 20 min. After the Triton X-100-soluble supernatant (*M,* monomeric) was removed, polymerized tubulin was extracted SDS buffer from the Triton X-100-insoluble material (*P,* polymeric) as described under "Experimental Procedures." An equivalent amount of each extract was resolved on a 10% SDS-PAGE, and samples were transferred to PVDF membrane for Western immunoblotting with anti-tubulin antibody, and then the filter was reprobed with anti-PLC-γ1 antibody for the indication of PLC-γ1 levels (*upper panel*). Quantitation of polymerized tubulin from three independent experiments was performed by image density analysis (*lower panel*). % Polymerization of tubulin = polymeric tubulin/monomeric tubulin + polymeric tubulin pool (mean ± S.D.).

the first possibility, we examined the PIP_2 hydrolyzing activity of PLC-γ1 in the presence or absence of β-tubulin. In the presence of tubulin (Fig. 6), the PIP_2 hydrolyzing activity of PLC-γ1 was increased about 1.6-fold under the conditions of a 1:2 molar ratio (PLC-γ1 to β-tubulin). β-Tubulin is expected to activate PLC-γ1, based on our immunoprecipitation experiments using COS-7 cells before and after agonist stimulation and the observation that the PLC-γ1-β-tubulin complex gradually increased in EGF-treated cells. Moreover, when we treated COS-7 cells with taxol, a microtubule stabilizer, the amount of β-tubulin was greatly reduced in the immunocomplexes precipitated by anti-PLC-γ1 (data not shown), suggesting that taxol inhibits β-tubulin translocation to the membrane. These results suggest that tubulin heterodimers, rather than assembled microtubules, promote PLC-γ1 activity in the plasma membrane. Moreover, when we examined *in vivo* PLC-γ1 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-γ1 activation in cells.

Although we did not directly investigate the mechanism for PLC-γ1 activation, we speculate that β-tubulin may modulate the phosphoinositide-binding affinity of PLC-γ1 in the membrane, because β-tubulin directly binds to PIP_2 (42). In this scenario, a plausible possibility for the PLC-γ1 activation mechanism can be deduced from that of the actin-binding protein, CapG (43, 44). Overexpression of CapG enhances PDGF-stimulated PLC-γ1 activity either by CapG-dependent transport of PIP_2 to PLC-γ1 or CapG-induced modulation of the V_{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 nPH_2-SH2-SH2-SH3-cPH_2 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 PIP_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 PIP_2 hydrolyzing activity of PLC-β1 (42). At low concentrations (nanomolar), tubulin activates PLC-β1 via Gα activation, whereas at higher concentrations (micromolar), tubulin inhibits its PLC-β1 activity by blocking the Gα by tubulin-PIP_2 complex and rendering the enzyme inaccessible for receptor-activated Gα (42, 46). In contrast, receptor-activated Gα 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 PIP_2 pool to membrane-localized PLC-γ1. In this way, the PLC-γ1 PH domains play a critical role in PIP_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. 2*D*). 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-SH3 fusion protein increased the [^{35}S]GTPγS-bound form of purified tubulin 0.3-fold as compared with the effects of GST (data not shown). Although these data indicate that the SH3 domain of PLC-γ1 has a GEF activity for tubulin, one piece of evidence might argue against a direct role for the SH3 domain in tubulin assembly. Previous reports indicated that the GEF activity of the SH3 domain for PIKE and dynamin-1 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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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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