Phospholipase C-γ1 Activation by Direct Interaction with β-Tubulin Isotypes

In-Bum Lee, Sung-Kuk Kim, Jang Hyun Choi[1], Pann-Ghill Suh[1] and Jong-Soo Chang*

Department of Life Science, College of Natural Science, Daejin University, Kyeonggido 487-711, Korea
[1]Department of Life Science, Pohang University of Science and Technology (POSTECH), Kyungbuk 790-784, Korea

Received March 23, 2006 / Accepted April 25, 2006

Phosphoinositide-specific phospholipase C-γ1 (PLC-γ1) has pivotal roles in cellular signaling by producing second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). Tubulin is a main component of microtubules and mitotic spindle fibers, which are composed of α- and β-tubulin heterodimers in all eukaryotic cells. In humans, six β-tubulin isotypes have been identified which display a distinct pattern of tissue expression. Previously we found that PLC-γ1 and one of four β-tubulin isotypes including β1, β2, β3 and β6, colocalized in COS-7 cells and cotranslocated to the plasma membrane to activate PLC-γ1 upon agonist stimulation. In the present study, we demonstrate that the remaining two, tubulin β4 and β5, also showed a potential to activate PLC-γ1. The phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolyzing activity of PLC-γ1 was substantially increased in the presence of purified β4 and β5 tubulin *in vitro*, whereas the activity was not promoted by bovine serum albumin, suggesting that tubulin β4 and β5 also activate PLC-γ1. Taken together, our results suggest that all the β-tubulin isotype activates PLC-γ1 activity to regulate cellular signaling.

Key words – Phospholipase C-γ1, β-tubulin isotype, immunocytochemistry, GST pull down assay

Introduction

PLC-γ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), which regulate protein kinase C (PKC) activity in a cell[1,10]. PLC-γ1 has two pleckstrin homology (PH) domains for protein-protein and protein-lipid interactions: one PH domain is located in the 150 N-terminal amino acid residues, the other is split by SH2-SH2-SH3 domain[2,6]. PH domains of signaling molecules are often involved in targeted translocation of molecules to cell membranes[5,8] and PH domains can also specifically bind cellular signaling proteins, such as the βγ-subunit of the heteromeric G-protein[14], PKC[16] and EF-1α[2]. Previously, we identified four β-tubulin isotypes including β1, β2, β3 and β6 as a binding partner of PH domain PLC-γ1[3].

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[4,9]. The mitotic spindle, which is a dynamic array of microtubules, is responsible for chromosome segregation into daughter cells during mitosis.

Here, we sought to examine tubulin β4 and β5 proteins that specifically interact with the PH domains of PLC-γ1, and showed for the first time that these β-tubulin isotypes substantially activate PLC-γ1 activity.

*Corresponding author
Tel : +82-31-539-1853, Fax : +82-31-539-1850
E-mail : jchang@daejin.ac.kr

Materials and Methods

Antibodies

Monoclonal anti-β-tubulin and anti-α-tubulin were purchased from Chemicon (Temecula, CA) and Sigma-Aldrich (St. Louis, MO). The horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Upstate Inc. (Lake Placid, NY). Monoclonal anti-FLAG M5 and polyclonal anti-PLC-γ1 were obtained from Sigma-Aldrich. Fluorescein-conjugated Affinipure goat anti-rabbit IgG and rhodamine-conjugated Affinipure goat anti-mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

In vitro binding assay with GST fusion proteins

Using rat PLC-γ1 cDNA [13] as the template, GST constructs for fusion proteins were generated by polymerase chain reaction (PCR) as previously described[2]. GST fusion proteins were expressed in *E.coli* and incubated the lysates with glutathione sepharose bead, and 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 ㎍/㎖ aprotinin, 10 ㎍/㎖ leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate), resolved by 10 % SDS-polyacrylamide gel electrophoresis (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-Mβ6 and Mα2, kindly provided by Dr. Sally Lewis, New York University) [15] were ligated into the *EcoRI/SalI* restriction site of the N-terminal epitope tagging vector, pFLAG-CMV-2 (Sigma-Aldrich). When expressed, all α- and β-tubulin cDNAs encoded an N-terminal 9 amino acid FLAG epitope tag. cDNA sequence encoding the amino-terminal PH domain (PH_1) and each SH domains of rat PLC-γ1 were ligated into the pGEX-5X-1 vector (Amersham Biosciences), as described previously [2, 3]. All constructs were prepared using the Qiagen Plasmid Maxi Kit (Qiagen Inc., Santa Clarita, CA) and confirmed by DNA sequencing of the ligation sites. For the expression of FLAG-tagged tubulin isotypes, COS-7 cells were transfected with the pCMV-FLAG-β4 and β5 using the Lipofectamine reagent (Gibco BRL, Gaithersburg, MD). Forty-eight hours after transfection, the cells were either harvested for immunoblotting or fixed for immunocytochemistry.

Immunofluorescent microscopy

COS-7 cells were seeded on glass coverslips in 6-well plates and transfected with 5 ㎕ of Lipofectamine and one of the following: 2 ㎍ of the pCMV-FLAG-β4 or β5 vector. Following transfection, the cells were grown for 2 days in Dulbecco's modified Eagle's medium (DMEM) with 10 % FBS. The cells were fixed at 37 ℃ for 10 min in 4 % paraformaldehyde and then incubated with affinity purified monoclonal anti-FLAG antibody for 1 hr at room temperature in a humidity chamber. Following complete washing with PBS, the cells were incubated with 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.

PLC-γ1 activity assay

PLC-γ1 activity was measured as described previously [7]. 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 PE in 50 mM HEPES buffer (pH 7.0). Reactions were performed for 20 min at 30 ℃ in 100 ㎕ final volume containing 10 ng PLC-γ1 and 2 mM Ca^{2+}, and terminated by addition of 1 ㎖ of chloroform/methanol/HCl (50:50:0.5) and 400 ㎕ 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 15 ng of β-tubulin to the PLC-γ1 assay mixture.

Results

Expression of mouse β-tubulin isotypes

Mouse cells have been shown to express at least 6 different β-tubulin isotypes (Mβ1 to Mβ6) in a tissue-restricted manner. Previously we determined which isotype of Mβ-tubulin specifically binds to PLC-γ1, and consequently we found that four FLAG-tagged Mβ1, Mβ2, Mβ3 and Mβ6 tubulin isotypes directly bind to the PH domain of PLC-γ1[3]. In the present study, we sought to determine whether the remaining β4- and β5-tubulin are capable of interaction with PH domain of PLC-γ1. We transiently transfected each recombinant DNA into COS-7 cells and examined the cells with anti-FLAG monoclonal antibody staining. As shown in Figure 1 and 2, both two transfected isotypes, Mβ4 and Mβ5, were expressed (Fig. 1) and efficiently assembled into microtubules (Fig. 2). In the case of the Mβ3 isotype, the protein was expressed but

BT : α-FLAG

Fig. 1. Expression of β-tubulin isotypes in COS-7 cells. Cells transiently transfected with N-terminal FLAG-tagged mouse α- and β-tubulin isotypes were harvested and immunoblotted with anti-FLAG antibody.

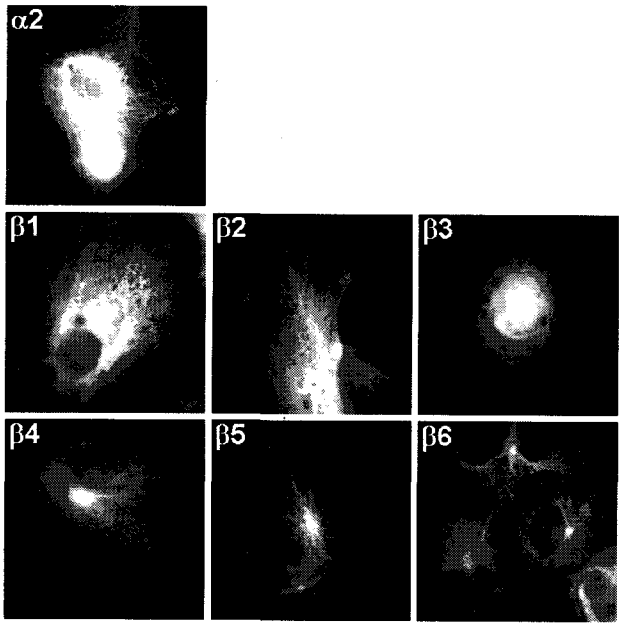


Fig. 2. Cells transfected with FLAG-tagged mouse α- and β -tubulin isotypes were visualized by immunofluorescence microscopy. cDNAs encoding FLAG-Mα2 and FLAG-Mβ1, -β2, -β3, -β4, -β5 and -β6 were transiently transfected into COS-7 cells. After 48 hr, the cells were fixed and stained with anti-FLAG antibody.

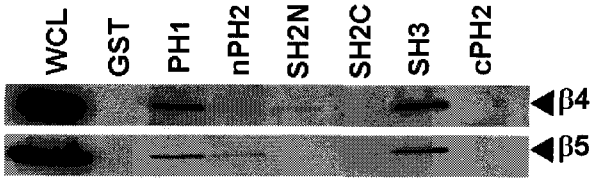
it was unable to assemble into microtubules, suggesting that the Mβ3 isotype likely exists as monomer or heterodimer with α-tubulin isotypes, as reported previously[3]. M α2-tubulin was also expressed in this system and shown to assemble into microtubules (Fig. 1 and 2). Together, these data indicate that the ectopically expressed tubulin isotypes behaved in a manner similar to the endogenous proteins.

Mβ4 and Mβ5 bind to PH and SH3 domain of PLC-γ1

To examine the binding specificity of the PH domains 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 Figure 3, GST-PH_1 and GST-nPH_2 associated with Mβ4- and Mβ5-tubulin isotypes and SH3 domain also showed high affinity for Mβ4- and Mβ5-tubulin, as shown by Western blotting, whereas SH2 domains (GST-SH2N and -SH2C) did not. α-Tubulin did not bind to the PLC-γ1 PH domains of in our *in vitro* binding assay.

Mβ4 and Mβ5 activate PLC-γ1 activity

To examine whether the Mβ4 and Mβ5 affect PLC-γ1 activity, we measured the PIP_2 hydrolyzing activity of



BT : α-FLAG

Fig. 3. PH domains of PLC-γ1 associate with β4-tubulin and β5-tubulin. 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 anti-FLAG antibody.

PLC-γ1 in the presence of purified Mβ4 and Mβ5. Mβ4 and Mβ5 were purified from the transiently transfected COS-7 cells according to the affinity purification system (Sigma-Aldrich) using anti-FLAG antibody. The purified M β4 and Mβ5 were pre-incubated with PLC-γ1 at 4 ℃ for 1 hr, and then [^{3}H]PIP_2 hydrolyzing activity of the mixture was measured. As shown in Figure 4, mouse tubulin substantially promoted PLC-γ1 activity, while purified BSA (control) did not. Mβ4 and Mβ5 activate PLC-γ1 activity approximately 1.5-fold.

Microtubule instability in cells overexpressing Mβ 4 and Mβ5

Since purified β4-tubulin and β5-tubulin promotes PLC-γ1

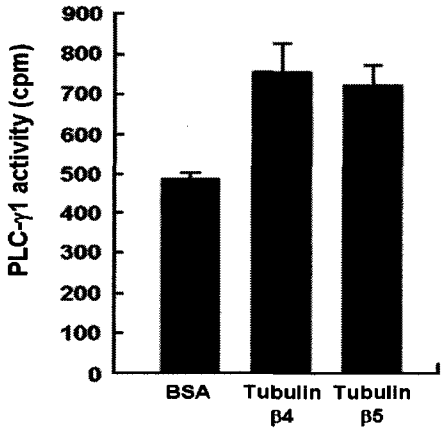


Fig. 4. Mβ4 and Mβ5 tubulin promote PLC-γ1 activity. The PIP_2 hydrolyzing activity of PLC-γ1 was measured in the presence of purified tubulin isotypes. PLC-γ1 and tubulin used in this experiment were purified from COS-7 cells (FLAG-PLC-γ1, -Mβ4 and -Mβ5). PLC-γ1 activity is expressed as the radioactivity of [^{3}H]IP_3. The data represent the average of duplicate determinations (mean ± range) from three experiments with similar results.

activity via protein-protein interactions, we next examined the dynamic instability of microtubule in cells overexpressing β4- and β5-tubulin. Microtubule coexists in both growing and shrinking states; these dynamic states interconvert frequently in vivo. So dynamic instability represents microtubule turnover that plays a critical role in the assembly of the spindle fiber and microtubule. To know whether the dynamic instability was changed in cells overexpressing β-tubulin isotypes, we examined the ratio between depolymerized tubulin to polymerized tubulin pool. As shown in Figure 5, polymerized pool was much higher than depolymerized pool in five β-tubulin isotypes including β1-, β2-, β4-, β5- and β6-tubulin, whereas depolymerized pool was much higher than that in cells overexpressing β3-tubulin. This result might because the existence of unpolymerized tubulin, which has been shown in Figure 2. Interestingly, cells expressing α2-tubulin showed higher proportion of depolymerized state than that

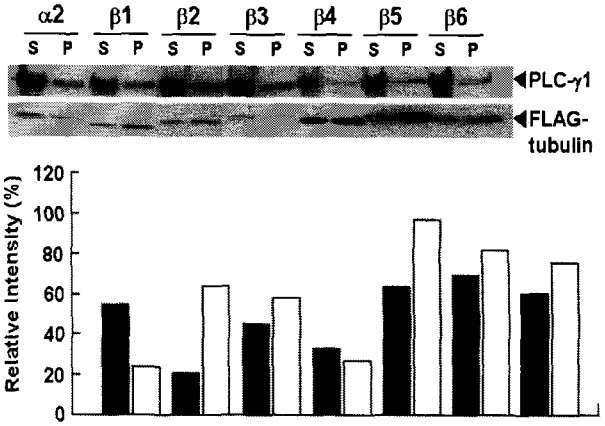


Fig. 5. Dynamic instability of microtubule in cells overexpressing tubulin isotypes. Quantitative Western immunoblotting was performed to examine the ratio between polymerized and depolymerized tubulin in COS-7 cells overexpressing each tubulin isotype. After transfection of the cells with pFLAG-Mβ1-Mβ6, depolymerized (monomeric) tubulin was extracted in 0.1 % Triton X-100 extraction buffer plus protease inhibitors at 37 ℃ for 20 min. After the Triton X-100-soluble supernatant (monomeric: M) was removed, polymerized tubulin was extracted SDS buffer from the Triton X-100-insoluble material (polymeric: P) [3]. 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-FLAG antibody, and then the filter was reprobed with anti-PLC-γ1 antibody for the indication of PLC-γ1 levels (*upper*). Quantitation of polymerized tubulin was performed by image density analysis (*lower*).

of polymerized, suggesting that α-tubulin isotype has different function in microtubule dynamics. Taken together, our results suggest that overexpression of α-tubulin isotype modulates dynamic instability of microtubule, while that of β-tubulin isotype did not affect microtubule instability.

Discussion

Our present study demonstrates that the PH domains of PLC-γ1 interact with β4- and β5-tubulin to promote enzyme activity. The PH domains of PLC-γ1 have been shown to facilitate membrane targeting via interactions with phosphoinositide[5] and activate enzymatic activity via protein-protein interactions[2].

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. During the cell cycle, tubulin polymerization and depolymerization are tightly regulated[4,11]. There are 6 different isotypes of β-tubulin in mammalian cells. We expressed all of these (Mβ1-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.

Our previous report had investigated PH_1 domain-mediated PLC-γ1/β-tubulin complex translocation in COS-7 cells[3]. Here, we showed that β4- and β5-tubulin also activate PLC-γ1 activity via direct binding. We examined the PIP_2 hydrolyzing activity of PLC-γ1 in the presence or absence of β-tubulin isotype. In the presence of β-tubulin (Fig. 4), the PIP_2 hydrolyzing activity of PLC-γ1 was increased about 1.5-fold under the conditions of a 1:2 molar ratio (PLC-γ1 to β-tubulin).

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 has been shown to directly binds to PIP_2[12]. 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.

Our data also shows that overexpression of tubulin isotype resulted in dynamics instability of microtubule. Dynamic instability was not changed in cells overexpressing β-tubulin isotypes, while it was modulated in cells over-

expressing α-tubulin isotype (Fig. 5), suggesting that α -tubulin isotype has different roles in the assembly.

Acknowledgments

We are grateful to Dr. Sally Lewis (New York University) for providing mouse α- and β-tubulin isotype cDNAs. This work was supported by Korean Council for University Education, grant funded by Korean Government (MOEHRD) for 2005 Domestic Faculty Exchange Program to J. -S. C., and also this study was undertaken during the Sabbatical Year Program awarded by Daejin University.

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초록 : 베타 튜불린에 의한 포스포리파제 C-감마1의 활성화

이인범 · 김성국 · 최장현[1] · 서판길[1] · 장종수*
(대진대학교 생명과학과, [1]포항공과대학교 생명과학과)

포스포리파제 C-감마1(phospholipase C-γ1; PLC-γ1)은 활성화될 경우 세포 내의 이차전령인 inositol 1,4,5-trisphosphate(IP_3)와 diacylglycerol(DG)을 생성하는 중요한 세포 신호전달 분자이다. 튜불린은 미세소관과 방추사의 주요 구성 단백질로서 알파형과 베타형의 두 가지 동위형이 있는데 이들은 모든 진핵세포에 존재하면서 이형 이합체를 형성한다. 이 중 베타형 튜불린은 사람의 경우 6종의 또 다른 동위형이 존재하는 것으로 밝혀졌는데 이들은 각 조직에서 그 발현양상이 서로 다르게 나타난다. 이전의 연구에서 우리들은 PLC-γ1과 4종의 베타 튜불린 동위형 즉, β1, β2, β3 및 β6이 세포 내에서 서로 결합할 수 있으며 또한 외부의 자극이 전달될 경우 이들 4종의 동위형이 PLC-γ1을 활성화시켜 준다는 사실을 보고한 바 있다. 이번 실험에서는 이전의 연구에서 조사하지 못하였던 베타 튜불린의 나머지 두 가지 동위형 즉, β4 및 β5가 PLC-γ1에 결합하여 PLC-γ1의 활성을 증가시켜줌으로서 세포 내에서의 신호전달계를 조절하고 있음을 확인하였다. 이 결과는 이전의 연구결과와 연관 지워볼 때, 6종의 모든 베타형 튜불린은 세포 외부의 자극이 있을 경우 PLC-γ1을 활성화시켜줌을 시사한다.