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Pleckstrin homology domain of phospholipase C-γ1 directly binds to 68-kDa neurofilament light chain

Sung-Kuk Kim[1], Jang Hyun Choi[2], Pann-Ghill Suh[2] and Jong-Soo Chang[1,3]

[1]Department of Life Science
College of Natural Science
Daejin University
Kyeonggido 487-711, Korea
[2]Department of Life Science
Division of Molecular and Life Science
Pohang University of Science and Technology (POSTECH)
Kyeongbuk 790-784, Korea
[3]Corresponding author: Tel, 82-31-539-1853;
Fax, 82-31-539-1850; E-mail, jchang@daejin.ac.kr

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Abbreviations: PLC, phospholipase C; NF, neurofilament; PH, pleckstrin homology; PIP_2, phosphatidylinositol 4,5-bisphosphate; NGF, nerve growth factor; PC12, pheochromocytoma 12; RIPA, radioimmunoprecipitation assay

Abstract

Phosphoinositide-specific phospholipase C-γ1 (PLC-γ1) has two pleckstrin homology (PH) domains: an amino-terminal domain (PH_1) and a split PH domain (PH_2). Here, we show that overlay assay of bovine brain tubulin pool with glutathione-S-transferase (GST)-PLC-γ1 PH domain fusion proteins, followed by matrix-assisted laser-desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), identified 68-kDa neurofilament light chain (NF-L) as a binding protein of amino-terminal PH domain of PLC-γ1. NF-L is known as a component of neuronal intermediate filaments, which are responsible for supporting the structure of myelinated axons in neuron. PLC-γ1 and NF-L colocalized in the neurite in PC12 cells upon nerve growth factor stimulation. *In vitro* binding assay and immunoprecipitation analysis also showed a specific interaction of both proteins in differentiated PC12 cells. The phosphatidylinositol 4, 5-bisphosphate [$PI(4,5)P_2$] hydrolyzing activity of PLC-γ1 was slightly decreased in the presence of purified NF-L *in vitro*, suggesting that NF-L inhibits PLC-γ1. Our results suggest that PLC-γ1-associated NF-L sequesters the phospholipid from the PH domain of PLC-γ1.

Keywords: neurofilament protein L; PC12 cells; phospholipase C gamma; phosphatidylinositol 4,5-diphosphate; protein interaction mapping

Introduction

PLC-γ1 plays a pivotal role in cellular signaling. Activated PLC-γ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), which regulate the release of Ca^{2+} from intracellular stores and activate protein kinase C (PKC), respectively (Berridge, 1993; Nishizuka, 1995). PLC-γ1 has two pleckstrin homology (PH) domains for protein-protein and protein-lipid interactions: one PH domain (PH_1) is located in the 150 N-terminal amino acid residues, the other (PH_2) is split by SH2-SH2-SH3 domain (Gibson *et al*., 1994). PH domains bind with high specificity and affinity to phosphoinositides such as PIP, PIP_2, PIP_3 and IP_3 (Lemmon and Ferguson, 2000), and the PH domains of signaling molecules are often involved in targeted translocation of molecules to cell membranes (Lemmon *et al*., 1996; Falasca *et al*., 1998). So, it is critical to identify and characterize the factors that regulate the PLC-γ1 activity by binding with PH domain of PLC-γ1. Here, we identify neurofilament light chain (NF-L) that specifically associate with the PLC-γ1 PH domain.

Neurofilaments (NF) are the most abundant cytoskeletal component in large myelinated axons during neuronal differentiation. They are heteropolymers composed of NF-L, NF-M and NF-H (Ching and Liem, 1993; Lee *et al*., 1993; Nakagawa *et al*., 1995). Each of them composed of three domains, an amino-terminal head domain, an α-helix rich rod domain, and carboxy-terminal tail domains (Fuchs and Weber, 1994). *In vitro* reconstitution studies observed that NF-L assembles to form the core filaments (Hisanaga and Hirokawa, 1990) and the resulting core filaments provide a scaffold to form intact neurofilament via cross-bridges either with NF-M or NF-H (Fuchs and Weber, 1994; Nakagawa *et al*., 1995). Recently, it is revealed that the transgenic mice overexpressing NF-L or NF-H resulted in abnormal assembly and aggregation of neurofilaments in neuronal cell bodies (Cote *et al*., 1993; Xu *et al*., 1993). Such neurofilament accumulation has been associated with neurodegenerative diseases such as Charcot-Marie-Tooth disease (Watson *et al*., 1994; Fabrizini *et al*., 2004),

Alzheimer's disease (Shepherd *et al*., 2002; Norgren *et al*., 2003) and amyotrophic lateral sclerosis (Hirano *et al*., 1984; Collard *et al*., 1995; Al-Chalabi and Miller, 2003).

Here, we present NF-L that specifically interact with the PH domain of PLC-γ1, and showed that NF-L inhibits PLC-γ1 activity by binding to PH domain of PLC-γ1.

Materials and Methods

Antibodies

Monoclonal anti-NF-L (mAb 1615) and polyclonal anti-NF-L were purchased from Chemicon (Temecula, CA). 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, polyclonal anti-PLC-γ1 and highly purified NF-L from bovine spinal cord were obtained from Sigma-Aldrich (St. Louis, Mo). 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 (Suh *et al*., 1988) as a template, glutathione-s-transferase (GST) constructs for fusion proteins were generated by polymerase chain reaction (PCR) as previously described (Chang *et al*., 2002). GST fusion proteins were expressed in *E.coli* and incubated the lysates with glutathione sepharose (GSH) 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 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) 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 enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Toroed, Norway) using monoclonal anti-FLAG and HRP-conjugated goat anti-mouse antibodies.

DNA construction and expression

For the expression in *E. coli*, cDNA sequence encoding the amino-terminal PH domain (PH_1) of rat PLC-γ1 (amino acids 25-145) (Suh *et al*., 1988) was ligated into the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Piscatawan, NJ) for GST-PH_1 fusion protein expression, as described previously (Chang *et al*., 2005). PCR-amplified rat cDNAs encoding NF-L (purchased from the American Type Culture Collection, ATCC, Rockville, MD) were ligated into the *Eco*RI/*Sal* I restriction site of pGEX-5X-1. For the expression of domain-specific NF-L fusion proteins, PCR-amplified cDNAs for the NF-L head domain (NF-L(H), amino acid residues 1-93), rod domain (NF-L(R), amino acid residues 93-397), head/rod domain (NF-L(H/R), amino acid residues 1-397), and tail domain (NF- L(T), amino acid residues 398-542) were separately inserted into the *Eco*RI/*Sal* I sites of pGEX-5X-1. All constructs were prepared using the Quiagen Plasmid Maxi Kit (Quiagen Inc., Santa Clarita, CA) and confirmed by DNA sequencing of the ligation sites.

Immunoprecipitation and immunoblotting

PC12 cells treated with 50 ng/ml nerve growth factor (NGF) were washed twice with phosphate- buffered saline (PBS) and lysed with radio immunoprecipitation assay (RIPA) buffer (20 mM Tris, 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 PMSF). For agonist stimulation, cells were serum starved for 24 h and then stimulated with 50 ng/ml NGF for 48 h. The lysate supernatants were precleared by incubation with Pansorbin (Calbiochem, La Jolla, CA) for 30 min. Precleared cell lysates were then incubated for 2 h with either polyclonal anti- NF-L antibodies or monoclonal anti-PLC-γ1 antibodies conjugated with affinity purified goat anti- mouse immunoglobulin G (IgG)-bound Pansorbin (Calbiochem). The immune complexes were collected by centrifugation, washed three times with ice-cold RIPA buffer, and then resolved by 10% SDS-PAGE and blotted to a PVDF membrane. The blot was probed with either anti-PLC-γ1 or anti- NF-L antibody 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 in 10% SDS-PAGE and transferred onto PVDF membrane. Nonspecific binding to the membrane was blocked by adding 2% skim milk in Tris-buffered Tween 20 (TBT) for 1 h at room temperature. The membranes were then incubated with GST-PH_1 and GST as a control (0.5 μg/ml) in blocking buffer for 14 h at 4°C. After washes in TBT buffer, the membranes were incubated with anti-GST antibody for 2 h at room temperature. After washing the membrane with TBT buffer again, bound proteins were detected by successive incubation with HRP-conjugated anti-goat antibody as a second antibody

using ECL detection system.

Immunofluorescent microscopy

PC12 cells were seeded on glass coverslips in 6-well plates, serum starved and treated 50 ng/ml NGF for 2 days in Dulbecco's modified Eagle's medium (DMEM) with 0.5% FBS. The cells were fixed at 37°C for 10 min in 4% paraformaldehyde and then incubated with polyclonal anti-NF-L or monoclonal anti-PLC-γ1 antibodies for 1 h at room temperature in a humidity chamber. Following complete washing with PBS, 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, Madison, WI) 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., Foster City, CA). Peptide mass maps were searched against theoretically derived maps from proteins found in the nonredundant protein database (NCBI) using the ProFound online program (www.proteometrics.com).

PLC-γ1 activity assay

PLC-γ1 activity was measured as described previously (Hepler *et al*., 1993). Briefly, the substrate was prepared as sonicated vesicles of 75 mM PIP_2, 75 mM [^{3}H] PI(4,5)P_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°C in 100 μl final volume containing 100 ng 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 NF-L on PLC-γ1 activity was examined by adding appropriate amounts of purified neurofilament mixtures to the PLC-γ1 assay mixture.

Results

The PH_1 domain of PLC-γ1 directly binds to NF-L

In our previous studies aimed at understanding the

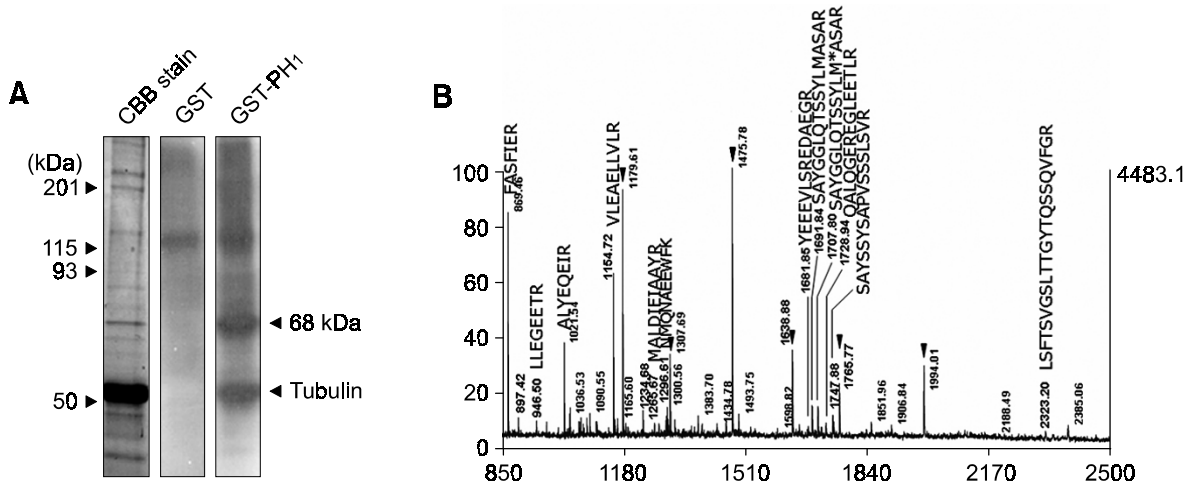

Figure 1. The PH domain of PLC-γ1 directly binds to NF-L. (A) Far Western blot hybridization reveals a direct interaction between PLC-γ1 and NF-L. Purified tubulin from bovine brain (2 μg per lane) was resolved by 10% SDS-PAGE, and either stained with Coomassie Brilliant Blue (CBB) or transferred to a PVDF membrane, probed with purified GST and GST-PH_1 fusion proteins, and detected with an HRP-conjugated goat anti-GST antibody. (B) MALDI-TOF mass spectrometric analysis of tryptic peptides from the unidentified 68-kDa protein. Tryptic peptides for keratin and oxidized methionine are indicated by ▼ and *, respectively.

Table 1. Tryptic peptides of NF-L identified by MALDI-TOF

Peptide sequence[a]	Start-end[b]	Monoisotopic mass ([M+H$^+$])	
		Theoretical	Experimental
SAYSSYSAPVSSSLSVR	37-53	1747.8	1747.88
FASFIER	100-106	868.9	869.46
VLEAELLVLR	116-125	1154.4	1154.72
ALYEQEIR	136-143	1021.1	1021.54
QALQGEREGLEETLR	157-171	1728.8	1728.94
YEEEVLSREDAEGR	177-190	1681.7	1681.85
NMQNAEEWFK	272-281	1296.4	1296.61
MALDIEIAAYR	380-390	1265.4	1265.67
LLEGEETR	392-399	946.0	946.50
LSFTSVGSLTTGYTQ-SSQVFGR	400-421	2323.5	2323.20

[a]Amino acid residues derived from the 68-kDa protein.
[b]Position of the amino acid residue in the deduced peptide sequence of bovine NF-L.

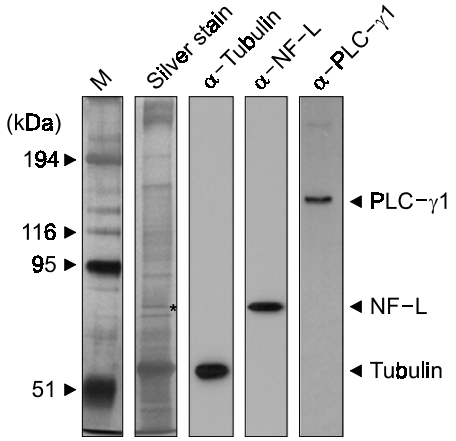

Figure 2. PLC-γ1 formed a complex with microtubule (MT) and neurofilament (NF). Purified tubulin from bovine brain (2 μg per lane) was resolved by 10% SDS-PAGE, transferred to a nylon membrane and probed with anti-tubulin, anti-NF-L or anti-PLC-γ1 antibodies, followed by an HRP-conjugated goat anti-mouse antibody for detection. The position of the NF-L band (determined by silver staining) is indicated with an asterisk (*).

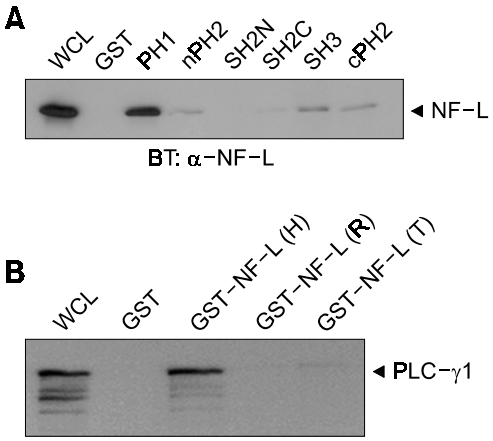

Figure 3. Domain specificity in PLC-γ1/NF-L interactions. (A) To examine the domain specificity of the NF-L association, different GST fusion proteins were incubated with PC12 cell lysates containing NF-L proteins. The bound proteins were resolved by 10% SDS-PAGE and immunoblotted with an anti-NF-L antibody. (B) The NF-L head domain interacts with PLC-γ1. The head (H), rod (R) and tail (T) domain GST fusion proteins were incubated with PC12 cell lysates for 1 h at 4°C, and the bound proteins were analyzed by Western blotting with anti-PLC-γ1. *WCL* indicates the whole cell lysates used for each pull-down experiment.

relationship between β-tubulin and PLC-γ1-dependent signaling (Chang *et al*., 2005), we detected a 68-kDa protein capable of specifically interacting with our GST-PLC-γ1-PH1 domain fusion protein in an overlay assay of purified bovine brain tubulin fraction (Figure 1A). In the present study, we focused on the interaction between the PH domain of PLC-γ1 and the 68-kDa protein. To identify this protein, we excised the gel band and analyzed it using MALDI-TOF mass spectrometry. Figure 1B shows the mass spectrum derived from a tryptic digest of this 68-kDa protein. Searching the peptide mass map against the NCBI protein database allowed us to identify the 68-kDa protein as bovine NF-L (Table 1). This identification was confirmed by Western blotting with monoclonal anti-NF-L (Figure 2).

As the bovine brain tubulin fraction contains NF-L, and PLC-γ1 binds to both β-tubulin and NF-L, we examined the co-existence of these three proteins in the bovine brain fraction. Western blotting of purified bovine brain tubulin with specific antibodies revealed that all three proteins were present in the tubulin pool (Figure 2), suggesting that PLC-γ1, tubulin and NF-L may exist as a trimeric complex in nerve cells. This is consistent with the results of microtubule (MT)/NF (Flynn and Purich, 1987; Hirokawa, 1982), MT/PLC-γ1 (Chang *et al*., 2005) and NF/PLC-γ1 interactions (in this study). Far Western blot analysis using a GST-PH1 fusion protein confirmed the interaction between PLC-γ1 and NF-L (Figure 1A), suggesting that this may be a direct interaction. Also, we examined the binding specificity of the PH domains to NF-L by comparing the binding reactions of GST fusion proteins including GST-PH1, -nPH2, -cPH2, -SH2, and -SH3. As shown in Figure 3A, Western blotting revealed that only GST-PH1 strongly associated with NF-L, whereas GST-nPH2, -cPH2,

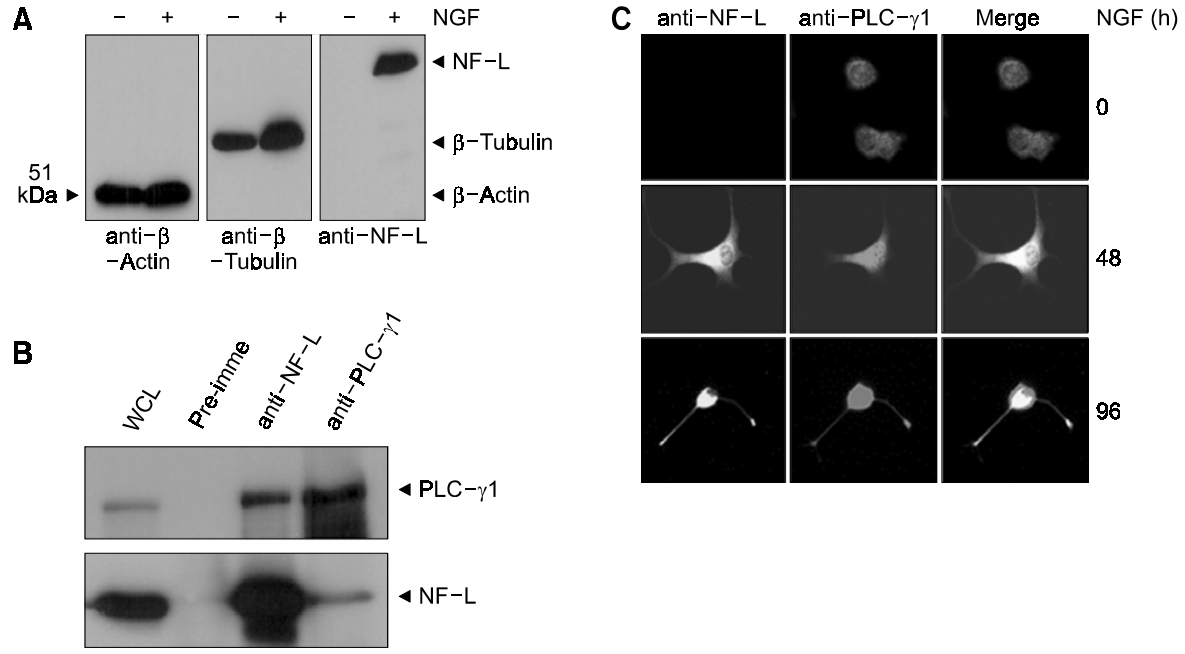

Figure 4. NGF-induced NF-L expressions in PC12 cells. (A) NF-L expression levels in PC12 cells treated with or without NGF (50 ng/ml) were examined by Western blotting. Serum-starved PC12 cells were treated with NGF for 48 h and then harvested for Western blotting with anti-β-actin, anti-β-tubulin and anti-NF-L. (B) Immunoprecipitation analysis of the *in vivo* interaction between NF-L and PLC-γ1. PLC-γ1 from differentiated PC12 cell lysates was coimmunoprecipitated with NF-L (upper), or NF-L was coimmunoprecipitated with PLC-γ1 (lower), using polyclonal antibodies in both cases. WCL indicates the whole cell lysates used for immunoprecipitation. (C) Colocalization of PLC-γ1 and NF-L in the neurite was assessed by double immunostaining for NF-L and PLC-γ1. PC12 cells treated with or without NGF (50 ng/ml) for 48-96 h were fixed with 4% paraformaldehyde for 10 min at 37°C and stained with a monoclonal anti-PLC-γ1 and a polyclonal anti-NF-L antibody, followed by rhodamine- and FITC-labeled secondary antibody staining, respectively.

-SH2N, SH2C and -SH3 did not.

Lastly, to identify the PLC-γ1 PH1 domain binding site on NF-L, we performed *in vitro* binding assays with the various GST-NF-L fusion proteins. As shown in Figure 3B, PLC-γ1 associated with GST-NF-L (H), but not GST-NF-L (R) and GST-NF-L (T), indicating that this binding occurs in the head domain of NF-L.

NGF induces NF-L expression and formation of PLC-γ1/NF-L complexes in PC12 cells

Rat pheochromocytoma PC12 cells differentiate into neuron-like cells in response to NGF treatment. To test the possible interaction of NF-L with PLC-γ1 following NGF treatment, we examined the effects of agonist stimulation on coimmunoprecipitation of PLC-γ1 with NF-L *in vivo*. As shown in Figure 4B, PLC-γ1 coimmunoprecipitated with NF-L and vice versa in NGF-treated PC12 cells, but not in untreated proliferating PC12 cells (data not shown). This might due to the lack of NF-L proteins in proliferating PC12 cell extracts, as shown by Western blotting (Figure 4A).

To examine the subcellular localizations of PLC-γ1 and NF-L in differentiated PC12 cells, we performed double immunostaining using anti-PLC-γ1 and anti-NF-L antibodies. As shown in Figure 4C, PLC-γ1 and NF-L were localized in neurite and cell bodies of differentiated PC12 cells. Overlays of the fluorescent microscopic images showed that the proteins dominantly colocalized in both locations. In contrast, untreated proliferating PC12 cells showed no NF-L immunostaining (Figure 4C).

$PI(4,5)P_2$ promotes the association between NF-L and PH_1 domain

Because the PH domain is known to be a phosphoinositides binding motif in numerous proteins (Lemmon and Ferguson, 2000), we next examined whether phosphoinositides are involved with the molecular interaction between the PLC-γ1 PH domain and NF-L. We performed a GST-PLC-γ1-PH_1 pull-down assay using PC12 cell lysates containing $PI(4,5)P_2$ in vesicle form. As shown in Figure 5, substantially more NF-L proteins bound to the GST-PLC-γ1-PH_1 domain in the presence of $PI(4,5)P_2$, compared to $PI(4,5)P_2$-untreated controls. These findings indicate that $PI(4,5)P_2$ promotes the

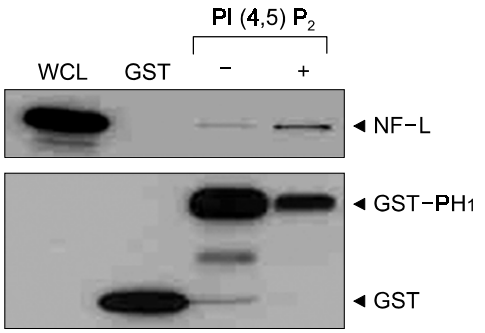

Figure 5. PI(4,5)P_2 promotes the interaction between the PH domain of PLC-γ1 and NF-L. GST-PH_1 domain fusion proteins were incubated with NGF-treated PC12 cell extracts (NF-L pool) in the presence of PI(4,5)P_2 (5 μg/100 μl reaction mixture), and bound proteins were analyzed by Western blotting with anti-NF-L (upper) and anti-GST (middle) antibodies. Two independent experiments showed similar results.

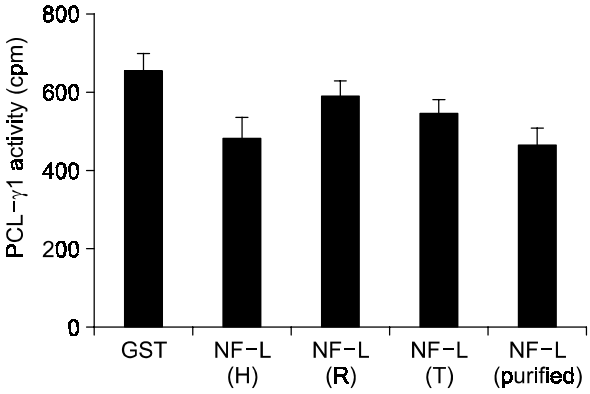

Figure 6. Purified NF-L inhibits PLC-γ1 activity. The PI(4,5)P_2 hydrolyzing activity of PLC-γ1 was measured in the presence of purified NF-L. The reactions were performed under the conditions of a 1:2 molar ratio (PLC-γ1 to NF-L). The PLC-γ1 and NF-L proteins used in this experiment were purified from insect sf9 cells and bovine spinal cord, respectively. GST-NF-L fusion proteins were prepared as described in "Experimental Procedures" 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.

association of PLC-γ1 with NF-L.

NF-L inhibits PLC-γ1 activity

To examine whether the complex formation affects PLC-γ1 activity, we measured the PI(4,5)P_2 hydrolyzing activity of PLC-γ1 in the presence of purified NF-L. The purified bovine NF-L mixture was pre-incubated with PLC-γ1 at 4°C for 1 h, and then [^{3}H]PI(4,5)P_2 hydrolyzing activity was measured. As shown in Figure 6, bovine NF-L slightly decreased PLC-γ1 activity, while purified BSA (control) did not. Moreover, GST-NF-L(H) also showed the inhibiting effect, and this inhibition probably caused by direct binding with PLC-γ1 PH_1 domain.

Discussion

Our present study demonstrates that NF-L interacts with the PH domain of PLC-γ1 to inhibit PLC-γ1 activity. The PH domains of PLC-γ1 have been shown to facilitate membrane targeting via interactions with phosphoinositide (Falasca *et al*., 1998; Kim *et al*., 2004) and activate enzymatic activity via protein-protein interactions (Chang *et al*., 2002). Previously, the amino terminal half of the split PH domain (nPH_2) of PLC-γ1 was shown to specifically bind EF-1α (Chang *et al*., 2002), the βγ-subunit of small G-protein (Gβγ) (Thodeti *et al*., 2000) and β-tubulin (Chang *et al*., 2005).

In our previous studies focused on β-tubulin/PLC-γ1 interaction (Chang *et al*., 2005), we unexpectedly found a 68-kDa protein that specifically interacts with PLC-γ1 PH_1 domain (Figure 1A) from bovine brain tubulin fraction and subsequently from NGF-treated PC12 cell extracts. In this study, we identified and characterized the 68-kDa protein as NF-L as a PLC-γ1 PH_1 domain-associated protein.

We sought the biological relevancy of the PLC-γ1/NF-L interaction by examination of the PI(4,5)P_2 hydrolyzing activity of PLC-γ1 in the presence or absence of NF-L. In the presence of NF-L (Figure 6), the PI(4,5)P_2 hydrolyzing activity of PLC-γ1 was decreased about 20%. We examined PLC-γ1 activity with different concentrations of NF-L, and the lowest activity was shown under the conditions of a 1:2 molar ratio (PLC-γ1 to NF-L). Although we did not directly investigate the mechanism for PLC-γ1 inactivation, we speculate that NF-L may sequester the phosphoinositides including PI(4,5)P_2 from the PH domain of PLC-γ1. This means that PLC-γ1 activity is not required for NF assembly, and suggests that binding of PH domain of PLC-γ1 to the head domain of NF-L may preclude NF assembly. This hypothesis is consistent with our previous finding that overexpression of either PLC-γ1 or its truncated molecule consisting of SH2-SH2-SH3, which contains a split PH domain (PH_2), prevented PC12 cell differentiation upon NGF treatment (Bae *et al*., 1998). Taken together with our previous finding that β-tubulin promotes PLC-γ1 activity (Chang *et al*., 2005), we propose that cytoskeletal proteins regulate PLC-γ1 activity, *i.e.* that PLC-γ1 activity is regulated by NF (a negative) and MT (a positive regulation)

We then sought to determine whether phosphoinositides affects PLC-γ1/NF-L interactions. In the pre-

sence of phosphoinositides, NF-L binding affinity for PH domain of PLC-γ1 was substantially increased (Figure 5), suggesting that the PH domain of PLC-γ1 may act for adaptor protein for neurofilament transport from cell body to the terminal in nerve cells. Two models have been suggested to account for neurofilament transport: i) neurofilament proteins are assembled in the cell body and transported into axons as fully assembled neurofilaments during axonal outgrowth (Baas and Brown, 1997) and ii) neurofilament proteins move as subunits (monomers or oligomers) and interact with previously assembled neurofilaments to form stable networks in the axon (Hirokawa *et al*., 1997). In either case, membrane-localized phosphoinositides including $PI(4,5)P_2$ may act as a terminal-anchor for transporting the neurofilament/PLC-γ1 complex, which has been formed in the cell body. This process might resemble the "cargo" model of UNC-104 (KIF1A) transport in *Caenorhabditis elegans* (Klopfenstein *et al*., 2002; Klopfenstein and Vale, 2004). This model suggests that the amino-terminal PH domain of PLC-γ1 regulates neurofilament transport, and is involved in neuronal differentiation along with phosphoinositides. Although future work will be required to determine the precise mechanism of PLC-γ1-mediated NF-L transport and assembly, it is possible that the PH domain of PLC-γ1 may recruit NF-L to the terminal.

In summary, our present results demonstrate that the PH domain of PLC-γ1 directly associates with NF-L to regulate cell differentiation. NF-L is associated with PLC-γ1 mainly in the cell body of PC12 cells, where PLC-γ1 is inactivated by NF-L.

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