

Phosphatidylinositol phosphates directly bind to neurofilament light chain (NF-L) for the regulation of NF-L self assembly

Sung-Kuk Kim¹, Ho Kim¹, Yong-Ryoul Yang²,
Pann-Ghill Suh² and Jong-Soo Chang^{1,3}

¹Department of Life Science
College of Natural Science
Daejin University
Kyeonggido 487-711, Korea

²School of Nano-Biotechnology and Chemical Engineering
Ulsan National Institute of Science and Technology
Ulsan 689-798, Korea

³Corresponding author: Tel, +82-31-539-1853;
Fax, +82-31-539-1850; E-mail, jchang@daejin.ac.kr
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Abbreviations: GST, glutathione S-transferase; NF-L, neurofilament light chain; PH, pleckstrin homology; PIP₂, phosphatidylinositol 4,5-bisphosphate; PtdInsP, phosphatidylinositol phosphate; PLC- γ 1, phospholipase C- γ 1

Abstract

Phosphatidylinositol phosphates (PtdInsPs) are ubiquitous membrane phospholipids that play diverse roles in cell growth and differentiation. To clarify the regulation mechanism acting on neurofilament light chain (NF-L) self assembly, we examined the effects of various PtdInsPs on this process. We found that PtdInsPs, including PI(4,5)P₂, directly bind to the positively charged Arg⁵⁴ of murine NF-L, and this binding promotes NF-L self assembly *in vitro*. Mutant NF-L (R53A/R54A) proteins lacking binding affinity to PtdInsPs did not have the same effect, but the mutant NF-L proteins showed greater self assembly than the wild-type in the absence of any PtdInsP. These results collectively suggest that Arg⁵⁴ plays a pivotal role in NF-L self assembly by binding with PtdInsPs.

Keywords: neurofilament protein L; phosphatidylinositol phosphates; phospholipase C γ

Introduction

Phosphatidylinositol phosphates (PtdInsPs) play multiple roles in cell growth and differentiation (Berridge, 1993; Nishizuka, 1995; Lemmon *et al.*, 1996; Carlton and Cullen, 2005), acting as: (i) precursors of second messenger molecules; (ii) effectors for the localization and assembly of protein molecules; and (iii) components of the cellular plasma membrane.

Neurofilaments (NFs), the most abundant cytoskeletal components in large myelinated axons, are obligate heteropolymers composed of NF-L, NF-M and NF-H (Ching and Liem, 1993; Lee *et al.*, 1993; Nakagawa *et al.*, 1995). Each NF is composed of three domains: an α -helix-rich rod domain in the middle, flanked by an N-terminal head domain and a C-terminal non- α -helical tail domain (Fuchs and Weber, 1994; Grant and Pant, 2000; Al-Chalabi and Miller, 2003). NF-L is known to self-assemble into core filaments that then form cross-bridges with NF-M or NF-H (Fuchs and Weber, 1994; Nakagawa *et al.*, 1995). Recent studies have revealed that overexpression or point mutation of NF-L can result in abnormal NF assembly and aggregation in neuronal cell bodies (Cote *et al.*, 1993; Xu *et al.*, 1993; Mathieu *et al.*, 1995; Carter *et al.*, 1996; Lee *et al.*, 2008). NF aggregations due to overexpression or point mutations of NF-L have been associated with neurodegenerative diseases such as Charcot-Marie-Tooth disease (Watson *et al.*, 1994; Fabrizi *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). Notably, phosphorylation of the NF-L head domain can inhibit self assembly or induce disassembly of polymerized NF-L (Sihag and Nixon, 1991; Guan *et al.*, 1992; Hisanaga *et al.*, 1994; Gibb *et al.*, 1996; Mukai *et al.*, 1996; Yates *et al.*, 2009). However, the factors and mechanisms that regulate NF assembly are not yet fully understood. Thus, we need to identify and characterize additional factors capable of regulating the cytoplasmic assembly, axonal transport and organization of NFs. Phospholipase C- γ 1 (PLC- γ 1) plays a pivotal role in cell growth and differentiation by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DG)

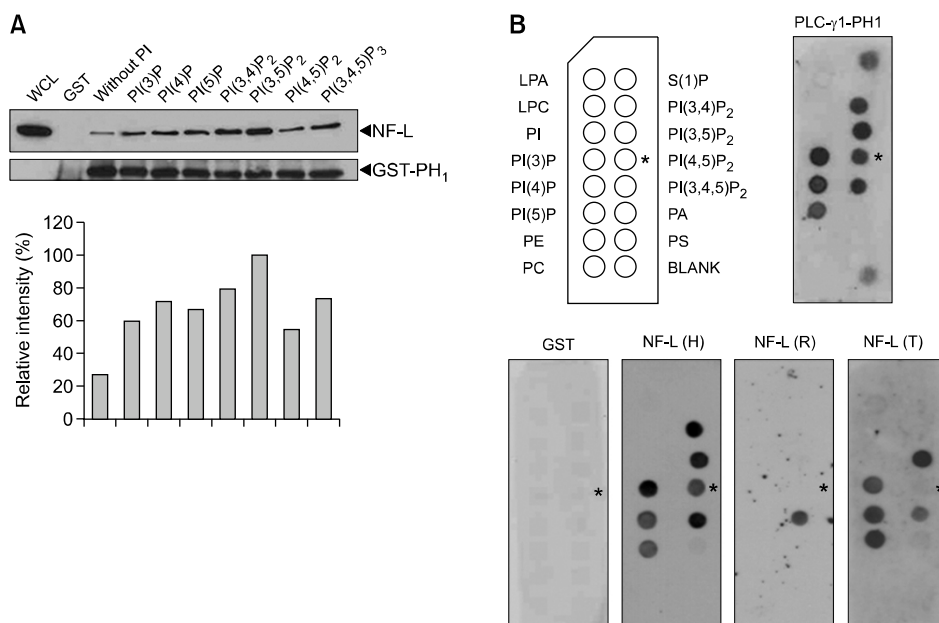


Figure 1. PtdInsPs promote the interaction between the PH1 domain of PLC- γ 1 and NF-L. (A) GST-PLC- γ 1-PH1 domain fusion proteins were incubated with NGF-treated PC12 cell extracts (NF-L pool) in the presence of different PtdInsPs (50 μ g/ml), and bound proteins were analyzed by Western blotting with anti-NF-L (upper) and anti-GST (middle) antibodies. The relative intensities of the NF-L bands were analyzed using image analysis software (Quantity One, Bio-Rad) (lower). Two independent experiments showed similar results. WCL represents whole cell lysate. (B) Dot-blot analysis shows that PtdInsPs directly bind NF-L. PIP strips were incubated with purified GST, GST-PH1, GST-NF-L (H), GST-NF-L (R) and GST-NF-L (T) fusion proteins (0.5 μ g/ml) in TBT buffer containing 2% non-fat skim milk for 14 h at 4°C. After washes with TBT buffer, the membranes were incubated with a goat anti-GST antibody for 2 h at room temperature. Finally, the bound proteins were detected with an HRP-conjugated anti-goat antibody. Each dot contained 100 pM of PI. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S(1)P, sphingosine-1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. The asterisk (*) represents the position of PI(4,5)P₂.

(Suh *et al.*, 1988; Berridge, 1993; Rhee, 2001; Shin *et al.*, 2002). PLC- γ 1 has two pleckstrin homology (PH) domains for protein-protein and protein-lipid interactions (Gibson *et al.*, 1994; Falasca *et al.*, 1998; Chung *et al.*, 2010): PH1 is located in the 150 amino acid residues closest to the N-terminus, while PH2 is located near the center of the molecule and is split by an SH2-SH2-SH3 domain (Gibson *et al.*, 1994; Chang *et al.*, 2002). PH domains bind with high specificity and affinity to phosphatidylinositol phosphates (PtdInsPs) such as PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ and PI(3,4,5)P₃ (Lemmon and Ferguson, 2000; Cozier *et al.*, 2004; Carlton and Cullen, 2005; Balla, 2005). During our study of the interaction between the PH domains of PLC- γ 1 and NF-L (Kim *et al.*, 2006), we found that NF-L also associates with PtdInsPs.

Here, we show for the first time that NF-L directly binds PtdInsPs to regulate NF-L self assembly. These findings provide important new insights into the roles of PtdInsPs in neuronal differentiation.

Results

PtdInsPs promote the association between NF-L and the PH1 domain of PLC- γ 1

In our studies aimed at understanding the relationship between NF-L and PLC- γ 1-dependent signaling, we found that the PH1 domain of PLC- γ 1 directly binds to NF-L (Kim *et al.*, 2006). Because the PH domain is known to be a PtdInsP-binding motif in numerous proteins (Lemmon and Ferguson, 1996; Klopfenstein *et al.*, 2002; Klopfenstein and Vale, 2004), we examined whether PtdInsPs could be involved in the molecular interaction between the PLC- γ 1 PH1 domain and NF-L. We performed a GST-PLC- γ 1-PH1 pull-down assay using nerve growth factor (NGF)-treated PC12 cell lysates containing different PtdInsPs in vesicle form including PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ and PI(3,4,5)P₃. As shown in Figure 1A, substantially more NF-L proteins bound to the GST-PLC- γ 1-PH1 domain in PtdInsP-harboring cells compared to untreated controls. This result led us to examine whether NF-L itself can bind

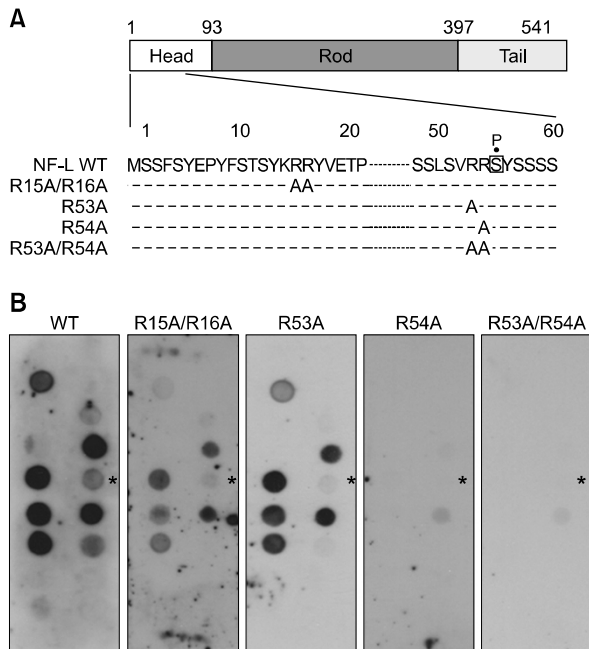


Figure 2. PtdInsPs directly binds to Arg⁵⁴ of NF-L. (A) Schematic representation of the NF-L head domain containing point mutations in arginine residues, which were replaced with the neutral amino acid, alanine. The boxed "S" represents a possible phospho-Ser. (B) Two site-directed point mutants of NF-L, R54A and R53A/R54A, had no binding affinity for PtdInsPs. Point-mutant GST-fusion proteins were used for dot-blotting as described in Figure 1 (B). The asterisk (*) represents the position of PI(4,5)P₂.

PtdInsPs.

PtdInsPs directly bind to NF-L

To examine the binding affinity and specificity of NF-L for phospholipids, we purified GST-fused NF-L proteins containing GST-NF-L(H), -NF-L(R) and -NF-L(T), which represent head (H), rod (R) and tail (T) domains of NF-L, from an *E. coli* expression system and applied the recombinant proteins to dot-blot analysis using Echelon strips (See 'Methods'). Both the NF-L(H) and NF-L(T) domains specifically bound all of the tested PtdInsPs with high affinity (Figure 1B), whereas the NF-L(R) domain showed a weak affinity for only PI(3,4,5)P₃. Thus, NF-L directly binds to phospholipids containing the phosphatidylinositol rings of PtdInsPs but not to other tested lipids, such as phosphatidylinositol (PI), phosphatidylethanol (PE), phosphatidylcholine (PC) and sphingosine 1-phosphate (S(1)P) (Figure 1B). NF-L(H) and NF-L(T) showed the highest affinity for PI(3,5)P₂, which strongly promoted the molecular interaction between NF-L and the PLC-γ1 PH1 domain (Figure 1A). NF-L(H) and NF-L(T) showed the lowest affinity to PI(4,5)P₂, which only moderately promoted

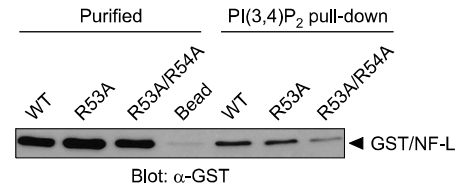


Figure 3. The R53A/R54A mutant shows reduced binding affinity for PIP₂. Bacterially expressed GST-NF-L proteins were incubated with PIP₂-bound agarose beads for 1 h at 4°C, and the bound proteins were quantified by Western blotting.

the interaction between NF-L and the PLC-γ1 PH1 domain (Figure 1A). The PLC-γ1 PH1 domain also showed high affinities for PI(3,5)P₂ and PI(3)P, and had a higher affinity to PI(4,5)P₂ than did the NF-L protein (Figure 1B).

Since our results revealed that the NF-L molecule binds to PtdInsPs but not to other phospholipids (e.g., PI, PC or PE), we speculated that the negatively charged phosphate groups in 3rd, 4th and 5th carbon positions of the inositol ring may supply the binding affinity to NF-L. If this is true, then the positively charged basic amino acid residues of NF-L may be involved in binding the phosphate group of the inositol ring. To determine the amino acid residue(s) in NF-L responsible for binding PtdInsPs, we constructed point mutants in which Arg¹⁵/Arg¹⁶, Arg⁵³, Arg⁵⁴ and Arg⁵³/Arg⁵⁴, one or two basic amino acids in the head domain of NF-L, were replaced with alanine (Ala; a neutral amino acid) (Figure 2A). As shown in Figure 2B, the R54A and R53A/R54A mutants showed almost no affinity for the tested PtdInsPs (Figure 2B), while the R53A and R15A/R16A mutants showed only slight decreases in their binding affinities for the tested PtdInsPs (Figure 2B). These results suggest that the positively charged Arg⁵⁴ is responsible for the binding of NF-L with PtdInsPs. The Ser⁵⁵ residue (S; boxed in Figure 2A) may be phosphorylated by some protein kinases (Guan *et al.*, 1992; Gibb *et al.*, 1996; Grant and Pant, 2000).

We also assessed the PIP₂ binding affinity for R53A/R54A mutant and wild-type NF-L using PIP-bound agarose beads (Figure 3). The beads were incubated with GST-R53A, -R53A/R54A or -wild-type NF-L for 1 h at 4°C, and then extensively washed with buffer. As shown in Figure 3, R53A/R54A mutant NF-L showed a substantially reduced affinity for PIP₂ compared with that of wild-type NF-L(H), suggesting that the head domain of NF-L lost its affinity for PIP₂ upon mutation of Arg⁵⁴ residue. In addition to NF-L head domain, there are so many basic amino acid residues in the tail domain of NF-L that shows a high affinity for PtdInsPs (Figure 1B). We then tried to identify the

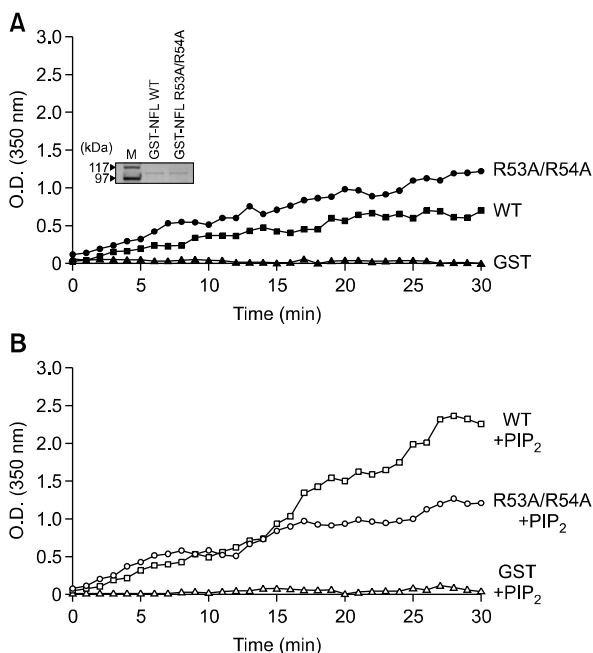


Figure 4. PtdInsP promotes NF-L assembly by direct binding to the Arg⁵⁴ of NF-L. (A) Comparison of NF-L self assembly between wild-type and R53A/R54A mutant NF-L *in vitro*. Bacterially expressed GST-NF-L proteins were incubated in NF-L polymerizing buffer and the optical density (OD) was measured over time. (B) PIP₂ affects NF-L self assembly. Wild-type and R53A/R54A mutant NF-L were incubated in the presence of PIP₂, and the OD was measured over time. Wild-type NF-L self assembly was promoted by PIP₂, whereas that of the mutant (which did not bind PIP₂) was not.

binding partner residues of PtdInsPs within the tail domain, none of 10 point mutants (substitutes alanine for arginine or lysine residue) showed decreased binding affinity for PtdInsPs. So we used only head domain mutant, NF-L(H)-R53A/R54A, for further experiments.

PIP₂ accelerates NF-L self assembly

Since purified NF-L can self assemble *in vitro* (Hisanaga and Hirokawa, 1990; Lee *et al.*, 1993), we next compared NF-L self assembly between R53A/R54A mutant and wild-type NF-L using the GST-fusion proteins. As shown in Figure 4A, R53A/R54A mutant NF-L showed higher self-assembly activity than wild-type NF-L, whereas the control GST protein showed basal activity (Figure 4A). Next, we compared the levels of NF-L self assembly between wild-type GST-NF-L and GST-NF-L-R53A/R54A mutant in the presence of PtdInsPs. The self-assembly reaction of the GST-NF-L-R53A/R54A mutant was unchanged in the presence of PIP₂, whereas that of wild-type NF-L was greatly increased by the presence of PIP₂ (Figure 4B), suggesting that binding of PIP₂ accelerates NF-L

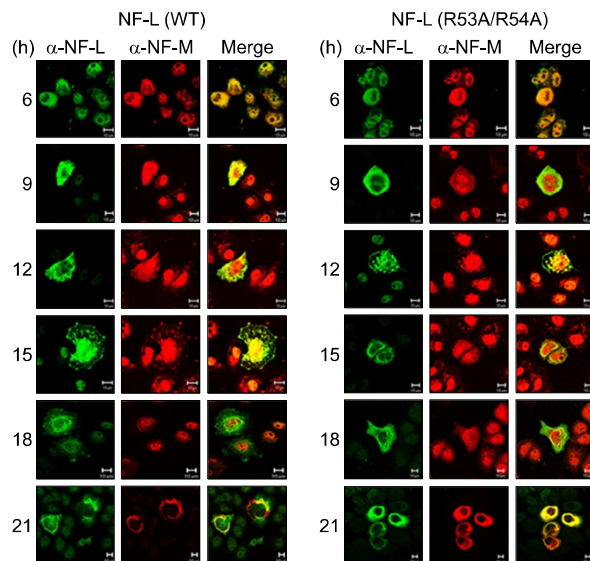


Figure 5. The R53A/R54A mutant shows accelerated NF assembly in intact cells. The cDNAs encoding rat NF-M/NF-L (wild set, left) or NF-M/NF-L-R53A/R54A (mutant set, right) were transiently co-transfected into SW13 cells (Vim⁻) grown on glass coverslips. The cells were fixed in 4% paraformaldehyde at the indicated times, and subjected to immunostaining with anti-NF-L and anti-NF-M antibodies, followed by detection using FITC- and rhodamine-conjugated secondary antibodies, respectively.

self assembly *in vitro*. We tested other PtdInsPs that showed binding to the NF-L head domain (Figure 1B), and found that their self-assembly-promoting effects on NF-L were similar to those of PIP₂ (data not shown).

The R53A/R54A mutant shows accelerated NF-L assembly *in vivo*

Finally, we compared the level of filament formation between the mutant and wild-type NF-L proteins in intact cells. The vimentin-deficient human adrenal carcinoma-derived SW13 cell line has been used for the examination of intermediate-filament formation (Lee *et al.*, 1993). Here, we examined the filament formation levels in SW13 cells co-transfected with vectors encoding rat NF-M with either wild-type NF-L (wild set) or NF-L-R53A/R54A (mutant set). The transfected cells were fixed and double stained with anti-NF-M and anti-NF-L antibodies at various time points. As shown in Figure 5, SW13 cells transfected with the mutant formed intermediate filaments within 15 h after transfection, whereas cells transfected with the wild-type construct needed at least 18 h to form filaments. The mutant set also developed rod-like thick filaments over time, while wild set showed thread-like filaments (Figure 5). This result suggests that *in vivo* intermediate-filament formation by NF-L

is regulated by binding between PtdInsPs and the Arg⁵⁴ residue.

Discussion

We herein show that the PtdInsPs directly interact with NF-L and regulate neuronal differentiation by binding with the NF-L head domain along with the PH1 domain of PLC- γ 1. We also examined whether PtdInsPs affect NF polymerization, and show for the first time that a number of PtdInsPs, including PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂ and PI(3,4,5)P₃, directly bind NF-L molecules (Figure 1B). All of the tested PtdInsPs bound the NF-L head and tail domains with different affinities, and were found to promote NF-L self assembly *in vitro*. Interestingly, among all tested PtdInsPs, PI(4,5)P₂ showed the poorest binding to NF-L.

Previously, researchers had hypothesized that charge-dependent polymerization (phosphorylation/dephosphorylation) of the head domain of NF-L is responsible for regulating NF-L self assembly (Sihag and Nixon, 1991; Hisanaga *et al.*, 1994; Nakamura *et al.*, 2000; Yates, 2009). The head domain of mammalian NF-L has an Arg-Arg-Ser (RRS) sequence (amino acid residues 53-55), where S⁵⁵ can be phosphorylated by protein kinases to preclude NF-L self assembly (Sihag and Nixon, 1991; Nakamura *et al.*, 2000; Yates *et al.*, 2009). These previous findings seem to indicate that binding of the negatively charged phosphate group from residue Ser⁵⁵ of the NF-L head domain precludes self assembly (Gibb *et al.*, 1996; Grant and Pant, 2000; Yates *et al.*, 2009).

Interestingly, our present findings suggests a new regulation mechanism wherein the positively charged Arg⁵⁴ acts as an adaptor for PtdInsPs, which function as positive effectors for NF-L assembly. Consistent with this hypothesis, we found that the R53A/R54A double mutant was resistant to PtdInsP-induced NF-L self assembly. However, in the absence of any PtdInsP, the R53A/R54A mutant was more efficient than the wild-type protein for self assembly both *in vitro* (Figure 4) and *in vivo*, as assessed by intermediate-filament formation in SW13 cells (Figure 5). The mutant-transfected SW13 cells showed a characteristic pattern of filament assembly, characterized by rapid self assembly and the formation of thick, rod-like filaments (Figure 5). Our observations that replacement of the positively charged arginine with a neutral amino acid increased the efficiency of NF-L self assembly may suggest that the positive charge of Arg⁵⁴ attenuates the self assembly of wild-type NF-L.

In sum, our present findings suggest that Arg⁵⁴

plays a key role in NF-L self assembly and neurofilament formation, likely via an association with PtdInsPs and/or the direct attenuation of NF-L self assembly. These observations provide important new insights into the roles of PtdInsPs in neuronal differentiation.

Methods

Antibodies and cell culture

Monoclonal anti-NF-L (mAb 1615) and polyclonal anti-NF-L were purchased from Chemicon (Temecula, CA). Monoclonal anti-NF-M (mAb RNF403) was purchased from MP Biochemicals (Aurora, OH). The horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Fluorescein-conjugated Affinipure goat anti-rabbit IgG and rhodamine-conjugated Affinipure goat anti-mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). All PtdInsPs were purchased from Sigma-Aldrich (St. Louis, MO), and the Echelon Strips (P-6001) and PIP-bound agarose beads (P-B045a) were purchased from Echelon Bioscience Inc. (Salt Lake City, UT).

PC12 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 5% horse serum and 100 U/ml penicillin-streptomycin. The human adrenal carcinoma SW13 cell line (Vim) was grown at 37°C in DMEM supplemented with 10% FBS.

Vector constructs and protein expression

For expression in *E. coli*, the cDNA sequence encoding the amino-terminal PH domain (PH1) of rat PLC- γ 1 (amino acids 25-145) (Suh *et al.*, 1988) was ligated into the pGEX-5X-1 vector (Amersham Pharmacia Biotech Inc. Piscataway, NJ) for expression of a GST-PH1 fusion protein, as previously described (Chang *et al.*, 2002). PCR-amplified rat cDNAs encoding NF-L (purchased from the American Type Culture Collection, Manassas, VA) were ligated into the *EcoRI/SalI* 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 *EcoRI/SalI* sites of pGEX-5X-1. The GST-NF-L-R54A and GST-NF-L-R53A/R54A point mutants were engineered by PCR-based point mutation (Takara Bio Inc. Kyoto, Japan). All constructs were prepared using a Qiagen Plasmid Maxi Kit (Qiagen Inc., Santa Clarita, CA) and confirmed by direct sequencing of the ligation and mutation sites.

The GST fusion proteins were expressed in *E. coli*, and lysates were incubated with glutathione sepharose (GSH) beads. The bound beads were washed extensively with Igepal buffer [20 mM Tris-Cl, pH 7.5, 1% Igepal CA-630, 300 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride

(PMSF) and 1 mM sodium orthovanadate], and the purified proteins were eluted with a buffer containing reduced glutathione.

To examine intermediate-filament formation in SW13 cells, cDNAs encoding rat NF-L (purchased from ATCC) and NF-M (kindly provided by Dr. S. Hisanaga, Tokyo Metropolitan University, with the permission of Dr. R. K. H. Liem, Columbia University) were ligated into the pCMV-FLAG (Clontech Laboratories, Mountain View, CA) and pREFA vectors (Chang *et al.*, 1997), respectively. The resulting constructs, pFLAG-NF-L and pREFA-NF-M, produced proteins that were FLAG-tagged at their N-termini. SW13 cells were transiently co-transfected with pFLAG-NF-L and pREFA-NF-M using a ProFection kit (Promega, Madison, WI), which applies the calcium-phosphate coprecipitation method. The cells were fixed at 37°C for 10 min in 4% paraformaldehyde for either immunostaining using anti-NF-L and anti-NF-M antibodies, or immunoblotting with an anti-FLAG antibody. For green fluorescent protein (GFP)-fused NF-L expression, PC12 cells were transiently transfected with constructs encoding GFP-NF-L or GFP-NF-L-R53A/R54A fusion proteins, using a ProFection kit.

Immunofluorescent microscopy

SW13 cells were seeded on glass coverslips in 6-well plates, transfected using a ProFection kit, and grown for 2 days in DMEM. The cells were fixed at 37°C for 10 min in 4% paraformaldehyde, and then incubated with affinity purified monoclonal anti-NF-M or polyclonal anti-NF-L antibodies for 1 h at room temperature in a humidified 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 photographed under a confocal laser-beam fluorescent microscope (Perkin-Elmer Inc., Waltham, MA).

Dot-blot analysis

The ability of the PH1 domain and NF-L proteins to bind different phospholipids was examined by dot-blot analysis using Echelon P-6001 strips (Echelon Bioscience Inc.). Each membrane was blocked with TBT buffer for 1 h and then incubated with purified GST, GST-PH1, GST-NF-L or point-mutant GST-NF-L fusion proteins (0.5 µg/ml) in TBT buffer containing 2% non-fat skim milk for 14 h at 4°C. After washes with TBT buffer, the membranes were incubated with goat anti-GST antibodies for 2 h at room temperature. The membranes were then washed extensively with TBT buffer, and then the bound proteins were detected with HRP-conjugated anti-goat antibodies and visualized using an ECL detection system.

In vitro assay of NF-L self-assembly

To assess NF-L polymerization *in vitro*, purified GST-NF-L or mutant NF-L fusion proteins obtained from *E. coli* were dissolved in 200 µl of NF-L polymerizing buffer (20 mM Tris-Cl, pH 7.0, 1 mM dithiothreitol, 1 mM MgCl₂, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 2 µg GST-NF-L) and incu-

bated for 0.5 h at 35°C, and then optical density was measured at 350 nm. To examine the effect of PtdInsPs on NF-L self assembly, appropriate amounts of phospholipid vesicles (Li *et al.*, 2006) were added before incubation and optical density was measured at 350 nm over time.

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