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Phospholipase D1 is an Important Regulator of bFGF-Induced Neurotrophin-3 Expression and Neurite Outgrowth in H19-7 Cells

Hye-Jin Choi · Brian Junho Chang · Joong-Soo Han

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Abstract The purpose of this study was to examine the role of phospholipase D1 (PLD1) in basic fibroblast growth factor (bFGF)-induced neurotrophin-3 (NT-3) expression and neurite outgrowth in H19-7 rat hippocampal neuronal progenitor cells. Overexpression of PLD1 increased bFGF-induced NT-3 expression, and dominant-negative-PLD1 or PLD1 siRNA abolished bFGF-induced NT-3 expression and neurite outgrowth. Treatment with bFGF activated the RhoA/Rho-associated kinase (ROCK)/c-jun N-terminal kinase (JNK) pathway, and bFGF-induced NT-3 expression was blocked by a dominant-negative RhoA as well as by a specific Rho-kinase inhibitor (Y27632) and a SAPK/JNK inhibitor (SP600125). Furthermore, bFGF-induced JNK activation was also blocked by Y27632. These results indicate that the RhoA/ROCK/JNK pathway acts as an upstream signaling pathway in bFGF-induced NT-3 expression. Also, phosphatidic acid, the product of PLD, increased NT-3 expression. We found that PLD regulated the RhoA/ROCK/JNK pathway, which then led to Elk-1 transactivation. When Elk-1 activity was blocked by Elk-1 siRNA, bFGF-induced NT-3 expression and neurite outgrowth decreased. NT-3 overexpression increased neurite outgrowth, indicating that NT-3 is important for neurite outgrowth. Taken together, these results suggest that PLD1 is an important regulator of bFGF-induced NT-3 expression and neurite outgrowth, which are mediated by the RhoA/ROCK/JNK pathway via Elk-1 in H19-7 cells.

Keywords Phospholipase D1 · RhoA · JNK · Elk-1 · NT-3 · Neurite outgrowth

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Introduction

Basic fibroblast growth factor (bFGF), a member of FGF family, has broad biological functions involving the regulation of cell growth, differentiation, and proliferation [1, 2]. Binding of bFGF to cell surface tyrosine kinase FGF receptors (FGFRs) triggers receptor dimerization and autophosphorylation of tyrosine residues [3], and this in turn creates binding sites for the SH2 domains of a number of transduction molecules such as Src and PLCγ. These may happen independently or synergistically to stimulate survival, mitogenesis, and differentiation [4, 5]. We previously reported that bFGF upregulates phospholipase D1 (PLD1) activity during neuronal differentiation in H19-7 rat hippocampal neuronal progenitor cells [6]. However, details of the intracellular pathway by which PLD activates neurite outgrowth are not fully understood.

PLD, which has two isoforms PLD1 and PLD2, is a phospholipid-metabolizing enzyme which catalyzes the hydrolysis of phospholipids, mainly phosphatidylcholine (PC), forming phosphatidic acid (PA) and free polar head group, choline. PA by itself can act as a cellular messenger and can be converted to diacylglycerol by PA phosphohydrolase (PAP) or to lysophosphatidic acid (LPA) by phospholipase A_2 (PLA_2). Both of these factors serve as second messengers that contribute to the effects of PLD. PA is associated with many aspects of mammalian physiology such as proliferation, survival, and differentiation [7, 8]. It is

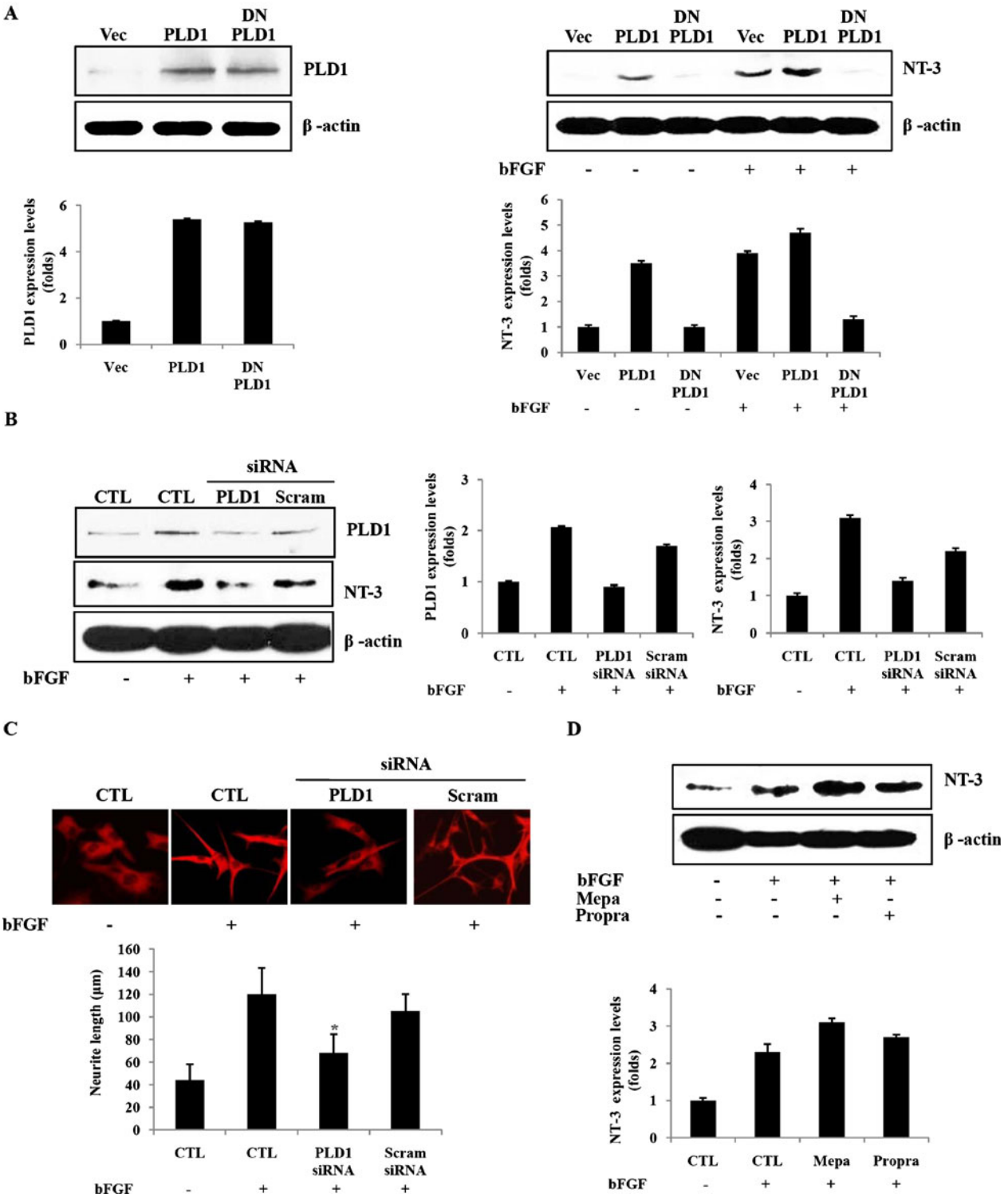


Fig. 1 Effect of PLD1 knockdown on bFGF-induced NT-3 expression and neurite outgrowth in H19-7cells. **a** Cells were infected with control retroviral *vector* and vectors harboring *PLD1* or *DN-PLD1*. At 72 h after infection, they were treated with bFGF (20 ng/ml) for 48 h. Cell lysates were analyzed by Western blot analysis using PLD1 and NT-3 antibodies. **b** Cells were grown at 33°C in DMEM containing 10 % FBS, transiently transfected with 100 nM PLD1 siRNA or scrambled siRNA as a control for 48 h, and then stimulated with bFGF (20 ng/ml) for 48 h. Expression levels of PLD1 and NT-3 were determined by Western blot analysis. **c** Cells were grown at 33°C in DMEM containing 10 % FBS, transiently transfected with 100 nM PLD1 siRNA or scrambled siRNA for 48 h, and then stimulated with bFGF (20 ng/ml) for 48 h. Immunocytochemical analysis with anti-Tuj1 antibody and neurite length measurements were performed as described in "Materials and Methods." **d** Cells were pre-treated with *Mepa* (mepacrine, 50 μM) or *Propra* (propranolol, 50 μM) for 30 min and then stimulated with bFGF (20 ng/ml) for 48 h. The expression level of NT-3 was determined by Western blot analysis. Band intensities were quantified using ImageJ software (NIH-http//rsb.info.nih.gov/ij/)

expressed in many functionally diverse brain areas, such as the spinal cord, brainstem, and cerebral cortex [9], and plays a critical role in neurite outgrowth of neural stem cells [10].

Rho GTPases are active in the regulation of signal transduction pathways required for neuronal differentiation [11], and differential activation of Rho GTPases contributes to the generation of morphological diversity in developing cortex [12]. RhoA and Rac1 can promote neurite outgrowth that regulates and stabilizes adhesion contacts with the cell matrix [13]. The RhoA downstream effector, Rho-associated kinase (ROCK), enhances neurite extension and growth cone movement [14, 15]. Activation of Rho family proteins contributes to neuronal differentiation in H19-7 cells [16] and regulates gene expressions by activating mitogen-activated protein kinase (MAPK) cascade [17–19]. Extracellular-regulated protein kinases (ERK), p38 MAPKs, and c-jun N-terminal kinases (JNK) are three groups of subfamilies of the MAPK family. Among the MAPK subtypes, JNK is especially crucial for cellular differentiation induced by external stimuli [20] and has also been implicated in survival, cellular stress, apoptosis, transformation, and

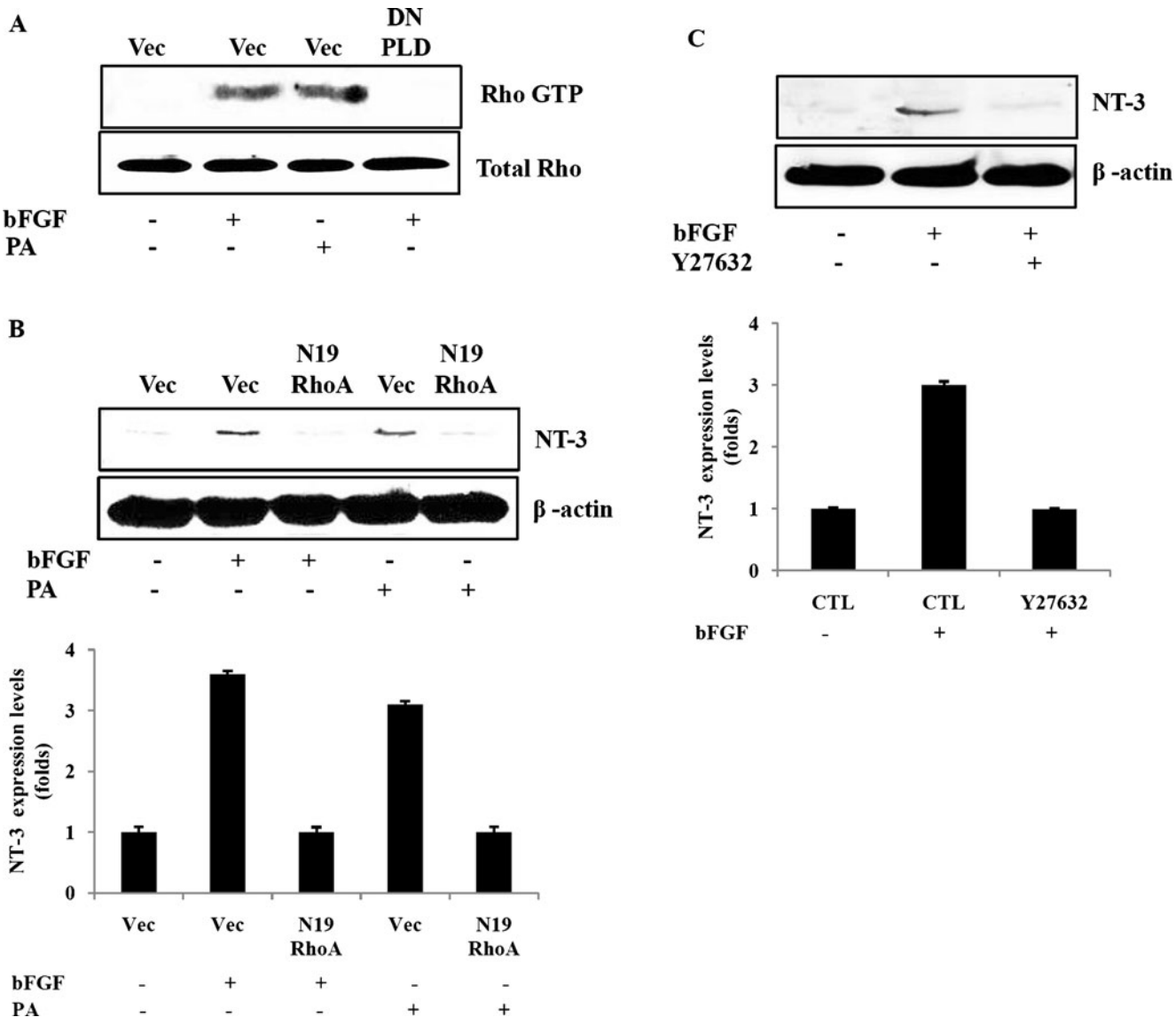


Fig. 2 Effect of RhoA/ROCK in bFGF-induced NT-3 expression in H19-7cells. **a** Cells were infected with control retroviral *vector* and vector harboring *DN-PLD1*; 72 h after infection, they were treated with PA (50 μM) or bFGF (20 ng/ml) for 15 min. Activated RhoA was measured as described in "Materials and Methods" by measuring its GTP-bound form. **b** Cells were transfected with 5 μg of either control vector or vector expressing a dominant-negative form of RhoA (*N19*); 48 h later, the cells were treated with bFGF (20 ng/ml) or PA (50 μM) for 48 h. The expression level of NT-3 was determined by Western blot analysis. **c** Cells were pretreated with Y27632 (30 μM) for 1 h and then stimulated with bFGF (20 ng/ml) for 48 h. The expression level of NT-3 was determined by Western blot analysis. Band intensities were quantified using ImageJ software (NIH-http//rsb.info.nih.gov/ij/)

morphogenesis [21]. In addition, JNK can phosphorylate Elk-1 to promote gene transcription [22]. Elk-1 activity is regulated by phosphorylation at Ser383 and occurs in association with cell stress and cytokine production and differentiation indicating that a variety of distinct signaling cascades are capable of stimulating Elk-1 activity [16, 22].

The formation of neurites in cortical neurons is regulated by neurotrophins [23]. Neurotrophin-3 (NT-3) is a member of the neutrophin family, which includes brain-derived neurotrophic factor (BDNF), neurotrophin-4.5, and nerve growth factor [24, 25]. NT-3 is expressed in neuronal cells, where it plays crucial roles in survival, development, and differentiation of the central and peripheral nervous systems [25, 26]. However, the molecular mechanisms of bFGF-induced NT-3 expression and the relationship between PLD and NT-3 are not well defined.

In the present study, we investigated the role of PLD in bFGF-induced NT-3 expression and neurite outgrowth mediated by Elk-1 through RhoA/ROCK/JNK pathway. We show that PLD is an important mediator in a bFGF-stimulated RhoA/ROCK-/JNK/Elk-1 pathway that leads to expression of NT-3 and neurite outgrowth in H19-7 cells.

Materials and Methods

Materials

Fetal bovine serum (FBS), penicillin/streptomycin solution, and Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and low glucose were purchased from WelGENE (Daegu, Korea), and bFGF was from R&D Systems (Minneapolis, MN, USA). Phosphatidylbutanol and 1-palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphate (PA) dissolved in chloroform were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Propranolol, mepacrine, and oleoyl-*sn*-glycero-3-phosphate (LPA) were from Sigma-Aldrich (St. Louis, MO, USA), and SP600125 and Y27632 were from Calbiochem (San Diego, CA, USA). [^{3}H]palmitic acid was from PerkinElmer Life Sciences. The silica gel 60A plates for TLC were purchased from Whatman (Clifton, NJ). Antibodies used were as follows: β-tubulin type III (TujI) monoclonal antibody (Babco; Richmond, CA), cy^{TM} 3-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch; PA, USA), PLD1 polyclonal antibody, p-SAPK/JNK polyclonal antibody, SAPK/JNK polyclonal antibody, Elk-1 polyclonal antibody, and p-Elk-1 polyclonal antibody (Beverly, MA, USA). NT-3 polyclonal antibody and β-actin monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RhoA activation kits were from Upstate Biotechnology (Temecula, CA).

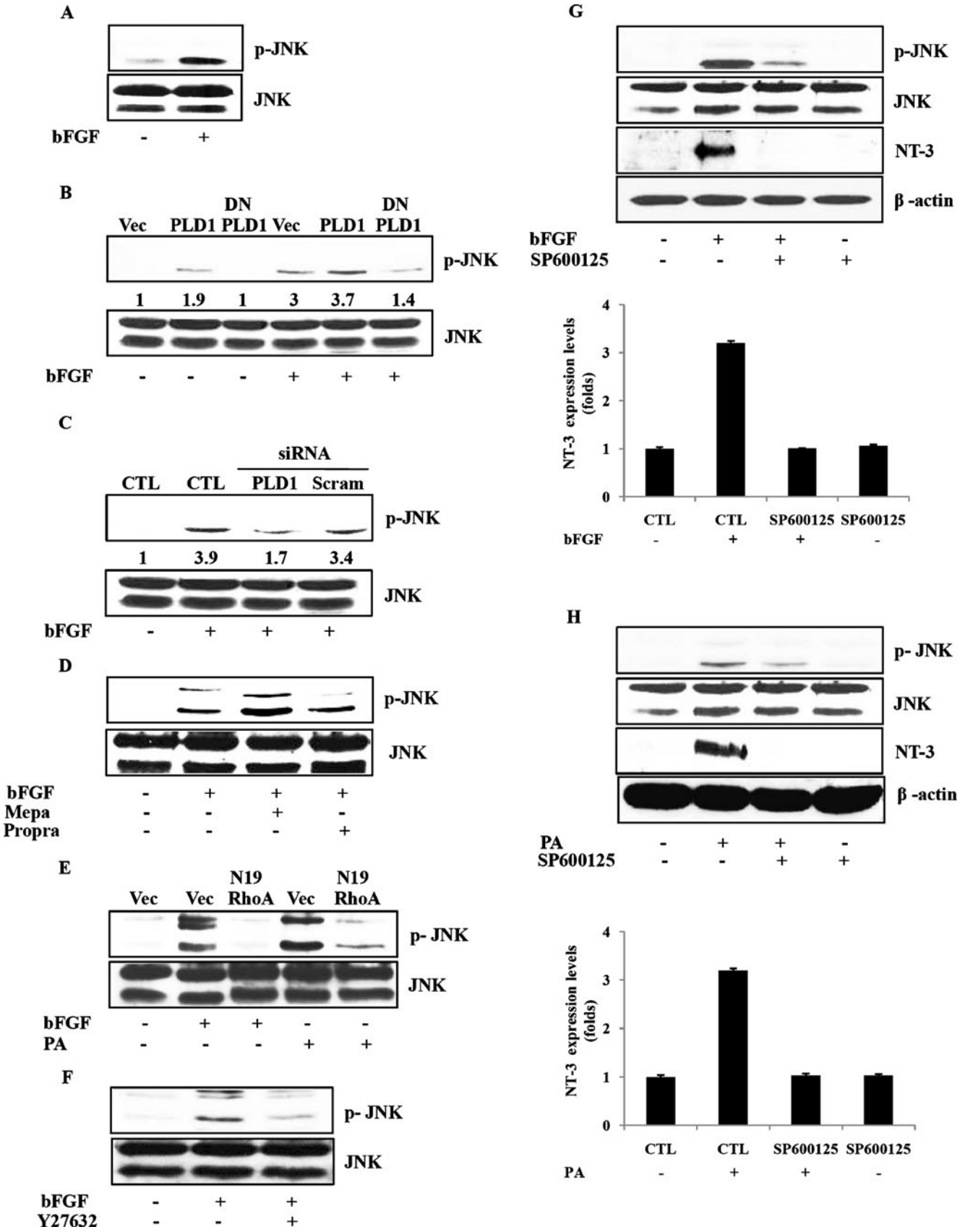
Fig. 3 JNK activation during bFGF-induced NT-3 expression is regulated by PLD1 in H19-7cells. **a** Cells were treated with bFGF (20 ng/ml) for 15 min and then harvested, and the amounts of total JNK and p-JNK were determined by Western blot analysis. **b** Cells were infected with control retroviral *vector* and vectors harboring *PLD1* or *DN-PLD1*; 72 h after infection, they were treated with bFGF (20 ng/ml) for 15 min. Cell lysates were analyzed by Western blot as before. **c** Cells grown at 33°C in DMEM containing 10 % FBS were transiently transfected with 100 nM PLD1 siRNA or scrambled siRNA for 48 h and stimulated with bFGF (20 ng/ml) for 15 min. Cell lysates were analyzed as above. **d** Cells were pretreated with *Mepa* (mepacrine, 50 μM) or *Propra* (propranolol, 50 μM) for 30 min then treated as above. **e** Cells were transfected with 5 μg of either vector control or vectors expressing dominant-negative form of RhoA (*N19*); 48 h later, the cells were treated with bFGF (20 ng/ml) or 50 μM PA for 15 min. Cell lysates were analyzed as above. **f** Cells were pretreated with Y27632 (30 μM) for 1 h and then treated as before. **g** Cells were pretreated with SP600125 (50 μM) for 1 h and then stimulated with bFGF (20 ng/ml) for 15 min (for p-JNK) and 48 h (for NT-3). Cell lysates were analyzed by Western blot analysis using total JNK, p-JNK, and NT-3 antibodies. **h** Cells were pretreated with SP600125 (50 μM) for 1 h and then stimulated with PA (50 μM) for 15 min (for p-JNK) and 48 h (for NT-3). Cell lysates were analyzed as before

Cell Culture

H19-7 cells were originally derived from rat hippocampal neurons and were conditionally immortalized by stable transfection with temperature-sensitive SV40 large T antigen. They were grown in DMEM supplemented with 10 % FBS and maintained at 33°C under G418 selection throughout. To induce differentiation, the cells were placed in serum-free defined medium (N2) and shifted to 39°C prior to treatment with differentiating agents. Differentiated cells were defined as cells with round and refractile cell bodies containing at least one neurite whose length was greater than the diameter of the cell body. H19-7 cells were kindly provided by Dr. K.C. Chung. (Yonsei University, Korea).

Western Blot Analysis

Cells were lysed in 20 mM Tris, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4. Samples containing 20 to 30 μg protein were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels (10–12 %), electrophoresed, and transferred to nitrocellulose membranes (Amersham Biosciences). After being blocked with 5 % dried skim milk for 1 h, the membranes were incubated with primary antibodies. The blots were further incubated with horseradish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p-JNK
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PA - + + -
SP600125 - - + +
NT-3 expression levels (folds)
0 1 2 3 4
CTL CTL SP600125 SP600125
PA - + + -

peroxidase-conjugated secondary antibody (1:2,000; New England Biolabs, Beverly, MA), and specific bands were detected by ECL (Ab FRONTIER).

Infection of H19-7 Cells

For transient infection, retroviral constructs of *vector*, *PLD1*, dominant-negative (*DN*)-*PLD1*,, and *NT-3* were added to H19-7 cells. After overnight culture, the virus-containing medium was replaced by a fresh complete medium. The infected H19-7 cells were plated on poly-L-lysine-coated dishes, and 72 h later, they were switched to N2 medium and the temperature was adjusted to 39°C. At the same time, bFGF was added to the N2 medium in order to induce differentiation.

Luciferase Assay

The *Elk-1*-dependent *trans*-reporting system was provided by Dr. Kang-Yell Choi (Yonsei University, Seoul, Korea). This incorporated a reporter vector (*pFR-Luc*) containing five tandem repeats of GAL4-binding elements and a basic promoter element (TATA box), followed by the coding sequence of firefly luciferase and a fusion transactivator plasmid (*pFA2-Elk-1*) expressing a trans-activator protein consistent with the yeast GAL4-binding domain and the activation domain of trans-activator Elk-1. For transient reporter assays, cells were cotransfected with reporter plasmid *p-FR-Luc* and *p-FA2-Elk-1*. When required, they were also transfected with the *PLD* or *DN-PLD1*. Transfection efficiencies were normalized by cotransfection with the *pCMV-β-gal* reporter; 48 h after transfection, the cells were stimulated with bFGF for 15 min. Levels of expression of the targets were analyzed by firefly luciferase assays. Luciferase activity was measured with a luminometer (Berthold, USA)

RhoA Activity Assay

For pull-down assays, H19-7 cells were lysed in 125 mM HEPES, pH 7.5, containing 750 mM NaCl, 5 mM EDTA, 5 % lgepal CA-630, 50 mM $MgCl_2$, 10 % glycerol, 10 μg/mL aprotinine, and 10 μg/mL leupeptin. Equal amounts of total protein from clarified lysates were incubated with GST-RBD (the Rho-binding domain of rhotekin) to precipitate GTP-bound RhoA, according to the manufacturer's instructions (Upstate Biotechnology). Precipitated GTP-bound RhoA were resolved by a 12 % SDS-polyacrylamide gel electrophoresis (PAGE), and membranes were immunoblotted with monoclonal antibodies specific for RhoA (3 μg/mL, Upstate Biotechnology); 30-μg samples of the lysates were also resolved by 12 % SDS-PAGE and immunoblotted to measure total amounts of RhoA.

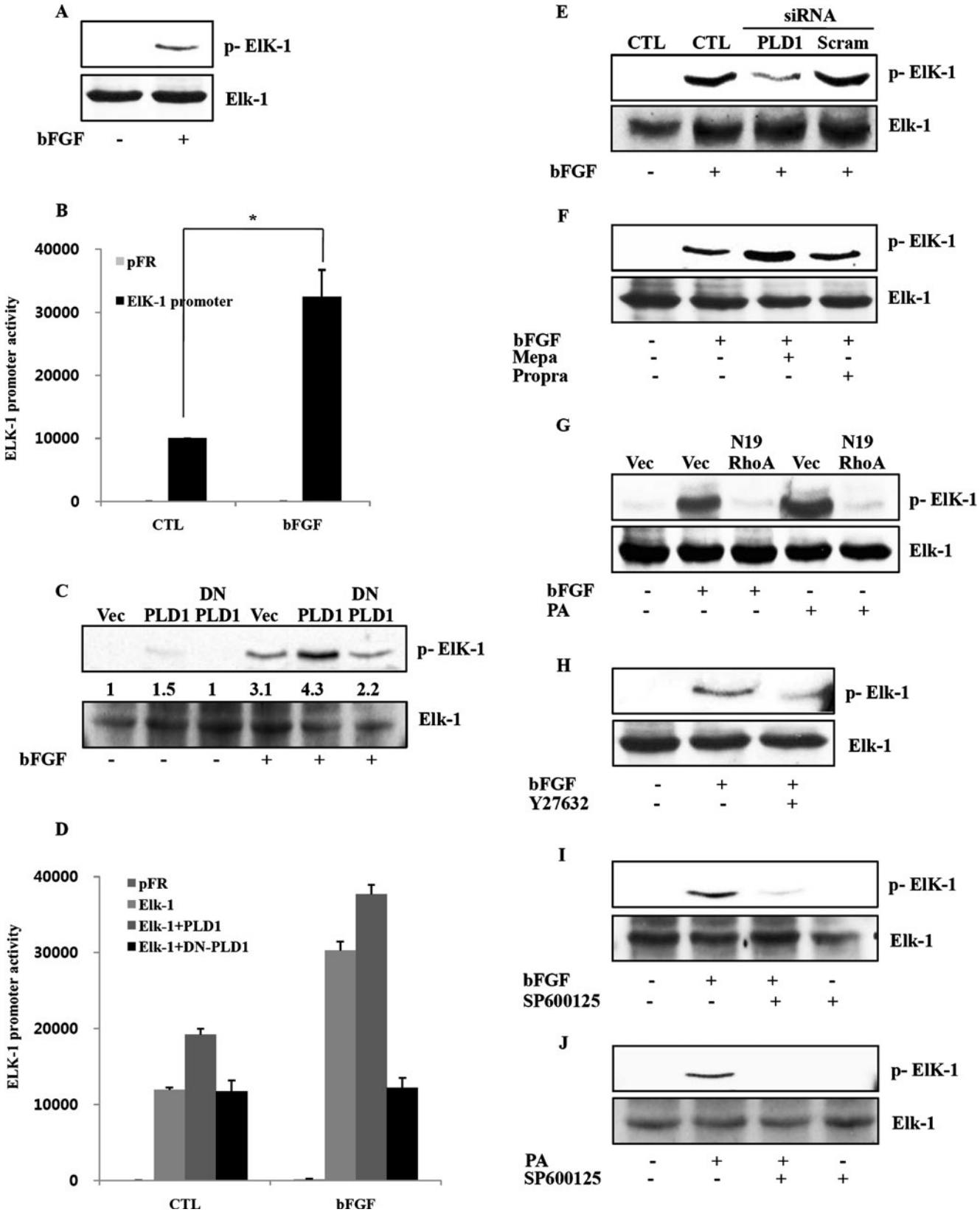
Fig. 4 Effect of PLD1 knockdown and exogenous PA treatment on bFGF-induced Elk-1 phosphorylation in H19-7cells. **a** Cells were treated with bFGF (20 ng/ml) for 15 min and harvested, and the amounts of total Elk-1 and p-Elk-1 were determined by Western blot analysis. **b** Cells were cotransfected with reporter plasmid *p-FR-Luc* along with *p-FA2-Elk-1*. Transfection efficiencies were normalized by cotransfection with the *pCMV-β-gal* reporter; 48 h after the transfection, the cells were stimulated with bFGF for 15 min. The level of expression from the target was analyzed by firefly luciferase assay depending on the reporter construct used. The luciferase activity obtained from luminometer (Berthold, USA). **c** Cells were infected with control retroviral *vector* and vectors harboring *PLD1* or *DN-PLD1*; 72 h after infection, they were treated with bFGF (20 ng/ml) for 15 min. Cell lysates were analyzed by Western blot analysis using total Elk-1 and p-Elk-1 antibodies. **d** Cells were cotransfected with reporter plasmid *p-FR-Luc* along with *p-FA2-Elk-1*.When appropriate, cells were cotransfected with the *PLD* or *DN-PLD1*. Transfection efficiencies were normalized by cotransfection with the *pCMV-β-gal* reporter; 48 h after the transfection, the cells were stimulated with bFGF for 15 min. The expression level of the target gene was analyzed by firefly luciferase assay depending on the reporter construct used. **e** Cells were grown at 33°C in DMEM containing 10 % FBS then transiently transfected with 100 nM PLD1 siRNA or scrambled siRNA for 48 h and stimulated with bFGF (20 ng/ml) for 15 min. Cell lysates were analyzed as in **c**. **f** Cells were pretreated with *Mepa* (mepacrine, 50 μM) or *Propra* (propranolol, 50 μM) for 30 min and then stimulated with bFGF (20 ng/ml) for 15 min and analyzed as in **e**. **g** Cells were transfected with 5 μg of either vector control or vectors expressing dominant-negative form of RhoA (*N19*); 48 h later, the cells were treated with bFGF (20 ng/ml) or 50 μM PA for 15 min and analyzed as in **g**. **h** Cells were pretreated with Y27632 (30 μM) for 1 h and then stimulated with bFGF (20 ng/ml) for 15 min, and then, cell lysates were analyzed by Western blot analysis using total Elk-1 and p-Elk-1 antibodies. **i** Cells were pretreated with SP600125 (50 μM) for 1 h and then stimulated with bFGF (20 ng/ml) for 15 min. Cell lysates were as before. **j** Cells were pretreated with SP600125 (50 μM) for 1 h and then stimulated with PA (50 μM) for 15 min. Cell lysates were analyzed as before

Small Interfering RNA

Rat Elk-1 siRNA was purchased from Ambion (Austin, TX, USA). A rat Elk-1 sequence (sense 5′-CUACUACUAU GAUAAGAAUtt-3′, antisense 5′-AUCUUAUCAUA GUAGU-AGcg-3′) was chosen for Elk-1 RNAi targeting. A negative control was carried out with Negative Control siRNA#2 (from Ambion). Cells were transfected with oligonucleotide siRNAs using siPORTTM NeoFX (Ambion) reagent according to the manufacturer's instructions. At the indicated intervals following transfection, cell lysates were assayed for gene silencing by Western blotting.

Measurement of Neurite Outgrowth

For culture experiments, cells at 1×10^5 cells/well were plated in a six-well plate in serum containing DMEM. After 24 h, the cells were switched to N2 medium and the temperature was adjusted to 39°C; they were then treated with bFGF for 48 h. Two hundred cells in randomly selected areas on each coverslip were manually scored by several investigators in a blinded manner. Length of a neurite was



defined as the distance between the cell periphery and the tip of the neurite, and total length of multiple neurites per cell was calculated. Clusters of cells were excluded from the morphometric analysis. Morphological characteristics were

quantified using SigmaScan Pro (SPSS, Chicago, IL). Unless mentioned otherwise, results are represented as the mean ± SE of three independent experiments, and statistical significance was evaluated using the two-tailed Student *t* test.

Immunocytochemistry

H19-7 cells were grown on poly-L-lysine-coated coverslips in DMEM containing 10 % fetal bovine serum at 33°C. After 24 h, they were switched to N2 medium and stimulated with 20 ng/ml bFGF. To investigate the effects of PLD1and NT-3 overexpression on neurite outgrowth, the cells were infected with *PLD1* and *NT-3* and incubated for further 72 h in N2 medium with bFGF. Thereafter, they were fixed in 4 % buffered paraformaldehyde for 20 min, washed three times for 5 min each with 0.1 % bovine serum albumin in phosphate buffered saline (PBS), permeabilized with 0.3 % Triton X-100 for 30 min, and blocked with 10 % goat serum in PBS for another 30 min. They were then immunostained for 1 h with a primary antibody (a 1:2,000 dilution of β-tubulin type III (Tuj1) rabbit polyclonal antibody) and washed three times for 5 min each with 0.1 % bovine serum albumin in PBS. For Tuj1 primary antibody, cy^{TM}3-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) was used as a secondary antibody (1 h in the dark), the cells were washed with distilled water, and coverslips bearing the immunostained cells were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA). Photographs of the cells were taken with a fluorescence microscope (Nikon, Japan), and the images were analyzed with ImageJ software (NIH-http//rsb.info.nih.gov/ij/).

Statistical Analysis

All experiments were performed at least in triplicate, and results are expressed as means ± SD. The significance of differences was assessed by an unpaired *t* test.

Results

PLD1 Acts as a Regulator of NT-3 Expression and Neurite Outgrowth in H19-7 Cells

Recent studies have revealed that neurotrophic factors play important roles in many biological process including differentiation, survival, and proliferation [27, 28]. We previously reported that PLD1 activation is involved in bFGF-induced neurite outgrowth [6]. To determine whether PLD1 is involved in NT-3 expression induced by bFGF, H19-7 cells were infected with vectors containing wild-type PLD1 or a catalytically inactive mutant of PLD1. Three days after infection, they were treated with bFGF. As shown in Fig. 1a, PLD1 overexpression potentiated NT-3 expression increased by bFGF, whereas knockdown of PLD1 with DN-PLD1 infection abolished NT-3 expression induced by bFGF, indicating that PLD1 is involved in bFGF-induced NT-3 expression. To confirm the role of PLD1 in bFGF-induced NT-3 expression, we examined the effects of PLD1 siRNA. Scrambled siRNA was transfected as a control. PLD1 siRNA abolished NT-3 expression and inhibited bFGF-induced neurite outgrowth (Fig. 1b, c). In general, PLD exerts its effects by generating PA which is degraded to diacylglycerol by PAP or to LPA/arachidonic acid by PLA_2. To determine whether PA itself regulates NT-3 expression, we pretreated cells with mepacrine (a PLA_2 inhibitor) or propranolol (a PAP inhibitor) and then stimulated them with bFGF. As shown in Fig. 1d, pretreatment with mepacrine or propranolol did not block bFGF-induced NT-3 expression, suggesting that PLA_2 or PAP metabolites of PA are not involved in NT-3 expression induced by bFGF treatment. These findings indicate that PLD1 is important for bFGF-induced NT-3 expression and neurite outgrowth and that PA itself, an end-product of PLD, participates in bFGF-induced NT-3 expression.

bFGF-Induced NT-3 Expression is Regulated by the RhoA/ROCK/JNK Pathway in H19-7 Cells

Rho GTPase activity is important in the regulation of neurite outgrowth, and activation of RhoA is required for neurite outgrowth [13]. To assess whether RhoA/ROCK is involved in the PLD1-mediated NT-3 expression, we examined the effect of PLD1 on activation of RhoA. The intracellular concentration of active GTP-bound Rho was measured as described in "Materials and Methods." As shown in Fig. 2a, RhoA activity increased in response to bFGF or PA. To confirm the role of PLD1 in bFGF-mediated RhoA activation, we used DN-PLD1 to reduce PLD1 activity. DN-PLD1 completely abolished RhoA activation induced by bFGF. To investigate the roles of RhoA in bFGF- or PA-mediated NT-3 expression, we transfected H19-7 cells with a dominant-negative mutant of RhoA and then treated the cells with bFGF or PA for 48 h. As shown Fig. 2b, the dominant-negative mutant reduced the increase of NT-3 expression by bFGF or PA. Next, to determine whether the Rho kinase, ROCK, a downstream target of RhoA, is involved in bFGF-induced NT-3 expression, we treated cells with Y27632, a specific inhibitor of Rho kinase. We found that Y27632 strongly inhibited NT-3 expression induced by bFGF (Fig. 2c), indicating that Rho/ROCK is important for bFGF-induced NT-3 expression via PLD1 activation in H19-7 cells.

Recent evidence indicates that treatment with bFGF activates JNK during neuronal differentiation [16]. As shown in

Fig. 3a, JNK phosphorylation was induced by bFGF. To determine whether PLD1 was involved in this effect, cells were infected with vectors containing wild-type and catalytically inactive mutant of PLD1. Figure 3b shows that overexpression of PLD1 increased JNK phosphorylation even in the absence of bFGF, whereas DN-PLD1 decreased bFGF-induced JNK phosphorylation. To confirm the role of PLD1 in bFGF-induced JNK activation, we showed that the effects of PLD1 siRNA also decreased bFGF-induced JNK phosphorylation (Fig. 3c). Next, to determine whether PA itself regulates JNK phosphorylation, we pretreated cells with mepacrine or propranolol and then treated them with bFGF. As shown in Fig. 3d, pretreatment with mepacrine or propranolol did not block bFGF-induced JNK phosphorylation, suggesting that PLA_2 or PAP metabolites of PA are not involved in JNK phosphorylation induced by bFGF treatment. Next, we transfected H19-7 cells with the dominant-negative mutant of RhoA and found that JNK phosphorylation by bFGF or PA was reduced (Fig. 3e). Similarly, Y27632, a specific inhibitor of Rho kinase, decreased JNK phosphorylation induced by bFGF (Fig. 3f). To further elucidate the role of JNK in bFGF-induced NT-3 expression, cells were pretreated with SP600125, a specific JNK inhibitor. The pretreatment of the cells with SP600125 inhibited bFGF-induced NT-3 expression (Fig. 3g). Furthermore, PA-induced NT-3 expression was also abolished by the pretreatment with SP600125 (Fig. 3h). Taken together, these results show that JNK is involved in bFGF-induced NT-3 expression and acts downstream of PLD1 and RhoA/ROCK.

Activation of Elk-1 is Controlled by the PLD1/RhoA/ROCK/JNK Pathway in H19-7 Cells

Elk-1 can activate transcriptional processes in response to MAPKs' activation [29, 30]. Since JNK contributes to growth factor-triggered Elk-1 phosphorylation during neurite formation [31], we examined Elk-1 phosphorylation during NT-3 expression and neurite outgrowth induced by bFGF. As expected, treatment of H19-7 cells with bFGF increased the phosphorylation of Elk-1 as well as the activity of its promoter (Fig. 4a, b). To investigate whether Elk-1 is related to PLD1 in bFGF-treated H19-7 cells, we infected the cells with vectors containing wild-type and catalytically inactive mutant of PLD1. As shown in Fig. 4c, d, bFGF-induced Elk-1 phosphorylation and its promoter activity

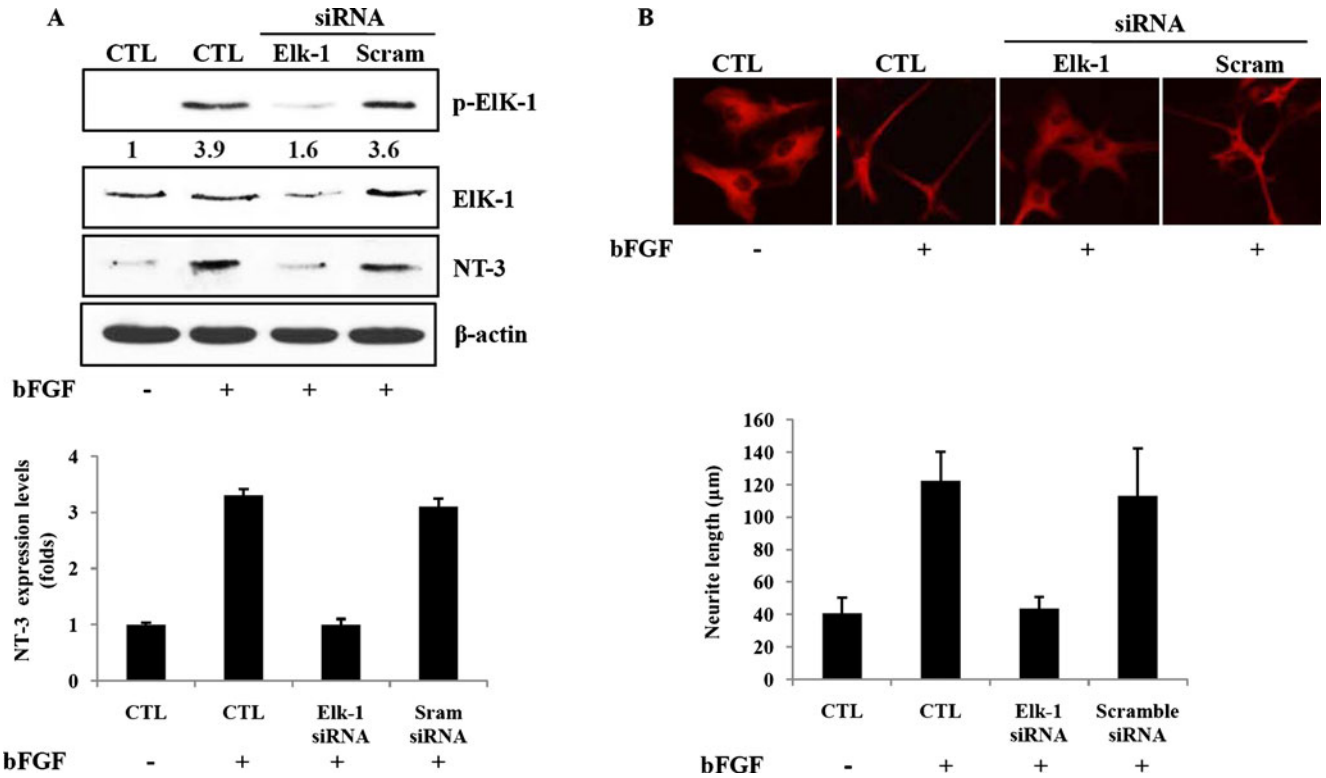


Fig. 5 Effect of Elk-1 phosphorylation on bFGF-induced NT-3 expression and neurite outgrowth in H19-7 cells. **a** Cells were transiently transfected with 100 nM Elk-1 siRNA or scrambled siRNA for 48 h and stimulated with bFGF (20 ng/ml) for 15 min (for p-Elk-1) and 48 h (for NT-3). Cells were harvested, and the amounts of total Elk-1 and p-Elk-1and NT-3 were determined by Western blot analysis. **b** Cells were grown at 33°C in DMEM containing 10 % FBS. The cells were transiently transfected with 100 nM Elk-1 siRNA or scrambled siRNA as a control for 48 h and then stimulated with bFGF (20 ng/ml) for 48 h. Immunocytochemical analysis with anti-Tuj1 antibody and neurite length measurement were performed as described in "Materials and Methods"

were greatly reduced by DN-PLD1 and also by PLD1 siRNA (Fig. 4e). To determine whether PA itself regulates Elk-1 phosphorylation, we pretreated the cells with mepacrine or propranolol. We found that they did not block bFGF-induced Elk-1 phosphorylation, suggesting that PLA_2 or PAP metabolites of PA are not involved in Elk-1 phosphorylation induced by bFGF treatment (Fig. 4f). Next, we asked whether the RhoA/ROCK/JNK pathway is involved in the bFGF-induced Elk-1 phosphorylation by examining the effect of the dominant-negative mutant of RhoA on Elk-1 phosphorylation; as anticipated, Elk-1 phosphorylation by bFGF or PA was dramatically reduced (Fig. 4g). Furthermore, as shown in Fig. 4h, i, bFGF-induced Elk-1 phosphorylation was inhibited by both Y27632 and SP600125 as was with the PA-induced Elk-1 phosphorylation (Fig. 4j). These results indicate that the PLD1/RhoA/ROCK/JNK pathway is crucial for bFGF-induced Elk-1 phosphorylation. In addition, when we knocked down the Elk-1 expression with Elk-1 siRNA, both bFGF-induced NT-3 expression and neurite outgrowth were completely abolished (Fig. 5a, b). This shows that Elk-1 phosphorylation is controlled by PLD1 through the RhoA/ROCK/JNK pathway activated by bFGF and also that Elk-1 phosphorylation regulates bFGF-induced NT-3 expression and neurite outgrowth in the H19-7 cells.

Overexpression of NT-3 Leads to Neurite Outgrowth in H19-7 Cells

Finally, we investigated the effect of NT-3 expression on bFGF-induced neurite outgrowth in the H19-7 cells. As shown in Fig. 6, overexpression of NT-3 by transient infection with NT-3(h)-EGFPBsd-CLBC3 (T3ebCD) led to more than a twofold increase in neurite length compared with the controls. This result indicates that NT-3 expression controls neurite outgrowth of H19-7cells.

Discussion

Neurotrophins are a unique family of polypeptide growth factors that influence the proliferation, survival, and differentiation of neurons and are associated with various neuronal disorders [32]. Neurotrophins regulate neuronal differentiation and neurite outgrowth in signaling pathway through Trk B and Trk C in cortical progenitor cells [33]. PLD is activated

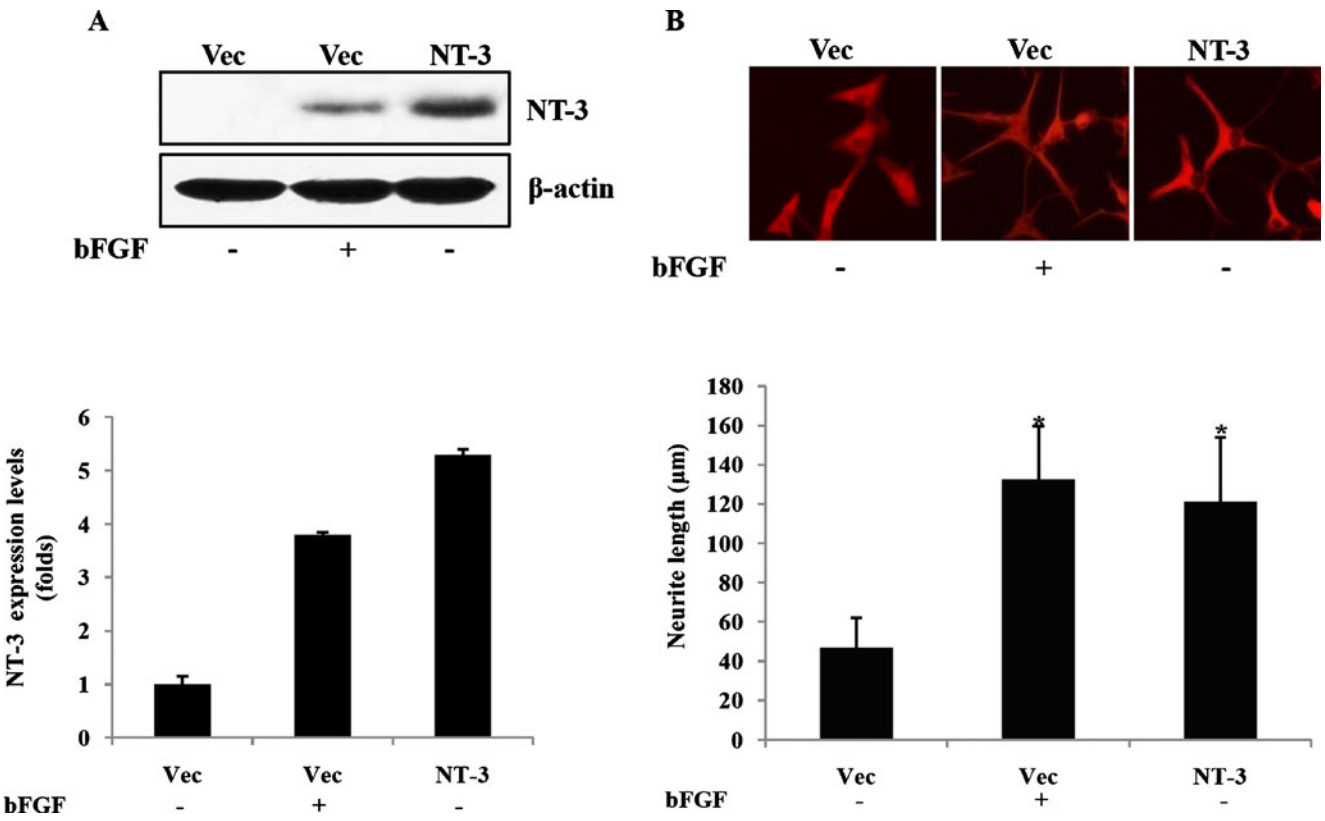


Fig. 6 Effect of NT-3 overexpression on bFGF-induced neurite outgrowth in H19-7 cells. **a** Cells were infected with control retroviral *vector* and a vector harboring NT-3(h)-EGFPBsd-CLBC3 (T3ebCD); 72 h after infection, they were treated with bFGF (20 ng/ml) for 48 h. Cell lysates were analyzed by Western blot analysis using NT-3 antibody. **b** Cells were treated as in **a**, and immunocytochemical analysis with anti-Tuj1 antibody and neurite length measurement were performed as described in "Materials and Methods"

in a wide variety of brain- and neuronal-derived cells, and it is involved in various neuronal signaling pathways [6, 34]. PLD is expressed in a variety of neuronal cells including PC12 cells, cerebellar neurons, hippocampal cells, and NIE-115 cells [6, 35, 36]. Previously, we reported that the PLD1-mediated Bcl-2 expression promotes neurite outgrowth by inducing neurotrophic factors such as NT4/5 and BDNF expression in H19-7 cells [37]. NT-3 is a potent neurotrophic factor and thus has a strong effect on neurite outgrowth [38, 39]. However, there are only few studies concerning the relation of PLD activation and bFGF-induced NT-3 expression in the nervous system. In this study, we provided some insight into the PLD1-regulated signaling pathway involving bFGF-induced NT-3 expression and neurite outgrowth. In the present study, we showed that downregulation of PLD1 decreased NT-3 expression and neurite outgrowth in response to bFGF (Fig. 1). In addition, PA increased the expression of NT-3. Clearly, PLD1 is an important regulator of bFGF-induced NT-3 expression and neurite outgrowth in H19-7 cells.

Next, we considered which of the signaling molecules affects bFGF-induced NT-3 expression and neurite outgrowth in response to activation of PLD1. Rho GTPases and related molecules play an important role in various aspects of neuronal development, including neurite outgrowth and differentiation, axon pathfinding, and dendritic spine formation and maintenance [12, 40–43]. Other studies indicate that the balance between Rac1/Cdc42 and RhoA activities is crucial for neuronal differentiation [44, 45]. Moreover, neurotrophins affect neurite outgrowth in cultured cortical neurons, and this is regulated by Rho GTPase [12]. However, the signaling pathway of RhoA activation in neuronal differentiation still remains largely unknown. Therefore, we tried to determine whether the RhoA/ROCK pathway is involved in PLD-mediated NT-3 expression and found that both bFGF and PA can activate RhoA and PLD1 knockdown completely blocked induction of RhoA activity (Fig. 2). Also, Y27632 blocked NT-3 expression induced by bFGF; thus, the RhoA/ROCK pathway is required for the stimulation of NT-3 expression.

Cross talk between PLD and MAPKs in response to agonists has been reported in a number of cell systems [6, 46, 47]. One report showed that PLD2 activation downstream of MAPK is required for NGF-induced neurite outgrowth [35]. Furthermore, prolonged activation of ERK is both necessary and sufficient for the differentiation of PC12 cells [48]. JNK also controls neuronal differentiation, indicating that neurite outgrowth by MAPK activation may depend on the cell type or agonist under investigation. Current evidence indicates that neurite outgrowth depends on JNK in the developing brain [20, 49, 50]. According to one report, bFGF activates JNK and this results in growth inhibition, neuronal differentiation, and apoptosis in neuroblastoma cells [51]. However, there has been no study regarding the relationship between PLD and JNK activation in the context of the induction of neuronal differentiation by bFGF. In the present study, we addressed that JNK is activated in response to bFGF stimulation, and this led to a hypothesis that bFGF-induced JNK activation may also be mediated via PLD1. Both DN-PLD1 and PLD1 siRNA blocked bFGF-induced JNK phosphorylation; moreover, PA directly increased JNK phosphorylation, suggesting that JNK is phosphorylated by PLD1 after pathway is activated by bFGF. According to a variety of reports on cellular systems, RhoA and ROCK can stimulate JNK activity [52, 53]. Based on that, we tested whether inhibition of RhoA/ROCK blocked bFGF-induced JNK phosphorylation and found that JNK was crucial for bFGF-induced NT-3 expression in H19-7 cells.

JNK is known to regulate several transcription factors: c-Jun, ATF-2, Elk-1. Among them, Elk-1 is a target of ERK, JNK, and p38 MAPK, which are all involved in diverse physiopathological forms of neuronal plasticity. Phosphorylation of Elk-1 by MAPK can occur on at least nine residues [54], two of them, Ser 383 and Ser 389, being crucial for transcriptional activity. Elk-1 is present in its resting state in the cytoplasm, where it colocalizes with mitochondrial proteins or microtubules and, when phosphorylated by MAPK, translocates to the nucleus where it

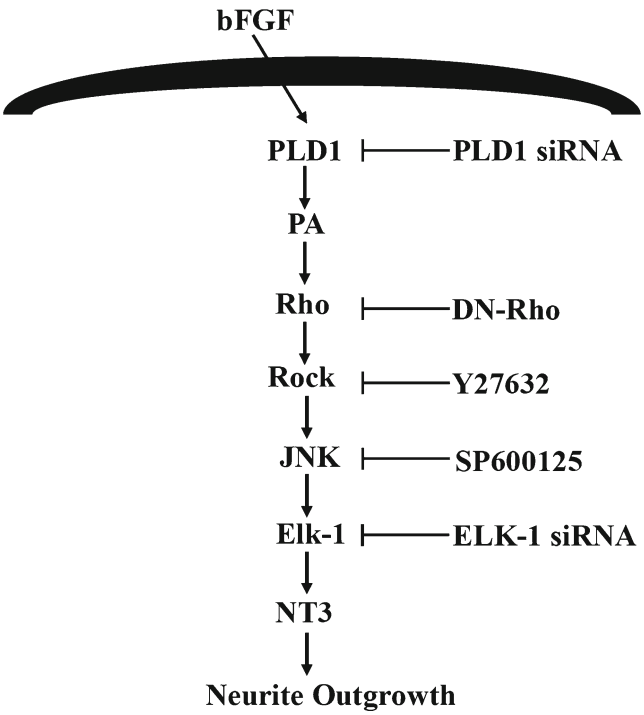


Fig. 7 Model of the PLD1 signaling pathway involved in bFGF-induced NT-3 expression and neurite outgrowth in H19-7 cells. The model suggests that bFGF-induced NT-3 expression in H19-7 cells results from PLD1 activation which activates the RhoA/ROCK/JNK/Elk-1 pathway and the resulting increase in NT-3 expression leads to neurite outgrowth

influences chromatin remodeling, SRE-dependent transcription, and neuronal differentiation [55]. Thus, it plays a dual role in neuronal function: proapoptotic within the cytoplasm and prodifferentiation within the nucleus. In this study, we demonstrated that bFGF-induced NT-3 expression and neurite outgrowth required the activation of Elk-1 through PLD1. First, we confirmed that Elk-1 was activated induced by bFGF or PA, and DN-PLD1 or PLD1 siRNA blocked Elk-1 phosphorylation and its promoter activity in response to bFGF. Moreover, inhibition of RhoA/ROCK and JNK decreased the phosphorylation of Elk-1 induced by bFGF. Knockdown of Elk-1 decreased bFGF-induced NT-3 expression as well as neurite outgrowth. These results suggest that Elk-1 transcriptional activity is required for NT-3 expression and neurite outgrowth in the PLD1-mediated RhoA/ROCK/JNK pathway in H19-7 cells. Finally, we identified the role of NT-3 in neurite outgrowth in H19-7 cells. Overexpression of NT-3 promotes neurite outgrowth without bFGF; this result addresses that NT-3 has an important effect on neurite outgrowth in H19-7 cells. In conclusion, the present study provides evidences that PLD1 regulates bFGF-induced NT-3 expression and neurite outgrowth through a RhoA/ROCK/JNK/Elk-1 signaling pathway in H19-7 cells (Fig. 7).

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