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Phospholipase D1 Activation Through Src and Ras Is Involved in Basic Fibroblast Growth Factor-Induced Neurite Outgrowth of H19-7 Cells

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Abstract Phospholipase D (PLD) is implicated in a variety of physiological processes that reveal it to be a member of the signal transducing phospholipases. We found that PLD1 is activated when basic fibroblast growth factor (bFGF) stimulates neurite outgrowth of an immortalized hippocampal cell line (H19-7). Overexpression of PLD1 in H19-7 cells dramatically elongated bFGF-induced neurite outgrowth and increased PLD activity. Transfection of DN-rPLD1 blocked bFGF-induced PLD activation and completely inhibited neurite outgrowth induced by bFGF, suggesting that PLD1 activation is important in bFGF-induced neurite outgrowth of H19-7 cells. PLD activation and neurite outgrowth induced by bFGF was dependent on phospholipase C gamma (PLC-γ) and Ca^{2+}, but not protein kinase C (PKC). Furthermore, inhibition of Src and Ras partially blocked bFGF-induced PLD activation and neurite outgrowth, respectively. Coinhibition of Src and Ras completely blocked bFGF-induced PLD activation, suggesting that Src and Ras independently regulate PLD1 activation. Interestingly, bFGF-induced PLD activation and neurite outgrowth did not require ERK1/2 activated by Ras. Taken together, this study demonstrates that bFGF activates PLD1 through PLC-γ activation, which leads to neurite outgrowth in H19-7 cells. Furthermore, our results show that PLD1 activation by bFGF is regulated by Src and Ras independently. J. Cell. Biochem. 101: 221–234, 2007.

Key words: phospholipase D1 (PLD1); neurite outgrowth; basic fibroblast growth factor (bFGF); Src; Ras; phospholipase C-γ (PLC-γ).

Basic fibroblast growth factor (bFGF) is a potent mitogenic factor that is also known to initiate changes important for neurite outgrowth, survival, and plasticity [Lo, 1995]. Mechanisms underlying these diverse actions of bFGF are not well understood. The bFGF receptor belongs to the tyrosine kinase family of membrane receptors and forms a subfamily of receptor tyrosine kinases. Four structurally related members of this family have been characterized, FGFR1-4 [Johnson and Williams, 1993]. FGF receptors (FGFR) are activated upon ligand binding and trans-phosphorylate each other in the FGF-induced receptor-dimer. Seven tyrosine residues in the FGFR intracellular domain have been shown to become phosphorylated upon ligand binding [Mohammadi et al., 1996]. The phosphorylated tyrosine residues from binding sites for signal transduction molecules, leading to initiation of specific signaling pathways and subsequent biological responses. Four signal transduction molecules have been shown to bind directly to the receptor: phospholipase C-γ (PLC-γ), Shb, Crk, and FGF receptor substrate 2 (FRS2) [Cross and Claesson-Welsh, 2001]. In the FGFR, only Tyr766 has been identified as a direct binding site for an

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SH2 domain of PLC-γ [Mohammadi et al., 1992]. The role of PLC-γ in FGFR signaling is not clear. However, inhibition of PLC-γ activation by mutation of the binding site in the FGFR partly reduced the FGF-induced mitogenecity. Furthermore, PLC-γ binding and activation of FGFR has been implicated in the regulation of Src kinase activity [Klint et al., 1995; Landgren et al., 1995]. Binding of PLC-γ to the activated FGFR leads to its translocation to the plasma membrane where it catalyzes the hydrolysis of phosphoinositol lipids to inositol phosphates and diacylglycerol (DAG), which in turn stimulate the increase of intracellular Ca^{2+} and protein kinase C (PKC), respectively [Divecha and Irvine, 1995]. The adaptor molecule Shb was shown to also bind to Y766. However, there was no apparent competition between PLC-γ and Shb for the binding site [Cross et al., 2002]. Crk binds the guanine nucleotide exchange factor C3G that has been shown to activate the monomeric GTPase Rap1 resulting in activation of extracellular signal-regulated protein kinase (ERK) [York et al., 1998]. The adaptor FRS2 is phosphorylated by the activated FGFR on multiple tyrosine residues. Thereby, four binding sites for the Grb2-Sos complex are created [Meakin et al., 1999] initiating the major pathway for the activation of the monomeric GTPase Ras, which promotes transient activation of ERK1/2.

Phospholipase D (PLD) hydrolyzes phospholipids, particularly phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. PA acts as a second messenger and can be converted to other messenger molecules, such as 1,2-diacylglycerol (DAG) and lysophosphatidic acid (LPA). In addition to hydrolysis, PLD also catalyzes specific transphosphatidylation reactions in which a primary alcohol acts as acceptor in place of H_2O [Exton, 1994]. The production of phosphatidylalcohol is often used as an indicator for the activity of PLD. Recent cDNA cloning studies have revealed the existence of at least two isozymes (PLD1, PLD2) in mammalian cells [Kodaki and Yamashita, 1997]. For PLD1, two alternatively spliced forms, PLD1a and PLD1b (PLD1b is a shorter form lacking 38 amino acids) have been identified [Nakashima et al., 1997]. The splicing does not affect the catalytic activity of PLD1. Both PLD1a and PLD1b have low basal activity and are activated in vitro by ADP ribosylation factor (Arf), Rho family GTP-binding proteins (G proteins), and PKC in the presence of phosphatidylinositol 4,5-biphosphate (PIP_2). By contrast, PLD2 has a very high basal activity in the presence of PIP_2 but is unresponsive in vitro to small G proteins and PKC [Sung et al., 1999]. In mammalian cells, PLD is activated by a variety of extracellular signals in a wide range of cells and is recognized to play an important role in signal transduction. The receptor-mediated PLD activation is thought to be involved in a variety of cellular responses including rapid responses such as secretion and superoxide generation, as well as long-term responses, such as proliferation, differentiation, and apoptosis [Nakashima and Nozawa, 1999; Kim et al., 2003]. The signal-dependent activation of PLD has been observed in the neuronal differentiation of neural stem cells [Yoon et al., 2005]. PLD is expressed in many functionally diverse brain areas, including the cerebral cortex, brainstem, cerebellar Purkinje cells, and spinal cord [Lee et al., 2000]. Upregulations of mRNA for both PLD1 and PLD2 have been reported in differentiated neuronal cells [Rujano et al., 2004]. Therefore, the identification of PLD activation as a novel signal transduction pathway engenders a special interest in the function of PLD in the nervous system. However, despite the potential role of PLD in differentiation, the current literature provides little information regarding the activation patterns of PLD, as well as the pathways mediating these responses in neuronal cells.

Brain development and neuronal cell differentiation are controlled by a complex program. As a model system for studying the differentiation of neuronal progenitor cells from the central nerve system (CNS), we utilized a conditionally immortalized hippocampal cell line (H19-7) by transducing rat E17 hippocampal cells with a retroviral vector expressing a temperature-sensitive simian virus 40 (SV40) large T antigen [Yang et al., 2004]. The H19-7 cells proliferate in a response to epidermal growth factor at permissive temperature (33°C) in the presence of active T and differentiate in response to bFGF at the non-permissive temperature (39°C) when T is inactivated. The differentiated H19-7 cells are resistant to mitogenic stimulation by serum or epidermal growth factor, they extend neurites, undergo morphological changes, express neuronal markers such as neurofilaments and brain type II sodium channels, and display action potentials

[Yang et al., 2004]. The differentiation ability of H19-7 cells in response to bFGF mimics the response of primary hippocampal cells and selected other neuronal cell types during late embryogenesis, since bFGF can act as a differentiating factor in certain regions of the CNS which express the bFGF receptor, such as the hippocampus [Yoshimura et al., 2001]. Neuronal cell lines that have been similarly immortalized with temperature-sensitive large T antigen have the capability of developing into region-specific neurons upon transplantation [Renfranz et al., 1991], indicating that the transient expression of large T antigen creates cell lines that are able to respond in vivo to environmental cues.

In this study, we tried to determine whether activation of PLD1 is required for neurite outgrowth of H19-7 cells and to identify other key signaling pathways. A specific inhibitor of MEK (PD98059), a Ras farnesyl transferase inhibitor (Manumycin-A), PLC-γ inhibitor (U73122), PKC inhibitor (bisindolylmaleimide), a Src family of tyrosine kinases (SFKs) inhibitor (PP2), a chelator of intracellular Ca^{2+} (BAPTA-AM), the dominant-negative mutant of PLD1, dominant-negative Src (DN-Src), and the dominant-negative mutant of Ras (N17Ras) were used to probe the signaling molecules that are essential in the neurite outgrowth process. Our study indicates that neurite outgrowth by bFGF requires the activation of PLD1, so we raised the possibility that the bFGF receptor kinase activates PLD1 via PLC-γ activation, which leads to neurite outgrowth. As a downstream signaling molecule of PLC-γ, Src and Ras regulate bFGF-induced PLD1 activation independently. We also found that the neurite outgrowth by bFGF occurs without MAP kinase signaling.

MATERIALS AND METHODS

Materials

Manumycin-A and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO), phosphatidylethanol (PEt) standard from Avanti Polar Lipids (Avanti, Alabaster, AL), and [9,10(n)-3H]-palmitic acid from Amersham Pharmacia Biotech (Amersham Place, Little Chalfont, Buckinghamshire, England). Fetal bovine serum (FBS), penicillin/streptomycin solution, and Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and low glucose was purchased from Gibco-BRL (Gaithersburg, MD). bFGF was purchased from R&D (Minneapolis, MN). U73122, bisindolylmaleimide, BAPTA-AM, PP2, and PD98059 were obtained from Calbiochem (San Diego, CA). Dominant-negative DN-Src (K296R/Y528F) was purchased from Upstate Biotechnology (Lake Placid, NY). pcDNA3.1-H-RasN17 was obtained from David Stokoe of ONYX Pharmaceuticals (Richmond, CA). A generated rabbit polyclonal antibody, designated PLD1063-1074, directed against a peptide in the C terminus of rat PLD1 (TKEAIVPMEVWT) was used in this experiment. Anti-GFP antibody was purchased from Roche (Indianapolis, IN), fluorescence-tagged secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA), and biotinylated secondary antibody was purchased from Vector Laboratories (Burlingame, CA). Silica gel 60A plates for TLC were obtained through Whatman (Clifton, NJ). All other chemical agents were of analytical trace amount. The dominant-negative mutants of PLD1 (EGFP-DN-rPLD1), EGFP-rPLD1, and anti-PLD antibody that recognized both PLD1 and PLD2 were kindly provided by Dr. D.S. Min (Pusan University, Korea).

Cell Culture

H19-7 cells were generated from rat hippocampal neurons [Yang et al., 2004]. They were conditionally immortalized by stable transfection with temperature-sensitive SV40 large T antigen. They were grown in DMEN plus 10% FBS and maintained at 33°C under G418 pressure throughout the experiments [Yang et al., 2004]. To induce neurite outgrowth, the cells were placed in N2 medium and shifted to 39°C prior to treatment with differentiating agents as previously described [Yang et al., 2004]. Differentiated cells were defined as cells with a rounded and refractile cell body containing at least one neurite whose length is greater than the diameter of the cell body. H19-7 cells were kindly provided by Dr. Chung K.C. (Yonsei University, Korea).

Transient Transfection of H19-7 Cells

H19-7 cells were transiently transfected with 10 μg of each pcDNA3.1-dominant-negative PLD1, EGFP-C1 (Vector), EGFP-rPLD1, DN-Src, and N17Ras plasmid using Nucleofector™ Kit (Amaxa). Transfected H19-7 cells were

seeded in a poly-L-lysine-coated dish. At 24 h after transfection, the cells were switched to N2 medium and the temperature adjusted to 39°C. At the same time, bFGF was treated to differentiate the cells in N2 medium.

Determination of PLD Activity

PLD activity was determined by the formation of PEt, the product of PLD-mediated transphosphatidylation, in the presence of ethanol following a previously described procedure [Kim et al., 2003]. The cells were allowed to differentiate for the indicated times in 6-well plates at a density of 1×10^5 cells/ml and were labeled radioactively with 1 μCi/ml of [^{3}H]-palmitic acid in serum-free medium for 24 h. The cells were then pre-treated with 1% (v/v) ethanol for 15 min before stimulation. Following bFGF stimulation for 15 min, the cells were quickly washed with ice-cold PBS and suspended in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer [1959], and PEt was separated by TLC using a solvent system of acetate/isooctane/acetic acid/water (110:50:20:100, by vol.). The regions corresponding to the authentic PEt bands were identified with 0.002% (w/v) primulin in 80% acetone, scraped and counted using a scintillation counter.

Immunocytochemistry

Cells were grown on poly-L-lysine-coated coverslips in DMEM containing 10% FBS at 33°C. The next day, the cells were switched to N2 medium and stimulated with bFGF at a final concentration of 10 ng/ml. At 48 h after induction, cells were fixed with 4% buffered paraformaldehyde for 20 min, washed with 0.1% bovine serum albumin in PBS three times for 5 min, permeabilized with 0.3% Triton X-100 for 30 min, and blocked with 10% goat serum in PBS for 30 min. The cells were then subsequently immunostained using primary antibody (1:400 dilution of GFP antibody and 1:2,000 dilution of PLD1 polyclonal antibody) for 1 h and washed with 0.1% bovine serum albumin in PBS three times for 5 min. This procedure was followed by consequent incubation for 1 h at room temperature with secondary antibodies (1:400 dilution of fluorescence-tagged and 1:200 dilution of biotinylated). Following incubation, the cells were extensively washed with distilled water, and lastly the coverslips were mounted on slides with Vectashield (Vector Laboratories). The cells were analyzed with a fluorescence microscope (Nikon), and the images were further processed using Photoshop 6.0 software. In order to eliminate any anomalies, all experiments were repeated at least three times.

Western Blot Analysis

Cells were firstly 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 PMSF, and 1 mM Na_3VO_4. Proteins of 10–20 μg were subsequently loaded onto SDS-polyacrylamide gels (12%), electrophoresed, and were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). After blocking with 5% dried skim milk for 1 h, the membrane was incubated with primary antibodies. The blots were further incubated with HRP-conjugated secondary antibody (1:2,000, New England Biolabs, Inc., Beverly, MA) and specific bands were detected through ECL (Amersham Pharmacia Biotech).

Measurement of Neurite Outgrowth

For culture experiments, they were plated in 6-well plate at 1×10^5 cells/well in serum-containing DMEM. At 24 h after transfection, the cells were switched to N2 medium and the temperature adjusted to 39°C, and then treated with bFGF for 48 h. Cells from randomly selected areas of at least five cultures from three independent experiments were photographed. Morphological characteristics were quantitated using Sigmascan Pro (SPSS, Chicago, IL). Clusters of cells were excluded from the morphometric analysis. The length of neurite was defined as the distance from the soma to the tip of the longest branch.

Statistical Analysis

All experiments were performed at least three to five times, and data were analyzed using one-way ANOVA. Data were considered to be significantly different at $P < 0.05$.

RESULTS

PLD Activation Is Involved in bFGF-Induced Neurite Outgrowth of H19-7 Cells

Like neuronal cells similarly generated by the transduction of temperature-sensitive large

T antigen, H19-7 cells have two properties, namely conditional proliferation and a capacity for differentiation after the cessation of division. First, to analyze the biological effect of bFGF on neurite outgrowth, H19-7 cells were shifted to 39°C to inactivate the simian virus 40 antigen, transferred to N2 medium for 48 h without bFGF, or treated with bFGF for 48 h and morphologically examined for the change in neurite outgrowth. Differentiated neurons are characterized by a rounded and refractile cell body containing at least one neurite that is longer than the diameter of the cell body; undifferentiated cells are flat with no extend neurites. H19-7 cells at 39°C without bFGF for 48 h showed no morphological phenotype of neurite outgrowth. Treatment with bFGF (10 ng/ml) in N2 medium at 39°C resulted in neurite outgrowth as characterized by neurite extension. bFGF stimulation for 48 h elicited formation of extremely elongated neurites (Fig. 1A). Furthermore, treatment with bFGF in N2 medium at 33°C, the temperature at which large T antigen is functional, showed no morphological phenotype of neurite outgrowth (Fig. 1A). As shown in Figure 1B, the percentage of cells with a differentiated morphology significantly increased by 78% after treatment of cells with bFGF for 48 h at 39°C, whereas only 22% of cells changed after bFGF treatment for 48 h at 33°C. Secondly, in the course of bFGF-induced neurite outgrowth, PLD activity was measured by labeling the cells with [^{3}H]-palmitic acid. bFGF treatment increased PLD activity maximally within 15 min (Fig. 1C). Taken together, these results imply that PLD activation might be involved in bFGF-induced neurite outgrowth of H19-7 cells.

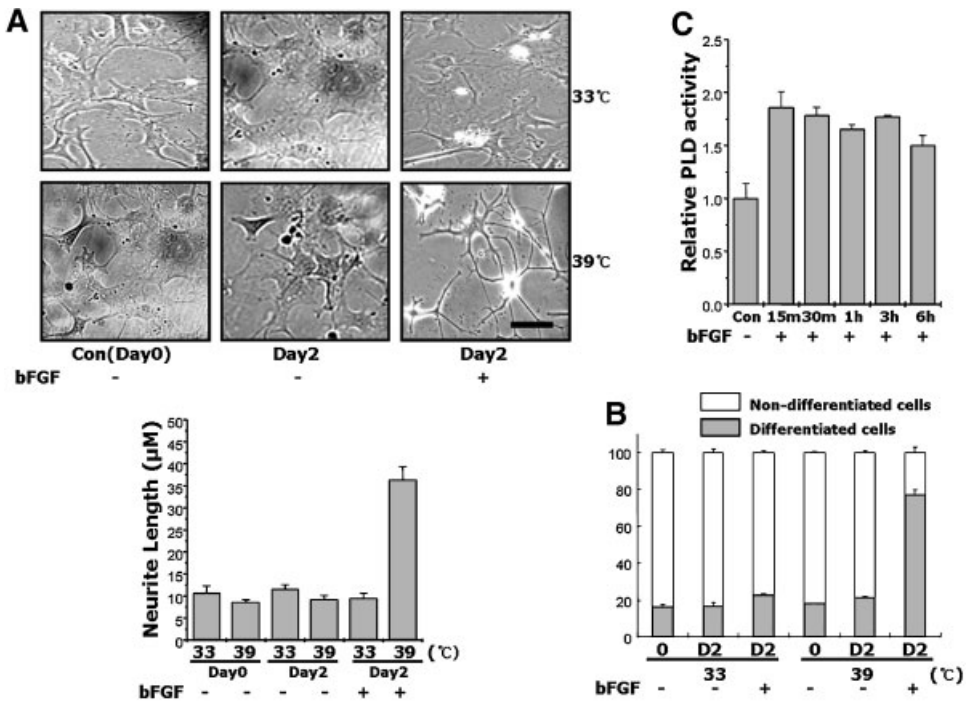

Fig. 1. Effect of bFGF on PLD activity and neurite outgrowth in H19-7 cells. **A**: Cells were grown at 33°C in DMEM containing 10% FBS, then switched to N2 medium for differentiation. The cells were subsequently treated with either bFGF in N2 media at 39°C or bFGF in serum-free DMEM at 33°C for 48 h. bFGF-induced neurite outgrowth of the cells was morphologically assessed. Scale bar, 10 μm. Magnification, 200×. bFGF-induced neurite outgrowth of the cells was morphologically assessed by the length of neurite outgrowth and visualized by phase-contrast microscopy. Cells from randomly selected areas of at least five cultures from three independent experiments were photographed. **B**: Cells from selected areas were photographed. The percentage of differentiated cells was obtained by dividing the number of differentiated, non-differentiated cells by the total number of cells. The total number of cells counted was 200 for the non-differentiated cells and differentiated cells in the replicate field. The results are the mean and the range of data from three independent experiments. **C**: Cells labeled with [^{3}H]-palmitic acid were treated with bFGF at 10 ng/ml for the indicated times in the presence of 1% ethanol. The amount of [^{3}H]-PEt was quantitated as described in ''Materials and Methods.'' Data are the means ± SE of three independent experiments.

Involvement of PLD1 in bFGF-Induced Neurite Outgrowth of H19-7 Cells

In order to assess which PLD isoenzyme is involved in bFGF-induced neurite outgrowth, we transfected cells with EGFP-rPLD1 and 2, and then examined its effects on bFGF-induced PLD activation and neurite outgrowth. After transfection, expression of PLD isozymes was monitered by successive immunobloting for PLD (Fig. 2A). The anti-PLD antibody recognized both PLD1 and PLD2. In unstimulated cells, overexpression of PLD1 or PLD2 led to higher basal activity of PLD. In bFGF-stimulated cells, overexpression of PLD1 but not PLD2 only increased the activity of PLD, compared with that of control cells (Fig. 2A). Basal activity of PLD also increased in the overexpression of PLD2. However, overexpression of PLD2 was without effect on the bFGF-induced PLD activation (Fig. 2A), demonstrating that bFGF receptor signaling is not coupled to PLD2 activation. The neurites in PLD1-expressing cells were longer than those in vector-expressing cells. bFGF stimulation for 48 h elicited formation of elongated neurites in PLD1 expressing cells (Fig. 2B). Furthermore, we also found that overexpression of PLD1 alone could elicit the formation of neurite outgrowth without bFGF in 2 days after transfection (Fig. 2B). In contrast to PLD1, overexpression of PLD2 was without effect on the bFGF-induced neurite outgrowth (data not shown). To investigate whether the enhancement by PLD1 of the bFGF-induced neurite outgrowth depends upon its lipase activity, N-terminal enhanced green fluorescence protein (EGFP)-tagged inactive rPLD1-V5 (DN-rPLD1) was examined

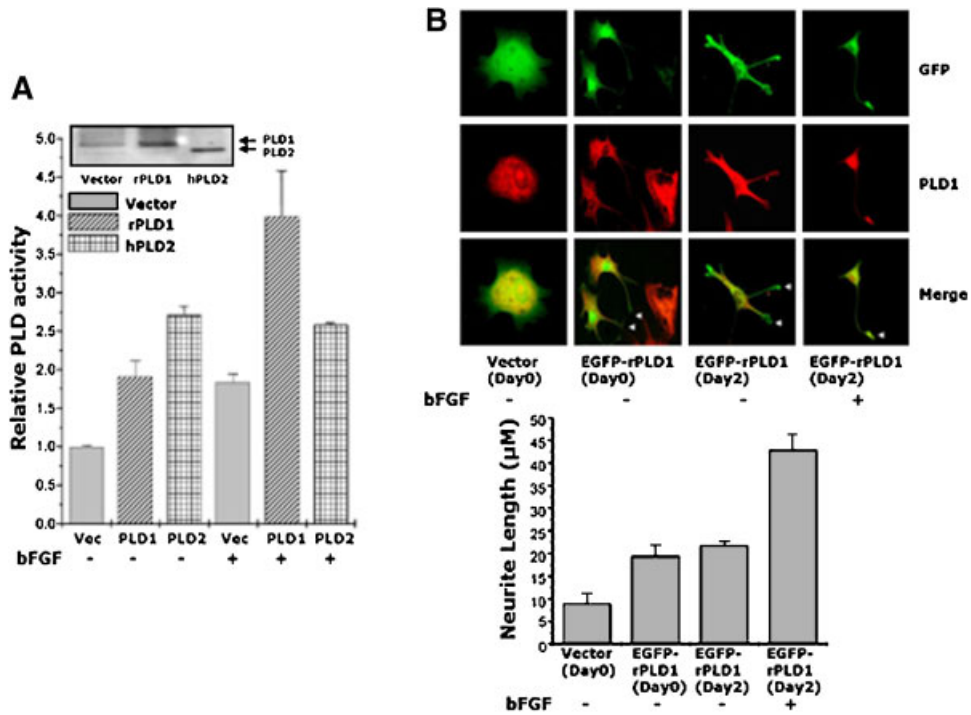

Fig. 2. Effect of transfection of PLD1 or PLD2 on bFGF-induced PLD activation and neurite outgrowth in H19-7 cells. **A**: Cells were transfected with EGFP-PLD1 or 2. Cells labeled with [^{3}H]-palmitic acid were treated with bFGF at 10 ng/ml for the indicated times in the presence of 1% ethanol. The amount of [^{3}H]-PEt was quantitated as described in ''Materials and Methods.'' Insert shows the overexpression of PLD1 and 2 revealed by immunoblot using anti-PLD antibody. Data are the means ± SE of three independent experiments. **B**: Cells were transfected with EGFP-PLD1. Cells were grown at 33°C in DMEM containing 10% FBS for 24 h, then switched to N2 medium for differentiation. Where indicated, the cells were subsequently treated with bFGF in N2 media at 39°C for 48 h. bFGF-induced neurite outgrowth of the GFP-positive cells was morphologically assessed by immunostaining using antibodies against GFP and PLD1. Immunostained GFP-positive cells were observed by a fluorescence microscope. Magnification, 200×. bFGF-induced neurite outgrowth of the GFP-positive cells was morphologically assessed by the length of neurite outgrowth and visualized by a fluorescence microscope. Cells from randomly selected areas of at least five cultures from three independent experiments were photographed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and EGFP-C1 was used as a marker for the transfected cells. DN-PLD1 was inducibly expressed by threefold over endogenous PLD1 (Fig. 3A). DN-rPLD1 transfected cells completely blocked the PLD activation stimulated by bFGF (Fig. 3A). Furthermore, when the activation of PLD was blocked, there was a remarkable inhibition of bFGF-induced neurite outgrowth (Fig. 3B), indicating that PLD1 activation is important for bFGF-induced neurite outgrowth of H19-7 cells.

PLC-γ Activation Is Important for bFGF-Induced Neurite Outgrowth via PLD1 Activation in H19-7 Cells

Next, we tried to determine how bFGF increases PLD activity. Stimulation of FGFR is known to result in the phosphorylation of tyrosine 766 and the recruitment and subsequent activation of PLC-γ [Cross et al., 2002]. In order to assess the role of PLC-γ in bFGF-induced neurite outgrowth via PLD1 activation, cells were pre-treated with a specific PLC-γ inhibitor, such as U73122, and the effects on bFGF-induced PLD activation and neurite outgrowth were examined. The pre-treatment of U73122 (10 μM) for 30 min completely inhibited bFGF-induced PLD activation (Fig. 4A). Furthermore, when the activity of PLC-γ was blocked, a remarkable inhibition of neurite outgrowth was observed (Fig. 5C), which implies that PLC-γ activation is located upstream of PLD1 and is important for bFGF-induced neurite outgrowth via PLD1 activation in H19-7 cells.

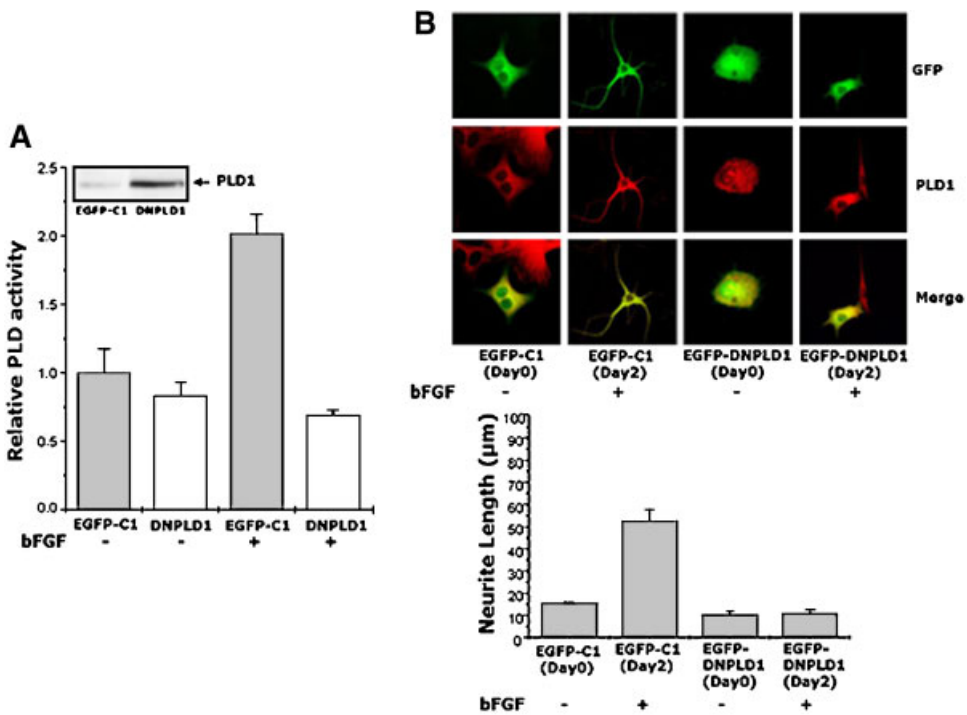

Fig. 3. Effect of DN-PLD1 on bFGF-induced PLD activation and neurite outgrowth in H19-7 cells. **A**: Cells were transfected with EGFP-C1 or constructs encoding dominant-negative PLD1 (EGFP-DN-PLD1). Cells labeled with [^{3}H]-palmitic acid were treated with bFGF at 10 ng/ml for the indicated times in the presence of 1% ethanol. The amount of [^{3}H]-PEt was quantitated as described in ''Materials and Methods.'' Insert shows the overexpression of DN-PLD1 revealed by immunoblot using anti-PLD1 antibody. Data are the means ± SE of three independent experiments. **B**: Cells were transfected with constructs encoding dominant-negative PLD1 (EGFP-DN-PLD1). Cells were grown at 33°C in DMEM containing 10% FBS for 24 h, then switched to N2 medium for differentiation. Where indicated, the cells were subsequently treated with bFGF in N2 media at 39°C for 48 h. bFGF-induced neurite outgrowth of the GFP-positive cells was morphologically assessed by immunostaining using antibodies against GFP and PLD1. Immunostained GFP-positive cells were observed by a fluorescence microscope. Magnification, 200×. bFGF-induced neurite outgrowth of the GFP-positive cells was morphologically assessed by the length of neurite outgrowth and visualized by a fluorescence microscope. Cells from randomly selected areas of at least five cultures from three independent experiments were photographed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

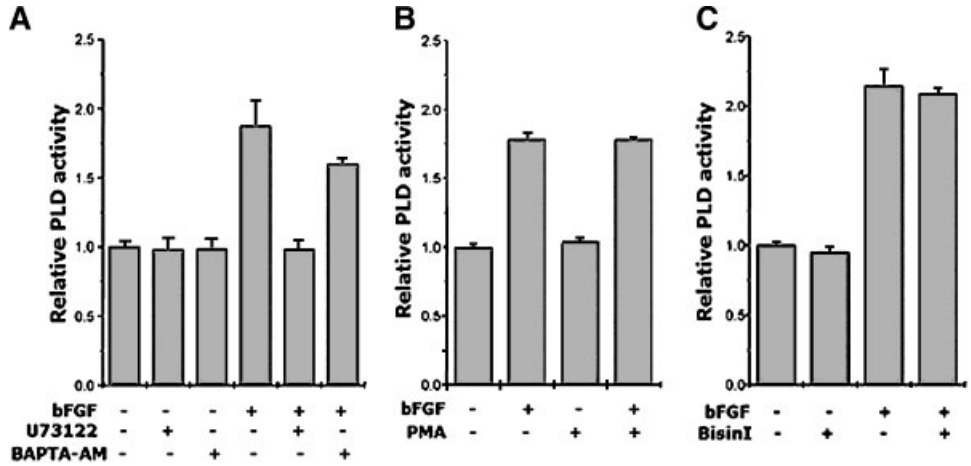

Fig. 4. Effect of the PLC-γ inhibitor U73122 and a chelator of intracellular Ca^{2+} BAPTA-AM on bFGF-induced PLD activation in H19-7 cells. **A**: Cells were pre-treated with 10 μM of the PLC-γ inhibitor, U73122 for 30 min and 10 μM of a chelator of intracellular Ca^{2+} (BAPTA-AM) for 1 h prior to bFGF stimulation. Cells labeled with [^{3}H]-palmitic acid were treated with bFGF at 10 ng/ml for the indicated times in the presence of 1% ethanol. The amount of [^{3}H]-PEt was quantitated as described in ''Materials and Methods.'' Data are the means ± SE of three independent experiments. **B**: Cells were pre-treated with 200 nM of PMA for 24 h prior to bFGF stimulation for effective downregulation of PKC activity. **C**: Cells were pre-treated with 4 μM of the PKC inhibitor, bisindolylmaleimide, for 15 min prior to bFGF stimulation. Cells labeled with [^{3}H]-palmitic acid were treated with bFGF at 10 ng/ml for the indicated times in the presence of 1% ethanol. The amount of [^{3}H]-PEt was quantitated as described in ''Materials and Methods.'' Data are the means ± SE of three independent experiments.

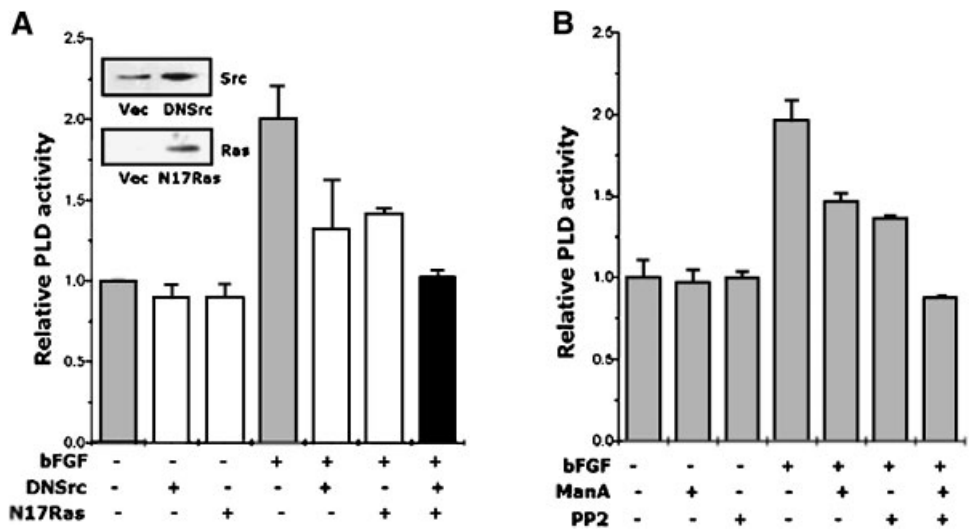

Fig. 5. Effect of Src and Ras inhibition on bFGF-induced PLD activation and neurite outgrowth in H19-7 cells. **A**: Cells were transfected with either DN-Src or N17Ras and cotransfected with DN-Src and N17Ras. **B**: Cells were pre-treated with 5 μM of a Src family of tyrosine kinases inhibitor, PP2, for 30 min or 1 μM of manumycin-A for 30 min and were pre-treated both PP2 and manumycin-A prior to bFGF stimulation. Cells labeled with [^{3}H]-palmitic acid were treated with bFGF at 10 ng/ml for the indicated times in the presence of 1% ethanol. The amount of [^{3}H]-PEt was quantitated as described in ''Materials and Methods.'' Insert (A) shows an overexpression of DN-Src and N17Ras revealed by immunoblot using Src and p21Ras antibody. Data are the means ± SE of three independent experiments. **C**: Cells were grown at 33°C in DMEM containing 10% FBS, then switched to N2 medium for differentiation. Cells were pre-treated with 10 μM of the PLC-γ inhibitor, U73122 for 30 min, 5 μM of a Src family of tyrosine kinases inhibitor, PP2, for 30 min 1 μM of manumycin-A for 30 min and were pre-treated both PP2 and manumycin-A prior to bFGF stimulation. Where indicated, the cells were subsequently treated with bFGF (10 ng/ml) in N2 media at 39°C for the indicated times and visualized by phase-contrast microscopy. bFGF-induced neurite outgrowth of the cells was morphologically assessed by the length of neurite outgrowth and visualized by phase-contrast microscopy. Cells from randomly selected areas of at least five cultures from three independent experiments were photographed. Scale bar, 10 μm. Magnification, 200×.

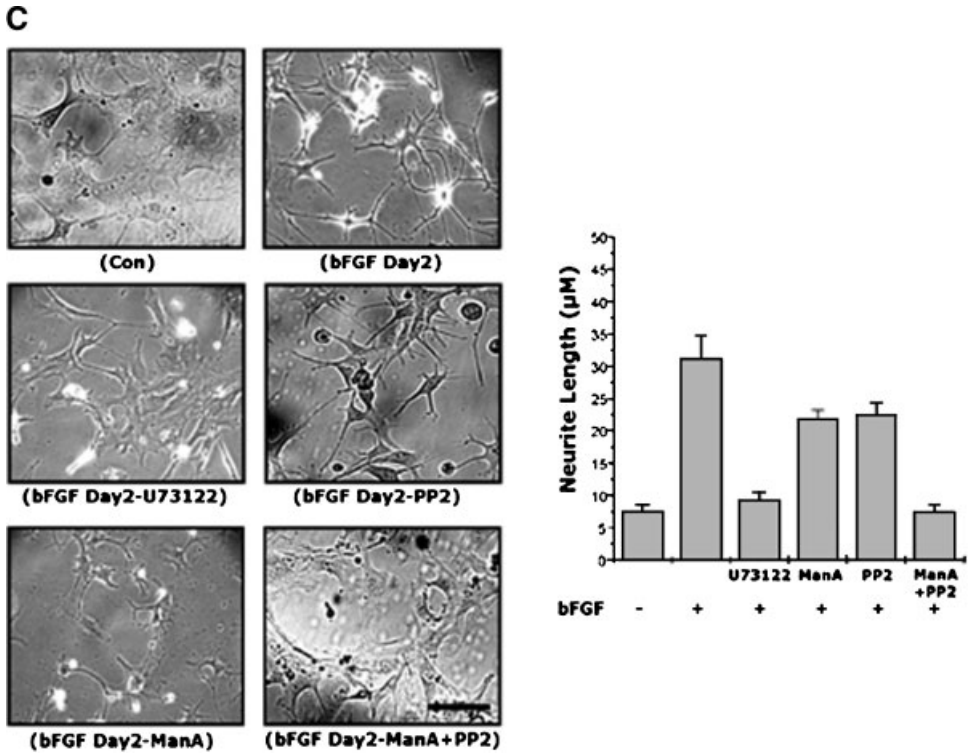

Fig. 5. (*Continued*)

Src and Ras, but not PKC, Is Required for bFGF-Induced Neurite Outgrowth via PLD1 Activation in H19-7 Cells

Activation of FGFR leads to the phosphorylation and activation of PLC-γ, thereby generating inositol triphosphate (IP_3), which is responsible for inducing the release of Ca^{2+} from intracellular stores, and DAG that is the physiological activator of PKC. We next examined the effects of PKC inhibition and Ca^{2+} chelation on bFGF-induced PLD1 activation. First, we examined the effect of PKC down-regulation on bFGF-induced PLD1 activation. Prolonged pre-treatment of the cells with PMA did not inhibit bFGF-induced PLD activation (Fig. 4B). The pre-treatment of bisindolylmaleimide (4 μM) as an inhibitor against the classical PKC isoform for 15 min also did not inhibit bFGF-induced PLD activation (Fig. 4C). However, the pre-treatment of an intracellular Ca^{2+} chelator BAPTA-AM (10 μM) for 1 h partially inhibited bFGF-induced PLD activation (Fig. 4A). These results suggest that intracellular Ca^{2+}, not PKC is involved in bFGF-induced PLD1 activation in H19-7 cells.

There is a report that calcium, which is mobilized in the response to PLC activation, is able to activate Src [Rusanescu et al., 1995], implying that Src may be involved in bFGF-induced neurite outgrowth via PLD1 activation in H19-7 cells. To further assess the role of Src in bFGF-induced neurite outgrowth via PLD1 activation, we examined the effects of DN-Src and a Src family of tyrosine kinases (SFKs) inhibitor, PP2, on bFGF-induced PLD1 activation and neurite outgrowth. The pre-treatment of PP2 (5 μM) for 30 min and the transfection of DN-Src partially inhibited bFGF-induced PLD activation (Fig. 5A,B). Furthermore, when PP2 was pre-treated, a remarkable inhibition of neurite outgrowth was observed (Fig. 5C). These results suggest that Src is required for bFGF-induced neurite outgrowth via PLD1 activation in H19-7 cells.

Activation of FGFR leads to the tyrosine phosphorylation of FRS2, allowing the binding of Grb2 that is required for activation of Ras. We thus hypothesized that a common link between bFGF-induced signaling and PLD1 activation may be the small GTPase from the Ras family. To investigate the involvement of Ras activation

in bFGF-induced neurite outgrowth via PLD1 activation, we performed a Ras inhibition study using manumycin-A (an inhibitor of Ras farnesyl transferase) and a dominant-negative form of Ras (N17Ras). As shown in Figure 5A,B, pretreatment of cells for 30 min with manumycin-A (1 μM) and transfection of N17Ras both partially abolished bFGF-induced PLD activation. Moreover, manumycin-A inhibited bFGF-induced neurite outgrowth of cells (Fig. 5C). Taken together, these results suggest that Ras is also an important mediator for bFGF-induced neurite outgrowth via PLD1 activation in H19-7 cells.

In this study, we found that DN-Src and N17Ras partially inhibited bFGF-induced PLD1 activation compared to vector transfected cells. We next examined the effects of the coinhibition of Src and Ras on bFGF-induced PLD1 activation. Cotransfection of DN-Src and N17Ras completely inhibited bFGF-induced PLD activation (Fig. 5A). Moreover, cotreatment of Manumycin-A and PP2 completely inhibited bFGF-induced neurite outgrowth and PLD activation (Fig. 5B,C), suggesting that Src and Ras independently mediated bFGF-induced PLD1 activation.

MAP Kinase Activation Is not Required for bFGF-Induced Neurite Outgrowth via PLD1 Activation

To examine whether MAP kinase activation is required for bFGF-induced neurite outgrowth via PLD1 activation, we initially determined the time course for bFGF-induced MAP kinase activation in H19-7 cells. bFGF-induced MAP kinase activation occurred rapidly and reached a maximum value 30 min after treatment of bFGF (Fig. 6A). Since the MEK inhibitor, PD98059, could efficiently suppress the activation of MAP kinases, we used it to test the requirement for MAP kinase activation in the bFGF-induced neurite outgrowth and PLD1 activation in H19-7 cells. Pre-treatment of PD98059 (20 μM) for 15 min completely blocked bFGF-induced MAP kinase activation (Fig. 6B), whereas it did not inhibit bFGF-induced PLD1 activation (Fig. 6C). As shown in Figure 6D, bFGF-induced neurite outgrowth did not change by treatment of the MEK inhibitor, suggesting that bFGF-induced MAP kinase activation and PLD1 activation are mediated through separate signaling pathways. Furthermore, MAP kinase activation is not required for bFGF-induced neurite outgrowth.

DISCUSSION

To distinguish between the signaling pathways that lead to differentiation as opposed to growth, it is important to define the initial signals that are required and/or sufficient for this process. To define the signaling pathways required for bFGF-induced neurite outgrowth, we used a conditionally immortalized rat hippocampal neuronal progenitor cell line, H19-7. Neurites extended in a time-dependent manner with bFGF treatment, indicating that these cells might be useful as a model system for hippocampal development and neurite outgrowth. PLD is involved in various neuronal signaling pathways, and is also thought to be an important mediator of neuronal function. PLD activation has been observed in a wide variety of brain and neuronal-derived cells, including primary neurons and glial cells, as well as neuroblastoma, glioma, astrocytoma, and pheochromocytoma cell lines [Nishida et al., 1992]. However, there are few studies concerning the relation of PLD activation and bFGF-induced neurite outgrowth in the nervous system. In the present study, our results indicate that bFGF activates PLD1 through PLC-γ activation, which leads to neurite outgrowth in H19-7 cells, and that Src and Ras independently mediate in bFGF-induced PLD1 activation.

The main objective of this study was to evaluate the potential activation of PLD1 by neurogenic bFGF in H19-7 cells. This enzyme is considered to play an important role as an effector enzyme in membrane lipid-mediated signal transduction. Accordingly, bFGF-induced PLD activation occurred in a fast and transient manner. Basal PLD activity in control cells was low, but increased during the progress of bFGF-induced neurite outgrowth, which is thought to transmit neurogenic signals leading to neurite outgrowth of H19-7 cells. However, when diverse changes of cell phenotype are induced by bFGF, the molecular mechanism and kinetics of cellular membrane perturbations are believed to be different. For example, PLD could change the properties of cellular membranes by altering their lipid composition through the conversion of PC to PA [Cockcroft, 2001]. Recent studies reported that PLD1 protein was predominantly localized in the

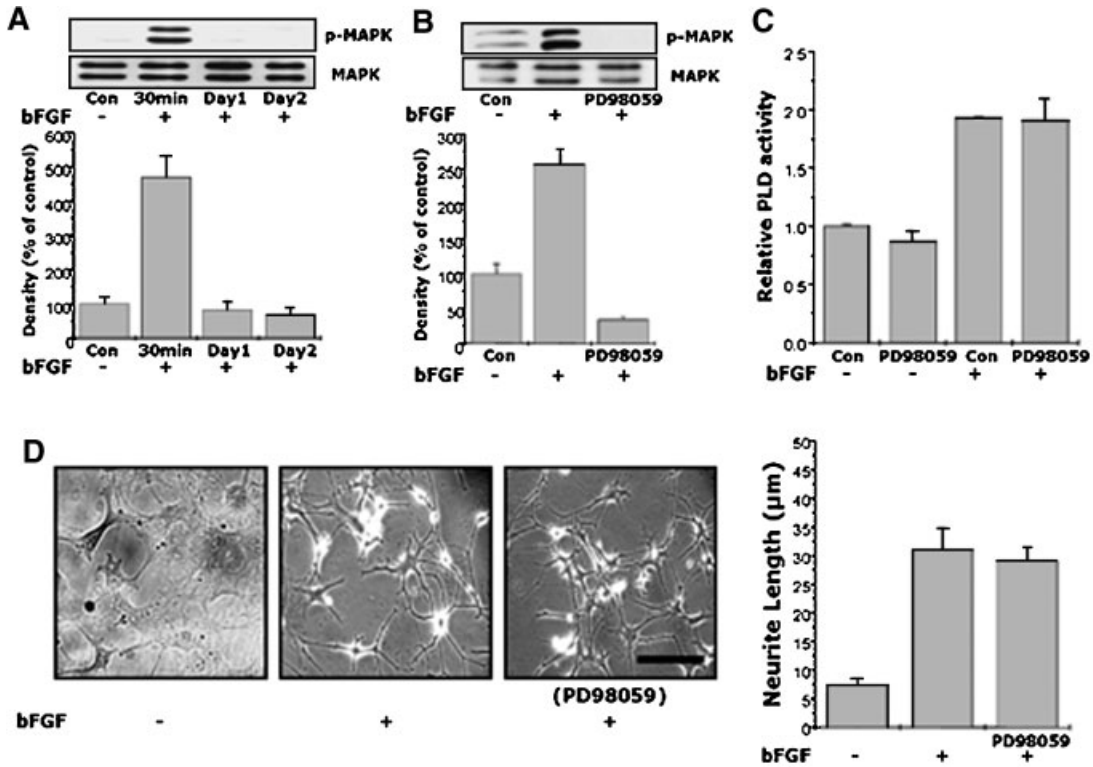

Fig. 6. Effect of the MEK inhibitor PD98059 on bFGF-induced PLD activation, neurite outgrowth, and MAP kinase phosphorylation in H19-7 cells. **A**: Cells were grown at 33°C in DMEM containing 10% FBS, then switched to N2 medium for differentiation. The cells were subsequently treated with bFGF (10 ng/ml) in N2 media at 39°C for the indicated times. **B**: Cells were pre-treated with 20 μM of PD98059 for 15 min prior to bFGF stimulation. Where indicated, the cells were subsequently treated with bFGF (10 ng/ml) in N2 media at 39°C for the indicated times. Cells were then lysed and immunoblotted using p-ERK antibody and ERK antibody. The intensity of bands was quantified using QuantityOne software (Bio-Rad). These experiments were repeated three times. **C**: Cells were pre-treated with 20 μM of PD98059 for 15 min prior to bFGF stimulation. Cells labeled with [^{3}H]-palmitic acid were treated with bFGF at 10 ng/ml for the indicated times in the presence of 1% ethanol. The amount of [^{3}H]-PEt was quantitated as described in ''Materials and Methods.'' Data are the means ± SE of three independent experiments. **D**: Cells were grown at 33°C in DMEM containing 10% FBS, then switched to N2 medium for differentiation. Cells were pre-treated with 20 μM of PD98059 for 15 min prior to bFGF stimulation. Where indicated, the cells were subsequently treated with bFGF (10 ng/ml) in N2 media at 39°C for the indicated times and visualized by phase-contrast microscopy. bFGF-induced neurite outgrowth of the cells was morphologically assessed by the length of neurite outgrowth and visualized by phase-contrast microscopy. Cells from randomly selected areas of at least five cultures from three independent experiments were photographed. Scale bar, 10 μm. Magnification, 200×.

neurite extension of PC12 cells [Min et al., 2001a], neurons [Lee et al., 2000], and areas of neurite in developing rat hippocampus [Min et al., 2001b]. Thus, the present finding that the bFGF-induced PLD activation is rapid and transient suggests that the major role of PLD activation seems to involve the generation of second messengers, such as IP_3, DAG, PA, and choline, which subsequently lead to the propagation of neurotrophic signals directly and cause membrane perturbation. Moreover, other reports suggest that PLD1 plays an important role in vesicle transport [Jones et al., 1999]. In order to extend neurites, cells must synthesize and transfer phospholipids to the plasma membrane during neurite outgrowth induced by neurotrophic signals. Therefore, it can be assumed that PLD1 activation is necessary for membrane transport to replenish the plasma membrane and may function in the drastic morphological changes associated with cellular processes such as cytoskeletal rearrangement. Interestingly, the overexpression of DN-rPLD1 mutants significantly inhibited bFGF-induced neurite outgrowth in H19-7 cells. In addition, EGFP-rPLD1-transfected cells showed fully formed neurites without bFGF. The results presented here suggest that PLD1 activation plays an important role in neurite outgrowth in H19-7 cells.

Upon binding to the receptor, bFGF also interacts with heparin proteoglycan sulfates

to promote dimerization and activation [Song et al., 2004], and receptor tyrosine kinases undergo autophosphorylation. Some of the phosphorylation sites acts as anchors for other signaling molecules, and other sites within the catalytic domain are required for activity. FGFs mediate their biological response by binding to and activating a family of four receptor tyrosine kinases designated FGFR1-4. The FGFRs are associated through their juxtamembrane domains with distinct docking proteins, termed Gab1 and FRS2, respectively, and it is likely that the signaling molecules recruited to the receptors via these adaptor proteins play an important role in initiating specific cascades. Gab1 has been found to similarly mediate EGF signaling [Fan et al., 2004]. In the case of bFGF, receptor stimulation leads to complex formation between FRS2 and a variety of signaling molecules including the adaptor Grb2 and the Ras activator Sos [Klint et al., 1999]. PLC-γ is another protein docked by activated FGFR1. A C-terminal site (Y766) of FGFR1 recruits PLC-γ and has been implicated in neurite outgrowth [Lundin et al., 2003]. For example, an analysis of FGFR1 and FGFR3 chimeras demonstrated that the juxtamembrane region is primarily responsible for neurite outgrowth in PC12 cells; the PLC-γ binding site induced only a very modest increase in neurite outgrowth [Lin et al., 1998]. Src kinases have been reported to associate with the bFGF receptor [Klint et al., 1999]. However, it should be noted that other investigators failed to detect a direct association between Src and the bFGF receptor, possibly suggesting a role for other Src family kinases [Landgren et al., 1995]. Generally, neurite outgrowth is accompanied by extensive reorganization of the cytoskeleton to initiate the extension of neuritic processes, and many of the known substrates of Src, such as $p130^{cas}$, appear to be associated with cytoskeletal rearrangement [Anneren et al., 2000]. There is a report suggesting that Src kinases are an important effector of the bFGF receptor, and act as a mediator of neurite outgrowth in H19-7 cells [Kuo et al., 1997]. Taken together, this complex appears to be the primary mechanism by which bFGF stimulates PLC-γ, Ras, MAP kinase, and Src.

We demonstrated that inhibitors of PLC-γ (U73122) completely blocked bFGF-induced PLD1 activation and neurite outgrowth in H19-7 cells. These results raise the possibility that bFGF activates a PLC-γ-mediated pathway related to PLD activation and neurite outgrowth. Studies with dominant-negative mutants in PC12 cells implicate Ras is important in this process [Rusanescu et al., 1995]. We demonstrated that pre-treatment of manumycin-A and transfection of N17Ras partially decreased bFGF-induced PLD activation. Also, bFGF-induced neurite outgrowth was inhibited by manumycin-A. These results suggest that Ras is also an important mediator of neurite outgrowth via PLD1 activation in H19-7 cells. However, is the activation of PLD1 by Ras direct or indirect? Luo et al. [1997] demonstrated that PLD1 associates directly with the small GTPase RalA, a downstream target of Ras, indicating that PLD1 activation by Ras may be mediated in RalA indirectly. Moreover, Ca^{2+}, which is increased by PLC-γ activation, is able to activate Src in PC12 cells [Chao et al., 1997]. DN-Src and a Src family of tyrosine kinases inhibitor, PP2, partially inhibited bFGF-induced PLD activation, and bFGF-induced neurite outgrowth was inhibited by treatment of PP2. Moreover, cotransfection of DN-Src and N17Ras completely inhibited bFGF-induced PLD activation. Therefore, we could conclude that both of Src and Ras might be required for PLD1-mediated neurite outgrowth and that Src and Ras independently regulate PLD1 activation.

The current model for neurite outgrowth is based on the finding in PC12 cells that prolonged activation of ERK is both necessary and sufficient for differentiation [Sagara et al., 2004]. However, other reports showed that prolonged activation of MAP kinase is insufficient for differentiation of immortalized hippocampal cells, even though prolonged activation of Raf-1 is sufficient [Chung et al., 1998]. This indicates that neurite outgrowth by MAP kinase activation may depend on the cell types or agonists under investigation. Furthermore, the other report showed that PLD2 activation downstream of MAP kinase is required for NGF-induced neurite outgrowth in PC12 cells [Watanabe et al., 2004]. However, we observed that MAPK pathways are not required for PLD1 activation and neurite outgrowth by bFGF in H19-7 cells. Accordingly, it is likely that MAP kinase signaling is only mediated in NGF-induced PLD2 activation signaling pathways.

In this study, we show that bFGF induces neurite outgrowth of H19-7 cells through sti-

mulation of the PLC-γ-Src-PLD1 and PLC-γ-Ras-PLD1 pathways independently. This study identifies PLD1 activation as a new target for bFGF-induced neurite outgrowth in H19-7 cells. To date, the molecular mechanisms underlying the cellular outcomes induced by differentiating factors remain unknown. Further study of downstream signaling pathways activated by PLD may give deeper insights into the mechanisms behind biological outcomes that are induced by differentiation factors.

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