Phospholipase D1 mediates bFGF-induced Bcl-2 expression leading to neurite outgrowth in H19-7 cells

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INTRODUCTION

FGFs (fibroblast growth factors) constitute a large family of peptide and protein hormones that influence a wide array of biological processes such as angiogenesis, embryogenesis, differentiation and proliferation [1]. Among FGFs, bFGF (basic FGF; also called FGF-2) plays an important role in differentiation and/or function of the nervous system by interacting with a specific cell-surface receptor [2–4] and is widely used to expand adult neuronal stem cells and NPCs (neural progenitor cells) in vitro [5]. Binding of bFGF to cell-surface tyrosine kinase FGF receptors triggers receptor dimerization and autophosphorylation of tyrosine residues, which leads to tyrosine phosphorylation of the adaptor protein, FRS2 (FGF receptor substrate 2) [6] and initiates the major pathway for activation of the monomeric GTase Ras. Activated GTase-Ras binds to p110α, the catalytic subunit of PI3K (phosphoinositide 3-kinase), and translocates PI3K to the cell membranes, resulting in a complex regulation of downstream signalling events [7]. PI3K has been shown to mediate the induction of neurite outgrowth of PC12 cells by NGF Ras-dependently [8–13], and PLCγ (phospholipase Cγ) is activated by the interaction of the PH (pleckstrin homology) domain of PLCγ with the PI3K product PI3P (phosphatidylinositol 3,4,5-trisphosphate) [14]. Activated PLCγ hydrolyses PIP2 (phosphatidylinositol 4,5-bisphosphate) to generate DAG (diacylglycerol) and IP3 (inositol 1,4,5-trisphosphate), resulting in PKC (protein kinase C) activation and Ca2+ release from internal stores [15]. Ca2+ is important for the PLD (phospholipase D) catalytic process [16], even though PKCα also activates PLD [17]. Although all of these Ras-mediated molecules are involved in bFGF-triggered signalling, previous studies have not clarified proper connections between them. The aim of the present study was to elucidate the precise relationship between Ras-mediated signalling and PLD1 activation during induction of neuronal differentiation by bFGF in immortalized hippocampal progenitor cells (H19-7 cells).

Bcl-2 is reported to be involved in neuronal cell differentiation [18,19] due to the correlation between expression of Bcl-2 in CNS (central nervous system) neurons and axon elongation in the developing brain [20], and a reduction in the ability of neurons to extend neurites in Bcl-2-deficient embryos [20,21]. Moreover, Bcl-2 is critical for neuronal commitment of mouse embryonic stem cells [22], even though it is a member of a large complex gene family of anti- and pro-apoptotic proteins. The signal transduction mechanisms involved in bFGF-induced neurite outgrowth have long been under intensive investigation, but the findings are inconclusive as to what the signalling role of Bcl-2 in this process is. In the present study, we investigated the relationship between bFGF-induced Bcl-2 expression and PLD1 signalling, and discovered that PLD1 is activated by bFGF through the following the PI3K/PLCγ pathway, indicating that bFGF-induced PLD activation took place through the Ras/PI3K/PLCγ pathway. Furthermore, PLD1 was required for activation of JNK (c-Jun N-terminal kinase), which led to activation of STAT3 (signal transducer and activator of transcription 3) and finally Bcl-2 expression. When Bcl-2 was overexpressed, neurite outgrowth was stimulated along with induction of neurotrophic factors such as brain-derived neurotrophic factor and neurotrophin 4/5. In conclusion, PLD1 acts as a downstream effector of bFGF/Ras/PI3K/PLCγ signalling and regulates Bcl-2 expression through JNK/STAT3, which leads to neurite outgrowth in H19-7 cells.

Key words: Bcl-2, c-Jun N-terminal kinase (JNK), phospholipase Cγ (PLCγ), phospholipase D1 (PLD1), Ras, signal transducer and activator of transcription 3 (STAT3).
Ras/P13K/PLCγ cascade and is involved in activation of JNK (c-Jun N-terminal kinase) followed by STAT3 (signal transducer and activator of transcription 3)-mediated Bcl-2 expression. Interestingly, overexpression of Bcl-2 led to a marked increase in the number of neurite-bearing H19-7 cells and in the expression of neurotrophic factors such as NT4/5 (neurotrophin 4/5) and BDNF (brain-derived neurotrophic factor).

**EXPERIMENTAL**

**Materials**

FBS (fetal bovine serum), penicillin/streptomycin solution, NB (neurobasal medium), B27 and DMEM (Dulbecco’s modified Eagle’s medium), L-glutamine and low glucose were purchased from Gibco. bFGF was from R&D Systems. Manunycin A, wortmannin, U73122, BAPTA/AM [1,2-bis-(o-aminophenoxy)ethane-N,N,N’,N’-tetra-acetic acid tetra-kis(acetoxymethyl ester)], Ro320432, PP2 and SP600125 were from Calbiochem. [3H]Palmitic acid was from PerkinElmer. PBT (L-α-phosphatidylbutanol) was from Avanti Polar Lipids and the silica gel 60A plates for TLC were purchased from Whatman. Antibodies used were as follows: anti-(β-tubulin type III) (Tuj I) monoclonal antibody (Babco); Cy3(TM) (indocarbocyanine)- conjugated AffiniPure goat anti-[rabbit IgG (H + L)] (Jackson ImmunoResearch); biotinylated secondary antibody (Vector Laboratories); anti-p-JNK/JNK rabbit polyclonal antibody, anti-Stat3 rabbit polyclonal antibody, anti-p-ERK (where ERK is extracellular-signal-regulated kinase) rabbit monoclonal/ERK rabbit polyclonal antibody, anti-p-PLCγ (Tyr197)/PLCγ rabbit polyclonal antibody, anti-p110α rabbit polyclonal antibody and anti-PLD1 and anti-PLD2 rabbit polyclonal antibodies (all from Cell Signaling Technology); and anti-NFα/β antibody (Abbiotec). Antibodies for Ras, PKCζ, Bcl-2, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), integrin α2 and β-actin were purchased from Santa Cruz Biotechnology, and Raf-1 RBD was from Upstate Biotechnology. All other chemicals were of analytical grade.

**Cell culture**

H19-7 cells were grown in DMEM with 10% (v/v) FBS and maintained at 33°C under G418 selection. To induce differentiation, the cells were cultured in N2 medium with bFGF (20 ng/ml) after starvation in N2 medium without bFGF at 39°C for 18 h. The cells were kindly provided by Dr K.C. Chung (Department of Biology, Yonsei University, Seoul, Republic of Korea).

**Preparation of cytosolic and membrane fractions**

Cytosol and membranes were prepared as previously described [23]. Briefly, cells were suspended in a buffer (50 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin and 50 mM Hepes/NaOH, pH 7.5), disrupted by sonication and centrifuged at 100,000 g for 1 h. The supernatant was used as the cytosolic fraction, and the pellet was resuspended in lysis buffer containing 1% (v/v) Triton X-100. After centrifuging the resuspended pellet at 100,000 g for 1 h, the supernatant was collected as the membrane fraction.

**Western blot analysis**

Cells were lysed in 20 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate and 1% Triton X-100, and samples containing 20–30 μg of protein were loaded on to SDS/polyacrylamide gels (10% or 12%), electrophoresed and transferred on to nitrocellulose membranes (Amersham Biosciences). After blocking with 5% (w/v) dried skimmed milk for 2 h, the membranes were incubated with primary antibodies followed by HRP (horseradish peroxidase)-conjugated secondary antibody (1:2000 dilution; New England Biolabs), and specific bands were detected by ECL (enhanced chemiluminescence) (Amersham Biosciences).

**Affinity precipitation of activated Ras**

Raf-1 RBD (Ras-binding domain) (Upstate Biotechnology) was used for affinity precipitation of activated Ras (GTP-Ras) according to the manufacturer’s protocol. Briefly, lysates (1 mg) were incubated with 10 μl of Raf-1 RBD–agarose for 30 min at 4°C. The beads were then boiled in sample buffer, electrophoresed and transferred on to a nitrocellulose membrane, which was probed with monoclonal anti-Ras antibody.

**Transient transfection of H19-7 cells with PLD1, DN (dominant-negative)-PLD1, STAT3, bcl-2 (rat Bcl-2) or siRNAs (small interfering RNAs)**

H19-7 cells were transiently transfected with 5 μg each of pcDNA3.1, pcDNA3.1-PLD1, pcDNA3.1-DN-PLD1, mCherry–IRES-3-CL (where IRES is internal ribosome entry site and CL is cationic lipids), mCherry–IRES3-CL–rbC2, pMSCV-HA-IRES-CL–EGFP (where EGFP is enhanced green fluorescent protein and HA is haemagglutinin) and pMSCV-HA-STAT3-IRES-CL–EGFP for overexpression experiments. PLD1 siRNAs (#1 and #2), PLD2 siRNAs (#1 and #2), STAT3 siRNA or mismatch siRNAs (#1 and #2) were introduced for knockdown experiments, using a Nucleofector™ kit (Lonza). The transfected cells were cultured in DMEM for 48 h at 33°C. The cells were then transferred into N2 medium at 39°C. At the same time, bFGF was added to the N2 medium in order to induce differentiation. PLD1 siRNA (#1) and PLD2 siRNA (#1) were from Dharmacon Research, and PLD1 siRNA (#2), PLD2 siRNA (#2) and STAT3 siRNA were from Bioneer. As a mismatch control, single base pair changes were introduced in PLD1, PLD2 and STAT3 siRNAs.

**RT (reverse transcription)–PCR**

cDNA was prepared from total mRNA extracted from H19-7 cells with TRIzol® reagent, and 2 μg of RNA was reverse-transcribed using random hexamer mixed primers. The resulting cDNA was amplified by PCR using the following primers: Bcl-2 forward, 5′-GCTCTAGAATGGCGCAAGCCGGGAGAAC-3′, and Bcl-2 reverse, 5′-ATAGTTTAGCGGCCGCTCACTTGTGGCCCAGG-3′; BDNF forward, 5′-GTTGACAGTATTTAGCACTGGTGACAGTATG-3′, and BDNF reverse, 5′-GGGTAGTTCGGCATTGC-3′; NT4/5 forward, 5′-CCTGTCGGTACGTTTCTCTG-3′, and NT4/5 reverse, 5′-CTGACGTAGCCACCGTCTCTG-3′. The PCR conditions for Bcl-2, BDNF and NT4/5 were as follows: denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. In each case we used 33 cycles. Amplified DNA fragments were identified on a 1.5% agarose gel and visualized by ethidium bromide staining.

**Determination of PLD activity**

PLD activity was measured by the formation of PBl, the product of PLD-mediated transphosphatidylation in the presence of butanol, as described previously [24]. Cells were allowed to differentiate for the indicated times in six-well plates at a density of 10⁵ cells/ml and were labelled with 1 μCi/ml [3H]palmitic acid in serum-free medium for 24 h. Then, cells were pretreated with 0.3% bFGF for 15 min, quickly
washed with ice-cold PBS and suspended in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer [24], and Pbt was separated by TLC using acetate/ethanol 2:2:4-trimethylpentane/acetic acid/water (110:50:20:100, by vol.) as a solvent system. Regions corresponding to authentic Pbt bands were identified with 0.002% primulin in 80% acetone, scraped and counted with a scintillation counter.

Immunoprecipitation

H19-7 cells were transfected with mCherry–IRE3-CL and mCherry–IRE3-CL-rBcl-2, and the cells were grown on poly-L-lysine-coated coverslips in DMEM containing 10% (v/v) FBS for 48 h at 33°C. They were then fixed in 4% (w/v) paraformaldehyde for 20 min, washed three times for 5 min each with 0.1% BSA in PBS, permeabilized with 0.3% Triton X-100 for 30 min, and blocked with 10% goat serum in PBS for 30 min. Next, they were immunostained for 1 h using a primary antibody [1:400 dilution of mouse monoclonal anti-Bcl-2 antibody and 1:2000 dilution of rabbit monoclonal anti-(β-tubulin type III) (Tuj1) antibody] and washed with 0.1% BSA in PBS three times for 5 min. Tuj1 primary antibody was detected with biotinylated anti-mouse IgG, followed by a streptavidin-conjugated secondary antibody. For the anti-Bcl-2 primary antibody, Cy3-conjugated AffiniPure goat anti-rabbit IgG (H + L) was used as the secondary antibody (1 h in the dark), cells were washed with distilled water, and coverslips bearing the immunostained cells were mounted on slides with Vectashield (Vector Laboratories). Photographs of the cells were taken under a fluorescence microscope (Nikon) and the images were analysed with ImageJ software (NIH; http://rsb.info.nih.gov/ij/).

Immunocytochemistry

To investigate the effects of rBcl-2 overexpression on neurite outgrowth, H19-7 cells were transfected with mCherry–IRE3-CL-rBcl-2, and the cells were grown outgrowth, H19-7 cells were transfected with mCherry–IRES3-CL-rBcl-2, and the cells were grown

PLD1 mediates bFGF-induced Bcl-2 expression in H19-7 cells

Our previous results [24] suggested that H19-7 cells underwent differentiation in response to bFGF stimulation producing PLD1. To investigate whether Bcl-2 expression is increased during differentiation induced by bFGF and is regulated by PLD1 and/or PLD2, H19-7 cells were cultured in N2 medium supplemented with bFGF. As shown in Figure 1(A), expression of Bcl-2 gradually increased over 24 h and then decreased. Next, to assess the role of PLD in Bcl-2 expression, we down-regulated PLD1 and PLD2 by introducing PLD1- and PLD2-specific siRNAs (#1 and #2) and then rescued PLD1 and PLD2 by overexpression of these PLD genes respectively. As shown in Figure 1(B), bFGF-induced Bcl-2 expression was completely abolished with treatment of PLD1 siRNAs, whereas PLD2 siRNAs had no effect on Bcl-2 expression. When we rescued PLD1 by transfection of this gene, bFGF-induced Bcl-2 expression was also rescued. We also confirmed the effect of PLD1 on Bcl-2 expression using PLD1 or DN-PLD1 transfection. PLD1 overexpression showed an 85% increase in Bcl-2 expression, whereas overexpression of DN-PLD1 significantly reduced Bcl-2 expression (50% decrease compared with the bFGF-treated control group) (Figure 1C). These results indicate that bFGF up-regulates Bcl-2 expression through PLD1 activation in H19-7 cells.

PLD activation by bFGF occurs via Ras/Pi3K/PLCγ signalling

According to our previous findings [25], PLD activity is maximal within 15 min of bFGF treatment in H19-7 cells. When we stimulated H19-7 cells with bFGF for 15 min, GTP-Ras and Src phosphorylation were increased (Figure 2A) and p110α (the catalytic subunit of PI3K), PLCγ and PKCa were all translocated from the cytosol to membranes (Figure 2B; 3.5-fold increases in p110α and PLCγ, and a 2.5-fold increase in PKCa). To further investigate whether these molecules regulate Bcl-2 expression, we pre-incubated cells for 30 min with manumycin A (a selective farnesyltransferase inhibitor of Ras) and with inhibitors of Src, Pi3K, PLCγ and PKCa (PP2, wortmannin, U73122 and Ro320432 respectively) before adding bFGF. Interestingly, all these inhibitors except for the Src and PKCa inhibitors, strongly inhibited Bcl-2 expression in response to bFGF (Figure 2C), indicating that Bcl-2 expression is regulated by Ras, Pi3K and PLCγ, but not by Src and PKCa. As expected, DN-Src overexpression did not affect Bcl-2 expression. PLCγ can cause the release of Ca2+ from intracellular stores by generating IP3 and can then activate PLD. We also examined the role of Ca2+ in Bcl-2 expression by pretreating cells with the intracellular Ca2+ chelator BAPTA/AM. Treatment of BAPTA/AM also blocked Bcl-2 expression induced by bFGF (Figure 2C). To determine whether these intermediates regulate PLD activity, we pre-incubated cells for 30 min with manumycin A, wortmannin, U73122 or BAPTA/AM before adding bFGF. PLD activation was blocked by each of these inhibitors (30% inhibition by manumycin A and 50% inhibition by wortmannin, U73122 or BAPTA/AM compared with the control containing only bFGF) (Figure 2D), indicating that PLD activity is regulated by Ras, Pi3K and PLCγ.

RESULTS

PLD1 mediates bFGF-induced Bcl-2 expression in H19-7 cells

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Figure 1  Effects of bFGF and PLD on Bcl-2 expression

H19-7 cells were grown at 33°C in DMEM containing 10% (v/v) FBS. (A) After serum-starvation in N2 medium at 39°C for 18 h, the cells were stimulated with bFGF (20 ng/ml). At the indicated times, the cells were lysed and proteins were analysed by Western blotting using an anti-Bcl-2 or anti-β-actin antibody. (B) H19-7 cells were transfected with PLD1 siRNAs (#1 and #2), PLD2 siRNAs (#1 and #2) or mismatch siRNAs (#1 and #2) and then rescued with the construct encoding PLD1 (left-hand panels) or PLD2 (right-hand panels), using a Nucleofector™ kit. After transfection with siRNAs and/or expression vector, the cells were cultured in N2 medium with bFGF (20 ng/ml) at 39°C for 24 h. Proteins were analysed by Western blotting using an anti-PLD1, anti-PLD2, anti-β-actin or anti-Bcl-2 antibody. (C) Cells grown at 33°C were transfected with PLD1 or DN-PLD1 (5 μg), and cultured in N2 medium with bFGF at 39°C for 24 h. Proteins were analysed by Western blotting using an anti-PLD1, anti-Bcl-2 or anti-β-actin antibody. Results are shown as fold changes from the basal value (without bFGF) and are means ± S.D. for three experiments. *P < 0.01 compared with the bFGF-treated control. si, siRNA.

PLD1 activates JNK, but not ERK

To test whether any MAPK (mitogen-activated protein kinase) is involved in the induction of Bcl-2 by PLD1, we examined the effects of PLD1 siRNAs on the phosphorylation of MAPK in response to bFGF. Western blotting showed that phosphorylation of ERK1/2 and JNK1/2 were both increased by bFGF; however, both of the PLD1 siRNAs (#1 and #2) decreased only JNK phosphorylation, not ERK phosphorylation. In particular, PLD1 siRNA (#2) inhibited JNK phosphorylation more effectively (Figure 3A; 95% and 85% inhibition of JNK1 and JNK2 phosphorylation respectively compared with the control containing bFGF). As expected, overexpression of PLD1 rescued the cells from inhibition of JNK phosphorylation. To see whether the molecules upstream of PLD1 (Ras, PI3K and PLCγ) regulate PLD1-mediated JNK phosphorylation, cells were then pretreated with each specific inhibitor for 30 min before bFGF-stimulation. All inhibitors of the upstream signalling molecules of PLD1 reduced JNK phosphorylation (Figure 3B). Treatment with the JNK inhibitor SP600125 completely blocked bFGF-induced Bcl-2 expression (Figure 3C). Taken together, these findings indicate that PLD1 is involved in activation of JNK, which in turn stimulates Bcl-2 expression.

STAT3 acts downstream of JNK and is required for Bcl-2 expression

A recent study suggested that STAT3 plays an important role in the differentiation of neurons in response to neurotrophic factors and growth factor [26]. We found that phosphorylation of STAT3 on Ser727 was increased by bFGF, and PLD1 knockdown with PLD1 siRNAs blocked STAT3 activation by bFGF, whereas overexpression of PLD1 rescued the cells from inhibition of STAT3 phosphorylation (Figure 4A). We also confirmed the role of PLD1 in phosphorylation of STAT3 on Ser727 using PLD1 or DN-PLD1 transfection. Overexpression of DN-PLD1 remarkably decreased phosphorylation of STAT3 (on Ser727) induced by bFGF, whereas PLD1 overexpression potentiated the phosphorylation of STAT3 (on Ser727) (Figure 4B). These results indicate that phosphorylation of STAT3 on Ser727 depends on PLD1 activation.
The inhibition of JNK activity by SP600125 also showed a complete blockage of STAT3 phosphorylation induced by bFGF, suggesting that STAT3 acts downstream of JNK (Figure 4C). We also investigated the ability of STAT3 to interact with JNK by immunoprecipitation assays. Cells were stimulated with bFGF, and, as shown in Figure 4(D), JNK was immunoprecipitated with STAT3. More precisely, it appears that the affinity of JNK and, as shown in Figure 4(D), JNK was immunoprecipitated with STAT3. We also investigated the ability of STAT3 to interact with JNK by immunoprecipitation assays. Cells were stimulated with bFGF, and, as shown in Figure 4(D), JNK was immunoprecipitated with STAT3. More precisely, it appears that the affinity of JNK activity by SP600125 also showed a complete blockage of STAT3 phosphorylation induced by bFGF, suggesting that STAT3 acts downstream of JNK (Figure 4C). We also investigated the ability of STAT3 to interact with JNK by immunoprecipitation assays. 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Overexpression of Bcl-2 leads to neurite outgrowth and increased expression of neurotrophic factors (NT4/5 and BDNF)

To determine the effect of Bcl-2 overexpression on neurite outgrowth, H19-7 cells were transiently transfected with mCherry–IRE3-CL-rBcl-2 and with mCherry–IRE3-CL as a control. The cells were cultured in DMEM containing 10% (v/v) FBS for 48 h at 33°C. Neurite-bearing cells were then counted under a fluorescence microscope after immunostaining with anti-β-tubulin and anti-Bcl-2 antibodies. As shown in Figure 5(A), the percentage of neurite-bearing cells was almost 5-fold greater in the rBcl-2-overexpressing cells than in the control cells. Neurotrophic factors such as NT4/5 and BDNF also increased dramatically in the rBcl-2-overexpressing cells (Figure 5B), suggesting that expression of Bcl-2 is intimately related to neurite outgrowth.

DISCUSSION

Bcl-2 is a well-known anti-apoptotic protein preventing the release of apoptogenic factors such as cytochrome c, Smac (second mitochondrial-derived activator of caspase)/DIABOL [direct IAP (inhibitor of apoptosis)-binding protein with low PI] and Omi. Apart from this anti-apoptotic function, many reports have demonstrated that Bcl-2 controls axonal elongation in the developing brain [20] and embryonic neurons [27]. PLD is involved in various neuronal signalling pathways, and is also thought to be an important mediator of neuronal function [25].
However, little is known about the relationship between PLD activation and Bcl-2 expression in neuronal differentiation. In the present study, in order to investigate the molecular mechanism for PLD1-mediated Bcl-2 expression and the role of Bcl-2 in neuronal differentiation in response to bFGF, a conditionally immortalized rat hippocampal neuronal progenitor cell line (H19-7) was used as a model system for the differentiation of neural progenitor cells [25]. The ability of H19-7 cells to differentiate in response to bFGF resembles the behaviour of primary hippocampal cells, and bFGF may act as a differentiation factor in regions of the CNS that express the bFGF receptor, such as the hippocampus [28].

bFGF-induced Bcl-2 expression reached a maximum within 24 h. Previously, we reported that transfection of PLD1, but not PLD2, increased PLD activity in bFGF-stimulated H19-7 cells, and that this in turn elicited neurite outgrowth [25]. A PLD-knockdown study with PLD1- or PLD2-specific siRNAs showed that bFGF-induced Bcl-2 expression is coupled with PLD1 activation in H19-7 cells. We also confirmed that PLD1 regulated Bcl-2 expression on the basis of the results from overexpression of DN-PLD1 or PLD1 (Figure 1). Therefore we tried to further elucidate the relationship between PLD1 signalling and Bcl-2 expression by bFGF. PLD is a key intermediate in receptor-mediated signalling and transduces signals from several molecules, in particular Ras, Src, PLCγ, PKCα and PI3K [25, 29–31]. Consequently, some of the proteins mentioned are activated by bFGF: PLCγ (directly, or via Src or PI3K), Ras (via FRS2), PI3K (via a Ras-dependent or Ras-independent pathway) and PKC (via PLCγ activation) [6–13, 32–34]. This is also the case in H19-7 cells. The use of inhibitors allowed us to demonstrate that among these proteins, Ras, PI3K and PLCγ were required for bFGF-induced Bcl-2 expression in H19-7 cells and that PLD acts downstream of them. Even though Ras inhibition reduced PLD activity less than inhibition of PI3K or PLCγ, all of these molecules participate in bFGF-induced PLD activation (Figure 2). Our previous study [25] showed that inhibition of Ras or chelation of intracellular Ca²⁺ resulted in partial reduction of bFGF-induced PLD activity. In the present study, PLD activity was completely abolished in BAPTA/AM-treated cells. A higher concentration of BAPTA/AM (50 μM) compared with the concentration used previously (10 μM) [25] was applied to maximize the effect of Ca²⁺ chelation on PLD activity. Surprisingly, PLD activity was reduced more dramatically, indicating that intracellular Ca²⁺ is also involved in bFGF-induced PLD activation in H19-7 cells. We were also able to show, by examining translocation of the various factors from the cytosol to membranes, that bFGF activated Ras, PI3K and PLCγ respectively.

Hence, we tried to identify the downstream molecules that are regulated by bFGF-induced PLD1 activation. Previous studies have demonstrated that ERK is stimulated by PLD1 activation [35, 36] and is associated with cell survival, proliferation and differentiation [37–39]. We have also reported previously, on the basis of examining the actions of PA in HeLa cells, that...
PLD1 mediates bFGF-induced Bcl-2 expression in neurite outgrowth

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Figure 4  Effects of PLD1, JNK and the Ras/PI3K/PLCγ signalling on Ser727 phosphorylation of STAT3, and of STAT3 on Bcl-2 expression in response to bFGF

(A) H19-7 cells were grown at 33 °C in DMEM containing 10% (v/v) FBS, transfected with PLD1-siRNAs (#1 and #2) or mismatch siRNAs (#1 and #2) and then rescued with the construct encoding PLD1. The cells were cultured in N2 medium containing bFGF (20 ng/ml) at 39 °C for 15 min and proteins were assayed by Western blot using an anti-p-STAT3 (Ser727), anti-STAT3 or anti-PLD1 antibody. (B) Cells grown at 33 °C were transfected with PLD1 or DN-PLD1 (5 μg). The cells were cultured in N2 medium with bFGF (20 ng/ml) at 39 °C for 15 min. Proteins were analysed by Western blotting using an anti-p-STAT3 (Ser727), anti-STAT3 or anti-PLD1 antibody. (C) Cells were treated with 50 μM SP600125 for 30 min. The cells were cultured in N2 medium with bFGF (20 ng/ml) at 39 °C for 15 min. Proteins were analysed by Western blotting using anti-p-STAT3 (Ser727) or anti-STAT3 antibody. (D) Lysates of H19-7 cells stimulated with bFGF (20 ng/ml) at 39 °C for 15 min were precipitated with an anti-STAT3 antibody at 4 °C for 3 h. The immunoprecipitates (IP) and the crude lysates were immunoblotted with an anti-p-STAT3 (Ser727), anti-STAT3, anti-p-JNK or anti-JNK antibody. (E) Cells were treated with 20 μM manumycin A (Man A), 50 μM wortmannin (Wort), 5 μM U73122 or 50 μM BAPTA/AM (Bapta-am) for 30 min, and cultured in N2 medium containing bFGF (20 ng/ml) at 39 °C for 15 min. Proteins were analysed by Western blotting using an anti-p-STAT3 (Ser727) or anti-STAT3 antibody. (F) H19-7 cells were grown at 33 °C in DMEM containing 10% (v/v) FBS, transfected with STAT3 siRNA or mismatch siRNA (#1 and #2) and then rescued with the construct encoding STAT3. After stimulation with bFGF (20 ng/ml) at 39 °C for 15 min (p-STAT3 (Ser727)) or for 24 h (Bcl-2), proteins were analysed by Western blotting using an anti-p-STAT3 (Ser727), anti-Bcl-2 or anti-β-actin antibody. Results are shown as fold change from the basal value (without bFGF) and are means ± S.D. for three experiments. *P < 0.01, compared with bFGF-treated control. The number above each lane in (B, C and E) indicates the mean intensity for three experiments. si, siRNA.

PLD1 is a candidate enzyme for ERK activation [40]. JNK is another candidate regulated by PLD [41] and is involved in differentiation [13,42–44]. In the present study, we showed that phosphorylation of JNK and ERK increased in response to bFGF. However, PLD1 siRNAs suppressed only JNK phosphorylation, not ERK phosphorylation. Although some studies have reported that PLD has an effect on ERK phosphorylation depending on cell types and agonists [36,40], in H19-7 cells, only JNK, not ERK, was regulated by PLD1 activated by bFGF. Furthermore, Bcl-2 expression was reduced by inhibiting JNK activity (Figure 3), indicating that PLD1 regulates bFGF-induced Bcl-2 expression through JNK.

ATF2 and c-Jun were suspected as the transcription factors activated by JNK for the expression of Bcl-2 because they frequently mediate JNK signalling. However, these molecules were not involved in Bcl-2 expression induced by bFGF in H19-7 cells (results not shown). Therefore we turned our attention to STAT3, since JNK phosphorylates STAT3 on Ser727 in response to EGF [45], and STAT3 is known to act as a transcription factor for Bcl-2 expression [40,46]. Exogenous PA promotes the phosphorylation of STAT3 on Ser727 via ERK activation, and this in turn induces Bcl-2 expression in HeLa cells [40]. We found that phosphorylation of STAT3 on Ser727 was increased by bFGF and completely abolished by PLD1 siRNA or DN-PLD1. As expected, overexpression of PLD1 potentiated the phosphorylation of STAT3 compared with the control group. Interestingly, JNK directly bound to STAT3, particularly after bFGF stimulation. Inhibition of JNK also completely blocked phosphorylation of STAT3 (on Ser727) induced by bFGF (Figure 4). Furthermore, STAT3 siRNA reduced bFGF-induced Bcl-2 expression. Taken together, PLD1, as a downstream molecule of Ras/PI3K/PLCγ, mediates bFGF-induced Bcl-2 expression via the JNK/p-STAT3 (Ser727) pathway.

Finally, our results show that Bcl-2 has an important function in neurite outgrowth in response to bFGF since the overexpression of Bcl-2 increased neurite-bearing cells and the production of
Figure 5  Effects of rBcl-2 overexpression on neurite outgrowth and expression of neurotrophic factors in H19-7 cells

(A) H19-7 cells were transiently transfected with 5 μg of mCherry–IRES3-CL or mCherry–IRES3-CL-rBcl-2. Transfected cells were plated on 24-well plates at 1.5 × 10^4 cells/well and cultured in DMEM containing 10% (v/v) FBS at 33°C for 48 h. After immunostaining with antibody against Bcl-2 or β-tubulin (magnification, ×200; scale bars, 20 μm) (left-hand panel), the cells bearing neurites were counted under a fluorescence microscope. Results in the right-hand panel are the percentage increase in numbers of cells with growing neurites. Cells were selected from random areas of at least three cultures. (B) After transfection with mCherry–IRES3-CL or mCherry–IRES3-CL-rBcl-2, the cells were cultured in DMEM containing 10% (v/v) FBS at 33°C for 48 h. mRNA was extracted from the cells using TRIzol® reagent. The expression levels of Bcl-2, NT4/5, BDNF or β-actin were determined by RT–PCR using specific primers and by Western blotting using an anti-BDNF, anti-NT4/5 or anti-β-actin antibody.

Figure 6  Signalling model of PLD1-mediated Bcl-2 expression and neurite outgrowth in response to bFGF

Our model suggests that PLD1 mediates bFGF-induced activation of Ras/PI3K/PLCγ signalling to Bcl-2 expression via JNK-dependent STAT3 phosphorylation in H19-7 cells. Increased Bcl-2 expression promotes neurite outgrowth by inducing NT4/5 and BDNF expression.
neurotrophic factors such as BDNF and NT4/5 (Figure 5). The findings of the present study are summarized in Figure 6.

AUTHOR CONTRIBUTION

Sung Nyo Yoon essentially designed and performed the experiments, and analysed and interpreted the data. Kang Sik Kim and Ju Hwan Cho performed the experiments and analysed data. Hye-Jin Choi assisted with the experiments. Sung-Joon Kwon contributed reagents, materials and analytical tools. Joong-Soo Han devised the overall project, interpreted the data and wrote the paper.

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REFERENCES


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