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Lipase Inactive Mutant of PLC-γ1 Regulates NGF-induced Neurite Outgrowth Via Enzymatic Activity and Regulation of Cell Cycle Regulatory Proteins

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Src homology (SH) domains of phospholipase C-γ1 (PLC-γ1) impair NGF-mediated PC12 cells differentiation. However, whether the enzymatic activity is also implicated in this process remains elusive. Here, we report that the enzymatic activity of phospholipase C-γ1 (PLC-γ1) is at least partially involved to the blockage of neuronal differentiation via an abrogation of MAPK activation, as well as sustained Akt activation. By contrast, Overexpression of WT-PLC-γ1 exhibited sustained NGF-induced MAPK activation, and triggered transient Akt activation resulting in profound inhibition of neurite outgrowth. However, lipase-inactive mutant (LIM) PLC-γ1 cells fail to suppress neurite outgrowth, although it contains intact SH domains, specifically enhancing the expression of cyclin D1 and p21 proteins, which regulate the function of retinoblastoma Rb protein. These observations show that the lipase inactive mutant of PLC-γ1 does not alter NGF-induced neuronal differentiation via enzymatic inability and the modulation of cell cycle regulatory proteins independent on SH3 domain.

Keywords: Differentiation, Nerve growth factor, Phospholipase C-γ1, PC12 cell

Introduction

NGF stimulation induces PC12 cells differentiation into a sympathetic neuron-like phenotype, and this effect is regulated by the sustained activation mitogen-activated protein kinase (MAPK) (Gomez *et al.*, 1990; Gotoh *et al.*, 1990; Traverse *et al.*, 1992), coincident with cell cycle arrest (Unsicjker *et al.*, 1978; Anderson *et al.*, 1986) and changes in the expression of cell cycle machinery proteins, including cyclin D1, a γ1 phase cyclin (Buchkovich and Ziff, 1994; Yan and Ziff, 1995) , and a p21 WAF1/CIP1/Sdi1 (p21), a cyclin-dependent kinase (Cdk) inhibitor (Yan and Ziff, 1995). Cyclin D1 forms complex with Cdk4 and Cdk6, which phosphorylate the retinoblastoma protein Rb, failing to repress cell proliferation. The p21 protein prevents the Cdk4/D1 and Cdk6/D1 complexes and preserves the hypophosphorylated state of Rb that prevents S phase entry. Thus, the relative expression of p21 and cyclinD1 may contribute cell differentiation.

Phospholipase C-γ1 (PLC-γ1) performs a pivotal role in both cell proliferation and differentiation. In mice, PLC-γ1 is crucial to embryonic development (Ji *et al.*, 1997). Microinjection of PLC-γ1 evokes DNA synthesis in quiescent NIH 3T3 cells, and Ras-induced DNA synthesis in NIH 3T3 cells (Smith *et al.*, 1989; Smith *et al.*, 1990). However, this mitogenic activity is independent of its phospholipase activity, but requires its SH3 domain (Smith *et al.*, 1994; Huang *et al.*, 1995). In addition, our previous study demonstrated that the enzymatic activity of PLC-γ1 regulates MAPK signaling (Rong *et al.*, 2003). Previous studies show that both Shc-mediated Ras-dependent and PLC-γ1-dependent pathways are essential for the NGF-induced differentiation of PC12 cells. Recent studies have demonstrated that the Src homology domain of PLC-γ1 inhibits the NGF-induced differentiation of PC12 cells (Bae *et al.*, 1998). Very recently, we found that overexpression of PLC-γ1 inhibits NGF-induced neurite outgrowth by proliferative activity of SH3 domain (Truong *et al.*, 2007). Despite LIM-PLC-γ1 possess whole intact SH2 and SH3 domains, lipase-inactive point mutant of PLC-γ1 is able to bare normal neurite outgrowth under NGF treatment (Truong *et al.*, 2007). However, none of these studies has explicitly defined the role of the phospholipase activity of PLC-γ1 in neuronal differentiation and how LIM induce normal neurite outgrowth with intact SH3 domain. Utilizing cells stably transfected with

Abbreviations: NGF, nerve growth factor; PLC-γ1, phospholipase C -γ1; LIM, lipase inactive mutant PLC-γ1

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an inducible form of wild-type and lipase-inactive mutant (H335Q) PLC-γ1, we demonstrate here that the lipase-inactive mutant of PLC-γ1 contribute neurite outgrowth trough regulating cell cycle regulatory proteins and an alteration of the balance between MAPK activation and Akt activation by its enzymatic inability.

Materials and Methods

Chemicals. Mouse monoclonal anti-HA and anti-Flag antibodies were purchased from Sigma. Antibodies that recognize Akt, MAPK/Erk (P44/42), Retinoblastoma (Rb) protein, and the phosphorylated forms of Akt (Ser473), MAPK (p44/42), and Rb were obtained from Cell Signaling. The anti-PCNA was purchased from Oncogene. Anti-cyclin D1 (DCS-6) was acquired from Santa Cruz. The anti-tubulin was purchased from Molecular Probes. NGF was purchased from Roche Diagnostics. All chemicals not included above were obtained from Sigma.

Cell cultures. PC12 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 5% horse serum, and 100 units penicillin-streptomycin at 37°C, under a 5% CO_2 atmosphere in a humidified incubator. The Flag-tagged WT-PLC-γ1 stably transfected PC12 cells (Tet-off cell line) were kindly provided by Dr. P. G.. Suh (Pohang University of Science and Technology, Korea) and the LIM-PLC-γ1 cells were graciously supplied by Dr. K. Ye (Emory Univ, USA). These transfected genes were grown in PC12 cell culture medium supplemented with 100 μg/ml G418, 100 μg/ml hygromycin B and 2 μg/ml tetracyclin. PLC-γ1 was induced for 24 h in tetracycline free medium.

Assay for phospholipase activity. *In vivo* PLC-γ1 activity assay was conducted as described by Rong *et al.* (2003). The induced and non-induced PLC-γ1 cells were labeled with myo-[2-^{3}H]inositol (2 μlCi/mL; PerkinElmer Life Sciences) in inositol-free medium for 24 h and were pretreated for 15 min with 20 mM LiCl, and then stimulated with NGF for 10 min. The reaction was terminated via the addition of 0.6 ml ice-cold 5% $HClO_4$. After 30 min on ice, the extract was eluted through a Bio-Rad Dowex AG 1-X8 anion exchange column. Total inositol phosphate was eluted with a solution containing 1 M ammonium formate and 0.1 M formic acid, and quantified via liquid scintillation counting.

Phase contrast microscopy and measurements of neurite outgrowth. Cells (1×10^4) treated for 5 days with 50 ng/ml of NGF in induced or non-induced condition. The cells were photographed and scored using an Axiovert 100 microscope (Carl Zeiss). Neuritic processes in excess of two cell bodies in length were counted as neurites, and a minimum of 5 random fields were photographed in each case. The lengths of the neurites were measured using the Axio Vision program.

Immunofluorescent staining. Cells were plated onto cover slips and induced in tetracycline free medium. Cells were fixed for 15 min with 10% formalin and treated for 15 min with permeabilizing solution (0.5% Tryton X-100 in 1X PBS). After primary antibody reaction for 1 h at room temperature, they were stained with the fluorescent dye-conjugated antibody, Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, 1 : 1000). DAPI was introduced 7 min.

Results

To determine the effects of the phospholipase activity of PLC-γ1 in NGF-induced neuronal differentiation, we employed tet-off PC12 cells which had been stably transfected with the inducible form of Flag-tagged wild-type (WT) and LIM-PLC-γ1, which was generated with a point mutation in His335 to Gln335 of PLC-γ1. The expression of Flag-tagged PLC-γ1 and endogenous PLC-γ1 was verified via culturing in tetracycline free medium for 24 h (Fig. 1A). We confirmed that the inducible LIM-PLC-γ1 could inhibit phospholipase activity (Fig. 1B). Upon 5 days of NGF treatment, almost 50% of the PC12 cells fully extended neurites. However, consistent with the findings of a previous report (Bae *et al.*, 1998; Truong *et al.*, 2007), WT-PLC-γ1 PC12 cells evidently inhibited neuritic outgrowth. Conversely, LIM-PLC-γ1 cells exhibited substantial neuritic extension, to a degree commensurate with the differentiation of naïve PC12 cells (Fig. 1C). Moreover, the neurite outgrowths in the WT-PLC-γ1 cells extended only short distances from the cell body, whereas the naïve PC12 cells and LIM-PLC-γ1 showed similar patterns of neurite growth, which were extended significantly, exhibiting neuritis of up to twice the previous length by day 5 of NGF treatment (Table 1). These observations, coupled with the observed diminished phospholipase activity in the LIM-PLC-γ1 cells, suggest that the phospholipase activity of PLC-γ1 is at least partially involved to inhibit the neuritic outgrowth of PC12 cells.

In order to evaluate further the effects of the LIM of PLC-γ1 in NGF-induced differentiation of PC12 cells, we evaluated the relative contribution of MAPK (Erk1 and Erk2) and Akt, the major intracellular signaling pathways involved in neuronal differentiation and proliferation. Although compelling evidence exists to suggest that the differentiation of PC12 cells is regulated by sustained MAPK activation, WT-PLC-γ1 cells evidenced more prominent MAPK activation than control PC12 cells. NGF treatment in these cells elicited further stimulation and prolonged activation. On the other hand, PI3K signaling was found to be intact in these cells, as compared with the control PC12 cells, as was shown by the observed Akt phosphorylation. By contrast, MAPK activity is diminished in the LIM-PLC-γ1 cells (Fig. 2A). However, phosphoinositide-3-kianse signaling remained the same in these cells, which was confirmed by Akt phosphorylation (Fig. 2B). Equal amounts of Flag-tagged PLC-γ1 proteins were expressed (Fig. 2C). Quantitative analysis of p-ERK1/2 and p-Akt were demonstrated in Fig. 2D and 2E respectively. Our previous report (Rong *et al.*, 2003) also indicated that MAPK signaling is abrogated in LIM-PLC-γ1 cells. These findings show that

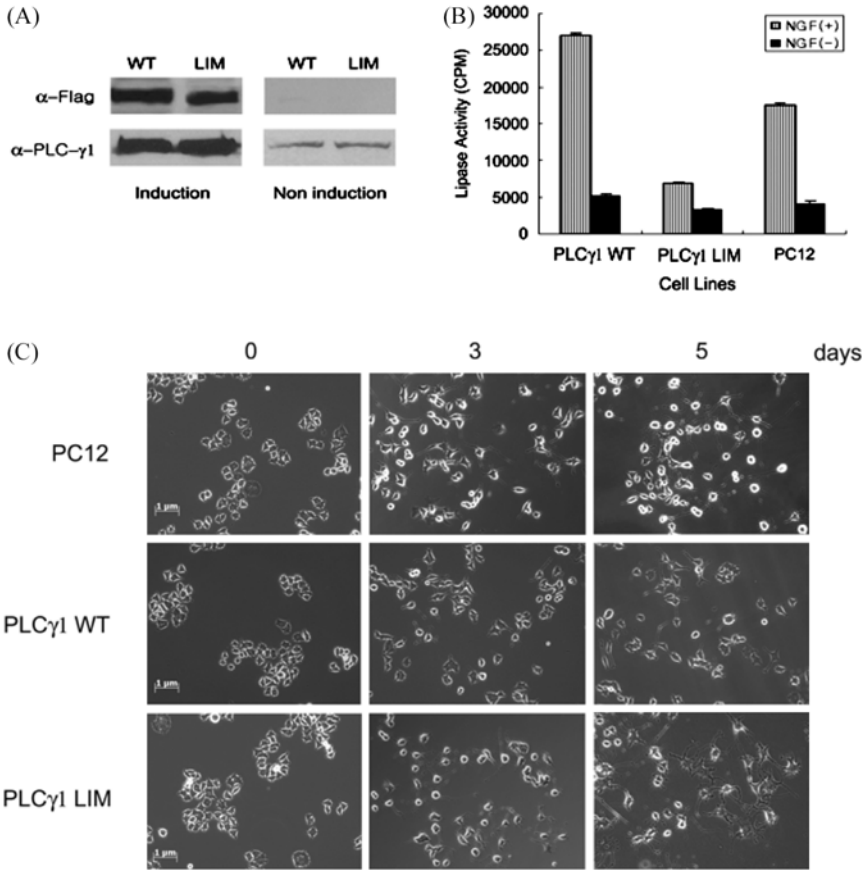

Fig. 1. Expression of Flag-PLC-γ1s and their effect in NGF-induced differentiation. (A) Immunoblotting analysis of Flag-PLC-γ1 in induced and uninduced PC12 cells with anti-Flag antibody. (B) Phospholipase activity assay. H335Q mutation abolished NGF-induced phospholipase activity of PLC-γ in stably transfected PC12 cells. Bars represent original counts per minute (CPM) values from three individual experiments. *$p < 0.01$ (C) Phase-contrast photomicroscopy. Photographs were acquired under a confocal microscope (×200) in random fields on the indicated day. Bar = 1 μm.

the longevity of MAPK activation might be insufficient to induce neurite extension from WT-PLC-γ1 cells. On the other hand, the abrogation of MAPK activation might not affect NGF-induced neurite outgrowth in LIM-PLC-γ1 cells. This, in turn, indicates that the differential activation of MAPK and Akt in the PLC-γ1 cells may contribute in some way to NGF-elicited neuronal differentiation.

NGF-induced differentiation of PC12 cells is link to alterations in the disposition of a variety of cell cycle-associated proteins (Bravo and Macdonald-Bravo, 1987; Peter and Herskowitz, 1994; Dobashi *et al.*, 1995). Multiple studies have shown that NGF does not block cell proliferation, but rather, retards it. NGF treatment resulted in a reduction of the proliferating cell nuclear antigen (PCNA), a molecular marker of S phase cells (Bravo and Macdonald-Bravo, 1987). Conversely, cyclin D1, γ1 phase specific cyclin protein (Buchkovich and Ziff, 1994; Yan and Ziff, 1995; Dobashi *et al.*, 1995) and p21, a negative regulator of the γ1 phase, are all greatly increased upon NGF treatment (Peter and Herskowitz, 1994). Moreover, NGF represses the phosphorylation level of Rb protein, which functions as a negative regulator of cell proliferation (Yan and Ziff, 1995). To demonstrate the effects of the phospholipase activity on the expression of cell cycle regulatory proteins, we monitored the protein levels in WT- and LIM-PLC-γ1 stable cell lines after NGF induction. Cyclin D1 expression was substantially diminished in WT-PLC-γ1 cells, but both the control PC12 cells and LIM-PLC-γ1 cells showed elevated expression of cyclin D1, affecting PCNA expression level (Fig. 3A). In addition, p21 protein levels were only slightly reduced in WT-PLC-γ1 cells, but were markedly increased in both the control and LIM-PLC-γ1 cells

Table 1. The length of neurite outgrowth

Cell lines	Length of neurite outgrowth (mm)
PC12	1.60 ± 0.13
PLC-γ1 WT	0.73 ± 0.06
PLC-γ1 LIM	1.67 ± 0.10

The data represent the average of a minimum of 200 cells and are expressed as means ± SD values of triplicate determinations from two experiments each ($p < 0.005$ by Student's t test).

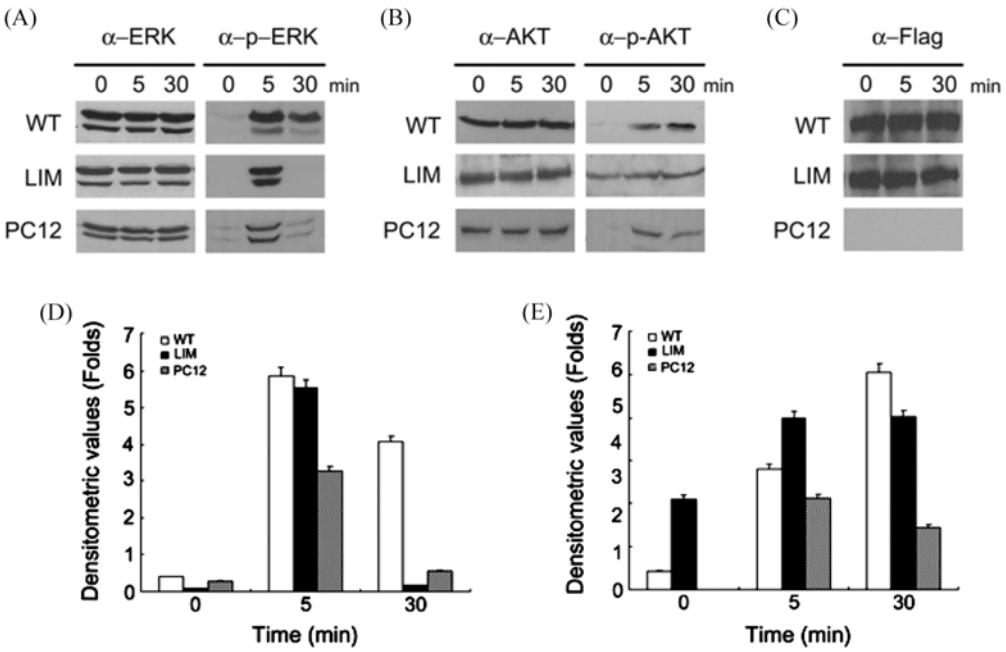

Fig. 2. MAPK activation but not Akt activation is abrogated in LIM-PLC-γ1 cells. Cells were cultured without tetracycline for 24 h before 100 ng/ml NGF was added and analyzed with phosphor-Erk (A) or phospho Akt antibody (B). (C) Similar protein induction was verified using anti-flag antibody. (D, E) The densitometric measurement of p-ERK1/2 and p-Akt was presented at bar graphs.

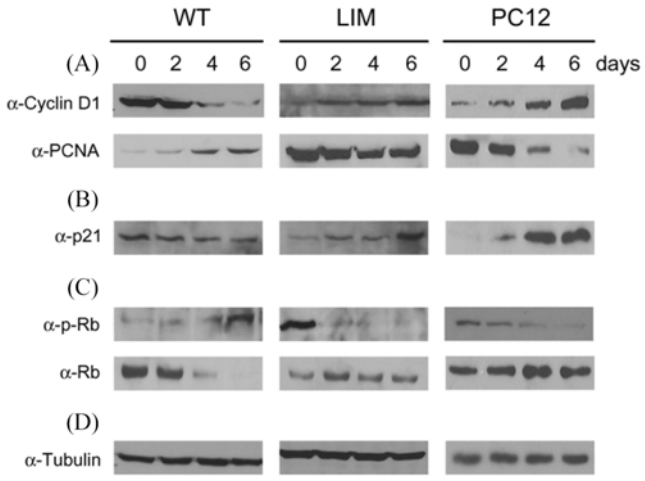

Fig. 3. Differential modulation of cell cycle regulatory proteins in PLC-γ1 cell lines. (A) Cells were cultured for 6 days with 100 ng/ml NGF, and harvested on the indicated day. The cell lysates were subjected to immunoblot analysis using anti-cyclin D1 antibody (upper panel) and anti-PCNA antibody (bottom panel), (B) anti-p21 antibody, (C) anti-phospho-Rb antibody and anti-Rb (unphosphorylated) antibody (D) Similar protein quantities were verified using anti-tubulin antibody.

(Fig. 3B). By contrast, phosphorylated Rb appeared in both control and LIM-PLC-γ1 NGF-untreated cells, but not the NGF-treated those cells. However, the WT-PLC-γ1 cells exhibited gradual elevations in the levels of phosphorylated Rb with NGF treatment (Fig. 3C). Similar quantities of proteins were employed in our western blotting analyses (Fig. 3D). Collectively, these data indicate that WT-PLC-γ1 and LIM-PLC-γ1 cells distinctively affect both the cell cycle and cell differentiation.

Previous studies have revealed that NGF exposure elicits the translocation of PLC-γ1 to the nucleus in both native PC12 cells and WT-PLC-γ1-treated cells (Ye *et al.*, 2002). To determine whether deficiencies in phospholipase activity affect the translocation process, we conducted immunofluorescent staining, using the anti-Flag antibody. Nuclear translocation was confirmed via DAPI staining. WT-PLC-γ1 evidenced significant degree of nuclear translocation in response to 10 min of NGF stimulation (data not shown), and manifested nearly 90% nuclear translocation after 30 min of NGF stimulation (Fig. 4). However, LIM-PLC-γ1 exhibited delayed nuclear translocation. We detected only minimal nuclear translocation of LIM-PLC-γ1 after 10 min of NGF treatment, although we observed an improved nuclear translocation, which was not exceeded 50% after 30 min of NGF stimulation. These results indicate that the phospholipase activity of PLC-γ1 may contribute to the early nuclear translocation of PLC-γ1, thereby negatively regulating neurite outgrowth.

Discussion

Previous study have shown that overexpression of PLC-γ1 suppresses NGF-induced PC 12 cell differentiation (Bae *et al.*, 1998), which proposed that the enzymatic activity of PLC-γ1 is not required for the inhibitory effect on PC12 cell

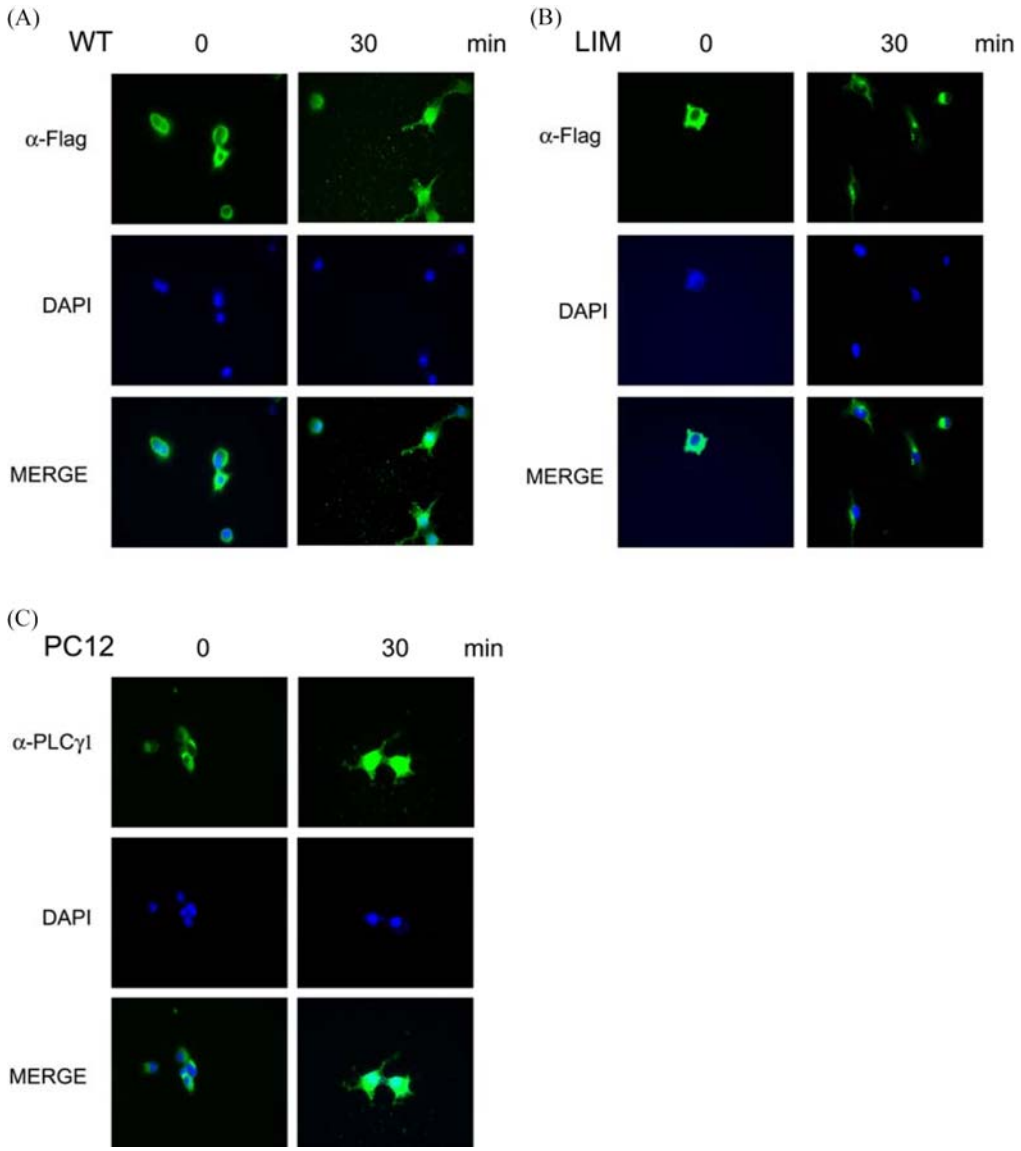

Fig. 4. NGF-mediated nuclear translocation is delayed in LIM-PLC-γ1 cells. After adding 100 ng/ml NGF, primary Flag antibody (1 : 1000), fluorescent dye-conjugated antibody Alexa Fluor 488 goat anti-mouse IgG, and DAPI were employed in the immunolocalization experiments. (a) WT-Flag-PLC-γ1 cells were translocated to the nucleus within 30 min of NGF. (b) LIM-Flag-PLC-γ1 cells exhibited delayed nuclear translocation. (c) Nuclear translocation of endogenous PLC-γ1 in naïve PC12 cell line confirmed with anti-PLC-γ1 antibody upon NGF treatment.

differentiation and we demonstrated that the mechanism governing the inhibition of NGF-mediated differentiation in PC12 cells by PLC-γ1 is primarily via proliferative activity of SH3 domain. In this report, we show that the phospholipase activity of PLC-γ1 is involved in NGF-induced differentiation in PC12 cells, although it is not primarily factor for differentiation. The induction of stable inducible lipase-inactive PLC-γ1 (H335Q) evidently induces a recurrence of neuritic outgrowth as compared with the wild-type PLC-γ1-transfected cells (Fig. 1C and Table 1).

Treatment of PC12 cells with fibroblast growth factor or NGF results in neurite outgrowth and the cessation of cell division (Green and Tischler, 1976), thereby inducing transient and sustained activation of MAPK. However, we were unable to observe any defects in MAPK activation in WT-PLC-γ1-treated cells (Fig. 2). Moreover, we observed abrogated MAPK activation in the LIM-PLC-γ1 cells. Nevertheless, Akt activation remained intact in both PLC-γ1 cell lines. These data showed that the activation of MAPK probably stimulates proliferation rather than differentiation in these PLC-γ1 cell lines, and it is not absolutely required for NGF-mediated morphological differentiation under these conditions, indicating that lipase activity is likely necessary for the inhibitory effects on the neurite outgrowth of PC12 cells.

As compared with control PC12 cells, cells expressing WT-PLC-γ1 are enriched in the S phase, whereas LIM-PLC-γ1 accumulate in cells in the G2 phase (data not shown). In addition, WT-PLC-γ1 and LIM-PLC-γ1 trigger different expression profiles of cell cycle regulatory proteins (Fig. 3). Overexpression of LIM-PLC-γ1 selectively activates p21 and cyclin D1. Cyclin D1 and p21 exert opposing effects on the cell cycle. Whereas cyclin D1 facilitates γ1 phase transition via the phosphorylation of the retinoblastoma protein, Rb (Sherr, 1995), the p21 protein maintains the hyopophosphorylated state of Rb which blocks S phase entry. In the present study, the pRb protein was found to be abundantly expressed in LIM-PLC-γ1 cells in the absence of NGF. The complete decline of the phosphorylated Rb level occurs after 2 days of NGF treatment. The hypophosphorylation of Rb by p21 may result in a barrier to PC12 cell proliferation, thereby resulting in differentiation. By contrast, we failed to detect any phosphorylated Rb in WT-PLC-γ1 cells at 4 days of NGF treatment. Instead, we detected phosphorylated Rb only after 6 days of NGF treatment. Presumably the phospholipase activity of PLC-γ1 modulates the effects of cyclin D1 and p21 during neuronal differentiation to modulate Rb phosphorylation, thereby inducing the regulation of gene expression for cell proliferation.

One of the more interesting aspects of the PLC-γ1 cell lines is that LIM-PLC-γ1, but not WT-PLC-γ1, delayed nuclear translocation via NGF exposure. Previous studies have shown that PLC-γ1 is crucial to cellular proliferation, and its mutant deficient in lipase activity also induces DNA synthesis, implying that the phospholipase activity of PLC-γ1 is not responsible for its mitogenic effect. In particular circumstances, the early nuclear translocation of PLC-γ1 may inhibit differentiation, and its lipase activity may contribute to its nuclear translocation during the initial stages of NGF treatment. Taken together, our findings show that the overexpression of PLC-γ1 mediates its cell differentiation-inhibitory function, via its enzymatic lipase activity.

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