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Phosphorylation of glycogen synthase kinase-3β at serine-9 by phospholipase Cγ1 through protein kinase C in rat 3Y1 fibroblasts

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Abbreviations: EGF, epidermal growth factor, GSK-3β, glycogen synthase kinase 3-β; PKC, protein kinase C; PLCγ1, phospholipase Cγ1

Abstract

Phospholipase Cγ1 (PLCγ1) plays an important role in controlling cellular proliferation and differentiation. PLCγ1 is overexpressed in some tumors, and its overexpression induces solid tumors in nude mice. However, the regulatory mechanisms underlying PLCγ1-induced cell proliferation are not fully understood. Here we show that overexpression of PLCγ1 highly phosphorylated glycogen synthase kinase-3β (GSK-3β) at serine-9 in 3Y1 fibroblasts. Inhibition of protein kinase C (PKC)s with GF109203X abrogated GSK-3β phosphorylation by PLCγ1. We also found that steady-state level of cyclin D1 protein, but not cyclin D1 mRNA, was highly elevated in response to serum stimulation in PLCγ1-transfected cells as compared with vector-transfected cells. Since GSK-3β is involved in cyclin D1 proteolysis in response to mitogenic stimulation, PLCγ1-mediated GSK-3β phosphorylation may function as a regulation of cyclin D1 accumulation in PLCγ1-overexpressing cells.

Keywords: cyclin D1; fibroblasts; glycogen synthase kinase 3; phospholipase C; protein kinase C

Introduction

The hydrolysis of membrane phospholipid, phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$), by phospholipase C (PLC) is one of the earliest key events in the regulation of various cell functions (Rhee and Bae, 1997). To date four types (β, γ, δ, and ε) and 11 PLC isozymes (β1-4, γ1-2, δ1-4, ε) are identified (Rhee and Bae, 1997). Among them, PLCγ1 isozyme is a direct substrate of the certain receptor tyrosine kinases such as epidermal growth factor (EGF) receptor and PDGF receptor (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Nishibe *et al.*, 1989; Wahl *et al.*, 1989) and may play critical roles in cell proliferation and differentiation (Smith *et al.*, 1989; Smith *et al.*, 1990; Valius and Kazlauskas, 1993; Roche *et al.*, 1996; Bae *et al.*, 1998; Kim *et al.*, 2000). Several papers have reported that PLCγ1 is overexpressed in some human hyperproliferative tissues, including breast carcinoma (Arteaga *et al.*, 1991), human skin under hyperproliferative conditions (Nanney *et al.*, 1992), colorectal carcinoma (Noh *et al.*, 1994; Lee *et al.*, 1995), familial adenomatous polyposis (Park *et al.*, 1994), and highly metastatic colorectal tumor cell lines (Yeatman *et al.*, 1994). Overexpression of PLCγ1 shows anchorage- independent growth in soft agar and induces tumors after injection into nude mice (Chang *et al.*, 1997; Smith *et al*, 1998). Recently, we have found that overexpression of PLCγ1 down-regulates the transcription factor *Egr-1* gene (Shin *et al.*, 2002), which plays a key regulatory role in cell growth, differentiation, and development (Cao *et al.*, 1992; Gashler and Sukhatme, 1995), and functions as a tumor suppressor (Huang *et al.*, 1994). These results suggest that PLCγ1 is a necessary component for mitogenic signaling pathway and that abnormal expression of PLCγ1 may be associated with tumor development in some tumors. However, it is not fully understood how PLCγ1 regulates cell proliferation.

Glycogen synthase kinase-3β (GSK-3β) is a protein kinase that regulates multiple biological processes,

including body patterning during embryonic development, protein synthesis, cell adhesion, cell proliferation, cell differentiation, and cell death (reviewed in Frame and Cohen, 2001). In resting state, GSK-3β is active and negatively regulates some transcription factors including c-Jun (Nikolakaki *et al.*, 1993), and NFAT (Beals *et al.*, 1997). GSK-3β also phosphorylates cyclin D1 at threonine-286 which is required for nuclear export and ubiquitin-dependent degradation in the cytoplasm (Diehl *et al.*, 1998). Upon growth factor stimulation such as insulin or EGF, GSK-3β is phosphorylated at N-terminal serine- 9 residue by protein kinase B (PKB, also called Akt) (Cross *et al.*, 1995), leading to inhibition of its kinase activity. GSK-3β is also a negative regulatory component of Wnt-dependent signaling. In Wnt signaling, control of β-catenin accumulation is critical for regulation of Wnt-dependent transcriptional activation. In the resting state, GSK-3β phosphorylates β-catenin, leading to subsequent degradation *via* the proteasome- dependent pathway (Aberle *et al.*, 1997; Ikeda *et al.*, 1998). When cells are activated by Wnt ligands, GSK-3β activity is inhibited through Dishevelled protein (Kishida *et al.*, 1999; Miller *et al.*, 1999; Smalley *et al.*, 1999). This leads to stabilization and accumulation of cytosolic β-catenin. The accumulated β-catenin interacts with Tcf/Lef family of transcription factors and translocates to nucleus to activate Wnt target genes, including c-*myc* and cyclin D1 (He *et al.*, 1998; Tetsu and McCormick, 1999).

In this study, we investigated whether PLCγ1 regulates the phosphorylation of GSK-3b in 3Y1 fibroblasts.

Materials and Methods

Materials and cell culture

Low-glucose Dulbecco's modified Eagle medium (L-DMEM) and fetal calf serum were purchased from Life Technologies (Gaithersburg, MD). GF109203X and EGF were obtained from Calbiochem (San Diego, CA). Antibodies against β- catenin, p42/p44 MAPK, phospho-p42/p44 MAPK (Thr202/Tyr204), phospho-GSK-3β (Ser9) were purchased from New England BioLabs (Beverly, MA). Antibodies against cyclin D1 was from Santa Cruz Biotechnology (Santa Cruz, CA). pCMV/flag-PLCγ1 plasmid, monoclonal anti-PLCγ1 antibody (F7 clone) and 3Y1 transfectants were gifts from Dr. Pann-Ghill Suh (Pohang University of Science and Technology, Pohang, Korea). 3Y1 rat fibroblast transfected with PLCγ1 cDNA (PLCγ1-3Y1) or vector only (Vector-3Y1) were maintained in L-DMEM supplemented with 10% fetal calf serum and 100 μg/ml hygromycin B as described previously (Chang *et al.*, 1997). Cells were starved by culturing in L-DMEM containing 0.5% serum for 24 h before stimulation with 20% serum or 100 ng/ml EGF.

Western blot analysis

Cells were lysed in 20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/ml leupeptin, and 1mM PMSF. Protein samples (20 μg of each) were electrophoresed in 10% SDS-polyacrylamide gel and transferred to nitrocellulose filters. Blots were incubated with primary antibodies and developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech., Piscataway, NJ). In some cases, the same blot was stripped by incubating in 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol for 30 min at 50°C, and reprobed with anti-Erk1/2 (1:2000) antibody for use as an internal control.

Northern blot analysis

Total RNA was isolated as described previously (Lee and Seo, 2002). Isolated total RNA (5-10 μg) was separated on 1.2% agarose gel containing 6% formaldehyde in 0.02 M Mops, pH 7.0, 8 mM sodium acetate, and 1 mM EDTA, then transferred to Hybond N^+ nylon membrane (Amersham Pharmacia Biotech., Piscataway, NJ) by the capillary method. Cross-linking was then performed by UV irradiation. cyclin D1 and GAPDH cDNAs were labeled with [α-^{32}P]dCTP (6,000 Ci/mmol, DuPont NEN) by standard random primer method. After prehybridization, blots were hybridized overnight at 42°C in Northern-Max hybridization solution (Ambion, Austin, TX). Blots were then washed with 2 SSC/0.1% SDS for 20 min at room temperature, 2 SSC/0.1% SDS at 42°C for 30 min, and 0.5 SSC/0.1% SDS for 30 min at 52°C. For rehybridization, the probes were stripped from the membrane by boiling in 0.1 SSC/0.5% SDS.

Tcf/Lef-1-dependent transcription assay

The pTOPflash reporter plasmid (Korinek *et al.*, 1998), which contains three copies of consensus Tcf/Lef-1-binding sites was purchased from Upstate Biotechnology (Lake Placid, NY). pTOPflash reporter (50 ng-1 μg) was transfected into HEK293T cells without or with expression plasmids (pCMV/flag-PLCγ1, pQNCII/flag-Dvl-1) using LipofectAMINE 2000 reagents according to manufacturer's instruction. pCMV/β-gal plasmid (100 ng) was included all the samples to monitor the transfection efficiency. At 24 h after transfection, cells were harvested and protein extracts were prepared by three cycles of freezing and thawing. One to two μg of protein was assayed for luciferase activity. Luminescence was measured using a luminometer model TD 2020 (Berthold, Tubingen, Germany). Trans-

fection efficiencies were normalized by a ratio of luciferase activity to β-galactosidase activity obtained from the same sample.

Results and Discussion

Overexpression of PLCγ1 enhances GSK-3β phosphorylation at serine-9 in response to mitogenic stimulation

Previous studies have demonstrated that GSK-3β is involved in the control of cell growth in response to growth factors stimulation (Diehl *et al.,* 1998; Alt *et al.,* 2000; Sears *et al.,* 2000). As PLCγ1 is overexpressed in some human cancer tissues, including colon and breast cancers (Yeatman *et al.,* 1994; Lee *et al.,* 1995), and has been implicated mitogenic signaling, we wondered if PLCγ1 is involved in mitogen-induced phosphorylation of GSK-3β. To determine the effect of PLCγ1 on GSK-3β phosphorylation, we used PLCγ1-transfected 3Y1 fibroblast cell lines (Chang *et al.*, 1997). Quiescent, serum-starved, vector-transfected 3Y1 cells (Vector-3Y1) and PLCγ1-transfected 3Y1 cells (PLCγ1-3Y1) were treated with 20% serum, and GSK-3β phosphorylation was assessed by Western blotting using anti-phospho-GSK-3β (Ser 9) antibody. Treatment with serum-induced time-dependent phosphorylation of GSK-3β at serine-9 in both transfectants. Increased phosphorylation of GSK-3β was evident at 30 min, after which the level of phosphorylation gradually reduced in Vector-3Y1 cells (Figure 1A). Basal level of GSK-3β phosphorylation was approximately 7-fold higher in PLCγ1-3Y1 cells compared with Vector-3Y1 cells. In PLCγ1-3Y1 cells, serum-induced increase of GSK-3β phosphorylation was occurred as early as 15 min and was sustained for more than 120 min after serum stimulation (Figure 1A). During this time course, notable increases in GSK-3β phosphorylation were observed in PLCγ1-3Y1 cells compared with Vector-3Y1 cells. When the serum-starved cells were treated with EGF for 30 min, phosphorylation of GSK-3β at serine-9 also strongly induced in PLCγ1-overexpressed cells as compared to Vector-3Y1 cells (Figure 1B). These data suggest that PLCγ1 may contribute to the elevation of mitogen-induced GSK-3β phosphorylation at serine-9.

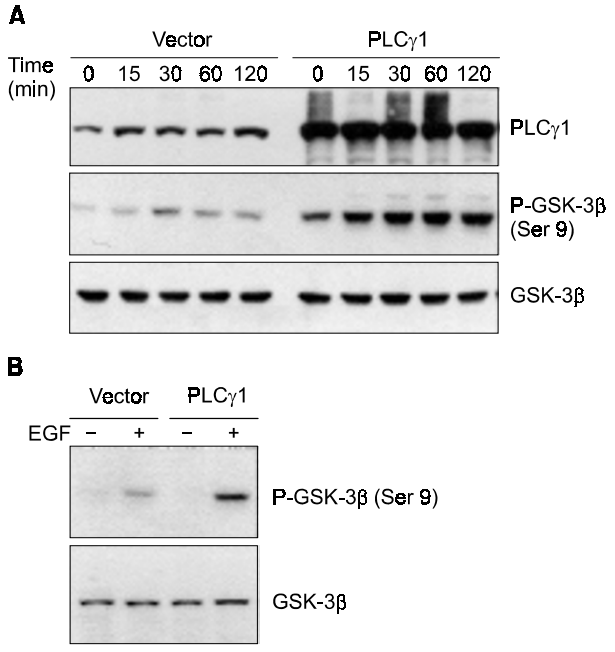

Figure 1. Overexpression of PLCγ1 enhances GSK-3β phosphorylation in response to mitogenic stimulation. (A) Effect of serum on serum-induced GSK-3β phosphorylation at serine-9. Vector-3Y1 cells (Vector) and PLCγ1-3Y1 cells (PLCγ1) were serum-starved with 0.5% for 24 h, and then treated with 20% serum for indicated times. (B) Effect of EGF on serum-induced GSK-3β phosphorylation at serine-9. Vector-3Y1 cells (Vector) and PLCγ1-3Y1 cells (PLCγ1) were serum-starved with 0.5% for 24 h, and then treated with 100 ng/ml EGF for 30 min. Cell extracts were prepared, and subjected to Western blotting with anti-PLCγ1, anti-phospho-GSK-3β (Ser 9), or anti-GSK-3β antibodies. Data are representative of two independent experiments.

Inhibition of PKC activity abrogates EGF-induced GSK-3β phosphorylation

It is well established that PLCγ1 is activated and hydrolyzes phosphatidyl inositol 4,5-bisphosphate ($PtdInsP_2$) to produce the two second messengers, *sn*-1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$) upon growth factors stimulation. DAG activates protein kinase C (PKC), whereas $InsP_3$

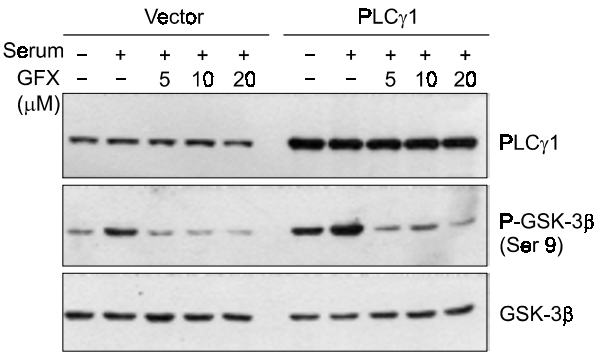

Figure 2. Inhibition of PKC abrogates serum-induced GSK-3β phosphorylation in 3Y1 cells. Vector-3Y1 cells (Vector) and PLCγ1-3Y1 cells (PLCγ1) were serum-starved with 0.5% for 24 h, and then treated with the indicated concentrations of GF109203X before 30 min of exposure to 20% serum. After an additional incubation for 30 min, cells were harvested and whole cell lysates were prepared for Western blotting with anti-PLCγ1 (top), anti-phospho-GSK-3β (Ser 9) (middle), or anti-GSK-3β (bottom) antibodies.

stimulates the release of Ca^{2+} from internal stores (Nishizuka, 1984). To determine whether PKC activity is required for PLCγ1-mediated increase of GSK-3β phosphorylation, we examined the effect of GF109203X, a selective inhibitor of PKCs (Toulle *et al.*, 1991), on the serum-induced GSK-3β phosphorylation. As shown in Figure 2, pre-incubation of GF109203X abrogated the serum-induced GSK-3β phosphorylation at serine-9 in a dose-dependent manner in both cell lines. This result suggests that PKC activity is necessary for PLCγ1-mediated GSK-3β phosphorylation. This conclusion was further supported by experiments demonstrating that down-regulation of PKC by long-term treatment with 1 μM phorbol 12-myristate 13-acetate (PMA) for 24 h failed to induce the GSK-3β phosphorylation (data not shown).

PLCγ1-mediated GSK-3β phosphorylation is not linked to Wnt signaling

Wnt signaling plays an important role in embryonic development (Cardigan, 1997) and deregulation of Wnt signaling considered as a significant factor causing cancer development (Bienz and Clevers, 2000). Control of β-catenin stability is central to Wnt signal pathway. Upon stimulation by Wnt signaling, the activity of GSK-3β is inhibited, leading to stabilization and accumulation of β-catenin in the nucleus. Accumulated β-catenin interacts with Tcf/lef-1 transcription factors to activate transcription of Wnt target genes such as c-*myc* and cyclin D1 (He *et al.*, 1998; Tetsu and McCormick, 1999). To investigate whether inhibition of GSK-3β by PLCγ1-mediated phosphorylation at serine-9 is linked to Wnt signaling, we examined cytosolic and nuclear β-catenin levels in Vector- and PLCγ1-3Y1 cells. After exposure to X-ray film, the relative intensities of β-catenin bands in each lane were determined by quantitative scanning densitometer. The amount of cytosolic free β-catenin in PLCγ1-3Y1 cells was much less than in Vector-3Y1 cells (Figure 3A middle panel and Figure 3B). Basal level of nuclear β-catenin in PLCγ1-3Y1 cells was reduced to 42% of that in Vector-3Y1 cells (Figure 3A bottom panel and Figure 3B). Translocation of β-catenin to the nucleus in response to serum stimulation is increased in a time-dependent manner after serum stimulation in Vector-3Y1 cells, but very marginal in PLCγ1-3Y1 cells (Figure 3A bottom panel). The amount of nuclear β-catenin in Vector-3Y1 cells was 5-fold higher than that in PLCγ1-3Y1 cells by 6 h of stimulation (Figure 3B). This finding suggested that PLCγ1-mediated phosphorylation of GSK-3β by serum is not associated with the regulation of β-catenin. The mechanism by which PLCγ1 decreases free β-catenin pools is not understood at present.

To further determine whether PLCγ1-mediated GSK-3β phosphorylation is not involved in Wnt signaling, we examined the effect of transient expression of PLCγ1 on Tcf/Lef-1-mediated transcription. pTOPflash reporter constructs, which contain three repeats of the Tcf/Lef-1 binding elements (Korinek *et al.*, 1997), was transfected into HEK293T cells. Transfection of pTOPflash reporter produced Tcf/Lef-1-dependent transcription in a DNA concentration-dependent manner (Figure 4A). Expression of flag-tagged Dvl-1, as a positive control, induced pTOPflash reporter activity by 3-fold, while PLCγ1 had no effect on the reporter activity (Figure 4B), suggesting that Tcf/Lef-1-mediated transcription is not dependent on PLCγ1. Taken together, these results suggest that PLCγ1-mediated GSK-3β phosphorylation is not linked to Wnt signaling.

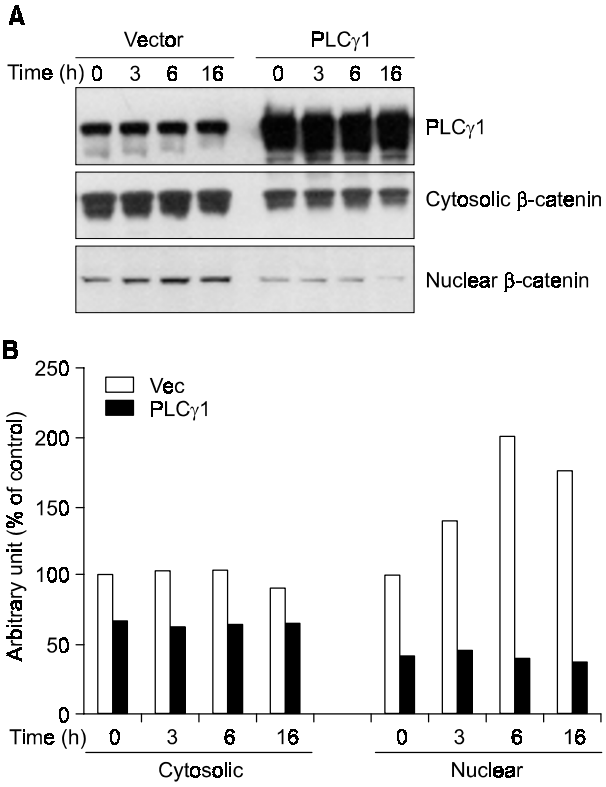

Figure 3. Overexpression of PLCγ1 is not associated with β-catenin accumulation. (A) Vector-3Y1 cells (Vector) and PLCγ1-3Y1 cells (PLCγ1) were serum-starved with 0.5% for 24 h, and then treated with 20% serum for indicated time periods. Cytosolic and nuclear fraction was prepared and subjected to Western blotting with anti-β-catenin antibody. (B) The relative band intensities of β-catenin in Figure 3A were measured by quantitative scanning densitometer and image analysis software, Bio-1D version 97.04.

Effect of PLCγ1 overexpression on serum-induced cyclin D1 expression

GSK-3β can reduce abundance of cyclin D1 protein

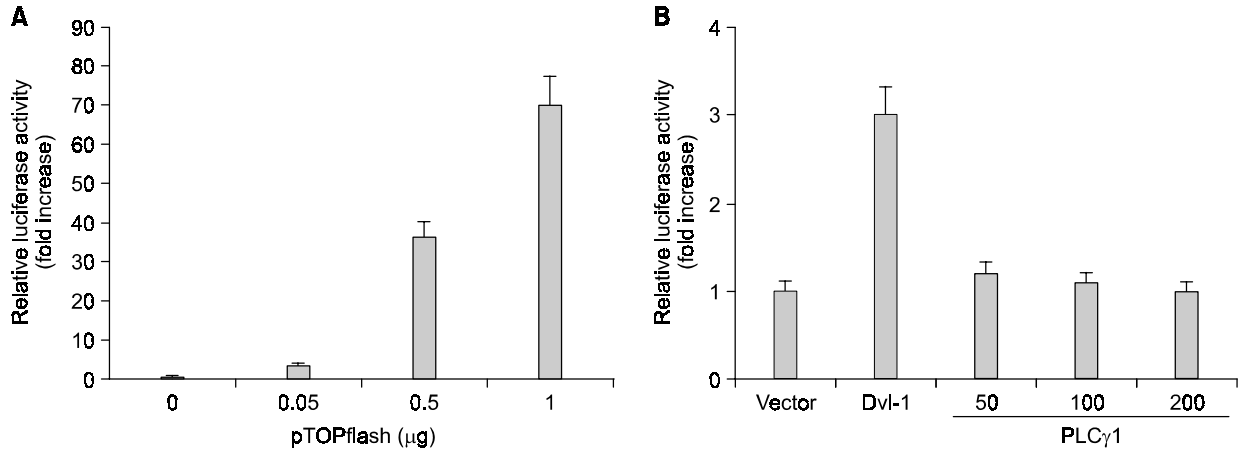

Figure 4. Overexpression of PLCγ1 is not linked to Wnt signaling. (A) Tcf/Lef-1-mediataed transcription activity was measured in HEK293T cells transfected with pTOPflash luciferase reporter (0.05-1 μg) and pCMV/β-gal plasmid (100 ng). (B) Effect of PLCγ1 on Tcf/Lef-1-dependent transcription. pTOPflash reporter (0.5 μg) was transfected with Dvl-1 (100 ng) or PLCγ1 (50-200 ng) expression vector into HEK293T cells. At 24 h after transfection, luciferase activity of cell lysates was measured and normalized with β-galactosidase activity. Error bars represent standard deviations from three independent experiments in duplicate.

through phosphorylation of cyclin D1 at threonine-286, which target for ubiquitination-dependent proteolytic degradation (Diehl *et al.*, 1998). GSK-3β is constitutively active in the resting state and its enzyme activity is inhibited by phosphorylation at serine-9 upon growth factor stimulation, resulting in accumulation of cyclin D1. Thus, we next examined whether increase of GSK-3β phosphorylation in PLCγ1-3Y1 cells regulates the cyclin D1 level. As shown in Figure 5A, cyclin D1 protein levels gradually increased up to 12 h after serum stimulation, and declined there after in Vector-3Y1 cells. After 36 h, amount of cyclin D1 protein had fallen back to near control levels. During this time course, notable increases in the cyclin D1 level were observed in PLCγ1-3Y1 cells compared with Vector-3Y1 cells. In contrast, cyclin E levels were not changed between two cell lines. To examine whether this increase in cyclin D1 protein levels results from a increase in mRNA levels, Northern blot analysis was performed. Induction of cyclin D1 mRNA by serum stimulation showed similar kinetics between two cell lines (Figure 5B). This finding suggests that PLCγ1 controls cyclin D1 abundance at the post-transcriptional level, probably through phosphorylation of GSK-3β.

In this report, we demonstrate for the first time that GSK-3β is a down-stream target of PLCγ1-dependent signals. PLCγ1 overexpression resulted in increase of GSK-3β phosphorylation at serine-9 by serum stimulation, which is abrogated by inhibition of PKC activity by treatment of GF109203X. It has been reported that GSK-3β is active in unstimulated state and phosphorylates cyclin D1 at threonine-286, thereby resulting ubiquitination and subsequent proteolytic de-

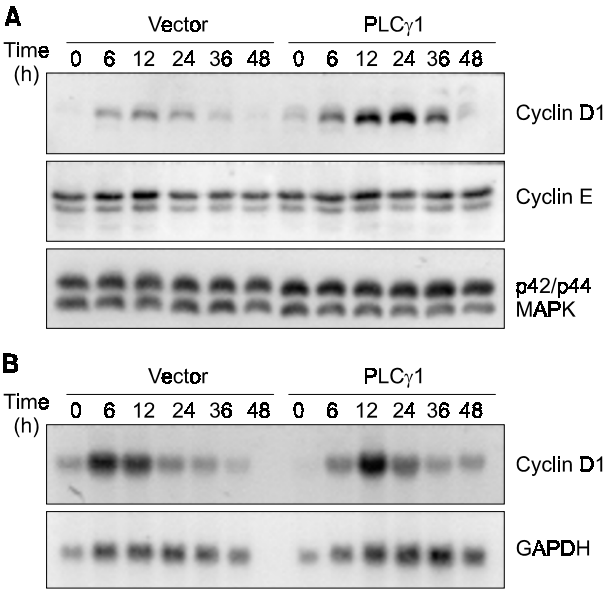

Figure 5. Up-regulation of cyclin D1 protein in PLCγ1-overexpressed 3Y1 cells. Vector-3Y1 cells (Vector) and PLCγ1-3Y1 cells (PLCγ1) were serum-starved with 0.5% for 24 h, and then treated with 20% serum for indicated time periods. (A) Proteins were extracted, separated on 10% SDS-polyacrylamide gels (20 μg/lane) and transferred to nitrocellulose membrane. Cyclin D1 (top), cyclin E (middle) and p42/p44 MAPK (bottom) protein levels were detected by Western blotting. (B) Total RNA was isolated from cells, electrophoresed on 1% agarose-gel (10 μg/lane), capillary transferred to a nylon membrane, and subjected to Northern blotting. The blot was hybridized with the ^{32}P-labeled cyclin D1 probe (top). The same blot was stripped and reprobed with ^{32}P-labeled GAPDH probe (bottom).

gradation of cyclin D1 (Diehl *et al.*, 1997; Diehl *et al.*, 1998). Upon mitogenic stimulation, GSK-3β is phosphorylated and inactivated through several signaling pathways including Ras, phosphatidylinositol-3-OH kinase (PI-3K) and PKB/Akt. Inhibition of GSK-3β activity results in the elevation of cyclin D1 level (Diehl *et al.*, 1998). This may enhance the activities of cyclin-dependent protein kinase (CDK)-4 and CDK-6, allowing them to phosphorylate pRb and stimulate cell cycle progression. Given the facts that (1) GSK-3β phosphorylation is increased by PLCγ1; (2) GSK-3β directly regulates cyclin D1 proteolysis in response to mitogenic stimulation; (3) Cylin D1 protein level, but not mRNA level, is up-regulated in PLCγ1-transfected cells, it is plausible to suggest that the functional role of PLCγ1-mediated GSK-3β phosphorylation may be associated with the accumulation of cyclin D1 in PLCγ1-overexpressing cells.

In summary, this study demonstrates that PLCγ1 mediate GSK-3β phosphorylation at serine-9 *via* PKC activation. Since phosphorylation of GSK-3β is responsible for the accumulation of cyclin D1 in response to mitogenic stimulation, PLCγ1 overexpression might contribute to mitogen-induced cell cycle progression through phosphorylation of GSK-3β at serine-9, at least in part.

Acknowledgments

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