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Phospholipase D activity is elevated in hepatitis C virus core protein-transformed NIH3T3 mouse fibroblast cells

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate acetate; PtdBut, phosphatidylbutanol

Abstract

Hepatitis C Virus (HCV) is associated with a severe liver disease and increased frequency in the development of hepatocellular carcinoma. Overexpression of HCV core protein is known to transform fibroblast cells. Phospholipase D (PLD) activity is commonly elevated in response to mitogenic signals, and has also been overexpressed and hyperactivated in some human cancer cells. The aim of this study was to understand how PLD was regulated in the HCV core protein-transformed NIH3T3 mouse fibroblast cells. We observed that PLD activity was elevated in the NIH3T3 cells overexpressing HCV core protein over the vector alone-transfected control cells, however, expression levels of PLD protein and protein kinase C (PKC) in the HCV core protein-transformed cells was similar to the control cells. Phorbol 12-myristate 13-acetate (PMA), which is known to activate PKC, stimulated PLD activity significantly more in the core protein-transformed cells, in comparison with that of the control cells. PLD activity assay using PKC isozyme-specific inhibitor and PKC translocation experiment showed that PKC-δ was mainly involved in the PMA-induced PLD activation in the core-transformed cells. Moreover, in cells overexpressing HCV core protein, PMA also stimulated p38 kinase more potently than that of the control cells, and an inhibitor of p38 kinase abolished PMA-induced PLD activation in cells overexpressing HCV core protein. Taken together, these results suggest that PLD might be implicated in core protein-induced transformation.

Keywords: HCV core protein; p38; PKC; PLD

Introduction

The hepatitis C virus (HCV) is the etiologic agent of acute and chronic hepatitis affecting more than 100 million people worldwide (Uchida, 1994). Chronic hepatitis is one of the leading causes of liver cirrhosis and hepatocellular carcinoma (Saito *et al.,* 1990). However, roles and mechanisms, of HCV in the development of this cancer has not been clearly elucidated. HCV, a member of the flavivirus family, has a 9.5-kb positive single-stranded RNA genome, which encodes a polyprotein that is processed into at least 10 different structural and nonstructural proteins (Ray *et al.,* 2001). Core protein is a structural protein of HCV and is derived from the N-terminus of the polypeptide and has a highly basic N-terminal region and a highly hydrophobic C-terminus. This multifunctional protein has also been implicated in hepatocyte proliferation and transformation. However, its role in cellular proliferation and tumorogenesis has not been well understood.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC), the major membrane phospholipid, to form phosphatidic acid (PA) and choline. PA generally recognized as the signaling product of PLD, functions as an effector in multiple physiological processes (Liscovitch *et al.,* 2000; Steed and Chow, 2001; Kim *et al.,* 2003). To date, two PLD isoforms, PLD1 and PLD2, have been cloned and characterized (Colley *et al.,* 1997; Hammond *et al.,* 1997). PLD1 has a low basal activity and is up-regulated by small G proteins (ARF, Rho and Ral), protein kinase C

(PKC), and phosphatidylinositol 4, 5-bisphosphate (PIP2) *in vitro*. In contrast, PLD2 has a high basal activity, requires PIP2, and is up-regulated by ARF and PKC. It was reported that the ARF-dependent nuclear PLD activity was increased in the S-phase of the regenerating rat liver after partial hepatectomy and also was much higher in AH 7974 cells than in the resting rat liver (Banno *et al.,* 1997). These results suggested that the nuclear ARF-dependent PLD activity may be associated with cell proliferation. It has been reported that PLD activity is significantly elevated in human cancer tissues (Uchida, 1994; Uchida *et al.,* 1997; Uchida *et al.,* 1999) as well as oncogene-transformed cells (Jiang *et al.,* 1994; Frankel *et al.,* 1999; Fiucci *et al.,* 2000). Moreover, overexpression of PLD isozymes in fibroblast cells was associated with neoplastic transformation and tumorigenesis (Min *et al.,* 2001). These results suggest that PLD might play a critical role in regulating cell responses that contribute to mitotic signaling and transformation. Recently, Jung *et al.* have demonstrated that overexpression of HCV core protein induce transformation of murine fibroblast cells (Jung *et al.,* 2003). In the present study, we demonstrate that PLD activity is upregulated in HCV core protein-transformed cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). The antibodies to PKC-α, PKC-δ, and PKC-ξ were purchased from Santa Cruz. PKC isozyme-specific inhibitors (Gö6976, Rottlerin) were from Calbiochem (San Diego, CA). Phospho-p38 and p38 antibodies were from Cell Signaling (Beverly, MA), and PD98059 and SB203580 were from Biomol (Plymouth, PA). Rabbit polyclonal antibody that recognizes both PLD1 and PLD2 was generated as described previously (Lee *et al.,* 2000). Phosphatidylbutanol (PtdBut) standard was from Avanti Polar Lipid. [9,10-^{3}H]myristate were purchased from Perkin-Elmer Life Sciences. Silica gel 60 A thin layer chromatography plates were from Whatman (Clifton, NJ). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were from Kirkegaard and Perry Laboratory (Gaithersburg, MD). The enhanced chemiluminescence (ECL) Western blotting detection kit was from Amersham Biosciences (Beverly, MA).

Establishment of stable cell lines

Stable NIH 3T3 cells overexpressing HCV core protein were established as described previously (Han *et al.,* 2002). Construction of pCI-neo-core K (genotype 1b) was made. Stable NIH 3T3 cell lines following transfection with the plasmid including HCV or empty vector were generated and the cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 500 μg/ml G418 under 5% CO_2.

Phospholipase D activity assay

PLD activity was assessed by measuring the formation of [^{3}H] PtdBut, the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol. Cells were subcultured in 6- well plates at 2×10^5 cells/well and serum-starved in the presence of 1 μCi/ml [^{3}H]myristic acid. After overnight starvation, the cells were washed three times with 5 ml of phosphate-buffered saline (PBS) and pre-equilibrated in serum-free DMEM for 1 h. For the final 10 min of preincubation, 0.3% butan-1-ol was included. At the end of the preincubation, cells were treated with agonists for the indicated times. The extraction and characterization of lipids by thin-layer chromatography were performed as previously described (Min *et al.,* 2002).

Immunoprecipitation

The trated cells were lysed with the lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenymethylsulfonyl fluoride, PMSF). The supernatant of cell lysate was collected by centrifugation at 10,000 *g* for 1 h, and incubated with anti-PLD or anti-PKC antibody complexed to protein A sepharose bead for 4 h at 4°C with gentle rocking. The immune complexes were collected by centrifugation and washed five times with a buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM Na_3VO_4, 10% glycerol and 1% Nonidet P-40) and resuspended in sample buffer. The final pellet was loaded onto a polyacrylamide gel for immunoblot analysis.

Western blotting

Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis on 8% gels and were transferred to a nitrocellulose membrane. The blots were then blocked with 5% non-fat milk and incubated with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using ECL.

Subcellular fractionation

The cells were harvested and washed twice with

ice-cold lysis buffer (20 mM Hepes, pH 7.4, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM of dithiothreitol, and 1 mM PMSF fluoride and 10 μg/ml leupeptin). The cells were then resuspended in lysis buffer and lysed by ten passages through a 25-gauge needle. Trypan blue staining of the lysate indicate > 95% disruption of the cells. The cell lysate was first spun at 500 *g* for 10 min to remove unbroken cells. The supernatant was then spun at 100,000 *g* for 1 h at 4°C to separate the cytosolic and membrane fractions. Membrane fractions were washed twice with the buffer to remove cytosolic proteins.

Results

PLD activity is enhanced in NIH 3T3 cells overexpressing HCV core protein

To understand how PLD activity is regulated in the HCV core protein-transformed cells, the basal PLD activity was compared in both core and vector alone transfected cells. The results shown in Figure 1A, indicated that PLD activity was much higher in the core protein-transformd cells than in the vector-transfected control cells. PMA known to activate both PKC isoforms and PLD, induced further activation of PLD significantly higher in the transformed cells than the control cell. Such increase of PMA-induced PLD activity in the core protein-transformed cells occurs in a time dependent manner (Figure 1B). These results demonstrated that PLD activity is up-regulated in the core protein-transformed cells.

PLD1 and PKC are expressed at a similar level in both core protein and vector-transfected cells

To examine whether up-regulation of PLD activity may be due to an enhanced expression of PLD or PKC protein, the expression levels of the both proteins were examined. The result (Figure 2) showed that both the vector and the core protein-transfected cells expressed a similar protein level of PLD1. However, PLD2 was not detected in either cells (data not shown), indicating that the PLD activity shown in these cells may be due mainly to PLD1. Examination of PKC isozyme expression with the isozyme-specific antibodies showed that PKC-α (a conventional PKC), PKC-δ (a novel PKC), and PKC-ξ (atypical PKC) were predominantly expressed. PKC-α was expressed more than other PKC isozymes in these cells. The vector or the core protein-transformed cells showed similar expression level of PKC isozymes. These results suggest that enhanced PLD activity in core protein-transformed cells may be not due to up-regulation of PLD and PKC proteins.

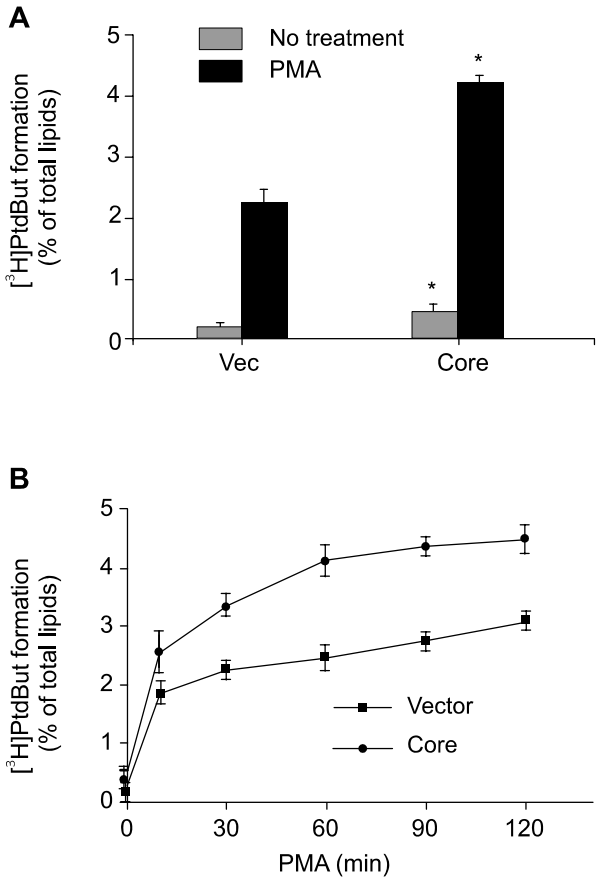

Figure 1. Overexpression of HCV core protein enhances PMA-induced PLD activity. (A) NIH3T3 fibroblasts overexpressing HCV core as well as vector-transfected cells were cultured in six-well plates, labeled with [^{3}H]myristate, and treated with PMA (100 nM) for 60 min. (B) Vector and core-transformed cells were treated with PMA for different times. *P < 0.05 compared to cells transfected with vector and treated with or without PMA. The radioactivity incorporated into phosphatidylbutanol was measured as described under "Materials and Methods". Results are the means ± S.D. of three independent experiments.

PKC-δ is mainly involved in PMA-induced PLD activation in HCV core protein-transformed cells

To identify which isozymes of PKC may be responsible on PMA-stimulated PLD activation in the NIH3T3 cells overexpressing HCV core protein, each PKC isozyme was suppressed by the use of specific inhibitors (Figure 3). Pretreatment of calcium dependent PKC inhibitor (Gö6976) suppressed PMA-induced PLD activation mildly. In contrast, PMA-induced PLD activation was inhibited dramatically in a dose-dependent manner by the pretreatment of cells with Rottlerin, PKC-δ specific inhibitor. These results show that in the core-transformed cells, PKC-δ might be mainly involved in PMA-stimulated PLD activation.

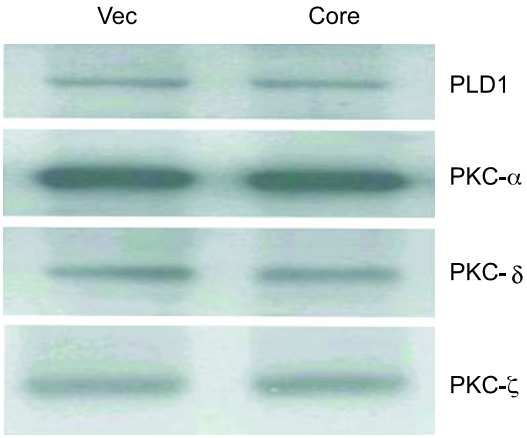

Figure 2. Expression of PLD1 and PKC isozymes in NIH3T3 fibroblasts. Vector or core overexpressing cells were lysed, and immunoprecipitates (IP) were prepared using anti-PLD, anti-PKC-α, anti-PKC-δ, or anti-PKC-ξ antibodies. Immune complexes were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer of proteins to nitrocellulose membrane and Western blotting (Blot) with the indicated antibody. The results shown are representatives of three separate experiments.

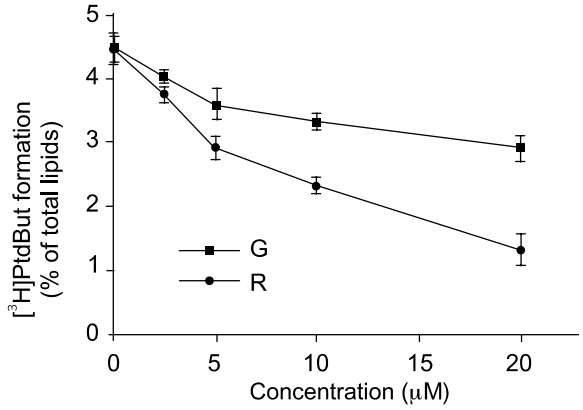

Figure 3. PKC-δ is mainly involved in the PMA-induced PLD activation in core-transformed cells. The core-transformed cells were pretreated with various concentration of Gö6976 (G) or Rottlerin (R) for 30 min and stimulated with 100 nM PMA for 60 min. The radioactivity incorporated into phosphatidylbutanol was measured as described under "Materials and Methods". Results are the means ± S.D. of three independent experiments.

PMA induced rapid translocation of PKC-δ from cytosol to membrane fraction than those of PKC-α in the core-transformed cells

To confirm that PKC-δ is involved in the PMA-induced PLD activation in the HCV core protein-transformed cells, translocation of the PKC isozymes were analyzed by the fractionation of subcellular component of the treated cells and distribution of respective isozymes was identified by western blot. As shown in Figure 4, treatment of PMA induced a gradual translocation of PKC-α from cytosol to membrane fraction

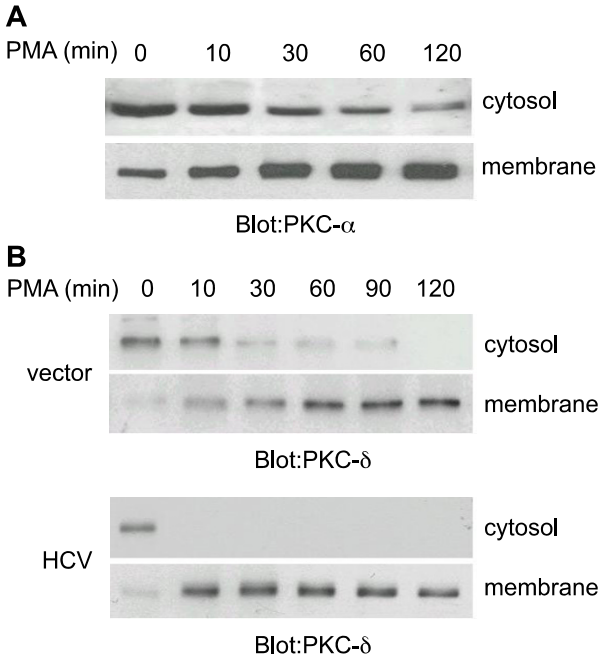

Figure 4. PMA induced more rapidly translocation of PKC-δ from cytosol to membrane fraction than that of PKC-α in core-transformed cells. (A) Core protein-transformed cells were treated with 100 nM PMA for the indicated times. (B) Vector-transfected cells and core protein-transformed cells were stimulated with 100 nM PMA for the indicated times. Lysates were fractionated into the cytosolic and membrane fractions. Each fraction was immunoblotted using antibodies specific for PKC-α and PKC-δ. The results shown are representatives of three separate experiments.

in a time dependent manner in the core protein-transformed cells. But the PKC-δ was rapidly translocated to the membrane fraction post 10 min PMA treatment, whereas a gradual translocation of PKC-δ was observed in the vector-transfected and PMA treated cells suggesting that PKC-δ is mainly responsible for the PMA-stimulated PLD activation in HCV core protein-transformed fibroblasts.

p38 MAPK is involved in PMA-induced PLD activation in HCV core protein-transformed cells

HCV core protein has been shown to activate both the extracellular signal-regulated kinase (ERK) and the p38 MAPK (Erhardt *et al.,* 2002; Tsutsumi *et al.,* 2003). A potential participation of these kinases in the PMA-induced PLD activation in HCV core protein-transformed cells was explored. As shown in Figure 5-A and -B, SB203580, p38 MAPK-specific inhibitor, caused a dose-dependent decrease in PMA-stimulated PLD activity but PD98059, ERK upstream inhibitor, did not. In addition, PMA induced a significant activation of p38 MAPK in both control cell and HCV core protein-transformed cells and pretreatment of

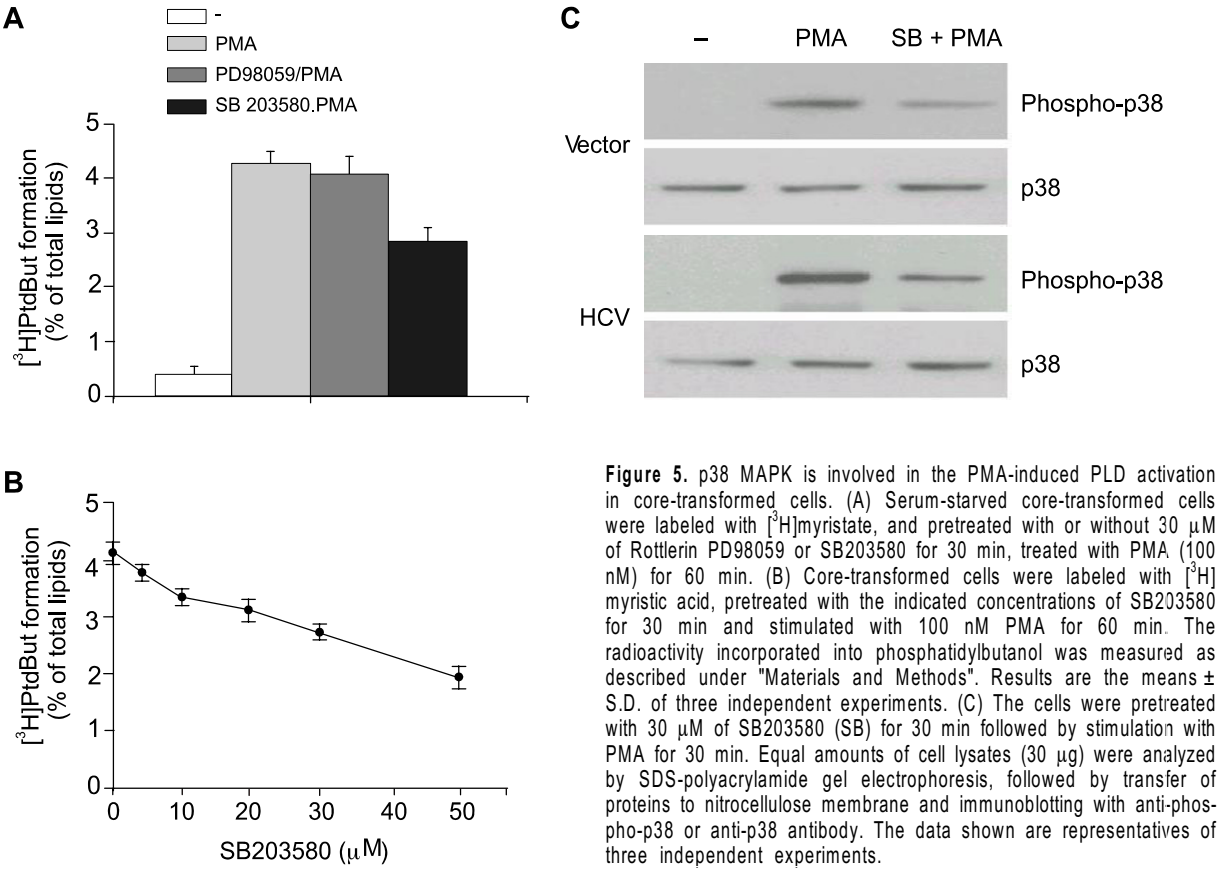

Figure 5. p38 MAPK is involved in the PMA-induced PLD activation in core-transformed cells. (A) Serum-starved core-transformed cells were labeled with [^{3}H]myristate, and pretreated with or without 30 μM of Rottlerin PD98059 or SB203580 for 30 min, treated with PMA (100 nM) for 60 min. (B) Core-transformed cells were labeled with [^{3}H] myristic acid, pretreated with the indicated concentrations of SB203580 for 30 min and stimulated with 100 nM PMA for 60 min. The radioactivity incorporated into phosphatidylbutanol was measured as described under "Materials and Methods". Results are the means ± S.D. of three independent experiments. (C) The cells were pretreated with 30 μM of SB203580 (SB) for 30 min followed by stimulation with PMA for 30 min. Equal amounts of cell lysates (30 μg) were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer of proteins to nitrocellulose membrane and immunoblotting with anti-phospho-p38 or anti-p38 antibody. The data shown are representatives of three independent experiments.

SB203580 inhibited PMA-induced p38 activation (Figure 5C). Interestingly, PMA stimulated p38 MAPK activation far greater extent in the HCV core protein-transformed cells than in control cells. Reprobing the immunoblot with anti-p38 MAPK antibodies showed the equal levels of proteins in each lane. These results suggest that p38 MAPK is involved in PMA-induced PLD upregulation in HCV core protein-transformed cells.

PMA induces activation of p38 MAPK *via* PKC in core-transformed cells

To determine whether PMA-induced p38 MAPK activation is exerted via PKC-δ, the cells were stimulated with PMA in the presence of Rottlerin, PKC-δ specific inhibitor. As shown in Figure 6, pretreatment with the Rottlerin suppressed PMA-induced p38 phosphorylation, suggesting that PMA induces activation of p38 *via* PKC-δ.

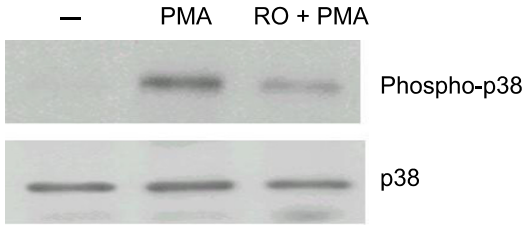

Figure 6. PMA induces activation of p38 MAPK via PKC-δ in core-transformed cells. Serum-starved core-transformed cells were pretreated with 10 μM of Rottlerin (Ro) for 30 min followed by stimulation with PMA for 30 min. Equal amounts of cell lysates (30 μg) were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer of proteins to nitrocellulose membrane and immunoblotting with anti-phospho-p38 or anti-p38 antibody. The data shown are representatives of three independent experiments.

Discussion

In this study, we demonstrated that an overexpression of HCV core protein in NIH3T3 fibroblasts upregulated PLD activity. PLD activity has been found to be markedly elevated in various cancer tissues (Uchida *et al.,* 1997; Uchida *et al.,* 1999; Zhao *et al.,* 2000), multidrug resistant cancer cells (Fiucci *et al.,* 2000), and transformed cells (Jiang *et al.,* 1994;

Frankel *et al.,* 1999). Tumorigenic transformation of fibroblasts was induced by overexpression of PLD isozyme (Min *et al.,* 2001) and transmodulation between PLD and c-Src enhanced cell proliferation (Ahn *et al.,* 2003). PLD, thus, may be closely associated with in the path of signal transduction leading to cellular proliferation and transformation. Hepatocellular carcinoma (HCC) is one of the most common human cancers in a worldwide basis. Among the several risk factors, chronic infection with either hepatitis C virus is closely related to the development of HCC. HCV core protein has been considered to play important roles during development of HCC by HCV (Koike *et al.,* 2002). Core protein demonstrated its tumorigenecity by inducing HCC *in vivo* in transgenic mice (Moriya *et al.,* 1998). As a multifunctional regulator, core protein is known to deregulate cell cycle checkpoints by modulating transcription of the p21 gene (Jung *et al.,* 2001). In addition, it can activate the expression of some proto-oncogenes (Ray *et al.,* 1997). Furthermore, it might modulate mitogenic intracellular signaling pathways as well as apoptotic pathways leading to cell death by interacting several signaling molecules (Shiravastava *et al.,* 1998; Marusawa *et al.,* 1999). Our earlier report of fibroblast transformation induced by an overexpression of HCV core protein adds to the diversified role of HCV core protein (Jung *et al.,* 2001). In this study, PMA-induced PLD activity was enhanced in the cells transformed with HCV core protein. Both control and core-transformed cells expressed PLD1 protein, but PLD2 isozyme was not detected. These cells showed no difference in the levels of PLD1 and PKC expressed. Inhibitor of PKC-ξ did not affect PMA-induced PLD activation (data not shown). Moreover, PMA induced rapidly translocation or activation of PKC-δ from cytosol to membrane fraction, whereas PKC-α was gradually translocated into membrane fraction, supporting PKC-δ is significantly involved in PMA-induced PLD activation in core-transformed cells. This indicates that the initial translocation of PKC-δ may be at least partly involved in the PMA-induced PLD activation and that other unidentified mechanism(s) associated with the initial activation of PKC-δ may play an important role in progressive PLD activation.

Protein kinases play a key role in the regulation of cell growth. There is an increasing evidence to suggest that the development of hepatocellular carcinoma might be associated with activation of the Ras/Raf/MAP kinase pathway (Ito *et al.,* 1998). On the other hand, there are numerous clinical and *in vitro* studies suggesting a role of the HCV, especially of the HCV core protein, in liver carcinogenesis (Chang *et al.,* 1998; Moriya *et al.,* 1998). Thus, one may speculate that the HCV core protein plays a key role in the development of human liver disease through activation of the MAP kinase pathway. Indeed, activation of ERK and p38 has been reported for the HCV core protein (Erhardt *et al.,* 2002; Tsutsumi *et al.,* 2003). However, the influence of the HCV core protein on MAP kinase and the influence of the MAP kinase on disease progression are not fully understood. Since both PLD and HCV core protein have been implicated in the cell proliferation and tumorigenesis, it is possible that PLD and HCV core protein could cross-react at different stages to make a cell pass some of the multi-step required for hepatocarcinogenesis. However, role(s) of PLD signaling in tumorigenesis of core protein-transformed cells requires further study. In the present study, we demonstrated upregulation of PLD activity in the core-transformed cells. These results might provide a clue to understand signaling network for the promotion and/or progression of cancers *via* PLD and HCV core protein.

Acknowledgment

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