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Phorbol Ester Up-regulates Phospholipase D1 but Not Phospholipase D2 Expression through a PKC/Ras/ERK/NFκB-dependent Pathway and Enhances Matrix Metalloproteinase-9 Secretion in Colon Cancer Cells*[S]

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Despite its importance in cell proliferation and tumorigenesis, very little is known about the molecular mechanism underlying the regulation of phospholipase D (PLD) expression. PLD isozymes are significantly co-overexpressed with cancer marker genes in colorectal carcinoma. Phorbol 12-myristate 13-acetate (PMA) treatment, as a mitogenic signal in colon cancer cells, selectively increases PLD1 expression in transcription and post-transcription. Moreover, experiments using intraperitoneal injection of PMA into mice showed selective PLD1 induction in the intestine and lung tissues, which suggests its physiological relevance *in vivo*. Therefore, we have undertaken a detailed analysis of the effects of PMA on the promoter activity of *PLD* genes. Protein kinase C inhibitors, but not a protein kinase A inhibitor, were found to suppress the up-regulation of PLD1 but not PLD2. Dominant-negative mutants of Ras, Raf, and MEK suppressed the induction and activity of PLD1. Moreover, depletion of the supposedly involved proteins reduced the endogenous PLD1 protein level. An important role for NFκB as a downstream target of ERK in PMA-induced PLD1 induction was also demonstrated using the inhibitor, small interfering RNA, chromatin immunoprecipitation assay, and site-specific mutagenesis. Furthermore, inhibitors of these signaling proteins and depletion of PLD1 suppressed PMA-induced matrix metalloproteinase-9 secretion and PLD1 induction. In conclusion, we demonstrate for the first time that induction of PLD1 through a protein kinase C/Ras/ERK/NFκB-dependent pathway is involved in the secretion of matrix metalloproteinase-9 in colorectal cancer cells.

Colorectal cancer (CRC)[2] is one of the leading causes of cancer death in much of the developed world, despite the facts that colonic malignancies can be effectively managed when detected early and that chemoprevention has shown some success in reducing the severity of the disease (1). Dysregulation of proliferation and apoptosis frequently occurs in the development of various human cancers. The unraveling of growth-regulatory pathways and the process of tumorigenesis has revealed new diagnostic and prognostic markers of disease as well as potential targets for therapeutic intervention. Therefore, the discovery of new cancer-associated genes and their mechanisms of action is important for overcoming CRC.

Phospholipase D (PLD) has emerged as a critical regulator of cell proliferation and survival (2). Abnormalities in PLD expression and activity have been observed in many human cancers (2). PLD hydrolyzes phosphatidylcholine, the predominant membrane phospholipid, and produces phosphatidic acid and free choline. Two mammalian isozymes of phosphatidylcholine-specific PLD (PLD1 and PLD2) have been identified and are differentially regulated. PLD1 is activated by protein kinase C (PKC) and small G proteins of the Rho and ADP-ribosylation factor families, whereas PLD2 is not directly activated by these regulators (3). We have reported that overexpression of the PLD isozymes causes anchorage-independent growth and neoplastic transformation of mouse fibroblasts (4). PLD1 has been reported as a critical downstream mediator of H-Ras-induced transformation (5). Mutation in the Ras oncogene is found in many human cancers (6) and can lead to the deregulated activation of PLD and important downstream signaling pathways, including Raf/MEK/ERK (7) and PI3K/Akt (8). Moreover, PLD has been shown to be involved in the resistance of cancer cells to chemotherapeutic drugs (9, 10). Despite gathering evidence regarding the regulation of PLD activity in cell function, little is

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[2] The abbreviations used are: CRC, colorectal cancer; PLD, phospholipase D; PMA, phorbol-12-myristate-13-acetate; DAG, diacylglycerol; PKC, protein kinase C; ChIP, chromatin immunoprecipitation; MMP-9, matrix metalloprotease-9; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.

known about the functional role and regulatory mechanisms of PLD expression.

Phorbol esters, such as phorbol-12-myristate-13-acetate (PMA), can substitute for diacylglycerol (DAG; the endogenous PKC activator) and function as tumor promoters. Phorbol esters have been reported to modulate diverse cellular responses, such as gene transcription, mitogenesis, and differentiation, tumor promotion, and apoptosis, through the PKC signaling pathway (11, 12). PMA protects cells from stimuli-induced apoptosis through a mechanism that is dependent on the activation of PKC (13). However, the signaling pathways involved in PMA-induced PLD expression have not been defined. HCT116 human colon cancer cells contain the most common K-ras mutation type found in colorectal cancers, the glycine to aspartate mutation at codon 12 (14). Because many growth factors utilize Ras-dependent signaling pathways, this cell line serves as a model system for studying alterations in gene expression that occur during the progression of CRC. Secretion of matrix metalloproteinases (MMPs) from cancer cells is an important stage of metastatic spread. MMPs hydrolyze components of the extracellular matrix and allow the invasion of cancer cells from their primary site to the circulation and secondary sites (15–17). Various stimuli, including PMA, have been shown to induce the secretion of MMP-9 from cancer cell lines (18), including human fibrosarcoma line HT 1080 (19, 20). Thus, we investigated the involvement of PLD1 induction in PMA-stimulated MMP-9 secretion.

The present study was conducted to define the cellular mechanisms that regulate the expression of PLD isozyme in human colon cancer cells. Here, we demonstrate for the first time that induction of PLD1 through the PKC/Ras/ERK/NFκB-dependent pathway is involved in the secretion of MMP-9 in colorectal cancer cells.

EXPERIMENTAL PROCEDURES

Materials—PMA and DAG were purchased from Sigma. Various inhibitors were obtained from Calbiochem. The oligonucleotides of NFκB-binding sites were purchased from Genotech (Daejeon, Republic of Korea). The siRNA of human *PLD1* (nucleotides 1486–1506, AAGGUGGGACGACAAUGAGCA) and *PLD2* (nucleotides 2665–2685, AAUGGGGAUGCGGAUAAAGGC) were purchased from Dharmacon Research Inc. (Lafayette, CO). The siRNAs of *PKCα*, *Ras*, and *ERK* were obtained from Cell Signaling Inc. (Beverly, MA). Dual luciferase assay kits were purchased from Promega (Madison, WI). Polyfect transfection reagent was purchased from Qiagen.

Cell Culture—Human colon cancer cells (HCT116, DLD1, LS174T, RKO, CaCO2, and SNU-C5) and breast cancer cells (SK-BR3, MCF7) were purchased from ATCC (Manassas, VA). HCT116, DLD1, SNU-C5, and MCF7 were incubated in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum with penicillin/streptomycin (Invitrogen). LS174T, RKO, CaCO2, and SK-BR3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Animals and Treatments—ICR female mice (6 weeks of age, weighing ~18–21 g) were obtained from Oriental Laboratories (Seoul, Republic of Korea). During this study, the mice received standard rodent chow and acidified tap water *ad libitum*. After acclimatization for 1 week, female mice were injected intraperitoneally with PMA three times at doses of 0.015 or 0.15 mg/kg over the course of 1 week ($n = 5$ samples). Controls received an equal volume of corn oil in the same manner.

Preparation of Human CRCs Samples and Immunohistochemistry—Formalin-fixed and paraffin-embedded samples were obtained from surgical resections of 122 colorectal cancers. The tumor stage was classified according to Dukes' criteria (21). There were 12, 47, 55, and eight cases with stage A, B, C, and D, respectively. Two pathologists screened histological sections and selected areas of the representative tumor cells. Three tissue cores (0.6 mm in diameter) were taken from each tumor sample and placed in a new recipient paraffin block using a commercially available microarray instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD) according to established methods (22). One cylinder of normal colonic mucosa adjacent to each tumor was also transferred to the recipient block. Immunohistochemical analysis was performed as described previously (23).

Cloning of the Promoter Regions of Human PLD1 and PLD2—Approximately 1.9- and 2.6-kb genomic DNA fragments covering the 5′ upstream regulatory sequences of human *PLD1* or *PLD2*, respectively, were amplified from human genomic DNA and cloned into a pGL4.14b luciferase reporter plasmid (Promega, Madison, WI) and were then used for promoter analysis. For further detail, see supplemental data.

Transient Transfection, Luciferase Assays—Cells were seeded onto 24-well plates at 1×10^5 cells/well and grown to 50–60% confluence. Transfection and luciferase assays were performed as described previously (12).

RNA Isolation and Reverse Transcription PCR (RT-PCR)—Total RNA was isolated from cells using TRIzol reagent (Sigma). First strand cDNA was synthesized using 5 μg of total RNA with Moloney murine leukemia virus reverse transcriptase (Promega, Mannheim, Germany). The reaction was incubated at 80 °C for 5 min and 42 °C for 90 min, and Moloney murine leukemia virus reverse transcriptase was inactivated at 95 °C for 5 min. The synthesized cDNA was amplified using *PLD1*, *PLD2*, and *GAPDH* primers with an Eppendorf thermocycler (Eppendorf Scientific, Westbury, NY). PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining. For PCR conditions and primer sequences, see supplemental data.

Immunoprecipitation and Western Blotting—The cells or tissues were analyzed by immunoblotting or immunoprecipitation as described previously (24). ECL was used to detect the signal. The following antibodies were used: anti-β-tubulin (Sigma); anti-NFκB (p65) antibody (Santa Cruz Biotechnology); anti-PKCα antibody (Santa Cruz Biotechnology); and anti-ERK antibody (Cell Signaling Inc., Beverly, MA). Rabbit polyclonal anti-PLD antibody that recognizes both PLD1 and PLD2 was generated as described previously (4).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP experiments were essentially performed as described previously (25), with minor modifications. For further details, see supplemental data.

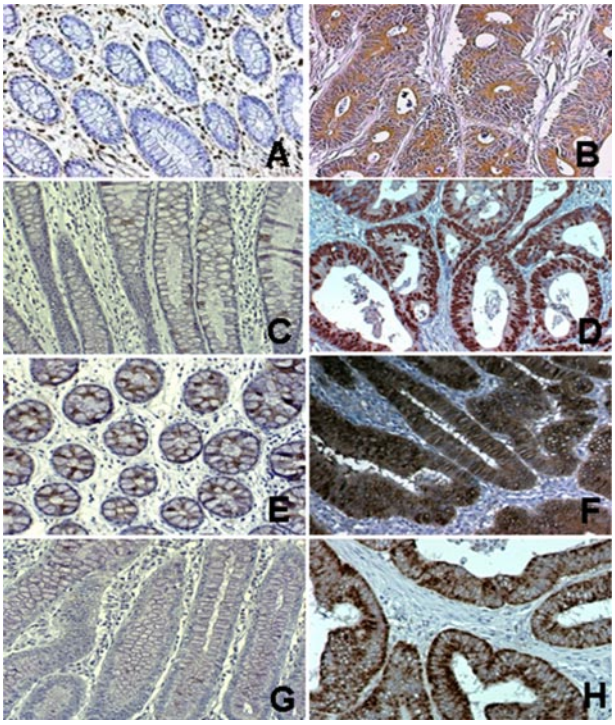

FIGURE 1. **Expression of PLD, Ki-67, β-catenin, and p-Akt in normal colonic mucosa and cancers.** A tissue microarray containing 122 human colorectal cancer tissues was subjected to immunohistochemical analyses with primary antibodies. A positive immunohistochemical reaction is indicated by diaminobenzidine (*brown staining*), and hematoxylin was used as a nuclear counterstain. Representative images from normal colonic mucosa and cancer tissue are shown. *A*, normal colonic mucosa. Note that immunoreactivity for PLD is not present in the normal colonic mucosal epithelial cells. Inflammatory cells in lamina propria demonstrate negative to weak staining. *B*, colon cancer. Note that tumor cells demonstrate strong labeling for PLD, whereas connective tissues in the stroma of cancer specimens are not stained for PLD. *C*, normal colonic mucosa showed immunonegativity for Ki-67. *D*, colon cancer cells showing strong nuclear staining for Ki-67. *E*, in normal colonic mucosa, β-catenin was lightly stained in the membranes of the colonic epithelial cells. *F*, colon cancers showed strong nuclear and/or cytoplasmic staining for β-catenin. *G*, normal colonic mucosa was immunonegative for p-Akt. *H*, colon cancer cells demonstrated strong cytoplasmic staining for p-Akt protein (original magnification was ×200).

PLD Activity Assay—PLD activity was assessed by measuring the formation of [^{3}H]phosphatidylbutanol, the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol as described previously (24).

Zymography—HCT116 cells were seeded in 12-well plates at 1×10^5 cells/ml and were washed and refreshed with serum-free Dulbecco's modified Eagle's medium for 24 h. The conditioned media of the cells were determined by 0.1% casein zymography. Zones of caseinolytic activity were detected as clear bands against a blue background.

Measurement of Cell Viability and Apoptosis—For the cell viability assay, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed. Absorbance was measured using a spectrophotometer at 540 nm, and viability was expressed relative to the control. Cell apoptosis was measured using an Annexin V binding assay (Molecular Probes), which was performed according to the protocol of the manufacturer. Annexin V-positive cells were quantitated. More than 200 cells were counted for each variable per experiment using fluorescence microscopy (Axiovert 200M, Zwiss, Germany).

TABLE 1

Relationship of PLD expression detected by immunohistochemistry and clinicpathological parameter in colorectal carcimoma

The correlation between PLD overexpression and clinicopathologic parameters involving tumor stage and lymph node metastasis was analyzed by immunohistochemistry using a tissue microarray containing 122 human colorectal carcinomas. In addition, the correlation between PLD expression and the expression of Ki-67, β-catenin, or p-AKT in these cancer samples was also analyzed for examination of proliferative capacity in CRCs with overexpression of PLD. A positive or negative sign means strong immunostained tissues and faint or negative immunostained tissues, respectively. L/N, lymph node.

	PLD		*p* value
	+	−	
Stage			>0.05[a]
A	6	6	
B	29	18	
C	33	22	
D	6	2	
L/N metastasis			0.4583[b]
+	39	22	
−	35	26	
Ki-67			0.0019[b]
+	63	29	
−	11	19	
β-Catenin			0.0000[b]
+	64	24	
−	10	24	
p-Akt			0.0000[b]
+	43	10	
−	31	38	

[a] Bartholomew test.
[b] χ^2 test.

Statistics—The results are expressed as means ± S.E. of the number of determinations indicated. Statistical significance of differences was determined by analysis of variance. The correlation between PLD expression and various clinicopathologic parameters and Ki-67, β-catenin, and phospho-Akt expression were assessed using the χ^2 and Bartholomew tests. A *p* value less than 0.05 was considered to indicate statistical significance.

RESULTS

PLD Isozymes Are Significantly Co-overexpressed with Cancer Marker Genes in Human Colorectal Cancer—The expression and activity of PLD isozymes have been reported to be highly up-regulated in many cancer tissues (2), but the number of cases in each study was small. Thus, using a tissue microarray containing 122 CRCs obtained from surgery, we attempted to investigate the expression of PLD isozymes and their association with clinicopathological parameters involving tumor stage, lymph node metastasis, and other cancer marker genes, which are important factors in prognosis. The expression level of PLD isozymes was assessed by immunohistochemistry. As shown in Fig. 1*A*, using the antibody to PLD that recognizes both PLD1 and PLD2, PLD expression was detected as faint or negative in normal colonic mucosa. Occasional weak staining of PLD was observed in inflammatory cells in lamina propria. No immunoreactivity was observed in connective tissues; thus, these tissues served as internal negative controls. Immunopositivity for PLD protein was clearly marked in colorectal tumor cells (Fig. 1*B*). The tumors were interpreted as negative when immunostaining was weak, as in the corresponding normal colonic mucosa, or when immunopositive cells represented <5% of the cancer cells. Table 1 summarizes the relationship between clin-

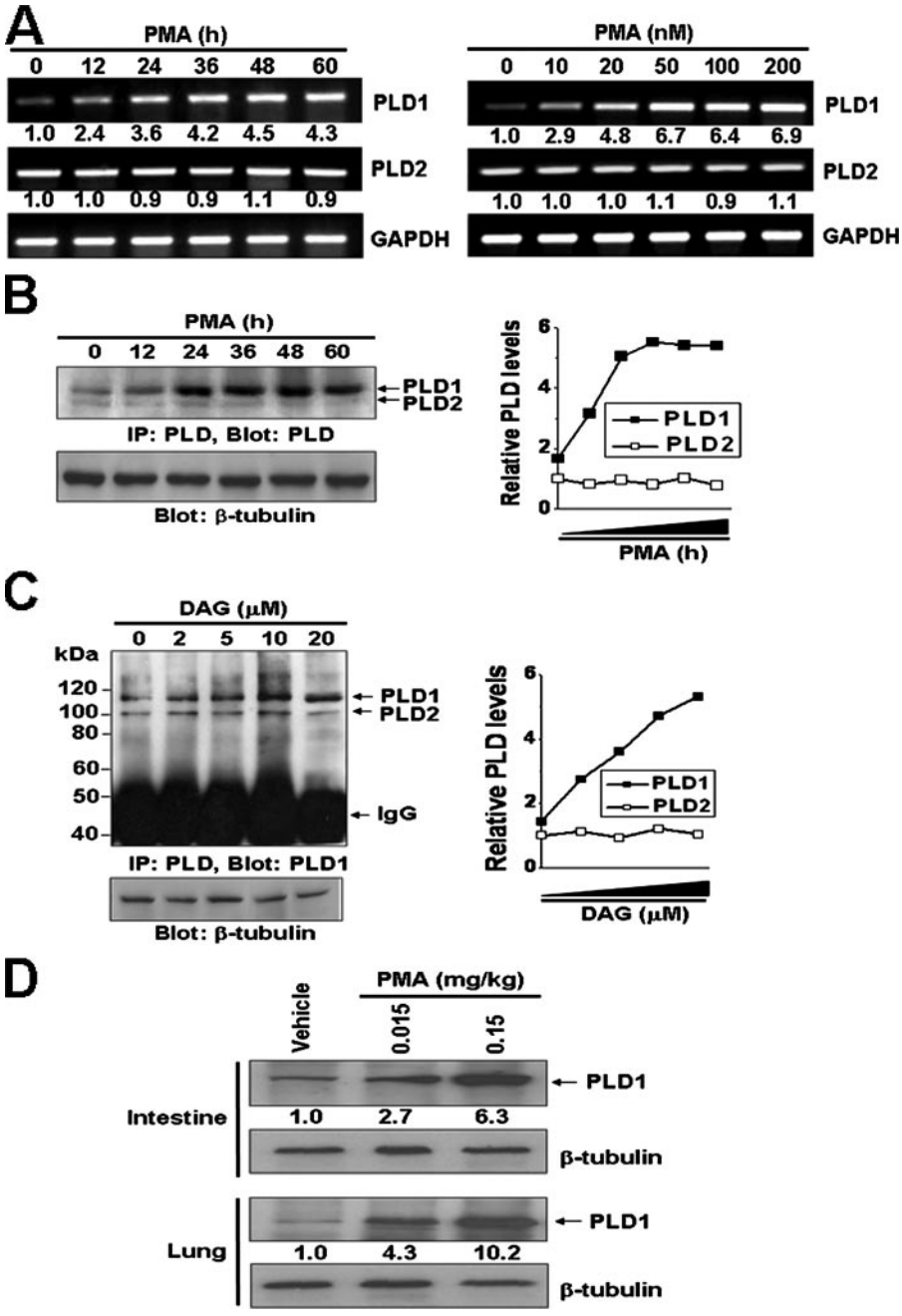

FIGURE 2. **PMA selectively up-regulates the expression of PLD1 in HCT116 cells and mice.** *A*, HCT116 cells were treated with vehicle or PMA (100 nM) for the indicated times or with various concentrations of PMA for 24 h. Expression of *PLD* isozymes and *GAPDH* was analyzed by RT-PCR. The levels of PLD expression were determined by densitometer analysis. The cells were treated with PMA (100 nM) for various times (*B*) or treated with various concentrations of DAG for 24 h (*C*). PLD expression was analyzed by immunoprecipitation and Western blot analysis. These blots are representative of results obtained from three experiments. *D*, mouse tissues obtained after peritoneal injection of PMA were analyzed for PLD expression using Western blot analysis. These blots are representative of results obtained from three experiments.

icopathological features and PLD expression in colorectal carcinoma. Overexpression of PLD isozymes was detected in 74 (60.1%) of 122 colorectal cancers (Table 1). However, no significant association was found between the PLD expression level and tumor stage or lymph node metastasis (Table 1). Additionally, to determine the proliferative capacity in CRCs with overexpression of PLD, we also examined the correlation between the expression of PLD and the expression of Ki-67, β-catenin, or p-AKT in cancer samples. We compared the expression pattern of the proteins in tumor cells and corresponding normal cells and interpreted results as being positive or showing overexpression when immunostaining was stronger than that of corresponding normal cells. In immunohistochemical analyses, corresponding normal colonic mucosa showed negative or weak staining for β-catenin, Ki-67, and p-Akt (Fig. 1, *C*, *E*, and *G*). PLD isozymes were significantly co-overexpressed with early stage cancer marker genes, such as Ki-67, β-catenin, or p-Akt in colorectal carcinoma (Fig. 1, *B*, *D*, *F*, and *H*, and Table 1). These findings suggest that PLD plays an important role in the progression of early stages of development of colon carcinogenesis.

PMA Up-regulates the Expression of PLD1, but Not PLD2, in Human Colon Cancer Cells and Mice—To investigate the signaling pathways responsible for PLD expression in CRC, we examined the effects of PMA on PLD expression in HCT116 cells. Treatment with PMA markedly increased *PLD1* mRNAexpressioninatime-anddose-dependent manner (Fig. 2*A*). In contrast, *PLD2* isozyme was not significantly affected by PMA. In addition, the induction of *PLD1* mRNA levels by PMA resulted in the corresponding induction of PLD1 protein (Fig. 2*B*). Moreover, DAG increased the expression of PLD1 protein, but not PLD2, in a dose-dependent manner (Fig. 2*C*). To assess the physiological relevance *in vivo*, we examined the PLD expression after intraperitoneal injection of PMA into five different mice. Strikingly, PMA treatment significantly induced the expression of PLD1 in the intestine and lung tissues, compared with vehicle (Fig. 2*D*). However, PLD2 expression in these tissues was not detected by anti-PLD antibody, which recognizes both PLD1 and PLD2. Taken together, these results demonstrate that PMA, which is known to activate PKC and then PLD, selectively up-regulates the expression of PLD1 in HCT116 cells as well as mice.

Cloning of the Promoter Region of Human PLD1 and PLD2 Genes—To determine how PMA is able to regulate PLD gene expression and what its transcriptional mechanism might be, we have cloned the promoter regions of human *PLD1* (pGL4-

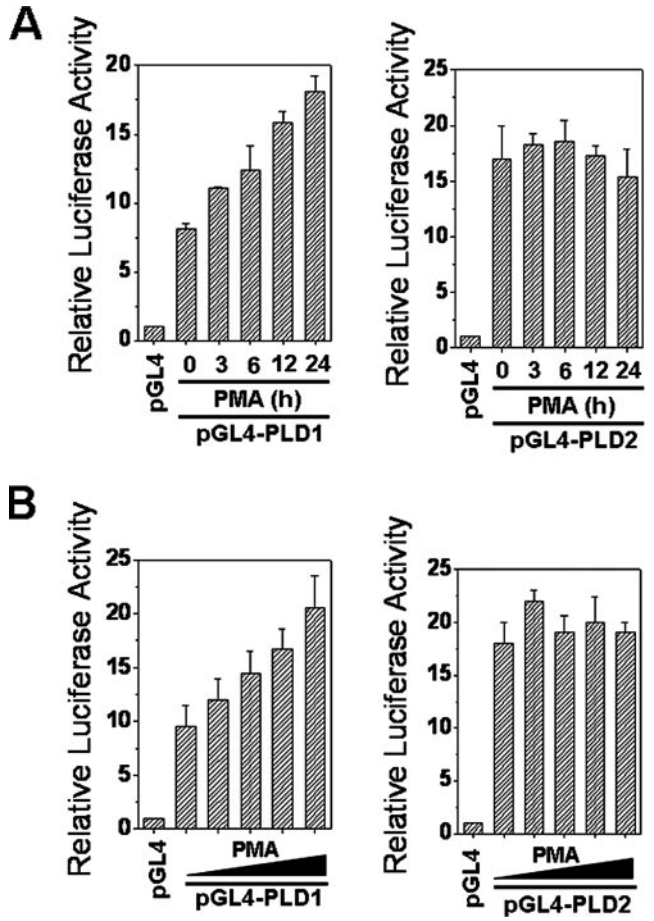

FIGURE 3. **PMA selectively stimulates the promoter activity of *PLD1* but not that of *PLD2*.** HCT116 cells were transiently transfected with the reporter constructs and treated with vehicle or 100 nM PMA for the indicated times (*A*) or with various concentrations of PMA (10, 20, 50, 100 nM) for 24 h (*B*), and luciferase activity was subsequently measured. A *Renilla* luciferase control vector was co-transfected to normalize the transfection efficiency. Values represent averages of four independent assays, and S.D. values are shown by *error bars*.

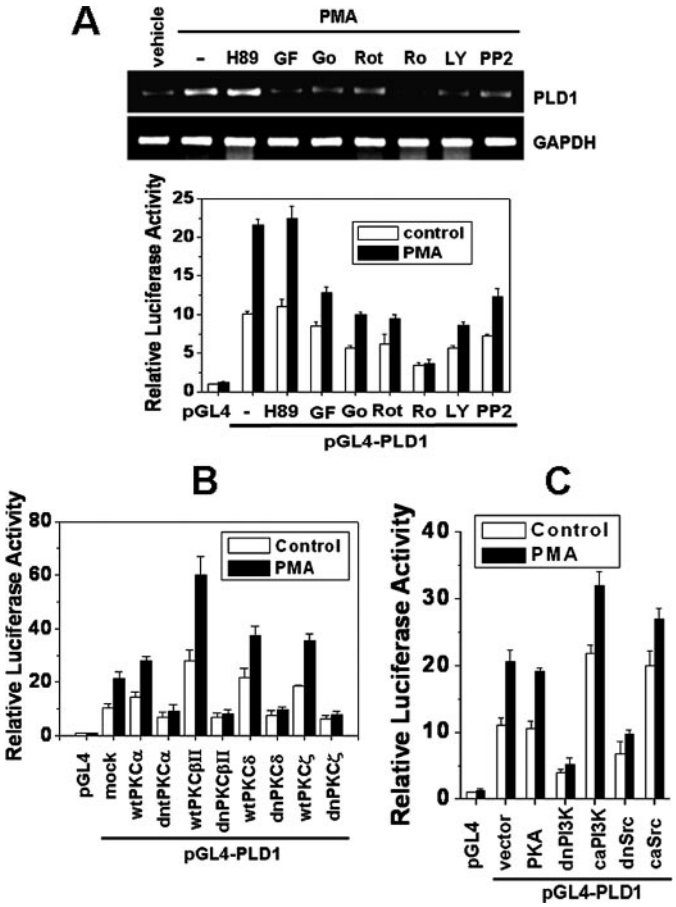

FIGURE 4. **PMA-induced PLD1 expression is dependent on PKC, PI3K, and Src.** *A*, HCT116 cells were pretreated with various inhibitors and treated with or without 100 nM PMA. The expression of *PLD1* and *GAPDH* was determined by RT-PCR analysis (*top*). The cells were transfected with pGL4-PLD1 reporter construct and pretreated with the indicated inhibitor for 1 h, followed by treatment with 100 nM PMA for 20 h (*bottom*). The concentrations of inhibitors used are as follows: 10 μM H89, 2 μM GF109203x (*GF*), 10 μM Gö-6976 (*Go*), 5 μM Rotterin (*Rot*), 5 μM R0-31-8220 (*Ro*), 20 μM LY294002 (*LY*), 10 μM PP2. *B* and *C*, the cells were co-transfected with pGL4-PLD1 and the indicated expression vectors, treated with 100 nM PMA for 24 h, and then measured for luciferase activity. *wt*, wild type; *dn*, dominant negative; *ca*, catalytically active. Results shown are means ± S.E. of three independent experiments.

PLD1; 1.9 kb) transcribed from exon 2 among two alternate transcripts of *PLD1* to be transcribed at two different transcription sites (exons 1 and 2) and human *PLD2* (pGL4-PLD2; 2.6 kb) transcribed from exon 1, on the basis of human genome data from the NCBI data base. Supplemental Fig. S1 shows the schematic diagrams of genomic DNA structure and the promoter regions of the human *PLD1* and *PLD2* genes.

PMA Increases the Transcriptional Activity of PLD1 Promoter—To investigate whether PMA increases the transcriptional activities of *PLD* genes, HCT116 cells were transiently transfected with *PLD-luc* and treated with PMA. PMA increased the promoter activity of *PLD1* in a time- and dose-dependent manner but did not increase the promoter activity of *PLD2* (Fig. 3, *A* and *B*). The *PLD2* promoter showed higher basal activity compared with that of *PLD1*. These results demonstrate that PMA selectively up-regulates the transcriptional activity of *PLD1* but not that of *PLD2*.

PMA-stimulated PLD1 Induction Is Mediated via the PKC Pathway—Therefore, to examine the signaling pathways leading to PMA-mediated PLD1 induction, HCT116 cells were pretreated with various PKC inhibitors (GF109203x, Go6976, Ro-31-8220, Rottlerin) and PI3K inhibitor (LY294001) or a protein kinase A inhibitor (H89) prior to the addition of PMA. As shown in Fig. 4*A*, PMA-induced *PLD1* mRNA expression was blocked by all of the PKC inhibitors, inhibitors of PI3K, and Src tyrosine kinase but not by a protein kinase A inhibitor. Furthermore, the effects of these inhibitors on mRNA expression of *PLD1* were correlated with the results obtained from the *PLD1* promoter assay, which suggests that the effects are mediated through an increase in transcription (Fig. 4*A*). To further confirm this observation, the cells were transfected with various constructs with *PLD1-luc*. Wild-type PKC-α, -βII, -δ, or -ζ stimulated *PLD1* promoter activity, but its dominant negative mutants suppressed both basal and PMA-stimulated promoter activities (Fig. 4*B*). Furthermore, dominant negative PI3K (p85) and Src, but not protein kinase A, inhibited PMA-stimulated *PLD1* promoter activity (Fig. 4*C*). However, dominant active PI3K (p110) and Src enhanced its activity. Taken together, these results, which utilized complementary approaches, dem-

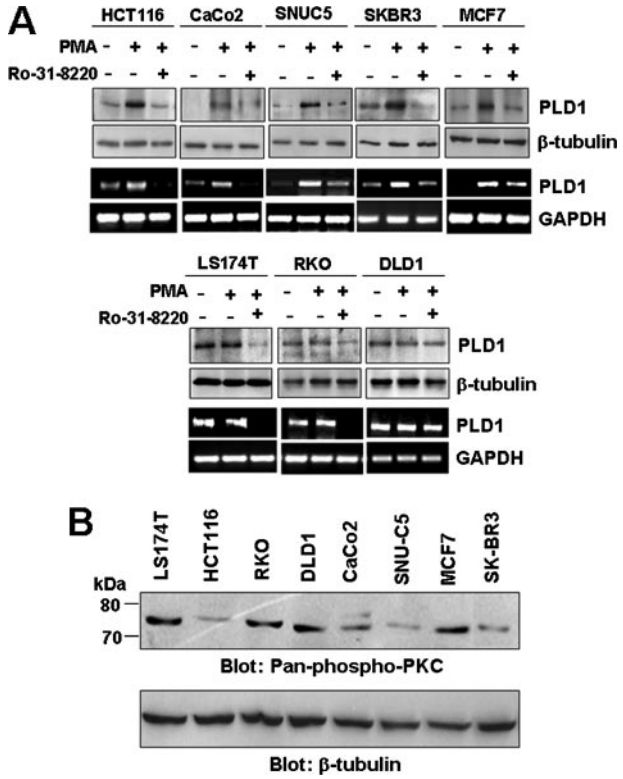

FIGURE 5. **Cell-specific differences in PMA-mediated PLD1 induction.** *A*, various cancer cells were pretreated with Ro31-8220 (5 μM) and stimulated with PMA (100 nM) for 24 h. The proteins were extracted and analyzed by Western blotting using antibody to PLD1 or β-tubulin. Total RNA was extracted, and expression for *PLD1* and *GAPDH* was determined by RT-PCR. *B*, to examine the endogenous PKC activation states, lysates from these cells were analyzed by immunoblotting using anti-pan-phospho-PKC antibody. These blots are representative of results obtained from three experiments.

onstrate a contributory role for these PKCs, PI3K, or Src in PMA-mediated PLD1 induction.

PMA-induced PLD1 Expression Occurs in Other Human Cancer Cell Lines—To examine whether PMA-induced expression of PLD1 is specific to the HCT116 cells or also occurs in other human cancer cells, the colon cancer cells (HCT116, CaCo2, LS174T, RKO, DLD1, and SNU-C5) and breast cancer cells (SK-BR3, MCF7) were treated with PMA, and *PLD1* expression was analyzed by RT-PCR and Western blot analysis (Fig. 5*A*). The induction of *PLD1* mRNA and protein was observed in the HCT116, CaCo2, SNU-C5, SK-BR3, and MCF7 cancer cells and was blocked by Ro31–8220. In contrast, PMA-induced PLD1 expression was not detected in LS174T, RKO, and DLD1 cells. Using antibody to pan-phospho-PKC, we found that the cells that were responsive to PMA (except for MCF7 cells) showed relatively low basal PKC activation compared with that of the cells that were unresponsive to PMA (Fig. 5*B*). For DLD1 cells, the PKC inhibitor did not greatly affect PLD1 expression. Although responsiveness to PMA may be partly related to the extent of basal PKC activation in different cancer cells, it is possible that these cell-specific differences in PMA-induced PLD1 expression might be related to differences in the genetic profiles of these tumors.

Ras/Raf/ERK Pathways Are Required for the Induction of PLD1 by PMA—We next investigated whether Ras, a downstream target of PKC (26), is also involved in the signaling cascade. As shown in Fig. 6*A*, expression of Ras-N17 (inactive mutant) significantly suppressed both basal and PMA-stimulated *PLD1* promoter activity. Conversely, expression with Ras-V12 (active mutant) significantly stimulated its promoter activity. Ras activates multiple parallel pathways that involve Raf, MEK, and ERK kinases (26). Like Ras, Raf enhanced *PLD1* promoter activity in a similar pattern (Fig. 6*A*). These results indicate that Ras and Raf are involved in PMA-induced PLD1 up-regulation. Moreover, we investigated the role of MEK/ERK, acting downstream of Ras, in the regulation of PLD1 expression. Pretreatment with MEK inhibitors (PD98059, U0126) and overexpression of the dominant negative mutant of MEK2, but not p38 inhibitor (SB203580), significantly blocked PMA-mediated *PLD1* expression, as measured by RT-PCR and a promoter assay (Fig. 6*B*). The JNK inhibitor had no effect on the induction of PLD1 (data not shown). PKC βII-stimulated PLD1 induction was suppressed by the expression of dominant negative mutants of Ras, Raf, MEK2, and ERK2 (Fig. 6*C*). Furthermore, we examined the relationship between enhanced PLD1 expression and PLD activity. As shown in Fig. 6*D*, transfection of PKC-βII, Ras-V12, catalytically active Raf, and MEK, but not its dominant negative mutant, enhanced PLD activity, which suggests that PKC and these proteins are responsible for an increase in PLD activity in the colon cancer cells. In addition, PMA increased the phosphorylation of ERK, Akt, and Src kinase in a time-dependent manner (data not shown). Moreover, we investigated whether depletion of the supposedly involved protein affects PLD1 expression. Using siRNA, the depletion of PKCα, Ras, and ERK2 reduced the endogenous PLD1 protein level (Fig. 6*E*). Taken together, these results suggest that PMA stimulated the induction of PLD1, and thus, the activity might be mediated via PKC/Ras/Raf/MEK/ERK signaling pathways in HCT116 colon cancer cells.

NFκB Induces PMA-mediated PLD1 Expression, but Not PLD2 Expression, and Increases PLD Activity—PMA is known to activate NFκB, which has been shown to be a downstream target gene of MEK/ERK (27). Thus, we investigated whether NFκB plays a role in PMA-stimulated PLD1 induction. As shown in Fig. 7*A*, pretreatment with PDTC, an NFκB inhibitor, and overexpression of the dominant negative mutant of IκBα (S32A/S36A) significantly blocked PMA-mediated PLD1 transcriptional expression. However, ectopic expression of NFκB (p65) enhanced the promoter activity, protein expression, and enzymatic activity of PLD1 in a dose-dependent manner but did not significantly affect those of PLD2 (Fig. 7, *B–D*). Furthermore, transfection of siRNA for *NFκB* (*p65*) decreased PMA-stimulated PLD1 protein expression (Fig. 7*E*). Taken together, these results demonstrate that NFκB is required for the PMA-induced expression and activation of PLD1.

Identification of NFκB-binding Sites Responsible for PMA-induced PLD1 Expression—Next, we attempted to examine the regions that are responsible for PMA-induced PLD1 expression. Treatment with PMA and ectopic expression of NFκB enhanced the promoter activity of PLD1 in all of the deletion constructs (Fig. 8*A*). We found four putative NFκB-binding sites in the *PLD1* promoter using bioinformatics (Fig. 8*B*). To determine if NFκB (p65) could directly bind to the *PLD1* promoter *in vivo* and to further define the NFκB response elements

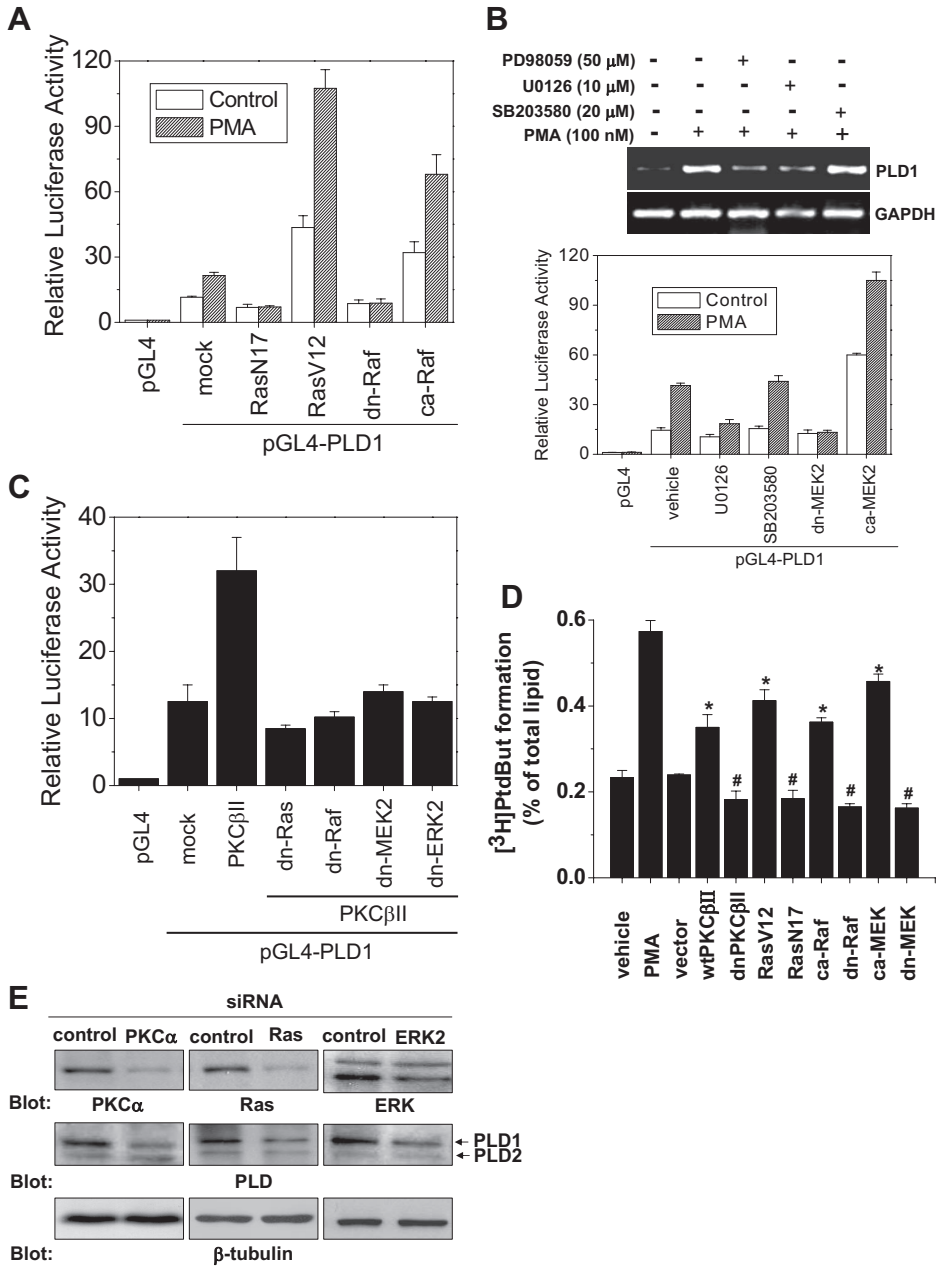

FIGURE 6. **PMA-induced PLD1 expression is mediated by a Ras/Raf/ERK pathway.** *A*, HCT116 cells were cotransfected with pGL4-PLD1 and the indicated expression vectors. After 16 h of transfection, the cells were treated with 100 nM PMA for 20 h, and the luciferase activity was then measured. *B*, the cells were pretreated with the indicated inhibitors and treated with PMA for 24 h. Expression of *PLD1* and *GAPDH* was analyzed by RT-PCR (*top*). The cells were transfected with pGL4-PLD1, pretreated with the indicated inhibitors, and treated with PMA for 20 h. After 16 h of cotransfection with pGL4-PLD1 plus the dominant negative or active mutant of MEK2, the cells were treated with 100 nM PMA for 20 h, and the luciferase activity was then measured (*bottom*). *C*, HCT116 cells were cotransfected with pGL4-PLD1 and the indicated expression vector. Activities were normalized to the reading for the *Renilla* luciferase internal control and are shown as the average of four assays with S.D. value shown as *error bars*. *D*, the cells were transfected with various constructs for 24 h, labeled with [^{3}H]myristate for 12 h, and treated with or without PMA (100 nM) for 1 h, and the PLD activity was then measured as described under "Experimental Procedures." Results shown are means ± S.E. of three independent experiments. *, $p < 0.01$; #, $p < 0.05$ compared with mock transfectants. *E*, the cells were transfected with siRNA specific for *PKCα*, *K-Ras*, and *ERK2* or control siRNA for 48 h. The lysates were immunoblotted with the indicated antibodies. These blots are representative of results obtained from three experiments.

(NFκB-1 and NFκB-4), showing especially strong binding of NFκB to the NFκB-1 site. These results are comparable with those of the deletion promoter assay. As a positive control, the binding of NFκB to the promoter of *c-IAP2* was enhanced by PMA (28). These data suggest that NFκB may play a role as an activator of the transcription of *PLD1*. Furthermore, mutagenesis of NFκB-1 and NFκB-4 sites attenuated PMA-induced *PLD1* promoter activity (Fig. 8*C*). These findings demonstrate the functional importance of NFκB in the PMA-induced induction of PLD1 in HCT116 cells.

PMA-induced PLD1 Induction Is Involved in the Secretion of MMP-9 via Ras/ERK/NFκB in Colon Cancer Cells—MMPs are well known to be involved in the invasion of tumor cells. PLD has been shown to be essential for the induction of MMP-9 expression by several stimuli (29–31). Thus, we examined whether PMA-induced PLD1 induction is involved in the secretion of MMP-9 in HCT116 colon cancer cells. As shown in Fig. 9*A*, inhibitors that were specific for Ras (L744832), ERK (U0126), and NFκB (PDTC), which are involved in the induction of PLD1 by PMA, suppressed PMA-induced MMP-9 secretion and PLD1 induction. Moreover, depletion of PLD1 inhibited PMA-induced MMP-9 secretion, as analyzed by zymography. Taken together, these results suggest that PMA-induced expression of PLD1 via a Ras/ERK/NFκB pathway might mediate invasiveness associated with the secretion of MMP-9 in HCT116 cells.

Depletion of PLD Decreases Cell Viability and Increases Apoptosis—Furthermore, we attempted to investigate the effect of the depletion of PLD isozyme on cell viability and apoptosis in cancer cells. As shown in Fig. 10, the depletion of PLD1 or PLD2 using siRNA decreased cell viability and increased apoptosis compared with those of control siRNA, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and Annexin V binding assay. Depletion of both PLD1 and PLD2 significantly suppressed cell viability and enhanced apoptosis compared with that of either PLD isozyme. Taken together, these results suggest that

to the promoter, a chromatin immunoprecipitation assay was carried out after treatment with PMA. PCR was performed using primers designed to amplify the four putative NFκB-binding regions, as shown in Fig. 8*B*. PMA enhanced the binding of p65 to the two NFκB-binding sites of the *PLD1* promoter

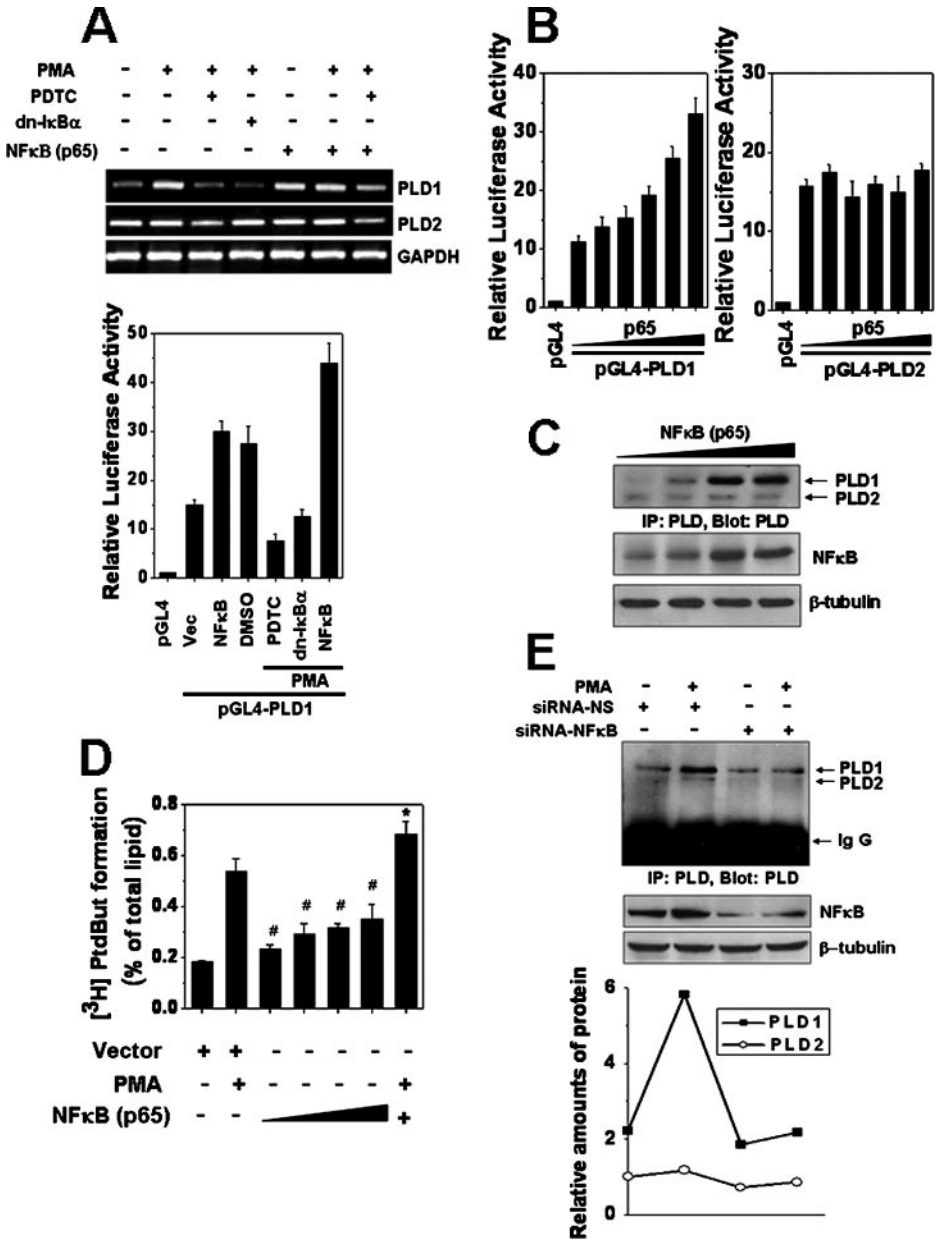

FIGURE 7. **NFκB is required for the transcriptional activation of *PLD1* by PMA.** *A*, HCT116 cells were preincubated for 30 min with PDTC (50 μM) and then treated with PMA (100 nM) for 24 h. Cells were transfected with the indicated expression vector. After 12 h, cells were treated with PMA or vehicle (*top*). The expression of *PLD* isozymes was examined using RT-PCR. The same methods were performed after transfection of pGL4-PLD1, and the luciferase activity was measured (*bottom*). *B*, the cells were cotransfected with *NFκB* (*p65*) at various doses (0, 50, 100, 200, 400, and 600 ng) and with pGL4-PLD, and the luciferase activity was then measured. *C*, HCT116 cells were transfected with *NFκB* (*p65*) at various doses (0, 0.5, 1, and 2 μg), and the lysates were immunoprecipitated or immunoblotted with antibodies to the indicated antibodies. *D*, the cells were transfected with *NFκB* (p65) at various doses for 24 h, labeled with [^{3}H]myristate for 12 h, and treated with or without PMA (100 nM) for 1 h, and the PLD activity was then measured as described under "Experimental Procedures." Results shown are means ± S.E. of three independent experiments. #, $p < 0.05$ *versus* vector; *, $p < 0.05$ *versus* PMA treatment. *E*, the cells were transfected with siRNA specific for *NFκB* or control siRNA (*NS*, nonspecific) and were treated with or without 100 nM PMA for 24 h. The lysates were immunoprecipitated and immunoblotted with anti-PLD antibody. The levels of PLD expression were determined by densitometer analysis. The expression levels of NFκB (p65) and β-tubulin proteins were determined by Western blot analysis. These blots are representative of results obtained from three experiments.

PLD might play some roles in the cell survival and antiapoptotic functions in cancer cells.

DISCUSSION

Abnormalities in the expression and activity of PLD have been reported in various human cancers using small numbers of tumor samples, but its clinicopathological significance remains unclear. In this study, using 122 colorectal carcinomas obtained from surgery, we found that PLD overexpression was detected in 74 (60.1%) of 122 colorectal cancers. However, no significant association was found between the PLD expression level and tumor stage or lymph node metastasis. Our observations indicated that PLD isozymes are significantly co-overexpressed in human CRCs with Ki-67, β-catenin, or p-Akt, which are involved in the early stages of the development of colon cancer. Thus, it is suggested that PLD may contribute to the progression of colorectal carcinoma in the clinical setting.

Despite extensive information regarding the regulation of PLD activity in cell function, the signaling mechanisms that regulate PLD expression in cancer remain undefined. In the present study, we demonstrate for the first time that activation of PKC by PMA enhances the expression of PLD1, but not that of PLD2, via an NFκB-dependent pathway in colon cancer cells and suppresses apoptosis. Selective induction of PLD1 by PMA was also confirmed in the tissues of mice injected with PMA, thus suggesting its physiological relevance. Moreover, PMA-induced expression of PLD1 was not noted in all of the cancer cell lines examined. The specificity of PLD1 induction in certain cancer cells might be partly associated with endogenous PKC activation states, depending on cell types, but there are the other possible explanations for this phenomenon, including different genetic profiles of cancer cells or the sensitivity of the assays.

In this study, we found for the first time that PMA selectively upregulates the induction of PLD1, but not PLD2, via a Ras/Raf/MEK/ERK/NFκB signaling pathway in HCT116 colorectal cancer cells. Elevated expression of these induced proteins also enhanced PLD activity. Moreover, depletion of these supposedly involved proteins reduced the PLD1 protein level. Recently, Kikuchi *et al.* (32) reported that Ewing's sarcoma fusion protein induces the expression of the *PLD2* gene but not that of the *PLD1* gene.

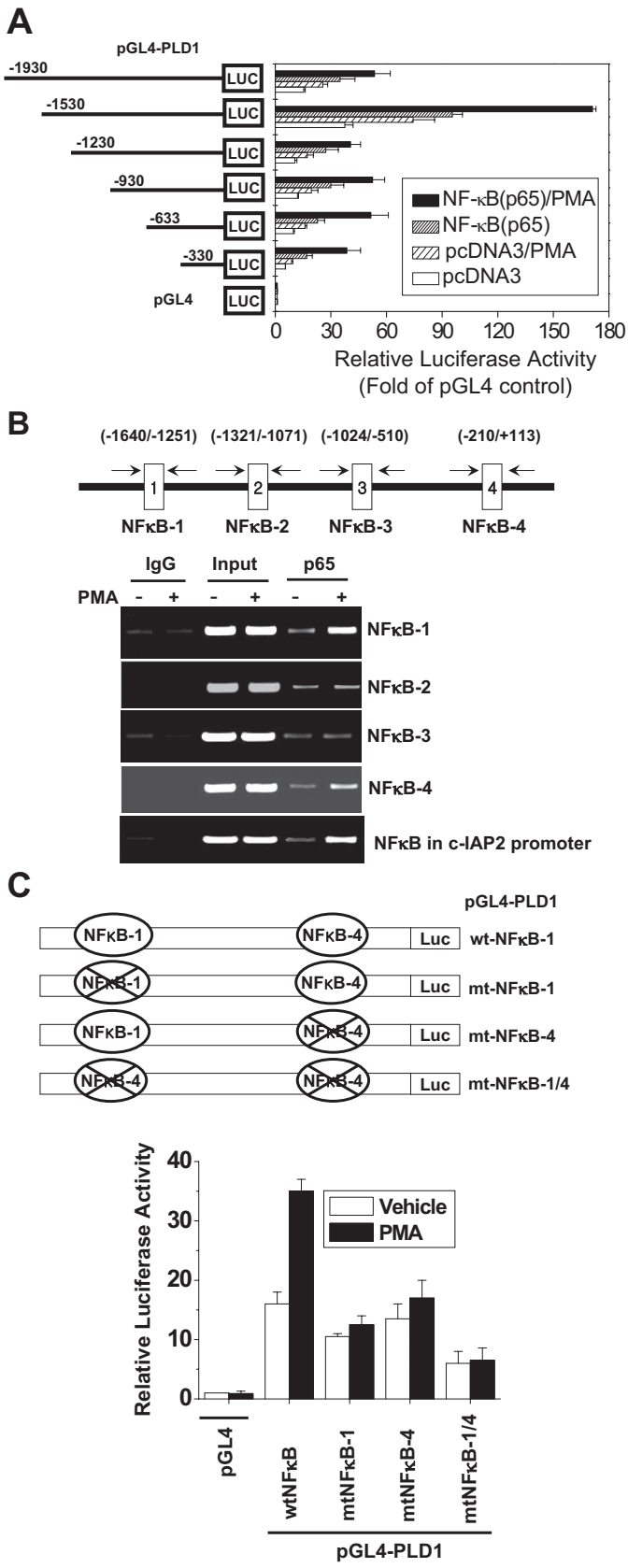

FIGURE 8. **Identification of NFκB elements responsive for PMA-induced PLD1 expression.** *A*, deletion analysis of pGL4-PLD1 in HCT116 cells. A schematic representation of pGL4-PLD1 reporter constructions containing various lengths is shown. The cells were cotransfected with pGL4-PLD1 and *NFκB* and were then stimulated with 100 nM PMA for 24 h. *B*, HCT116 cells were treated with 100 nM of PMA for 6 h. A ChIP assay using the indicated primers and antibodies was performed for identification of *NFκB*-binding sites present in the *PLD1* promoter. A ChIP analysis of *c-IAP2* promoters was performed as a control. *C*, HCT116 cells were transfected with wild-type pGL4-PLD1 or mutant pGL4-PLD1 and were then treated with or without 100 nM PMA for 24 h. The luciferase activity was measured. Results shown are means ± S.E. of three independent experiments.

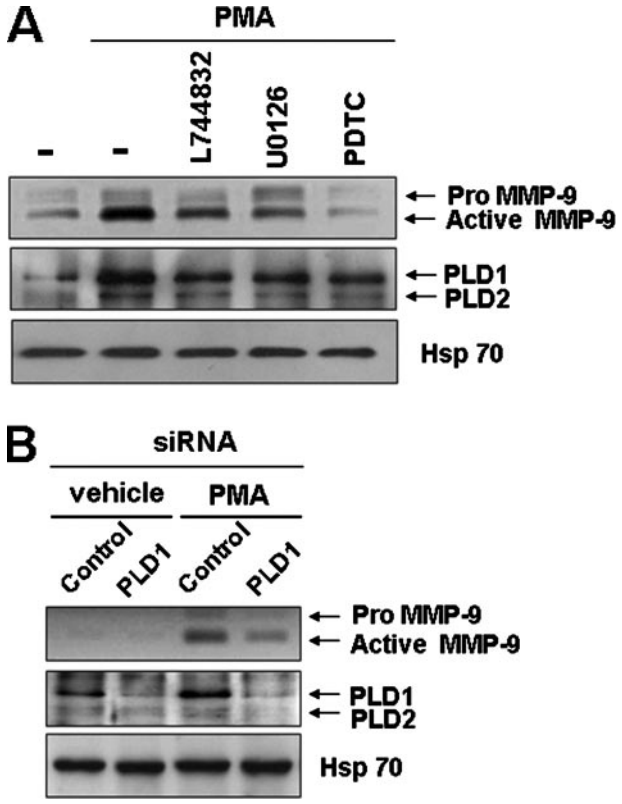

FIGURE 9. **PMA-induced PLD1 induction is involved in the secretion of MMP-9 via Ras/ERK/NFκB in colon cancer cells.** *A*, HCT116 cells were pretreated with or without L744832 (10 μM), U0126 (10 μM), and PDTC (50 μM) and treated with 50 nM PMA for 24 h. *B*, the cells were transfected with siRNA for control or *PLD1* and were then treated with or without 50 nM PMA for 24 h. The conditioned media were collected and subjected to casein zymography. The expression levels of proteins in the cell lysates were determined by Western blotting using the indicated antibodies. Data are representative of three independent experiments.

NFκB has been reported to regulate transcription of a number of target genes related to tumorigenesis and cell survival, including TRAF1, TRAF2, cIAP-1, cIAP-2, and FLIP (28, 33, 34). Analysis of the *PLD1* promoter shows the presence of two functional NFκB sites; the binding of these sites to the *PLD1* promoter is enhanced by PMA, and the mutation of these sites suppresses PMA-induced PLD1 expression. NFκB plays a role in the enhanced survival of colon cancer cells, and inhibition of NFκB sensitizes colon cancers that are resistant to chemotherapy-induced apoptosis (35). These results suggest that NFκB activation is a critical step in the tumor-promoting effect of PMA. Despite these important findings, the signaling pathways involved in PMA regulation of genes that are directly involved in cell death or death resistance remain largely unknown. Our current study shows that expression of PLD1 is regulated through the PKC-stimulated activation of NFκB.

The secretion of MMP-9 from cancer cells (36, 37) leads to hydrolysis of the extracellular matrix, thus enabling cells to

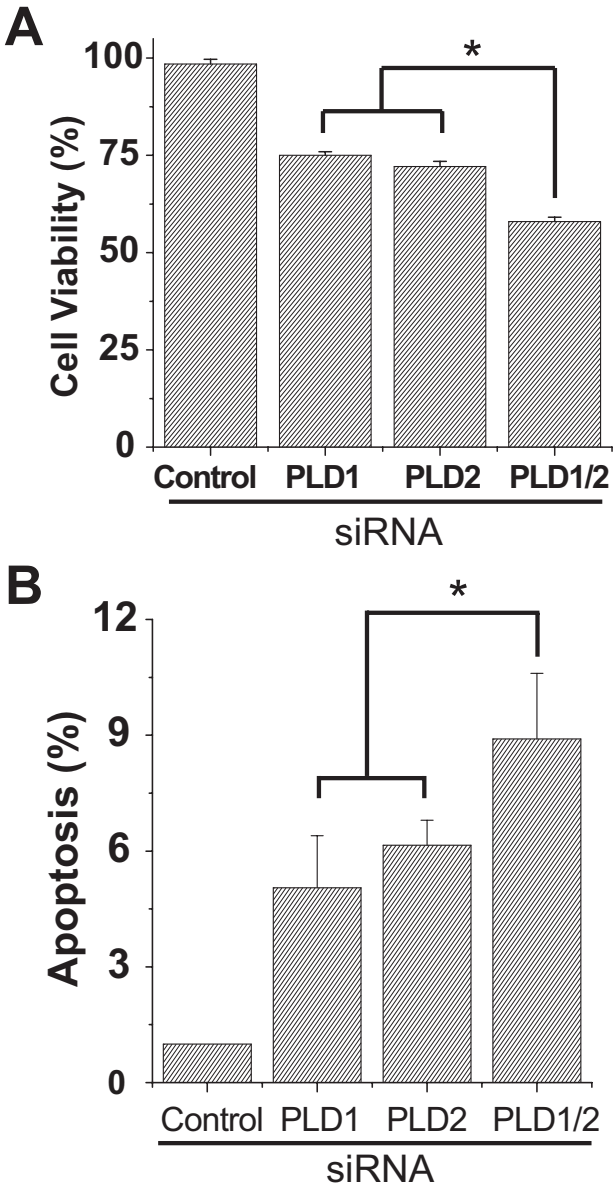

FIGURE 10. **Depletion of PLD increases apoptosis and reduces cell viability.** HCT116 cells were transfected with control siRNA or siRNA specific for *PLD1* or *PLD2*. *A*, cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as described under "Experimental Procedures." *, $p < 0.05$ *versus* either small interfering PLD transfection. *B*, apoptosis was measured using an Annexin V staining kit. Results shown are means ± S.E. of four independent experiments. *, $p < 0.05$ *versus* either siPLD transfection.

break out of their primary site into the circulation and from there to secondary sites. MMP-9 secretion is induced by various agonists that differ among cell lines (36). MMP-9 is secreted in response to PMA. PLD is known to be essential for the induction of MMP-9 secretion by several stimuli, including PMA. We found that inhibition of the proteins (Ras/ERK/NFκB) involved in the PMA-stimulated induction of PLD1 suppresses PMA-induced MMP-9 secretion and PLD1 protein level. Reduced expression of PLD1 using siRNA also inhibited PMA-stimulated secretion of MMP-9. Furthermore, depletion of PLD decreased cell viability and increased apoptosis, thus suggesting the involvement of PLD in survival and anti-apoptosis.

In conclusion, we demonstrate selective induction of PLD1 in human colon cancer cells and mice by PMA. Taken together, our current study is, to our knowledge, the first to demonstrate that the induction of PLD1 through a PKC/Ras/ERK/NFκB pathway is involved in the secretion of MMP-9 associated with invasiveness in colon cancer cells. In addition, our finding identifies PLD1 as a novel downstream target molecule of NFκB, which contributes to tumorigenesis. Since PLD has emerged as a critical regulator of cell proliferation and invasion, the selective modulation of PLD expression levels in certain cancers might be useful in sensitizing resistant cancers to the effects of anti-cancer drugs.

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Supplementary Data

Supplementary Methods

Rapid amplication of 5′ complementary DNA ends

The transcription start sites of the hPLD1 were mapped by using the CapFishing™ Full-length cDNA Premix Kit (Seegene, Korea) according to the manufacturer's instructions. This technique involved the incorporation of a "CapFishing™ adaptor" onto the 5′ end of the reverse-transcribed cDNA for the 5′ RACE analysis. The 5′ end of hPLD1 was then amplified by using a standard PCR protocol. Primers were designed so that a relatively large section of the coding region (120 ~ 280 bp) was amplified during the nested PCR, along with the 5′ UTR. Reaction products were analyzed by 1.5% agarose gel electrophoresis, purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). A purified 5′ RACE fragment was cloned into the pGEM-T Easy vector (Promega) and sequenced.

Cloning of promoter regions of exon 2 from hPLD1 and site-directed mutagenesis

A 1930-bp promoter region of hPLD1 transcribed from exon 2 was amplified by PCR using primers based on the genomic DNA sequence of the hPLD1 gene. The PCR product was purified using gel extaction Kit (Qiagen) and subcloned into the KpnI/BglII site in pGL4-14b-basic reporter vector with luc reporter gene (Promega, Madison, WI, USA). The PCR-based method was used to clone serially deleted PLD1 promoter constructs into the pGL4-14b reporter vector at the KpnI and BglII site. The primers were used as follow :

-1530 5′- GGGTACCCACCAGCGAGGTGCATTCTAAAC -3′,

-1230 5′- GGGTACCCGAGACTCATTGAGTTATAATAAGGGC -3′,

-930 5′- GGGTACCGATTTGCAGTAAGAAAGGAACATGTGGC -3′,

-630 5′- GGGTACCGTCTTTCGGAATAGGTATATTAATCAATTT -3′,

-333 5′- GGGTACCGCTTTCCCAAACCAATCTCCCTTG -3′

The same antisense oligonucleotide primer that corresponded to +1 (5′-GAAGATCTTAGGAAAGAAGAAAACGGTTACAAAGACTTAG -3′) was used for the generation of the clones. All PCR products were sequenced in both directions by

using primers designed from a known sequence. A site-specific mutation was introduced into the NFκB (for mtNFκB1, forward primers, 5′- CCT TCG CTC CCA TTT TTC AAT TTA GAA ACA CAG TCT CTT TCT C -3′ and reverse primer, 5′- GAG AAA GAG ACT GTG TTT CTA AAT TGA AAA ATG GGA GCG AAG G -3′; for mtNFκB4 forward primers, 5′- GTC TGG ATT CAA ATT GTG CAA TAG AGT TTT ATG TTA TCA AAA TGC -3′ and reverse primer, 5′- GCA TTT TGA TAA CAT AAA ACT CTA TTG CAC AAT TTG AAT CCA GAC -3′ binding sites on PLD1 promoter by Quick Change Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA) according to the manufacturer's instructions.

Cloning of the hPLD2 promoter region

A 2,671-bp of hPLD2 promoter region was amplified by PCR using primers based on the genomic DNA sequence of the hPLD2 gene. The PCR product was purified gel extaction Kit (Qiagen) and subcloned into the Kpn I/Bgl II site in pGL4-14b reporter vector with luc reporter gene (Promega, Madison, WI, USA) and then sequenced in both directions by using primers designed from a known sequence.

RT-PCR analysis.

PCR reactions were cycled as follows: initial denaturation at 95°C for 2 min and then 30 cycles for PLD1, PLD2 and GAPDH at 95°C for 40 sec, 58°C for 40 sec, and 72°C for 40 sec. Forward primer of GAPDH was 5′- GTG GTC TCC TCT GAC TTC AAC -3′, and the reverse primer was 5′- TCT CTT CCT CTT GTG CTC TTG -3′ with 197 bp amplified fragments. The forward primer of PLD1 gene was 5′- TGT CGT GAT ACC ACT TCT GCC A -3′, and the reverse primer was 5′- AGC ATT TCG AGC TGC TGT TGA A -3′ with 531 bp amplified fragments. The forward primer of PLD2 gene was 5′- CAT CCA GGC CAT TCT GCA C -3′, and the reverse primer was 5′- GTG CTT CCG CAG ACT CAA GG -3′ with 412 bp amplified fragments.

Detailed Chip assay

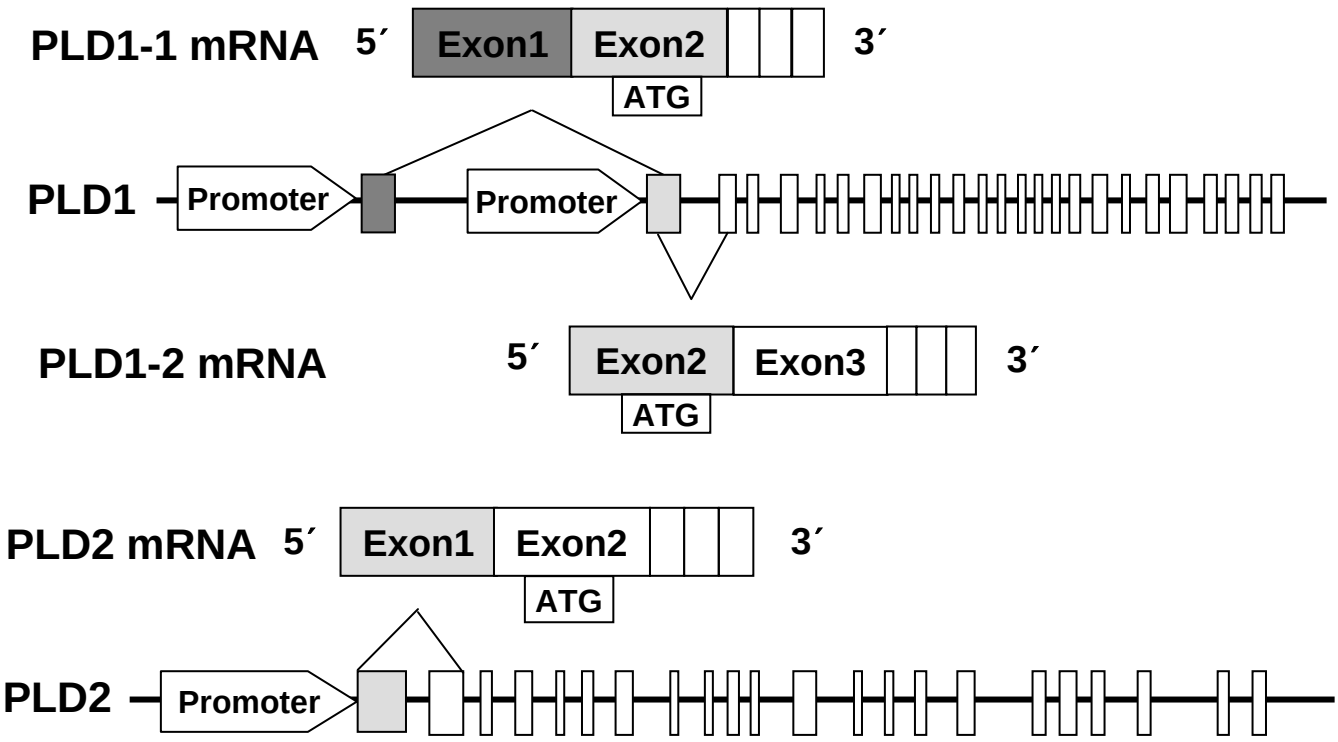
HCT116 cells treated with or without PMA were used for the crosslinking with 1% paraformaldehyde in phosphate-buffered saline for 10 min. The cells were scraped and collected by centrifugation. Cells were lysed in lysis buffer, and normal rabbit IgG or anti-NFκB (p65) antibody was added and incubated for 8 h at 4 ^{o}C. The immnunocomplexes were extracted 3 times with 1% SDS, 0.1M $NaHCO_3$, and crosslinking was reversed by incubating at 65 ^{o}C overenight. The saved chromatin input

fraction was also processed in the same manner. The samples were then digested with proteinase K at 45 ^{o}C for 2 h, and extracted with phenol/chloroform/isoamylalcohol. DNA was purified by ethanol precipitation, and resuspended in 20 µl of nuclease-free water. As a positive control, the c-IAP2 promoter region was amplified by PCR using primers 5′-GCA ATG ATC GTC CTC TCT ATA TGG-3′ (forward) and 5′-GTC TCA CGC TGT CTT TTA AAT GC-3′ (reverse). The PLD1 promoter regions were amplified by PCR using primers; for NFκB1 (-1640 ~ -1251 bp), 5′- CCT TAT CTA TAA AGA GGG GAT GGC- 3′ (forward), 5′- GTC GGG AGA AAG AGA CTG TGT TTG G -3′ (reverse), for NFκB2 (-1321 ~ -1071 bp), 5′- ATG TCT GGC CAA ACC AGA ACC - 3′ (forward), 5′- CAA GGT CTT AGG CTT CTT GAG AAT G -3′ (reverse), for NFκB3 (-1024 ~ -510 bp), 5′- GCA CCC ATA TCA GGT GCT CCT TAA TC -3′ (forward), 5′- CTC TGT AGG TGA TAT CCC AAG C -3′ (reverse), for NFκB4 (-210 ~ -113 bp), 5′- CAC ACA GAG CAG GCT GAA TTG -3′ (forward), 5′- GCT CAG ATC ATC CGT CTT TAC C -3′ (reverse). Amplification was carried out for 38 cycles, which was determined to be within the linear range. PCR products were analyzed by 2 % agarose gel electrophoresis with ethidium bromide staining.

Figure legends

S1. Diagrammatic representation of hPLD1 and hPLD2 promoter constructs. **A,** Two alternate transcripts of hPLD1 are transcribed from exon 1 and exon 2, respectively. hPLD2 is transcribed from exon 1. **B,** Transcription start site transcribed from exon 2 and exon 1 was represented as +1 (transcript 2) and -55 (transcript 1), respectively. Total RNA isolated from HCT116 cells was subjected to a 5′ RACE reaction using 5′ adaptor primer mix and 3′ PLD1-specific antisense primer. Following amplification, the 5′ RACE products were visualized as two alternate transcripts on 1.5% agarose gels stained with ethidium bromide.

A


PLD1-1 mRNA
5′
Exon1
Exon2
3′
ATG
PLD1
Promoter
Promoter
PLD1-2 mRNA
5′
Exon2
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Promoter

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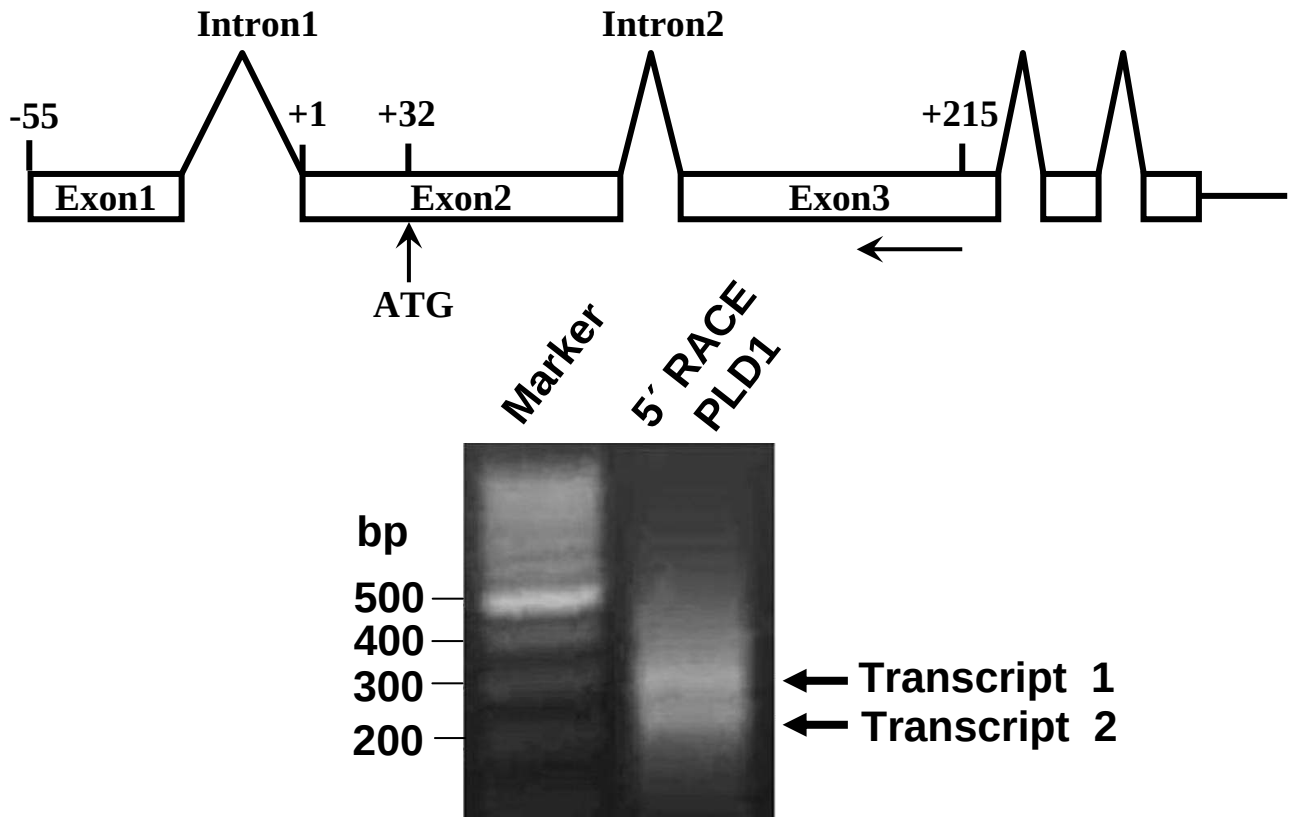
Intron1
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-55
+1
+32
+215
Exon1
Exon2
Exon3
ATG
Marker
5′ RACE PLD1
bp
500
400
300
200
Transcript 1
Transcript 2

Mechanisms of Signal Transduction:

Phorbol Ester Up-regulates Phospholipase D1 but Not Phospholipase D2 Expression through a PKC/Ras/ERK/NFκB-dependent Pathway and Enhances Matrix Metalloproteinase-9 Secretion in Colon Cancer Cells

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