

Phospholipase D Prevents Etoposide-Induced Apoptosis by Inhibiting the Expression of Early Growth Response-1 and Phosphatase and Tensin Homologue Deleted on Chromosome 10

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Abstract

Phospholipase D (PLD) has emerged as a critical regulator of cell proliferation and survival signaling. We show for the first time that elevated expression of PLD isozymes attenuates expression of the tumor suppressors early growth response-1 (Egr-1) and the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) tumor suppressor and apoptosis during etoposide treatment. When formation of phosphatidic acid was inhibited by overexpression of catalytically inactive PLD during etoposide treatment, expression of Egr-1 and PTEN and the apoptotic effect of etoposide were not inhibited. This suggests that PLD inhibits expression of these tumor suppressors and inhibits apoptosis. Deletion of a specific Egr-1-binding site present in the PTEN promoter blocked etoposide-induced PTEN activity and elevated expression of PLD decreased the sensitivity to apoptosis induced by ectopic expression of Egr-1. Etoposide-induced activation of Akt was potentiated by overexpression of PLD and PLD-stimulated suppression of Egr-1 was blocked by inhibition of phosphatidylinositol 3-kinase/Akt survival pathway at the both transcriptional and posttranscriptional levels. These results show that survival signals generated by PLD attenuate expression of Egr-1 by activation of phosphatidylinositol 3-kinase signaling pathway and induction of PTEN by Egr-1, which confers resistance to apoptosis. (Cancer Res 2006; 66(2): 784-93)

Introduction

Tissue homeostasis is maintained by a delicate balance between apoptosis and cell growth. Tumor growth results when these mechanisms are deregulated. Apoptosis is regulated by numerous genes. The identification and characterization of the function of these genes will thus help to define the process of cell death and identify possible targets for therapeutic intervention.

The early growth response-1 (Egr-1) transcription factor (also known as NGFI-A, TIS8, Krox-24, and Zif268) is a member of the

immediate-early gene family and involved in the regulation of cell growth and apoptosis. Recent reports have implicated the *Egr-1* gene in tumor suppression (1, 2). Overexpression of Egr-1 in transformed cells suppresses the growth of cells cultured in soft agar and tumor growth in nude mice (3, 4). Egr-1 is down-regulated in several types of neoplasia and tumor cells (3, 5). These studies suggest that the decrease in Egr-1 may cause loss of cellular homeostasis and that it may play a pivotal role in tumorigenesis. Clearly, the identification of Egr-1-responsive genes would constitute a significant step toward understanding the mechanistic basis of the role of Egr-1. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) exercises its role as tumor suppressor by negative regulation of the phosphatidylinositol (PI) 3-kinase/Akt (PI3K/Akt) signaling pathway (6, 7). Recently, it has been reported that Egr-1 activates the *PTEN* gene during UV irradiation and etoposide treatment (8). Despite the significance of Egr-1 and PTEN for cancer and apoptosis, much less is known about the mechanism responsible for the regulation of expression of these tumor suppressors.

Many lines of evidence have established that deregulation of growth-promoting proto-oncogenes is involved in cancer progression. PI3K/Akt is the most studied survival-signaling pathway (9). A phospholipid-modifying enzyme, phospholipase D (PLD), has recently been implicated in the generation of survival signals. PLD catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. There are two PLD isozymes, PLD1 and PLD2 (10, 11), both of which have been implicated in mitogenic signaling (12). PLD has emerged as a critical regulator of cell proliferation and survival; it also prevents apoptosis (13). Although it has been suggested that abnormal expression of PLD isozyme may be associated with tumor development in some tumors, a detailed role of PLD overexpression has not been fully elucidated. Recently, we reported that overexpression of the PLD1 and PLD2 isozymes causes anchorage-independent growth of cells in soft agar and represses expression of the *p21* gene via p53-dependent and p53-independent pathways (14, 15). We also found that transmodulation between c-Src and PLD contributes to the promotion of cellular proliferation by amplifying mitogenic signaling pathways (16). Elevated expression of PLD increases MDM2 expression and suppresses p53 protein stabilization (17). PLD also prevents apoptosis in cells transformed by v-Src and in the MDA-MB-231 human breast cancer cell line (18). Thus, the paradigm that is emerging for PLD is that it provides the signal that inhibits apoptotic cascades. However, the mechanism by which PLD promotes cell survival is still unclear. In this report, we present

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evidence showing that survival signals generated by PLD inhibit expression of the proapoptotic tumor suppressor Egr-1 and that induction of PTEN by Egr-1 confers resistance to apoptosis.

Materials and Methods

Materials. DMEM, fetal bovine serum (FBS), and LipofectAMINE Plus were purchased from Life Technologies, Inc. (Rockville, MA). Dual luciferase assay kits were from Promega (Madison, WI). Etoposide, cisplatin, Adriamycin, and rapamycin were from Calbiochem (Nottingham, United Kingdom). The antibodies to Egr-1 and PTEN were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against phospho-Akt and Akt were obtained from Cell Signaling Technology (Boston, MA). Polyclonal antibody that recognizes both PLD1 and PLD2 was generated as previously described (19).

Cell culture. NIH 3T3 mouse fibroblasts overexpressing wild-type PLD1 or PLD2 were described previously (14). NIH 3T3 mouse fibroblasts overexpressing catalytic inactive mutant of PLD1 or PLD2 were obtained by transfection, using LipofectAMINE Plus reagent (Life Technologies) according to instructions of the manufacturer. Transfected cells were selected with G418 (500 µg/mL) for 21 days at 37°C. All cells were maintained in DMEM supplemented with 10% FBS.

Construction of plasmids. The pEBS1⁴luc construct was generously provided by Dr. Seung Joon Baek (University of Tennessee, Knoxville, TN). The Egr-1 promoter reporter construction p-668egrLuc [by inserting 688 bp region (from -668 to +1) of the human *Egr-1* gene into a pGL2-basic luciferase plasmid] has been described (20). The full-length human PTEN promoter (*PTEN-luc*) and its mutant constructs, including *PTEN-luc* deleted in three putative Egr-1 binding sites (Δ 117) and individually mutated three putative Egr-1 binding sites (mut A, mut B, and mut C), were generously provided by Dr. Ian de Belle (The Burnham Institute, Cancer Research Center, La Jolla, CA). Catalytically inactive PTEN (C124S) cDNA were generously provided by I.S. Kang (Kyung Hee University, Seoul, South Korea). pGL2-Ecadherin and pGL2-2XSp1 were generously provided by Dr. Kyung Lib Jang (Pusan National University, Busan, South Korea).

4',6-Diamidino-2-phenylindole staining. 4',6-Diamidino-2-phenylindole (DAPI) staining was done as described previously (21). Before staining, the cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and then washed with PBS. DAPI was added to the fixed cells for 1 hour, after which they were examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei.

Measurement of phosphatidylserine expression using fluorescein-labeled (FITC) Annexin V. Annexin V binding and PI uptake were assessed by flow cytometry using a commercial kit (Boehringer Ingelheim Bioproducts, Indianapolis, IN) according to the instructions of the manufacturer. Briefly, $\sim 2.5 \times 10^5$ cells for each sample were washed twice in PBS and the pellet was resuspended in 200 µL of the binding buffer provided in the kit. A 5 µL of the fluorescein-labeled (FITC)-Annexin V kit stock solution were added to the cell suspension (1 µg/mL final concentration) and incubated for 10 minutes at room temperature in the dark. Then, the cells were washed in PBS and resuspended in 190 µL of binding buffer plus 10 µL of the PI stock solution (1 µg/mL final concentration). The cells were immediately analyzed with a FACScan flow cytometer equipped with an excitation laser line at 488 nm and Cell Quest software. The FITC-Annexin V (green fluorescence) and the PI (red fluorescence) were collected on a log scale through a 530 ± 20 and 575 ± 15 nm band pass filter, respectively (22).

Analysis of DNA fragmentation. The DNA fragmentation study was done as described elsewhere (18). In brief, cells were gently lysed for 30 minutes at 4°C in a buffer containing 5 mmol/L Tris buffer (pH 7.4), 20 mmol/L EDTA, and 0.5% Triton X-100. After centrifugation at $12,000 \times g$ for 5 minutes, supernatants containing the soluble fragmented DNA were collected and extracted with phenol/chloroform/isoamyl alcohol (25:24:1, by volume) and analyzed electrophoretically on 1.8% agarose gel containing ethidium bromide.

PLD assay. PLD activity was assessed by measuring the formation of [³H]phosphatidylbutanol, the product of PLD-mediated transphosphatidy-

lation, in the presence of 1-butanol. Cells were subcultured in six-well plates at 2×10^5 per well, and then incubated in the presence of 1 Ci/mL [³H]myristic acid. After overnight labeling, the cells were washed thrice with 5 mL of PBS and pre-equilibrated in serum-free DMEM for 1 hour. PLD catalyzed transphosphatidylation in presence of 0.5% 1-butanol, and the extraction and characterization of lipids by TLC were done as previously described (16).

Western blot analysis. Protein levels of PLD, Egr-1, PTEN, and β -tubulin were determined by immunoblotting equal amounts of proteins with specific antibodies. The bands were developed with an enhanced chemiluminescence system (ECL kit, Amersham, Buckinghamshire, United Kingdom).

Transfection and Luciferase assay. Cells were plated in 12-well plates at 1×10^5 per well and grown to 50% to 60% confluence. Plasmid mixtures containing 0.25 µg of promoter linked to luciferase, 0.25 mg of expression vector, and 0.025 µg of pGL2 (Promega) were transfected by LipofectAMINE Plus according to the protocol of the manufacturer. After 24 hours of transfection, the cells were treated with or without etoposide for 3 hours. Following stimulation, cells were washed with PBS and lysed in luciferase lysis buffer. The activities of *firefly* and *Renilla* luciferase in the cellular extracts were measured using the dual-luciferase reporter assay system. Relative luciferase activity was obtained by normalizing the firefly luciferase activity against the internal control *Renilla* luciferase activity.

Results

Elevated expression of PLD1 or PLD2 suppresses etoposide-induced apoptosis. The DNA-damaging anticancer drug, etoposide, is a topoisomerase II inhibitor. It is currently in clinical use and induces apoptotic cell death (23). To examine the role of PLD in the survival signal response to etoposide, we established NIH 3T3 mouse fibroblast cells that overexpressed PLD isoforms. We found that apoptosis induced by the etoposide was suppressed in cells that overexpressed PLD1 or PLD2, compared with that of control cell, using DNA fragmentation assay (Fig. 1A). Taken together, these data suggest that both PLD1 and PLD2 confer resistance to etoposide-induced apoptosis. To examine whether elevated PLD activity could provide a survival signal for overcoming etoposide-induced apoptosis, we used cells that had been stably transfected with vectors, wild-type PLD1, wild-type PLD2, or the catalytically inactive mutant of PLD1 (K898R) or PLD2 (K758R). These mutants can block the effects of PLD signaling (24). The expression of PLD isoforms was confirmed by Western blot analysis (Fig. 1B). We used an Annexin V binding assay to quantify etoposide-induced apoptosis (Fig. 1B). Cells stained with Annexin V conjugate (*M2*, right side) are apoptotic cells; viable cells (negative for both Annexin and PI) are shown on the left side (*M1*). Etoposide induced cell death in $\sim 25\%$ of vector-transfected cells but the effect of etoposide was attenuated in cells that overexpressed PLD1 ($\sim 9\%$) and PLD2 ($\sim 8\%$). Moreover, the effects of PLD1 and PLD2 were clearly activity dependent because expression of the catalytically inactive forms of PLD1 and PLD2 did not inhibit apoptosis. Therefore, we suggest that PLD activity is involved in the suppression of etoposide-induced apoptosis. After application of etoposide, we treated the cells with DAPI, which specifically stains nuclei, and observed the cells using fluorescence microscopy. Furthermore, DAPI staining showed that treatment with phosphatidic acid, a product of PLD activity, prevented etoposide-induced apoptosis in cultured NIH 3T3 fibroblasts and C6 glioma cells (Fig. 1C). This inhibition of apoptosis by phosphatidic acid was also confirmed using the Annexin V-PI flow cytometry assay (Fig. 1D) in C6 glioma cells. In addition, we found that blocking PLD activity with 1-butanol, but not 3-butanol, increased the apoptotic activity of etoposide in C6 glioma cancer cells as shown

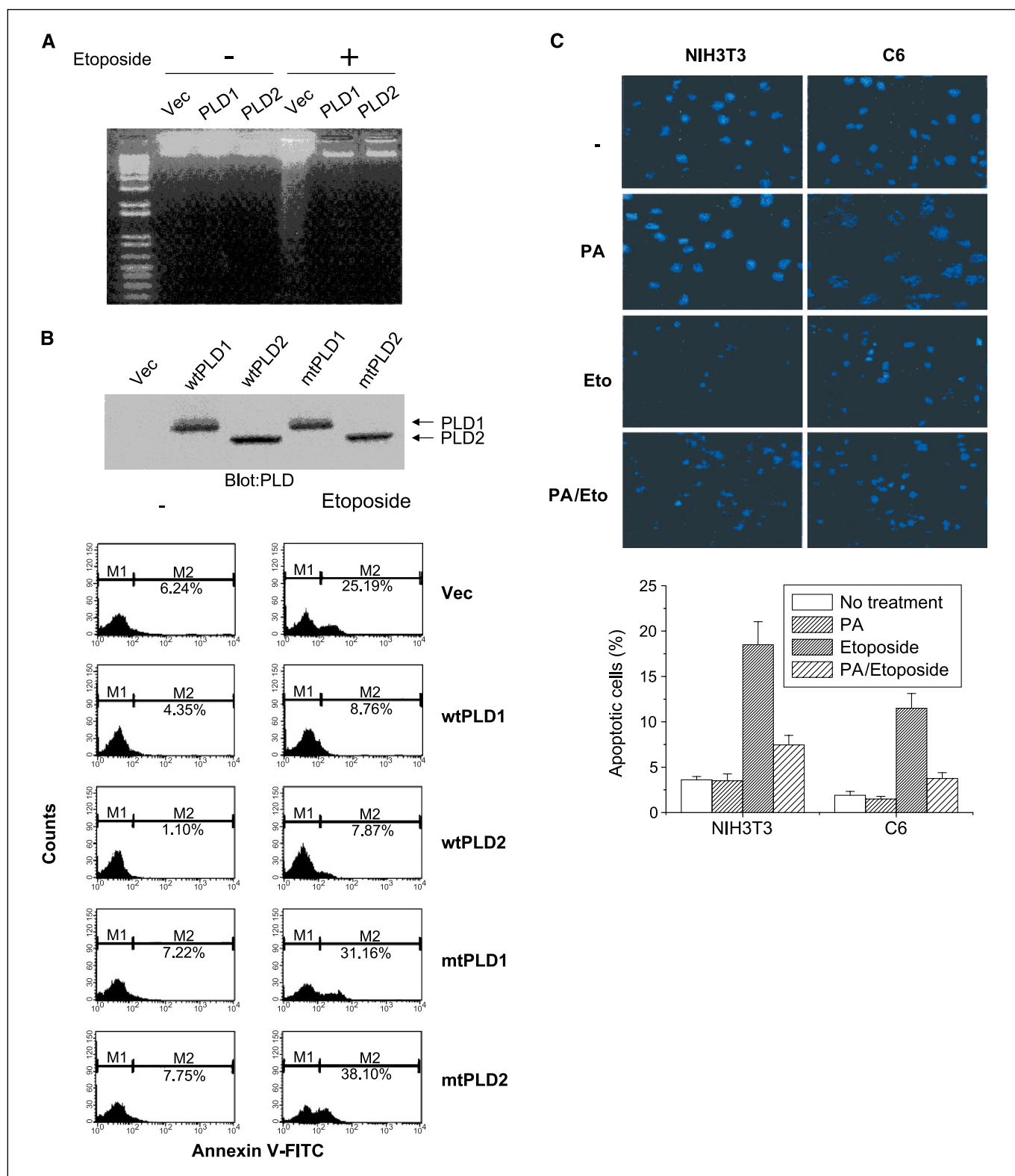


Figure 1. PLD isozymes suppress etoposide-induced apoptosis. *A*, fragmentation of genomic DNA after treatment with etoposide (25 μ M/L) for 16 hours. Fragmented DNA was extracted and analyzed on 2% agarose gel. *B*, *top*, expression of PLD in NIH 3T3 vector (Vec) cells and NIH 3T3 cells that overexpressed wild-type PLD1, mutant PLD1, wild-type PLD2, or mutant PLD2; *bottom*, cells were treated with or without etoposide (25 μ M/L) for 16 hours and analyzed with a FACScan flow cytometer equipped with an excitation laser line at 488 nm and Cell Quest software. M2, apoptotic channel. The FITC-Annexin V (green fluorescence) and the PI (red fluorescence) were collected on a log scale through 530 \pm 20 and 575 \pm 15 nm bandpass filters, respectively, as described in Materials and Methods. *C*, *top*, cultured NIH 3T3 fibroblasts and C6 glioma cells were treated with or without 25 μ M/L etoposide for 16 hours in the presence or absence of 50 μ M/L phosphatidic acid. Apoptosis was characterized by condensation and fragmentation of nuclei using DAPI staining; wt, wild type; mt, mutant; *bottom*, data in DAPI staining are expressed as a percentage of apoptotic cells relative to control (untreated) cells and means \pm SD were derived from three independent experiments.

in Fig. 1E. These data strongly suggest that PLD activity confers the resistance to etoposide.

Etoposide induces Egr-1 expression, which is suppressed by elevated expression of PLD isozymes. The mechanism through which etoposide exerts its antitumorigenic effect may involve the up-regulation of antitumorigenic or proapoptotic proteins. Because PLD isozymes are overexpressed in human cancers, such as colon and breast cancers (21–29), the roles of PLD1 and PLD2 in regulating the expression of the Egr-1 was examined. The Egr-1 protein level in mouse fibroblasts that stably overexpress PLD1 or PLD2 was examined to determine whether the decrease of Egr-1 expression in response to etoposide is associated with overexpression of PLD isozymes (Fig. 2A). Three different clonal cell lines were used for PLD1 and PLD2 to eliminate random gene aberration during transfection. Vector-transfected cells were grown under the same conditions and used as controls. As shown in Fig. 2A, etoposide induced Egr-1 expression. Treatment of cells that overexpressed PLD1 or PLD2 with etoposide decreased Egr-1 expression compared with empty vector-transfected cells. This suggests that elevated expression of PLD isozymes is sufficient to decrease the Egr-1 response to etoposide. Furthermore, we investigated the relative expression of Egr-1 and PLD in normal human mammary cells and three breast cancer cell lines (Fig. 2B–D). PLD1 expression and its activity were significantly increased in breast cancer cells compared with that in normal mammary cells (Fig. 2B and C). During etoposide-induced apoptosis, Egr-1 protein expression was suppressed in the breast cancer cell lines (MDA-MB231, SKBR3, and SCC1370). These results suggest that elevated expression and activity of PLD contributes to the down-regulation of Egr-1 in breast tumor cells.

Elevated expression of PLD suppresses etoposide-induced transactivation of a reporter with Egr-1 response elements. The transactivation of Egr-1 in etoposide-treated cells was

determined using an Egr-1-responsive reporter. The plasmid pEBS1⁴luc contained four copies of Egr-1 response elements linked to the basal promoter, followed by a luciferase reporter gene (30). We confirmed that the overexpression of Egr-1 protein and some anticancer drugs induced transactivation of a reporter with Egr-1 response elements, pEBS1⁴luc (data not shown). Various anticancer drugs, including etoposide, cisplatin, adriamycin, and rapamycin, also induced Egr-1 protein expression, which was suppressed in cells that overexpressed PLD (Fig. 3A). Taken together, these data show that etoposide and some anticancer drugs not only induce Egr-1 expression but also transactivation of a reporter with Egr-1 response elements. To examine the role of PLD in the transactivation activity of Egr-1, we used wild-type PLD1 and wild-type PLD2, and their catalytically inactive mutants, PLD1 (K898R) and PLD2 (K758R). As shown in Fig. 3B, etoposide-induced transactivation of Egr-1 was suppressed in wild-type cells that overexpressed PLD1 or PLD2 but not in cells containing the overexpressing catalytically inactive mutants of these isozymes. These results suggest that PLD activity is involved in the suppression of etoposide-induced transactivation of Egr-1.

Etoposide-induced PTEN expression is down-regulated by elevated expression of PLD isozymes and this is mediated via Egr-1. We next examined whether the effect of PLD on etoposide-induced Egr-1 was also reflected at the level of Egr-1-targeted gene expression. Recently, it has been reported that PTEN is up-regulated by Egr-1 after irradiation or etoposide and that Egr-1 can directly regulate PTEN, triggering the initial step in the apoptotic pathway (8). PTEN, a known phosphatase and tumor suppressor, is inactive in several human cancers and in disorders that are characterized by a predisposition to cancer (31). Therefore, we tried to investigate whether etoposide induces PTEN and whether its expression is attenuated in cells that overexpress PLD isozymes. We examined the temporal kinetics of etoposide-induced Egr-1

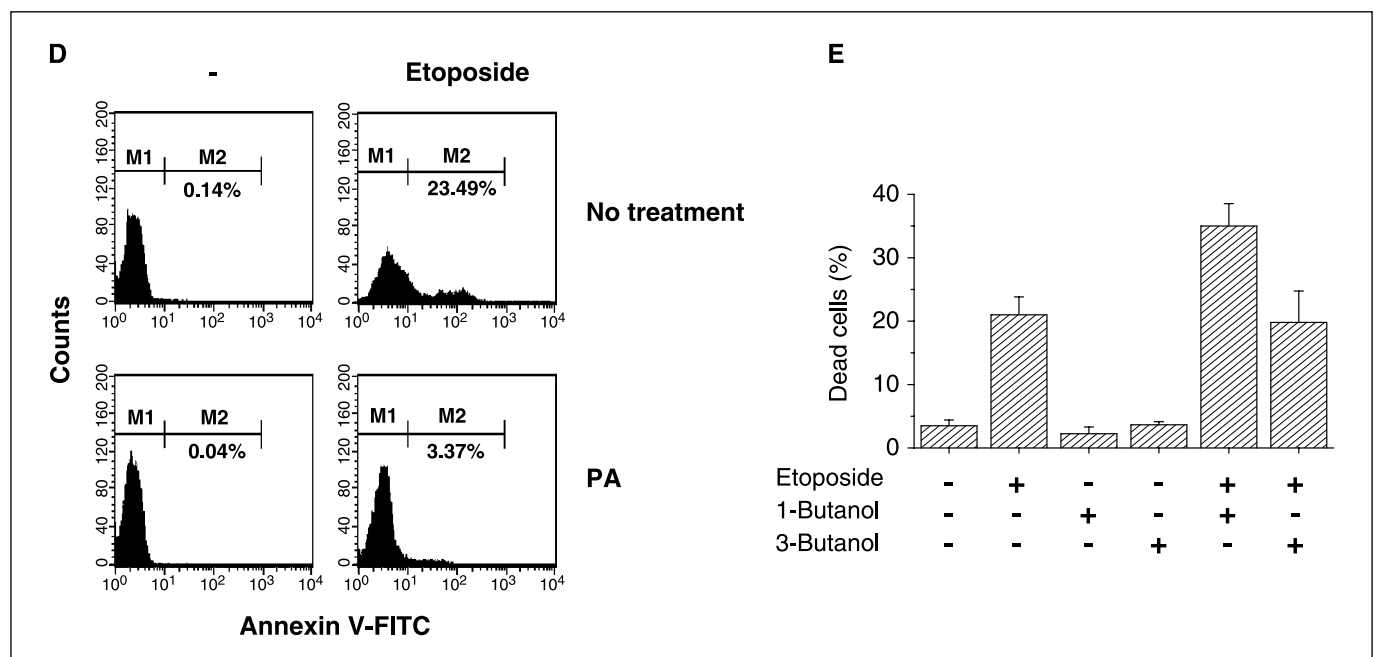


Figure 1 Continued. D, FACS analysis using Annexin V and PI staining in NIH 3T3 fibroblasts treated with or without 25 μ M etoposide (Eto) for 16 hours in the presence or absence of 50 μ M phosphatidic acid (PA). E, C6 glioma cells were treated with or without etoposide in the presence of 0.5% 1-butanol or 3-butanol. Dead cells were determined 30 hours later by trypan blue staining. Detached and trypsinized cells were pooled and incubated with 0.2% trypan blue. Cell death is shown as a percentage of blue cells. Columns, mean of three independent experiments; bars, SD.

and PTEN expression. Egr-1 protein levels after treatment with etoposide increased in vector cells, peaked at 30 to 60 minutes, and declined thereafter (Fig. 4A). A significant decline in Egr-1 protein level was observed in cells that overexpressed PLD1 or PLD2. Induction of PTEN in response to etoposide increased gradually in vector cells and peaked at 2 hours. PTEN level decreased thereafter and showed a somewhat delayed expression pattern compared with that of Egr-1. As with Egr-1, expression of PTEN by etoposide was down-regulated in cells that overexpressed PLD1 or PLD2 compared with control cells, whereas α -tubulin was similar in the three cell lines. These results indicate that PLD has a suppressive effect on the expression of PTEN.

Virolle et al. (8) reported that the region upstream of the translation start site is GC rich and contains several potential binding sites for the Egr-1 transcription factor, one of which is required for the inducible transactivation of PTEN by Egr-1. To determine the mechanism by which Egr-1 regulates PTEN expression, we used a luciferase reporter plasmid (PTEN-luc) containing a 2 kb genomic DNA fragment corresponding to the PTEN promoter and its 5' upstream regulatory sequence. To investigate whether the putative Egr-1-binding sites are involved in the regulation of the *PTEN* gene, PTEN-luc was transiently

transfected into mouse fibroblast cells with or without exogenous Egr-1 expression and incubated in the presence or absence of etoposide. Expression of PTEN-luc in mouse fibroblasts was greatly stimulated by exogenous Egr-1 expression or etoposide treatment, indicating that Egr-1 is able to stimulate PTEN promoter activity (Fig. 4B). The full-length PTEN promoter has numerous putative Egr-1 binding sites. To determine which sites are responsible for Egr-1 stimulation, a series of deletions reported by Virolle et al. (8) was used. Deletion of a 117 bp GC-rich region containing three putative Egr-1 binding sites (Δ 117) in the context of the full-length PTEN regulatory sequence (8) eliminated stimulation, narrowing the search to this short piece of DNA. We used the mutant constructs of three Egr-1 binding sites (EBSA, EBSB, and EBSC), which were individually mutated to give the mut A, mut B, and mut C constructs (8). Mutation of EBSB and EBSC did not affect induction of the PTEN promoter in response to etoposide or exogenous Egr-1 expression, whereas mutation of EBSA abolished the effect of both (Fig. 4B). These results showed that nine nucleotides between positions -947 and -939 constitute a functional *cis*-acting element necessary and sufficient for PTEN promoter stimulation by both transiently transfected Egr-1 and etoposide-stimulated endogenous Egr-1. These results were correlated

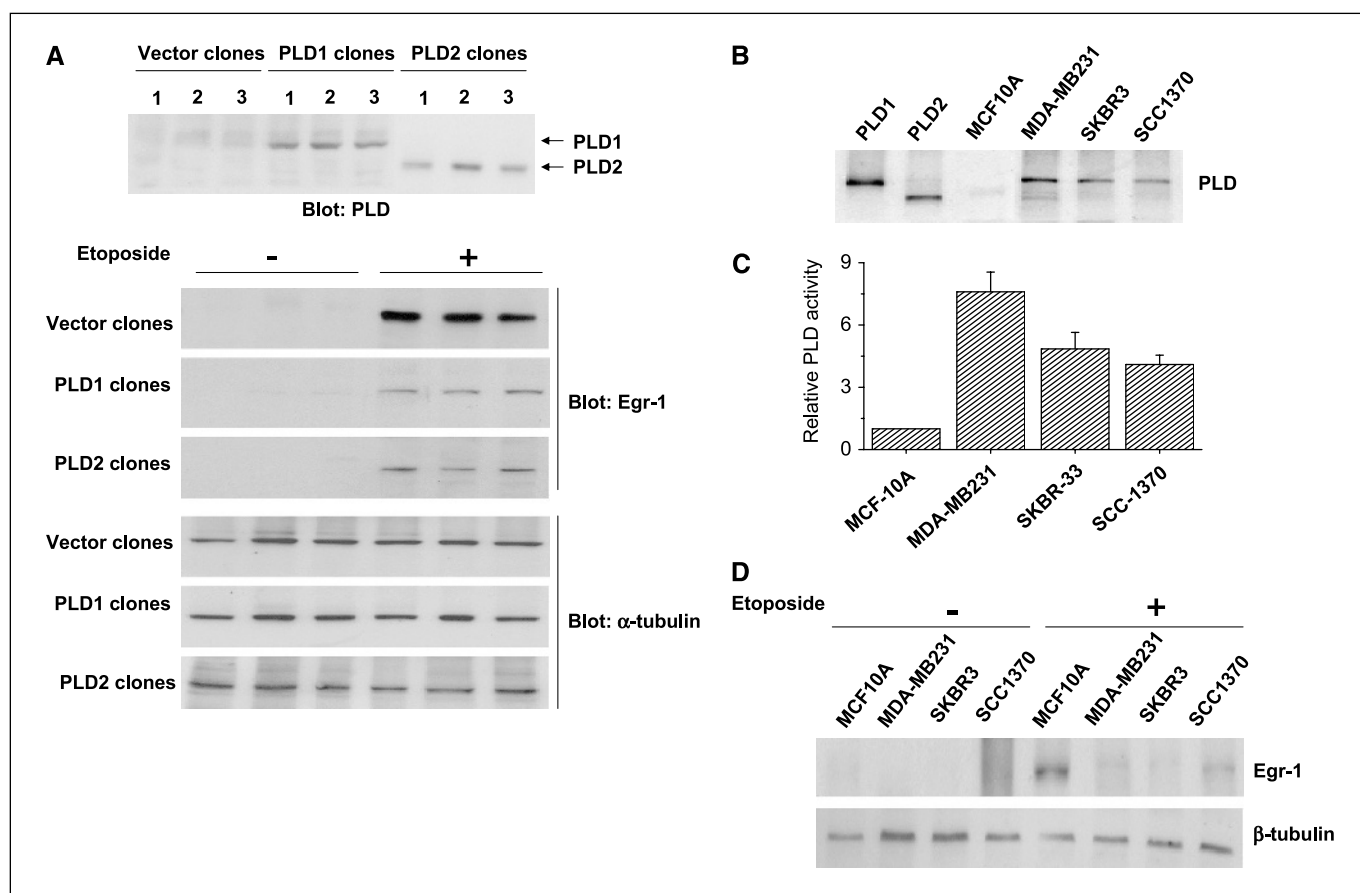


Figure 2. A, down-regulation of Egr-1 in PLD1- or PLD2-overexpressed NIH 3T3 cells and human mammary carcinoma cells. *Top*, lysates from three different NIH 3T3 clones overexpressing vector, PLD1, or PLD2 (clones 1, 2, and 3) were analyzed for PLD expression by Western blot. *Bottom*, cells were treated with 50 μ M etoposide for 2 hours. The extracted proteins were immunodetected with anti-Egr-1 or anti- β -tubulin antibody. These blots are representative of results obtained from three experiments. B, lysates of normal mammary (MCF-10A) and human mammary carcinoma cells (MDA-MB231, SKBR-3, and SCC-1370) were prepared and analyzed for PLD expression by Western blot analysis. C, PLD activity in mammary cells was determined as described in Materials and Methods. The PLD activity in the cell lines was normalized to that in MCF-10A cells, which was assigned a value of 1. Columns, mean of three independent experiments; bars, SD. D, after treatment with etoposide (50 μ M/L) for 2 hours, the cells were analyzed for Western blot using antibody to Egr-1. The same blot was stripped and reprobed with antibody to β -tubulin. These blots are representative of results obtained from three experiments.

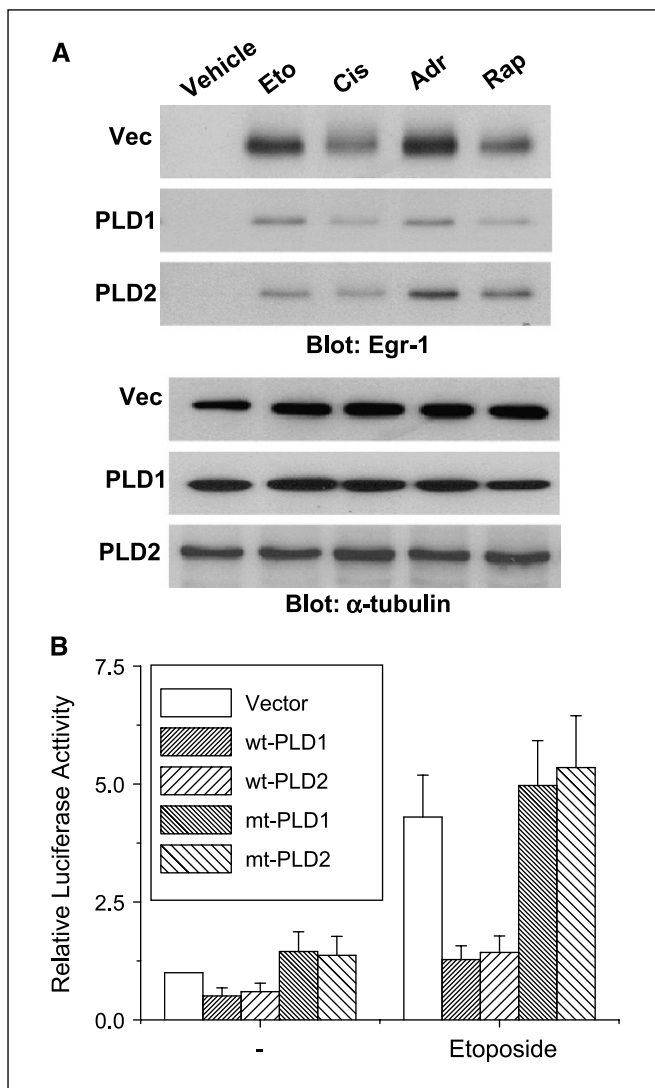


Figure 3. Elevated expression of PLD suppresses etoposide-induced transactivation of a reporter with Egr-1 response elements. **A**, vector and PLD1- or PLD2-transfected cells were treated with 25 $\mu\text{mol/L}$ etoposide, 2 $\mu\text{mol/L}$ adriamycin, 5 $\mu\text{mol/L}$ cisplatin, and 2 $\mu\text{mol/L}$ rapamycin. After 3 hours of incubation, the cell lysates were analyzed by Western blotting using antibody to Egr-1. Blots were stripped and reprobed with antibody to β -tubulin. These blots are representative of results obtained from three experiments. **B**, NIH 3T3 fibroblasts stably transfected with the vectors, wild-type PLD1, and wild-type PLD2, and their catalytically inactive mutants, PLD1 (K898R) and PLD2 (K758R), were transfected with pEBS1⁴luc for 24 hours and then treated with or without etoposide (50 $\mu\text{mol/L}$) for 3 hours. Luciferase activity was assayed as described in Materials and Methods. Columns, mean of three independent transfections; bars, SD.

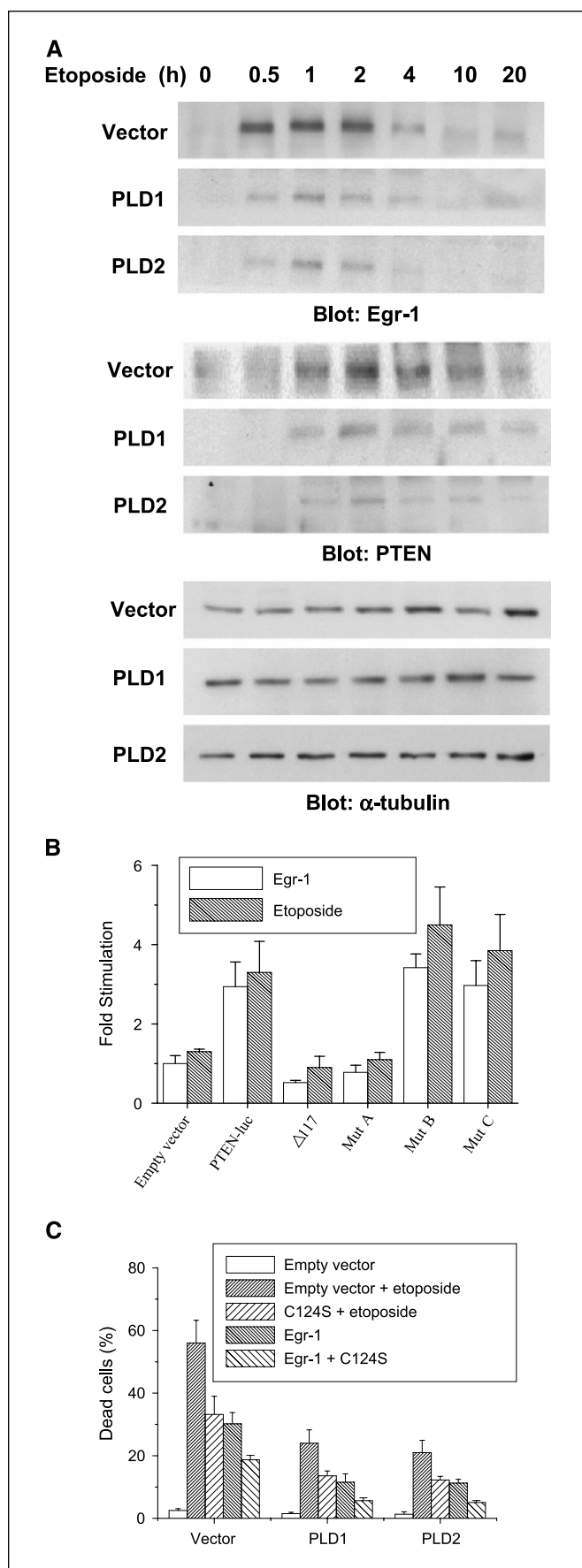
with those obtained from Virolle et al. (8). Egr-1 and PTEN are proapoptotic and their overexpression results in increased susceptibility to apoptosis (8, 32). To examine whether overexpression of these genes affects cell survival directly, we tested the viability of mouse fibroblast cells that overexpressed vector, PLD1, or PLD2. As shown in Fig. 4C, ectopic expression of Egr-1 caused a significant increase of cells that stained positive for trypan blue. Furthermore, elevated expression of PLD resulted in decreased sensitivity of fibroblasts to etoposide-induced apoptosis. This was shown to be mediated by Egr-1 and PTEN using overexpression of Egr-1 or the catalytically inactive form of PTEN (the Cys¹²⁴ \rightarrow Ser construct). Therefore, the ability of both PLD1 and PLD2 to

suppress Egr-1 expression and Egr-1 induction of PTEN partly explains the ability of PLD to suppress apoptosis.

PLD activity is involved in the down-regulation of etoposide-induced Egr-1 and PTEN expression. Data presented in Fig. 1 suggested to us that elevated PLD activity prevents etoposide-induced apoptosis. We then examined whether PLD activity affects Egr-1 and PTEN expression in etoposide-treated cells. As shown in Fig. 5A, treatment of vector-transfected cells with etoposide for 2 hours induced Egr-1 and PTEN protein expression. In cells overexpressing wild-type PLD1 and PLD2, etoposide-induced Egr-1 and PTEN protein expression were significantly suppressed compared with that in vector-transfected control cells. Because there was no decrease of these proteins in response to etoposide in cells expressing either of the catalytically inactive mutants of PLD, the effect is clearly activity dependent. This activity dependency was further confirmed by measuring the promoter activities of the *Egr-1* and *PTEN* genes. A luciferase reporter plasmid containing the -668 to +1 genomic fragment corresponding to the *Egr-1* promoter (p-688egrLuc) was transfected into the cells expressing vector, wild-type PLD1 or PLD2, or the inactive mutants of PLD1 and PLD2. As shown in Fig. 5B, luciferase activity was increased in vector cells after treatment with etoposide. However, when cells expressing wild-type PLD1 and PLD2 were treated with etoposide, the luciferase activity of the *Egr-1* promoter was decreased compared with that of vector cells. On the other hand, etoposide treatment of cells expressing inactive mutants of PLD1 and PLD2 showed luciferase activity similar to that of vector cells.

To confirm that PLD activity is involved in the transcriptional activation of PTEN, the full-length PTEN promoter was transfected into these cells. As shown in Fig. 5C, luciferase activity in response to etoposide was increased in vector cells and suppressed in cells expressing wild-type PLD1 and PLD2. However, the decrease in etoposide-induced luciferase activity in cells expressing wild-type PLD1 and PLD2 was not observed in cells expressing the catalytically inactive mutants of PLD1 and PLD2. A role for PLD in the pathway leading to suppression of etoposide-induced Egr-1 expression was further substantiated by a trial in which 1-butanol was used to block phosphatidic acid production by PLD. Cells were stimulated with etoposide in the presence of 1% 1-butanol or 3-butanol. As shown in Fig. 5D, 1-butanol did not decrease etoposide-induced Egr-1 expression. An identical concentration of 3-butanol, an inactive analogue for PLD-mediated phosphatidic acid formation, had no significant effect. This suggests that PLD activity is involved in the suppression of etoposide-induced Egr-1. Taken together, these results show that transcriptional activity of the *Egr-1* and *PTEN* genes and their protein expression is attenuated by a PLD-dependent pathway. In addition, we did the experiments using additional gene reporters to show that the effect of PLD on Egr-1 is specific. As shown in Fig. 5E, PLD1 repressed the luciferase activity of the *Egr-1* promoter compared with vector but did not affect the luciferase activity of E-cadherin, cyclin D1, or Sp1, suggesting that the effect of PLD on Egr-1 is not a result of generalized transcriptional repression.

PLD-induced suppression of Egr-1 is mediated by PI3K/Akt survival pathway. It was reported that overexpression of PLD prevented drug-induced apoptosis via the activation of PI3K and extracellular signal-regulated kinase (ERK) survival signaling pathways (17, 33). We therefore examined the effect of inhibitors of mitogen-activated protein/ERK kinase (U0126) and PI3K (LY294002)



on Egr-1 expression in PLD-overexpressed cells. Surprisingly, we found that LY294002, but not U0126, inhibited the PLD-suppressed induction of Egr-1 by etoposide (Fig. 6A and B) using Western blot and Egr-1 promoter assay. Figure 6C and D shows that although overexpression of PLD1 or PLD2 have no effect on the total expression levels of Akt, elevated expression of PLD1 and PLD2 stimulated activation of etoposide-induced Akt via PI3K. These results suggest that PLD-induced suppression of Egr-1 is mediated by activation of PI3K/Akt survival pathway at the transcriptional and posttranscriptional levels.

Discussion

This study shows for the first time that the tumor suppressors Egr-1 and PTEN are novel targets for PLD signaling. We showed that elevated expression and activity of PLD in several tumor cell lines and PLD-transformed cells caused suppression of Egr-1 and PTEN expression. Despite progress in identifying and characterizing the signaling mechanism that regulates the expression of the *Egr-1* and *PTEN* genes, much less is known about the mechanism responsible for the loss of expression of these tumor suppressors. Suppression of Egr-1 or PTEN expression by PLD isozymes is a novel mechanism for counteracting apoptosis and growth suppression. Thus, both PLD1 and PLD2 apparently provide survival signals that suppress the activation of Egr-1 and PTEN.

In addition to enhancing cell proliferation, elevated PLD expression was shown to prevent cell cycle arrest and apoptosis. Although the exact mechanism by which PLD contributes to cell survival is unknown, it seems to be involved in the survival signaling events that overcome cell cycle arrest and default apoptotic programs. Treatment of cells with DNA-damaging agents, such as etoposide, can initiate a series of events that results in programmed cell death. In the present study, after using three different approaches [DAPI staining, DNA fragmentation, and fluorescence-activated cell sorting (FACS) analysis], we concluded that PLD activity is involved in the prevention of etoposide-induced apoptosis. Overexpression of wild-type PLD1 or PLD2 but not of the catalytically inactive mutants of PLD1 and PLD2, protects against etoposide-induced apoptosis. Furthermore, the addition of exogenous phosphatidic acid, the enzymatic product of PLD, reverses the apoptotic effects of etoposide. It seems that phosphatidic acid from PLD1 or PLD2 has antiapoptotic effects on cells. These findings led us to propose that the protection that PLD confers against etoposide-induced apoptosis may be due to its effect on proapoptotic protein(s).

Figure 4. Etoposide-induced PTEN expression is down-regulated by elevated expression of PLD isozymes and this is mediated via Egr-1. A, vector and PLD1- or PLD2-transfected cells were treated with 25 μ M etoposide for the indicated time. The cell lysates were analyzed by SDS-PAGE followed by transfer of proteins to nitrocellulose membranes and immunoblotting with anti-Egr-1 or anti-PTEN antibody. Blots were stripped and reprobed with antibody to α -tubulin. The data shown are representative of three independent experiments. B, the wild-type and mutated constructs were transfected into NIH 3T3 fibroblasts stimulated or not by etoposide or exogenous Egr-1, and assayed as described in Materials and Methods. Columns, mean of three independent experiments; bars, SD. C, the cells overexpressing vector, PLD1, or PLD2 were transfected with Egr-1 and the catalytically inactive form of the PTEN (Cys¹²⁴ \rightarrow Ser) expression vector in the absence or presence of etoposide. Empty vector was transfected as a negative control. Dead cells were determined 24 hours later by trypan blue staining. Detached and trypsinized cells were pooled and incubated with 0.2% trypan blue. Cell death is shown as a percentage of blue cells. Columns, mean of three independent experiments; bars, SD.

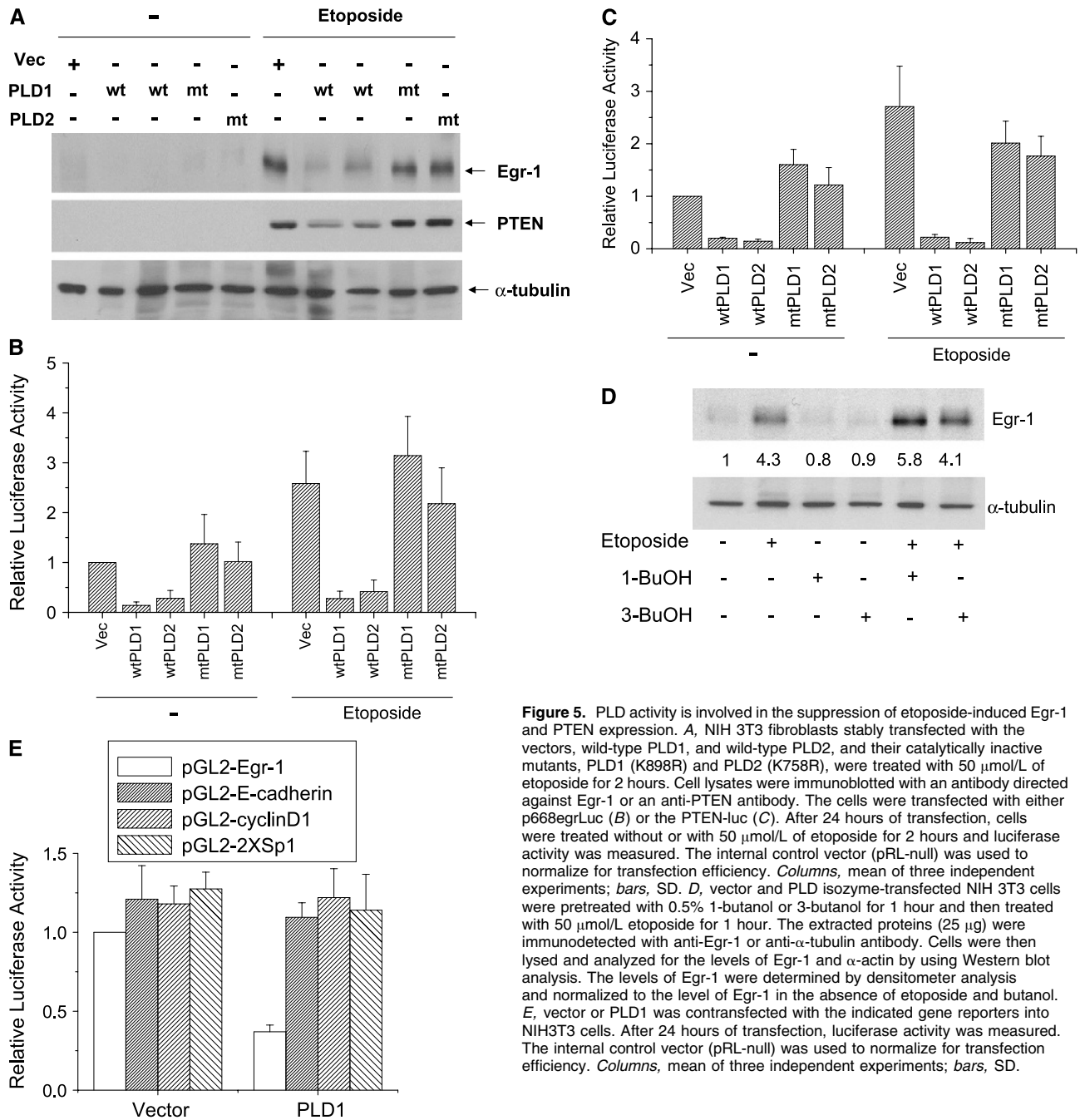


Figure 5. PLD activity is involved in the suppression of etoposide-induced Egr-1 and PTEN expression. **A**, NIH 3T3 fibroblasts stably transfected with the vectors, wild-type PLD1, and wild-type PLD2, and their catalytically inactive mutants, PLD1 (K898R) and PLD2 (K758R), were treated with 50 μ M of etoposide for 2 hours. Cell lysates were immunoblotted with an antibody directed against Egr-1 or an anti-PTEN antibody. The cells were transfected with either p68egrLuc (**B**) or the PTEN-luc (**C**). After 24 hours of transfection, cells were treated without or with 50 μ M of etoposide for 2 hours and luciferase activity was measured. The internal control vector (pRL-null) was used to normalize for transfection efficiency. **Columns**, mean of three independent experiments; **bars**, SD. **D**, vector and PLD isozyme-transfected NIH 3T3 cells were pretreated with 0.5% 1-butanol or 3-butanol for 1 hour and then treated with 50 μ M etoposide for 1 hour. The extracted proteins (25 μ g) were immunodetected with anti-Egr-1 or anti- α -tubulin antibody. Cells were then lysed and analyzed for the levels of Egr-1 and α -actin by using Western blot analysis. The levels of Egr-1 were determined by densitometer analysis and normalized to the level of Egr-1 in the absence of etoposide and butanol. **E**, vector or PLD1 was cotransfected with the indicated gene reporters into NIH3T3 cells. After 24 hours of transfection, luciferase activity was measured. The internal control vector (pRL-null) was used to normalize for transfection efficiency. **Columns**, mean of three independent experiments; **bars**, SD.

To gain a better understanding of the mechanisms responsible for the antiapoptotic effect of PLD, we measured the expression of tumor suppressor proteins and found that etoposide stimulates the expression of Egr-1. Evidence was presented recently (1, 3, 16, 17) that *Egr-1*, a member of the immediate-early gene response family, is a proapoptotic protein or a tumor suppressor gene (34), and the overexpression of Egr-1 results in increased susceptibility to apoptosis when induced by an apoptotic agent (18). Our data show that elevated expression of wild types of PLD, but not catalytically inactive mutants of PLD, suppresses etoposide-induced Egr-1 expression. The evidence implicating PLD activity as an important

component of these functional events relies on the use of alcohols. Importantly, inhibition of phosphatidic acid formation through the addition of the primary alcohol 1-butanol did not suppress etoposide induction of Egr-1 expression and increased the apoptotic activity of etoposide in cancer cells. Therefore, our findings imply that PLD activity is an important factor in the suppression of etoposide-induced Egr-1 expression.

The elevated expression and activity of PLD in breast cancer tissues suggests that PLD may play a role in the progression of breast cancer (25, 28). It was also suggested that PLD activity or components of PLD signaling should be targeted to kill breast cancer

cells that exhibit elevated PLD activity (35). We found that etoposide-induced Egr-1 protein was down-regulated in mammary carcinoma cell lines compared with nontransformed mammary epithelial cells, whereas the expression and activity of PLD1 is increased in human mammary carcinoma cell lines. PLD1 and Egr-1 levels were inversely correlated in breast cancer cells during apoptosis, suggesting that abnormal elevation of PLD1 expression probably contributes to the down-regulation of Egr-1 in breast tumor cells.

One logical mechanism by which Egr-1 may exert antitumorigenic effects is transcriptional up-regulation of antitumorigenic genes. Like Egr-1, *PTEN*, which is a well-known tumor suppressor gene, also has growth-suppressing activities and it is reasonable to speculate that PTEN could be regulated, at least in part, by Egr-1. Virolle et al. (8) have recently reported that PTEN is up-regulated by Egr-1 after irradiation and etoposide, and that Egr-1 binds specifically to the

Egr-1 binding site of PTEN. We also showed that stimulation of PTEN expression by etoposide treatment was in concord with the expression of Egr-1 in mouse fibroblasts. We showed that nine nucleotides located between positions -947 and -939, the same site as the Egr-1 binding site of PTEN after UV irradiation (8), constitute a functional *cis*-acting element necessary and sufficient for PTEN promoter stimulation by both transiently transfected Egr-1 and endogenous etoposide-stimulated Egr-1. As in the case of Egr-1, elevated expression of wild-type PLD, but not that of the catalytically inactive mutants of PLD, suppressed etoposide-induced PTEN expression, suggesting that PLD activity was centrally involved in suppression of etoposide-induced PTEN expression. Furthermore, ectopic expression of Egr-1 caused apoptosis of fibroblast cells. Inhibition of endogenous PTEN activity by overexpressing the catalytically inactive form of PTEN leads to a decrease in the

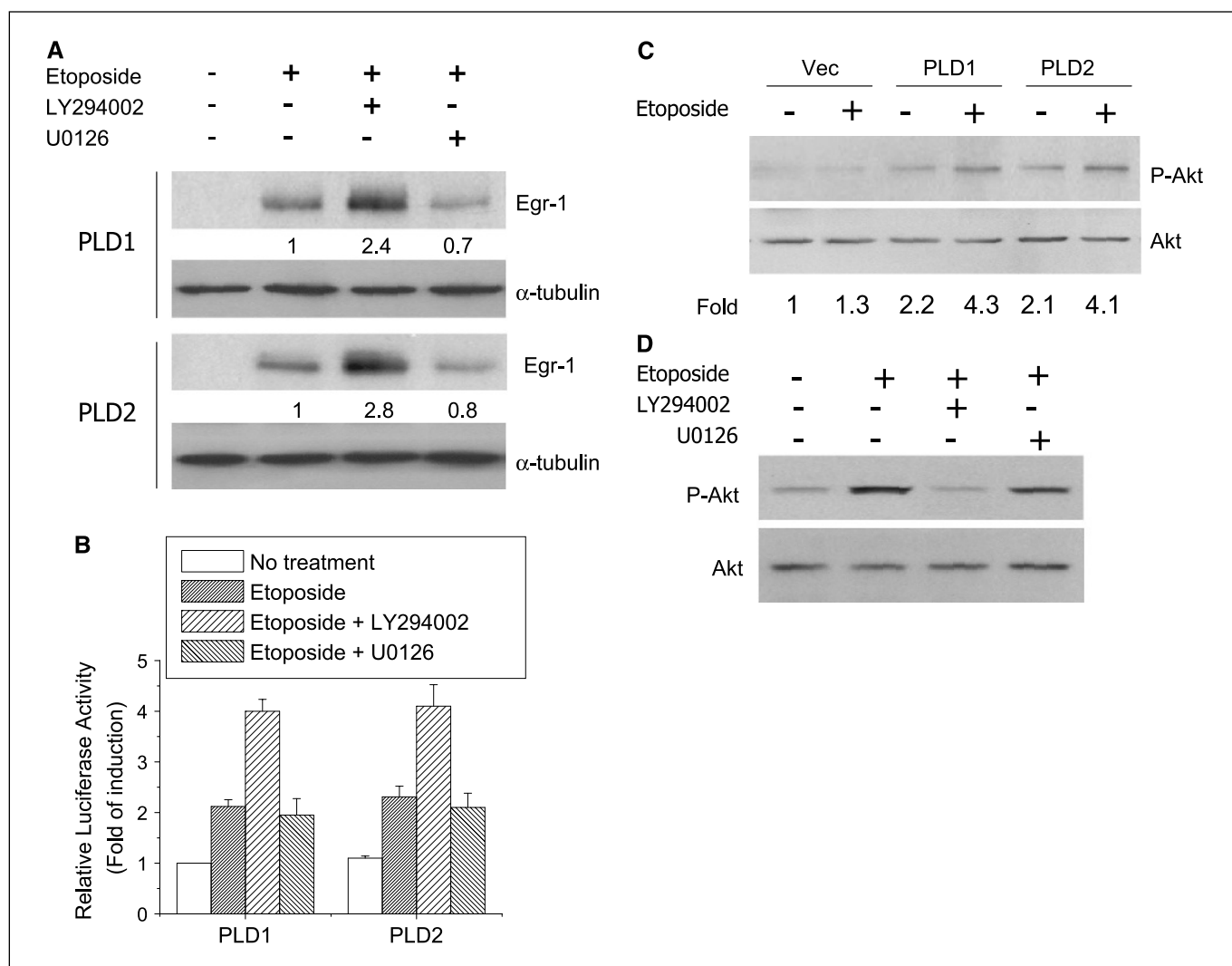


Figure 6. PLD-induced suppression of Egr-1 is mediated by PI3K/Akt survival pathway. **A**, PLD1 or PLD2-overexpressed cells were preincubated with LY294002 (20 μ M) and U0126 (20 μ M) for 1 hour and then treated with 50 μ M etoposide for 1 hour. Cells were then lysed and analyzed for the levels of Egr-1 and α -actin by using Western blot analysis. The levels of Egr-1 were determined by densitometer analysis and normalized to the level of Egr-1 in the presence of only etoposide. **B**, PLD1- or PLD2-overexpressed cells were transfected with p668egrLuc. After 24 hours of transfection, the cells were preincubated with LY294002 (20 μ M) and U0126 (20 μ M) for 1 hour and then treated without or with 50 μ M of etoposide for 2 hours, and luciferase activity was measured. **C**, vector-, PLD1-, and PLD2-transfected cells were treated with or without 50 μ M etoposide for 2 hours, and cell lysates were subjected to Western blot analysis using antibodies raised against phosphorylated Akt and Akt. The levels of phosphorylated Akt were determined by densitometer analysis. **D**, PLD1-overexpressed cells were preincubated with the indicated inhibitors for 1 hour and then treated with etoposide for 2 hours. The cell lysates were analyzed by Western blot using the indicated antibodies. Representative of three experiments.

sensitivity of the fibroblast to etoposide-induced apoptosis, which is mediated by Egr-1. Although this phenomenon was also observed in cells that overexpress PLD, elevated expression of PLD allowed the cells to survive this death signal. Therefore, the ability of both PLD1 and PLD2 to suppress Egr-1 and its induction of PTEN may partly explain the ability of PLD to suppress apoptosis. Interestingly, PLD-induced suppression of Egr-1 is sensitive to PI3K inhibitor and mediated by activation of PI3K/Akt survival pathway at the transcriptional and posttranscriptional levels.

Recently, it has been reported that PLD elevates the basal expression of MDM2 and suppresses stabilization of p53 during comptonectin-induced apoptosis (17). However, we could not detect any significant changes in the expression of MDM2 and p53 proteins in mouse fibroblast cells during etoposide-induced apoptosis. This could be a consequence of differing cell backgrounds, as the activity of Egr-1 is dependent on multiple factors (36). Our finding that PLD functions as a negative modulator of gene expression of the tumor suppressors Egr-1 and PTEN during apoptosis opens a new avenue for identifying the antiapoptotic roles of PLD in cancer biology.

The involvement of PLD in many aspects of cell proliferation, survival, and metastasis (14, 25–28, 37) suggests that PLD or targets of PLD signaling can prove to be valuable targets for therapeutic intervention in cancers. Despite some significant advantages inherent to the PLD isoforms as target for small molecule inhibitors, the development and evaluation of PLD inhibitors is still at an early stage of advancement. Because blocking of PLD activity increase the apoptotic activity of anticancer drug, development of inhibitor specific for PLD as potential drug target may be useful for cancer treatment.

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Phospholipase D Prevents Etoposide-Induced Apoptosis by Inhibiting the Expression of Early Growth Response-1 and Phosphatase and Tensin Homologue Deleted on Chromosome 10

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