Phorbol myristate acetate-induced Egr-1 expression is suppressed by phospholipase D isozymes in human glioma cells

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Abstract Early growth response-1 (Egr-1) is involved in the regulation of cell growth. Here, we found that overexpression of phospholipase D (PLD) isozymes decreased tumor promoter phorbol myristate acetate (PMA)-induced Egr-1 expression and transactivation in glioma cells. Suppression of PMA-induced Egr-1 was dependent on the expression level of PLD isozymes. Overexpression of catalytically inactive PLD, treatment with PA, and prevention of PA dephosphorylation by 1-propranolol significantly suppressed PMA-induced Egr-1 expression. PLD-induced suppression of Egr-1 was reversed by inhibition of phosphatidylinositol 3-kinase (PI3K). Taken together, these results suggest that elevated expression and activity of PLD attenuate PMA-induced Egr-1 expression via PI3K pathway.

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1. Introduction

Transcription factor early growth response factor-1 (Egr-1) is involved in the regulation of cell growth in response to mitogens [1]. The fact that phorbol myristate acetate (PMA) stimulated Egr-1 biosynthesis [2] was in agreement with function of Egr-1 in cell growth. However, in other circumstances, Egr-1 is induced very early in the apoptotic process [3]. Egr-1 has been found to be decreased in several tumors [4–6]. It was suggested that functional loss of Egr-1 may contribute to tumorigenic potential [7]. Despite progress in characterizing the signaling that positively regulate the Egr-1 gene expression, much less is known about negative regulatory mechanism responsible for loss of Egr-1 expression.

Phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline, has been implicated in the generation of survival signals. There are two PLD isoforms, PLD1 and PLD2 which are regulated by PKC and small G proteins [8]. PLD is overexpressed in human cancers [8] and prevent etoposide-induced apoptosis by suppression of the expression of Egr-1 [9].

Malignant glioma, are refractory to classical chemotherapy and radiotherapy and have a poor prognosis [10]. Egr-1 expression is significantly down-regulated in human glioma tissues compared with normal brain [6].

To investigate the signaling mechanism for altered expression of Egr-1, we examined whether PLD-mediated signaling affects PMA-induced Egr-1 expression in glioma cells. In the present study, we demonstrate for the first time that PMA-induced Egr-1 expression is suppressed by elevated expression of PLD in glioma cells.

2. Materials and methods

2.1. Materials

Dual luciferase assay kits were from Promega (Madison, WI). The antibodies to Egr-1 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal antibody that recognizes both PLD1 and PLD2 was generated as previously described [11]. Anti-β-tubulin antibody and phosphatidic acid were from Sigma (St. Louis, MO), and various inhibitors were from Biomol (Plymouth Meeting, PA).

2.2. Construction of plasmids

The pEBS1\textsuperscript{1}Luc construct was generously provided by Dr. Seung Joon Baek (University of Tennessee, USA). The Egr-1 promoter repporter construction (p-668egrLuc inserted into a pGL2-basic luciferase plasmid) has been described [9].

2.3. Cell culture and transfections

U87 MG human astroglioma was obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum. U87 cells were transiently transfected with expression plasmid using LipofectAMINE Plus (Invitrogen) according to manufacturer’s instructions. U87 cells overexpressing stably wild type PLD isozyme were obtained by transfection.

2.4. PLD activity assay

PLD activity was assessed by measuring the formation of [\textsuperscript{3}H] phosphatidylbutanol (PdBBut), the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol. After 20 h transfection, U87 cells in 6-well plates were serum-starved in the presence of 2 μg/ml [\textsuperscript{3}H] myristic acid for overnight. The cells were preincubated with 0.3% 1-butanol for 10 min and then treated with agonist. The extraction and characterization of lipids by thin-layer chromatography were performed as previously described [9].
2.5. Western blot
Cell extracts were separated in 8% SDS–polyacrylamide gel and the proteins were blotted onto a nitrocellulose membrane. Subsequently, membranes were incubated with specific antibodies. Antigen–antibody complexes were visualized after incubating the membrane with horse-radish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence.

2.6. Luciferase assay
After 24 h of transfection with pGL2-Egr-1, the cells were treated with PMA for 12 h. 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.

3. Results
3.1. PMA-induced Egr-1 expression is suppressed by elevated expression of PLD isozymes in glioma cells
Egr-1 expression is suppressed in astrocytic tumors compared with normal brain tissue and loss of Egr-1 expression may provide an important maker of glial cell malignancy [12]. Since PLD is elevated in human cancer tissues [8], the role of PLD in regulating expression of Egr-1 was examined. To investigate whether Egr-1 is down-regulated by PLD in glioma cells, we established U87 MG human glioma cells expressing PLD1 and PLD2. Egr-1 protein level peaked 30 min after PMA stimulation and declined thereafter (Fig. 1). A significant decline in Egr-1 protein level was observed by elevated expression of PLD isoymes compared with control cells. Three different U87-PLD isozyme clones showing different expression level of PLDs were used to eliminate the possibility of random gene aberrations during the transfection procedure. PMA-induced Egr-1 expression was inversely correlated with expression level of PLD isozymes (Fig. 2A). Moreover, PMA-induced Egr-1 promoter activity was decreased by elevated expression of PLD isozymes (Fig. 2B). These results suggest that elevated protein expression of PLD isoymes is
involved in the suppression of PMA-stimulated Egr-1 expression at transcriptional and post-transcriptional levels.

3.2. Elevated expression of PLD suppresses PMA-induced transactivation of a reporter with Egr-1 response elements

The transactivation of Egr-1 in PMA-treated cells was determined using an Egr-1-responsive reporter. The plasmid pEB-S14luc contained four copies of Egr-1 response elements linked to the basal promoter, followed by a luciferase reporter gene [13]. PMA-induced transactivation of Egr-1 was suppressed by elevated expression of PLD isozymes (Fig. 3), suggesting that PLD isozymes are involved in the suppression of PMA-stimulated transactivation of Egr-1.

3.3. PLD activity is involved in the suppression of PMA-induced Egr-1 expression

We examined whether PLD activity affects PMA-induced Egr-1 expression. Although PMA-induced Egr-1 promoter activity was reduced by expression of wild type PLD, PMA treatment of cells expressing inactive mutants of PLD isozymes (K898R for PLD1, K758R for PLD2) did not suppress Egr-1 promoter activity (Fig. 4A). Moreover, 1-butanol was used to block phosphatidic acid production by PLD, by formation of phosphatidylbutanol through the transphosphatidylation reaction. 1-Butanol did not suppress PMA-induced Egr-1 expression (Fig. 4B). An identical concentration of 3-butanol, an inactive analogue for PLD-mediated phosphatidic acid formation, had no significant effect. Furthermore, propranolol, PA phosphohydrolase inhibitor, suppressed PMA-induced Egr-1 expression (Fig. 4C). Furthermore, dioctanoyl PA significantly inhibited PMA-induced Egr-1 expression (Fig. 4D). These results demonstrate that PLD activity is importantly involved in the suppression of PMA-induced Egr-1.

3.4. PLD-induced suppression of Egr-1 by PMA are dependent upon PI3K

To investigate how PLD suppresses PMA-induced Egr-1 expression, we examined the effect of various inhibitors. PMA-induced Egr-1 expression was suppressed by MEK inhibitors (PD98059, U0126), JNK inhibitor (SP60125), and PKC inhibitor (Go6976), but not inhibitors of p38MAPK and PI3K (Fig. 5A). These results were also confirmed by Egr-1 promoter assays (Fig. 5B). Recently, we have reported that PI3K inhibitor (LY294002) inhibited PLD-suppressed induction of Egr-1 by etoposide [9]. Therefore, we examined whether PI3K was involved in the suppression of PMA-induced Egr-1 expression by PLD. PMA-induced Egr-1 expression was significantly increased in a dose-dependent manner.

Fig. 3. Elevated expression of PLD suppresses PMA-induced transactivation of a reporter with Egr-1 response elements. Vector, PLD1 or PLD2-overexpressed cells were transfected with pEBS14luc for 24 h and then treated with 100 nM PMA for 12 h. Luciferase activity was assayed as described in Section 2. Results show means ± S.D. of three independent experiments.

Fig. 4. PLD activity is involved in the suppression of PMA-induced Egr-1 expression. (A) U87 glioma cells were cotransfected with Egr-1 promoter and the indicated expression vectors, treated with 100 nM of PMA for 12 h and luciferase activity was measured. Results show means ± S.D. of three independent experiments. (B) Glioma cells were pretreated with 0.5% 1-butanol or 3-butanol for 30 min and then treated with 100 nM PMA for 1 h. The lysates were immunodetected with the indicated antibody. The levels of Egr-1 were determined by densitometer analysis. The cells were pretreated with the indicated concentration of 1-propranolol (C) and PA (D) for 1 h and treated with PMA for 1 h. The extracted proteins were immunodetected with anti-Egr-1 or β-tubulin antibody. The data shown are representative of three independent experiments.
by PI3K inhibitor in glioma cells overexpressing PLD iso- 
zymes, but not in vector-transfected cells (Fig. 5C). PMA-in-
duced Egr-1 promoter activity was also increased by 
pretreatment with 20 μM LY294002 in PLD-expressed cells, 
but not in control cells (Fig. 5D). Moreover, elevated expres-
sion of PLD isozymes stimulated activation of PMA-induced 
Akt, downstream of PI3K as assessed by immunoblot analysis 
using antibody to phospho-Akt (Ser-473) (Fig. 5E). These re-
results suggest that PLD-induced suppression of PMA-induced 
Egr-1 expression is mediated via PI3K/Akt pathway.

4. Discussion

The present study shows that Egr-1 is a novel target for PLD 
signaling. We showed that elevated expression and activity of 
PLD in glioma cells caused suppression of PMA-induced 
Egr-1 expression.

PMA is one of the strongest stimuli to induce Egr-1 expres-
sion. Since PMA has been shown to elicit both growth promo-
tion and growth inhibition in different cells, it has been 
suggested that PKC may participate in negative feedback of 
growth. It has been reported that PMA can enhance the growth 
inhibitory activity of negative growth factors like transforming 
growth factor-β in human prostate cancer cells [14]. Our 
preliminary study shows that PLD is overexpressed in human 
glioblastoma tissues compared with normal brain tissues. Our 
results demonstrate that PMA-induced Egr-1 expression was 
inversely correlated to the amount of PLD isozymes expressed, 
suggesting that abnormal elevation of PLD expression contrib-
utes to the down-regulation of PMA-induced Egr-1 expression 
in glioma cells. Using the experiments including expression of 
catalytically inactive mutants of PLD, addition of primary 
alcohol, and treatment of propranolol and PA, our findings 
imply that PLD activity is importantly involved in the suppres-
sion of PMA-induced Egr-1 expression.
The identification of downstream target genes of Egr-1 will help us to understand signal-transcription coupling mediated by Egr-1. Antitumorigenic genes such as p53 [15], PTEN [16] and nonsteroidal anti-inflammatory drug-activated gene-1 [17] were identified as Egr-1 target genes. Interestingly, PLD-induced suppression of Egr-1 is sensitive to PI3K inhibitor and mediated by activation of PI3K pathway. The involvement of PLD in many aspects of cell proliferation and survival suggests that PLD or target of PLD signaling can prove to be valuable targets for the therapeutic intervention in cancers.

The identification of Egr-1 target genes in glioma cells will be informative in determining PMA-induced signaling cascade in these cells downstream of Egr-1. Loss-of-function experiment of Egr-1 are further required to elucidate the exact role in these cells downstream of Egr-1. The mechanism of growth control by Egr-1 via PLD-mediated signaling pathway in glioma cells will be informative in determining PMA-induced signaling cascade in these cells downstream of Egr-1. Loss-of-function experiment of Egr-1 are further required to elucidate the exact role in these cells downstream of Egr-1. The involvement of PLD in many aspects of cell proliferation and survival suggests that PLD or target of PLD signaling can prove to be valuable targets for the therapeutic intervention in cancers.

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