Down-regulation of phospholipase D during differentiation of mouse F9 teratocarcinoma cells

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Abstract Phospholipase D has been recognized as playing an important role in signal transduction in many types of cells. We investigated the expression of phospholipase D during the differentiation of F9 embryonal teratocarcinoma cells. The ADP ribosylation factor-dependent phospholipase D activity, as measured by an in vitro assay, and H_2O_2-induced phospholipase D activity and phospholipase D protein content in whole cells were decreased during the differentiation of F9 cells induced by a combination of dibutyryl cyclic AMP and all-*trans* retinoic acid. In contrast, these changes were not observed when cells were induced by retinoic acid. These results suggest that down-regulation of phospholipase D protein is associated with differentiation of F9 cells to a parietal endoderm lineage.

Key words: Phospholipase D; F9 teratocarcinoma; Differentiation; Down-regulation

1. Introduction

Phospholipase D (PLD) plays an important role in membrane lipid-mediated signal transduction. PLD catalyzes the hydrolysis of phospholipids, usually phosphatidylcholine (PC), to generate phosphatidic acid (PA) and choline. PA may act directly as a signaling molecule or can be further metabolized to form other messenger molecules, 1,2-diacylglycerol and lysophosphatidic acid. PLD has been implicated in a wide range of physiological processes and diseases including inflammation, secretion, mitogenesis, neuronal and cardiac stimulation, diabetes and the respiratory burst in neutrophils [1]. However, the precise role of PLD in cellular functions is still poorly understood. Mouse F9 teratocarcinoma cells have been used widely as a model system for the study of differentiation related to tumorigenesis and early events in the mouse development [2]. F9 stem cells show a very low spontaneous differentiation. However, they can be differentiated into visceral extra-embryonic endoderm in response to retinoic acid (RA). Because cancer is a disease involving loss of cellular growth control and disruption of cell differentiation, there has been much interest in the role of RA in controlling patterns of gene expression during the differentiation of F9 teratocarcinoma stem cells. RA is a strong tumor cell differentiation agent and has been used as an anti-cancer drug. On the other hand, when RA and dibutyryl cyclic AMP (dbcAMP) are added to culture media, F9 cells differentiate into a second phenotype that morphologically and biochemically resembles parietal endoderm cells [3,4] which synthesize large quantities of extracellular matrix constituents including laminin, entactin and proteoglycan. Although the mechanism whereby RA regulates gene expression and differentiation is not completely understood, it is likely that high affinity RA receptor and/or cellular RA binding proteins are involved [5,6]. In addition, there might be a transmembrane signal transduction that provides, in part, a biochemical basis for the development of the RA-induced differentiation.

It has been reported that expression of phospholipase C-γ [7] or G-protein-coupled receptor [8] is negatively regulated in the differentiation of F9 teratocarcinoma stem cells into parietal endoderm. In the light of that work, PLD may be one of the intracellular signaling elements involved in the differentiation of F9 cells. Therefore, in order to examine the involvement of PLD during the differentiation of F9 cells, we investigated the expression of PLD by a PLD activity assay and Western blot analysis during cell differentiation. In the present study, we found that RA and dbcAMP reduced the ADP ribosylation factor 3 (ARF3)-dependent activation of PLD as well as H_2O_2-induced PLD stimulation during differentiation. Furthermore, protein levels of PLD1 were also concomitantly decreased during differentiation into parietal endoderm, but only RA had no effect on the activity and expression of PLD during differentiation into visceral endoderm, suggesting that down-regulation of PLD relates to differentiation of F9 teratocarcinoma cells into parietal endoderm.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics were obtained from Gibco. dbcAMP and RA were from Sigma. Horseradish peroxidase-conjugated anti-rabbit IgG was from KPL. The ECL Western blotting detection kit was from Amersham. Anti-peptide antibody to PLD1 was generated in rabbits using the C-terminal dodecapeptide of PLD1 coupled to keyhole limpet hemocyanin with glutaraldehyde. Human ARF3 was co-expressed with myristoyltransferase in *Escherichia coli* and purified using DEAE-Sephacel and Superdex 75 column chromatography as described by Weiss et al. [9].

2.2. Cell culture

F9 cells were maintained in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were cultured on plates pre-coated with 1% gelatin.

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Abbreviations: PLD, phospholipase D; ARF, ADP ribosylation factor; RA, retinoic acid; dbcAMP, dibutyryl cyclic AMP; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PtdBut, phosphatidylbutanol

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2.3. Differentiation of F9 cells

Differentiation into parietal endoderm was induced by addition of 10^{-7} M RA and 10^{-3} M dbcAMP to the medium. Cells were harvested after 1, 2, 3 and 5 days of treatment. F9 cells were also differentiated into visceral endoderm by growing them in suspension in petri dishes with medium containing 10^{-7} M RA. RA-treated cells were harvested after 1, 2, 3 or 7 days.

2.4. Measurement of PLD activity in F9 cell lysates

The cells were washed twice with buffer A (20 mM HEPES, pH 7.2, 250 mM sucrose, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride) and resuspended in buffer A. The cells were lysed by 10 passes through a 27 gauge needle on ice. After unbroken cells and nuclei were removed by centrifugation at $900\times g$ for 5 min, the resulting supernatant was used as the cell lysate for experiments. PLD activity was measured by the formation of [^{3}H]phosphatidylbutanol (PtdBut) from [^{3}H]palmitoyl-PC in the presence of 1% butanol as described previously [4]. Phospholipid vesicles containing phosphatidylethanolamine/phosphatidylinositol 4,5-bisphosphate/PC (16:1.4:1) were used as substrate [10].

2.5. In vivo assay of PLD

Undifferentiated or differentiated cells were serum-starved in DMEM for 24 h before the start of the assay. For the final 20 h of serum starvation, the cells were labelled with 1 μCi/ml [9,10-^{3}H]-myristic acid. The cells were washed and pre-incubated with 0.3% butane-1-ol for 10 min. The cells were treated with H_2O_2 for 30 min and PtdBut formation was measured as described in [10,11]. Radioactivity incorporated into total phospholipids was measured and used to normalize the results.

2.6. Immunoprecipitation

Cells treated with RA and dbcAMP for the indicated times were lysed with radio immunoprecipitation assay buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). After incubation for 30 min in an ice-bath, cells were completely broken by sonication and then, the lysate supernatant was pre-cleared by incubation with pre-immune IgG and protein A Sepharose for 30 min. Pre-cleared cell lysates were incubated with anti-PLD1 antibody and 30 μl of 50% slurry of protein A Sepharose for 4 h. The immune complex was collected by centrifugation and washed five times with an ice-cold buffer containing 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM Na_3VO_4, 10% glycerol, 1% Nonidet P-40, followed by addition of SDS sample buffer and boiling.

2.7. Western blot analysis

The recovered protein was resolved on a SDS-polyacrylamide gel electrophoresis (PAGE) gel and blotted to a PVDF membrane. The blot was probed with anti-PLD1 antibody. Immunoreactive bands were visualized by chemiluminescence using horseradish peroxidase-conjugated anti-rabbit IgG and ECL reagent (Amersham).

3. Result and discussion

Although it has been suggested that PLD may be involved in cell growth and differentiation [12–14], the relationship between the expression of PLD and cellular differentiation has not been well elucidated. PLD activity has been shown to increase with granulocytic differentiation of HL60 cells [15] and with differentiation of rat C6 glioma cells to the astrocytic phenotype [16]. Several factors have been suggested to underlie this increased activity [13,17], including changes in the amounts of known PLD activators, such as PIP_2, ARF and Rho family members or protein kinase C, or in the level of PLD mRNA. However, the most straightforward possibility that protein levels of PLD itself are regulated during differentiation remains untested, since an antibody against PLD for

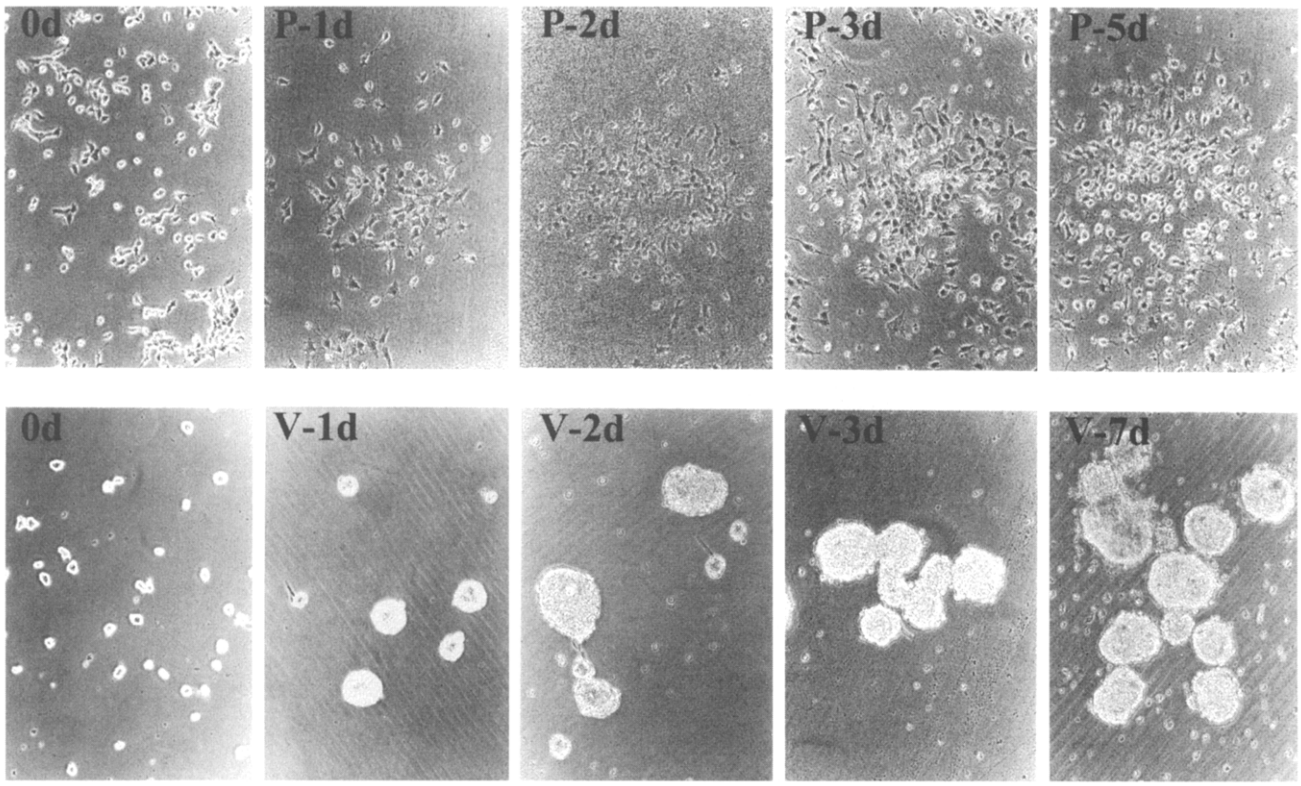
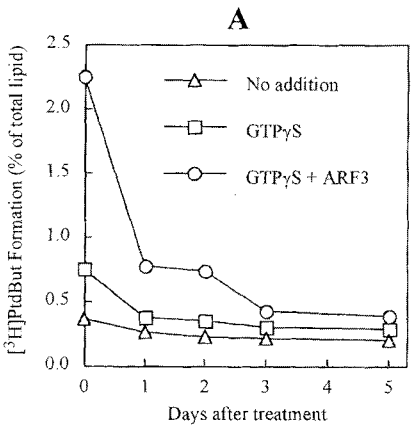
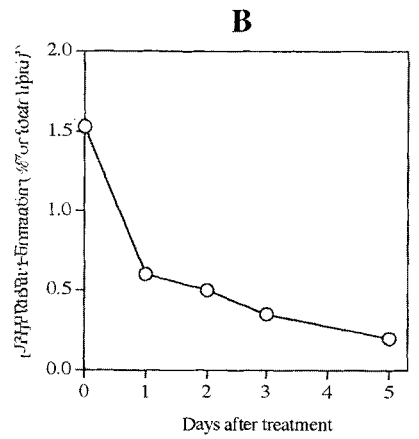


Fig. 1. Morphological changes during the differentiation of F9 cells. F9 stem cells (0d) were differentiated into parietal endoderm (upper panel) by RA or into visceral endoderm (lower panel) by RA and dbcAMP as described in Section 2. Parietal endoderm cells treated for 1 (P-1d), 2 (P-2d), 3 (P-3d) or 5 (P-5d) days; Visceral endoderm cells treated for 1 (V-1d), 2 (V-2d), 3 (V-3d) or 7 (V-7d) days.





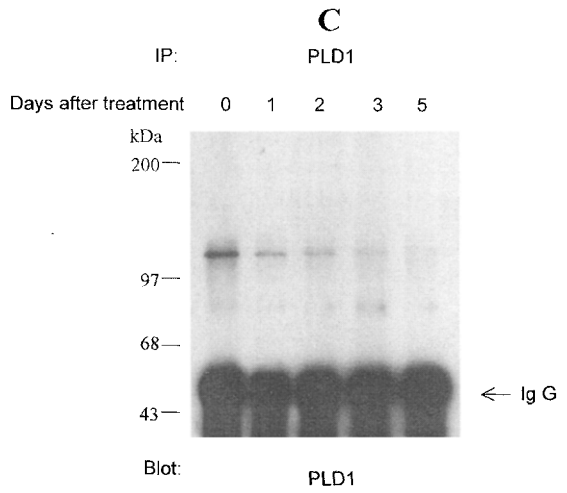
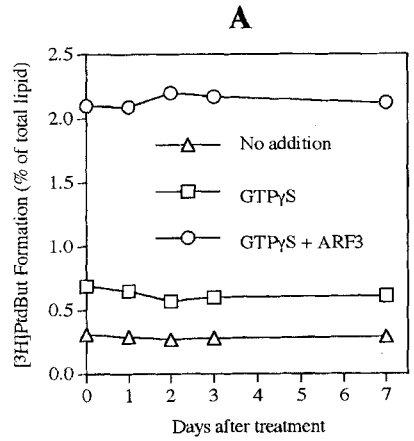


Fig. 2. Down-regulation of PLD during differentiation into parietal endoderm of F9 cells. (A) F9 cells were incubated with RA and dbcAMP for the indicated periods of time. Cells were lysed and PLD activity in lysates was determined by measuring the ratio of formation of [^{3}H]PtdBut and radioactivity incorporated into total phospholipid. (B) Cells treated without or with RA and dbcAMP for the indicated periods were labelled with [^{3}H]myristate. The labelled cells were pre-treated with vanadate (100 μM) for 20 min and then stimulated with 500 μM H_2O_2 for 20 min. The radioactivity incorporated into PtdBut was measured as described in Section 2. (C) Cells treated with RA and dbcAMP for the indicated periods of time were lysed and immunoprecipitates (IP) were prepared using anti-PLD1 antibody. The immunoprecipitates were then subjected to Western blotting with the same antibody.

Western blot analysis was not available. We have used the mouse F9 teratocarcinoma cell line to study the role of PLD expression during differentiation of F9 cells. F9 cells were progressively differentiated into parietal endoderm cells by treatment of RA/dbcAMP for 1, 2, 3 and 5 days and into visceral endoderm by treatment of RA for 1, 2, 3 and 7 days. Differentiation was monitored by morphological changes (Fig. 1). Cellular aggregation in suspension cultures was typical for the visceral phenotype, while parietal endoderm often exhibited cellular processes [18]. Differentiation was measured by an increase in the level of mRNA expression of laminin [19]. After exposure of F9 cells to RA/dbcAMP for various periods, ARF3-dependent PLD activity in the lysate of F9 cells was measured by using the exogenous substrate. In undifferentiated F9 cells, PLD activity was increased by addition of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and ARF3 (Fig. 2A). However, after 1 day of RA/dbcAMP treatment, a significant decrease of PLD activity was observed. It is known that morphological differentiation into cells resembling parie-



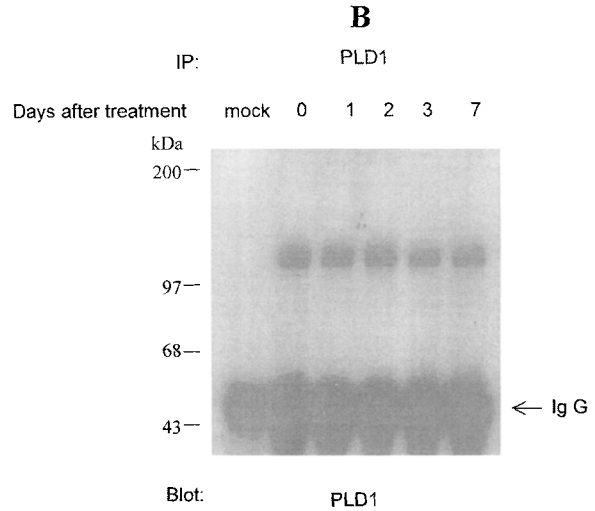


Fig. 3. Regulation of PLD during the differentiation of visceral endoderm. (A) Differentiation of F9 cells was induced with RA for the indicated periods of time. Cells were lysed and PLD activity in lysates was determined as described in Fig. 2. (B) Cells treated with RA for the indicated periods of time were lysed and immunoprecipitates (IP) were prepared using anti-PLD1 antibody. 'Mock' represents a mock-precipitated control. The immunoprecipitates were then subjected to Western blotting with the same antibody.

tal endoderm is complete after 5 days of treatment with RA and dbcAMP. After treatment for 5 days, PLD activity, in response to recombinant ARF3, was greatly suppressed in our reconstitution assay in comparison to PLD activity in undifferentiated cells. Furthermore, we measured H_2O_2-induced PLD activation by labelling F9 cells with [^{3}H]myristic acid. Recently, we reported that the combination of H_2O_2 and vanadate stimulates PLD activity in Swiss 3T3 cells [20]. PLD activation was markedly increased by H_2O_2 and vanadate in undifferentiated F9 cells. However, while F9 cells differentiated into parietal endoderm cells with the treatment of RA/dbcAMP, H_2O_2-induced PLD activation was abolished (Fig. 2B). In contrast, changes in ARF-dependent PLD activity were not observed during differentiation into visceral endoderm (Fig. 3A). These results demonstrate that PLD activity decreased during differentiation of F9 cells into parietal endoderm but not into visceral endoderm. There are several possibilities that can interpret this finding and one of them deals with the change of the level of PLD protein itself. In order to analyze the relationship between the induction of differentiation and PLD expression in F9 cells, we examined the protein level of PLD during the different stages of differentiation. Immunoprecipitation and Western blot analysis revealed that the expression level of PLD decreased dramatically after 1 day of RA/dbcAMP treatment and was barely detectable after 5 days (Fig. 2C). Thus, the decrease in PLD activity in the differentiated cells is most likely due to the decreased level of PLD protein. Taken together, our results, obtained by a PLD activity assay, immunoprecipitation and Western blotting by anti-PLD1 antibody, indicate that PLD is down-regulated during RA/dbcAMP-induced differentiation. In contrast, the protein level of PLD showed no significant changes after treatment with RA which differentiates F9 cells into visceral endoderm (Fig. 3B). To investigate PLD down-regulation in a transcriptional context, we examined the level of PLD mRNA by Northern blot analysis. However, the level of PLD mRNA was below the threshold of detectability. Recently, in contrast to our results, it has been reported that GTPγS-dependent PLD activity increased time-dependently during differentiation of C6 cells induced by dbcAMP [16] and HL60 cells induced by RA/dbcAMP [15]. A different regulation of PLD isozymes during differentiation of cells may be due to cell type specificity. In conclusion, we have found that the level of PLD protein markedly decreased during RA/dbcAMP-induced differentiation. From these results, it may be assumed that down-regulation of PLD could be an important step in differentiation of F9 cells by RA/dbcAMP.

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