Down-regulation of phospholipase C-γ1 during the differentiation of U937 cells

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Abstract Phospholipase C (PLC)-γ1 and -γ2 play a pivotal role in signal transduction for cell proliferation and differentiation. The enzyme activity and protein level of PLC-γ1 were markedly decreased in the human histiocytic leukemia U937 cell line during the differentiation process which is induced by phorbol 12-myristate 13-acetate (PMA) but those of PLC-γ2 were not altered. Northern blot analysis showed that the levels of PLC-γ1 and -γ2 transcripts were not changed. These results suggest that the expression of PLC-γ1 during the PMA-induced differentiation may be down-regulated by post-transcriptional processing.

Key words: Differentiation; PMA; Phospholipase C-γ1; Phospholipase C-γ2; Down-regulation; U937 cell

1. Introduction

Phosphoinositide-specific phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and thereby produces the intracellular messenger molecules, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) in response to the binding of various ligands to their surface receptors in signal transduction pathway [1]. Several distinct PLC isozymes have been purified and cloned from a variety of mammalian tissues [1]. Among the three known types of PLC isozymes (PLC-β, -γ, -δ), the PLC-γ isozymes contain the *src* homology domains (SH2 and SH3) in addition to common X and Y regions found in all the PLC isozymes [1].

PLC-γ1 is activated by phosphorylation of specific tyrosine residues by growth factor receptors with intrinsic tyrosine kinase activities such as platelet-derived growth factor (PDGF) receptor [2], epidermal growth factor (EGF) receptor [3], fibroblast growth factor (FGF) receptor [4]. These data imply that the activation of PLC-γ1 might be one of the important pathways involved in mitogenic signals triggered by these growth factors. Some evidences have also demonstrated that PLC-γ1 participated in cellular growth signal transduction. Smith et al. [5] found that microinjection of PLC-γ1 into quiescent NIH 3T3 cells induced the acute dose-dependent induction of DNA synthesis. Moreover, the experiments with PDGF receptor mutants that fail to associate with some signaling molecules confirmed the possible role of PLC-γ1 as downstream effector of mitogenic signal triggered by PDGF [6]. Recently, we have found that PLC-γ1 is highly expressed in human tissues obtained from colorectal cancer [7], and familial adenomatous polyposis [8], supporting the view that PLC-γ1 plays an important role in controlling cellular proliferation.

The human cell line U937 [9], when cultured in the presence of phorbol 12-myristate 13-acetate (PMA), undergoes growth arrest and promotes terminal differentiation from monoblasts to monocyte/macrophage-like cells [10,11]. It is important to elucidate the molecular mechanism inducing the growth arrest of differentiated cells. However, little is known about which molecules regulate the inhibition of cell growth in differentiated cells. In this study, to determine which PLC-γ isozymes are involved in differentiation-induced cellular growth arrest, we examined the change of the expression level of PLC-γ1 and PLC-γ2 in PMA-treated U937 cells as a function of time.

2. Materials and methods

2.1. Materials

PMA, Pansorbin and wheat germ phosphatidylinositol were purchased from Calbiochem (San Diego, CA). 3-Phosphatidyl[2-^{3}H]inositol (17.9 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Alkaline phosphatase-conjugated antibodies to mouse IgG were from Kirkegaard & Perry Inc. (Gaithersburg, MD).

2.2. Cell culture

U937 cells were cultured in RPMI 1640 medium (Life Technologies; Grand Island, NY) supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, UT).

2.3. Induction of differentiation

Differentiation of U937 cells was induced by adding 5 nM PMA (Calbiochem, San Diego, CA) as described [10,11]. Cell proliferation was checked by counting the viable cell with Trypan blue dye exclusion. Differentiation into macrophage-like cells was assessed by morphology and by nitroblue tetrazolium (NBT) assay as described previously [12].

2.4. Immunoprecipitation and immune complex activity assay

For immunoprecipitation, monoclonal antibodies to PLC-γ1 [13] or PLC-γ2 (Lee, Y.H. et al., unpublished data) were prepared as described previously. Cells (7×10^6) were lysed with 20 mM HEPES, pH 7.2, containing 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 7 μg each of monoclonal antibodies to PLC-γ1 or PLC-γ2 was added to the lysates. After incubation for 30 min in an ice-bath, 30 μl of 20% solution of formaline-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem, San Diego, CA) precoated with rabbit anti-mouse Ig (Sigma, St. Louis, MO) were added and incubated for 2 h in an ice-bath. For the enzyme activity assay of PLC-γ1 and PLC-γ2, immunoprecipitates were prepared as described above. After washing three times with a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, immunecomplexes were resuspended in 100 μl of the same buffer. The enzyme activity of PLC-γ1 or PLC-γ2 was determined in the 200 μl assay mixture containing 50 μM PI (20,000 cpm, [^{3}H]PI), 1 mM EGTA, 10 mM $CaCl_2$, 0.1% sodium deoxycholate, 50 mM HEPES (pH 6.8) and 10 ml of resuspended immunecomplexes. The assay mixture was incubated with at 37°C for 10 min and terminated as described previously [14].

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2.5 Western blot analysis

For the Western blot analysis, immunoprecipitates were prepared as described above. The immunecomplexes were washed five times with 50 mM Tris-HCl, pH 8.5, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 150 mM NaCl. The precipitated proteins were heated at 95°C for 3 min with Laemmli cooking buffer and resolved by 8% SDS-PAGE, transferred to nitrocellulose, and proved with the indicated monoclonal antibodies for 4 h. Immunoreactive proteins were incubated with alkaline phosphatase conjugated goat anti-mouse IgG + IgA + IgM (1:2,000; Kirkegaard and Perry Lab.) and developed with 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium substrate system (Kirkgaard and Perry, Gaithersburg, MD) as described previously [8].

2.6. Radioimmunoassay

To determine the protein level of PLC-γ1 or -γ2 in the control or differentiated cells, radioimmunoassay was performed according to the method as described by Suh et al. [18]. Briefly, the primary antibody-coated 96-well microtiter plates (Immulon-2) were prepared by adding 1.5 μg of each monoclonal antibody (anti-PLC-γ1, F7-2; anti-PLC-γ2, γ2-1-4) per well. After blocking the unsaturated plastic surface with 0.5% bovine serum albumin, 50 μl of various concentrations of purified PLC-γ1 or -γ2 standard and crude cell lysates (100 μg/ml) were added to the wells and incubated for 2 h at 37°C. Cell lysates were prepared as described in immunoprecipitation and immunecomplex activity assay. After the plates were washed five times with phosphate-buffered saline containing 0.05% Tween-20, secondary antibody (anti-PLC-γ1, D7-3; anti-PLC-γ2, γ2-2-3) labeled with ^{125}I (20,000 cpm/well) was added and incubated for 4 h at room temperature. After five washing with above buffer, ^{125}I-radioactivity of each well was measured. Standard PLC-γ1 or -γ2 was purified from HeLa cell extracts overexpressing each PLC isozymes by using vaccinia virus expression system. Secondary antibody was radioiodinated using IODO-BEADS (Pierce Chemical Co.) according to the manufacturer's instructions.

2.7. Northern blot analysis

Total RNA was extracted from U937 cells treated without or with PMA for 72 hours using acid phenol method as described by Chomoczynski and Saccai [15]. Fifteen μg of total RNA was separated on 1% agarose formaldehyde gel and blotted onto nylon membrane. Hybridization and washing were performed in the conditions described by Church and Gilbert [16]. Briefly, the membrane was incubated in the hybridization solution (1% bovine serum albumin, 7% SDS, 0.5 M NaH_2PO_4, pH 7.0, 1 mM EDTA, 2×10^6 cpm/ml ^{32}P-labeled probe) at 65°C overnight. Washing was performed in 0.1% SDS and 1 × SSC at 70°C for 1 h. The membrane was exposed to X-ray film to identify the bands.

3. Results and discussion

PLC-γ1 and -γ2 are well known substrates of some growth factor receptor kinases [2–4] and are involved in the cellular growth signaling [5–8]. Although it is generally accepted that activation of PLC-γ1 causes induction of DNA synthesis and cellular proliferation [5,6], the relationship between the expression of PLC-γ isozymes and cellular differentiation has not been elucidated well. We have used the human U937 histiocytic leukemia cell line to study the role of expression of PLC-γ1 and -γ2 during differentiation of hematopoietic cells. When treated with PMA, U937 cells can be induced to differentiate along a monocyte/macrophage pathway [10,11]. In this experiment, PMA-induced differentiation was monitored by morphological change, viable cell counting and NBT reduction assay (data not shown). In order to see whether differentiation of U937 cells modulates the PLC-γ1 and -γ2 activities, we examined PLC activity in immunecomplex. After exposure of U937 cells to PMA for various periods of times, extracted proteins were immunoprecipitated with monoclonal antibodies to PLC-γ1 or -γ2 , and then PI-hydrolyzing activity in immunoprecipitates

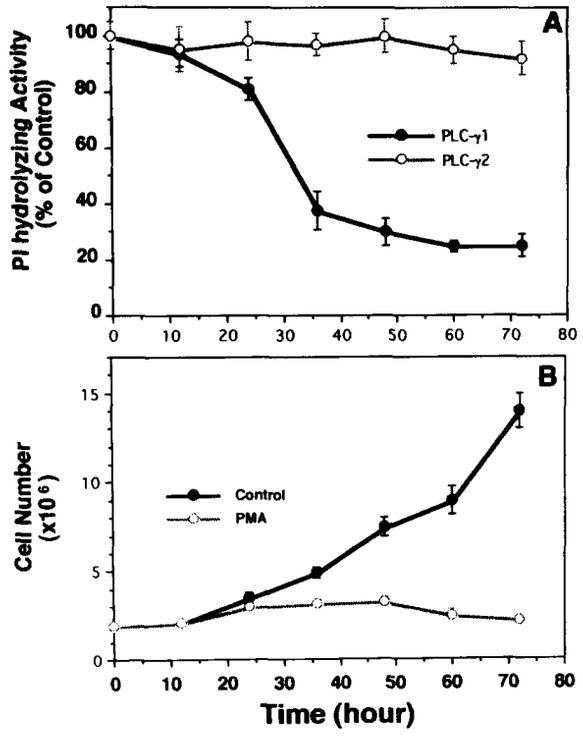

Fig. 1. Effect of PMA on U937 cells. A, U937 cells were treated with 5 nM PMA for various times. After immunoprecipitation of cell extracts with anti-PLC-γ1 (l) or anti-PLC-γ2 (m) antibody, enzyme activity in immunecomplex was measured in the assay mixture containing 50 μM PI (20,000 cpm, [^{3}H]PI) and immunecomplexes at 37°C (mean ± S.D., n = 3). B, U937 cells were seeded at a concentration of 2×10^6 cells and treated without (l) or with (m) 5 nM PMA for various times. On the hours noted, viable cells were counted in a hemocytometer chamber. Viability was determined by 0.1% Trypan blue dye exclusions.

was measured. After 24 h of PMA treatment, a significant decrease of PLC-γ1 activity was observed (Fig. 1A). The decrease was even more significant after 60 h treatment with PMA. The activity of PLC-γ1 was 81.2 ± 4.2% and 30.0 ± 5.0% of control after 24 and 48 h treatment with PMA, respectively. In contrast, no significant change in the activity of PLC-γ2 was observed during the time-course of PMA-induced differentiation. The rate of proliferation of PMA-treated cells was compared with that of control cells by determining the number of cells after the addition of PMA to cultures. Control U937 cells with the initial cell number of 2×10^6 proliferated exponentially in liquid culture. However, no proliferation and the complete cessation of cell division in PMA-treated cells became evident after 24 h as shown in Fig. 1B. This result demonstrated that the time course of the loss of proliferation of PMA-treated cells was closely correlated with that of the decrease of the PLC-γ1 activity, suggesting that the reduction of PLC-γ1 activity may be an important step in PMA-induced differentiation of U937 cells.

In order to analyze the relationship between the induction of differentiation and PLC-γ1 or -γ2 expression in U937 cells, we examined the protein levels of PLC-γ1 and -γ2 during a time course of PMA-induced differentiation. Protein level of PLC-γ1 was 64.1 ± 5.2 ng/ml and 14.7 ± 1.2 ng/ml at 0 and 60 h after PMA treatment, respectively, when determined by radioimmu-

noassay (Fig. 2A). Western blot analysis also revealed that PLC-γ1 decreased dramatically after 36 h of PMA treatment (Fig. 2A inset), whereas that of PLC-γ2 showed no significant change (Fig. 2B). This result is in agreement with the changes of the activities in immunecomplexes as shown in Fig. 1. Therefore, the decrease of PLC-γ1 activity during PMA-induced differentiation was clearly due to the down-regulation of protein level of PLC-γ1.

Recently, we have found that considerably high level of PLC-γ1 is detected in some colon cancer [7] and familial adenomatous polyposis tissues [8], suggesting that up-regulation of PLC-γ1 may be involved in cellular proliferation associated with tumor progression. Thus, the regulation of PLC-γ1 expression may play an important role in decisive designation of cellular proliferation and growth arrest. In contrast, steady-state expression of PLC-γ2 may be related to its physiological role in differentiated monocyte/macrophages. Indeed, PLC-γ2 is highly expressed in cells of hematopoietic origin, such as promyelocytic leukemia HL60 cells, histiocytic leukemia U937 cells and B cell lines [17–19]. Moreover, PLC-γ2 is involved in some functional characteristics such as CD40-mediated signaling which plays an important role in B cell survival, memory and immunoglobulin isotype switch [20] and immunoglobulin G Fc

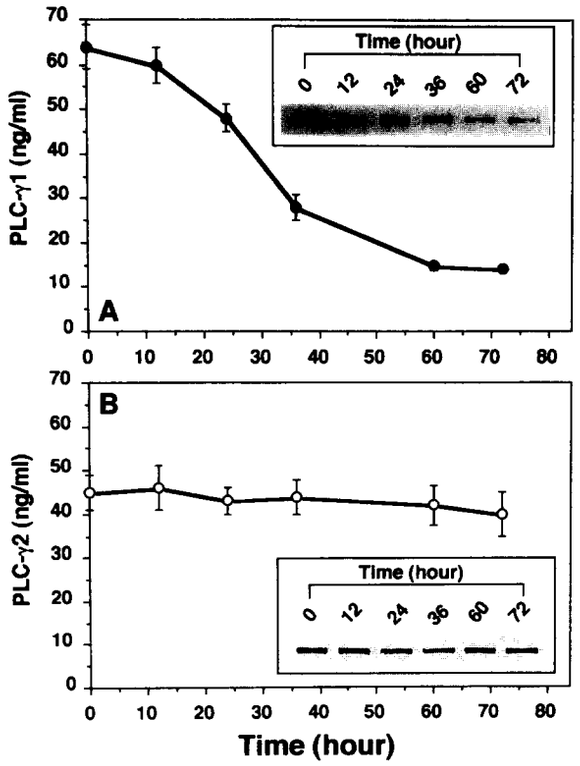

Fig. 2. Quantitation of PLC-γ1 or -γ2 by radioimmunoassay during the PMA-induced differentiation of U937 cells. U937 cells were treated with 5 nM PMA for various times. The extracts (300 μg/50 μl) were incubated with each anti-PLC-γ1 (A) or -γ2 (B) antibody-coated microtiter plate and the antibody-PLC complex was quantitated by adding ^{125}I-labeled secondary antibody (20,000 cpm/well) in phosphate buffered saline containing 5% bovine serum albumin. All samples were assayed in triplicate. *Insets:* Western blot analysis of PLC-γ1 (A) or -γ2 (B) expression. Cell lysates were immunoprecipitated and immunecomplexes were subjected to 8% SDS-polyacrylamide gels, transferred onto nitrocellulose membrane, and probed with relevant antibodies followed by alkaline phosphatase conjugated goat anti-mouse Ig.

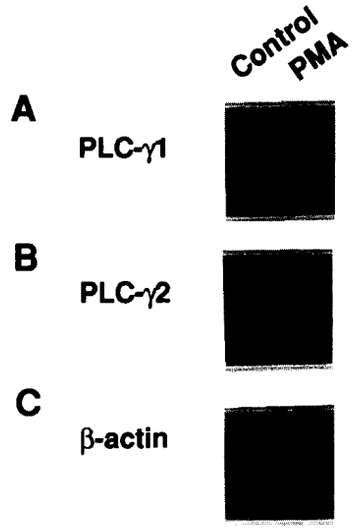

Fig. 3. Northern blot analysis of PLC-γ1 or -γ2 expression in U937 cells before and after differentiation with PMA. U937 cells were treated without *(Control)* or with *(PMA)* 5 nM PMA for 72 h. Total RNA was purified by acid phenol methods. Fifteen μg of total RNA was hybridized to the ^{32}P-labeled cDNA of PLC-γ1 (A) or PLC-γ2 (B). Hybridization of the same filter with β-actin probe(C) is shown as a control.

receptor (FcγRIIIA)-initiated signal transduction that mediates the antibody-dependent cellular toxicity in natural killer cells [21].

We ask whether the down-regulation of PLC-γ1 protein level came from transcriptional or translational regulation. The effect of PMA on the level of PLC-γ1 transcript was examined. U937 cells were treated without or with PMA for 72 h and then the level of PLC-γ1 transcript was examined with Northern blot analysis using the cDNA of PLC-γ1 as a probe. As shown in Fig. 3, mRNA level of PLC-γ1 remained unchanged during PMA-induced differntiation. Therefore, this result suggests that differentiation-induced down-regulation of PLC-γ1 protein may be controlled by the posttranscriptional events rather than by transcriptional level.

In conclusion, we have found that the level of PLC-γ1 protein markedly decreased but not PLC-γ2 during PMA-induced differentiation process. From these results, it could be assumed that down-regulation of PLC-γ1 may be an important step in differentiation of U937 cells by PMA.

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