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Regulatory role of diacylglycerol kinase γ in macrophage differentiation of leukemia cells☆

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Abstract

Although nine diacylglycerol kinase (DGK) isozymes have been identified, our knowledge of their individual functions is still limited. Here we report that the levels of DGKγ mRNA/protein in human leukemia HL-60 and U937 cells were rapidly and markedly decreased upon cellular differentiation into macrophages. In contrast, the enzyme expression remained almost unchanged in granulocytic differentiation pathway. Interestingly, the overexpression of wild-type or constitutively active DGKγ, but not its kinase-dead mutant, markedly inhibited phorbol ester-induced cell attachment and nonspecific esterase activity, which are hallmarks of macrophage differentiation. We noted in this case that no effects were observed for the corresponding constructs of a closely related isozyme, DGKα. Prior to the cell attachment, phorbol ester induced translocation of DGKγ from the cytoplasm to the cell periphery, resulting in its co-localization with F-actin together with protein kinase Cδ. The results suggest that DGKγ negatively regulates macrophage differentiation through its catalytic action operating on the cytoskeleton.

Keywords: Diacylglycerol kinase; Macrophage differentiation; Phorbol ester; Cytoskeleton; HL-60 cells; U937 cells; Diacylglycerol; Phosphatidic acid

It is well recognized that a variety of lipid mediators of both glycero- and sphingolipid origins participate in the regulation of a wide range of biological processes. The cellular concentrations of such signaling lipids are strictly regulated by the action of metabolic enzymes. Diacylglycerol (DG) kinase (DGK) phosphorylates DG to yield phosphatidic acid (PA). DG is known to be an activator of conventional and novel protein kinase Cs (PKCs), chimerins, Unc-13, and Ras guanyl nucleotide-releasing protein [1,2], and PA has been reported in a number of studies to modulate phosphatidylinositol-4-phosphate kinase, Raf-1 kinase, atypical PKC, and many other important enzymes [3]. DGK thus appears to participate in modulating the balance between two bioactive lipids, DG and PA.

Mammalian DGK is known to exist as a large protein family consisting of nine isozymes classified into five subtypes according to their structural features [4–7]. These isozymes can be characterized by the presence of a variety of regulatory domains of known and/or predicted functions, clearly indicating their distinct functions and regulatory mechanisms. The type I DGKs presently consisting of α, β, and γ-isozymes contain two sets of Ca^{2+}-binding EF-hand motifs at their N-termini. The basic structures and amino acid sequences of the three isozymes, although encoded by separate genes, are highly similar to each other to an extent that is almost superimposable. Although physiological implications of the existence of three DGK isozymes with basically the same structures remain largely unknown, the distinct

☆ *Abbreviations:* CA, constitutively active; DG, diacylglycerol; DGK, diacylglycerol kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; KD, kinase-dead; PA, phosphatidic acid; PBS, phosphate-buffered saline; PKC, protein kinase C; RT-PCR, reverse transcriptase-polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; WT, wild-type.

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tissue- and cell-dependent expression patterns detected for these isozymes suggest their differentiated functions in particular types of cells [4–7]. Moreover, we recently reported that the EF-hand motifs of the type I DGKs have properties distinct from each other with respect to affinities for Ca^{2+} and to Ca^{2+}-induced conformational changes [8]. Among the type I DGKs, DGKα has recently been a subject of intensive investigations [9–12]. However, the functions of the type I DGKs other than the α-isozyme have not yet been defined and it also remains unclear whether various functions described for DGKα are redundantly shared by other type I isozymes.

In the present work we examined the expression patterns and the effects of overexpression of the two species of the type I DGKs (α and γ-isozymes) during HL-60 cell differentiation into monocytes/macrophages or granulocytes. We demonstrate that DGKγ but not DGKα negatively regulates specifically the macrophage differentiation pathway, and for the first time show that, even among the type I isozymes sharing very similar structures, each member can be assigned with functions distinct from each other.

Materials and methods

Cell culture. HL-60 (human promyelocytic leukemia) and U937 (human promonocytic leukemia) cells were grown at a cell density of $0.5–2 \times 10^6$ cells/ml as suspension in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Life Technologies), penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37 °C in a 5% CO_2 humidified atmosphere. The cell differentiation was initiated by adding 10 nM (for HL-60 cells) or 32 nM (for U937 cells) 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Biomol Research Laboratories) or 1.5% dimethyl sulfoxide (DMSO) (Sigma) at a cell density of 2×10^5/ml.

Reverse transcriptase-polymerase chain reaction. Total RNA isolation, reverse transcription, and PCR amplification were performed as described [13]. The specific primers used were as follows: human DGKα [14]: forward primer 5′-TCCCTTCTGGAGGGTGGTCGG-3′ (nucleotide positions 413–433), reverse primer 5′-CTCCAGACCC AGCAGCACTAG-3′ (694–674); human DGKγ [15]: forward primer 5′-TCCCTGCTGGAGACGGGGAGG-3′ (1021–1041), reverse primer 5′-ATCCATCCCCAGGAGCACCAG-3′ from (1302–1282). For normalization, human glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA was simultaneously amplified.

Western blot analysis. HL-60 cells (4×10^6) were resuspended in 0.4 ml of lysis buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaF, 1 mM dithiothreitol, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin, pepstatin, aprotinin and soybean trypsin inhibitor, and FOY 305 (Ono Pharmaceutical, Osaka, Japan). Western blot analysis for DGK enzyme proteins was performed as described previously [16].

Plasmids and transfection. The cDNAs encoding wild-type (WT) DGKα, constitutively active (CA) DGKα (Δ1–196) [17], DGKγ-WT [15], and DGKγ-CA (Δ1–259) were amplified by PCR and subcloned into pEGFP-C3 using *Xho*I/*Pst*I site (DGKα) or *Hin*dIII/*Sal*I site (DGKγ). The kinase-dead (KD) versions of green fluorescent protein (GFP)-DGKα and -DGKγ were generated replacing in their catalytic domains Gly435 and Gly494, respectively, with Asp using the Quickchange Site-directed Mutagenesis Kit (Stratagene). HL-60 cells were transiently transfected with Tfx-50 transfection reagent (Promega) as described by the manufacturer. U937 cells were transiently transfected by electroporation (300 V, 950 μF) according to the instructions from the manufacturer (BioRad Laboratories).

Microscopy. U937 cells were collected and fixed on poly-L-lysine-coated glass coverslips with 3.7% formaldehyde–phosphate-buffered saline (PBS) for 20 min, permeabilized with 0.1% Triton X-100–PBS for 5 min, and blocked in 1% bovine serum albumin–PBS for 30 min. For visualization of F-actin, cells were incubated with Alexa 594-conjugated phalloidin (Molecular Probe) for 1 h, and then washed five times with PBS at room temperature. Confocal microscopy was performed as described previously [16].

Separation of adherent and nonadherent cells. After TPA treatment for 24 h, nonadherent cells were separated by rocking. Culture dishes with the adherent cells were gently washed three times with PBS and then incubated in 0.05% trypsin/0.02% EDTA at 37 °C for 5 min. The cells were suspended by pipetting. The number of nonadherent and adherent cells exhibiting green fluorescence was counted using a hematocytometer.

Cytochemical analysis of nonspecific esterase activity. After TPA treatment for 18 h, U937 cells were collected and fixed with 3.7% formaldehyde/60% acetone. Cytochemical stain of the cells having nonspecific esterase activity was performed according to the instructions from the manufacturer (Muto Pure Chemicals, Tokyo, Japan). The percentage of cells containing the intracellular reddish brown deposits was determined under light microscopy.

Results

Distinct expression patterns of DGKα and DGKγ during leukemia cell differentiation

We found that when HL-60 cells were treated with TPA, the DGKγ mRNA rapidly decreased to an almost undetectable level (Figs. 1A and B). The suppression of DGKγ expression was almost completed as early as 3 h after TPA treatment and remained suppressed thereafter. When the same experiment was performed using another leukemia cell line, U937, essentially the same results were obtained (data not shown). In marked contrast, the mRNA level was affected to a much lesser extent during granulocyte differentiation induced by DMSO. In accord with the expression patterns of DGKγ mRNA, the treatment with TPA, but not with DMSO, rapidly decreased the protein levels of DGKγ at day 1 (Figs. 1C and D). The results suggest that the suppression of DGKγ expression occurs specifically at the early stages of macrophage differentiation. The decrease of DGKγ enzyme protein was not much marked when compared to that of its mRNA. The results, however, suggest that the cellular DGKγ content needs to be kept lower than a certain threshold level to initiate cellular differentiation.

In contrast to DGKγ, a moderate increase of the DGKα mRNA was observed in the presence of TPA by day 2 (Fig. 2). We also confirmed that the protein level of DGKα significantly increased by TPA at day 2 (data not shown). The DMSO treatment did not affect significantly DGKα expression (Fig. 2). We also noted that the mRNA level of a type II isoform, DGKδ, decreased

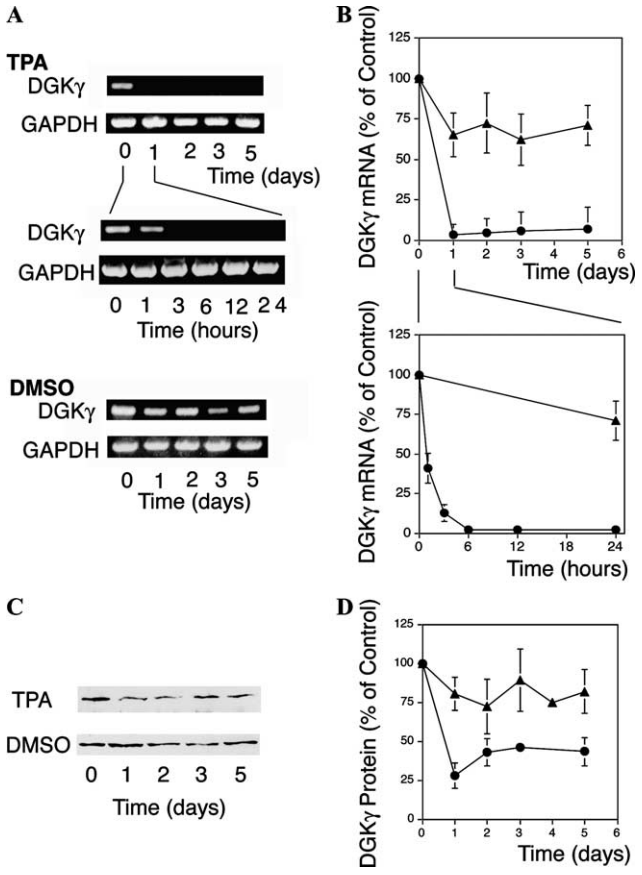

Fig. 1. The mRNA and protein levels of DGKγ during HL-60 cell differentiation induced by TPA- or DMSO-treatment. HL-60 cells were cultured in the presence of 10 nM TPA or 1.5% DMSO for indicated time periods. (A) The mRNA levels of DGKγ and GAPDH were analyzed by RT-PCR (25 cycles). Upper and lower panels in each set show 282 and 700-bp cDNA fragments amplified for DGKγ and GAPDH, respectively, in agarose gel electrophoresis. Typical results are shown. (B) The visualized bands of DGKγ mRNA from TPA- (circles) or DMSO- (triangles) treated cells were quantitated by densitometry and normalized for GAPDH amplification. The values of untreated cells are set at 100 and the data are means ± SD ($n = 4$). (C) The cell homogenates (20 μg of protein each) were separated by SDS–polyacrylamide gel electrophoresis and the DGKγ protein (88 kDa) was detected by Western blotting using anti-human DGKγ polyclonal antibodies. Typical results are shown. (D) The visualized bands of DGKγ protein from TPA- (circles) or DMSO- (triangles) treated cells were quantitated by densitometry. The values of untreated cells are set at 100 and the relative values are presented as means ± SD ($n = 4$–8).

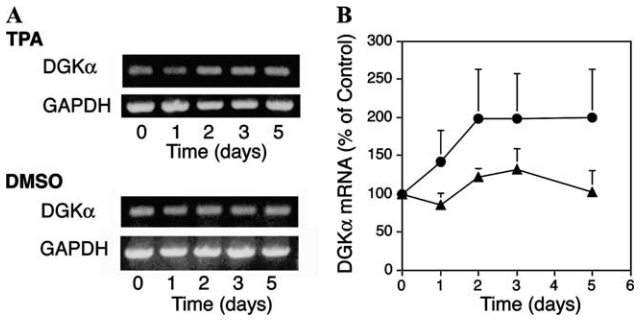

Fig. 2. The mRNA levels of DGKα during HL-60 cell differentiation induced by TPA- or DMSO-treatment. (A) HL-60 cells were cultured in the presence of 10 nM TPA or 1.5% DMSO for indicated periods. The mRNA levels of DGKα and GAPDH were analyzed by RT-PCR (25 cycles). Upper and lower panels in each set show 282 and 700 bp-cDNA fragments amplified for DGKα and GAPDH cDNAs, respectively. Typical results are shown. (B) The visualized bands of DGKα mRNA from TPA (circles)- or DMSO (triangles)-treated cells were quantitated by densitometry. The values of untreated cells are set at 100 and the values are means ± SD ($n = 4$).

only slightly at day 5 after TPA treatment (not shown). The results thus clearly indicated that the markedly suppressed expression occurred specifically for the γ-species among DGK isozymes.

We next attempted to see whether the marked change of DGKγ expression was indeed related to monocytic differentiation or simply reflected the pharmacological action of TPA. As observed for TPA treatment, another monocyte-inducer, sodium butyrate [18], also caused a marked reduction of the DGKγ mRNA levels while not affecting the DGKα expression (Table 1). Another neutrophil-inducer, all-*trans* retinoic acid [18], decreased DGKγ expression to a much lesser extent similarly as observed for DMSO treatment.

Effects of overexpression of DGKγ and its mutants on macrophage differentiation

To explore the physiological implications of the suppression of DGKγ expression, we next investigated the effects of overexpression of DGKγ and its mutants on cellular differentiation. Most of HL-60 cells trans-

Table 1
Effects of various differentiation inducers on the mRNA levels of DGKα and DGKγ in HL-60 cells

Inducers	DGKα mRNA (% of control)		DGKγ mRNA (% of control)	
	1 day	3 days	1 day	3 days
None	100	100	100	100
TPA	110.9 ± 13.0	180.5 ± 58.5	3.5 ± 0.9	6.2 ± 1.9
Sodium butyrate	100.0 ± 4.3	101.9 ± 13.0	21.4 ± 6.2	6.1 ± 3.2
DMSO	98.9 ± 13.3	121.2 ± 15.8	65.3 ± 5.9	58.0 ± 15.1
All-*trans* retinoic acid	95.4 ± 13.7	101.4 ± 20.5	57.3 ± 17.2	48.8 ± 12.9

HL-60 cells were cultured in the presence of 10 nM TPA, 3 mM sodium butyrate, 1.5% DMSO, or 1 μM all-*trans* retinoic acid for indicated periods. The mRNA levels of DGKα, DGKγ, and GAPDH (for normalization) were analyzed by RT-PCR (25 cycles). The visualized bands were quantitated by densitometry. The values of untreated cells are set at 100. Values are means ± SD ($n = 4$).

fected with the vector alone became attached to the tissue culture dishes by day 1 after treatment with TPA, thus exhibiting the phenotype of macrophage differentiation (Fig. 3A). The cell attachment was significantly inhibited by the expression of DGKγ-WT and this inhibition was markedly augmented by DGKγ-CA. The KD mutant failed to affect the cell attachment, thus indicating that the catalytic activity of DGKγ is essential for its inhibitory effects. Transfections of the corresponding DGKα constructs totally failed to affect the cell attachment. Moreover, as observed for HL-60 cells, the attachment of U937 cells was considerably inhibited by the expression of DGKγ-WT and this inhibition was significantly enhanced by DGKγ-CA (Fig. 3B). Trypan blue staining showed that more than 95% of the floating cells were viable, indicating that the inhibition of cell adherence or nonspecific esterase activity (see later) had not been caused by cytotoxicity of the overexpressed enzymes. Furthermore, in separate experiments employing COS-7 cells overexpressing wild-type DGKγ for 2 days, we did not detect significant cell death when assessed using cell death detection ELISA (data not shown).

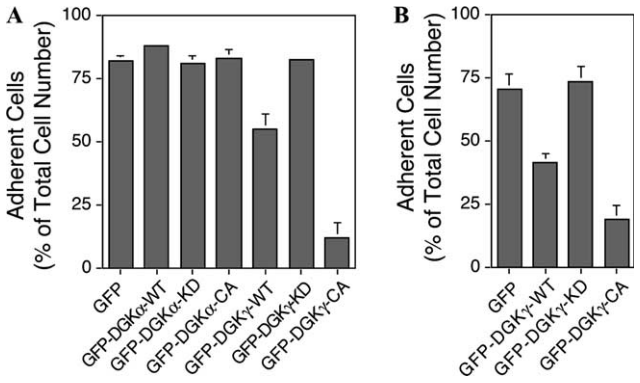

Fig. 3. Effects of overexpression of DGKα, DGKγ, and their mutants on adherence of leukemia cells during macrophage differentiation. HL-60 (A) and U937 (B) cells were transfected with the expression plasmids indicated 24 h before TPA stimulation and the number of floating and attached cells that exhibited green fluorescence was determined 24 h after the stimulation. The total number of cells counted in a single experiment ranged from 102 to 165 for HL-60 cells and from 117 to 216 for U937 cells. The average values ± SD ($n = 3$) of repeated experiments are shown. We confirmed that more than 95% of floating cells were viable (see text).

In addition to the cell adherence, we examined nonspecific esterase activity as a macrophage-differentiation marker [19]. We confirmed that about 50% of U937 (Table 2) and 80% of HL-60 (data not shown) cells developed nonspecific esterase activity when cultured with TPA for 18 h. The expression of DGKγ-WT reduced significantly the appearance of nonspecific esterase-positive U937 cells (Table 2). This reduction became more marked when the CA mutant was expressed. However, DGKγ-KD and DGKα-CA failed to affect the enzyme activity. Taken together, it is now clear that the cellular content of DGKγ needs to be lowered to a certain level to initiate and sustain macrophage differentiation.

Co-localization of DGKγ with F-actin and PKCδ

The cytoskeletal reorganization is known to be critically involved in the macrophage differentiation of leukemia cells [20] and in this context rat brain DGKγ was reported to be recovered in the Triton X-100-insoluble cytoskeletal fraction [21]. Thus, we next analyzed the enzyme distribution in U937 cells during macrophage differentiation in conjunction with the cytoskeleton (F-actin). Immunofluorescence analysis demonstrated that DGKγ and its mutants (not shown) were distributed at both the cytoplasm and the cell periphery in the absence of TPA (Fig. 4A). In CHO cells [22], DGKγ was shown to be translocated to the plasma membranes upon TPA-treatment. In the present work, DGKγ and its mutants partly translocated to the cell periphery and exclusively co-localized with F-actin after TPA-treatment. In contrast, DGKα-CA and its WT- and KD-enzymes (not shown) showed a diffuse cytoplasmic pattern and they gave only a moderate co-localization with F-actin even in the presence of TPA.

It is known that PKCδ is essential for macrophage differentiation of leukemia cells [23] and that its interaction with F-actin is important for regulating actin redistribution in leukocytes [24]. As shown in Fig. 4B, although DGKγ and PKCδ appeared to be only partly co-localized in the absence of TPA, the distribution patterns of DGKγ and PKCδ became essentially superimposable in U937 cells treated with TPA. This result further suggests functional relevance of DGKγ to the cytoskeletal reorganization.

Table 2
Effects of overexpression of DGKγ- and DGKα-mutants on nonspecific esterase activity of U937 cells during macrophage differentiation

	Nonspecific esterase-positive cells (% of total cell number)				
	GFP alone	GFP-DGKα-CA	GFP-DGKγ-WT	GFP-DGKγ-KD	GFP-DGKγ-CA
−TPA	6.6 ± 2.0	3.5 ± 2.3	5.2 ± 4.9	5.5 ± 1.6	3.3 ± 2.4
+TPA	50.8 ± 1.9	50.6 ± 3.7	28.4 ± 6.6	52.8 ± 2.7	9.7 ± 5.8

U937 cells were transfected with the expression plasmids indicated 24 h before TPA stimulation. The number of nonspecific esterase-positive cells that exhibited green fluorescence was determined 18 h after the stimulation. The total numbers of cells counted in a single experiment ranged from 25 to 50. The average values (% of total cell number) ± SD ($n = 4$) of repeated experiments are shown.

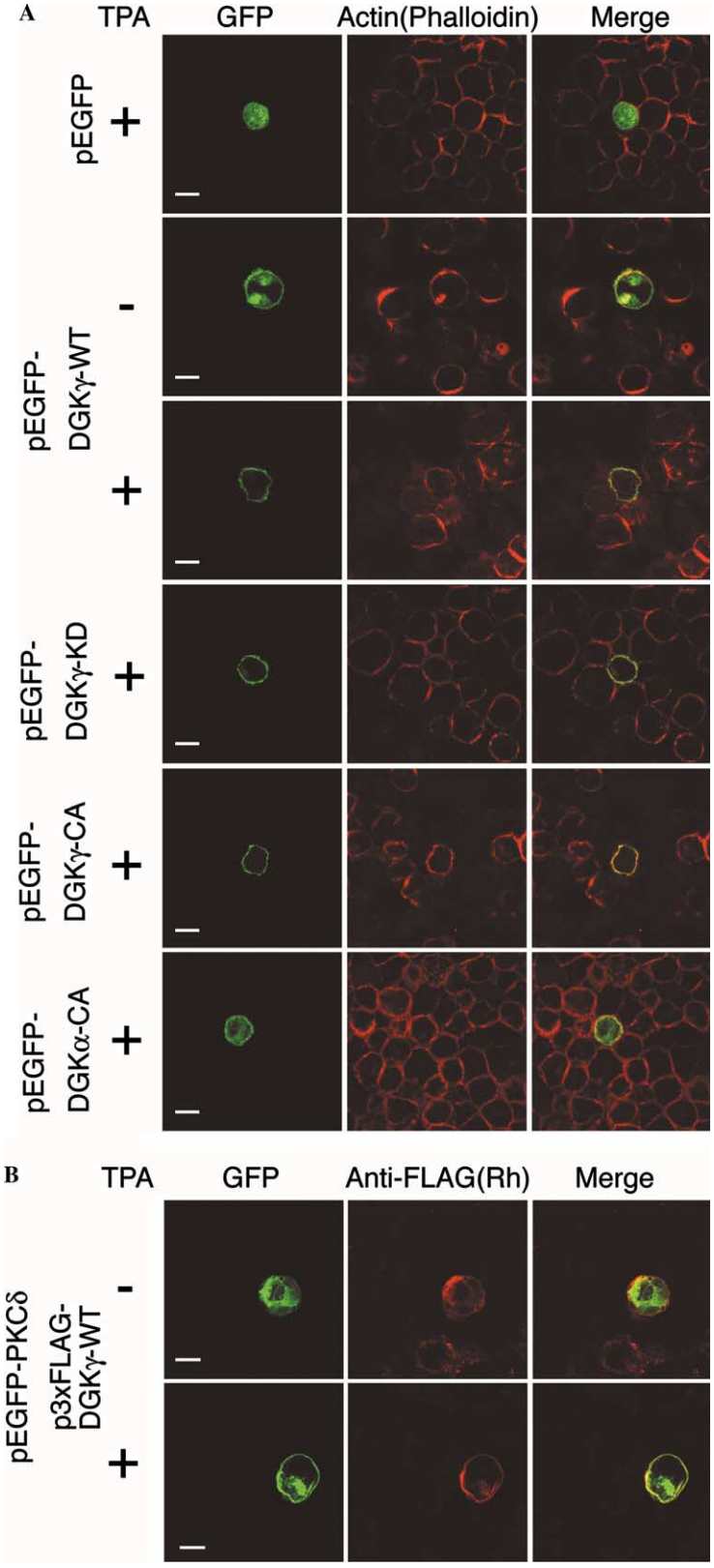

Fig. 4. Co-localization of DGKγ with actin and PKCδ in U937 cells. U937 cells were transfected with the plasmids indicated 24 h before TPA stimulation. Eighteen hours after the stimulation with 32 nM TPA, floating cells were fixed. F-actin was labeled with rhodamine-conjugated phalloidin (A). DGKγ was labeled with anti-FLAG antibodies and rhodamine (*Rh*)-conjugated secondary antibodies (B). GFP (green) and rhodamine (red) fluorescence were visualized by confocal laser scanning microscopy. Representative cell images reproducibly obtained for repeated transfection experiments are given. Bar, 10 μm.

Discussion

Lineage-specific blood cell differentiation and maturation are multistage processes involving a signal transduction cascade that entails many different proteins. The precise mechanisms whereby the blood lineages specifically develop are largely unknown. Because cell differentiation is accompanied by a loss of cell-proliferative potential, many genes exemplified by c-*myc* [25] were down-regulated (or up-regulated) along both monocyte/macrophage- and granulocyte-differentiation pathways. However, only a few genes such as proteoglycan core protein [26] and 55-kDa tumor necrosis factor receptor [27] genes have been reported so far to be decreased specifically during the macrophage differentiation. In this study, we showed that the rapid (within 3 h) decrease of DGKγ expression was specific for the induction of macrophage differentiation. This DGKγ isozyme protein at a certain reduced level should be essential for the early stages of cellular differentiation. Thus, it is possible that DGKγ is a novel therapeutic target in the area of leukemia.

It was of interest to note that the inhibitory effects of overexpressed DGKγ on the phenotypes of macrophage differentiation such as cell attachment and nonspecific esterase activity could become dominant over those of presumably potent reagent, phorbol ester, suggesting that DGKγ acts downstream of PKC or other phorbol ester-receptors. The TPA-induced signal transduction leads to drastic reorganization of the cytoskeletal networks, which closely links to the acquisition of the macrophage-specific phenotype [20]. In this study, we confirmed that DGKγ co-localized with the cytoskeletal component, F-actin. Although targets of DG and/or PA and precise roles of DGKγ in the differentiation of leukemia cells are still unclear, this localization pattern suggests that the isozyme is involved in the cytoskeletal reorganization. Tolias et al. [28] recently showed an interaction of unidentified DGK(s) with Rac1 that controls actin assembly. Because DGKγ was co-localized with actin, this isoform may be one of the unidentified DGK(s) noted previously. Because it has been reported that the cytoskeleton participates in the regulation of gene expression [29,30], DGKγ may indirectly regulate the macrophage differentiation through affecting gene expression of important proteins such as adhesion molecules and lysosomal enzymes.

In this paper, we demonstrated for the first time that DGKγ may act as a negative regulator at early stages of the phorbol ester-induced signal transduction cascade that results in macrophage differentiation of leukemia cells. On the other hand, increased expression levels of DGKα at later stages are likely to be necessary for differentiated macrophages. DGKα has been described to play important regulatory roles in T-cell proliferation [9,10], in the movement of endothelial cells induced by hepatocyte growth factor [11], and in the induction of hypoxia-inducible transcription factor 1 [12]. Thus, it is likely that, even among the type I isozymes sharing very similar structures, each DGK isoform has a differentiated function at its specific operating sites, although commonly accomplished by attenuating DG-signal and/or generating PA-signal.

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