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Differentiation of HL-60 Cells to Granulocytes Involves Regulation of Select Diacylglycerol Kinases (DGKs)

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Abstract Diacylglycerol Kinases (DGKs) are a family of enzymes that regulate the levels of different pools of diacylglycerol (DAG), affecting DAG-mediated signal transduction. Since DAG is known to play several important regulatory roles in granulocyte physiology, we investigated the expression pattern of DGK isoforms throughout differentiation of HL-60 cells to granulocytes. HL-60 cells were incubated with 1.25% dimethyl-sulfoxide (DMSO) to initiate differentiation and total RNA isolated at different time points. DGK expression was assessed through Northern blot, end-point PCR, and real-time PCR. The non-selective inhibitors R59022 and R59949 were used to block DGK at different time points throughout differentiation. CD11b and GPI-80, reactive oxygen species (ROS) generation, changes in the cell cycle, and apoptosis were used as markers of differentiation. Of the nine isoforms of DGK evaluated (α, δ, ε, γ, ζ, β, θ, ι, η), only five (α, δ, ε, γ, and ζ) were expressed in HL-60 cells. DGKα was virtually absent in non-differentiated cells, but was markedly upregulated throughout differentiation. The other isoforms (δ, ε, γ, and ζ) were expressed in undifferentiated HL-60 cells but were substantially decreased throughout differentiation. Non-selective blocking of DGK with R59022 and R59949 led to acceleration of differentiation, reducing the time necessary to observe upregulation of CD11b, GPI-80 and generation of ROS by 50%. Likewise, the cell cycle was disrupted when DGK isoforms were inhibited. These results provide evidence that DGK levels are dynamically regulated throughout differentiation and that expression of DGKs play an important regulatory function during the differentiation of neutrophils. J. Cell. Biochem. 94: 774–793, 2005.

Key words: gene expression profiling; signal transduction; cell cycle; apoptosis; leukocyte differentiation antigens; human

Diacylglycerol (DAG) is an important lipid second messenger, which has been shown to be key molecule(s) in signal transduction and the regulation of important cell processes [Topham and Prescott, 1999; van Blitterswijk and Houssa, 1999, 2000]. Distinct pools of DAG are generated in different cell compartments by activation of phospholipases C and D [Koo et al., 1983; Lad et al., 1984; Gewirtz et al., 1998]. Specifically, in the pathway mediated by phospholipase C, hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) generates the secondary messengers inositol 1,4,5 triphosphate and *sn*-1,2-diacylglycerol; the former induces release of intracellular calcium stores whereas the latter activates protein kinase C (PKC) by increasing its affinity for calcium [Berridge and Irvine, 1984; Biden et al., 1984; Burgess et al., 1984]. This regulatory pathway has been shown to be central to phagocyte biology [Burgess et al., 1984; Cooke et al., 1987; Badwey et al., 1988]. Specifically, in human polymorphonuclear neutrophils (PMN), stimulation with formyl peptides and other agonists results an increase in intracellular DAG, which is time and agonist concentration-dependent [Honeycutt and Niedel, 1986]. DAG is the natural activator of PKC, leading to NADPH oxidase assembly and sustained generation of superoxide anion [Rider and Niedel, 1987]. Intracellular DAG levels are

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tightly regulated by the Diacylglycerol Kinases (DGKs), a family of enzymes ubiquitous to many species, the function of which is to metabolize DAG by conversion to phosphatidic acid (PA) [Kato and Takenawa, 1987; Topham and Prescott, 1999]. A number of DGK isoforms have been characterized (α, ζ, ϵ, γ, δ, β, η, ι, θ) that are coded by different genes [Endele et al., 1996; Hart et al., 1999] and seem to be involved in the regulation of DAG pools generated in different cell compartments. DGKs have been grouped according to their structural similarities [Topham and Prescott, 1999], some presenting variants of the same isoform [Ding et al., 1997; Sakane et al., 2002; Murakami et al., 2003]. The expression of different DGK isoforms is tissue specific [Sakane et al., 1996; Houssa et al., 1997; Ding et al., 1998], which is related to the different DAG molecular species (fatty acid side chain substitutions) found in resting and stimulated cells [Divecha et al., 1991]. The variety of DGK isoforms characterized thus far underlines the regulatory importance of DAG as a secondary messenger and the diversity and complexity of the regulation of lipid-mediated signal transduction.

Cellular DAG content and composition has also been implicated in cancer and as a possible modulator of the cell cycle, based upon its dynamic regulation after addition of mitogenic agents [Lavie and Agranoff, 1996; Martelli et al., 2000]. It has been observed that a rapid change in the levels of nuclear DAG occurs in the human leukemia cell-line, HL-60, after addition of polar solvents, leading to a progressive increase of DAG throughout differentiation [Neri et al., 2002]. Since DGKs act as the natural regulators of DAG, we sought to temporally characterize the expression of DGKs during HL-60 cell differentiation. Here we show that select DGKs are expressed in HL-60 cells and that these isoforms are dynamically regulated throughout differentiation. In a further series of experiments we show that pharmacological inhibition of DGK can accelerate the differentiation of HL-60 cells to neutrophil-like cells.

MATERIALS AND METHODS

Cell Culture and Isolation of Peripheral Blood Neutrophils

HL-60 cells (ATCC, Manassas, VA) were cultured in Iscove's medium supplemented with 10% heat inactivated fetal bovine serum (HIFBS) at 37°C in a 5% CO_2 atmosphere until confluent. To initiate differentiation, 7.5×10^6 cells were centrifuged ($100 \times g$) and transferred to new flasks containing 15 ml of prewarmed Iscove's medium supplemented with 10% HIFBS and 1.25% dimethyl-sulfoxide (DMSO) [Collins et al., 1978]. Control non-differentiated (ND) cells were processed in the same way except for the addition of DMSO. On the third day, cells in all groups received an additional volume of media either containing only serum (ND) or DMSO. Cell viability was evaluated using trypan blue exclusion and changes in morphology were assessed visually after Giemsa-Wright's stain. All experiments were performed within six passages. Peripheral blood neutrophils were obtained from three healthy volunteers as previously reported [Kalmar et al., 1988]. Briefly, approximately 50 ml of peripheral blood was obtained by venipuncture and stored in heparinized tubes. Neutrophils were isolated after gradient centrifugation and isotonic lysis of red blood cells. After two washes in Ca^{2+}/Mg^{2+}-free PBS, cells were immediately processed for further experimentation.

Reactive Oxygen Species (ROS) Production

Two assays of ROS production were employed. Oxidation of Dihydrorhodamine (DHR123) and reduction of nitro-blue-tetrazolium (NBT) were carried out as previously reported [Ujihara et al., 1998; Walrand et al., 2003]. Before each assay, cells were washed twice in Ca^{2+}/Mg^{2+}-free PBS and resuspended in fresh 10% HIFBS media for 24 h at 37°C and 5% CO_2 atmosphere. For oxidation of DHR123, 5×10^5 cells were washed, resuspended in PBS and transferred to microtubes. DHR123 was added to the tubes to a final concentration of 5 μM and incubated on a shaker for 15 min at 37°C and 5% CO_2 atmosphere. PMA was added to a final concentration of 500 nM and cells were incubated on a shaker for an additional 5 min at 37°C and 5% CO_2. Cells were then kept on ice protected from light until fluorescence was measured in a flow cytometer (FACScan). For reduction of NBT, 5×10^5 cells were resuspended in PBS containing NBT (200 nM) and PMA (500 nM) and cells were incubated on a shaker in a humidified atmosphere at 37°C containing 5% CO_2. After 30 min, 1N HCl was added and microtubes were centrifuged for 5 min at $12{,}000 \times g$. The supernatant was discarded and the formazan precipitate resus-

pended in 200 μl of DMSO and transferred to 96-well plates. Absorbance of the precipitates was analyzed in a plate reader at 572 nm.

Flow Cytometric Assessment of GPI-80 and CD11b

Flow cytometry was used to assess the degree of differentiation of the HL-60 cells using two different neutrophil markers: GPI-80 and CD11b. Cell aliquots (1×10^6) were washed once in PBS, and resuspended in 500 μl of Ca^{2+}/Mg^{2+}-free PBS containing 2% FBS/0.05% sodium azide. Aliquots were then incubated for 30 min at RT with mouse monoclonal antibodies to human GPI-80 (Medical and Biological Laboratories Co., Nagoya, Japan) or CD11b (BD Biosciences, San Diego, CA) at a 1:500 dilution. After two washes in PBS, cells were centrifuge for 2 min at 100 × g, the supernatant was decanted, and the cells were resuspended in 50 μl of 1:50 dilution of FITC-labeled goat secondary antibody (Santa Cruz, Santa Cruz, CA) and incubated at RT for 15 min in the dark. After three washes in PBS, cells were resuspended in 500 μl of PBS and kept at 4°C. Stained cells were analyzed using a Flow Cytometer, and side-scatter and forward-scatter dot plots were generated (CellQuest Software). Peripheral blood neutrophils were used as references and FL-1 histogram plots of gated populations were generated.

Bromodeoxyuridine (BrdU) Incorporation Assay

In order to evaluate cell proliferation, incorporation of BrdU into DNA was assessed. One-hundred microliter of freshly passed cells at a density of 2×10^5 cells/ml were centrifuged (100 × g) and resuspended in 1 ml of media containing the DGK inhibitors R59022 and R59949 (Calbiochem, San Diego, CA) at final concentrations of 5, 10, 30, and 50 μM. Controls included cells incubated in 10% HIFBS Iscove's media only, DMSO (1.25%), the DNA-polymerase inhibitor Aphidicolin (5 μg/ml), cells with no BrdU and media only (blank). Cell suspensions were then transferred to 96-well plates and incubated in a 5% CO_2 atmosphere, at 37°C. After 24 h, 100 μl of BrdU (Oncogene, San Diego, CA) was added to each well and further incubated at 37°C in a 5% CO_2 humidified atmosphere. After an additional 24 h, cells were fixed, DNA denatured, and cells incubated at room temperature with mouse anti-BrdU primary antibody for 60 min. Following incubation with goat anti-mouse/horseradish peroxidase conjugate, the colorimetric reaction was precipitated through the addition of tetra-methylbenzidine substrate. The reactions were stopped by the addition of 2.5N sulfuric acid and the plate was read immediately using a dual wavelength (450–595 nm) spectrophotometer.

RNA Purification

For reverse transcriptase-PCR (RT-PCR) and real-time PCR, cells cultured in DMSO-containing medium for different time intervals and control non-differentiated cells (sampled at the same time intervals) were separated in 10^7 cell aliquots, centrifuged at 100 × g for 5 min, and RNA extracted using size exclusion chromatography and in-column DNase I treatment (Absolutely RNA, Stratagene, La Jolla, CA) according to the manufacturer's instructions. RNA concentrations were spectrophotometrically determined at 260/280 nm and samples stored at −80°C. The quality of RNA was assessed by separation of 5 μg aliquots in 1% formaldehyde denaturing gels. Sharp 28s and 18s ribosomal RNAs without smearing was obtained. Lower concentrations were analyzed by Sybr-Gold (Molecular Probes, Eugene, OR) staining. For Northern blotting, the same procedures were employed except that approximately 3×10^7 cells were lysed at each time point. After elution, RNA was concentrated by incubation with 0.5M ammonium acetate, pH 5.5, and 2.5 volumes of absolute ETOH at −20°C. After 24 h, cells were centrifuged, the supernatant discarded, and the pellet washed in 70% ETOH. Pellets were then dried at room temperature, resuspended in RNase free water at a concentration of 5 mg/ml, and stored at −80°C.

RT-PCR

Primers containing restriction sites (BamHI and EcoRI) close to the 5′ ends were designed for all DGK isoforms and specificity verified using BLAST (NCBI) (Table I). After reverse transcription of 2 μg of total RNA with random primers (Superscript III, Invitrogen), approximately 300 ng of cDNA was used as a template in 50 μl PCR reaction mixtures containing 300 nM forward and reverse primers, 1.5 mM $MgCl_2$, and 1 U of Taq proofreading mixture (Platinum Taq High Fidelity, Invitrogen). Mixes were incubated at 94°C for 3 min and

TABLE I. Recombinant Custom Primers Used for Amplification of DGKs

Gene	Accession	Primer sequence	Amplicon size
β-actin	461167	**F** 5′-GCC**ggatcc**CCCAGCACAATGAAGATCAA-3′ **R** 5′-GCT**gaattc**ATGGCAAGGGACTTCCTGTA-3′	473
α	NM201444	**F**-5′-GGT**ggatcc**AGGAGACCCCATGGGG-3′ **R**-5′-GGC**gaattc**GCATGGGCATCTGGTTC-3′	331
β	NM004080	**F** 5′-AAT**ggatcc**TGATCATGATGGAACCGTGT-3′ **R** 5′-GAT**gaattc**CACCAAACACAATGCAGTCC-3′	419
δ	D73409	**F** 5′- TAT**ggatcc**GCAGGACTTTGAGAACT-3′ **R** 5′- CAG**gaattc**TCAGCAGCTTTTCATCCAG-3′	415
ε	BC022297	**F** 5′-CCT**ggatcc**TTTCCCCCGCCCGGCTTC-3′ **R** 5′-ATT**gaattc**GGCCTCTACACCACTC-3′	680
γ	NM001346	**F** 5′-AGC**ggatcc**GCACGATTAAAATTACTC-3′ **R** 5′-AGG**gaattc**TGGGGCTCTTGGAG-3′	850
ι	NM004717	**F** 5′-AGG**ggatcc**CAGGAGGGGAAATGTAAGCA-3′ **R** 5′-CAA**gaattc**GCAAGGGTTTCATGAGAGGA-3′	358
η	NM152910	**F** 5′-AGT**ggatcc**CATCCCTAGCTACGCAGGAG-3′ **R** 5′-GTC**gaattc**GGGATGGATGTACAGGTTGG-3′	385
θ	NM001347	**F** 5′-ATG**ggatcc**AGGATCACGACACCCATCAC-3′ **R** 5′-ACG**gaattc**CGGAACTGGCTTCTTCTCAC-3′	350
ζ	NM201532	**F** 5′-GAC**ggatcc**GGCCGCGCTGCAG-3′ **R** 5′-TTG**gaattc**TCCTGGGCTCGGTCG-3′	550

then subjected to a three-step PCR protocol (94°C-1′, 55°C-30″, and 68°C-1′) for 28 cycles, followed by a final incubation at 68°C for 8 min. PCR aliquots were separated in 1% agarose gels and stained with ethidium bromide. Expected products (331–850 bp) were gel-purified (Qiaquick, Qiagen, Valencia, CA) and stored at −20°C for further restriction nuclease treatment, cloning, and sequencing. Primer validation and PCR optimization were carried out using cDNA reverse transcribed from human brain and testis total RNA (Ambion, Austin, TX).

Real-Time PCR

cDNA first strand synthesis was carried out from 200 ng of total RNA primed with random hexamers and reverse transcribed using AML-V reverse transcriptase in 10 μl reaction mixtures (final concentration of 20 ng/μl of cDNA). Reaction mixtures were subjected to 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min in a thermal cycler (ABI 9700, Applied Biosystems, Foster City, CA). Reverse transcription products were prepared in 10 ng duplicates and real-time PCR performed using primers and TaqMan probes labeled with FAM-MGB dye (Applied Biosystems, Assays-on-Demand and Assays-by-Design) for the DGK isoforms identified by Northern blot and RT-PCR as being expressed in HL-60 cells (isoforms α, δ, γ, ε, and ζ). DGK standards for isoform α were prepared from a 10-fold dilution of human brain cDNA, a tissue known to express this isoform [Topham and Prescott, 1999]. For isoforms δ, ε, γ, and ζ, total RNA was used as a source of cDNA, since end-point PCR showed that these isoforms are expressed. β-actin was selected as an endogenous control and amplified using pre-formulated VIC-TAMRA labeled TaqMan probes (Applied Biosystems, Endogenous Control). The source of cDNA used for standard preparation was the same as for the target DGK, e.g., either brain or HL-60 total RNA. Quantification was performed in an automated thermalcycler (ABI Prism 7000) and results analyzed through a software interface and Excel spreadsheet for calculation of relative expression (DGK/β-actin). Data was obtained from three separate experiments run in duplicate 1 week apart.

Northern Blotting

Twenty micrograms of each sample was fractionated on 1.2% formaldehyde denaturing gels overnight at 18 V and quality checked through ethidium bromide staining of 18s and 28s ribosomal RNA. RNA was then transferred to nylon membranes by vacuum blotting for 2 h at 5 inches of Hg. Membranes were then air-dried, UV-crosslinked (Stratagene) at 120 milijoules for 30 s and briefly washed in DEPC-treated water. For synthesis of RNA probes, PCR products amplified from human brain and HL-60 cells with recombinant primers (Table I) were incubated with BamHI and EcoRI (Roche Molecular, Indianapolis, IN) overnight (50 U each). After clean up of the enzymatic reaction using size exclusion chromatography, PCR products were cloned by incubation with linearized (EcoRI/BamHI) T3/T7 p18 plasmid (Ambion) and 5 U of T4 DNA ligase (Roche Molecular)

overnight at 4°C. Plasmids were then used to transform chemically competent *E. coli* (Invitrogen) and propagated on ampicillin-containing agar plates incubated at 37°C overnight. Positive clones were identified by PCR and cultured overnight in LB media. Recombinant plasmids containing the target DNA sequences were isolated (Qiagen) and stored at −20°C. Plasmid DNA aliquots were sequenced (Boston University Genetics Core) to confirm the identity of the fragments. For RNA probe synthesis, approximately 1 μg of DGK recombinant plasmid was linearized with XbaI downstream to the T7 promoter and incubated at 37°C with T7 RNA polymerase (5 U), dCTP, dATP, dGTP, and α-^{32}P-labeled dUTP (Perkin-Elmer, Boston, MA) for 1 h. The in vitro transcription reaction mix was then treated with DNase I and cleaned up using size fractionation columns (Nucaway, Ambion). Membranes were pre-hybridized for 30 min at 68°C and then incubated overnight at 68°C with labeled anti-sense RNA in hybridization buffer. After stringency washes, membranes were exposed to X-ray film for 24–72 h at −80°C.

Flow Cytometry for Cell Cycle Assessment

Cells were starved for 24 h in serum-free media, centrifuged at 100 × g for 10 min, and incubated in 10% HIFBS containing 1.25% DMSO plus R59022 or R59949. Control cells were incubated with DMSO only. After 1, 3, and 6 days, HL-60 cell aliquots (1×10^6 cells/ml) were centrifuged and resuspended in 1 ml cold PBS and drop wise fixed with 2 ml of methanol with constant vortexing. After incubation on ice for 1 h, cells were washed twice in cold PBS and resuspended in 1 ml of propidium iodide (PI, 10 mg), 0.1 ml Triton X-100 (Sigma, St. Louis, MO) and 3.7 mg EDTA in 90 ml of Ca^{2+}/Mg^{2+}-free PBS and 500 μl of RNase I (100 μg/ml). PI-stained cell preparations were analyzed in a Flow Cytometer using CellQuest software for DNA content assessment. Flow cytometer linearity, coefficient of variation, and alignment were evaluated using chicken erythrocyte nuclei, calf thymus nuclei, and fluorescent beads (DNA QC particles, B&D Cytometry Systems, San Jose, CA) before running samples. Doublet discrimination was performed using non-synchronized HL-60 and peripheral blood cells and the voltage adjusted so that singlets (2n) were positioned in channel 200 ± 5 and 4n cells in channel 400. Ten-thousand events were collected and dot plots (FL-2W vs. FL-2A) were used to gate singlets (low FL-2W). Histograms (FL2-A vs. cell count) were generated and percentage of cells within each cell cycle phase was then determined.

Propidium Iodide and Annexin-V Staining

Quantitation of apoptotic cells was assessed by dual staining and FACS, using Annexin V-FITC for phosphatidylserine [Vermes et al., 1995] and PI for DNA [Davies et al., 1994], according to the manufacturer's instructions (BD Biosciences). Briefly, 10^6 cells were washed twice in cold PBS and resuspended in 0.1M Hepes/NaOH pH 7.4, 1.4M NaCl, 25 mM $CaCl_2$. One hundred microliter of the solution was transferred to a 5 ml tube and incubated at RT with 5 μl of Annexin V-FITC and 5 μl of PI for 15 min in the dark. Controls included unstained cells, cells stained only with PI and Annexin V-FITC, control HL-60 cells, and cells treated with camptothecin (Sigma) at a final concentration of 5 μM. Controls were used to determine basal levels of apoptotic cells, set compensations and properly locate quadrants for cell population discrimination.

Statistics

All experiments were run in triplicate, using 3 batches of cells differentiated 1 week apart. Data were collected and descriptive statistics used in order to determine distribution patterns and normality. One-way ANOVA was used for multiple comparisons with Bonferroni's post-hoc test to assess inter and intragroup variation. Spearman correlation and linear regression analysis were carried out to establish correlations between selected variables. Significance was set at $P < 0.05$ for all experiments.

RESULTS

Expression of DGK Isoforms in DMSO-Induced Differentiation of HL-60 Cells

As a first approach to characterize DGK isoform expression in HL-60 cells, we sought to assess expression on a qualitative basis. To this end, RT-PCR and Northern blotting using riboprobes were employed. RT-PCR analysis showed that of the nine isoforms described to date, only five (α, δ, ε, γ, ζ) were expressed in HL-60 cells (Fig. 1a). Throughout differentiation, a striking difference in the levels of message for

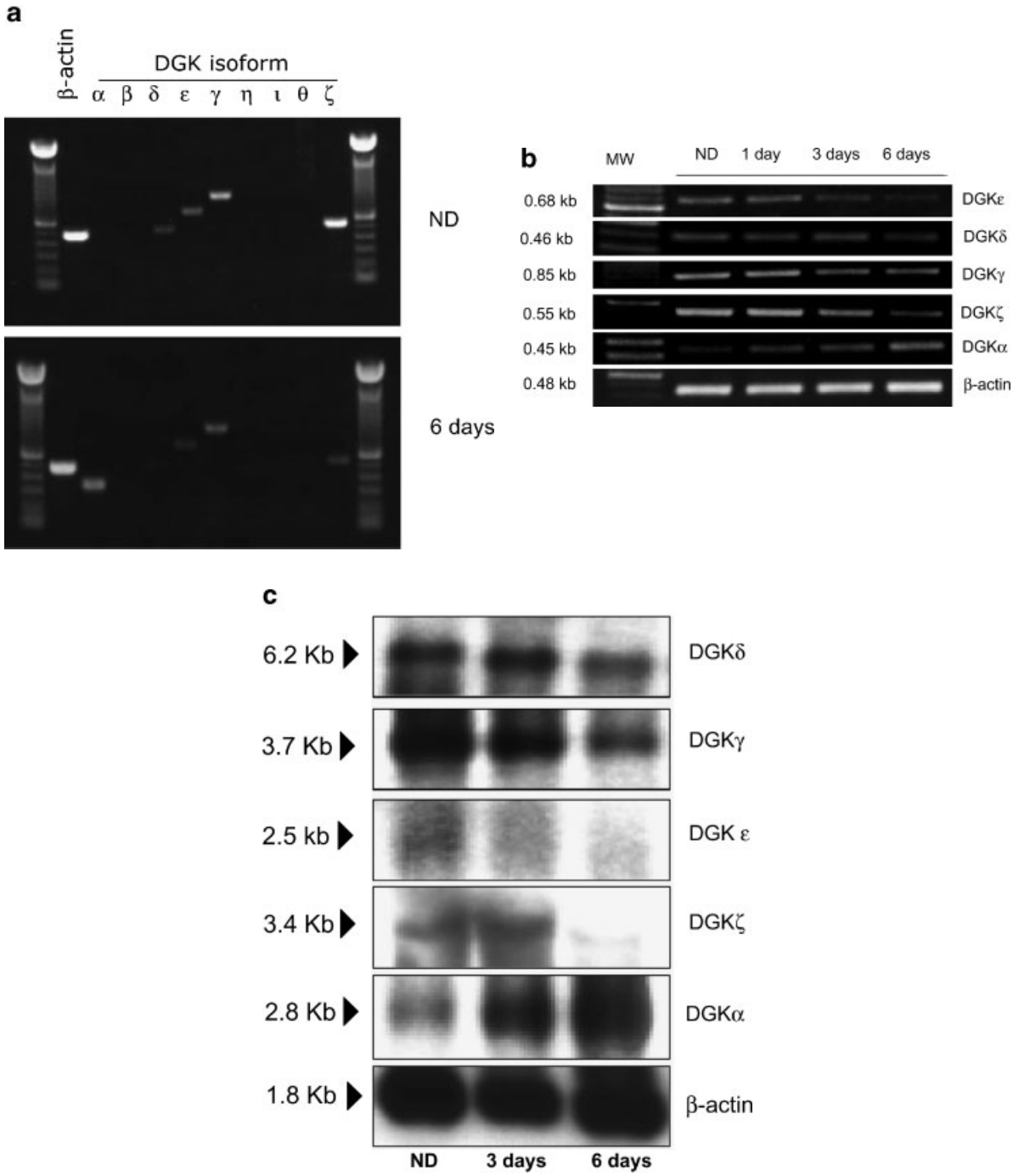

Fig. 1. Differential expression of DGK isoforms. RT-PCR revealed that in undifferentiated HL-60 cells, DGK isoforms δ, ε, γ, and ζ were expressed while DGKα was virtually absent (**a**, **upper panel**). After 6 days of incubation with 1.25% DMSO, there was a decrease in the message for isoforms δ, ε, γ, and ζ, and DGKα was significantly upregulated (**a, lower panel**). The RT-PCR based kinetics of expression of DGKs in differentiating cells are shown in (**b**) and Northern blot in (**c**).

the identified isoforms was observed; isoforms δ, ε, γ, and ζ were downregulated, whereas the α isoform was upregulated as HL-60 cells differentiated into neutrophil-like cells (Fig. 1b). Northern blotting of RNA isolated from non-differentiated cells and cells incubated with 1.25% DMSO for 3 and 6 days showed a similar trend (Fig. 1c).

Quantitative Assessment of Differentially Expressed DGK Isoforms

Real-time PCR was used to confirm and more accurately assess early changes in message levels after incubation of HL-60 cells with DMSO (30′, 1 h, 3 h, and 6 h) and throughout differentiation (1–6 days). DGKα showed a progressive increase in the levels of message throughout differentiation, but no significant changes were observed within the first 6 h (Fig. 2a). On a long-term basis, there was a progressive increase in DGKα message, especially after the third day of incubation with DMSO (Fig. 2b). The progressive upregulation of DGKα observed was highly correlated with the increase in production of PMA-induced ROS over time ($r = 0.849$) (Fig. 3). Linear regression revealed that 76.8% and 85.2% of the variability of DGKα expression and ROS production could be explained by the DMSO incubation time, respectively ($r^2 = 0.768$ and 0.852). The other expressed isoforms (δ, ε, γ and ζ) decreased throughout differentiation (Fig. 2b). Their downregulation was evident within hours of incubation with DMSO (Fig. 2a) but changes were not significant within the first 6 h. On a long-term basis, mRNA levels for DGK δ, ε, and ζ decreased throughout differentiation reaching their lowest levels at the 6th day. DGKγ decreased more rapidly, a 54% decrease within the first 24 h, and then remained fairly steady until day 6. Comparisons between peripheral blood PMNs obtained from three individuals and 6-day differentiated HL-60 cells revealed similar levels of mRNA for DGKα ($P > 0.01$) suggesting that DGKα expression in DMSO-treated promyelocytic cells is similar to peripheral blood PMN (Fig. 4). No statistically significant differences in the mRNA levels for isoforms δ, ε, and ζ were observed between 6-day differentiated HL-60 cells and PMN. However, cells incubated with DMSO for 6 days still presented DGKγ levels that were significantly higher that those observed in PMN ($P < 0.01$).

Inhibition of DGKs and Expression of CD11b and GPI-80

Throughout differentiation, HL-60 cells demonstrated a decrease in most of the expressed DGKs (δ, ε, γ, ζ). This observation, along with previous data that revealed an inverse relationship between the levels of nuclear DAG and the rate of proliferation [Neri et al., 2002], suggest that co-blockage of DGK in cells incubated with DMSO would accelerate differentiation. To better assess this hypothesis the DGK inhibitors R59022 [Cooke et al., 1987; Gomez-Cambronero et al., 1987] and R59949 [Jiang et al., 2000] were used. First, the maximum non-toxic concentrations of R59022 and R59949 were determined. BrdU was used to access proliferation since it is incorporated into replicating DNA [Dolbeare et al., 1983]. Figure 5 shows that proliferation was substantially inhibited by R59022 and R59949 at concentrations higher than 30 μM. In separate experiments, cell aliquots were incubated with DMSO and either R59022 or R59949 to a final concentration of 30 μM. Assessment of neutrophil cell surface markers CD11b and GPI-80 showed that in the first day of incubation no significant changes were observed among groups (Fig. 6a,b). Further evaluation at 3 days, however, revealed that expression of CD11b and GPI-80 markers was significantly increased in inhibitor-treated cells. This difference was observed with both inhibitors, although the expression pattern of the markers was temporally different. GPI-80 was predominantly increased between the third and the 6th day in DMSO treated cells, whereas CD11b showed changes after 24 h of incubation. These findings corroborate the results of others who showed that expression of GPI-80 is very specific for HL-60 cells differentiated with DMSO, but shows a temporally different expression pattern when compared to CD11b [Takeda et al., 2003]. Comparisons revealed that cells treated with DGK inhibitors presented a significant increase in the expression of CD11b and GPI-80 at 3 days (45% and 33% for R59022 and 29% and 15% for R59949, respectively). No significant differences were observed when comparing 3-day differentiated cells incubated with inhibitors and DMSO with those of DMSO-only at 6 days. These findings suggest that cells treated with inhibitors exhibited an acceleration of differentiation accompanied by a decrease in proliferation. At 6 days, however, there were no significant differences among groups.

ROS Production

To better characterize functional changes in differentiating HL-60 cells, DHR123 and NBT assays were used to measure generation of ROS, which increases with HL-60 cell differentiation [Levy et al., 1990] DHR123 measures H_2O_2

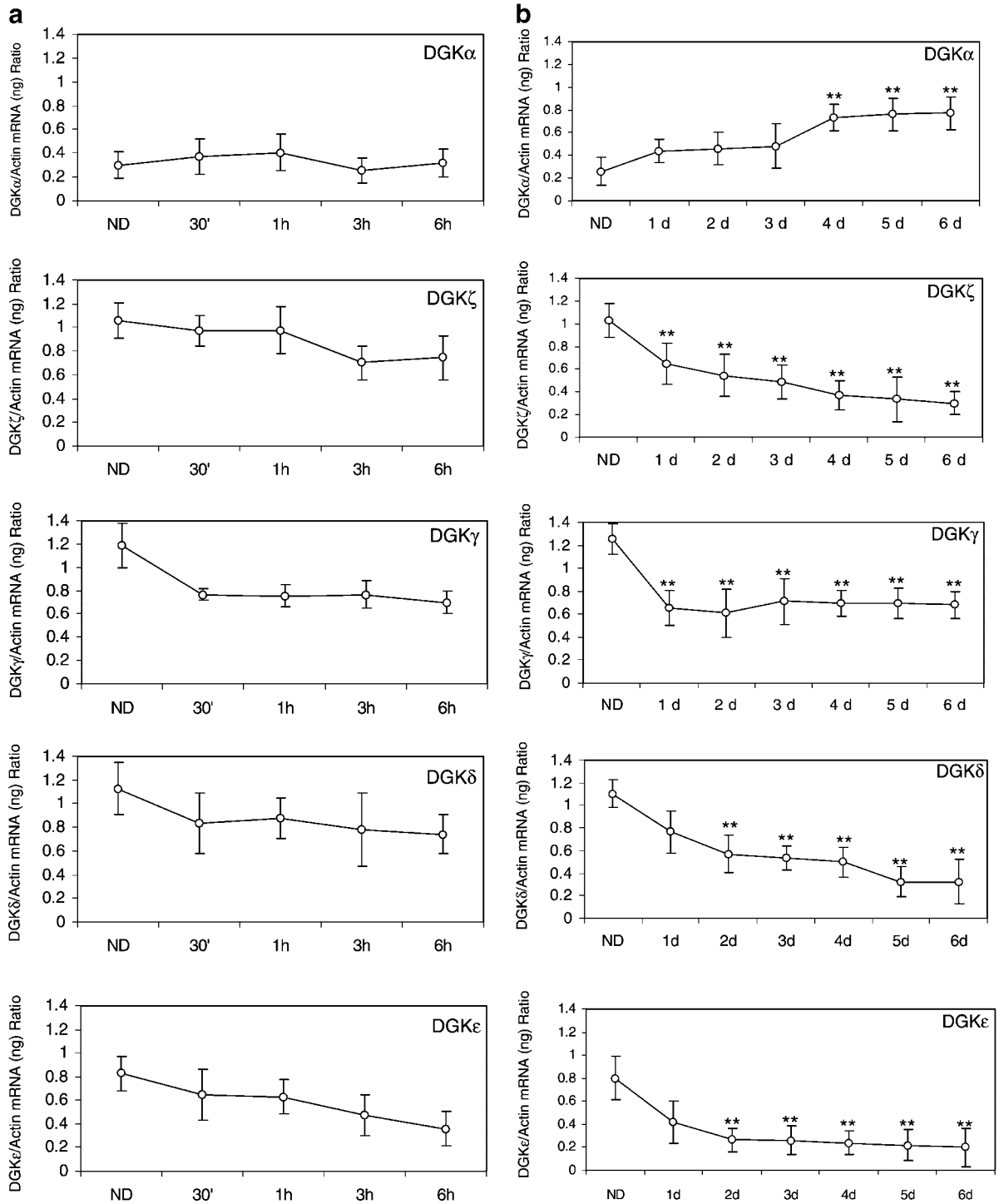

Fig. 2. Quantitative assessment of differentially expressed DGK isoforms. DGKα message levels were significantly lower compared to other isoforms and did not show important changes within the first 6 h of differentiation (**a**). Long-term analysis showed that DGKα expression was upregulated after 24 h and reached significance between days 3 and 4, with no further significant changes until day 6 (**b**). There was a significant decrease of DGKγ, which was more pronounced after 24 h ($P < 0.01$) but remained virtually unchanged until the 6th day. Isoforms δ, ε, and ζ, were more gradually and progressively decreased, which was observed until the 6th day of incubation with DMSO. Results represent means and SD of three batches of cells differentiated 1-week apart, run in duplicate in 96-well plates ($n = 6$; $^{**}P < 0.01$).

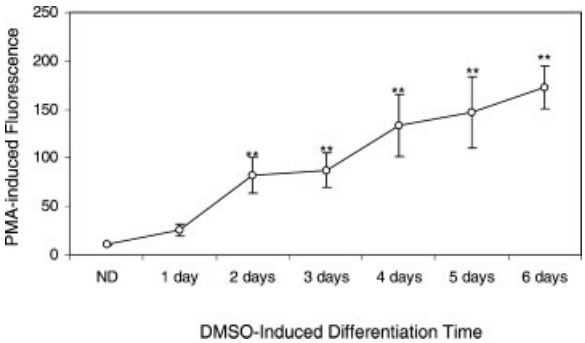

Fig. 3. ROS production throughout the course of differentiation. Cells were stimulated with PMA and ROS production assessed by oxidation of Dihydrorhodamine 123 as reported in ''Materials and Methods.'' Differences reached significance between days 2 and 3 and progressed throughout the differentiation period until day 6. The increase in ROS production was highly correlated with the expression of DGKα ($r = 0.849$; $^{**}P < 0.01$).

production [Walrand et al., 2003], whereas NBT reflects upstream events, since it is directly dependent on NADPH oxidase activity and superoxide production [Absolom, 1986]. For both approaches, at 3 days, cells differentiated with both inhibitors produced significantly higher levels of ROS than cells differentiated with DMSO only (Fig. 7a,b). These results further suggest that incubation with DGK inhibitors accelerates DMSO-induced differentiation of HL-60 cells. These findings, combined with the surface marker experiments, suggest that at 3 days most HL-60 cells co-incubated with DMSO and DGK inhibitors are terminally differentiated, e.g., the differentiation time is reduced by 50%.

Assessment of the Cell Cycle

In order to better characterize the changes in differentiation by HL-60 cells treated with DGK inhibitors, we analyzed changes in DNA content in order to assess changes in the cell cycle. The results are shown in Figure 8a,b. No statistically significant changes in DNA content within 24 h of incubation for any of the cell cycle phases were observed. Likewise, no significant differences were observed when comparisons were made to undifferentiated HL-60 cells at each time point (not shown). Nevertheless, after 3 days, significant differences were observed in cells treated with inhibitors; the number of cells re-entering the cycle (S phase) was particularly reduced. Likewise, the fraction of 4n cells stalled at G_2 and putatively undergoing mitosis (M phase) was significantly reduced in

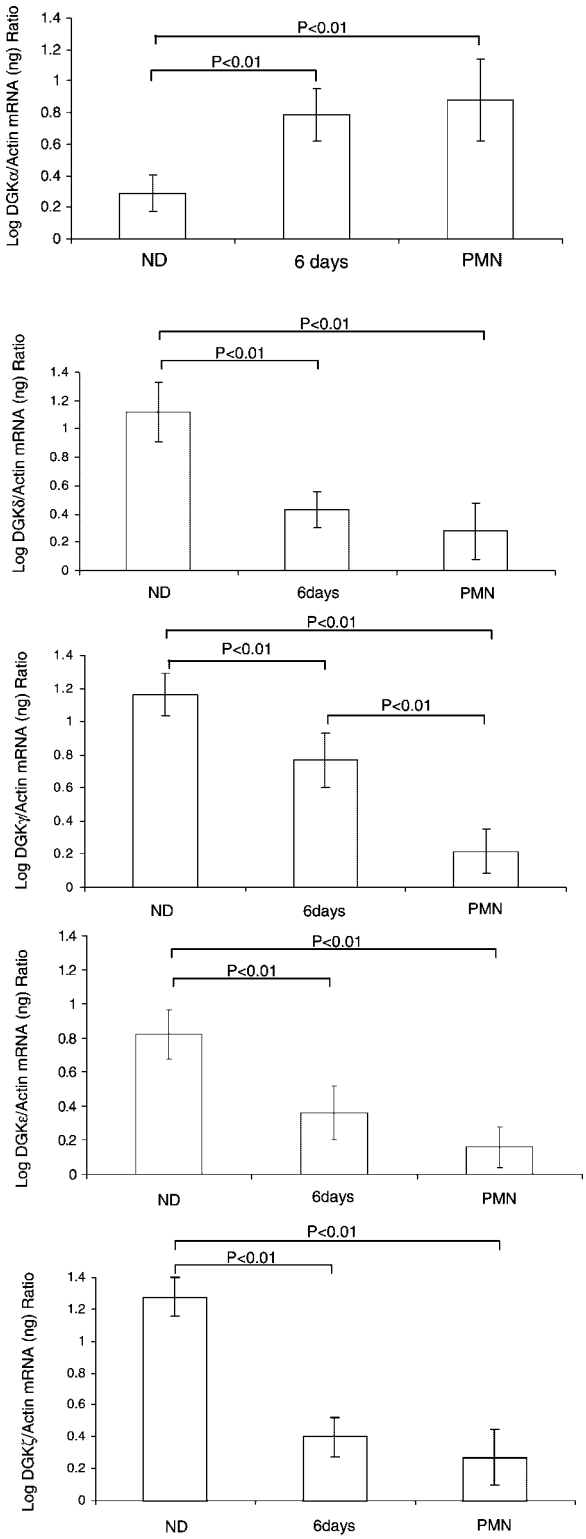

Fig. 4. Differentially expressed DGK mRNA levels in HL-60 cells and peripheral blood neutrophils (PMN). Real-time PCR revealed that, except for DGKγ, which was higher, the levels of all other expressed isoforms were similar in resting differentiated HL-60 cells and PMN. Results represent mean ± SD of three batches of cells differentiated one-week apart run in duplicate.

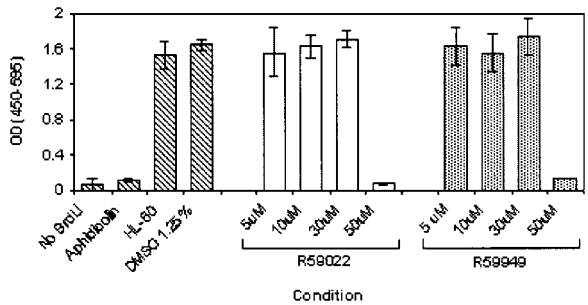

Fig. 5. Determination of maximum non-toxic concentrations of inhibitors R59022 and R59949. HL-60 cells were incubated with different concentrations of DGK blockers for 24 h and accessed for incorporation of bromodeoxyuridine (BrdU) as described. Incubations with either R59022 or R59949 at concentrations higher than 30 μM abrogated cell proliferation at levels similar to the DNA-polymerase inhibitor aphidicolin (5 μg/ml).

the groups treated with inhibitors, reflecting the decrease in the S phase and implying that proliferation and accumulation of cells in the G_2 phase was markedly affected when compared to DMSO-only. At 3 days, cells treated with inhibitors resembled those of DMSO-only treated cells at 6 days ($P > 0.01$). At 6 days, the number of cells stalled at G_2 or undergoing mitosis was still significantly reduced in the R59022 group when compared to DMSO and R59949 groups.

Assessment of Apoptosis

An increased number of cells in the pre-G_0/G_1 phase, i.e., an aneuploid cell population reflecting apoptosis, was observed for all groups throughout differentiation and particularly in the R59022 group. PI/Annexin V staining and FACS were used to assess apoptosis. With this approach, three cell populations can be identified; dead cells stain with both dyes; live cells not committed to apoptosis are negative for both dyes and cells in the early stages of apoptosis will show positive staining for Annexin V and are negative for PI. After generation of dot plots and proper setting of quadrants, cells not undergoing apoptosis (negative for Annexin and PI) and those undergoing early apoptosis changes but not necrotic (positive for Annexin and negative for PI) were gated and histogram plots generated (Fig. 9a). No significant changes were observed for any of the groups at day 1 (Fig. 9b), nor were changes observed when compared to control non differentiated cells at all time points (not shown). At 3 days cells incubated with R59022 presented a significant increase in the number of apoptotic cells, a trend not observed for DMSO-only and R59949 treated groups (Fig. 9b). At 6 days there was a significant increase in the number of apoptotic cells for all groups, but there were more apoptotic cells in the R59022 group. The same trend was observed in the assessment of dead cells using the trypan blue exclusion method (not shown).

DISCUSSION

In this study, we report that messenger RNA of select DGKs is dynamically regulated during differentiation of HL-60 cells. Of note, when HL-60 cells are co-incubated with a polar solvent and pharmacological inhibitors of DGKs, differentiation to neutrophil-like cells occurs more rapidly. A variety of DAG pools are generated by different cells in different cell compartments, which is presumably the reason for the diversity of DGKs involved in the regulation of DAG levels [Topham and Prescott, 1999]. This is corroborated by the fact that differences in the regulation of DAG and DGK activity seem to be both cell and agonist-type specific [Hurttia and Leino, 1996]. In this context, profiling the specific isoforms expressed in different cell types is a key approach to better characterize regulatory mechanisms involved in DAG-mediated signal transduction. In a previous publication, we demonstrated that select DGK isoforms were expressed in peripheral blood neutrophils [Oyaizu et al., 2003]. Nevertheless, since PMN are mature, fully differentiated cells, it was not clear whether other isoforms played a role in earlier stages of differentiation. HL-60 cells have been extensively used as a model for neutrophil developmental studies, since they can express the phenotype of granulocytes upon incubation with polar solvents [Collins et al., 1978; Bernardo et al., 1990]. Our results revealed that non-differentiated, pro-myelocytic cells expressed isoforms δ, ε, γ, and ζ, whereas the α isoform was virtually absent. Nevertheless, once differentiation was initiated, DGKα was gradually upregulated, becoming more evident between the 3rd and 4th days. This finding has functional implications, since DGKα seems to be involved in the control of a granulocyte DAG pool generated in the cytosol by phospholipase C-mediated hydrolysis of PIP_2, which is known to mediate activation of PKC and NADPH Synthase [Muid et al., 1988; Perkins et al., 1995; Cipres et al., 2003]. Activation of this pathway leads to the produc-

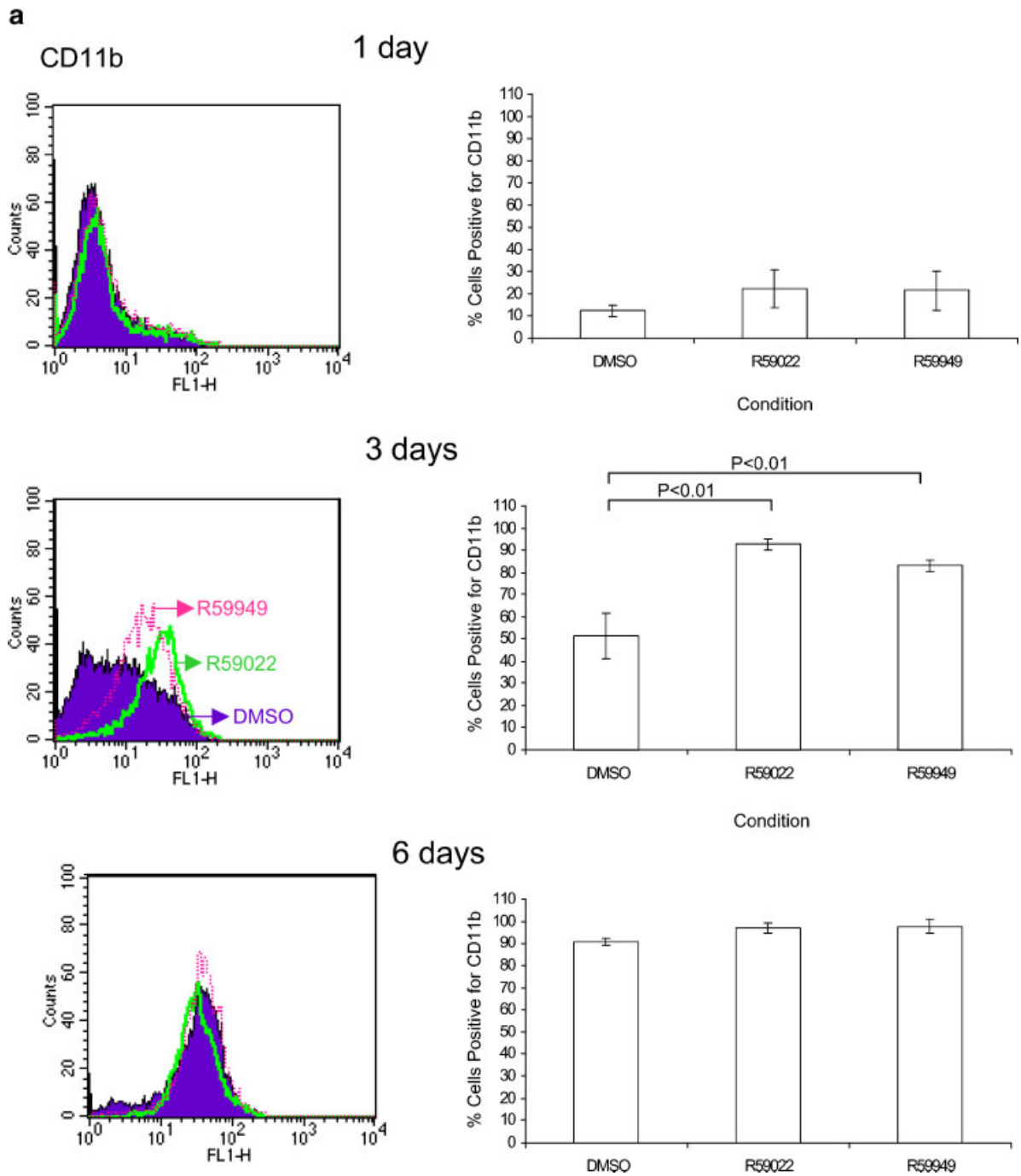

Fig. 6. Expression of granulocyte markers on HL-60 cells. FACS analysis of the expression of CD11b and GPI-80 showed that at day 1 of co-incubation with DMSO and either R59022 or R59949, the percentage of cells expressing CD11b and GPI-80 above the expression threshold ($>10^1$) was the same for all groups (**a**, **b**). At 3 days, however, this number was significantly higher for cells co-incubated with DMSO and either R59022 or R59949. At 6 days the levels of the markers were the same for all treatment conditions, suggesting that all groups reached the same differentiation status. Bars represent means and standard deviations of three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tion of ROS, notably superoxide, an important product in the oxidative killing pathway of neutrophils [Bergstrand et al., 1992]. The observed upregulation of DGKα, which was highly correlated to ROS production, suggests that during differentiation cells develop means by which they can respond to chemoattractants, produce ROS, and kill bacteria, but also

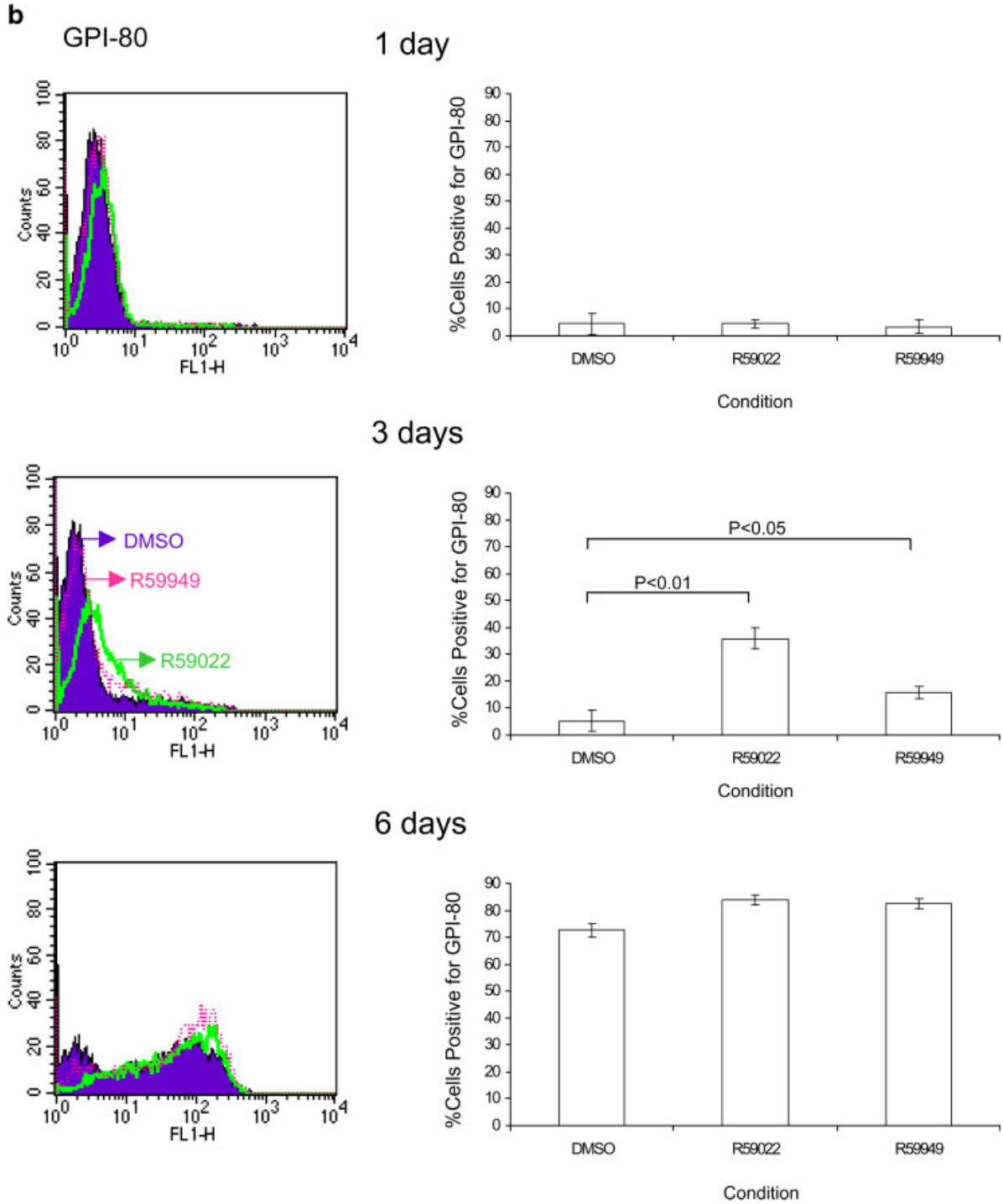

Fig. 6. (*Continued*)

regulatory mechanisms to counteract this process. This is extremely important since sustained production of ROS can also play a role in tissue destruction [Gavioli et al., 1987; McCord, 2002].

It is noteworthy that in our experiments, we used PMA, which directly activates PKC, leading to activation of NADPH oxidase [Heyworth and Badwey, 1990]. This is not a DAG-mediated process. The choice of PMA was based on the fact that most agonists that trigger this pathway (i.e., fMLP among others) bind G-protein coupled receptors that activate PKC through DAG. These same receptors are upregulated in HL-60 cells as differentiation proceeds [Prossnitz et al., 1993]. Therefore, using G-protein

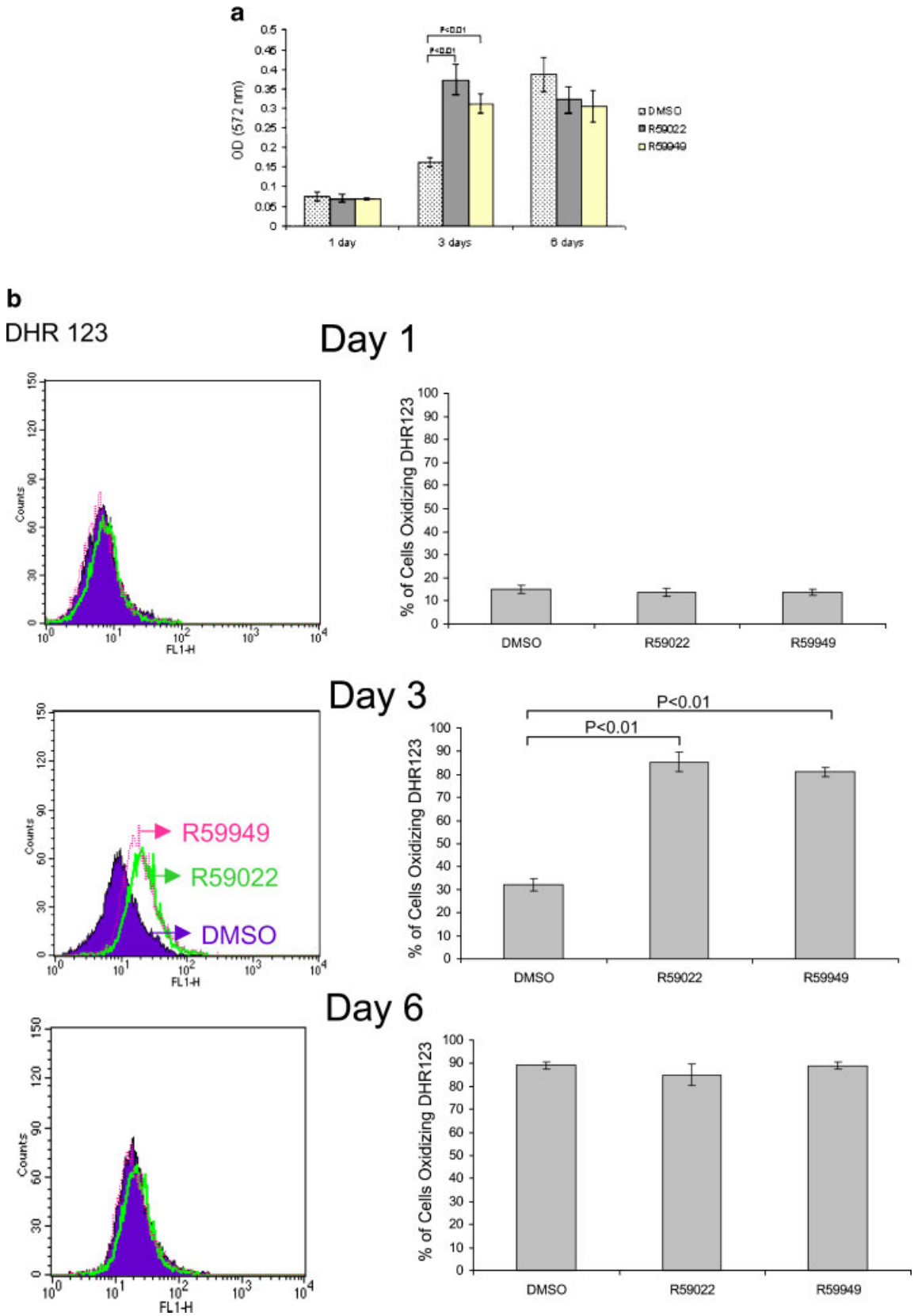
a
OD (572 nm)
P<0.01
P<0.01
DMSO
R59022
R59949
1 day
3 days
6 days
b
DHR 123
Day 1
Counts
FL1-H
% of Cells Oxidizing DHR123
DMSO
R59022
R59949
Day 3
P<0.01
P<0.01
R59949
R59022
DMSO
Day 6

Fig. 7.

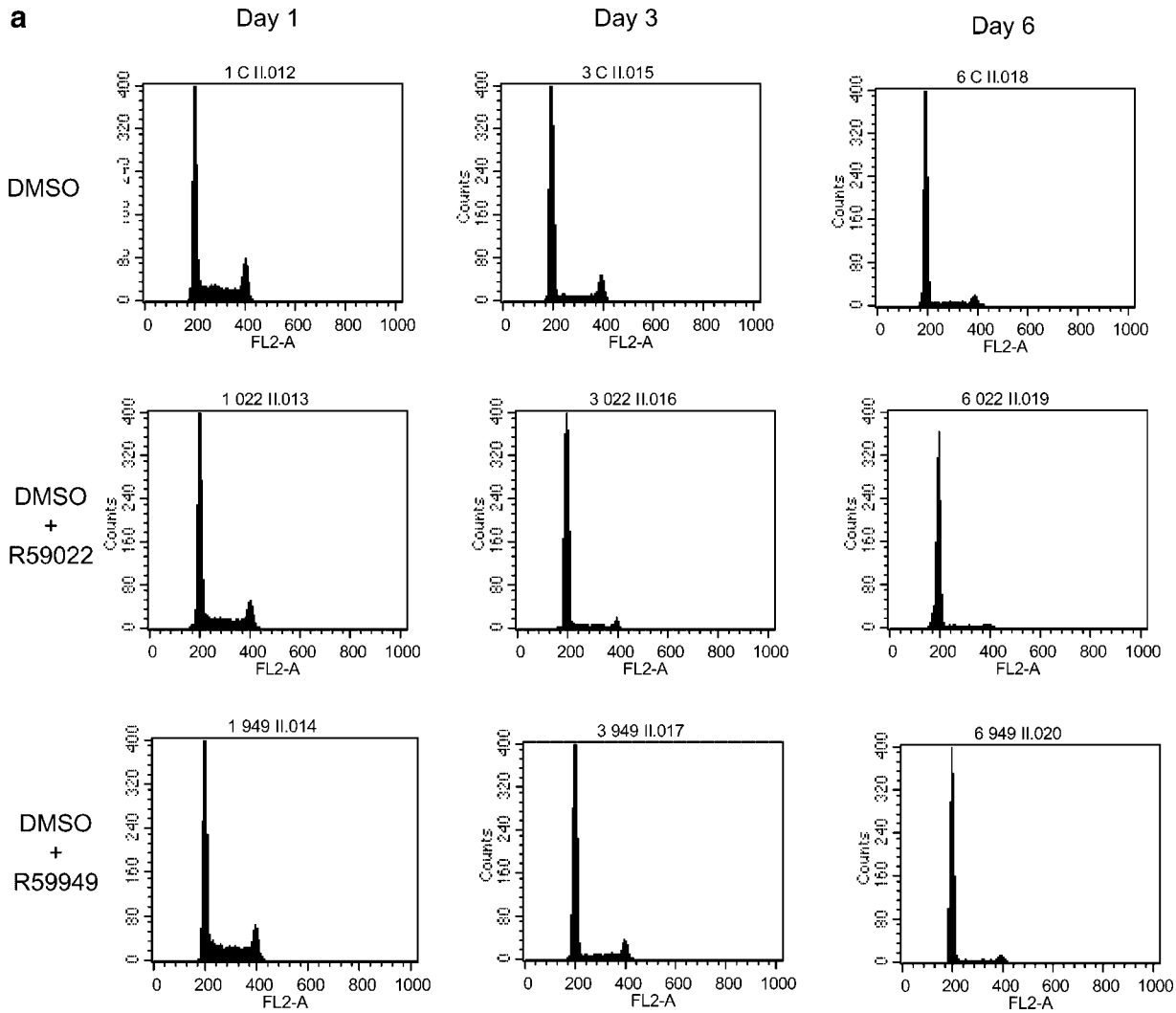

Fig. 8. Cell cycle assessment. Histograms generated for each experimental group were used to calculate the percentage of cells in each phase of the cell cycle (**a**). Results revealed no significant differences for any of the cell cycle phases at day 1 (**b**). Nevertheless, at day 3 there was a numeric increase in the percentage of cells at the G_0/G_1 phase, which was significant only for R59022. The number of cells progressing into the cell cycle (S phase) at 3 days was significantly decreased for cell populations incubated with both inhibitors, a trend also observed for cells stalled at the G_2 checkpoint or putatively undergoing mitosis (M). At 6 days, the population of cells in each of the cell cycle phases reached similar levels, with the exception of R59022-incubated cells in the G_2/M phase, which was significantly decreased as compared to DMSO-only treated cells. The percentage of cells at the S and G_2/M phases treated with inhibitors reached, at 3 days, levels that resembled 6-day DMSO-only treated cells ($P > 0.01$).

coupled receptor agonists; any variation in ROS would ultimately reflect changes in expression levels of the receptors, since receptor occupation is an upstream limiting step.

Other isoforms, namely δ, ε, γ, and ζ decreased with differentiation. All isoforms, except γ, were gradually downregulated throughout the first 4 days of differentiation and showed little if any

Fig. 7. ROS production as a functional marker of HL-60 differentiation. Cells were incubated with DMSO and either R59022 or R59949 and then ROS production assessed at 1, 3, and 6 days. Reduction of nitro-blue-tetrazolium (NBT) (**a**) showed no significant differences at day 1, but at day 3 a significant increase in ROS production was observed for R59022 and R59949 as compared to the DMSO-only group (2.31- and 2-fold increase, respectively). At day 6, the group treated with DMSO-only reached the same level of ROS production as the inhibitor groups. Similar results were also observed when oxidation of DHR123 was assessed (**b**); FACS histograms show no significant differences within 24 h, however, at 3 days a clear shift to the right was observed for groups treated with R59022 and R59949. At 6 days there were no significant differences between groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

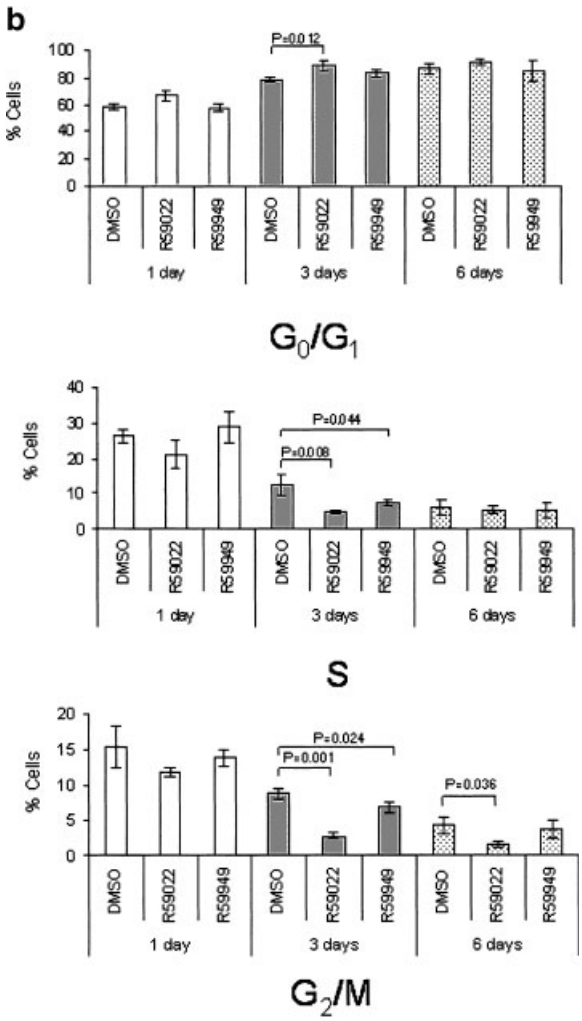

Fig. 8. (*Continued*)

significant change afterwards. These findings suggest that, at least in pro-myelocytic cells, there is temporal importance for different isoforms expressed. Isoforms only modestly expressed in mature resting cells may still play a role in granulocyte function, but seem to mainly mediate processes associated with cell proliferation and homeostasis in earlier phases of neutrophil development. Comparisons between resting differentiated HL-60 cells and peripheral blood PMN showed similar levels of message for isoforms α, δ, ε, and ζ. This provides a further line of evidence suggesting that DMSO-differentiated pro-leukemia cells share a high degree of phenotypic resemblance with PMN [Sham et al., 1995; Hauert et al., 2002]. The exception was DGKγ. This isoform showed the fastest decrease in mRNA levels within the first 24 h of differentiation but remained virtually unchanged after that. Comparisons between 6-day differentiated HL-60 cells and PMN showed that the message for DGKγ was still significantly higher in the HL-60 cells. A possible explanation for this finding is related to the variety of phenotypes observed in a HL-60 cell population undergoing differentiation. Previous reports have shown that induced and uninduced HL-60 cells are somewhat heterogeneous, with mixed cell populations at varying stages of differentiation [Collins et al., 1978; Harris and Ralph, 1985]. Once incubated with differentiating compounds, there is an increase in the number of cells progressively assuming the specialized phenotype of a granulocyte, which can be determined by cell fractionation and elutriation [Bernardo et al., 1990] as well as microscopy [Collins et al., 1978]. In practice, this implies that some fractions of the differentiating population probably still retain proliferative capacity even after 6 days of incubation with DMSO, compared to fully differentiated mature PMN. Taken together, these findings imply that downregulation of DGKγ is necessary for differentiation to occur. A recent finding published during the course of the present investigation seems to support this hypothesis: HL-60 cells overexpressing DGKγ and TPA-induced to differentiate into macrophages had a significant decrease in attachment. Since attachment is a hallmark of the induced-macrophage phenotype, this finding suggests that a negative regulation of differentiation is mediated by DGKγ [Yamada et al., 2003]. Protein analysis was not carried out here because antibodies to the target isoforms were unavailable to us.

Our findings suggest that differentiation of HL-60 cells to a granulocyte phenotype involves a decrease in the levels of select DGK isoforms, suggesting that specific pools of DAG were no longer tightly regulated. The downregulation of select DGK isoforms could just reflect the gene expression profile of granulocytes or eventually imply that downregulation of DGK and, as a consequence, increased levels of specific DAG pools are necessary for differentiation to proceed. In a recent publication, Neri et al. [2002] observed that DMSO incubation induced an increase in DAG mass in HL-60 cells and that independently regulated sources of DAG generated by PLC and PLD seem to exist in the nucleus. Our findings strongly support these findings at a different level; whereas the aforementioned work assessed changes at the

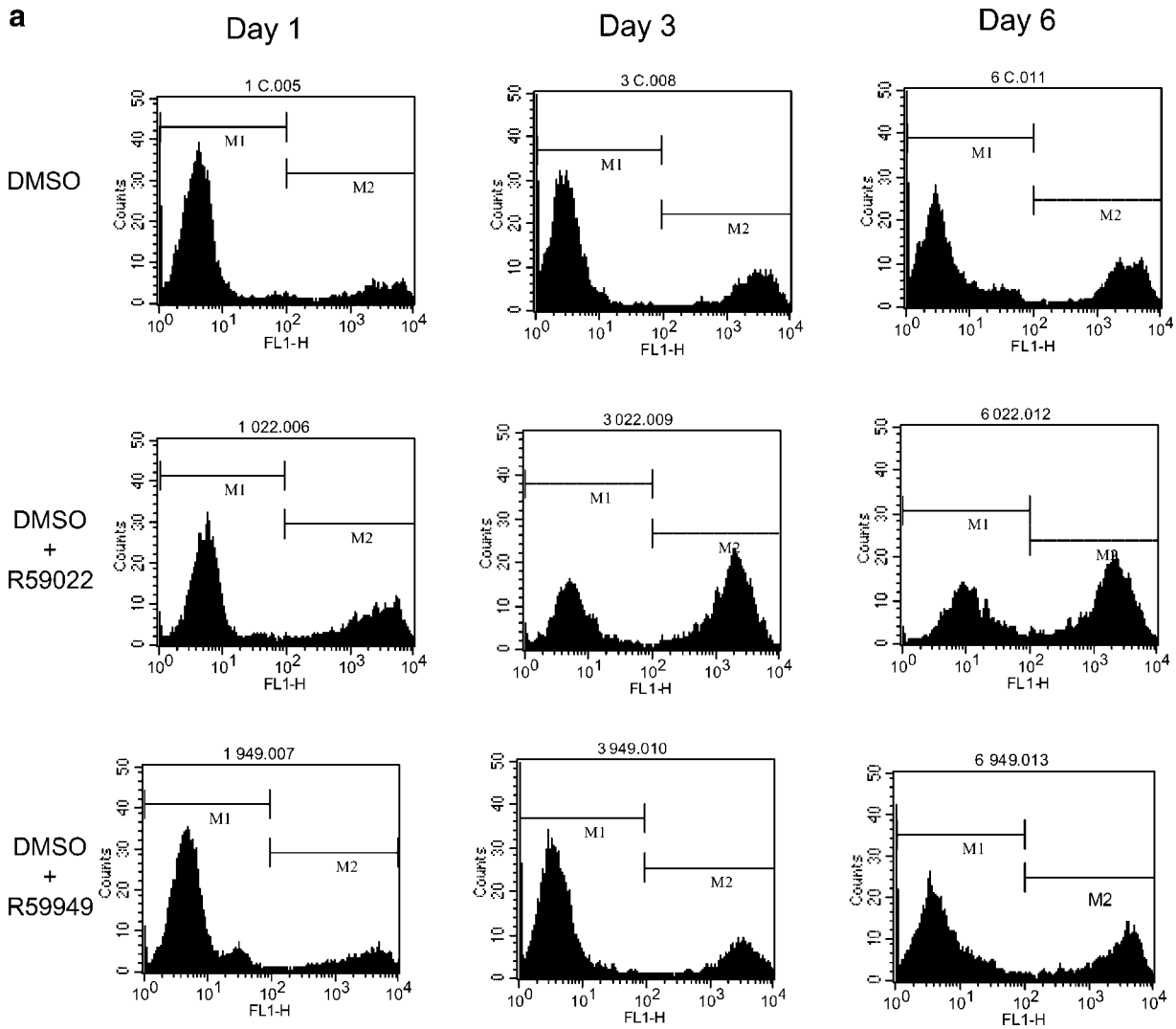

Fig. 9. Assessment of Apoptosis. Only cell populations negative for both dyes (non-apoptotic populations-M1) and positive for Annexin V/negative for PI (early apoptotic cells-M2) were included in the analysis (**a**). The percentage of cells negative (−) and positive (+) for apoptosis in each experimental group are shown (**b**). At day 1, no significant differences in the number of apoptotic and viable cells were observed for any of the groups. At day 3, there was a significant decrease in the number of viable (+) cells in the R59022-treated group, which was significantly higher that those in DMSO-only and R59949-treated groups. At 6 days, there was an overall increase in the number of apoptotic cells in all groups but a significantly higher number of R59022-treated cells were apoptotic compared to the other groups.

substrate level, i.e., DAG mass, our approach focused on the enzymes involved in the regulation of the substrate. These findings led us to hypothesize that a decrease in the expression of select DGK isoforms is necessary for specific mechanisms that lead to differentiation to be activated. Therefore, attenuation of DGKs should release DAG and select PKC isoforms from tight regulation leading to acceleration of differentiation. To test this hypothesis we used the pharmacological DGK inhibitors R59022 and R59949. These compounds have been used to investigate the role of DAG and PKC in signal transduction in different cell systems [de Chaffoy de Courcelles, 1990; Flores et al., 1999] and their effectiveness at specific concentrations has been confirmed by DGK activity and immunoprecipitation assays [Cooke et al., 1987; Ohtsuka et al., 1990; Flores et al., 1996]. Using deletion mutants, Jiang et al. [2000] showed that DGK inhibitors mediate attenuation of DGK activity by binding to the catalytic domain. Functionally, these compounds have been shown to substantially increase the production of superoxide in fMLP-stimulated neutrophils [Gomez-Cambronero et al., 1987; Mege et al.,

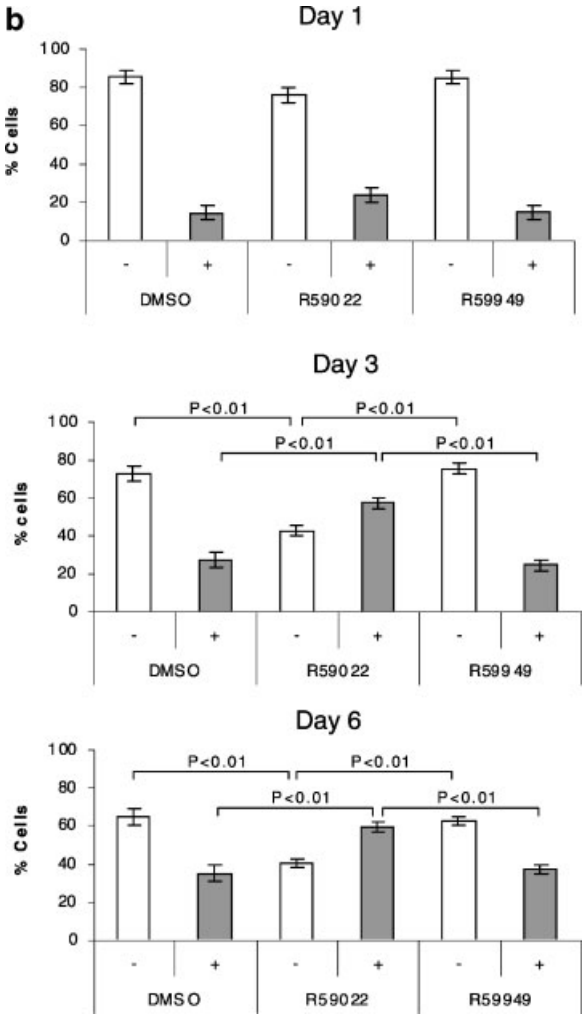

Fig. 9. (*Continued*)

1988]. Our results showed that co-incubation of HL-60 cells with DMSO and either R59022 or R59949 reduced the time necessary for promyelocytic cells to express a granulocytic phenotype by 50%. Analysis of DNA content was also used to assess changes in phenotype since differentiating cells are prevented to re-enter the cell cycle [Krishan, 1975; Johnson and Walker, 1999]. Our findings at the phenotypic level were also corroborated by changes in the cell cycle, since a drastic decrease in the number of cells proceeding to the S and G_2/M phase was observed when DGKs were blocked. At day 3, the group incubated with inhibitors showed that DNA replication was reduced by 50% as well. Previous findings have also suggested an important role for DGK in the proliferation of lymphocytic cell lines [Flores et al., 1999]. DGKα was identified as the main isoform involved in the regulation of proliferation in cells co-treated with IL-2. However, even though translocation and immunoprecipitation assays showed nuclear and cytosolic DGKα to be regulated by IL-2, and that R59022 and R59949 were able to affect these processes [Flores et al., 1996], it was not clear to what extent other isoforms were also being simultaneously activated. It has been shown that DGKs not only show subtype-specific translocation, which may depend on the type of extracellular signal, but also that more than one isoform may be activated by a single signal [Shirai et al., 2000]. Considering that different nuclear DAG pools may be generated in HL-60 cells [Neri et al., 2002], the involvement of an isoform other than α and/or even an orchestrated activation of multiple DGKs should not be ruled out. Also noteworthy is the fact that R59022 and R59949 do not seem to inhibit a single isoform [Jiang et al., 2000], which suggests that a specific isoform is not the solely responsible for the process. Nevertheless, lymphocytes and neutrophils are biologically different and caution should be taken when comparing the results of different methods and cell lines used.

An interesting finding was the increase in apoptotic cells throughout incubation with DGK inhibitors. While an overall increase in the number of apoptotic cells is expected to occur as differentiation proceeds, R59022 incubation resulted in more apoptotic cells than R59949, which did not differ from DMSO-only treated cells. A possible explanation for this finding is that R59022 and R59949 block different DGKs isoforms. R59949 attenuates calcium-dependent DGKs (α, β, γ), while R59022 affects a broader array of isoforms, including Ca-independent isoforms [Jiang et al., 2000]. In a previous report, camptothecin, a drug known to induce DNA damage and apoptosis by blocking topoisomerase I [Johnson and Walker, 1999], induced an increase in PKCα and PLD-mediated DAG pools in the nucleus of HL-60 cells [Martelli et al., 2000]. From these observations, it is reasonable to hypothesize that specific DGK isoforms that are attenuated only by R59022 are regulating pathways leading to cell death. There is precedent for this suggestion in the report that R59949 increases apoptotic events in mouse thymocytes [Outram et al., 2002]. In the absence of DMSO, incubation of cells with R59022 and R59949 in concentrations up to 30 μM did not induce differentiation or

changes in the cell cycle and apoptosis (not shown), implying that alone these compounds do not induce differentiation. Our findings suggest that inhibition of specific DGKs acted as a modulating co-factor, which allowed specific genes to be activated/suppressed more rapidly. It is not clear, however, by what mechanism polar solvents turn on and off transcription of specific combinations of genes that ultimately cause HL-60 cells to develop a neutrophil-like phenotype. In this context, our results as well as those of others [Balciunaite et al., 2000; Luo et al., 2003] point out that regulation of select DAG pools/PKC isoforms through differential expression of specific DGKs plays a key role. It is not yet clear if there is downregulation of specific isoforms or a single isoform is playing a more crucial role. However, DGKζ, which is downregulated throughout differentiation of HL-60 cells, has been shown to translocate to the nucleus where it also regulates DAG pools involved in the cell cycle [Topham et al., 1998; Luo et al., 2003]. Among the other isoforms identified in HL-60 cells, DGKε has been shown to concentrate in the cytosol and metabolize only arachidonate-containing DAG [Thirugnanam et al., 2001], and DGKδ has recently been associated with the negative regulation of endoplasmic-reticulum to Golgi traffic by inhibiting formation of ER export sites [Nagaya et al., 2002]. Nevertheless, no direct evidence of modulation of the cell cycle/apoptosis has been reported thus far.

In summary, we have demonstrated that the expression of select DGKs is dynamically regulated throughout differentiation of a pro-leukemia cell line. Furthermore, co-incubation of HL-60 cells with pharmacological inhibitors of DGK and a polar solvent can accelerate the differentiation of HL-60 cells to granulocytes. These findings provide additional evidence for the importance of this enzyme family in the regulation of diverse cell functions.

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