Journal of Cellular Biochemistry 101:411–424 (2007)

Protein Kinase Cα Is Differentially Activated During Neonatal and Adult Erythropoiesis and Favors Expression of a Reporter Gene Under the Control of the $^{A}\gamma$ Globin-Promoter in Cellular Models of Hemoglobin Switching

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Abstract PKCα was found to be expressed (mRNA and protein) throughout the in vitro maturation of primary human erythroblasts but its activity (phosphorylation levels and nuclear localization) was consistently higher in cells derived from human neonatal rather than adult blood. Since the $\gamma/\gamma+\beta$ globin expression ratio represented the major difference between neonatal and adult erythroblasts (58 ± 12 vs. 7 ± 3, respectively), we tested the hypothesis that PKCα might affect γ-globin expression by measuring the levels of $^{A}\gamma$- or β-promoter-driven reporter activity in erythroid cells stably (GM979) or transiently (K562, primary adult and neonatal erythroblasts) transfected with a dual μLCRβprRluc$^{A}\gamma$pr-Fluc reporter in the presence of transient expression of either the constitutively active (sPKCα) or catalytically inactive (iPKCα) PKCα. As further control, GM979 cells were incubated with the PKC inhibitor rottlerin (30 μM). In all the cells analyzed, sPKCα significantly increased (by two- to sixfold) the levels of luciferase activity driven by the $^{A}\gamma$-promoter and the $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R) expression ratio. In GM979 cells, rottlerin inhibited (by 50%) the $^{A}\gamma$-driven luciferase activity and the $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R) expression ratio. These results suggest that different PKC isoforms may exert ontogenetic-specific functions in erythropoiesis and that modulation of PKCα might affect the activity of $^{A}\gamma$-promoter-driven reporters. J. Cell. Biochem. 101: 411–424, 2007. © 2007 Wiley-Liss, Inc.

Key words: PKCα; Hb switching; erythropoiesis; thalassemia

The switch from the fetal ($\alpha_2\gamma_2$) to the adult ($\alpha_2\beta_2$) pattern of hemoglobin (Hb) expression in erythroid cells represents one of the most studied examples of developmental control of gene expression [Stamatoyannopoulos and Grosveld, 2001; Stamatoyannopoulos, 2005]. The interest to this phenomenon is partly due to the fact that fetal Hb ameliorates the clinical course of the disease in patients with β chain hemoglobinopathy. Indeed, induction of fetal Hb is one of the therapeutic approaches already used in the treatment of these disorders [Stamatoyannopoulos and Grosveld, 2001; Stamatoyannopoulos, 2005]. Experiments in erythroid

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: Italian Ministry of Industrial and University Research; Grant numbers: RBNE0189JJ 006, RBNE01SP72 003, CEA 2001-2003.

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Received 30 August 2006; Accepted 4 October 2006
DOI 10.1002/jcb.21189

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cell cultures [Papayannopoulou et al., 1976, 1977, 1978; Kidoguchi et al., 1978; Clarke et al., 1979; Umemura et al., 1990] and in vivo studies in baboons [Umemura et al., 1988] and in humans [Papayannopoulou et al., 1980] have clearly shown that adult erythroid progenitor cells have the potential for fetal globin expression but this potential is lost during downstream erythroid differentiation [Papayannopoulou et al., 1977; Stamatoyannopoulos and Grosveld, 2001; Stamatoyannopoulos, 2005]. The enhancement of fetal Hb in erythroid cell cultures provides a tool for identification of fetal Hb inducers which could potentially be used for treatment of patients with β-thalassemia and sickle cell disease.

Both negative and positive extrinsic modulators of fetal Hb production have been described. An inhibitor is present in the fetal sheep serum [Papayannopoulou et al., 1984], while inducers are present in selected batches of fetal bovine serum (FBS) [Constantoulakis et al., 1990; Fujimori et al., 1990; Migliaccio et al., 1990] or are produced by accessory cells [Terasawa et al., 1980; Darbre et al., 1981; Javid and Pettis, 1983]. Over the years, GM-CSF [Gabbianelli et al., 1989], IL-3 [Gabbianelli et al., 1990], and TGF-β [Bohmer et al., 2000] have all been described to induce fetal Hb in vitro. However, induction of fetal Hb by IL-3 has not been observed in vivo following administration of this growth factor to primates [Umemura et al., 1989]. More recently, stem cell factor (SCF) [Peschle et al., 1993; Gabbianelli et al., 2000], a cytokine that synergizes with erythropoietin (EPO) in the induction of erythroid differentiation [Migliaccio et al., 1991], has also been proposed as a possible modulator of fetal Hb production. It is not clear whether these factors increase fetal Hb production directly, by interacting with the transcriptional complex that activates the γ promoter, or indirectly, by modulating the kinetics of erythroblast maturation. In fact, at the beginning of their maturation, all the erythroid cells have the potential to produce high levels of fetal Hb [Papayannopoulou et al., 1979; Chui et al., 1980]. Growth factors that would accelerate, by skipping divisions, or delay, by sustaining self-renewal, the transition through this phase [Muta et al., 1995; Wojda et al., 2003], are expected to alter the fetal Hb content of mature cells. Since growth factors act through intracellular signaling pathways, those which activate fetal Hb expression should do so by delivering signals that do not involve pathways, such as JAK2/STAT5 and PI3K/AKT, triggering mainly proliferation and differentiation [Constantinescu et al., 1999].

Protein Kinase C (PKC) represents a family of serine-threonine kinases involved in the regulation of many cellular functions [Hofmann, 1997]. At least 9 different genes, encoding a total of 12 different PKC isoforms, have been described in mammals. All these isoforms share a conserved PKC motif and a similar serine/threonine-protein kinase domain. These enzymes exert their biological functions as intermediaries of the signal transduction machinery and become phosphorylated, and hence activated, in response to a variety of stimuli. Their activation is associated with translocation either to the cell membrane or to the nucleus, where they phosphorylate specific substrates [Hofmann, 1997; Ohno and Nishizuka, 2002]. On the basis of differences in structure, modality of induction, and Ca^{++}-requirements, the different PKC isoforms have been divided into three classes: conventional (α, βI, βII, and γ), which are Ca^{++}-dependent and are activated by both diacylglycerol and phosphatidyl-serine; novel (δ, ε, η, μ, and θ), which are Ca^{++}-independent and are regulated by either diacylglycerol or phospholipids; and atypical (ζ and λ/ι), which are Ca^{++}-dependent and activated by phosphatidylserine [Hofmann, 1997]. Each of these isoforms is expressed with a cell-specific pattern, although individual cell types usually express more than one PKC [Ohno and Nishizuka, 2002].

Human multipotent $CD34^{pos}$ progenitor cells express all of the PKC isoforms [Bassini et al., 1999; Oshevski et al., 1999]. Commitment of these cells along a specific lineage [Rossi et al., 1996; Lumelsky and Schwartz, 1997; Pierce et al., 1998; Kovanen et al., 2000; Racke et al., 2001; Mirandola et al., 2006], as well as their subsequent maturation along the lineage [Kovanen et al., 2000; Mirandola et al., 2006], requires suppression of specific PCK activities (in the case of the erythroid lineage, it requires suppression of PKCε) [Li et al., 1996; Bassini et al., 1999]. Other PKC isoforms, instead, exert a positive control on hematopoiesis. In fact, nonspecific PKC inhibitors impair the ability of bone marrow progenitor cells to form erythroid colonies in vitro [Jenis et al., 1989], probably by blocking EPO-induced activation of RAF1, ERK-1 and -2, and AP-1 [Patel and

Sytkowski, 1995; Devemy et al., 1997], as well as upregulation of c-Myc [Devemy et al., 1997]. On the other hand, inhibitors specific for PKCα impair the ability of erythroid cells to respond to EPO [Myklebust et al., 2000] and to phosphorylate EPO-R, STAT5, GAB1, ERK1/2, and AKT [von Lindern et al., 2000].

This study was designed to test the hypothesis that one of the PKC isoforms might be involved in the regulation of fetal Hb expression. First, we compared expression and phosphorylation state of different PKC isoforms during the in vitro differentiation of neonatal and adult erythroblasts, that express mainly fetal and adult Hb, respectively. This screening identified one conventional (PKCα) and one novel (PKCδ) PKC isoform differentially phosphorylated, and hence activated, during the differentiation of neonatal and adult erythroblasts. We next observed that rottlerin, a chemical suppressor of PKC activity, inhibited (by 50%) the activity of the reporter gene driven by the $^{A}\gamma$-promoter in GM979 cells stably transfected with the μLCRβprRluc$^{A}\gamma$prFluc reporter [Skarpidi et al., 2000], suggesting that PKC might be involved in the regulation of the $^{A}\gamma$-promoter activity. To clarify whether such an effect was mediated by a specific isoform, we evaluated the effect of forced expression of PKCα and PKCδ on the activity of reporter genes driven by either the β- or the $^{A}\gamma$-promoter in experimental models of Hb switch [cells stably (GM979 cells) or transiently (K562 cells and primary human erythroblasts) transfected with the μLCRβprRluc$^{A}\gamma$prFluc reporter] [Skarpidi et al., 2000]. Ectopic increase of PKCα activity increased the activity of the reporter gene driven by the $^{A}\gamma$-promoter in all of the cellular models investigated. Similar modulation of PKCδ activity had no effect on the expression of the reporter genes. These results suggest that PKCα is likely involved in the delivery of an external signal that either directly or indirectly upregulates fetal globin gene expression.

MATERIALS AND METHODS

Human Subjects

Peripheral blood was collected from normal adult donors and from post-delivery umbilical cords according to guidelines established by the institutional ethical committee for human subject studies of the Ospedale Civile, Pescara, Italy.

Culture of Primary Erythroblasts

Human erythroblasts were amplified in HEMA, a two-phase liquid culture that allows their massive amplification in vitro [Migliaccio et al., 2002]. Briefly, adult and neonatal light density cells were separated by Ficoll (Hypaque, Amersham Pharmacia Biotec, Uppsala, Sweden) and then cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad, CA) containing FBS (20%; Hyclone, Logan, UT), 4% Bovine Serum Albumin (vol/vol, BSA), 10 ng/ml SCF (Amgen, Thousand Oaks, CA), 1 U/ml EPO (Epoetina alfa, Dompe' Biotec, Milan, Italy), 1 ng/ml IL-3 (Bouty, Milan, Italy), 10^{-6} M dexamethasone and 10^{-6} M estradiol (both from Sigma, St. Louis, MO). After 10–11 days, the cells were collected and allowed to differentiate for additional 4 days with EPO (1 U/ml) and human recombinant insulin (10 ng/ml, Calbiochem, Darmstadt, Germany).

Phenotypic Analysis

Cell morphology was analyzed according to standard criteria on cytocentrifuged (Shandon, Astmoor, England) smears stained with May-Grünwald-Giemsa. The antigenic profile was analyzed by flow cytometry with EPICS XL (Beckman Coulter, Fullerton, CA), according to standard protocols. Briefly, cells were resuspended in Ca^{++} and Mg^{++}-free phosphate buffered saline supplemented with 1% (wg/vol) BSA, 2 mM EDTA, and 0.01% NaN_3, and labeled on ice with fluorescein isothiocyanate (FITC)-conjugated CD71 and CD235a (antiglycophorin A, Immunotech, Milano, Italy). Cells incubated with the corresponding irrelevant isotype-matched antibodies were used for gating non-specific fluorescence, and dead cells were excluded by propidium iodide staining (5 μg/ml) (Sigma).

Cell Lines

K562 cells were grown in RPMI 1640 containing 10% (vol/vol) FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine (all from Invitrogen). The μLCRβprRluc$^{A}\gamma$prFlucGM979 cell line was obtained by stably transfecting GM979 cells with a dual luciferase reporter containing a 3.1-kb μLCR cassette including the DNAse I hypersensitive core of the 5′ hypersensitive sites HS1, HS2, HS3, and HS4, linked to 315-bp of the human β-globin promoter and 1.4-kb of the $^{A}\gamma$-globin promoter

driving the Renilla (R) and the Firefly (F) Luciferase gene, respectively [Skarpidi et al., 2000]. μLCRβprRluc^{A}γprFlucGM979 cells were maintained in RPMI 1640 containing 10% (vol/vol) FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 400 μg/ml of G418 as described [Migliaccio et al., 2005].

Cell Manipulations

μLCRβprRluc^{A}γprFlucGM979 cells were treated for 6 h with the PKC inhibitor rottlerin (Calbiochem) solubilized in DMSO (final concentration 0.1% vol/vol) at the concentration of either 5 or 30 μM. These concentrations were chosen because reported by Gschwendt et al. [1994] to specifically inhibit either PKCδ (IC50 = 5 μM) or PKCα (IC50 = 30 μM). A following study by Davies et al. [2000], although failed to confirm such isoform specificity, indicated rottlerin as an effective PKC inhibitor with IC50 = 20 μM. Negative controls were represented by cells incubated with DMSO alone. μLCRβprRluc ^{A}γprFlucGM979 cells were transiently transfected by electroporation using the Electro Square Porator ECM 830 (BTX, Holliston, MA) with 30 μg of expression vector containing either sPKCα and iPKCα or sPKCδ and iPKCδ inserted in the pcDNA3 backbone with a C terminal Influenza Virus Hemagglutinin (HA) tag of nine amino acids [Soh et al., 1999]. Additionally, human primary erythroblasts and K562 cells were co-transfected with the PKCα mutants (27 μg) and the double μLCRβprRluc^{A}γprFluc luciferase reporter gene (3 μg). Luciferase activity was measured 24 h after transfection. Transfection efficiency was calculated on the basis of the number of fluorescent cells obtained in parallel samples transfected with a 30 μg of control plasmid containing pGFP (Clontech Laboratories, Mountain View, CA), as determined with EPICS XL. Necrotic cells were excluded from the analysis by propidium iodide (5 μg/ml) staining. Twenty-four hours after transfection, the percent of GFP positive K562 and μLCRβprRluc^{A}γprFlucGM979 cells and primary erythroblasts was 81.3 ± 5, 62.3 ± 7, and 39.3 ± 10, respectively.

Immunocytochemistry

Primary erythroblasts were spun onto coverslip, fixed (4% paraformaldehyde/phosphate-buffered saline, Invitrogen), saturated/permeabilized for 30 min with NET gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.05% NP-40, 0.25% Carrageenan Lambda gelatine, 0.02% NaN3), and incubated for 60 min with antibodies specific for PKCα (sc-8393) or P-PKCα (sc-12356) (Santa Cruz Biotechnology, Santa Cruz, CA, 1:50 dilution). After several washes, the slides were incubated again for 45 min with appropriate secondary FITC-conjugated antibodies (Sigma). Nuclei were counterstained with Dapi (Sigma). Negative controls were represented by samples incubated with the secondary antibody only. Slides were observed with an Axioskop 40 (Carl Zeiss, Oberkochen, Germany) and images acquired with a CoolSNAP*cf* digital CCD camera (PhotoMetrics, Huntington Beach, CA).

Western Blot Analysis

Cells were lysed in 50 mM Tris-HCl pH = 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 0.25% sodiun deoxicolate, 1 mM NaF, 0.5 mM Phenylmethanesulfonilfluoride, 1 μg/ml Aprotinin, 1 μg/ml Leupeptin, 1 mM sodium orthovanadate. Proteins were separated by electrophoresis on polyacrylamide denaturing gels (Invitrogen) that were blotted onto nitrocellulose membrane (Amersham Life Science, Little Chalfont, UK). The blots were sequentially incubated with primary and secondary peroxidase-conjugated antibodies. The antibodies used in this study were represented by anti-PKCα (sc-8393), anti-PKCδ (sc-937), and peroxidase conjugated-anti HA (3F10) monoclonals and by antibodies that recognize the activated form of PKCα (phosphorylated at Ser657, sc-12356) and PKCδ (phosphorylated at Thr507, sc11770) [Kikkawa et al., 2002; Nakashima, 2002] (all from Santa Cruz Biotechnology, with the exception of 3F10, from Roche, Penzberg, Germany). Immunoreactive bands were detected with the Chemiluminescence Luminol System (Santa Cruz Biotechnology) and densitometry was performed with the GelDoc 1000 (BioRad, Milan, Italy).

Dual Luciferase Assay

^{A}γ-Firefly (^{A}γ-F) and β-Renilla (β-R) luciferase activities were determined in triplicate using the Dual Luciferase Reporter Assay System (Promega, Madison, WI), as described by the manufacturer. Luminescence was measured with the Lumat LB9507 Luminometer (EG&G Berthold, Wellesley, MA) and expressed in Arbitrary Fluorescence Units (AFU). Results

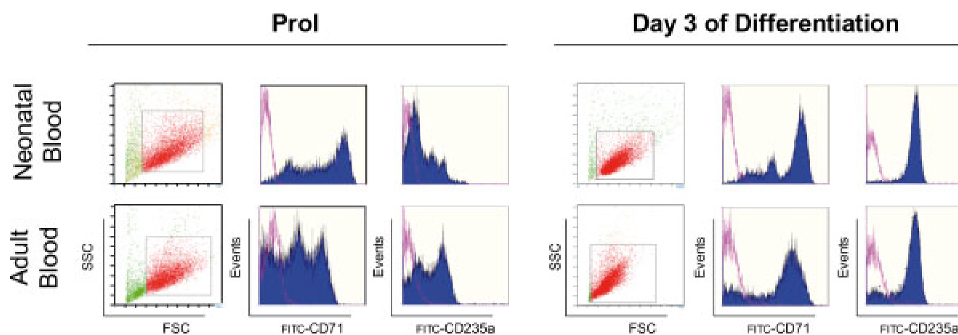


Fig. 1. Antigenic profile of erythroid cells in the proliferative (days 8–12) and in the differentiative (day 3) phase of HEMA culture seeded with mononuclear cells from either neonatal or adult blood, as indicated. Events in the forward (FSC) and side (SSC) scatter gate corresponding to erythroid cells were further analyzed for CD71 and CD235a expression, as indicated. Isotype controls are indicated by purple lines. In the proliferation phase, the majority of neonatal and adult cells expressed high levels of CD71 and low levels of CD235a. The cells became CD71^{high}, CD235a ^{high} after 3 days of exposure to EPO. Similar results were observed in at least three independent cord blood and adult blood experiments. The mean (±SD) observed in multiple experiments is presented in Table I. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

are presented both as absolute values and as activity ratios ($^{A}\gamma$-F AFU/$^{A}\gamma$-F AFU + 2β-R AFU).

RNA Isolation and Expression of PKC Isoforms by Multiple Gene RT-PCR and of Globin Genes by Quantitative Real-Time PCR

RNA was prepared with TRIZOL and reverse transcripted with 250 ng random primers, 1 μl dNTP (10 mM), 1 μl RNase OUT (recombinant RNase inhibitor 40 U/ml) (Invitrogen), as described by the manufacturer. Expression of the PKC isoforms was determined with a commercial multiple gene RT-PCR (PH-0131A, SuperArray Bioscience Co, Frederick, MD). Quantitative real-time PCR for γ- and β-globin transcripts was carried out in a 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), using a specific Kit. The level of γ- and β-globin mRNA was expressed in arbitrary units, using the amplification of GPDH as calibrator, according to the following algorithm: $\Delta Ct = [CtX - CtGPDH]$, where Ct is the threshold cycle and X is either γ- or β-globin, and presented as $2^{-\Delta Ct}$. The $\gamma/\gamma + \beta$ expression ratio was calculated as $2^{-\Delta Ct}\gamma/2^{-\Delta Ct}\gamma + 2^{-\Delta Ct}\beta$.

Statistical Analysis

Statistical analysis was obtained with the Origin 5.0 software for Windows (Microcal Software, Inc., Northampton, MA).

RESULTS

Expression of the γ- and β-Globin Genes During the Maturation of Primary Human Erythroblasts in HEMA Culture

As previously shown [Migliaccio et al., 2002], adult progenitor cells generated high numbers (fold increase ~3.7-fold) of CD71^{pos} CD235a^{low} pro-erythroblasts within 10–11 days of HEMA culture (Fig. 1 and Table I). When switched to a differentiation medium containing only EPO, these pro-erythroblasts progressed in maturation giving rise to mature CD71^{high}CD235a^{high} cells (Fig. 1 and Table I). Adult pro-erythroblats already expressed high levels of both γ- ($2^{-\Delta Ct} = 0.35 \pm 0.2$) and β-globin ($2^{-\Delta Ct} = 1.3 \pm 0.4$) mRNA (Table II). Their further maturation in EPO, was associated with a significant increase (~50%) in total mRNA content, reflected into increased levels of expression for both γ- (by twofold, not

TABLE I. Flow Cytometry Analysis of Primary Human Erythroblasts Obtained in HEMA Culture From Either Neonatal or Adult Peripheral Blood

Source	Cells	Fold increase	Erythroblast gate (%)	CD235a^{low}(%)	CD235a^{high} (%)
Neonatal	Proliferative phase	5.0 ± 5.3	85 ± 5	51 ± 8	24 ± 13
Blood	Day 3 of differentiation	None	90 ± 3	13 ± 6*	77 ± 9*
Adult	Proliferative phase	3.7 ± 2.6	76 ± 8	40 ± 7	29 ± 15
Blood	Day 3 of differentiation	None	94 ± 1	11 ± 6*	81 ± 9*

Cell frequencies statistically different ($P < 0.05$) between proliferative and differentiative cultures are indicated with an asterisk.

TABLE II. Globin Gene Expression in Primary Human Erythroblasts Obtained in HEMA Culture From Either Neonatal or Adult Peripheral Blood

Source	Cells	Total mRNA $\mu g/10^6$ cells	γ-globin mRNA $2^{-\Delta Ct}$	β-globin mRNA $2^{-\Delta Ct}$	$\gamma/\gamma+\beta$ (%)	$^{A}\gamma$-F[a] (AFU $\times 10^3$)	β-R (AFU $\times 10^3$)	$^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R) (%)
Neonatal	Proliferative phase	12.2 ± 0.8	2.6 ± 1.9	0.33 ± 0.09	79 ± 7	5.7 ± 2.2	65.5 ± 24.8	4.78 ± 2.99
Blood	Day 3 of differentiation	8.4 ± 0.4	0.7 ± 0.3*	0.45 ± 0.06	58 ± 12	n.d.	n.d.	n.d.
Adult	Proliferative phase	24.3 ± 3.2	0.35 ± 0.2**	1.3 ± 0.4	28 ± 8	0.4 ± 0.1*	31.0 ± 8.3	0.67 ± 0.09*
Blood	Day 3 of differentiation	33.7 ± 2.0*	0.74 ± 0.4	17.8 ± 8.9*/**	7 ± 3*/**	n.d.	n.d.	n.d.

Statistically different ($P < 0.05$) with respect to the corresponding cells in the proliferative phase (*) or with respect to the corresponding cells from neonatal blood (**).
[a]Mean ($\pm$SD) of 3 separate neonatal blood and 11 separate adult blood experiments.

statistically significant) and β- (by >10-fold, $P < 0.01$) mRNA. Consequently, the $\gamma/\gamma+\beta$-expression ratio decreased with maturation from 28 ± 8 of the pro-erythroblasts to the 7 ± 3 ($P < 0.05$) of mature cells (Table II).

Under HEMA culture conditions, the light density fraction of neonatal peripheral blood generated pro-erythroblasts as efficiently as the adult cells (fold increase ~fivefold). Also in this case, cells with the $CD71^{pos}$ $CD235a^{low}$ pro-erythroblast phenotype were generated by days 10–11 and when switched to EPO, the cells acquired the mature $CD71^{high}$ $CD235a^{high}$ phenotype in 3 days (Fig. 1 and Table II). Slight differences were observed in the levels of antigens expressed by adult and neonatal pro-erythroblasts. Neonatal cells expressed higher levels of CD71 (the transferring receptor) and lower levels CD235a (glycophorin A). However, at day 3 of differentiation, neonatal and adult erythroblasts were antigenically similar (Fig. 1).

Pro-erythroblasts obtained from neonatal blood contained half the amount of mRNA present in adult cells (12.2 ± 0.8 vs. 24.3 ± 3.2, $P < 0.05$) and their subsequent maturation was not associated with increased levels of total mRNA per cell (Table II). As expected, neonatal pro-erythroblast expressed levels of γ-globin mRNA ~1-log higher than those of β-globin. However, in contrast with the adult cells, their further maturation was not associated with significant changes in expression of γ- or β-globin. Consequently, their $\gamma/\gamma+\beta$ expression ratio remained high (58%) with maturation (Table II).

Expression of the Different PKC Isoforms During the Maturation of Neonatal and Adult Erythroblasts

Primary human pro-erythroblasts, both adult and neonatal, expressed all of the PKC isoforms investigated and, with the exception of PKCε and ζ, all of them remained expressed throughout the subsequent maturation in differentiation media (Fig. 2).

A good correlation was observed between the PKC isoforms expressed at the mRNA level and those expressed at the protein level. In fact, with the exception of PKCε and ζ that become undetectable as soon as (24 h), the cells were switched to differentiation media, all of the other proteins, and in particular PKCα and

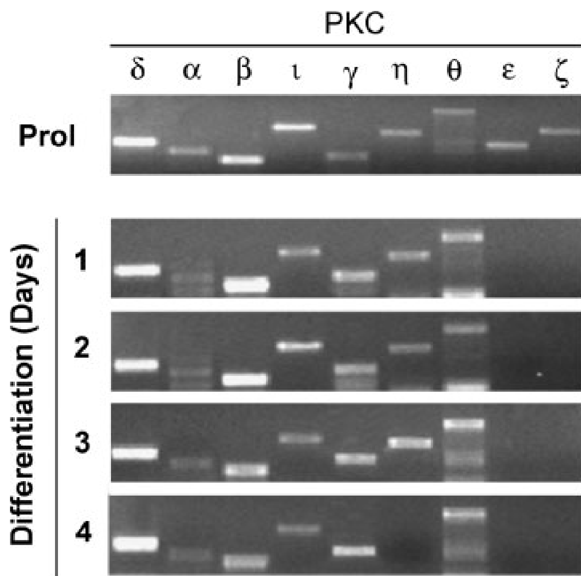


Fig. 2. Multigene RT-PCR analysis for the expression of nine different PKC isoforms during the differentiation of primary adult erythroblasts. Primary pro-erythroblasts (Prol) expressed all of the PKCs analyzed. The expression of most of them persisted throughout the 4 days of maturation, with the exception of PKCε and ζ, that became undetectable after 24 h. Similar results were obtained with primary neonatal erythroblasts.

δ, remained expressed during the maturation of pro-erythroblasts into CD71^{high}CD235a^{high} cells (Figs. 3, 4 and results not shown).

The activation state (defined as ratio between total and phosphorylated form) of two PKC isoforms, PKCα and PKCδ, was differently modulated during the maturation of erythroid cells obtained from adult and neonatal blood (Figs. 3 and 4). In the case of PKCα, adult and neonatal pro-erythroblasts expressed similar P-PKCα/PKCα ratio (Fig. 3). The ratio remained constant during the maturation of erythroblasts obtained from adult blood, but progressively increased during the maturation of the neonatal cells (Fig. 3A,B). Immuno-cytochemistry revealed that P-PKCα was preferential localized

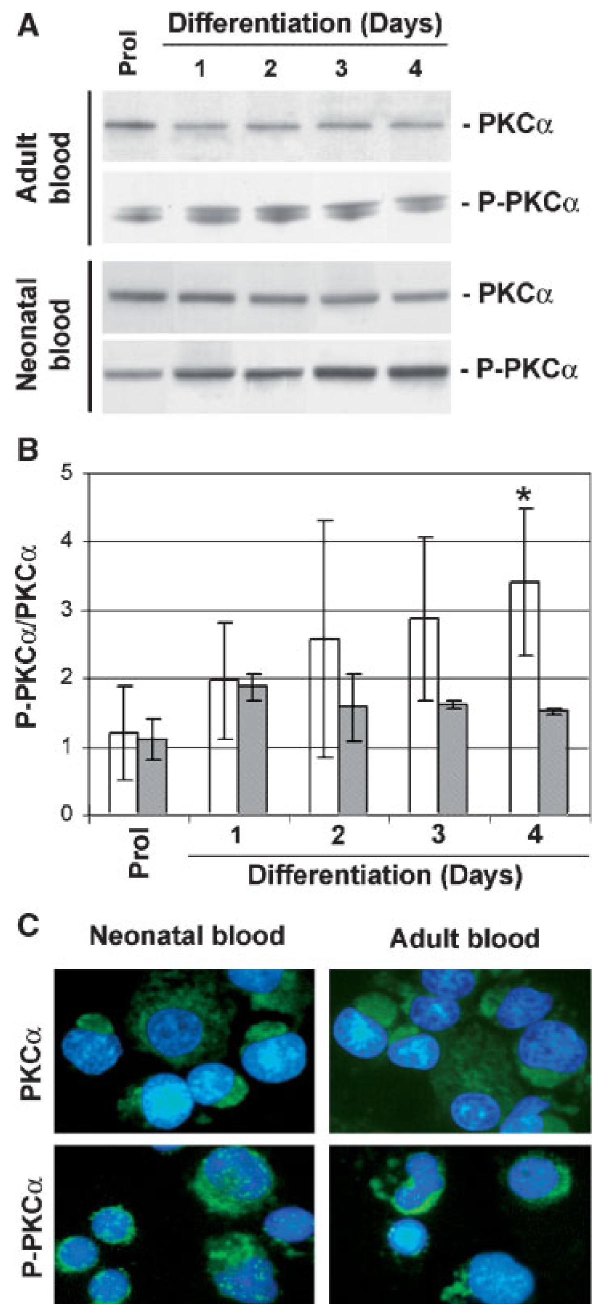


Fig. 3. PKCα is preferentially phosphorylated and translocated to the nucleus during the maturation of neonatal erythroblasts. **A**: Western blot analysis for total (PKCα) and phosphorylated (P-PKCα) PKCα during the maturation of primary pro-erythroblasts derived from either adult or neonatal blood, as indicated. Pro-erythroblasts (Prol) were analyzed either as such, or during 4 days of maturation in cultures containing only EPO. Equal amounts of protein (20 μg) were loaded in each line. Blots are representative of those obtained in three independent experiments. **B**: P-PKCα/PKCα ratio during the maturation of erythroblasts obtained from either neonatal (white columns) or adult (gray columns) blood, as indicated. Results are expressed as mean (±SD) of at least three independent experiments per experimental point. * Statistically different ($P < 0.01$, by ANOVA) with respect to the corresponding cells in the proliferative phase of the culture. **C**: Immunocytochemistry comparing the cellular localization of PKCα and P-PKCα in neonatal and adult pro-erythroblasts, as indicated. Nuclei were counterstained in blue by DAPI. The anti-PKCα antibody (green fluorescence) stained the cytoplasm of both neonatal and adult erythroblasts. Also, the anti-P-PKCα antibody reacted both with neonatal and adult pro-erythroblasts. In the case of neonatal erythroblasts, however, most of the staining was localized in clusters within the nucleus. Original magnifications 64×. Similar results were obtained in three additional experiments.

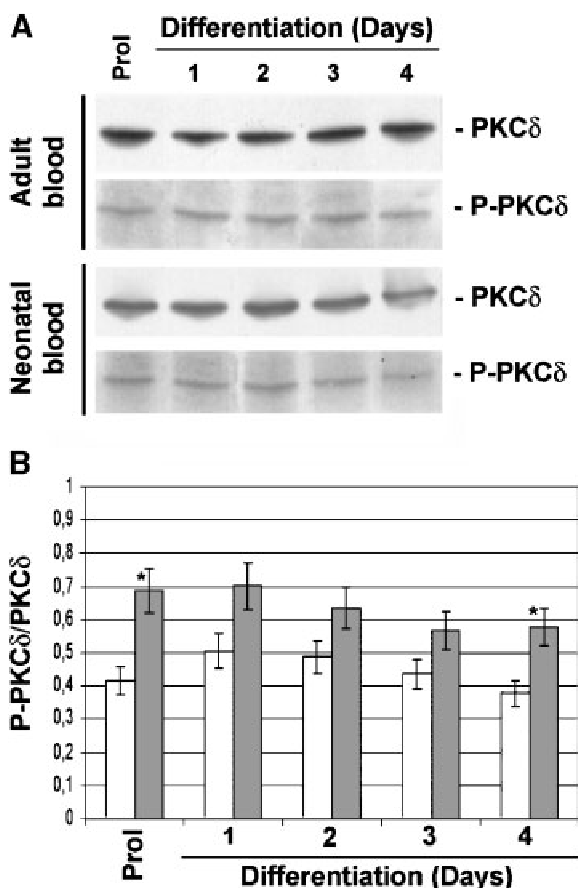


Fig. 4. Neonatal pro-erythroblasts express twofold less P-PKCδ than the corresponding adult cells. **A**: Western Blot analysis for the expression of total (PKCδ) and phosphorylated (P-PKCδ) PKCδ during the differentiation of primary adult and neonatal pro-erythroblasts, as indicated. Equal amounts of protein (20 μg) were loaded in each line. The same samples as presented in Figure 3A. Blots are representative of those obtained in three independent experiments. **B**: P-PKCδ /PKCδ ratio during the maturation of pro-erythroblasts derived from neonatal (white columns) or adult (gray columns) blood. Neonatal pro-erythroblasts expressed levels of P-PKCδ significantly lower than those expressed by their adult counterparts. In both cases, the phosphorylation levels of PKCδ remained constant with maturation. However, the differences in phosphorylation levels between neonatal and adult erythroblasts were statistically significant only at day 4. Results are expressed as mean (±SD) of at least three independent experiments. *Statistically different ($P < 0.01$, by paired *t*-test) from those expressed by adult cells at the corresponding phase of the culture.

in the nucleus of neonatal cells while it was similarly distributed between the cytoplasm and the nucleus of the adult ones (Fig. 3C).

On the other hand, pro-erythroblasts obtained from neonatal blood expressed levels of P-PKCδ/PKCδ significantly lower (~twofold) than those expressed by the corresponding adult cells (Fig. 4). These differences persisted throughout the subsequent maturation of the

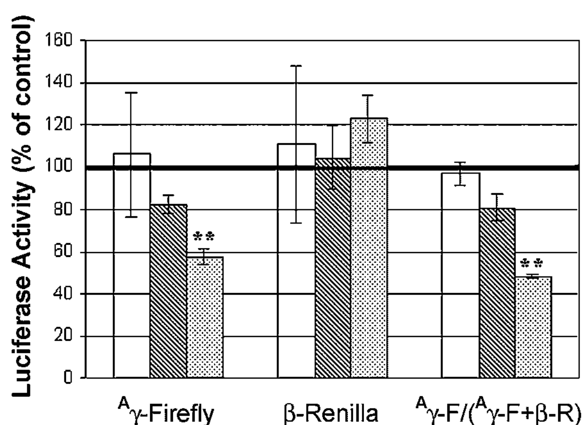


Fig. 5. Rottlerin (30 μM) decreases the luciferase activity driven by the $^{A}\gamma$-globin promoter in μLCRβprRluc$^{A}\gamma$prFlucGM979 cells. μLCRβprRluc$^{A}\gamma$prFlucGM979 cells were incubated with either 5 (hatched bars) or 30 (dotted bars) μM of rottlerin. Cells incubated with DMSO alone were used as control (white bars). Values are expressed as percent of basal luciferase activity (thick horizontal line) expressed by untreated cells, and presented as mean (±SD) of three separated experiments performed in triplicate. Values statistically different ($P < 0.001$, by ANOVA) from untreated controls are indicated by **.

cells in differentiation media, although were statistically significant only at day 4 (Fig. 4).

Effects of Loss of PKC Function on the Level of Expression of a Dual Luciferase Reporter Gene Stably Transfected in GM979 Cells

The association between differences in γ-globin expression and differential activation of PKCα and PKCδ observed in neonatal and adult pro-erythroblasts, suggested to us that either one of these two PKC isoforms could be involved in the regulation of the globin genes. To test this hypothesis, a first series of experiments measured the level of $^{A}\gamma$-Firefly expressed by μLCRβprRluc$^{A}\gamma$prFluc GM979 cells incubated with the PKC inhibitor rottlerin. Indeed μLCRβprRluc$^{A}\gamma$prFlucGM979 cells incubated with 30 μM of rottlerin expressed significantly less (by 50%) reporter activity driven by the $^{A}\gamma$-promoter and decreased $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R) ratio (Fig. 6), than untreated cells. Rottlerin had no significant effects on the viability of the cells for the duration of the assay. This result suggested that PKC might be involved in $^{A}\gamma$-promoter activation.

Effects of Gain of PKCα and PKCδ Function on the Level of Expression of a Dual Luciferase Reporter Gene in Cellular Models of Hb Switching

To identify whether the PKC involved in $^{A}\gamma$-promoter activation was the PKCα and/or

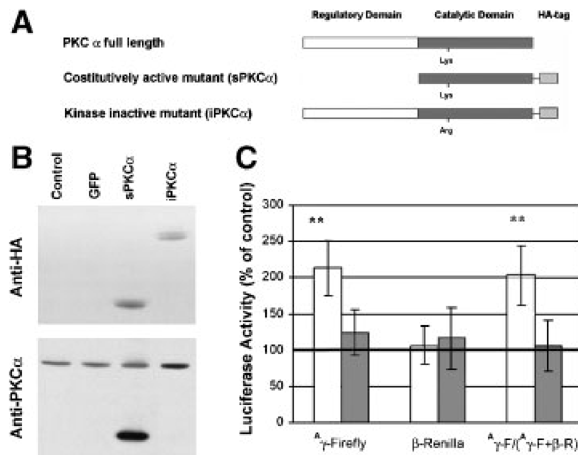


Fig. 6. Transient transfection of sPKCα increases the expression of a reporter gene driven by the $^{A}\gamma$-promoter in μLCRβprRluc$^{A}\gamma$prFluc GM979 cells. **A**: Structures of the two HA-tagged PKCα mutants used in the study. The sPKCα construct (346 pb) codifies the catalytic domain of the enzyme. The iPKCα construct (670 bp) encodes the full-length protein with a point mutation leading to the Lys to Arg substitution at position 368 of the catalytic domain that impairs its kinase activity [Nakashima, 2002]. **B**: Western blot analysis for the expression of the PKCα mutants in transiently transfected μLCRβprRluc$^{A}\gamma$prFlucGM979 cells. Cells were lysed 24 h after transfection and the proteins resolved by SDS–PAGE, blotted, and probed with either an anti-HA or anti-PKCα antibody, as indicated. Negative controls were represented by mock (control) or GFP-transfected cells. Equal amounts of protein (20 μg) were loaded in each line. The HA-tag was expressed only by cells transfected with either sPKCα or iPKCα. The anti-PKCα antibody recognized the full length PKCα in all the samples, but detected a band of lower molecular weight only in cells transfected with sPKCα. On the other hand, the intensity of the band corresponding to the full length PKCα was stronger in cells transfected with iPKCα. **C**: Analysis of the luciferase activities driven by the $^{A}\gamma$- and β-promoter in μLCRβprRluc$^{A}\gamma$prFlucGM979 cells transiently transfected with sPKCα (white columns) or iPKCα (gray columns). Values are expressed as percent of the basal luciferase activity (thick horizontal line) expressed by untreated cells or by cells transfected with an irrelevant vector (GFP) (100% luciferase activity $= 1.4 \pm 0.67 \times 10^{5}$ AFU for $^{A}\gamma$-Firefly, $13 \pm 1.8 \times 10^{6}$ AFU for β-Renilla and $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R) $= 0.5 \pm 0.1$). Results are expressed as mean (±SD) of six separated experiments performed in triplicate. Values statistically different ($P < 0.01$ by ANOVA) from controls are indicated by **.

PKCδ, expression vectors containing the HA-tagged sPKCα, and the iPKCα, as negative control, were transiently transfected into GM979 cells stably transfected with the μLCRβprRluc$^{A}\gamma$prFluc reporter and in K562 cells and into adult and neonatal erythroblasts transiently co-transfected with the dual reporter gene.

As shown by Western blot analysis with the anti-tag and anti-PKCα antibody, μLCRβprRluc$^{A}\gamma$prFlucGM979 cells transfected with sPKCα and iPKCα produced comparable levels of a protein whose molecular weight correspond to that of the expected ectopic product (Fig. 6B). Twenty-four hours after transfection, μLCRβprRluc$^{A}\gamma$prFluc GM979 cells transfected with sPKCα, but not those transfected with iPKCα, expressed significantly higher (~twofold) levels of $^{A}\gamma$-Firefly. Consequently, also the $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R) ratio expressed by the cells was increased (Fig. 6C). In parallel experiments, μLCRβprRluc$^{A}\gamma$prFlucGM979 cells were transiently transfected with vectors encoding either the constitutively active or the kinase inactive forms of PKCδ (sPKCδ and iPKCδ, respectively). These constructs, although expressed by the transfected cells at levels comparable to those obtained with the PKCα mutants, had no effect on the activity of the reporter genes (Supplementary Fig. 1).

In a second series of experiments, K562 cells and adult and neonatal erythroblasts were transiently co-transfected with the dual μLCRβprRluc$^{A}\gamma$prFlu**c** reporter gene and the PKCα mutants (Fig. 7). The luciferase produced by the cells was analyzed 24 h later. K562 cells and primary human erythroblasts transfected with sPKCα expressed five- to sixfold-more $^{A}\gamma$-Firefly than controls transfected with the reporter alone or in association with iPKCα. Consequently, also in this case, the $^{A}\gamma$-F/($^{A}\gamma$-F + 2β-R) ratio expressed by the cells transfected with sPKCα significantly increased over controls (Fig. 7).

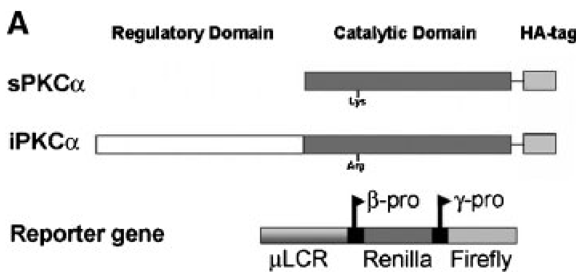
DISCUSSION

Human erythroblasts were generated very efficiently in HEMA cultures seeded with either neonatal or adult cells (Fig. 1 and Table I). In both cases, high numbers (>75%) of $CD71^{high}$ cells were generated by days 10–11 and, when switched to EPO, the cells progressed in maturation in 3–4 days to acquire the phenotype $CD71^{high}$ and $CD235a^{high}$. Quantitative RT-PCR analysis for globin mRNA confirmed that neonatal pro-erythroblasts express significantly more γ-globin than adult cells (Table II). It also confirmed that, in the case of adult cells, pro-erythroblasts express higher $\gamma/\gamma + \beta$ mRNA ratios than the mature ones. The changes in $\gamma/\gamma + \beta$ ratio observed both between cells of different ontogenetic stage and between those at different levels of maturation were due to changes in β-globin mRNA levels rather than in

those of γ-globin (Table II). This phenomenon has been documented before with direct quantitations of globin mRNA species using solution DNA/RNA hybridization [Farquhar et al., 1981].

All of the PKC isoforms were expressed by human pro-erythroblasts and, with the exception of PKCε and PKCζ, remained expressed throughout maturation (Fig. 2). The lack of PKCε expression within 24 h of the induction of maturation is consistent with previously published results [Li et al., 1996; Bassini et al., 1999]. An ontogenetically distinctive pathway of PKC activation was identified during the maturation of primary neonatal and adult erythroblasts. In fact, the conventional PKCα and the novel PKCδ isoform were differentially phosphorylated, and hence activated, in neonatal and adult cells (Figs. 3 and 4). In the case of PKCα, the levels of phosphorylation in neonatal and adult cells were similar at the pro-erythroblast stage but increased with maturation only in the neonatal ones (Fig. 3A,B). Furthermore, immunocytochemistry revealed a preferential nuclear localization of PKCα in neonatal pro-erythroblasts (Fig. 3C). In the case of PKCδ, the enzyme was more phosphorylated in adult than in neonatal erythroblasts throughout maturation (Fig. 4). Interestingly, among all of the PKC isoforms investigated, PKCα and PKCδ are those implicated in SCF signaling. PKCα is responsible for delivering the SCF signal that renders erythroid cells EPO-responsive [von Lindern et al., 2000]. On the other hand, although the role of PKCδ in erythropoiesis has not been established as yet, this PKC isoform triggers mast cell proliferation in response to SCF [Jelacic and Linnekin, 2005]. It is possible that the differential activation of these PKC isoforms in neonatal and adult cells is, at least in part, a reflection of their different sensitivity to this growth factor [Miller et al., 1997; Kurata et al., 1998].

The different phosphorylation pattern of PKCα and PKCδ during the maturation of neonatal and adult erythroblasts, prompted us to analyze whether chemical suppression of PCK activity or forced expression of either one of the



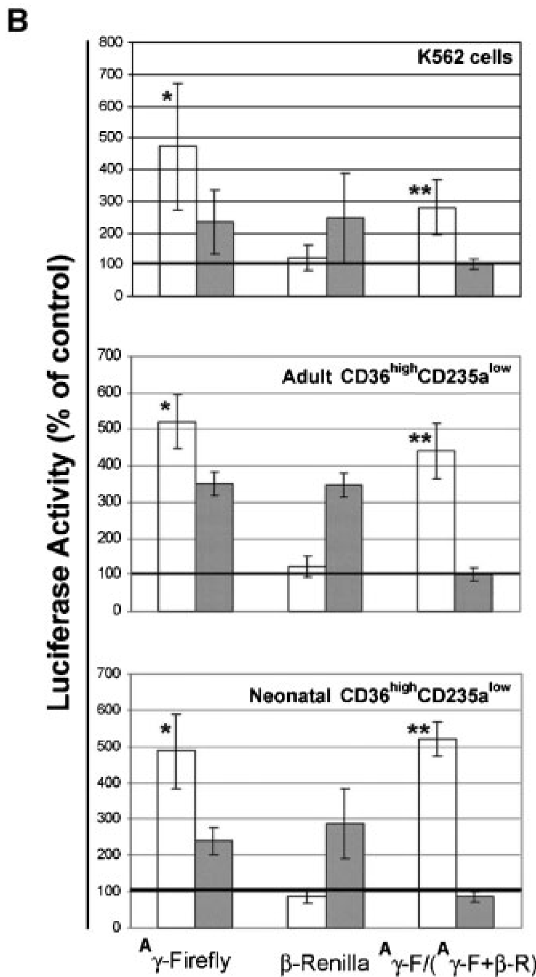


Fig. 7. sPKCα increases the reporter activity driven by the ^{A}γ-promoter in transient co-transfection assays with K562 cells and with adult or neonatal pro-erythroblasts. **A**: Structure of the HA-tagged PKCα mutants and of the double reporter gene used in the co-transfection experiments (see legend of Fig. 3 and Skarpidi et al., 2000 and Nakashima, 2002, for further details). **B**: Analysis of the reporter activity driven by the ^{A}γ- and β-globin promoter in K562 cells and in primary adult and neonatal erythroblasts co-transfected with the μLCRβprRluc^{A}γprFluc construct and either sPKCα (white bars) or iPKCα (hatched bars), as indicated. Values are expressed as percent of the luciferase activity (100%, thick horizontal line) observed in cells transfected with the reporter only. The basal activity (100%) of the reporter genes in K562 cells was: $2 \pm 0.9 \times 10^6$ AFU for firefly, $8 \pm 5 \times 10^6$ AFU for renilla and ^{A}γ-F/(^{A}γ-F + 2β-R) = 13.6 ± 4.2. The basal activity of the reporter genes in adult and neonatal pro-erythroblasts is reported in Table I. Results are presented as mean (±SD) of three separate experiments performed in triplicate. *($P < 0.05$) and ** ($P < 0.001$) indicate values statistically different (by ANOVA) from controls.

two isoforms would alter the levels of expression of reporter genes driven by the β- or the $^{A}\gamma$-promoter in cellular models of Hb switching. These models relay on the μLCRβprRluc$^{A}\gamma$pr-Fluc reporter specifically designed for studies of Hb regulation [Skarpidi et al., 2000]. The observation that rottlerin reduces the levels of $^{A}\gamma$-driven reporter activity in μLCRβprRluc$^{A}\gamma$prFlucGM979 cells supported the hypothesis that PKCs might affect the activity of the $^{A}\gamma$-promoter. On the other hand, ectopic expression of sPKCα increased the activity of the reporter gene driven by the $^{A}\gamma$-promoter in all the cellular models of Hb switching investigated [cells stably (GM979) or transiently (K562 and primary erythroblasts) transfected with the reporter] (Figs. 5 and 7). The increases were of similar magnitude whether the reporter was present in the cells in the integrated (GM979 cells) or episomal (K562 cells) form. Since similar manipulations of PKCδ were ineffective (Supplementary Fig. 1), we propose PKCα as the PKC specifically responsible for the activation of the $^{A}\gamma$-driven reporter activity. Since the activity of a reporter, especially in its episomal form, is independent from the rate of cell proliferation/maturation, these results suggest that the levels of PKCα activity might directly affect the phosphorylation, and therefore the activity, of factors directly or indirectly interacting with the $^{A}\gamma$-globin promoter. As such, they provide a proof of principle that it is possible for external stimuli to activate this promoter by activating the PKCα signaling.

Gain of function experiments in transiently co-transfection reporter assay with genes under the control of suitable promoters have provided important information on the protein–DNA interactions that regulate gene expression. Because of the high number of cells required, these assays relay on cell lines whose nuclear environment may not correspond to that of "normal" cells. Thanks to the high numbers of pro-erythroblasts obtained in HEMA cultures, we could refine this experimental design to analyze the effects of ectopically expressed genes on the activity of co-transfected reporters in primary cells. Interestingly, robust levels of expression were observed when the double luciferase reporter was transiently transfected in these cells (Table II). Furthermore, expression of the reporter genes, although not completely equivalent to that of the endogenous genes, was ontogenetically correct. In fact, expression of both endogenous and ectopically $^{A}\gamma$-driven gene was higher in primary neonatal than in adult pro-erythroblasts, while expression of both the endogenous and ectopically driven β-driven gene was similar in the two cell types (Table II).

Recently, it has been reported that the Special AT-rich binding protein 1 (*SATB1*) gene, when forcely expressed for at least 3 days, induces the expression of the endogenous ε gene in primary erythroblasts [Wen et al., 2005]. To analyze the effect of PKCα on the expression of the endogenous γ- and β-globin genes, we attempted similar experiments by transient expression of sPKCα (or sPKCδ) in primary erythroblasts and by their constitutive overexpression in CD34^{pos} cells, used then, to seed HEMA culture. Most of the primary pro-erythroblasts survived the first 24 h after transfection with sPKCα, allowing us to perform the experiments described in Figure 7. Very few of them, however, were alive 48–72 h later, that is, when the cells should begin to divide. Furthermore, while human CD34^{pos} cells generate erythroblasts under HEMA conditions at least as efficiently as mononuclear blood cells [Migliaccio et al., 2002], those transfected with sPKCα, but not those transfected with irrelevant controls, died in few days (results not shown). The failure to modulate the levels of PKCα in primary cells because of cell death is not surprising, giving the critical role exerted by this enzyme in the transduction of SCF signaling [Haslauer et al., 1999, Myklebust et al., 2000]. It is possible that proper erythroid differentiation depend on such a tight range of PKCα activity, that massive increases, or decrements, of its activity, such as those obtained by gain of function experiments, are incompatible with cell survival. Indirect proof that PKCα might have a physiological role in fetal Hb induction is provided by reports indicating that hypoxia, a known inducer of fetal Hb expression [DeSimone et al., 1982], induces expression of its target genes, EPO in human hepatocellular carcinoma cells and Egr-1 in endothelial cells [Ohigashi et al., 1999; Lo et al., 2001], through the PCKα signaling. Further studies, which will attempt to modulate PCKα activity by either physiological stimuli or siRNA technology, will clarify the role of this enzyme on fetal Hb expression in primary cells.

In conclusion, we describe an ontogenetic-specific regulation of the activity of PKCα and δ during the maturation of primary human

erythroblasts and provide data with transient transfection assays suggesting that external stimuli might activate γ-globin expression through the PKCα pathway.

ACKNOWLEDGMENTS

Human SCF was provided by Amgen (Thousand Oaks, CA, MTA No. 19982634-005). Expressing vectors codifying for PKC mutants were kindly provided by J.W. Soh, Department of Chemistry, Inha University, Korea.

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