Mechanism for fetal globin gene expression: Role of the soluble guanylate cyclase–cGMP-dependent protein kinase pathway

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Communicated by Yuet Wai Kan, University of California, San Francisco, CA, December 18, 2000 (received for review June 26, 2000)

Despite considerable concerns with pharmacological stimulation of fetal hemoglobin (Hb F) as a therapeutic option for the β-globin disorders, the molecular basis of action of Hb F-inducing agents remains unclear. Here we show that an intracellular pathway including soluble guanylate cyclase (sGC) and cGMP-dependent protein kinase (PKG) plays a role in induced expression of the γ-globin gene. sGC, an obligate heterodimer of α- and β-subunits, participates in a variety of physiological processes by converting GTP to cGMP. Northern blot analyses with erythroid cell lines expressing different β-like globin genes showed that, whereas the β-subunit is expressed at similar levels, high-level expression of the α-subunit is preferentially observed in erythroid cells expressing γ-globin but not those expressing β-globin. Also, the levels of expression of the γ-globin gene correlate to those of the α-subunit. sGC activators or cGMP analogs increased expression of the γ-globin gene in erythroleukemic cells as well as in primary erythroblasts from normal subjects and patients with β-thalassemia. Nuclear run-off assays showed that the sGC activator protoporphyrin IX stimulates transcription of the γ-globin gene. Furthermore, increased expression of the γ-globin gene by well known Hb F-inducers such as hemin and butyrate was abolished by inhibiting sGC or PKG activity. Taken together, these results strongly suggest that the sGC–PKG pathway constitutes a mechanism that regulates expression of the γ-globin gene. Further characterization of this pathway should permit us to develop new therapeutics for the β-globin disorders.

Elevated expression of fetal hemoglobin (Hb F) substantially benefits patients with the β-globin disorders (1, 2). There have been continued concerns with the molecular mechanisms that regulate expression of the γ-globin gene, as well as with pharmacological agents that stimulate Hb F production. A number of studies have been performed to investigate the mechanisms for transcriptional regulation of the γ-globin gene. Cis-acting DNA elements lying at flanking regions of the γ-globin gene have been extensively defined by *in vitro* and *in vivo* studies (3–5), and transcription factors binding to such DNA elements have been characterized in detail (6, 7).

Since the first administration of 5-azacytidine to β-thalassemic patients, considerable concerns have been directed to chemical agents that efficaciously stimulate Hb F production (8). Recent multicenter studies with hydroxyurea (HU) have shown that administration of this drug to patients with sickle cell anemia significantly increases Hb F production and improves clinical symptoms by reducing the frequency of pain crisis (9, 10). Butyrate compounds have been administered to patients with β-thalassemia (11, 12) or sickle cell anemia (13), and further large-scale studies seem to be necessary to evaluate the clinical efficacy of the compounds. Despite a number of clinical trials with Hb F-inducing agents, little is known about the molecular and cellular basis of their mode of action.

Regarding the mechanism by which expression of the γ-globin gene is induced by pharmacological agents, we hypothesized that Hb F-inducing agents such as HU and butyrates might employ intracellular second messengers to exert their biological effects on the γ-globin gene. To identify potential second messengers, we studied molecular effects on hemoglobin synthesis of hemin, which has been used to induce hemoglobin synthesis (14, 15). Whereas hemin has been shown to regulate hemoglobin synthesis by modulating the activity of heme-regulated eukaryotic initiation factor 2α kinase (16), previous studies clearly showed that hemin induces the accumulation of globin mRNA in erythroid cells (15, 17). This result suggests that heme stimulates hemoglobin synthesis at the transcriptional level as well. We explored cytosolic proteins whose functions are controlled by heme and which are involved in intracellular pathways, and focused on soluble guanylate cyclase (sGC). This enzyme requires heme as a prosthetic group to produce cGMP. The enzyme activity is affected by the heme synthetic pathway, because porphyrins such as protoporphyrin IX (PPIX) and zinc PPIX are well characterized regulators for sGC activity (18). cGMP acts as an intracellular second messenger by activating cGMP-dependent protein kinase (PKG), which is involved in various physiological processes, including vascular smooth muscle relaxation, peripheral and central neurotransmission, platelet aggregation, and phototransduction (19). To date, roles of the sGC–PKG pathway have not been studied in erythroid cells.

We report here that erythroid cells expressing the γ-globin gene, but not those expressing the β-globin gene, abundantly express sGC subunits, the levels of which are similar to or higher than those in lung, where sGC subunits are expressed at the highest level previously observed (20). Second, we show that activation of the sGC–PKG pathway results in the induction of γ-globin gene expression in erythroleukemic cells as well as in primary erythroblasts. Third, we demonstrate that induced expression of the γ-globin gene by hemin or butyrate requires activation of the sGC–PKG pathway. These results suggest an intracellular pathway in erythroid cells that plays a role in the regulation of γ-globin gene expression.

Materials and Methods

Chemicals. Hemin, PPIX, butyric acid, arachidonic acid, and LY83583 were purchased from Sigma. cGMP analogs, KT5823, and 3-isobutyl-1-methylxanthine were obtained from Calbiochem–Behring. Arginine butyrate was prepared by adjusting 0.1 M butyric acid to pH 7.4 by using 0.5 M arginine. PPIX was

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Abbreviations: Hb F, fetal hemoglobin; HU, hydroxyurea; sGC, soluble guanylate cyclase; PPIX, protoporphyrin IX; PKG, cGMP-dependent protein kinase.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.041599798. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.041599798

dissolved in 153 mM arginine/40% 1,2-propanediol/10% ethanol as described (21).

Cell Lines and Tissue Culture. Two independent K562 cell lines, K562A and K562B, were provided by Y. W. Kan (University of California, San Francisco). A murine erythroleukemic (MEL) cell line (DS19) was given by S. Sassa (Rockefeller University, New York). Human–MEL cell hybrid lines, A181γ and A181β, were described previously (22). Cells were grown in MEM α containing 10% (vol/vol) FCS (Intergen, Purchase, NY), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. K562 cells were treated for 3 days with various chemicals. To suppress sGC or PKG activity, cells were pretreated for 12 h with sGC or PKG inhibitor before adding other chemicals.

Culture of Primary Erythroblasts from Normal Subjects or β-Thalassemic Patients. Fifty milliliters of peripheral blood were drawn from normal adults and β-thalassemic patients after receipt of informed consent. Primary erythroblasts were amplified by the two-phase liquid culture of Fibach *et al.* (23). After the phase 1 culture (day 5), the cells were resuspended in fresh MEM α with 30% (vol/vol) FCS, 1% BSA, 10 μM 2-mercaptoethanol, 1 μg/ml cyclosporin A, and 1 unit/ml erythropoietin (Janssen-Cilag, High Wycombe, Bucks, U.K.). Also, 10 ng/ml stem cell factor (Genzyme) and 0.5 μM 8-(4-chlorophenylthio)-cGMP were added to the medium. The cells were harvested on day 19, and total RNA was isolated and subjected to globin mRNA analyses.

Isolation of Total RNA and Northern Analysis. Total RNA was extracted by the method of Chomczynski and Sacchi (24). Rat lung total RNA was purchased from Ambion, Austin, TX. Twenty micrograms of total RNA was fractionated in 1% formaldehyde/agarose gels and transferred to nitrocellulose membranes (Millipore) as described (25). Filters were hybridized with probes for rat sGC α- or β-subunit at 37°C in hybridizing solution (25% formamide/5 $\times$ SSPE/5 $\times$ Denhardt's solution/0.1% SDS/100 μg/ml salmon sperm DNA; ref. 26). cDNA probes for rat sGC subunits were provided by M. Nakane (Abbott).

Analysis of Globin mRNAs by Primer Extension. Primer extension analyses were performed as described (22). The primers for ε- and γ-globin mRNA were the same as those described previously (22). The labeled primer for human 28S RNA was diluted 100-fold to make its signal intensities comparable to those of globin genes. The sequence of the primer for human 28S RNA was 5′-GGGAATCCTGGTTAGTTTCTTTTCCTCC-3′ (27). Dried gels were exposed to PhosphorImager screens and signals were quantitated by using the IMAGEQUANT program (Version 1.2 for Macintosh).

Nuclear Run-Off Assay. Nuclear run-off assays were performed as described previously (28). Nuclei were isolated by treating cells (50×10^6) with lysis buffer (10 mM Tris, pH 7.4/2 mM $MgCl_2$/3 mM $CaCl_2$/3 mM DTT/0.5% Nonidet P-40), and suspended in reaction buffer (20 mM Tris/100 mM KCl/4 mM $MgCl_2$/2 mM DTT/20% glycerol/0.4 mM ATP/GTP/CTP/100 μCi [α-^{32}P]UTP). Assay reaction was performed at 30°C for 15 min. After isolation of RNA, labeled RNAs were hybridized with cDNAs immobilized on nitrocellulose filters. cDNAs for γ-globin and β-actin were cloned into pCR II (Invitrogen) by reverse transcriptase–PCR.

Measurement of cGMP Concentrations in K562 Cells. Five million cells were grown for different periods of time in the medium containing hemin (20 μM) or arginine butyrate (1 mM). 3-Isobutyl-1-methylxanthine was added to the culture medium at a final concentration of 0.5 mM 30 min before harvesting the cells.

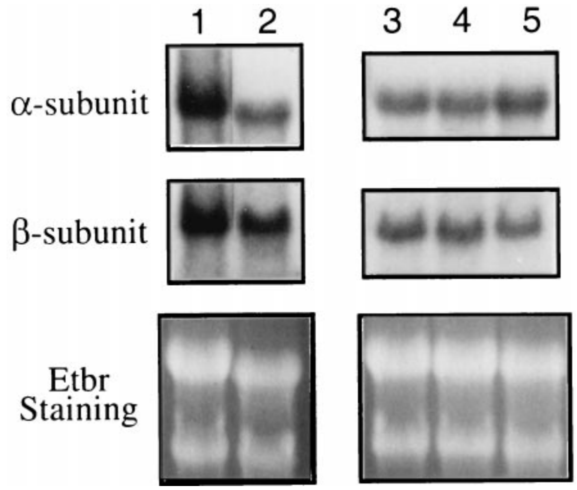

Fig. 1. sGC subunits are expressed at high levels in erythroid cells. Twenty micrograms of total RNA was fractionated in 1% formaldehyde/agarose gels. Gels stained with ethidium bromide are shown at the bottom. Lane 1, K562; lane 2, rat lung; lane 3, K562; lane 4, HEL; lane 5, fetal liver.

Intracellular cGMP concentrations were measured by using the cGMP enzyme-immunoassay system (Amersham Pharmacia) according to the protocol of the supplier.

Results

Erythroleukemic Cells Express Both α- and β-Subunits at High Levels. We first determined by Northern blotting whether sGC subunits are expressed in erythroleukemic cells. K562A cells, which express the γ-globin gene at a high level, expressed both the α- and β-subunits at comparable levels (Fig. 1, lane 1). Because sGC is expressed at the highest level in lung as well as in brain (20), we compared the levels of expression of sGC subunits in K562A cells with those in rat lung. Although the β-subunit is expressed at comparable levels in K562A cells and rat lung, the level of expression of the α-subunit in K562A cells is more than 5-fold higher than that of rat lung (Fig. 1, lanes 1 and 2). HEL cells and fetal liver, both of which express the γ-globin gene, also expressed both subunits at levels similar to those seen in K562A cells (Fig. 1, lanes 3–5). These results indicated that besides high expression in lung and brain, both α- and β-subunits are expressed at high levels in erythroid cells, suggesting an important role of sGC in erythroid lineage cells.

High or Moderate Expression of the α-Subunit Is Associated with Erythroid Cells Expressing a γ-Globin Gene. To study further the expression of sGC subunits in erythroid cells, we determined by Northern blotting whether the levels of expression of sGC subunits vary depending on globin gene programs expressed in erythroid cells. MEL cells were used as erythroid cells expressing an adult globin gene program. As shown in Fig. 2, the level of expression of the α-subunit was extremely low in MEL cells compared with that of K562A cells (lanes 1 and 2), whereas the β-subunit is expressed at comparable levels between the two cell lines. To exclude the possibility that reduced expression of the α-subunit in MEL cells is caused not by the differences of globin gene programs of erythroid cells, but by the differences of cellular characteristics between K562 and MEL cells, we examined the expression of sGC subunits in two human-MEL cell hybrids, A181γ and A181β, which express the human γ- and β-globin genes, respectively, and thus represent erythroid cells with distinct globin gene programs (29). Although the β-subunit is expressed at similar levels in the two hybrids, a marked difference was again seen in the levels of expression of the α-subunit (Fig. 2, lanes 4 and 5). Compared with K562 cells, the

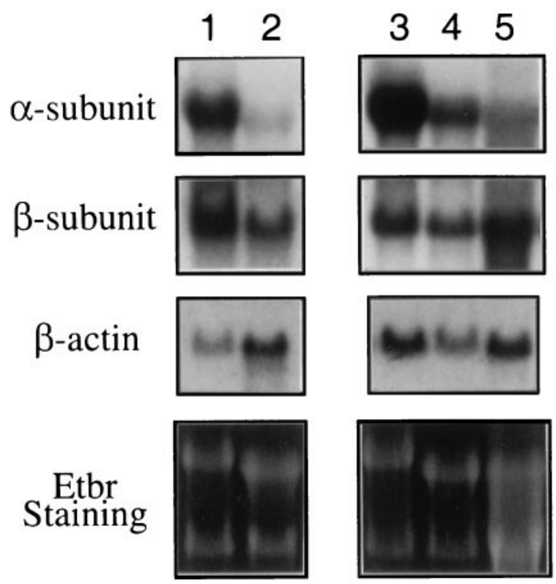

Fig. 2. Erythroid cells transcribing a γ-globin gene express the sGC α-subunit at moderate or high levels. Total RNA (20 μg) was analyzed in 1% formaldehyde/agarose gels. Lane 1, K562A; lane 2, MEL; lane 3, K562A; lane 4, A181γ; lane 5, A181β.

A181γ cells expressed the α-subunit at a moderate but significant level, but extremely low or no expression of the α-subunit was observed in the A181β cells. These results suggested that high or moderate level expression of the α-subunit is preferentially associated with erythroid cells expressing a γ-globin gene.

Levels of Expression of the γ-Globin Gene Are in Proportion to Those of the α-Subunit. To determine whether basal levels of γ-globin gene expression in erythroid cells might be determined by those of the α-subunit, we examined two independent K562 cell lines, K562A and K562B, in which the γ-globin genes were expressed at different levels. Primer extension analyses showed that the level of γ-globin mRNA of K562A is about four times higher than that of K562B, whereas a small difference in the levels of α-globin mRNA was seen between the two cell lines (Fig. 3 lanes 3 and 4). By Northern blotting, no appreciable difference was observed in the levels of expression of the β-subunit between the two cell lines, but the level of expression of the α-subunit of K562A was significantly higher than that of K562B (lanes 1 and 2). Thus, there was a positive correlation in the base line expression between the γ-globin gene and the α-subunit.

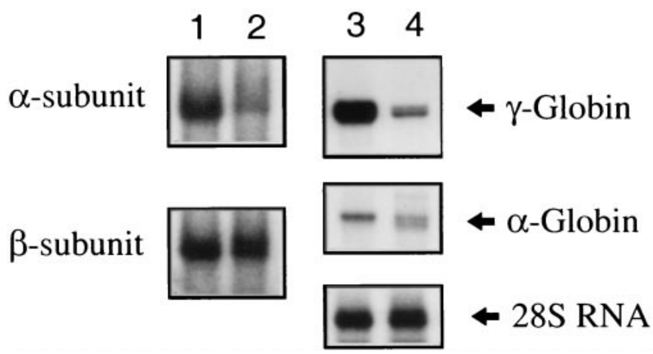

Fig. 3. Levels of expression of the γ-globin gene correlate to those of the sGC α-subunit. Total RNA prepared from two K562 lines was analyzed by Northern blotting. Lane 1, K562A; lane 2, K562B. mRNAs for γ- and α-globin were quantitated by primer extension. Lane 3, K562A; lane 4, K562B. 28S RNA was used as internal control. The sizes of extension products were 110 bp for γ-globin, 89 bp for α-globin, and 80 bp for 28S RNA.

Expression of the γ-Globin Gene Is Induced by Activating the sGC–PKG Pathway in Erythroleukemic Cells and Primary Erythroblasts. We next examined whether expression of the γ-globin gene can be induced in erythroleukemic cells by activating the sGC–PKG pathway. As shown in Fig. 4*A*, the levels of γ-globin mRNA in K562B were increased 5- to 6-fold over the control level by the treatment with PPIX, which is a strong sGC activator (18). Expression of γ-globin mRNA was also increased by treating with 10 μM arachidonic acid, an sGC activator that is structurally distinct from PPIX (ref. 30; data not shown). The induced expression of γ-globin mRNA by PPIX, however, was abolished by preincubating the cells with the sGC inhibitor LY83583 (31), or with KT5823, which is a selective inhibitor of PKG (ref. 32; Fig. 4*A*, lanes 4 and 5). These results suggested that both sGC and PKG are involved in PPIX-induced expression of the γ-globin gene. K562B cells were then incubated with various concentrations of 8-Br-cGMP (1 μM to 1 mM), which is a cell membrane-permeant cGMP analog that activates PKG. Again, the levels of expression of γ-globin mRNA were increased about 3- to 4-fold by cGMP treatment (Fig. 4*B*). We next examined how the level of expression of the α-globin gene is changed by cGMP. As shown in Fig. 4*C*, expression of the α-globin gene was increased about 1.5-fold by cGMP treatment in K562B cells, whereas a more than 4-fold increase was seen for the γ-globin gene. This result suggests that expression of the γ-globin gene is induced in a preferential manner by cGMP. To verify that induction of γ-globin gene expression by PPIX occurs at the level of transcription, we performed nuclear run-off assays. The rate of transcription of the γ-globin gene was increased about 6-fold 6 h after the addition of PPIX, and was reduced to the control level within 24 h of incubation (Fig. 4*D*).

Next, effects of a cGMP analog on γ-globin gene expression were examined by using primary erythroblasts (Fig. 4*E*). In normal subjects, the ratio of γ-globin mRNA to non-α-globin mRNA was increased 5- to 6-fold over the control level by the addition of a membrane-permeant cGMP analog. For the first and third β-thalassemic patients, the γ-globin mRNA ratios were improved from 44% to 68% and from 91% to 95%, respectively, because of the increase in γ-globin mRNA expression by cGMP treatment. In the second patient, however, the increase in the level of γ-globin mRNA expression was subtle but the level of β-globin mRNA was concomitantly decreased, which resulted in an improvement of the ratio of γ-globin mRNA to non-α-globin mRNA from 26% to 52%.

sGC and PKG Are Required for Induced Expression of the γ-Globin Gene by Hemin or Butyrate. Hemin and butyrate are potent Hb F-inducing agents for erythroleukemic cells and primary erythroblasts (17, 33, 34). We examined whether these chemicals require sGC activity to induce γ-globin gene expression. Both hemin and arginine butyrate increased γ-globin mRNA expression about 4-fold over the control level in K562B cells (Fig. 5*A*, lanes 3 and 6). To suppress sGC activity, K562B cells were treated for 12 h with the sGC inhibitor LY83583 before adding hemin or butyrate. As shown in Fig. 5*A*, lanes 4 and 7, both hemin and butyrate failed to induce γ-globin mRNA expression in the LY83583-treated cells. Also, a significant decrease in γ-globin mRNA expression was observed in the cells treated with LY83583 alone (Fig. 5*A*, lane 2). Experiments were also performed to determine whether PKG activity is necessary for the induction of γ-globin gene expression by these chemicals. Again, both chemicals were unable to induce expression of the γ-globin gene in the cells treated with a PKG inhibitor (Fig. 5*B*, lanes 4 and 6). To substantiate these results, we measured intracellular cGMP levels in K562B cells treated with hemin or butyrate. As

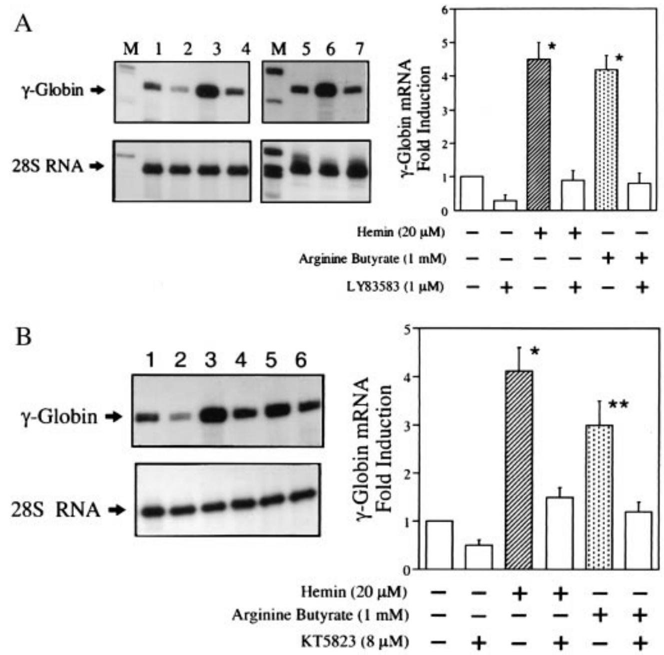

Fig. 5. sGC and PKG are indispensable for the induced expression of the γ-globin gene by Hb F-inducing agents. (*A*) Induced expression of the γ-globin gene by hemin or butyrate is abolished by an sGC inhibitor. Cells were treated with 1 μM LY83583 (sGC inhibitor) for 12 h and then incubated with hemin or arginine butyrate for 3 days. (*Left*) Northern blot. Lanes M, molecular weight marker; lane 1, control; lane 2, 1 μM LY83583; lane 3, 20 μM hemin; lane 4, 1 μM LY83583 + 20 μM hemin; lane 5, control; lane 6, 1 mM arginine butyrate; lane 7, 1 μM LY83583 + 1 mM arginine butyrate. (*Right*) Comparison of mRNA levels. *, $P < 0.001$. (*B*) A selective PKG inhibitor inhibits the induction of γ-globin gene expression by hemin or butyrate. Cells were treated with 8 μM KT5823 (PKG inhibitor) for 12 h and then incubated with hemin or butyrate for 3 days. (*Left*) Northern blots. Lanes M, moecule weight markers; lane 1, control; lane 2, 8 μM KT5823; lane 3, 20 μM hemin; lane 4, 8 μM KT5823 + 20 μM hemin; lane 5, 1 mM arginine butyrate; lane 6, 8 μM KT5823 + 1 mM arginine butyrate. *, $P < 0.001$; **, $P < 0.05$.

shown in Fig. 6, intracellular cGMP levels were increased 4- to 5-fold 1–3 h after stimulation by hemin or butyrate. These results indicated that sGC as well as PKG are indispensable for induced expression of the γ-globin gene by hemin or butyrate.

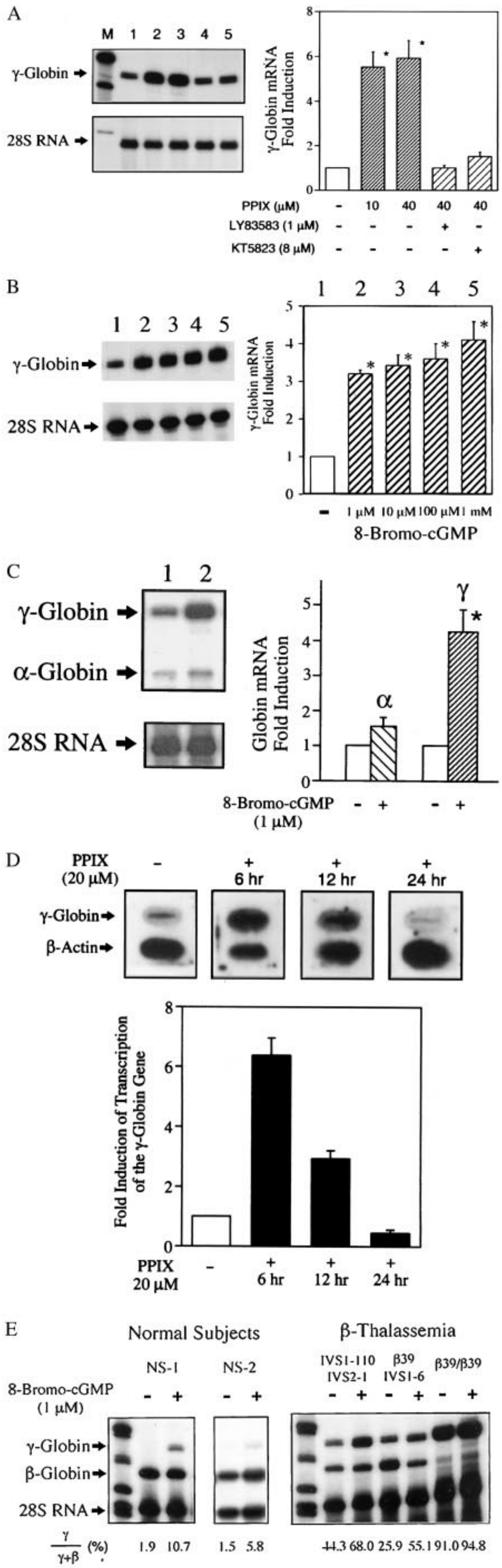

Fig. 4. Expression of the γ-globin gene is induced by activating sGC. (*A*) sGC activators induce γ-globin gene expression in erythroleukemic cells. K562B cells were incubated with PPIX for 3 days. Inhibitors of sGC or PKG were added 12 h before adding PPIX. (*Left*) Northern blot. Lane M, molecular weight marker; lane 1, control; lane 2, 10 μM PPIX; lane 3, 40 μM PPIX; lane 4, 1 μM LY83583 + 40 μM PPIX; lane 5, 8 μM KT5823 + 40 μM PPIX. (*Right*) The levels of γ-globin mRNA of PPIX-treated cells were compared with those of the control cells. *, $P < 0.001$. (*B*) A cell membrane-permeant cGMP analog induces γ-globin gene expression. K562B cells were treated with 8-Br-cGMP (1 μM to 1 mM) for 3 days. (*Left*) Northern blot. Lane 1, control; lane 2, 1 μM 8-Br-cGMP; lane 3, 10 μM, 8-Br-cGMP; lane 4, 100 μM, 8-Br-cGMP; lane 5, 1 mM 8-Br-cGMP. (*Right*)The levels of γ-globin mRNA of cGMP-treated cells were compared with those of the control cells. *, $P < 0.001$. (*C*) cGMP preferentially induces γ-globin gene over the α-globin gene in K562 cells. (*Left*) Northern blot. Lane 1, control; lane 2, 1 μM cGMP. (*Right*) Comparison of mRNA levels. *, $P < 0.05$, γ-globin vs. α-globin. (*D*) PPIX increases transcription of the γ-globin gene. (*Upper*) K562B cells were treated with 40 μM for different periods of time. (*Lower*) The level of transcription of the γ-globin gene in the control cells was set to 1. The level of transcription of the γ-globin gene in the cells treated by PPIX for 6 h was significantly higher than that of the control cells. $P < 0.001$. (*E*) cGMP improves the ratio of γ-globin to non-α-globin in primary erythroblasts. Primary erythroblasts were amplified and treated with 8-Br-cGMP as described in *Materials and Methods*. The genotypes of β-thalassemic patients are shown on top.

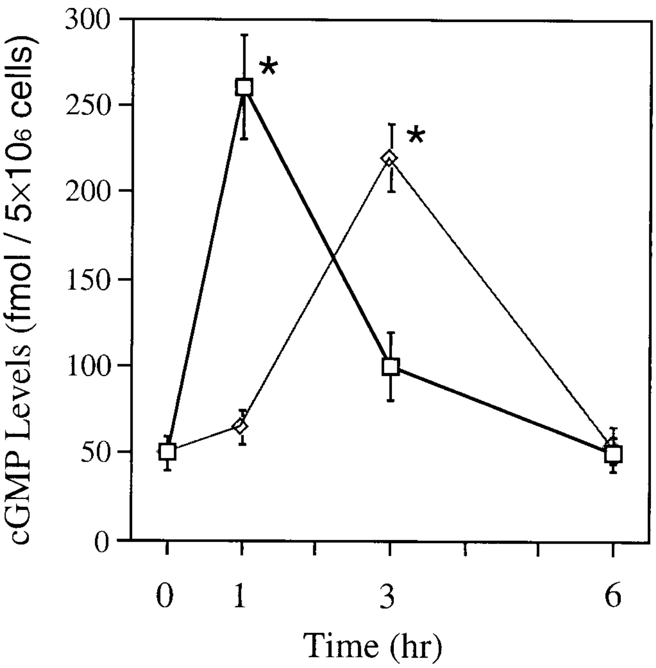

Fig. 6. Intracellular cGMP levels increase by the treatment of hemin or butyrate. K562B cells were treated for different periods of time with 20 μM hemin (□) or 1 mM arginine butyrate (◇). The experiment was done in triplicate. *, $P < 0.05$.

Discussion

Regarding the mechanisms of action of S stage-specific compounds such as 5-azacytidine and HU, alteration in erythroid-regeneration kinetics after cytotoxic events is postulated to underlie increased expression of Hb F (35, 36). However, the mechanisms are unclear by which the γ-globin gene is activated under rapid erythropoiesis and erythroid progenitors with an active Hb F program are selectively recruited. On the other hand, butyrate is an inhibitor of histone deacetylase (37) and is thereby assumed to activate expression of the γ-globin gene by altering chromatin structure. It is not clear, however, how the chromatin structure around the γ-globin gene is selectively changed by butyrate. Moreover, such an assumption is questioned by a recent study showing that chemical agents such as trapoxin, helminthsporium carbonum toxin, and trichostatin A, which are more potent histone deacetylase inhibitors than butyrate, do not necessarily demonstrate stronger induction of γ-globin gene expression in both erythroleukemic cells and primary erythroblasts (38).

As mentioned above, we propose a model in which Hb F-inducing agents stimulate γ-globin gene expression through intracellular pathways. This study demonstrated that the sGC–PKG pathway plays a role, at least in part, in the regulation of γ-globin gene expression in both erythroleukemic cells and primary erythroblasts. First, we showed high level expression of sGC subunits in erythroid cells expressing a γ-globin gene but not those expressing a β-globin gene. Also, the levels of expression of the γ-globin gene were found to correlate with those of the sGC α-subunit. Because heterodimerization of both subunits is essential for sGC activity (20), erythroid cells expressing a γ-globin gene seem to have higher sGC basal activities than those expressing a β-globin gene. Thus, erythroid cells might require high sGC activities to express the γ-globin gene. Next, we showed that both sGC activators and cGMP induce γ-globin gene expression in K562 cells and primary erythroblasts. Conversely, induced expression of the γ-globin gene by the sGC activator PPIX is abolished by inhibiting either sGC or PKG. Moreover, we also demonstrated by using K562 cells that the increased expression of γ-globin mRNA by hemin or butyrate was suppressed by sGC or PKG inhibitors, indicating that this pathway is involved in hemin- or butyrate-induced expression of the γ-globin gene. It should be confirmed, however, that these results are reproducible for primary erythroblasts grown *in vitro*. Taken together, these observations strongly suggest that the sGC–PKG pathway provides a mechanism that regulates the expression of the γ-globin gene in erythroid cells.

In hematopoietic cells sGC is known to modulate a variety of cellular functions of neutrophils and platelets. For instance, stimulation of sGC activity increases chemotaxis and degranulation in neutrophils (39, 40) and decreases platelet aggregation (41, 42). In erythroid cells, however, nothing is known about the roles of sGC. This study has demonstrated that sGC is a key enzyme for an intracellular pathway which is essential for induced expression of the γ-globin gene. To date, several intracellular signaling pathways have been identified in erythroid cells, but all of the pathways have been found to primarily regulate expression of the β-globin gene but not of the γ-globin gene (43, 44). Thus, the sGC–PKG pathway is likely to be the first intracellular pathway that is known to be involved in the regulation of γ-globin gene expression.

This study showed that both hemin and butyrate stimulate γ-globin gene expression by activating the sGC–PKG pathway. How do they activate the sGC–PKG pathway? Abraham *et al.* (45) reported that expression of heme oxygenase-1 is induced in K562 cells treated with hemin. We speculate that treatment of erythroid cells with hemin would induce heme oxygenase-1 expression, which in turn catabolizes heme to biliverdin, iron, and carbon monoxide. The resultant product, carbon monoxide, is a stimulator for sGC (46, 47). Thus, hemin might stimulate sGC activity by producing carbon monoxide. In contrast, butyrate is a short-chain fatty acid. Because fatty acids such as arachidonic acid and linoleic acid are known to stimulate sGC activity (30, 48), butyrate might directly activate sGC. Butyrate-responsive DNA elements that we identified in the γ-globin gene promoter might convey signals of the sGC–PKG pathway to the γ-globin gene (49). It will be interesting to examine whether such DNA elements bind new transcription factors in response to sGC activators or cGMP treatment. Alternatively, as reported by Mori *et al.* (50), butyrate might activate the sGC–PKG pathway indirectly by suppressing cyclic nucleotide-specific phosphodiesterases. As shown in Fig. 6, the intracellular cGMP level in K562 cells treated by hemin increased within 1 h, while cGMP production in K562 cells treated with butyrate was delayed. This result might suggest an indirect mechanism for the activation of the sGC–PKG pathway.

In summary, this study has demonstrated that the sGC–PKG pathway has substantial consequences on γ-globin gene expression in erythroid cells. This pathway seems to be involved in induced expression of the γ-globin gene by other important Hb F-inducing agents such as hemin and butyrate. Further characterization of this pathway should enable us not only to elucidate the mechanisms by which expression of the γ-globin gene is induced in the adult stage, but also to identify novel and safe therapeutics for the β-globin disorders.

We thank M. Nakane for rat sGC probes, Y. W. Kan for K562 cell lines, G. Stamatoyannopoulos and T. Papayannopoulou for human–MEL hybrids and fetal livers, S. Sassa for MEL cells, and A. Milunsky for his support. This study was supported in part by a Grant-in-Aid Award from the American Heart Association, and by the Italian Ministero dell'Universitá e della Ricerca Scientifica e Tecnologica.

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