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Erythroid Kruppel-like factor (EKLF) is recruited to the γ -globin gene promoter as a co-activator and is required for γ -globin gene induction by short-chain fatty acid derivatives

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Abstract

Objectives—The erythroid Kruppel-like factor (EKLF) is an essential transcription factor for β -type *globin* gene switching, and specifically activates transcription of the adult β -*globin* gene promoter. We sought to determine if EKLF is also required for activation of the γ -*globin* gene by short-chain fatty acid (SCFA) derivatives, which are now entering clinical trials.

Methods—The functional and physical interaction of EKLF and co-regulatory molecules with the endogenous human globin gene promoters was studied in primary human erythroid progenitors and cell lines, using chromatin immunoprecipitation (ChIP) assays and genetic manipulation of the levels of EKLF and co-regulators.

Results and conclusions—Knockdown of EKLF prevents SCFA-induced expression of the γ -*globin* promoter in a stably expressed μ LCR β_{pr} R $_{luc}^A$ γ_{pr} F $_{luc}$ cassette, and prevents induction of the endogenous γ -*globin* gene in primary human erythroid progenitors. EKLF is actively recruited to endogenous γ -*globin* gene promoters after exposure of primary human erythroid progenitors, and murine hematopoietic cell lines, to SCFA derivatives. The core ATPase BRG1 subunit of the human SWI/WNF complex, a ubiquitous multimeric complex that regulates gene expression by remodeling nucleosomal structure, is also required for γ -*globin* gene induction by SCFA derivatives. BRG1 is actively recruited to the endogenous γ -*globin* promoter of primary human erythroid progenitors by exposure to SCFA derivatives, and this recruitment is dependent upon the presence of EKLF. These findings demonstrate that EKLF, and the co-activator BRG1, previously demonstrated to be required for definitive or adult erythropoietic patterns of *globin* gene expression, are co-opted by SCFA derivatives to activate the fetal *globin* genes.

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SPP and DVF are inventors on one or more patent applications relevant to this report. SPP and DVF hold equity in, and are consultants for, a corporate entity developing therapeutics for disorders of hematopoiesis.

Keywords

erythropoiesis; fetal hemoglobin; gamma globin; globin gene switching; hemoglobinopathy; sickle cell disease; thalassemia

Pharmacological reactivation of developmentally silenced fetal globin expression is a promising targeted molecular approach to the treatment of the β -hemoglobinopathies, sickle cell anemia, and β -thalassemia. During human hematopoietic development, the first β -type *globin* gene expressed in blood cells formed within the yolk sac is the embryonic (ϵ)-*globin* variant. As the primary site of hematopoiesis migrates to the fetal liver, it is paralleled by a switch to fetal (γ)-*globin* gene expression. A terminal switch in expression occurs, with adult (β)-*globin* gene expression predominating once hematopoiesis moves permanently to the bone marrow. Mice lack a fetal globin gene equivalent and display only a single switch, from embryonic ($\beta H1$, $\beta H0$, $\epsilon\gamma$)-*globin* genes in the yolk sac to the adult (β minor- and β major-) *globin* genes in the fetal liver, adult bone marrow, and spleen. The relative proportions of expression of the genes comprising the β -*globin* cluster and the temporal sequence of their expression are regulated by the interactions of both ubiquitous and erythroid-specific transcription factors and complexes with the *cis*-acting elements residing in the promoters of each of these genes, and the interactions of these promoter-protein complexes with the upstream β -*globin* locus control region (β LCR), designated the active chromatin hub (1-5).

Agents capable of reactivating fetal *globin* gene expression *in vitro* or *in vivo* fall into several major groups, including cytotoxic/chemotherapeutic agents (*e.g.*, hydroxyurea or azacytidine) and histone deacetylase (HDAC) inhibitors (*e.g.*, the short-chain fatty acids [SCFAs] butyrate and valproate). More recently, a new class of SCFA derivatives has been developed, which efficiently reactivates γ -*globin* gene expression *in vivo* and *in vitro* in multiple species, but which does not possess any HDAC-inhibitory activity, and certain of these compounds are now entering clinical trials for the hemoglobinopathies [(6-11) and S.P. Perrine, M.S. Boosalis, D.V. Faller, unpublished data]. These compounds have been shown to relieve active repression of the silenced γ -*globin* promoter by selectively displacing a repressor complex containing HDAC3 and NCoR (11), but the way in which these agents then facilitate transcriptional activation of the γ -*globin* promoter has not yet been fully elucidated.

The *erythroid Kruppel-like factor* (*EKLF/KLF1*) gene is a erythroid cell-specific transcription factor, containing a DNA-binding domain (DBD) located at its C-terminus, composed of three 'Kruppel-like' C2H2 zinc finger motifs, and a proline-rich transactivation domain at its N-terminus (12, 13). EKLF preferentially binds to, and activates, the CACCC motif located in the adult β -*globin* promoter (12-14). EKLF is essential for adult β -*globin* gene transcription, as mice homozygous null for EKLF die at E14.5–E15, the time of hemoglobin switching, because of a severe deficiency in β -*globin* production (15, 16). EKLF also binds to the β LCR and β -*globin* promoters, and is required for direct interactions between the β LCR and the β -*globin* gene in humans, or the β major (β major) gene in mice (17, 18).

EKLF is critical for the recruitment of a number of transcription factors or co-activators to the β -*globin* gene promoter in hematopoietic progenitor cells, including p45, CBP, and SWI/SNF complexes, which then contribute to locus chromatin activation and β -*globin* gene potentiation (19). Mammalian SWI/SNF complexes consist of ~15 subunits and contain either BRM or BRG1 as the core ATPase, along with diverse BRG1-associated factors (BAFs). SWI/SNF complexes can serve as coactivators or co-repressors depending upon the

constituent BAFs (20-22), induce the partial unwrapping of DNA from the nucleosome, and can promote both octamer sliding and transfer to neighboring DNA (23). SWI/SNF is required for the developmental regulation of the human β -globin locus (24-26). EKLF recruits an erythroid-specific BRG1-containing SWI/SNF chromatin remodeling complex to the β -globin locus (27). This EKLF-BRG1 interaction appears to be crucial for EKLF transcriptional activity. The absence of EKLF leads to reduced DNase I hypersensitive site (HS) formation at the mouse β maj and human β -globin promoters (19, 28), and mice expressing a mutant hypomorphic BRG1 exhibit abnormal definitive erythroid cell differentiation, which resembles the phenotype observed in EKLF-knockout mice (29).

While required for the adult switch to high-level β -globin expression, EKLF is not essential for early hematopoietic differentiation. Yet, the CACCC-binding EKLF protein is expressed promiscuously in early hematopoietic progenitor cells and cell lines (19, 30). The human fetal (γ) and embryonic (ϵ) and the murine embryonic ($\epsilon\gamma$) globin gene promoters have distal core CACCC sites (at -145 and -114, respectively) that are very similar to the adult β -type promoter proximal core CACCC site (at -90). EKLF has been reported to transactivate the human embryonic globin gene ϵ through the embryonic/fetal CACCC site (31). In addition, EKLF occupies the $\epsilon\gamma$ 2- and β h1-globin promoters in primitive murine erythroid cells (32). Furthermore, the CACCC sites in the human γ -globin and the murine $\epsilon\gamma$ -globin promoters appear essential for maximal induction or reactivation by SCFAs (33-35).

We demonstrate herein that EKLF is required for activation of the γ -globin gene by a new class of SCFA derivatives, which do not have intrinsic HDAC-inhibitory activity. EKLF is actively and selectively recruited to the γ -globin gene promoter by exposure to these compounds. The SWI/SNF complex chromatin-modifying core ATPase BRG1 is required for γ -globin induction by these SCFA derivatives, and is actively recruited to the γ -globin promoter in an EKLF-dependent fashion. This is the first description of a necessary role for EKLF and BRG1 in γ -globin gene expression.

Materials and methods

Primary cell cultures and chromatin immunoprecipitation (ChIP) assays

Primary erythroid cells were enriched from cord blood (approved for study by the Institutional Review Board of the Boston University School of Medicine) as previously described (11). ChIP was carried by cross-linking 2×10^7 cells in the control and each experimental condition (11). Briefly, 450 μ l of the sonicated extract was immunoprecipitated with anti-EKLF antibody 7B2 (36) or with an anti-BRG1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or irrelevant antibodies (anti-Ikaros antibody, anti-TR2/TR4 antibody, and anti- β -actin antibody). The immunocomplexes were collected using Protein A/G agarose beads (Santa Cruz Biotechnology), washed, and reverse cross-linked. The immunoprecipitated DNA fragments were purified and amplified with primers specific for the γ -globin promoter. Primers specific for the human β -globin and γ -globin promoters and the human β -actin and *c-fos* promoters (used as a controls) were previously described (11, 20).

Gene silencing and quantitative RT-PCR

Endogenous murine or human EKLF and BRG1 proteins were ablated with gene-specific siRNA oligomers (Ambion, Austin, TX, USA) transfected into cultured primary human erythroid progenitor cells (day 11), or cultured murine GM9790 cells, using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). The EKLF siRNA sequences were directed to exon 2, a region with no homology to other *Kruppel*-like genes. Specificity of these siRNA for EKLF was demonstrated by their lack of effect on WT-1 mRNA levels

(the most related of the other *Kruppel*-like genes). Cells were assayed for mRNA and protein levels 24 h following transfection (11). cDNA synthesis was carried out using the Superscript III First Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA, USA), as described previously (11). For each primer pair used, homogeneity of the product was confirmed on an agarose gel prior to real-time PCR. Quantitative-PCR was carried out using the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) by the $\Delta\Delta C_t$ method. Primers used for detecting gene transcript were as follows: EKLF: 5' CGGACACACAGGATGACTTC 3' and 5' GTTGGTGAGGAGGAGATCCA 3'; β -actin: 5' TCCCTGGAGAAGAGCTACGA 3' and 5' AGCACTGTGTTGGCGTACAG 3'; β -globin: 5' CTGAGGAGAAGTCTGCCGTTA 3' and 5' GAGGTTGTCCAGGTGAGCCA 3'; and γ -globin: TCACAGAGGAGGACAAGGCTA and 5' GAGATCATCCAGGTGCTTT 3'.

Luciferase reporter assay

The GM979 cell line, a murine cell line containing a stably integrated human LCR-*globin* promoter-dual luciferase reporter construct was a generous gift of Dr. George Stamatoyannopoulos, and the dual luciferase assay was carried out as described (9, 37). Results are reported as a ratio of firefly luciferase/renilla luciferase, and were normalized to the levels observed in vehicle-treated control cells.

Co-immunoprecipitation and immunoblotting

Co-immunoprecipitation assays were carried using the Seize Primary Mammalian Immunoprecipitation Kit (Pierce, Rockford, IL, USA), according to the manufacturer's recommendations. Antibody to BRG1 (Santa Cruz Biotechnology) was covalently coupled to beads and used as the bait. Immunoprecipitates were recovered, resolved by SDS-PAGE, and immunoblotted with anti-EKLF antibody 7B2, or anti-BRG1 antibody. For quantitation of protein levels, images were photographed (Olympus digital camera D-550, Melville, NY, USA) and the density of each band was calculated using NIH Image software (<http://rsb.info.nih.gov/ni-image>).

Results

The erythroid-specific Kruppel-like transcription factor is required for activation of the γ -globin gene by SCFAs

Multiple studies have indicated the importance of the CACCC motif in fetal or embryonic *globin* gene promoters for efficient induction of the genes by SCFAs (33-35, 38). Therefore, we first determined whether the erythroid-specific CACCC-box binding factor EKLF was required for γ -globin induction by SCFAs. Using the GM979 luciferase reporter system, which is a validated model system comprised of a murine cell line with a stably integrated dual-luciferase reporter construct containing the $\mu LCR\beta_{pr}R_{luc}^{\Delta\gamma_{pr}F_{luc}}$ cassette, and detects only potent inducers of the upstream $\Delta\gamma$ -globin gene promoter (39), we found that knockdown of murine EKLF by siRNA significantly reversed the transcriptional induction of the γ -globin gene by an SCFA, designated RB7 (3-2ox-2H chromen-3yl benzoic acid) (11). Efficient EKLF knockdown (>75%) by a specific murine *EKLF* siRNA (directed against exon 2 of murine *EKLF*, a region which bears no homology to other *Kruppel*-like gene family members) is demonstrated in Fig. 1, panel A. The resulting inhibition of induction of the γ -globin promoter-luciferase reporter is shown in Fig. 1, panel B ($P < 0.01$). Identical levels of abrogation of γ -globin induction by RB7 were found when using a different siRNA (also directed against exon 2 of EKLF) to knockdown EKLF (not shown).

Similarly, knockdown of human EKLF by siRNA abrogated the transcriptional induction by SCFA derivatives of the endogenous human γ -globin genes in primary human erythroid progenitor cells. Greater than 75% knockdown of human EKLF protein and mRNA in these

primary human erythroid progenitor cells by a human EKLF-specific siRNA was demonstrated by immunoblotting and quantitative RT-PCR, respectively (Fig. 2, panels A and B). *γ-globin* mRNA transcript levels were induced by exposure to RB7, but this induction was blunted by knockdown of human EKLF by a EKLF-specific siRNA (Fig. 2, panel B). (We have previously demonstrated that RB7 exposure produces a reciprocal decrease in *β-globin* transcripts when inducing *γ-globin* transcripts (11).) In contrast, exposure to a control (scrambled) siRNA did not abrogate the induction of *γ-globin* mRNA.

Transcription factor EKLF is actively recruited to the endogenous *γ-globin* gene promoter by the SCFA derivatives

Occupancy of the proximal regions of the endogenous *γ-globin* or *β-globin* promoters by EKLF in cultured human erythroid precursors was examined by ChIP assays. Exposure of these primary cells to a SCFA derivative (RB7) increased ELKF occupancy at the *γ-globin* promoter by ~2.5-fold (Fig. 2, panels C and D). In contrast, occupancy at the *β-globin* promoter was unaffected. No occupancy of EKLF was found at the *β-actin* promoter under any experimental conditions after 40 amplification cycles, which therefore served as a control for specificity. As further specificity controls, parallel ChIP assays of the endogenous *γ-globin* genes using a irrelevant antibodies [pooled IgG or anti-*β-actin* (Fig. 3), or *α-Ikaros* or *α-TR2/TR4* antibodies (not shown)] produced no amplified products detectable by gel analysis or by quantitative PCR at 40 amplification cycles.

Similar to the results obtained after exposure to RB7, exposure of primary human erythroid progenitors to another SCFA derivative, 3-({4-nitro-1H-pyrazol-1-yl}methyl)benzoic acid (RB14), at concentrations, which induce transcription of the endogenous *γ-globin* gene (data not shown), also resulted in recruitment of transcription factor EKLF to the endogenous *γ-globin* gene promoter (Fig. 3A). In contrast, no such recruitment to the *β-globin* gene promoter was induced by RB14. Total cellular levels of EKLF protein and transcript were unaltered by exposure to SCFA derivatives (Fig. 2, panels A and B, and Fig. 3, panel B). Knockdown of endogenous EKLF in cultured human erythroid precursors by a specific siRNA prevented recruitment of EKLF to the *γ-globin* promoter after exposure to SCFA derivatives (Fig. 2, panels C and D), coincident with preventing induction of the gene (Fig. 2).

The BRG1 protein is required for *γ-globin* gene induction by SCFA derivatives

EKLF is known to be able to recruit the chromatinmodifying complex SWI/SNF to facilitate transcriptional activation of the *β-globin* gene. SWI/SNF complexes in erythroid cells contain *brahma-related gene 1* (BRG1) as their catalytically active ATPase component. Using co-immunoprecipitation analyses, we found that exposure of human primary erythroid cells to the SCFA derivatives RB7 and RB14 induced a physical association between EKLF and BRG1 (Fig. 4A).

We next demonstrated that knockdown of human BRG1 protein by siRNA abrogated the transcriptional induction of the endogenous human *γ-globin* gene in primary human erythroid progenitor cells by SCFA derivatives (Fig. 4B). *γ-globin* mRNA transcripts were induced by RB7 and RB14 in cells treated with a control (non-specific) siRNA vector, but this induction was blunted by knockdown of human BRG1 by a specific siRNA vector. Greater than 75% knockdown of BRG1 was demonstrated by immunoblotting (Fig. 4B, insert) and by measurement of mRNA levels by quantitative RT-PCR (data not shown).

BRG1 is actively recruited to the *γ-globin* gene promoter by exposure to SCFA derivatives

Chromatin immunoprecipitation assays demonstrated that BRG1 is actively recruited to the *γ-globin* gene promoter of primary human erythroid cells after exposure to the SCFA

derivative RB7 (Fig. 5, top panel). In contrast, association of BRG1 to the β -globin promoter was not altered by exposure to RB7 (Fig. 5, lower panel).

Knockdown of endogenous EKLK levels in cultured human erythroid precursors prevented recruitment of BRG1 to the γ -globin gene promoter by RB7 (Fig. 5), coincident with abrogation of the induction of the gene (Fig. 2). This finding suggests that recruitment of BRG1 to the γ -globin gene promoter is dependent upon occupancy by the transcription factor EKLK.

Discussion

Our data demonstrate that exposure of murine cell lines or primary human erythroid progenitor cells to certain HbF-inducing SCFA derivatives results in recruitment of EKLK to endogenous, or stably transfected, γ -globin gene promoters. Since its discovery in 1993 (12), EKLK has been considered a transcription factor whose primary globin-related gene target is adult (β)-globin. Even though EKLK is present in erythroid cells of all developmental stages, and the CACCC box is present in the promoters of embryonic (ϵ)-, fetal (γ)-, and adult (β)-globin genes, only β -globin gene expression is severely reduced in EKLK-null mice (15, 16). Transgenic mice deficient in EKLK die *in utero* at the time of embryonic to adult hemoglobin switching (28, 40). In EKLK heterozygotes harboring a human β -globin yeast artificial chromosome transgene, human β -globin expression was dramatically reduced, while γ -globin transcripts were elevated approximately fivefold. Conversely, over-expression of EKLK in transgenic mice results in premature globin gene switching (41).

One major action of EKLK plays in β -globin gene expression is stabilizing the interaction of the β -globin gene with the LCR. Specifically, EKLK is necessary for hypersensitivity at, and participation of, the β LCR and the β -globin promoter in the active chromatin hub, which is comprised of direct chromatin interactions between the β LCR and local β -like globin gene promoter regulatory elements (17). The gene competition model of globin switching posits that competition between the individual β -globin-like gene promoters for interaction with the LCR determines which gene is expressed (2). Genes within the β -globin cluster switch their interaction with the active chromatin hub during development, correlating with the switch in their transcriptional activity (5). With deletion of the β -globin promoter, including the CACCC site, the balance is shifted in erythroid cells from a predominantly β -globin gene–LCR interaction to favor instead a γ -globin gene–LCR interaction and a higher level of γ -globin expression, providing indirect support for the competition model (3, 42). If the β -globin-specific actions of EKLK are due simply to its physical localization to the β -globin gene promoter, thereby facilitating competition for the β LCR, we speculate that the ability of the new SCFA derivatives to induce recruitment of ELKF to the γ -globin promoter may simply increase its ability to compete with the β -globin promoter for participation in the active chromatin hub, resulting in reactivation of gene expression. Indeed, in previous studies, the effects of these SCFADs on γ -globin chain mRNA and protein expression both *in vitro* and *in vivo* have demonstrated a reciprocal reduction in β -globin expression accompanying the increase in γ -globin expression [(6-11) and S.P. Perrine, M.S. Boosalis, D.V. Faller, unpublished data]. Studies are underway to confirm SCFA derivative-induced, EKLK-dependent association of the γ -globin gene with the β LCR.

Although most studies have indicated that EKLK within the β -globin cluster is found predominantly at the CACCC motif in the β -globin promoter, as well as in the β LCR (12), the proximal promoters of all of human and mouse β -like and α -like globin genes contain CACCC motifs that are important for transcriptional activation in transient reporter assays and transgenic mice (43-47). The CACCC box in the fetal/embryonic globin gene promoters is believed to play a functionally important role in the activity of the isolated and the

endogenous genes. Deletion of this motif in the γ -globin promoter significantly reduces activity of this promoter (48, 49), and deletions of fetal (γ)-globin gene promoter CACCC sites in transgenic mice impair normal patterns of expression in the fetal stages of development (33, 50). In addition, the CACCC-box-dependence of many other erythroid gene promoters has been demonstrated *in vitro* and *in vivo* (51-59). *In vitro* methylation interference studies indicate that EKLF has eightfold greater affinity for the proximal CACCC box in the β -globin promoter (CCA-CACCC) than for the CACCC box in the γ -globin promoter (CTCCACCC) (14). Yet, EKLF is reported to bind to embryonic/fetal globin gene ($\epsilon\gamma$ 2 and β h1) promoters in primitive erythroid cells, as assayed by ChIP, and this binding is lost in definitive erythroid cells, leading to the suggestion that EKLF activates $\epsilon\gamma$ 2- and β h1-globin gene expression at this primitive developmental stage (32). This greater affinity of EKLF for the β -globin CACCC box might explain our findings in endogenous gene ChIP assays that depletion of ELKF by siRNA had a differentially greater effect on EKLF occupancy at the γ -globin promoters compared with the β -globin promoter.

Our identification of EKLF as a critical transcription factor mediating the induction of γ -globin gene transcription by SCFAs may provide a molecular basis for the previously reported importance of the CACCC motif in fetal and embryonic globin gene promoters with respect to their responsiveness to SCFA derivatives. Multiple putative butyrate- and SCFA-response elements have been described in embryonic and fetal globin genes. We and others have implicated specific promoter sequences (33-35, 38, 60, 61) and signal transduction pathways (8, 62). A consistent finding has been a diminution of basal or SCFA-induced expression when the fetal CACCC site is altered. Pace *et al.* (33, 34) demonstrated that the proximal region of the γ -globin gene, -36 to -201, which includes the CACCC site, as well as a more distal sequence, at -822 to -893, were SCFA-responsive in transgenic mice and murine erythroleukemia cells. We reported that the proximal γ -globin gene promoter of patients who had been treated with butyrate-like compounds *in vivo* (38) showed novel footprints at four sites, indicating the inducible binding of new proteins. Similarly, both the $\epsilon\gamma$ -CACCC site at ~114 bp and enhancer sequences (HS2) from the β LCR were shown to be essential for maximal SCFA-mediated transcriptional induction (35).

How might exposure to SCFAs increase the association of EKLF for the γ -globin promoters in definitive erythroid cells? Post-translational modification of EKLF, particularly acetylation, is one possibility. Acetylation of EKLF, with resultant transcription activation and recruitment of chromatin-remodeling complexes *in vitro*, has been well-characterized at the adult β -globin gene locus (63). Dempsey *et al.* (35) have suggested that one molecular mechanism through which SCFAs may act is by modification of 'adult' and constitutive transcription factors, such as EKLF and specificity proteins 1 and 3 (SP1, SP3), in a way that allows transactivation of an embryonic/fetal globin gene promoter during adult erythroid differentiation. Certain SCFAs, such as butyrate and propionate, are known inhibitors of class I and II HDAC function (26, 64, 65). Gel-shift analyses of binding activity from SCFA-induced MEL cell nuclear extracts showed *in vitro* binding by transcription factors SP1, SP3, and EKLF at the $\epsilon\gamma$ -CACCC site (35). EKLF, SP1, and SP3 are all able to recruit HDACs (66-68), and HDAC inhibitors can further transactivate EKLF and H/FATs at the adult β -globin gene promoter (63). While the newest SCFA derivatives described in this report do not possess intrinsic HDAC inhibitory activity, they are able to displace HDAC3 selectively from the γ -globin promoter, and this displacement is sufficient to cause local histone acetylation and allow transcriptional activation of the γ -globin gene (11). It is therefore possible that loss of an active class I HDAC (HDAC3) from the γ -globin promoter is sufficient to facilitate occupancy by EKLF, perhaps through alterations in its acetylation, and this mechanistic hypothesis is currently under investigation. Other investigators have suggested that changes in the level of expression of EKLF protein may

redirect its β -type *globin* gene promoter CACCC-motif binding preferences (32). However, we did not detect any changes in the total cellular levels of EKLF in murine or human erythroid cells after exposure to SCFA derivatives.

Another possibility is that changes in local chromatin configurations induced by exposure to SCFAs might facilitate new binding of EKLF to the γ -*globin* gene CACCC motif. Histone H3 hyperacetylation is a hallmark of developmental-specific potentiation of the human β -*globin* gene in erythroid progenitors (69, 70). The direct inhibition of HDAC activity or the indirect, but equivalent, action of displacing HDAC3 at the γ -*globin* promoter might be expected to produce local changes in chromatin. Indeed, we have recently reported significant increases in the acetylation of histones H3 and H4 at the γ -*globin* gene promoter, with no significant changes at the β -*globin* promoter, induced by the new SCFAs, which displace HDAC3 locally. Interestingly, however, during developmental gene switching the differential binding of EKLF to embryonic/fetal and adult globin gene promoters does not appear to result from changes in chromatin configuration (32).

EKLF influences chromatin organization at the β -*globin* gene promoter (19, 27). Studies of mice transgenic for the β -*globin* locus showed that the absence of EKLF expression resulted in an altered chromatin structure at the respective globin promoters, coincident with the reduction of β -*globin* and fivefold increases in, and prolongation of, γ -*globin* gene expression (17, 28). The SWI/SNF complex, and specifically BRG1, is necessary and sufficient for targeted chromatin remodeling and transcriptional activation by EKLF *in vitro* (63). The DBD of EKLF interacts with native and recombinant SWI/SNF in solution and when bound to DNA, SWI/SNF enhances EKLF binding to chromatin templates and remodels adjacent chromatin over at least one nucleosome (63). Acetylation of EKLF *in vivo* by p300/CBP increases the affinity of EKLF for SWI/SNF (27, 63, 71, 72). We have shown in this report that BRG1 is also required for induction of γ -*globin* gene expression by SCFAs, and that BRG1 is recruited to the γ -*globin* gene promoter in an EKLF-dependent fashion. We further demonstrate here that the SCFA derivatives increase the physical association of BRG1 and EKLF in cell lysates, and speculate that the direct or indirect effects of SCFA derivatives on deacetylase activity locally at the γ -*globin* gene promoter may enhance the association of EKLF with BRG1 at this locus, facilitating chromatin remodeling and transcriptional activation.

In conclusion, this is the first description of a necessary role for EKLF and BRG1 in γ -*globin* gene expression. It is noteworthy that the local changes in acetylation of histones H3 and H4 at γ -*globin* promoter, the EKLF-dependent recruitment of BRG1, and the local recruitment of pol II to the γ -*globin* gene promoter induced by exposure to SCFAs, detailed in this and our previous report (11), parallels what is seen during recruitment of an EKLF-BRG1 complex to the β -*globin* promoter during development switching.

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Reagents

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Figure 1.

Effect of erythroid Kruppel-like factor (EKLf) knockdown in murine cells on transcriptional induction of a human γ -globin promoter. Panel (A) immunoblot showing knockdown of endogenous EKLf protein levels in GM979 cells. Cells were transfected with either a scrambled siRNA (siC) or with siRNA directed towards the open reading frame of EKLf (siEKLf), and treated with RB7 or vehicle. β -actin protein levels (lower panel) serve as a loading control. Panel (B) dual luciferase reporter assay. γ -globin gene/ β -globin gene induction measured as a ratio of firefly luciferase to renilla luciferase activities in GM979 cells treated with control- or EKLf-specific siRNA, with or without exposure to RB7. Values are normalized to results obtained from cells that were transfected with a control siRNA (siC), and represent an average of four experiments. Error bars represent the standard error of the mean (SEM). * $P < 0.01$, compared with siRNA control.

Figure 2.

Effects of knockdown of endogenous erythroid Kruppel-like factor (EKLf) in cultured human erythroid precursors on globin gene induction and promoter occupancy by SCFA derivatives. Panel (A) immunoblot showing EKLf protein levels in cells cultured under control conditions (C), and in the presence of RB7 at 20 μ M (RB7) with 400 nM of siRNA control (RB7 siControl), or with 400 nM siEKLf (RB7 siEKLf). The blot was stripped and re-probed with an antibody to β -actin to serve as a loading control. Panel (B) EKLf transcript (top panel) and γ -globin transcript (bottom panel) levels from cells treated under the same conditions described for panel A. γ -globin gene induction measured by γ -globin mRNA transcript levels in primary human erythroid progenitor cells by quantitative RT-PCR, relative to β -actin mRNA levels. Values are normalized to results obtained from cells that were transfected with a control siRNA (siC), and represent an average of four experiments. Panel (C) ChIP assay for EKLf at the endogenous globin gene promoters under the conditions described in panel A. Photograph of an ethidium-stained agarose gel showing amplification of DNA fragments purified following ChIP using primers for γ -globin, β -globin, and β -actin promoters. 'Input' shows starting material prior to chromatin IP. The column on the right side of this panel shows amplified products from input DNA, without immunoprecipitation, using the primers for the γ -globin, β -globin or β -actin promoter regions. Panel (D) quantitative PCR following ChIP assay under the experimental conditions used for panel C. Relative association of EKLf with endogenous γ - and β -globin promoters. Values are normalized to cells cultured under control (vehicle) conditions, and are averaged from independent experiments. In panels B and D, error bars represent the standard error of the mean (SEM). * $P < 0.01$, compared with siRNA control. As specificity controls, parallel ChIP assays using pooled IgG, or anti- β -actin, anti-Ikaros or anti-TR2/TR4 antibodies produced no amplified products by quantitative PCR at 40 amplification cycles (Fig. 3).

Figure 3.

Panel (A) chromatin immunoprecipitation (ChIP) assays at the endogenous β -type *globin* gene promoters: Relative association of erythroid Kruppel-like factor (EKLf) at the β -*globin* promoter (left side) and γ -*globin* promoter (right side) after human erythroid progenitors were cultured in the presence of short-chain fatty acid derivatives RB7 (at 20 μ M) or RB14 (20 μ M). Results are normalized to the vehicle-treated control culture sample (C) for either the β -*globin* or γ -*globin* primers sets, arbitrarily set at 100% for each control, and represent an average of independent experiments. Panel (B) as a specificity control, parallel ChIP assays using pooled IgG or anti- β -actin antibodies produced no amplified products by quantitative PCR at 40 cycles. Similarly, parallel ChIP assays using anti-Ikaros or anti-TR2/TR4 antibodies produced no amplified products by quantitative PCR at 40 cycles (not shown). Panel (C) relative EKLf transcript levels in erythroid progenitors cultured under control conditions (C), or in the presence of short-chain fatty acid derivatives RB7 (20 μ M) or RB14 (20 μ M), measured by quantitative RT-PCR relative to β -*actin* transcript levels. Values are normalized to control conditions. Results are averaged from independent experiments. In all panels, error bars represent the standard error of the mean (SEM). * $P < 0.01$, compared with control.

Figure 4.

Panel (A) inducible co-immunoprecipitation of EKLF/BRG1. Human erythroid progenitors were cultured either under control (vehicle) conditions (C) or in the presence of RB7 or RB14, then lysed and proteins were immunoprecipitated with an anti-BRG1 antibody, separated by PAGE, and immunoblotted with an anti-BGR1 or an anti-EKLF antibody. BRG1 protein levels in the immunoblot demonstrate equal loading. Relative amounts of EKLF protein co-immunoprecipitating with BRG1 under each condition are plotted, after quantitation, in arbitrary units. Panel (B) effects of knockdown of endogenous BRG1 protein in human erythroid progenitors on γ -globin gene induction. Immunoblot showing BRG1 protein levels in RB7- and RB14-treated cultures. Cells were treated with either a scrambled siRNA (siControl) or BRG1-specific siRNA (siBRG1) (both at 400 nM). Immunoblot of β -actin levels serves as a loading control. γ -globin mRNA transcripts were quantified by real-time quantitative RT-PCR after primary erythroid cells were cultured either in control conditions (C), or in presence of short-chain fatty acid derivatives RB7 (left) or RB14 (right). Values were normalized to levels under control conditions. In both panels, error bars represent the standard error of the mean (SEM). * $P < 0.01$, compared with siRNA control.

Figure 5.

Chromatin immunoprecipitation (ChIP) assays of BRG1 at the endogenous β -type *globin* gene promoters in human primary erythroid progenitor cells. Quantitative PCR of immunoprecipitated complexes showing relative association of BRG1 with the γ -*globin* promoter (top left plot) and β -*globin* promoter (bottom left plot); RB7: Chromatin from RB7-treated cultures (20 μ M); RB7 (siControl): Chromatin from RB7-treated cultures, transfected with scrambled siRNA (400 nM); RB7 (siEKLF): Chromatin from RB7-treated cultures, transfected with an siRNA specific for EKLF (400 nM). As specificity controls, no products were amplified at 40 PCR cycles after IP with the BRG1-specific antibody, using primers specific for the *c-fos* promoter (right plot). Results represent an average of two independent experiments. Error bars indicate the standard error of the mean (SEM). * $P < 0.01$.