Erythroid Inhibition by the Leukemic Fusion AML1-ETO Is Associated with Impaired Acetylation of the Major Erythroid Transcription Factor GATA-1

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Abstract

Human acute myeloid leukemias with the t(8;21) translocation express the AML1-ETO fusion protein in the hematopoietic stem cell compartment and show impairment in erythroid differentiation. This clinical finding is reproduced in multiple murine and cell culture model systems in which AML1-ETO specifically interferes with erythroid maturation. Using purified normal human early hematopoietic progenitor cells, we find that AML1-ETO impedes the earliest discernable steps of erythroid lineage commitment. Correspondingly, GATA-1, a central transcriptional regulator of erythroid differentiation, undergoes repression by AML1-ETO in a nonconventional histone deacetylase–independent manner. In particular, GATA-1 acetylation by its transcriptional coactivator, p300/CBP, a critical regulatory step in programming erythroid development, is efficiently blocked by AML1-ETO. Fusion of a heterologous E1A coactivator recruitment module to GATA-1 overrides the inhibitory effects of AML1-ETO on GATA-1 acetylation and transactivation. Furthermore, the E1A-GATA-1 fusion, but not wild-type GATA-1, rescues erythroid lineage commitment in primary human progenitors expressing AML1-ETO. These results ascribe a novel repressive mechanism to AML1-ETO, blockade of GATA-1 acetylation, which correlates with its inhibitory effects on primary erythroid lineage commitment. (Cancer Res 2006; 66(6): 2990-6)

Introduction

The chromosomal translocation t(8;21) occurs in ~25% of cases of human acute myeloid leukemia (AML) with neutrophilic differentiation (French-American-British type M2; ref. 1). As compared with t(8;21)-negative cases of AML M2, t(8;21)-positive AMLs show increased granulopoiesis and diminished erythropoiesis (2, 3). Because the translocation occurs within the pluripotent hematopoietic stem cell (HSC; refs. 4, 5), this skewed lineage output most likely reflects a developmental abnormality caused by the translocation-associated fusion protein AML1-ETO (AE). Animal models for AE expression in the HSC compartment, either by gene targeting or retroviral transduction, have recapitulated the lineage skewing found in patients, with mice showing erythroid hypoplasia combined with granulocytic hyperplasia (6, 7). Studies in *ex vivo* cultured cell lines and primary progenitors confirm that AE inhibition of erythroid maturation occurs in a cell-autonomous manner (8–10). However, the developmental stage and molecular mechanism for this inhibition have remained undefined.

The AE fusion incorporates the DNA-binding runt domain from AML1 (RUNX1) and almost the entirety of ETO. Wild-type RUNX1 binds to a RUNX consensus sequence TGT/cGGY and regulates target genes involved in cell cycle control, HSC ontogeny, and adult myeloid and megakaryocytic differentiation (11–14). ETO seems to function as a multimeric scaffolding factor binding a variety of proteins including corepressors, transcription factors, kinase regulators, and nuclear matrix elements (13). A domain in ETO conserved from the *Drosophila* orthologue Nervy, known as the NHR4 (Nervy homology region 4) zinc finger domain, engages the corepressors NCoR and SMRT as well as the histone deacetylases (HDAC) 1 to 3. The AML1-ETO fusion gene may thus recruit HDACs and corepressors to RUNX binding sites, repressing target genes normally activated in the course of hematopoietic differentiation (15). However, recent results have also directed attention to leukemogenic contributions by ETO regions amino terminal to the NHR4 zinc finger (16), regions known to bind repressors and corepressors such as PLZF, Bcl-6, Gfi-1, and mSin3a (17–20) and also known to subvert the subnuclear trafficking of RUNX1 (21). As an additional mechanism, AE may also introduce repressive methylation into target promoter/enhancer sequences through recruitment of DNA methyltransferase 1 (DNMT1; ref. 22).

Importantly, the erythroid differentiation program occurs in a RUNX-independent manner (12), i.e., erythroid differentiation does not require RUNX factors, and erythroid promoters/enhancers generally lack RUNX binding sites. Therefore, altered RUNX1 target gene regulation most likely cannot account in any direct manner for the defects in erythroid development associated with AE. Shimada et al. have shown AE to dysregulate genes that do not represent normal target genes of RUNX1, presumably through indirect mechanisms involving protein-protein interactions (23). We and others have previously shown the ability of the RUNX1 runt domain to physically and functionally interact with the erythroid master regulatory transcription factor GATA-1 (11, 24), raising the possibility of GATA-1 repression by AE in t(8;21) leukemias through direct physical interaction.

In the current study, using a transient transfection system which provides rapid AE expression in purified primary human early progenitors, we found that AE blocked the earliest discernable steps of erythroid lineage commitment in a manner dependent on the NHR4 zinc finger. Correspondingly, AE repressed the transcriptional function of GATA-1 in a manner dependent on the NHR4 zinc finger but independent of HDAC and DNMT activity. We identified a novel inhibitory mechanism in which AE prevented GATA-1 acetylation by the coactivator p300. Previous studies have shown acetylation of GATA-1 by p300/CBP to be required for GATA-1 transcriptional activity and programming of erythroid differentiation (25, 26). As proof of functional significance, we employed a novel strategy of

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doi:10.1158/0008-5472.CAN-05-2944

enforcing GATA-1 coactivator recruitment/acetylation, which overrode the inhibitory effects of AE on transcriptional activation and permitted rescue of primary progenitor cells from the AE block in erythroid lineage commitment.

Materials and Methods

Plasmids. Expression vectors for intact AE and ΔZnf in pCMV5 were provided by Dr. Scott Hiebert (Vanderbilt Cancer Center, Nashville, TN; ref. 27). pCMV-p300HA was provided by Dr. David Livingston (Dana-Farber Cancer Institute, Boston, MA). pCMV-E1A, expressing Ad5 E1A was provided by Dr. Eileen White (Rutgers University, Piscataway, NJ; ref. 28). The reporter plasmid for GATA-1 transcriptional activity, αIIb-598-Luc, has been previously described (11). Stable expression constructs for AE and ΔZnf were generated by subcloning *Xba*I fragments, derived from the CMV plasmids (27), into pBluescript II KS. *Xho*I-*Not*I fragments released from pBluescript subclones were cloned into the corresponding sites of the pLRT retroviral vector (29). A similar approach was employed for AE R174Q, except that PCR mutagenesis introduced the runt domain mutation prior to subcloning into pBluescript. The expression construct for EG, pCMV-EG, was generated by overlap PCR, incorporating a full-length GATA-1 coding sequence preceded by the Ad5 E1A sequence encoding amino acids 1 to 89. PCR products initially cloned into pCR2.1-TOPO were subcloned as *Bam*HI-*Xba*I fragments into *Bgl*II-*Xba*I sites of pCMV5. Acetylation assays employed a vector expressing FLAG-GATA-1, generated by subcloning a *Bam*HI-*Xho*I fragment of full length, PCR amplified GATA-1 into the corresponding sites of pCMV-Tag 2B. Constructs were verified by sequencing.

Cell culture and transfections. Human $CD34^+$ cells at >98% purity were derived at the National Hematopoietic Cell Processing Core (PEGT-HCPC, NIH grant HL 66947) directed by Dr. Shelly Heimfeld (Fred Hutchinson Cancer Research Center). In brief, granulocyte colony-stimulating factor mobilized peripheral blood mononuclear cells from normal donors underwent purification using CliniMACS magnetic beads (Miltenyi Biotec, Auburn, CA). All experiments with human cells were approved by the University of Virginia Human Investigations Committee. Prestimulation medium consisted of SFEM (Iscove's modified Dulbecco's medium with 20% BITS 9500; Stem Cell Technologies, Vancouver, BC), 1% penicillin streptomycin amphotericin (Life Technologies, Rockville, MD), and 2 mmol/L L-glutamine with the following cytokines added: stem cell factor at 100 ng/mL, thrombopoietin at 100 ng/mL, FLT3-ligand at 100 ng/mL, and interleukin 3 at 20 ng/mL (all from Peprotech, Rocky Hill, NJ). Erythroid differentiation medium consisted of SFEM with 4.5 units/mL human erythropoietin (Amgen, Inc., Thousand Oaks, CA) and 25 ng/mL stem cell factor. The human erythroleukemic cell line K562 was maintained in RPMI 1640 with 10% fetal bovine serum and 1% penicillin streptomycin amphotericin. HEK293T, as well as the retroviral packaging lines, Phoenix and FLYRD18, were maintained in DMEM with 10% fetal bovine serum, 1% penicillin streptomycin amphotericin, and 2 mmol/L L-glutamine. Culture conditions for all cells consisted of 37°C, 5% CO_2, and humidified air.

Human $CD34^+$ cells grown for 48 hours in prestimulation medium were transfected using the Amaxa nucleofection technology (Amaxa Biosystems, Inc., Germantown, MD). Briefly, 7.5 to 10.0 × 10^5 cells resuspended in 100 μL of the mixture provided in the human CD34 cell nucleofector kit were combined with 3.75 μg total DNA, transferred to the provided cuvettes, and electroporated using program U-08. The DNA consisted of 1.5 μg of each expression construct and 0.75 μg of the pEYFP-C1 marker plasmid (Clontech, San Diego, CA). pCMV5 parent vector was included where required to normalize the DNA total. Immediately after electroporation, cells were transferred to 3 mL of prewarmed erythroid differentiation medium in a six-well plate followed by culturing for the indicated durations. HEK293T, as well as the retroviral packaging lines, Phoenix and FLYRD18, were transfected by calcium phosphate precipitation as previously described (30). To generate K562 clones expressing either green fluorescent protein (GFP), AE, ΔZnf, or R174Q in the pLRT vector, retroviral supernatants from Phoenix and FLYRD18 transfections were employed for spinoculation as previously described (30). Selection in 8 μg/mL blasticidin S hydrochloride (Calbiochem, La Jolla, CA) was followed by immunoblot screening of clones. Transient cotransfections of K562 cells for luciferase reporter assays were done as previously described (11); experiments using trichostatin A (TSA) or 5-aza-2′-deoxycitidine (DCB; both from Calbiochem) added compounds or control solvents (DMSO or 5% acetic acid, respectively) just prior to transfection.

Immunoprecipitation and immunoblot. 293T cells, treated from 24 to 48 hours posttransfection with 200 nmol/L TSA, were extracted for 20 minutes in ice-cold 50 mmol/L Tris-HCl (pH 7.6), 350 mmol/L NaCl, 0.5% NP40, 0.5 mmol/L DTT, 1 mmol/L EDTA, 10 mmol/L sodium butyrate, and protease inhibitor cocktail (Roche, Mannheim, Germany). Extracts, adjusted to 150 mmol/L NaCl and precleared by centrifugation, were immunoprecipitated with rabbit polyclonal anti-acetyl lysine (Upstate Cell Signaling Solutions, Charlottesville, VA) overnight at 4°C. Immune complexes collected on protein G-agarose beads (Pierce, Rockford, IL) for 1 hour at 4°C were subsequently washed repeatedly in extraction buffer containing first 150 mmol/L NaCl then 300 mmol/L NaCl. Immune complexes underwent standard immunoblotting with monoclonal rat anti-GATA-1 (N6, Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-HA (Covance, Berkeley, CA), and polyclonal rabbit anti-runt homology domain (RHD, Oncogene Research Products, San Diego, CA). Immunoblotting of K562 cells for GATA-1 (N6) and tubulin employed whole cell lysates of transiently transfected cells as previously described (11).

Flow cytometry, benzidine, and luciferase assays. For flow cytometry of transfected human $CD34^+$ cells, standard staining was done using combinations of APC-anti-CD34 plus PE-anti-CD36 or APC-anti-GPA plus PE-anti-CD13 (BD PharMingen, San Diego, CA). Negative controls for setting of gates included isotype-matched antibody conjugates and nontransfected GFP^- cells. For all experiments, GFP^+ populations were analyzed on a FACSCalibur instrument (Becton Dickinson, San Jose, CA) using FlowJo software (Treestar, Inc., Ashland, OR) as previously described (30). Benzidine staining for hemoglobin was done as described (30) on K562 clones induced 4 days with 60 μmol/L hemin (BioChemika Fluka, Switzerland) plus 0.5 ng/mL transforming growth factor-β (R&D Systems, Minneapolis, MN). Luciferase assays for reporter gene activity and β-galactosidase assays for normalization of transfection efficiency were done as previously described (11).

Results

AE inhibits primary erythroid lineage commitment. Erythroid lineage commitment occurs rapidly during *ex vivo* culture of primary human $CD34^+$ progenitor cells, making standard retroviral transduction suboptimal for analysis of this process (31). We have adopted an approach employing minimal prestimulation followed by nucleofection to permit efficient transgene expression (20-50% of cells) within 2 days of initiating cultures. In Fig. 1*A* (*bottom*), a time course of control vector–transfected cells is shown at 24, 48, and 72 hours post-nucleofection. As previously described, the $CD34^+$ $CD36^-$ compartment encompasses primitive multipotential progenitors; the $CD34^+$ $CD36^+$ compartment comprises the erythroid-restricted progenitors, BFU-E and early CFU-E; and the $CD34^-$ $CD36^+$ compartment contains late CFU-E progenitors as well as proerythroblasts and erythroblasts (31). Figure 1*A* shows the orderly progression of vector-transfected cells through these stages over the course of 24 to 72 hours in erythroid medium. By contrast, AE-transfected cells (Fig. 1*A, top*) showed minimal developmental progression, with retention of the majority of the cells in the $CD34^+$ $CD36^-$ compartment. Costaining of cells for Annexin V showed only a minor increase in apoptosis associated with AE expression, insufficient to account for the erythroid inhibition (data not shown). Thus, these results suggest that AE blocks erythroid differentiation at an early stage, prior to evidence of lineage commitment.

One possibility is that AE specifically affects CD34 and CD36 expression while preserving other aspects of erythroid differentiation. We have recently found that early erythroid differentiation

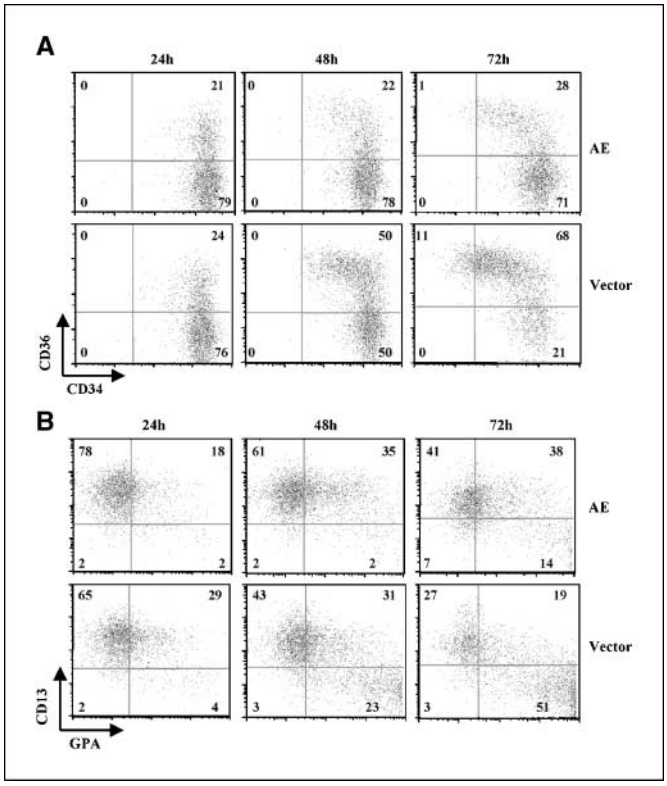

Figure 1. AML1-ETO inhibition of the earliest identifiable steps of human erythroid lineage commitment. *A* and *B,* primary human CD34$^+$ hematopoietic progenitors were transfected with either pCMV5 (*Vector*) or pCMV-AML1-ETO (*AE*) followed by culture in erythroid differentiation medium for the indicated intervals. All transfections included pEYFP-C1 for gating on transfectants, and results are shown for the gated EYFP$^+$ population. Similar results were obtained in three independent experiments. *A,* flow cytometric analysis for expression of CD34 and CD36. *B,* flow cytometric analysis for GPA and CD13.

includes tightly coordinated down-regulation of CD13, dimly expressed on multilineage progenitors, and up-regulation of glycophorin A (GPA), whose intensity correlates with erythroid maturation. Figure 1*B* (*bottom*) illustrates the continuous transition of vector-transfected cells over 24 to 72 hours from a CD13^{dim} GPA$^-$ phenotype to a CD13$^-$ GPA^{bright} phenotype. AE-transfected cells showed minimal down-regulation of CD13 as well as defective up-regulation of GPA. These results confirm a comprehensive disruption of early erythroid differentiation by AE.

The critical role of NHR4 Znf for erythroid inhibition by AE. To assess the structural requirements for AE inhibition of erythroid differentiation, several deletion mutants were initially analyzed. Unfortunately, many of the deletion mutants showed poor expression relative to intact AE and could not be rigorously evaluated for their repressive capacity.[1] Using stably transfected or transduced K562 cells, intact AE could be compared with two mutants, as well as with wild-type RUNX1, all of which showed equivalent or greater expression than AE (Fig. 2*A*). The ΔZnf mutant consists of an isolated NHR4 deletion, and the R174Q mutant converts a key arginine within the Runt domain of AE, thereby eliminating DNA binding (32). K562 clones expressing intact AE showed the expected impairment in erythroid induction (Fig. 2*A*). By contrast, expression of the ΔZnf mutant failed to block erythroid differentiation, possibly even enhancing the percentage of hemoglobin-expressing (benzidine-positive) cells. Clones expressing the AE R174Q mutant manifested a block in erythroid differentiation equivalent to or greater than the block associated with intact AE expression (Fig. 2*A*). Thus, target gene regulation by AE seems to be dispensable for mediating erythroid blockade. In separate experiments, K562 cells transduced with the MIG retroviral vector or a

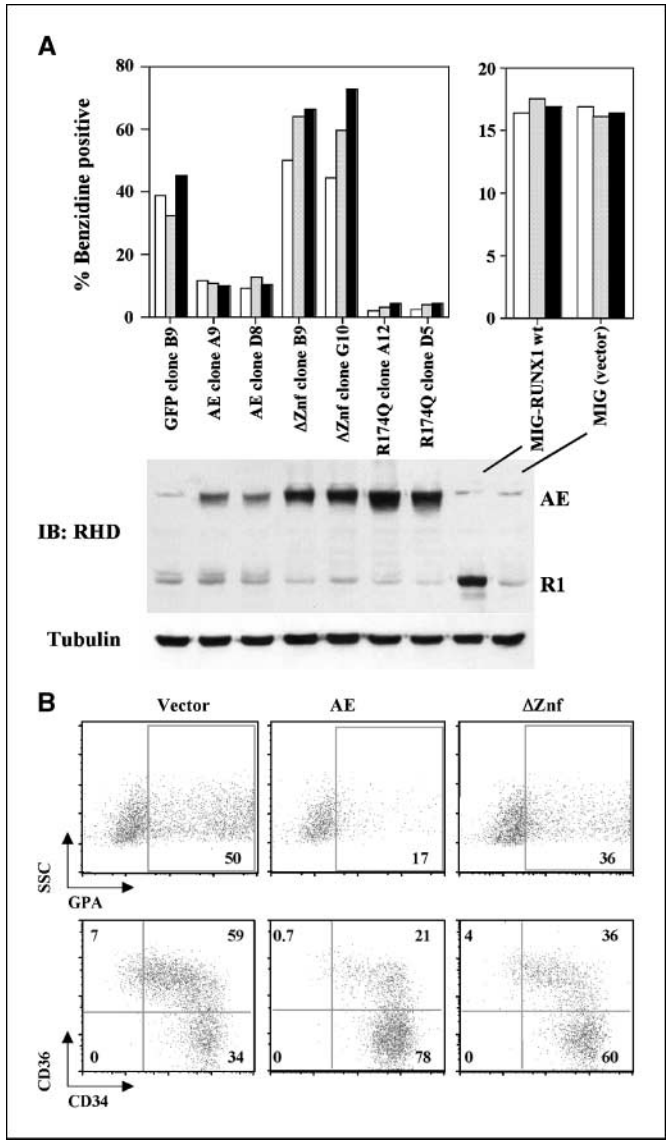

Figure 2. Requirement of the NHR4 zinc finger domain for AE inhibition of erythroid differentiation. *A,* effect of stably transfected AML1-ETO on erythroid differentiation of human erythroleukemic cells. K562 clones expressing full-length or mutant forms of AML1-ETO were induced to undergo erythroid differentiation followed by benzidine staining to detect the percentage of cells expressing hemoglobin. *Right,* K562 cells transduced with parent vector (*MIG*) or RUNX1 retrovirus and sorted for GFP expression underwent erythroid induction followed by benzidine staining. Results are shown for three separate experiments. Whole cell lysates underwent immunoblotting with an antibody recognizing the runt homology domain (*RHD*) found in RUNX1 (*R1*) and AML1-ETO (*AE*). *B,* involvement of the NHR4 zinc finger in inhibition of primary erythroid lineage commitment by AML1-ETO. Primary human CD34$^+$ hematopoietic progenitors were transfected with either CMV5 (*Vector*), pCMV-AML1-ETO (*AE*), or pCMV-AML1-ETO ΔZnf (*ΔZnf*) followed by culture in erythroid medium for 72 hours. Flow cytometric analysis of EYFP$^+$ transfectants was done as in Fig. 1. Similar results were obtained in three independent experiments.

[1] Unpublished data.

MIG-RUNX1 retrovirus were purified by sorting for GFP$^+$ cells, as described (11). Despite strong expression, wild-type RUNX1 caused no inhibition of erythroid differentiation, as compared with vector-transduced control cells (Fig. 2*A*).

The importance of the NHR4 Znf in erythroid inhibition was confirmed in primary human CD34$^+$ progenitor cells using the system described in Fig. 1. In this transient expression system, only the ΔZnf mutant showed expression equivalent to intact AE.[1] Whereas intact AE almost completely blocked erythroid commitment and differentiation, cells expressing ΔZnf retained GPA up-regulation, CD36 up-regulation, and CD34 down-regulation (Fig. 2*B*). It should be noted that the erythroid differentiation of ΔZnf-expressing cells was slightly impaired compared with vector controls, suggesting that in primary cells, domains other than NHR4 may also contribute to the inhibition. Similar results were obtained in three independent experiments.

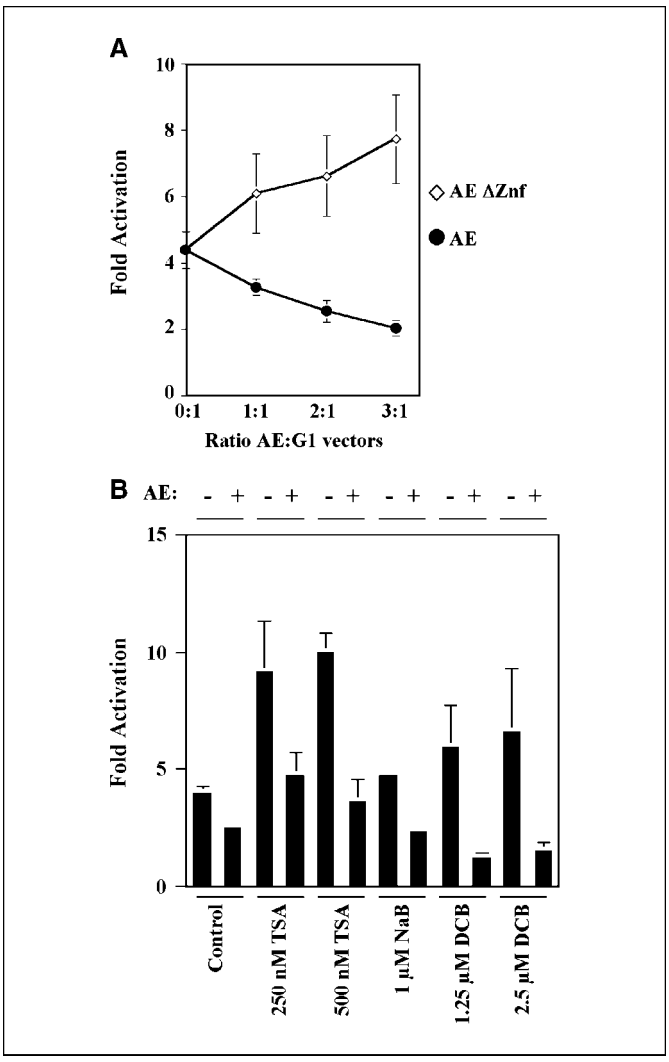

Figure 3. AML1-ETO repression of GATA-1 is dependent on the NHR4 Znf but is independent of HDAC and DNMT. *A,* dose-response curves of GATA-1 repression by intact AML1-ETO (*AE*) and by the zinc finger deletion mutant (*AE ΔZnf*). K562 cells were transiently cotransfected with expression vectors for GATA-1 plus either AE or AE ΔZnf; included in transfections were the GATA-1-responsive Luciferase reporter plasmid (αIIb-598-Luc), and a β-galactosidase expression vector (pCMV-β-gal) for normalization. Results depict fold activation of luciferase activity relative to that obtained with reporter plasmid alone. *Points,* average of three independent experiments; *bars,* ±SE. *B,* HDAC and DNMT inhibitors fail to reverse AE repression of GATA-1. Cells transfected with GATA-1 expression vector ± pCMV-AML1-ETO (*AE:* − +) were treated with the indicated doses of the HDAC inhibitor TSA, another HDAC inhibitor sodium butyrate (NaB), or the DNMT inhibitor, DCB. Transfections and luciferase assays were carried out as in (*A*).

AE repression of the erythroid transcription factor GATA-1 by a nonconventional pathway. In previous work, we showed the physical and functional interaction of GATA-1 with the RUNX1 runt domain (11). Because of its important role in programming erythroid differentiation and because of its ability to bind AE, GATA-1 represented an appealing target for AE inhibition of erythroid differentiation. Consistent with results in Fig. 2, the NHR4 zinc finger was necessary for AE repression of GATA-1 in standard luciferase reporter assays (ref. 11; Fig. 3*A*). It should be noted, however, that 2-fold variation in transient transfection experiments may represent a marginal difference due to potential interexperimental variations.

To test whether AE could repress GATA-1 transcriptional function in a classic HDAC-dependent manner, the effects of the HDAC inhibitor TSA were examined. Up to 500 nmol/L of TSA failed to diminish the fold-repression of GATA-1 function by AE, despite augmenting basal GATA-1 transcriptional activity by 2- to 3-fold (Fig. 3*B*). Recent data have identified an additional pathway for AE repression involving the recruitment of DNMT1 followed by promoter DNA methylation (22). A feature of this pathway comprises reversibility by the DNMT inhibitor decitabine (DCB), with 2.5 μmol/L of DCB releasing the interleukin-3 gene promoter from AE repression in Kasumi cells (33). This same dosage of DCB actually enhanced the fold repression of GATA-1 by AE, as well as enhancing basal GATA-1 activity (Fig. 3*B*). Therefore, AE repression of GATA-1 occurs by a novel mechanism independent of previously implicated HDACs or DNMT.

Enforced coactivator docking on GATA-1 overrides AE repression. One established mechanism for GATA factor repression consists of interference with regulation by coactivators (34, 35). To address whether AE might also interfere with GATA-1 regulation by coactivators, we engineered a novel GATA-1 mutant. In particular, the p300/CBP-binding module of the adenovirus E1A protein (amino acids 1-89) underwent fusion to full-length GATA-1 to create the EG fusion protein (Fig. 4*A*). EG was expressed at similar levels to GATA-1 in transient transfections (Fig. 4*B*) and retained the ability to activate GATA-dependent reporter plasmids at levels similar to wild-type GATA-1 (Fig. 4*C*). In contrast to wild-type GATA-1, EG showed virtually complete resistance to the repressive effects of AE. These results suggest that AE might interfere with the proper regulation of GATA-1 function by coactivators and that providing an artificial coactivator docking site could override this interference.

AE inhibits p300 acetylation of GATA-1. p300/CBP-mediated acetylation of GATA-1 is necessary for GATA-1 function in reporter plasmid activation and programming of erythroid differentiation (25, 26). Therefore, the effects of AE on GATA-1 acetylation were directly examined. 293T cells variably cotransfected with expression vectors for GATA-1, p300, and AE were analyzed for GATA-1 acetylation by immunoprecipitation with an antibody to acetyl-lysine (AcK), followed by immunoblotting for GATA-1 (Fig. 5*A*). GATA-1 expressed without p300 showed weak acetylation which was abrogated by AE. GATA-1 coexpressed with p300 showed enhanced acetylation which was also abrogated by AE coexpression. AE did not nonspecifically repress acetylation in that p300 autoacetylation remained unaffected by AE coexpression (immunoprecipitation, AcK; immunoblotting, HA).

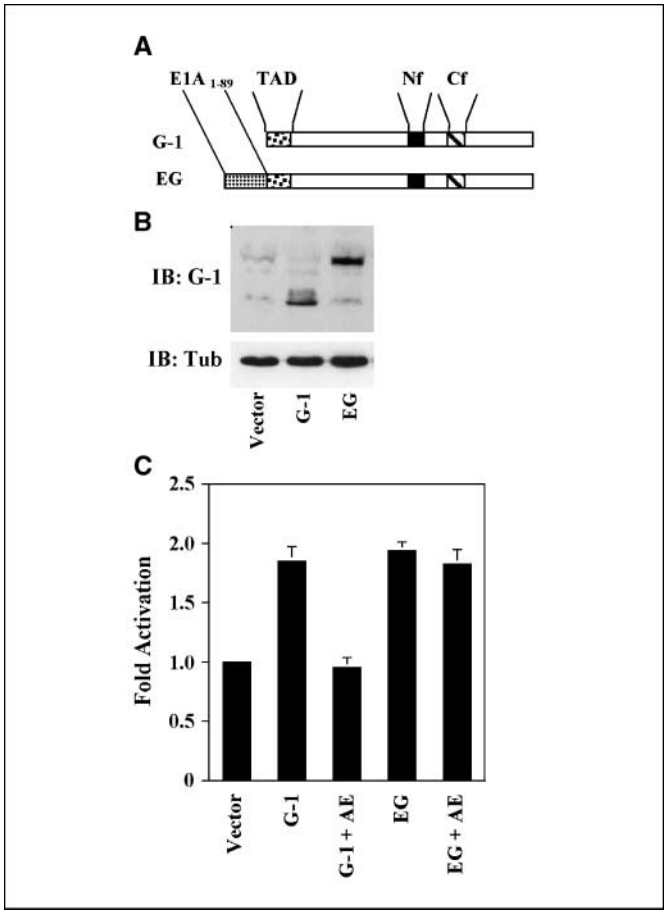

Figure 4. Enforced coactivator docking on GATA-1 prevents AML1-ETO repression. *A,* diagrams of wild-type GATA-1 (*G-1*) and E1A-GATA-1 fusion (*EG*), showing the GATA-1 transcriptional activation domain (*TAD*), amino-terminal zinc finger (*Nf*), carboxyl-terminal zinc finger (*Cf*), and heterologous coactivator docking module (E1A amino acids 1-89). *B,* immunoblot of transiently transfected K562 cells. *C,* comparison of wild-type GATA-1 (*G-1*) and E1A-GATA-1 fusion (*EG*) for repression by AML1-ETO (*AE*). Transfections and luciferase assays were conducted as in Fig. 3.

Further evidence against nonspecific inhibition of acetyltransferase activity by AE derived from experiments employing the EG fusion (Fig. 5*B*). Coexpression with p300 caused robust EG acetylation, which was minimally inhibited by AE expression. This finding correlates with the functional data in which EG transcriptional activity showed minimal repression by AE (Fig. 4*C*). Direct immunoblotting of the cellular extracts used for immunoprecipitation showed no effect of AE on overall stability of wild-type GATA-1 or EG ("Input" Fig. 5*A* and *B*). Because the NHR4 Znf domain played a critical role in inhibiting erythroid differentiation and GATA-1 function, its role in the inhibition of GATA-1 acetylation was also examined. In contrast to the potent inhibition of GATA-1 acetylation by full-length AE, the ΔZnf deletion mutant failed to inhibit GATA-1 acetylation by p300 (Fig. 5*C*). Wild-type RUNX1, which fails to inhibit erythroid differentiation (Fig. 2*A*) or GATA-1 function (11), also did not inhibit GATA-1 acetylation by p300 (Fig. 5*C*).

Enforced coactivator docking on GATA-1 overrides AE inhibition of primary erythroid differentiation. To determine whether the primary erythroid inhibition illustrated in Figs. 1 and 2*B* related to the ability of AE to repress GATA-1, we assessed GATA-1 or EG for reversal of the effects of AE on human $CD34^+$ progenitor cells (Fig. 6). Coexpression of wild-type GATA-1 caused no reversal of AE inhibition of erythroid differentiation. By contrast, coexpression of EG clearly reversed the inhibitory effects of AE. Although this reversal was not complete, cells cotransfected

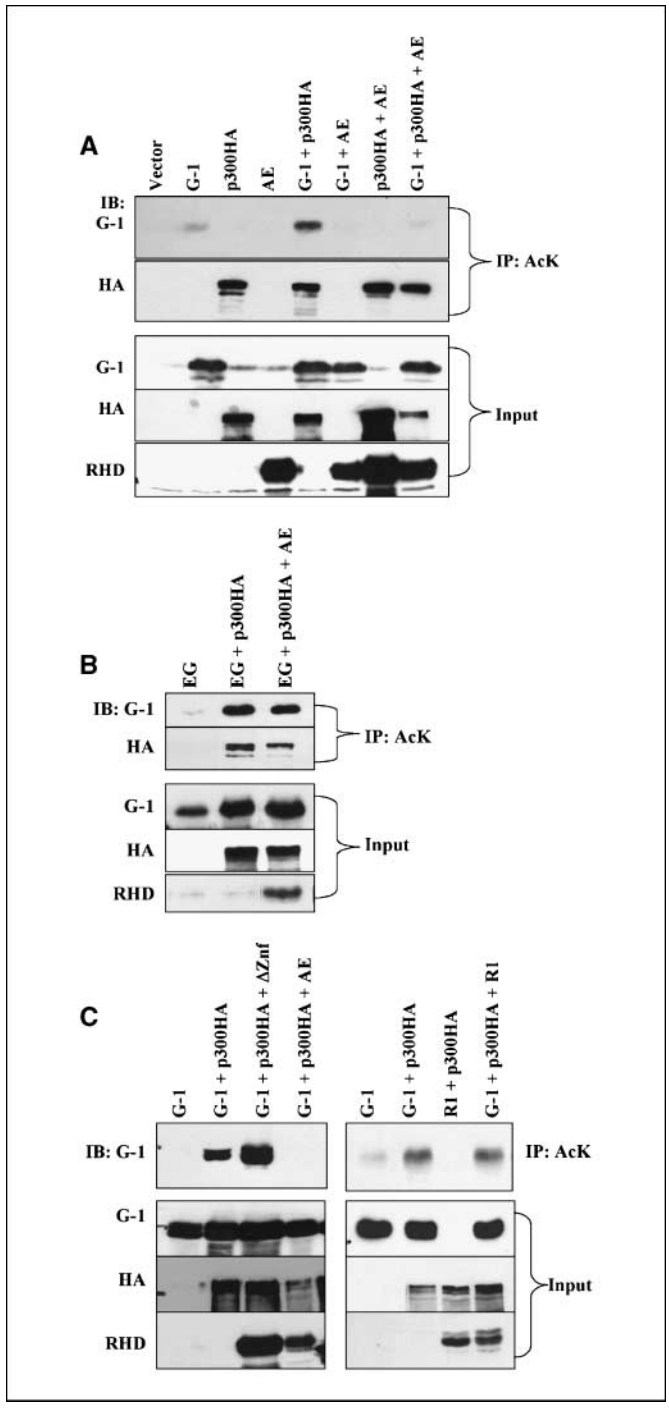

Figure 5. AML1-ETO blocks the acetylation of GATA-1 by p300. *A,* acetylation of GATA-1 coexpressed with p300 and AML1-ETO. 293T cells transfected with the indicated expression constructs underwent immunoprecipitation with rabbit anti-acetyl-lysine followed by immunoblotting for GATA-1 (*G-1*) and HA-tagged p300 (*HA*). Input extracts were immunoblotted for GATA-1, p300HA, and AML1-ETO (*RHD*). *B,* acetylation of E1A-GATA-1 (*EG*) coexpressed with p300 and AML1-ETO. Transfections, immunoprecipitations, and immunoblotting were conducted as in (*A*). *C,* acetylation of GATA-1 coexpressed with p300 and either AML1-ETO ΔZnf (*ΔZnf*), full-length AML1-ETO (*AE*), or RUNX1 (*R1*). Assays were done as in (*A*).

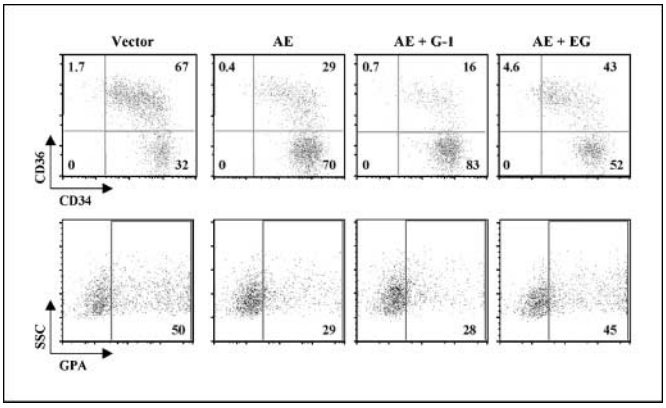

Figure 6. Reversal of AML1-ETO inhibition of primary erythroid lineage commitment by enforced coactivator docking on GATA-1. Primary human $CD34^+$ hematopoietic progenitors were transfected as indicated, cultured in erythroid medium for 48 hours, and analyzed by flow cytometry as in Fig. 1. *AE,* AML1-ETO; *G-1,* wild-type GATA-1; *EG,* E1A-GATA-1 fusion depicted in Fig. 4*A*. Similar results occurred in three separate experiments.

with AE + EG expression vectors showed similar percentages of cells in the most mature compartments ($CD34^-CD36^+$ and GPA^{bright}), as compared with control vector transfectants. Similar results were obtained in three independent experiments.

Discussion

Erythroid inhibition by AE occurs in human AML cases with t(8;21) and in mouse model systems expressing AE in the HSC compartment (2, 3, 6, 7). These murine systems recapitulate the finding of AE expression in HSC in human clinical samples (4, 5). Previous experiments with retrovirally transduced primary human progenitor cells have shown AE expression to promote the proliferation of early myeloid progenitors (36) and to impair erythroid colony formation as well as erythropoietin-independent erythroid maturation in suspension culture (10). Using an approach that provides rapid transgene expression in uncommitted cells, we find that AE clearly inhibits the earliest identifiable stages of erythroid lineage commitment in erythropoietin-containing medium, including the down-regulation of dim CD13 expression. Notably, the *RUNX1* promoter, which drives AE expression in leukemia cells, undergoes silencing shortly after erythroid lineage commitment (37). Therefore, the inhibitory effects of AE on erythropoiesis in patients with t(8;21)-positive AML most likely occur prior to lineage commitment or very early in erythroid differentiation.

AE exerts many of its leukemogenic effects through the direct repression of RUNX1 target genes, a mechanism recently supported in a *Drosophila* genetic model system (38). This direct repression occurs by at least two distinct biochemical pathways, histone deacetylation within target genes (39) and DNA methylation within target promoters/enhancers (22). Emerging evidence also supports indirect pathways through which AE can influence the expression of genes not normally regulated by RUNX1 (23). For example AE may block granulocytic differentiation through disruption of a protein complex containing ETO-2 and N-CoR, an effect dependent on the NHR4 zinc finger domain of AE (40). In addition, AE physically interacts with and silences E proteins, bHLH transcription factors previously implicated in cell cycle regulation (41). Furthermore, alterations in the subnuclear trafficking of AE, as compared with RUNX1, may contribute per se to the aberrant granulopoiesis seen in t(8;21) AML (21, 42).

Several findings support an indirect mechanism for AE inhibition of erythroid differentiation, with GATA-1 as a critical target. First, the NHR4 deletion mutant, ΔZnf, showed loss of function both for GATA-1 repression and for erythroid inhibition. Second, the R174Q AE mutant lacking DNA binding retained the capacity for erythroid repression. We have previously found that RUNX1 R174Q retains GATA-1 binding in coimmunoprecipitation experiments.[2] Third, fusion of a heterologous p300/CBP docking module to GATA-1 conferred resistance to AE and permitted reversal of AE inhibition of erythroid lineage commitment. However, repression of GATA-1 alone may not suffice for this effect because knockout mice have shown erythroid lineage commitment in the absence of GATA-1, most likely driven by up-regulated GATA-2 (43). It is thus possible that AE could target both GATA-2 as well as GATA-1 in precommitted progenitor cells.

The mechanism for AE repression of GATA-1 involves blockade of GATA-1 acetylation. The acetylation of two lysine clusters flanking the carboxyl terminal GATA zinc finger is critical for GATA-1 transcriptional programming of erythroid development (25, 26). As with AE inhibition of erythropoiesis and GATA-1 transactivation, the NHR4 zinc finger domain was also required for AE inhibition of GATA-1 acetylation. Our results suggest a prevention of GATA-1 acetylation rather than an induction of GATA-1 deacetylation, due to lack of reversal with HDAC inhibitors. Wild-type RUNX1 has also been found to possess leukemogenic properties when overexpressed (44), possibly due to its ability to recruit corepressors such as SUV39H1 and mSin3a (45, 46). Our results, however, showed no inhibition by wild-type RUNX1 of erythroid differentiation or of GATA-1 acetylation.

AE induces multiple transcriptional programs that could potentially interfere with GATA-1 function, including activation of the Notch and Wnt pathways (47, 48). We and others have recently shown that the downstream targets of Notch signaling, hairy-enhancer-of-split factors, directly bind and repress GATA factors (30, 35, 49). This repression seems to occur through interference with p300/CBP regulation of GATA factors (35). Therefore, AE blockade of GATA-1 function and erythroid lineage commitment could be indirectly mediated by Notch signaling. Several findings, however, argue against such a mechanism. First, AE R174Q lacks the capacity to regulate target genes, a function likely necessary for the activation of Notch (47), but retained repression of erythroid differentiation. Second, coexpression of a Notch pathway inhibitor, the dominant-negative mastermind mutant (50), failed to affect AE repression of GATA-1.[2] Third, treatment of cells with a pharmacologic γ-secretase inhibitor (GSI XVII, Calbiochem), which prevents Notch activation, also failed to affect AE repression of GATA-1.[2] Thus, AE most likely acts through a novel, NHR4 zinc finger–dependent mechanism involving direct interference with GATA-1 acetylation by the p300/CBP coactivating complex.

Acknowledgments

Received 8/18/2005; revised 12/13/2005; accepted 12/22/2005.

Grant support: NIH grants CA100057 and CA93735 (A.N. Goldfarb).

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We thank Dr. Shelly Heimfeld for provision of cells; Drs. Scott Hiebert, Eileen White, and David Livingston for plasmids; Dr. Gerd Blobel and Joanne Lannigan for advice on acetylation assays and flow cytometry, respectively; and Dr. Ivailo Mihaylov for helpful comments on the manuscript.

[2] K.E. Elagib, unpublished data.

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