Expression of the Transcriptional Repressor Gfi-1 Is Regulated by C/EBPα and Is Involved in Its Proliferation and Colony Formation–Inhibitory Effects in p210BCR/ABL-Expressing Cells

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Abstract

Ectopic expression of CAAT/enhancer binding protein α (C/EBPα) in p210BCR/ABL-expressing cells induces granulocytic differentiation, inhibits proliferation, and suppresses leukemogenesis. To dissect the molecular mechanisms underlying these biological effects, C/EBPα-regulated genes were identified by microarray analysis in 32D-p210BCR/ABL cells. One of the genes whose expression was activated by C/EBPα in a DNA binding–dependent manner in BCR/ABL-expressing cells is the transcriptional repressor Gfi-1. We show here that C/EBPα interacts with a functional C/EBP binding site in the Gfi-1 5′-flanking region and enhances the promoter activity of Gfi-1. Moreover, in K562 cells, RNA interference–mediated downregulation of Gfi-1 expression partially rescued the proliferation-inhibitory but not the differentiation-inducing effect of C/EBPα. Ectopic expression of wild-type Gfi-1, but not of a transcriptional repressor mutant (Gfi-1P2A), inhibited proliferation and markedly suppressed colony formation but did not induce granulocytic differentiation of BCR/ABL-expressing cells. By contrast, Gfi-1 short hairpin RNA–transduced CD34+ chronic myeloid leukemia cells were markedly more clonogenic than the scramble-transduced counterpart. Together, these studies indicate that Gfi-1 is a direct target of C/EBPα required for its proliferation and survival-inhibitory effects in BCR/ABL-expressing cells. Cancer Res; 70(20); 7949–59. ©2010 AACR.

Introduction

The transcription factor CAAT/enhancer binding protein α (C/EBPα) plays an essential role in regulating the balance between differentiation and proliferation during the early stages of myelopoiesis (1, 2).

In many types of myeloid leukemia, C/EBPα is mutated with reduced activity or its expression is lowered (3, 4), suggesting that decreased C/EBPα expression/activity is important for leukemogenesis.

Several mechanisms have been implicated in the inactivation of C/EBPα in myeloid leukemia. Mutations in the NH2 and COOH terminus reduce the functional levels of C/EBPα and have the potential to generate mutant proteins with dominant-negative activity (5, 6); these mutant proteins promote the development of acute leukemia when expressed from the C/EBPα gene locus (7, 8).

C/EBPα expression/activity is also inhibited by transcriptional, posttranscriptional, and posttranslational mechanisms (9–12). In myeloid cells transformed by the p210BCR/ABL oncoprotein, expression of C/EBPα is repressed at the translational level by mitogen-activated protein kinase–dependent phosphorylation and stabilization of the RNA binding protein hnRNPE2, which binds the 5′-untranslated region of C/EBPα mRNA and inhibits its translation (13, 14).

Regardless of the mechanisms responsible for C/EBPα loss of function, ectopic expression of C/EBPα in myeloid leukemia lines and in primary blast cells induces differentiation and inhibits proliferation (9, 13, 15, 16), further emphasizing the importance of genetic and/or functional inactivation of C/EBPα for leukemogenesis and the therapeutic potential of restoring expression of functional C/EBPα in leukemic cells.
Mechanistically, it is unclear how ectopic expression/activation of C/EBPα exerts its antileukemic effects in p210BCR/ABL-expressing cells; although the antiproliferative effects of C/EBPα depend, in part, on interaction with cell cycle-regulatory and chromatin-remodeling proteins (17–20), granulocytic differentiation is only induced by DNA binding and transcription activation–competent proteins in vitro and in leukemic mice (16, 21). Consistent with this, leukemogenesis is suppressed more potently by transcription activation–competent C/EBPα than by a DNA binding–deficient mutant (16).

However, it is unclear whether transcription-regulated C/EBPα targets may be, in part, responsible for its effects on cell proliferation and survival. We searched for transcriptionally regulated, biologically relevant targets of C/EBPα by probing oligonucleotide microarrays with RNA isolated at early time points after activation of wild-type or DNA binding–deficient C/EBPα in 32D-p210BCR/ABL cells. One of the genes whose expression was activated by C/EBPα in a DNA binding–dependent manner is the transcriptional repressor Gfi-1, which is important for maintenance of hematopoietic stem cells and for differentiation of late granulocytic progenitors (22–26). Moreover, transformation of hematopoietic stem cells engineered to express a DNA binding–deficient C/EBPα mutant is associated with reduced expression of Gfi-1 (8), raising the possibility that low levels of Gfi-1 are also expressed in acute myeloid leukemia (AML), with C/EBPα mutations, potentially reducing the “quiescence” of these AML stem cells.

We show here that Gfi-1 is a direct C/EBPα target and that its expression is required for C/EBPα-dependent inhibition of proliferation but not induction of differentiation in K562 cells. Consistent with these findings, expression of wild-type Gfi-1 inhibited proliferation and colony formation of BCR/ABL-expressing cell lines and primary chronic myelogenous leukemia (CML) cells, whereas Gfi-1 short hairpin RNA (shRNA)–transduced CD34+ CML cells were markedly more clonogenic of the scramble-transduced counterpart.

### Materials and Methods

#### Plasmids

p42C/EBPα-ER and K298E C/EBPα-ER, cloned in the Xhol-EcoRI–digested MigRI vector, were previously described (16).

MigRI-Gfi-1 and MigRI-Gfi-1 P2A were the kind gift of Dr. Zhu (Laboratory of Immunology, NIH).

MigRI-Gfi-1-ER was generated by PCR as follows: The MigRI-Gfi-1 and MigRI-Gfi-1 P2A were the kind gift of Dr. Zhu (University of Chicago, Chicago, IL). MigRI-Gfi-1 P2APGK-GFP.WPRE were generated by cloning Gfi-1 (PCR amplified from the wild-type or mutant MigRI-Gfi-1 plasmids) at the Xhol-EcoRV restriction sites upstream of the PGK-GFP cassette. The proximal promoter of the mouse Gfi-1 gene (−500 to +150) cloned into the pGL3-basic vector was the kind gift of Dr. P. Laslo (University of Chicago, Chicago, IL). pGL3-Gfi-1 (−500 to +150) with two substitutions (TCTGAAGCAA into TCTGAAACAAG) of the three more conserved nucleotides of the putative C/EBPα binding site (C/EBP-MUT pGL3 Gfi-1) was generated by site-directed mutagenesis from wild-type Gfi-1 (−500 to +150) pGL3 plasmid.

#### Cell cultures and viral infection

32D-p210BCR/ABL and derivative cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L l-glutamine, and penicillin/streptomycin (100 μg/mL each). K562 cells and derivative cell lines were cultured in IMDM supplemented with heat-inactivated FBS. The 32D-p210BCR/ABL-C/EBPα-ER and the K562-C/EBPα-ER lines were established in our laboratory and used in previously published studies (16, 27). The other derivative cell lines were established in the laboratory for this study. The identity of each cell line was authenticated by expression of p210BCR/ABL and C/EBPα-ER by sequence analysis. Fresh leukapheresis or peripheral blood samples were obtained with written informed consent from patients with CML in chronic phase (CP; n = 5) or blast crisis (BC; n = 1). Samples were enriched for CD34+ cells using CliniMACS (Miltenyi Biotec) according to the manufacturer’s instructions. Purity of CML CD34+ cells was >95%, and >95% were Ph+ as assessed by dual-color, dual-fusion fluorescence in situ hybridization.

Peripheral blood [granulocyte colony-stimulating factor (G-CSF) mobilized] normal CD34+ cells were purchased from StemCell Technologies. Normal and CML CD34+ cells were cultured in StemSpan SFEM medium (StemCell Technologies) supplemented with StemSpan CC100 [20 ng/mL interleukin-3 (IL-3) and IL-6, 100 ng/mL Kit ligand, 100 ng/mL Flt-3 ligand; StemCell Technologies] and 50 ng/mL thrombopoietin (StemCell Technologies).

For retroviral infections, Phoenix cells (kind gift of G.P. Nolan, Stanford University School of Medicine, Stanford, CA) were transiently transfected with the indicated plasmids. The infectious supernatant was collected 48 hours later and used to infect (a 48-hour procedure) p210BCR/ABL-expressing cells. Twenty-four hours later, infected cells were sorted (EPICS Profile Analyzer; Coulter) for green fluorescent protein (GFP) expression.

Lentiviral stocks were prepared by transient cotransfection of 293T cells with the transfer vector; the pCMVΔR8.74 encoding Gag, Pol, Tat, and Rev; the pMD.G plasmid encoding VSV-G; and Profection Mammalian Transfection according to Promega guidelines. The infectious supernatant was
collected 48 hours later and used to infect (a 48-hour procedure) p210BCR/ABL-expressing cells. Infected cells were selected by GFP positivity or puromycin resistance. CD34+ CML cells were transduced with the scramble or the Gfi-1 shRNA lentivirus (TRCN000020468, Open Biosystems) after testing the titer of the infectious supernatants by assaying puromycin-resistant colony formation of K562 cells. Lentivirally transduced CD34+ CML cells were assayed for colony formation in puromycin (3 μmol/L)—supplemented methylcellulose plates.

**Cell proliferation, colony formation, and differentiation assays**

For proliferation (counts of viable cells) and colony formation assays, GFP-positive Gfi-1–transduced p210BCR/ABL cells were washed with PBS and seeded at a cell density of 10^5/mL or plated in methylcellulose (250-μL). ABL cells were washed with PBS and seeded at a cell density of 10^6/mL were mixed with 2 μg of lentivirus human Gfi-1 shRNAs (TRCN0000020466, Open Biosystems).

The solution was added to Amaxa electrode cuvettes and electroporated in an Amaxa Nucleofector II using program T-16 for K562 cells.

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP Assay kit (U.S. Biochemical Corp.). Exponentially growing C/EBPα-ER-32D-p210BCR/ABL cells (4 × 10^7; untreated and 4-HT treated, 24 hours) were cross-linked with 1% formaldehyde, incubated for 30 minutes at 37°C, and treated with glycine (final concentration, 125 mmol/L; 5 minutes at room temperature). Cells were then washed with ice-cold PBS and resuspended in 1 mL of lysis buffer with a protease inhibitor cocktail and sonicated at 28% power for three pulses of 10 seconds each in a Branson Sonifier 450 (Branson Ultrasonics). Chromatin was precleared with 50 μL of protein A–agarose beads for 60 minutes at 4°C with rotation, and precleared lysates were immunoprecipitated with the anti-C/EBPα antibody (8 μg; Santa Cruz Biotechnology) at 4°C overnight with rotation. Immunoprecipitations without antibody (no-antibody control) or an anti-rabbit IgG (8 μg; Thermo Scientific Pierce) were included with each experiment. Immune complexes were collected with 50 μL of protein A–agarose beads for 60 minutes at 4°C with rotation (except for 10 μL of supernatant of the no-antibody control saved as input) and washed once with 1 mL of each of low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.0), and 150 mmol/L NaCl], high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.0), and 1.5 mol/L NaCl], and LiCl wash buffer [250 mmol/L LiCl, 1% NP40, 1% sodium deoxycholate, 1 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 8.0)] and twice with 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA. Immune complexes were next eluted using freshly prepared elution buffer (1% SDS and 0.1 mol/L NaHCO_3). Cross-links were reversed by heating at 65°C overnight in the presence of 0.2 mol/L NaCl. DNA was recovered using PrepEase DNA Clean-Up kit (U.S. Biochemical). C/EBPα DNA (2 μL) was used as a template for real-time quantitative PCR using the Gfi-1 promoter (sense, 5′-GGGACTTTAGTTTCTGAGACG-3′; antisense, 5′-GGTGGCTCAGGGACTATGTG-3′, from −259 to −58) and G-CSFR promoter (sense, 5′-TAGCTACCGATTTCCCTCA-3′; antisense, 5′-GCAAGTTCCCCAAAATCTC-3′, from −174 to +8) primers.

** Luciferase reporter assays**

For luciferase assays, 293T cells were plated into six-well plates at 5 × 10^4 per well and cultured overnight. Cells were transfected the following day with 1.5 μg of Gfi-1 (−500 to +150) pGL3 or C/EBP-MUT Gfi-1 (−500 to +150) pGL3, 1/50 of Renilla reporter, and ΔOurf/CEBPα-HA (p24 C/EBPα) or K298C/C/EBPα-HA expression plasmid as required for a total of 6 μg/well DNA using the Profection Mammalian Transfection System (Promega). After 48 hours, cells were washed and lysed and levels of firefly and Renilla luciferase activities were measured using a dual-luciferase assay kit (Promega). All
transfections were performed in triplicates in three independent experiments.

Statistical analyses
Means were compared using the unpaired two-tailed Student’s t test. A P value of <0.05 was considered statistically significant in all calculations.

Results
Activation of C/EBPα induces Gfi-1 expression in p210BCR/ABL-expressing cells
Ectopic expression of C/EBPα promotes granulocytic differentiation, inhibits proliferation, and suppresses leukemogenesis of p210BCR/ABL-expressing cells (13, 16).

For optimal C/EBPα-dependent suppression of leukemogenesis, proliferation inhibition and differentiation induction are both required, as the DNA binding–deficient K298E C/EBPα mutant, which fails to induce granulocytic differentiation (16, 21), is less effective of transcriptionally competent C/EBPα (16).

To identify potential mechanisms involved in the leukemia-suppressive effects of C/EBPα, microarray hybridization studies were performed using RNA from 4-HT–treated 32D-p210BCR/ABL cells transduced with MigRI C/EBPα-ER or MigRI K298E C/EBPα-ER. Total RNA was extracted at 12 hours after 4-HT treatment in three separate experiments and hybridized onto the Affymetrix MOE430_2 array, as described (27).

We identified several genes whose expression was down-regulated by wild-type C/EBPα but not by K298E C/EBPα, including the transcription factors c-Myb and GATA-2 (27). Instead, the transcription repressor Gfi-1 was among those whose mRNA levels were upregulated by C/EBPα in a DNA binding–dependent manner.

To confirm the microarray hybridization data, we assessed Gfi-1 mRNA levels in 4-HT–treated 32D-p210BCR/ABL cells expressing wild-type or DNA binding–deficient K298E C/EBPα-ER. Levels of Gfi-1 mRNA were upregulated as early as 6 hours after 4-HT treatment in cells expressing wild-type C/EBPα, but not in cells expressing the K298E mutant (Fig. 1A).

Figure 1. Expression of Gfi-1 is induced by C/EBPα in BCR/ABL-expressing cells. A, levels of Gfi-1 mRNA measured by real-time PCR in 4-HT–treated C/EBPα-ER-32D-p210BCR/ABL or K298E C/EBPα-ER-32D-BCR/ABL cells. Western blot shows expression of Gfi-1 in (B) 4-HT–treated wild-type C/EBPα-ER-32D-p210BCR/ABL or K298E C/EBPα-ER-32D-BCR/ABL cells and (C) 4-HT–treated C/EBPα-ER–transduced CD34+ CML cells.
Consistent with the changes in mRNA expression, Gfi-1 protein levels increased on activation of wild-type C/EBPα, but not of the K298E mutant (Fig. 1B). We also tested whether activation of C/EBPα induced Gfi-1 expression in primary CML cells. To this end, CD34+ cells from a CML-BC patient (M351T mutation) were retrovirally transduced with MigRI C/EBPα-ER, GFP sorted, and treated with 4-HT to activate C/EBPα. After 24 and 48 hours, lysates were prepared and used for Western blotting to assess Gfi-1 levels. Expression of Gfi-1 was induced on C/EBPα activation (Fig. 1C), consistent with the results in 32D-p210BCR/ABL cells.

The Gfi-1 promoter is activated by C/EBPα

To determine whether the effect of C/EBPα on Gfi-1 expression is due to a transcriptional mechanism, we first assessed expression of Gfi-1 pre-mRNA in nRNA of untreated and 4-HT–treated C/EBPα-ER-32D-BCR/ABL cells. As shown in Fig. 2A, levels of Gfi-1 pre-mRNA transcripts were increased approximately 3- to 5-fold on 4-HT treatment. We then performed luciferase assays in 293T cells cotransfected with wild-type or K298E C/EBPα expression plasmid and a Gfi-1 5′-flanking region (−500 to +150) luciferase plasmid (28), which contains a high- and a low-score putative C/EBP binding site based on search of transcription factor binding site databases (29). In three separate experiments, wild-type C/EBPα induced an ∼3-fold increase (P = 0.001) in Gfi-1–dependent luciferase activity (Fig. 2B, top), suggesting that Gfi-1 is a C/EBPα target.

To further investigate the role of C/EBPα in transcriptional activation of Gfi-1, we performed ChIP assays to determine
Figure 3. Expression of Gfi-1 is required for C/EBPα-dependent proliferation inhibition in K562 cells. A and E, Western blot shows Gfi-1 expression in 4-HT–treated scramble or Gfi-1 shRNA–transduced or Gfi-1 shRNA–transfected C/EBPα-ER/K562 cells. Histograms show proliferation of 4-HT–treated scramble (B and F) or Gfi-1 shRNA–transduced or Gfi-1 shRNA–transfected C/EBPα-ER-K562 cells (C and G) and methylcellulose colony formation of 4-HT–treated scramble or Gfi-1 shRNA–transduced or Gfi-1 shRNA–transfected C/EBPα-ER-K562 cells (D and H). Expression of Gfi-1 was detected by anti-Gfi-1 goat polyclonal antibody (N-20; Santa Cruz Biotechnology). For cell count assays, cells were seeded at 10^5/mL; for colony formation assays, 1,000 cells/plate were seeded. Data (expressed as % inhibition of 4-HT–treated versus untreated cells) are represented as the mean ± SE of two (B–D) or three (F–H) independent experiments. Asterisk denotes that the differences in cell counts between Gfi-1 and scramble shRNA K562 cells (compare C and G and B and F) are statistically significant (P = 0.001 and 0.002, respectively).
whether C/EBPα binds to the Gfi-1 promoter. Because expression of Gfi-1 is rapidly induced on C/EBPα activation in 32D-p210BCR/ABL cells, ChIP assays were performed in untreated 32D-p210BCR/ABL-C/EBPα-ER cells and in cells treated for 24 hours with 4-HT to activate C/EBPα; thereafter, the binding of C/EBPα to the Gfi-1 promoter was assessed by real-time quantitative PCR amplification of a segment of the Gfi-1 5′-flanking region, which includes the high-score putative C/EBP binding site (nucleotides −247 to −238, underlined in Fig. 2C, top).

As shown in Fig. 2C (bottom), activation of C/EBPα by 4-HT treatment led to its interaction with nucleotides −259 to −58 of the Gfi-1 promoter. As control, C/EBPα bound to a segment of the G-CSFR promoter (−174 to +8) containing a canonical C/EBP binding site (30).

To further assess the functionality of this C/EBP binding site at nucleotides −247 to −238 (Fig. 2C) of the Gfi-1 promoter, we generated a mutated (TCTGAAGCAA into TCTGAAACA) Gfi-1 (−500 to +150) luciferase plasmid and performed luciferase assays on cotransfection of wild-type or K298E C/EBPα and the mutated Gfi-1-Luc reporter plasmid in 293 cells; as shown in Fig. 2B (bottom), the mutated promoter was no longer transactivated by wild-type C/EBPα, confirming that the segment of the Gfi-1 5′-flanking region bound by C/EBPα in the ChIP assays contains a functional C/EBP binding site.

C/EBPα was also unable to transactivate a shorter Gfi-1 promoter (−41 to +150) lacking putative C/EBP binding sites (data not shown).

Expression of Gfi-1 is required for C/EBPα-dependent inhibition of proliferation and colony formation of K562 cells

To determine whether expression of Gfi-1 is required for the biological effects of C/EBPα in BCR/ABL-expressing cells, C/EBPα-ER K562 cells (27) were transduced or transfected with Gfi-1 shRNA lentiviruses, and levels of Gfi-1, numbers of viable cells, colony formation, and differentiation were assessed after 4-HT treatment (250 nmol/L) to activate C/EBPα.

In C/EBPα-ER K562 cells transduced with a scramble (a murine Gfi-1 shRNA with seven mismatches relative to the human sequence) or a Gfi-1 shRNA (TRCN0000020468), 4-HT treatment induced an increase in Gfi-1 expression in the scramble, but not in the Gfi-1 shRNA–transduced, cells (Fig. 3A).

Activation of C/EBPα markedly reduced the number of scramble-transduced K562 cells (Fig. 3B), whereas there was only a modest effect in Gfi-1 shRNA–transduced K562 cells (Fig. 3C); activation of C/EBPα in scramble-transduced K562 cells markedly suppressed colony formation (≈85% inhibition), whereas it was less effective (≈50% inhibition) in Gfi-1 shRNA–transduced cells (Fig. 3D).

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Comparable, although less strong, effects (probably reflecting residual expression of Gfi-1) were also observed on 4-HT treatment of C/EBPα-ER K562 cells transfected with a different Gfi-1 shRNA lentivirus (TRCN0000020466; Fig. 3D–H).

These cells were also used to assess the role of Gfi-1 expression in C/EBPα-dependent induction of granulocytic differentiation.
On 4-HT treatment, scramble shRNA–transfected K562 C/EBPα-ER cells showed morphologic features of granulocytic differentiation and increased levels of CD11b and CD15 (Fig. 4); in cells transfected with the Gfi-1 shRNA, treatment with 4-HT induced morphologic differentiation and an increase in CD11b and CD15 levels comparable with those in the control cells (Fig. 4), indicating that increased Gfi-1 expression is not required for C/EBP-dependent induction of differentiation in K562 cells.

**Effects of Gfi-1 in BCR/ABL-expressing cell lines**

To assess the effects of Gfi-1 in p210BCR/ABL-expressing cell lines, 32D-p210BCR/ABL and K562 cells were transduced with MigRI-Gfi-1 or MigRI-Gfi-1 P2A (a mutant deficient in constitutive Gfi-1 expression). Colony formation of BCR/ABL-expressing cells was assessed (Fig. 5). Colony formation was reduced in MigRI-Gfi-1–transduced cells compared with MigRI-Gfi-1 P2A–transduced cells, indicating that Gfi-1 expression inhibits colony formation in BCR/ABL-expressing cells.

**Figure 5.** Effect of constitutive Gfi-1 expression on colony formation of BCR/ABL-expressing cells. Colonies (triplicate plates) were counted 7 d after plating the indicated number of retrovirally transduced (GFP-positive) 32D-p210BCR/ABL (A) or K562 (B) cells. Representative of three experiments.

**Figure 6.** Effect of ectopic Gfi-1 expression on colony formation of CD34+ CML cells. Colony formation and Gfi-1 expression of (A) human CD34+ normal cells transduced with wild-type or P2A Gfi-1 lentivirus and (B) CD34+ cells from CML-CP samples transduced with wild-type or transcriptional repressor mutant Gfi-1 P2A lentivirus (experiments I and II) or retrovirus (experiment III). In experiment II, cells were also transduced with the empty vector lentivirus. Cells (5 × 10⁴ normal CD34+; 2.5 × 10⁴, 5 × 10⁴, or 1 × 10⁵ for CML CD34+, experiments I, II, and III, respectively) were plated after GFP sorting. Colonies were scored 12 d after plating.
transcriptional repression) and GFP-positive cells were used for cell counts of viable cells, colony formation, and differentiation assays.

Cells expressing wild-type Gfi-1 proliferated less than those expressing the Gfi-1 P2A mutant (data not shown). The effects of wild-type Gfi-1 were even more striking in clonogenic assays: in both cell lines, Gfi-1–expressing cells formed fewer colonies (~80% less) than cells expressing the Gfi-1 P2A mutant (Fig. 5A and B). However, ectopic Gfi-1 expression did not induce granulocytic differentiation, as 32D-p210BCR/ABL and K562 cells remained morphologically undifferentiated and did not express the differentiation marker Gr-1 (32D-p210BCR/ABL cells) or CD11b and CD15 (K562 cells; data not shown).

We also tested the effects of Gfi-1 after conditional activation of the chimeric Gfi-1-ER protein in BCR/ABL-expressing cells. To this end, 32D-p210BCR/ABL and K562 cells were transduced with the MigRI-Gfi-1-ER retrovirus, sorted for GFP expression, and treated with 4-HT to activate Gfi-1. Compared with untreated cells, 4-HT–treated cells showed reduced proliferation (Supplementary Fig. S1A and C) and were markedly less clonogenic when plated in methylcellulose (Supplementary Fig. S1B and D). Inhibition of colony formation on Gfi-1 activation was ~66% in 32D-BCR/ABL cells and 80% in K562 cells. 4-HT treatment of parental or MigRI-transduced K562 cells had no effect on cell numbers or colony formation (Supplementary Fig. S1E and F).

Modulation of Gfi-1 expression affects colony formation of CD34+ CML cells

The effect of ectopic Gfi-1 expression was also tested in normal and CML CD34+ cells using methylcellulose colony formation assays. Compared with cells expressing the P2A Gfi-1 mutant, expression of wild-type Gfi-1 in normal CD34+ cells induced a decrease in colony formation (Fig. 6A); the inhibitory effect of Gfi-1 was also noted in CML-CP CD34+ cells. In three separate experiments, cells transduced with wild-type Gfi-1 were markedly less clonogenic (approximately 65–75% inhibition) than those transduced with Gfi-1-P2A (Fig. 6B).

Colony formation of lentivirus-transduced CD34+ cells from CML sample 2-221 was similar to that of cells transduced with the Gfi-1-P2A mutant, suggesting that the inhibitory effect of wild-type Gfi-1 in CD34+ cells is not artificially enhanced as a result of stimulatory effects of the Gfi-1 P2A mutant reported for murine hematopoietic progenitors (31).

Hematopoietic stem cells from Gfi-1 knockout mice show reduced quiescence and enhanced self-renewal (22, 23), but it is unknown whether Gfi-1 expression is also required to restrict proliferation of normal or transformed human stem cells/early progenitors.

To address this issue, CD34+ cells from patients (n = 3) with CML-CP were transduced with stocks of a scramble or a Gfi-1 shRNA lentivirus (TRCN0000020468, capable of inducing optimal Gfi-1 downregulation in K562 cells; see Fig. 3A) pretested for comparable viral titers and plated in methylcellulose (5.0 × 10^5 to 2 × 10^5 cells/plate) in the presence of a cytokine cocktail and puromycin (3 μmol/L). Colonies were scored 7 to 10 d later. Histograms show number of colonies from Gfi-1- or scramble shRNA–transduced cells. Inset shows Gfi-1 expression (detected by Western blotting) in scramble- and Gfi-1 shRNA–transduced CD34+ CML cells. Cell lysates were obtained at the end of the lentiviral infection (before plating in puromycin-supplemented methylcellulose), possibly explaining the residual Gfi-1 expression in the Gfi-1 shRNA–transduced sample.
(Fig. 7). Methylcellulose plates seeded with $2 \times 10^5$ cells/plate were solubilized with PBS, and cells were recovered and counted. Viable cells from plates seeded with Gfi-1 shRNA–transduced CML cells were much more numerous than those from plates seeded with scramble-transduced cells ($360,000 \pm 60,000$ versus $35,000 \pm 25,000$).

Together, these findings support the important role of Gfi-1 expression in restricting the proliferation of early CML progenitors.

Discussion

To identify mechanisms responsible for the antileukemia effects of wild-type C/EBPα, we searched for biologically relevant transcription-regulated C/EBPα targets by probing oligonucleotide microarrays with RNA from 32D-p210BCR/ABL cells expressing the 4-HT–regulated wild-type C/EBPα or the K298E DNA binding–deficient mutant.

One of the genes whose expression was induced by C/EBPα in a DNA binding–dependent manner is the transcription repressor Gfi-1. This gene attracted our attention because of its reported involvement in maintenance of hematopoietic stem/progenitor cells and in terminal differentiation of myeloid precursors (22–26, 32).

Moreover, a functional relationship between C/EBPα and Gfi-1 is suggested by a recent study showing that murine hematopoietic stem cells engineered to express a DNA binding–deficient C/EBPα mutant exhibit reduced quiescence and, among several quiescence genes, express lower levels of Gfi-1 than the normal counterpart (8).

We show here that Gfi-1 is a direct transcriptional target of C/EBPα, and its expression is required for the inhibitory effects of C/EBPα on proliferation and colony formation of p210BCR/ABL-expressing cells but not for induction of granulocytic differentiation.

Consistent with these findings, ectopic expression of Gfi-1 also failed to induce granulocytic differentiation of 32D-p210BCR/ABL and K562 cells, whereas it was highly effective in inhibiting proliferation and suppressing colony formation of p210BCR/ABL cells, including CD34+ cells from CML patients.

These observations are apparently at odds with the notion that differentiation induction, not proliferation inhibition, depends on the activity of transcriptionally competent C/EBPα (16). Thus, the distinction in the biological effects of C/EBPα between transcription-dependent and transcription-independent mechanisms involved in induction of granulocytic differentiation and inhibition of proliferation, respectively, needs to be reassessed, as this study shows that transcription mechanisms play also an important role in C/EBPα-dependent proliferation inhibition.

The importance of Gfi-1 expression for controlling the proliferation and survival of BCR/ABL-expressing cells was not limited to cells conditionally expressing a functional C/EBPα; Gfi-1 shRNA–transduced CD34+ CML cells were markedly more clonogenic than the scramble-transduced counterpart, supporting the essential role of Gfi-1 in restricting the proliferation of hematopoietic stem/progenitor cells as revealed by studies with murine Gfi-1 knockout hematopoietic cells (22, 23).

Although, at the moment, it is unclear whether normal and CML stem cells exhibit differential sensitivity to perturbation of Gfi-1 expression, understanding the mechanisms responsible for the biological effects of Gfi-1 could, conceivably, lead to the development of therapeutic strategies exploiting its effects.

The effects of Gfi-1 depend on its activity as transcriptional repressor, as the P2A Gfi-1 mutant (which lacks a functional SNAG transcriptional repressor domain) had modest or no effects on proliferation and colony formation of p210BCR/ABL-expressing cells.

Preliminary microarray analysis data on Gfi-1 activation in K562 cells reveal that expression of several genes with established roles in hematopoietic stem cell proliferation and survival (31, 33–37) was downregulated (c-Myb, STAT5, and Mcl-1) or upregulated (FOXO3a and PTEN).

Although it is unknown whether these are direct or indirect Gfi-1 targets, it seems likely that the effects of Gfi-1 in BCR/ABL-expressing cells could be recapitated by strategies able to manipulate their expression. Indeed, coexpression of Mcl-1, STAT5B, and c-Myb almost completely reversed the antiproliferative effect of Gfi-1 in K562 cells (data not shown).

In summary, we have identified Gfi-1 as a novel transcription target of C/EBPα, which seems to play an important role as mediator of its inhibitory effects on proliferation and colony formation of p210BCR/ABL-expressing cells. These findings and those reported previously (27) are consistent with a model of C/EBPα function whereby it regulates, directly and indirectly, a network of transcription factors whose balanced expression might be necessary for controlling the proliferation versus the differentiation fate of early hematopoietic progenitors.

The fact that C/EBPα is structurally or functionally inactive in the majority of cases of AML (3, 4) further attests its essential role at the nodal points at which a balance in hematopoietic cell proliferation and differentiation needs to be maintained to prevent a potentially damaging disruption of tissue homeostasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Expression of the Transcriptional Repressor Gfi-1 Is Regulated by C/EBP α and Is Involved in Its Proliferation and Colony Formation—Inhibitory Effects in p210BCR/ABL-Expressing Cells

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