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Phosphorylation of Serine 21 Modulates the Proliferation Inhibitory More Than the Differentiation Inducing Effects of C/EBPα in K562 Cells

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ABSTRACT

The CCAAT/enhancer binding protein α (C/EBPα) is a transcription factor required for differentiation of myeloid progenitors. In acute myeloid leukemia (AML) cells expressing the constitutively active FLT3-ITD receptor tyrosine kinase, MAP kinase-dependent phosphorylation of serine 21 (S21) inhibits the ability of C/EBPα to induce granulocytic differentiation. To assess whether this post-translational modification also modulates the activity of C/EBPα in BCR/ABL-expressing cells, we tested the biological effects of wild-type and mutant C/EBPα mimicking phosphorylated or non-phosphorylatable serine 21 (S21D and S21A, respectively) in K562 cells ectopically expressing tamoxifen-regulated C/EBPα-ER chimeric proteins. We show here that S21D C/EBPα-ER induced terminal granulocytic differentiation of K562 cells almost as well as wild-type C/EBPα-ER, while S21A C/EBPα-ER was less efficient. Furthermore, wild-type C/EBPα suppressed the proliferation and colony formation of K562 cells vigorously, while S21D and S21A C/EBPα mutants had more modest anti-proliferative effects. Both mutants were less effective than wild-type C/EBPα in suppressing endogenous E2F-dependent transactivation and bound less E2F-2 and/or E2F-3 proteins in anti-C/EBPα immunoprecipitates. Together, these findings suggest that mutation of S21 more than its phosphorylation inhibits the anti-proliferative effects of C/EBPα due to reduced interaction with or impaired regulation of the activity of E2F proteins. By contrast, phosphorylation of serine 21 appears to have a modest role in modulating the differentiation-inducing effects of C/EBPα in K562 cells. J. Cell. Biochem. 113: 1704–1713, 2012.

KEY WORDS: TRANSCRIPTION FACTOR; PROTEIN COMPLEXES; CELL CYCLE; GRANULOCYTE DIFFERENTIATION; LEUKEMIA; PROLIFERATION; PHOSPHORYLATION

The CCAAT/enhancer binding protein α (C/EBPα) is a member of the leucine zipper family of transcription factors that regulate the proliferation and differentiation of various cell types [Landschulz et al., 1989; Hendricks-Taylor and Darlington, 1995].

In the hematopoietic system, its expression is detected in early myeloid progenitors and it increases only in myeloid cells undergoing granulocytic differentiation [Scott et al., 1992; Radomska et al., 1998; Miyamoto et al., 2002]: consistent with

Abbreviations used: C/EBPα, CCAAT/enhancer binding protein α; AML, acute myeloid leukemia; S21, serine 21; CML, chronic myelogenous leukemia; 4-HT, 4-hydroxytamoxifen; RT-Q-PCR, real-time quantitative polymerase chain reaction.

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this pattern of expression, loss of C/EBPα in vivo leads to mice with: (i) increased frequency of hematopoietic stem cells; (ii) block of the transition of common myeloid progenitors (CMPs) into granulocyte-monocyte precursors (GMPs), and (iii) lack of mature granulocytes but not monocytes [Wang et al., 1995; Zhang et al., 2004].

The requirement of C/EBPα in the myeloid lineage is, in part, due to its ability to transactivate the expression of myeloid-specific genes [Keeshan et al., 2003], but its inhibitory effect on cell proliferation has been also shown to be required for differentiation [Umek et al., 1991; Wang et al., 2001]. The mechanisms of C/EBPα-mediated cell cycle arrest include interaction with and inhibition of the activity of the cell cycle regulatory proteins CDK2/CDK4 and E2Fs [Timchenko et al., 1996; Pedersen et al., 2001; Wang et al., 2001], binding to the SWI/SNF chromatin remodeling complex [Slomiany et al., 2000], and upregulation of p21waf1/cip1 expression [Porse et al., 2001].

The expression and/or the activity of C/EBPα is consistently impaired in acute myeloid leukemia (AML) [Tenen, 2003], suggesting that its genetic or functional inactivation is an important event for leukemogenesis [Tenen, 2003; Nerlov, 2004]. Molecular mechanisms responsible for the inactivation of C/EBPα include mutations in the N- and C-terminus, inhibition of transcription or translation resulting in reduced protein levels, or post-translational modifications which render C/EBPα protein non-functional. Decreased transcription of C/EBPα has been described in AML cells with the t(8;21) translocation which generates the AML1-ETO chimeric protein [Westendorf et al., 1998]. AML1-ETO can also physically interact with C/EBPα blocking transcription of C/EBPα-regulated genes [Pabst et al., 2001]. In myeloid precursors transformed by the p210BCR/ABL oncoprotein and in blast cells of myeloid blast crisis of chronic myelogenous leukemia (CML) decreased expression of C/EBPα depends, in part, on inhibition of C/EBPα mRNA translation [Perrotti et al., 2002; Chang et al., 2007]. Other subtypes of AML including the t(3;21), which generates the AML-1-MDS-1-Evi-1 fusion protein, and the inv(16) which generates the chimeric CBFB-SMMHC protein are also associated with reduced expression of C/EBPα [Helbling et al., 2004, 2005].

In a recent study, Keeshan et al. [2006] reported that Trib2 functions as an oncogene inducing AML in mice through the association with and the proteasome-dependent degradation of wild-type C/EBPα leading to a relative increase in the expression of the proliferation-stimulatory p30 C/EBPα isoform.

The activity of C/EBPα can be also modulated by post-translational modifications. In particular, phosphorylation of serine 21 (S21) is mediated by the extracellular signal receptor kinase (Erk1/2) [Ross et al., 2004] and the p38 MAP kinase [Geest et al., 2009]. In leukemias harboring the FLT-3-ITD mutation, constitutive FLT-3 kinase activity results in sustained Erk1/2 phosphorylation of C/EBPα S21; this post-translational modification has been shown to suppress the ability of C/EBPα to induce granulocytic differentiation, possibly explaining the differentiation block associated with this type of AML [Radomska et al., 2006].

To obtain additional insights on the role of serine 21 phosphorylation for the activity of C/EBPα, we tested the biological effects of 4-hydroxytamoxifen (4-HT)-regulated wild-type or serine 21 C/EBPα mutants in K562 cells. We report here that the phosphomimetic S21D C/EBPα mutant is nearly as effective as the wild-type form in inducing differentiation of K562 cells and that the S21A non-phosphorylatable mutant, while less potent, is still able to promote granulocytic differentiation. Instead, either mutation generates proteins with lower anti-mitotic activity due to defective inhibition of E2F activity and/or defective protein interaction.

MATERIALS AND METHODS

PLASMIDS

MigRI-C/EBPα-ER and MigRI-C/EBPα-HA were previously described [Ferrari-Amorotti et al., 2006]. MigRI-S21D- and S21A C/EBPα-ER or C/EBPα-HA derivatives were obtained by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The following primers were used: S21D forward primer 5′-AGCCACCTCCAGGACCCCCCG-CACGC-3′ and S21D reverse primer 5′-GCGTGCGGGGGGTCCT-GGAGGTGGCT-3′; S21A forward primer 5′-AGCCACCTCCAGG-CCCCCCCGCACGC-3′ and S21A reverse primer 5′-GCGTGCGGGG-GGGCCTGGAGGTGGCT-3′. p-GL3 E2F 6X TATA LUC was a kind gift of Dr K. Helin (Biotech Research and Innovation Centre, University of Copenhagen N, Denmark). pTK-GCSF-R-luciferase was a kind gift of Dr D.G. Tenen (Harvard Institutes of Medicine, Cambridge, MA).

CELL CULTURE

K562 and derivative cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine. 293T and Phoenix cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine.

For retroviral infections, amphotropic Phoenix cells were transiently transfected with the indicated plasmids. The infectious supernatant was collected 48 h later and used to infect (a 48-h procedure) K562 cells. Twenty-four hours later, cells were sorted (EPICS Profile Analyzer; Coulter, Hialeah, FL) for green florescent protein (GFP) expression.

CELL PROLIFERATION AND DIFFERENTIATION ASSAYS

For proliferation assays, cells were washed with phosphate-buffered saline (PBS) and treated with 4-HT (100 nM; Sigma). Viable cells were counted by trypan blue exclusion.

For colony formation assays, cells were pretreated (1 h) with 100 nM 4-HT and plated in methylcellulose (5×10^2/plate) in presence of 100 nM 4-HT. Colonies were counted 6 days later. Differentiation was monitored by May-Grunwald/Giemsa staining and by detection of the differentiation markers CD11b and CD15 [Ferrari-Amorotti et al., 2006]. Images were visualized using an Olympus CK2 microscope with a 40×/0.65 numeric aperture objective, and were photographed using an Olympus SC35 type 12 camera (Olympus). JPEG images were viewed using Adobe Photoshop (Adobe Systems), and contrast adjustments were made.

IMMUNOPRECIPITATION AND WESTERN BLOT ANALYSIS

For immunoprecipitation, C/EBPα-ER-K562 cells (2.5×10^7) were treated with 250 nM of tamoxifen. Cells were harvested 12 h after,

washed once with 1 PBS and lysed (10^7 cells/100 μl lysis buffer) in buffer containing (150 mM Tris [pH 7.6], 50 mM NaCl, 0.1% NP40, 1 mM phenylmethyl sulfonyl fluoride [PMSF], sodium vanadate [Na_3VO_4] supplemented with a cocktail of serine and cysteine protease inhibitors [complete, EDTA-free; Roche]) for 30 min, rotating at 4°C, and the lysates were clarified by centrifugation at 14,000 rpm for 10 min. Supernatants were precleared with 40 μl 1:1 slurry of protein G plus agarose (Oncogene Research Products) in lysis buffer. The precleared supernatants were incubated with the anti-C/EBPα antibodies (40 μg, sc-61, Santa Cruz Biotechnology, Inc.) with a 1:1 slurry protein G plus agarose overnight rotating at 4°C. Immunoprecipitations without antibody (no antibody control) and an anti-rabbit IgG were included with each experiment. The beads were added to a lysate-Ab mix for 1 h, rotating, at 4°C, washed extensively (six times) in lysis buffer and the pellet was resuspended in 2× protein-loading buffer. Immunoprecipitated proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto a nitrocellulose membrane, and immunoblotted using anti-C/EBPα (sc-61 Santa Cruz, Inc.), anti-E2F1 (sc-251 Santa Cruz, Inc.), anti-E2F2 (sc-633 Santa Cruz Biotechnology, Inc.), or anti-E2F3 antibodies (sc-878 Santa Cruz Biotechnology, Inc.). Proteins were detected using enhanced chemiluminescence (ECL) (Amersham).

For Western blot, 5×10^6 cells were centrifuged for 5 min at 1,200 rpm, washed in PBS, lysed in 50 μl of 2× Laemmli sample buffer, and boiled at 100°C for 10 min. Twenty-five to 30 μl of sample were loaded on 10% SDS–PAGE gel. After blocking in 5% milk/TBST (TBST: 25 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20), membranes were stained with primary antibodies in 2.5% milk/TBST for 1 h at room temperature and with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h. Signals were detected by enhanced chemiluminescence and quantified by ImageJ software (National Institutes of Health). The primary antibodies were: rabbit p-C/EBPα Ser21 (#2841, 1:1,000; Cell Signaling), rabbit C/EBPα (sc-61, 1:500 Santa Cruz Biotechnology, Inc.), pERK (1:1,000 Cell Signaling), and β-actin (sc-47778, 1:1,000 Santa Cruz Biotechnology, Inc.). All secondary antibodies (rabbit and mouse) were HRP-conjugated (Santa Cruz Biotechnology, Inc.) and diluted 1:5,000.

LUCIFERASE ASSAY

293T cells were transiently transfected using ProFection Mammalian Transfection System-Calcium Phosphate (Promega, Madison, WI) with 3 μg reporter plasmid pTK-G-CSFR-luciferase (which contains 4 C/EBPα binding sites from the G-CSF receptor promoter), 3 μg of the indicated C/EBP expression plasmid, and 1/50 Renilla luciferase plasmid to account for variation in transfection efficiencies. K562-C/EBPα-ER cells (1×10^6) were transfected using the Cell Line Nucleofector™ Kit V (Amaxa) and the Amaxa Nucleofector (program T-016), according to the manufacturer's instructions. Cells were transfected with 4 μg of reporter plasmid E2F 6X TATA LUC and 0,02 μg of Renilla. Forty-eight hours after transfection, K562 were split 1:5 and treated with 250 nM 4-HT for 8 h. Firefly and Renilla luciferase activity was recorded on a luminometer using the Dual-Luciferase Reporter Assay System (Promega). Results are expressed as fold activation relative to empty vector-transfected (293T) or untreated (K562) cells after correction for Renilla luciferase activity and are representative of three individual experiments (performed in triplicate).

REAL-TIME QUANTITATIVE PCR

For real-time quantitative (RT-Q)-PCR, total RNA was isolated using the RNeasy Mini kit (Qiagen), 2 μg was reverse transcribed and the resulting first-strand cDNA used as PCR template. All reactions were done in triplicate. Primer pairs designed using the ABI Primer Express software are: GCSF-R forward primer (5′-GGCCACCAA-CAGTACAGTCC-3′) and reverse primer (5′-GTTCCACAGAGG-CAGGTGAG-3′). RT-Q-PCR was done using Go Taq PCR Master Mix (Promega) on a MyIQ thermocycler (Bio-Rad) and quantified using MyIQ software (Bio-Rad). HPRT, a housekeeping gene with constant expression, was used as an internal control to normalize input cDNA. Primer pairs designed using the ABI Primer Express software are: HPRT forward primer (5′-AGACTTTGCTTTCCTTGG-TCAGG-3′) and reverse primer (5′-GTCTGGCTTATATCCAACA-CTTCG-3′).

CELL CYCLE ANALYSIS

For cell cycle analysis, C/EBPα-ER K562 cells were seeded at a density of 1×10^5 cells/ml and left untreated or treated with 100 nM 4-HT. Two days later, cells were labeled with propidium iodide (PI) and analyzed by flow cytometry on a FACScan (Becton Dickinson). Cell cycle distribution was determined by DNA content analysis of PI-stained nuclei, as described [Ferrari-Amorotti et al., 2006].

BrdU INCORPORATION ASSAY

5-Bromodeoxyuridine (BrdU) incorporation assay was done with the APC BrdU Flow kit (BD Pharmingen). Untreated and 4-HT-treated (100 nM, 48 h) C/EBPα-ER-K562 cells were labeled with BrdU for 30 min. Cells were then fixed, stained with APC-labeled anti-BrdU antibody and 7-AAD, and analyzed by flow cytometry to determine the number of S phase cells on a FACScan (Becton Dickinson).

REAGENTS

The ERK1/2 pathway inhibitor PD184352 was kindly provided by Dr. Antonio Bonati (University of Parma Medical School, Italy). PD184352 was reconstituted in DMSO at 10 mM and used at a final concentration of 1 μM. 4-HT (Sigma Aldrich) was dissolved in EtOH at a final concentration of 50 mM.

STATISTICS

Data (presented as the means + SD of two or three experiments) were analyzed for statistical significance by the unpaired, two-tailed Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

PHARMACOLOGIC INHIBITION OF THE ERK1/2 PATHWAY TRIGGERS A DECREASE OF C/EBPα SERINE 21 PHOSPHORYLATION IN K562 CELLS ECTOPICALLY EXPRESSING C/EBPα-ER

Transformed myeloid progenitor cells expressing the constitutively active FLT3-ITD tyrosine kinase receptor exhibit a block in differentiation which appears to be mediated by inactivation of

C/EBPα due to ERK1/2-dependent phosphorylation of serine 21 [Ross et al., 2004; Radomska et al., 2006]. To test whether C/EBPα is also phosphorylated in an ERK1/2-dependent manner in cells transformed by the p210BCR/ABL oncogenic tyrosine kinase, we assessed serine 21 phosphorylation in K562 cells ectopically expressing C/EBPα-ER after treatment with PD 184352, an inhibitor of the ERK1/2 pathway. After treatment with the MEK inhibitor, levels of C/EBPα serine 21 phosphorylation were markedly decreased at 6 and 12 h (Fig. 1A); at 24 h, expression of serine 21 phosphorylation showed a rebound above the barely detectable levels detected at 12 h, probably reflecting the reduced activity of the inhibitor (Fig. 1A). Phosphorylation of serine 21 was only in part affected by differentiation; upon treatment with 4-HT which rapidly induces granulocytic differentiation of C/EBPα-ER-K562 cells, phosphorylation of serine 21 was only modestly reduced (Fig. 1B), suggesting that suppression of serine 21 phosphorylation is not an absolute requirement for the differentiation-inducing effects of C/EBPα in K562 cells.

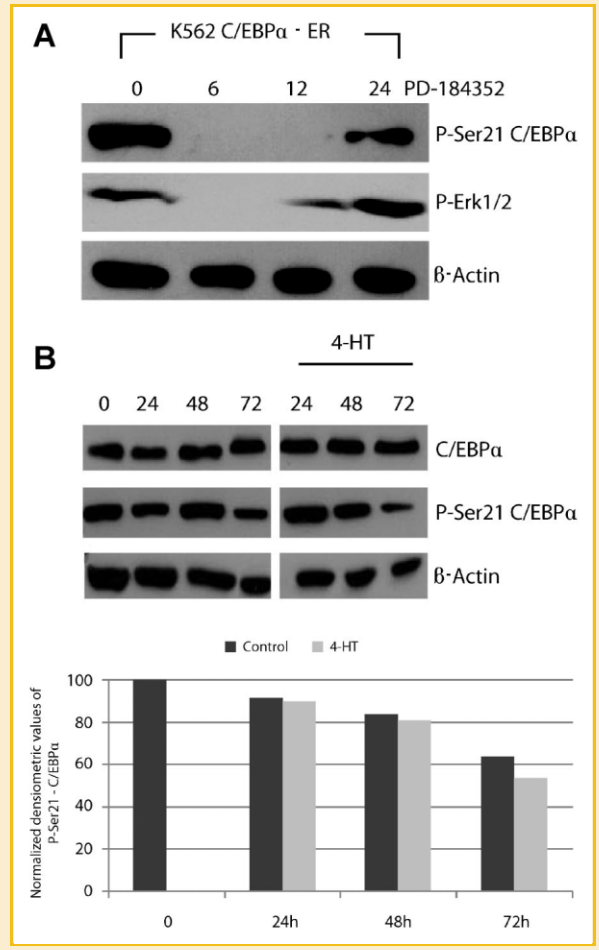

Fig. 1. ERK1/2-dependent phosphorylation of C/EBPα serine 21 in K562 cells. A: Western blot shows levels of P-Ser21 C/EBPα in PD184352-treated C/EBPα-ER K562 cells. Cells were cultured in the presence of 1 μM of PD184352 for 24 h. Blot was sequentially stained with anti P-Ser21 C/EBPα, anti-P-ERK1/2 and β-actin antibodies; (B, top) Western blot shows C/EBPα serine 21 phosphorylation upon 4-HT treatment of C/EBPα-ER K562 cells. Cells were cultured in the absence or in the presence of 100 nM 4-HT for 72 h. Blot was sequentially stained with anti P-ser21 C/EBPα, C/EBPα and β-actin antibodies. B, Bottom: Quantification of phosphorylated C/EBPα normalized to total C/EBPα protein is shown (bottom).

EFFECTS OF WILD-TYPE AND SERINE 21 C/EBPα-ER MUTANTS ON DIFFERENTIATION OF K562 CELLS

We first assessed the effect of serine 21 mutant C/EBPα on the differentiation of K562 cells upon transduction with MigRI-GFP retroviruses expressing the constitutively active wild-type or the S21D or S21A C/EBPα mutant. At 0, 24, 48, and 72 h post-transduction, GFP-positive cells were assessed for expression of the CD15 granulocyte differentiation marker; as shown in Supplementary Figure 1, the percentage of CD15+ cells was essentially identical in the wild-type and the C/EBPα mutant-transduced (GFP-positive) cells, suggesting that phosphorylation of C/EBPα has no (or only modest) effects on K562 cell differentiation. Since activation of C/EBPα-ER in K562 cells induces granulocytic differentiation rapidly and efficiently [D'Alo' et al., 2003; Ferrari-Amorotti et al., 2010], we further investigated the role of MAP kinase-dependent phosphorylation of serine 21 in K562 derivative cell lines transduced with retroviral plasmids expressing mutant C/EBPα mimicking phosphorylated or non-phosphorylatable serine 21 (S21D and S21A, respectively) (Fig. 2A). Immunoblot analysis of transduced K562 cells revealed that wild-type and serine 21 mutant C/EBPα are expressed at similar levels and that the phosphorylated form is present only in cells expressing wild-type C/EBPα, since the faint band detected in S21D C/EBPα-ER-K562 cells reflects cross-reaction of the antibody with phosphomimetic S21D C/EBPα (Fig. 2B).

The effects of wild-type and mutant C/EBPα on differentiation were assessed by monitoring morphology and the expression of granulocyte differentiation markers. Upon 4-HT treatment, activation of wild-type or S21D C/EBPα-ER induced an almost complete terminal granulocytic differentiation of K562 cells as indicated by the high frequency of segmented nuclei (approximately 67% and 49% of terminally differentiated cells in wild-type and S21D C/EBPα-ER K562 cells, respectively), while S21A C/EBPα-ER was less efficient as indicated by the presence of doughnut-shaped nuclei in most cells and segmented nuclei in approximately 28% of the cells (Fig. 2C, Table I).

Analysis of granulocytic differentiation markers confirmed these morphological changes: activation of wild-type or S21D mutant C/EBPα-ER led to a marked increase of CD11b and CD15 expression (Fig. 2D,E), although there were modest but statistically significant differences in the effects induced by these two proteins. By contrast, S21A C/EBPα-ER was less effective of wild-type C/EBPα or the S21D mutant (Fig. 2D,E), although some of the differences between the two mutant proteins were not statistically significant.

Consistent with these data, levels of G-CSFR mRNA detected by real-time PCR were more abundant upon activation of C/EBPα-ER or S21D C/EBPα-ER than of S21A C/EBPα-ER (Fig. 2F).

EFFECTS OF WILD-TYPE AND SERINE 21 MUTANT C/EBPα ON THE GCSF-R PROMOTER

To further assess the effects of wild-type and S21 C/EBPα mutants, we performed luciferase assays on the C/EBPα-responsive G-CSFR promoter. We measured luciferase activity in 293T and K562 cells

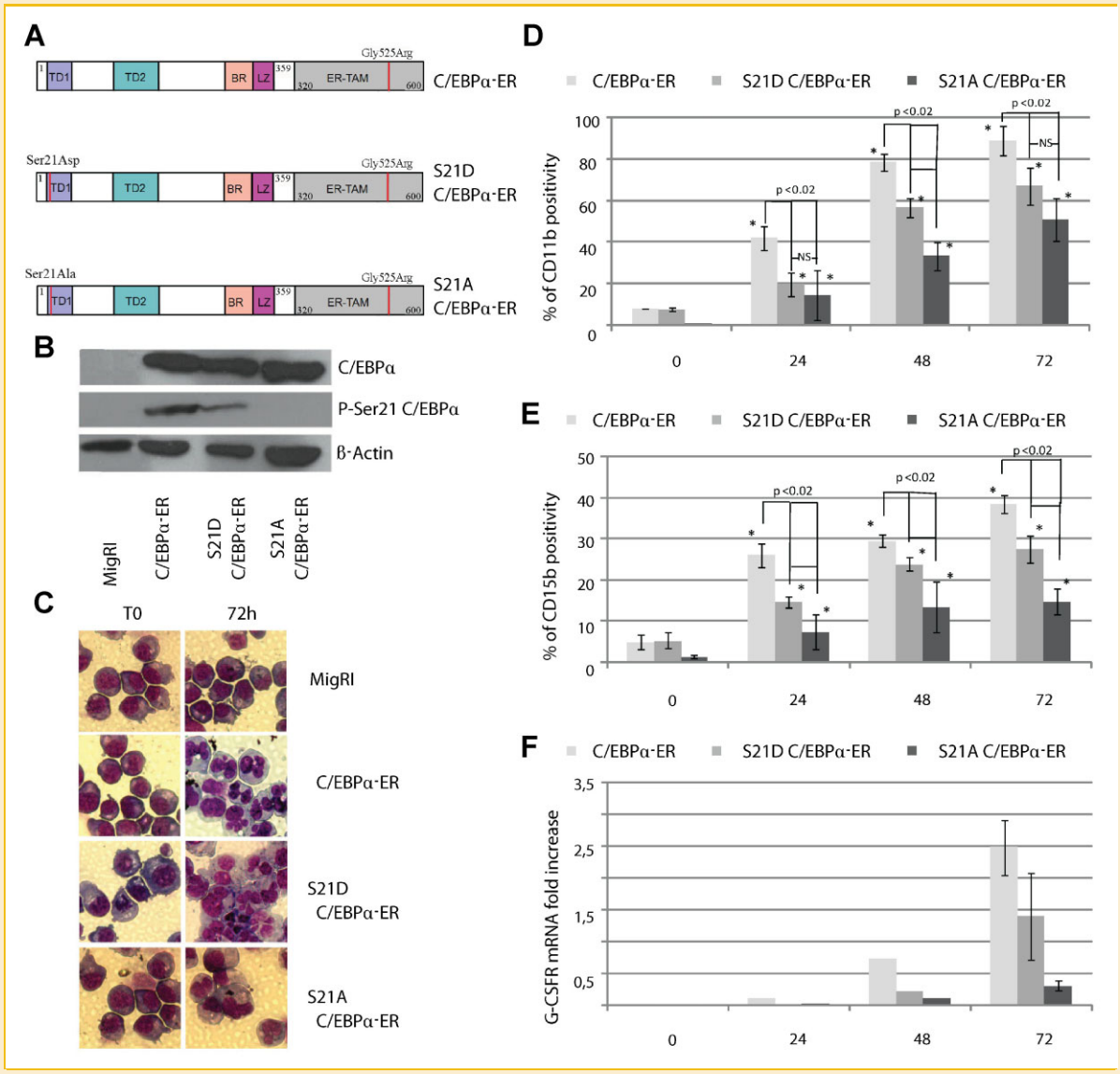

Fig. 2. Effects of wild-type and mutant C/EBPα on differentiation of K562 cells. A: Schematic diagram of C/EBPα-ER proteins; (B) Western blot shows levels of total and P-Ser21 C/EBPα in C/EBPα-ER K562 cells; expression of β-actin was monitored as loading control; (C) light microscopy images of May-Grunwald-stained untreated or 4-HT-treated C/EBPα-ER K562 cells; original magnification 40×. Counts of differentiated cells, summarized in Table I, were performed in at least 10 fields; (D,E) CD11b and CD15 positivity, respectively. Values represent the mean + SD of three independent experiments. $^{*}P < 0.05$ relative to T0. NS indicates that the difference in the % CD11b positivity of 4-HT treated S21D- and S21A C/EBPα-ER-expressing cells is not significant; statistical significance was calculated using unpaired, two-tailed Student's *t*-test; (F), GCSF-R mRNA expression assessed by real-time-Q-PCR in 4-HT-treated (24, 48, and 72 h) C/EBPα-ER K562 cells. HPRT expression was used as internal control. Results are reported as normalized-fold increase of G-CSFR mRNA expression. Error bars denote SD of normalized means of three independent experiments performed in triplicate. $^{*}P < 0.05$ relative to T0. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

co-transfected with the G-CSFR promoter-luciferase reporter (G-CSFR/Luc) plasmid, the Renilla luciferase control plasmid to account for variation in transfection efficiency and C/EBPα-HA plasmids or the MigRI empty vector. Results are expressed as fold activation relative to vector alone after normalization for Renilla luciferase activity (Fig. 3A,B).

The assays show that wild-type and serine 21 C/EBPα mutants are essentially undistinguishable in the ability to transactivate the C/EBPα-responsive G-CSFR promoter in 293T and K562 cells (Fig. 3A,B).

Thus, activation of a C/EBPα-regulated promoter does not correlate with morphological and molecular differentiation.

EFFECTS OF WILD-TYPE AND S21 MUTANT C/EBPα-ER ON THE PROLIFERATION OF K562 CELLS

The effects of wild-type and S21 mutant C/EBPα on cell proliferation were tested by cell counts, DNA content analysis, 5-bromodeoxyuridine (BrdU) labeling and colony formation assays. wild-type C/EBPα-ER markedly suppressed proliferation of K562

TABLE I. Frequency of Undifferentiated and Differentiated Myeloid Cells in 4-HT-Treated C/EBPα-ER-K562 Cells

72 h	C/EBPα-ER	C/EBPα-ER 4-HT	S21D C/EBPα-ER	S21D C/EBPα-ER 4-HT	S21A C/EBPα-ER	S21A C/EBPα-ER 4-HT
Blasts	43% (121)	/	36% (115)	1.23% (4)	41.10% (125)	4.60% (14)
Promyelocytes	47% (133)	/	46.70% (149)	8.90% (29)	41.30% (126)	19.50% (60)
Myelocytes	9.30% (26)	11% (44)	16.35% (52)	13.00% (42)	10.60% (32)	21.80% (67)
Metamyelocytes	0.70% (2)	22.3% (79)	0.95% (3)	28.70% (93)	6.60% (20)	25.70% (79)
Bands	/	15.1% (56)	/	16.60% (54)	0.30% (1)	16.60% (51)
Segments	/	51.6% (191)	/	31,50% (102)	/	11.80% (36)
Total	100% (282)	100% (370)	100% (319)	100% (324)	100% (304)	100% (307)

Number of cells scored are in parentheses.

cells; by contrast, the S21D- and S21A C/EBPα-ER mutant inhibited K562 cell proliferation less effectively (Fig. 4A).

DNA content analysis of K562-C/EBPα-ER cells treated with 4-HT for 48 h was consistent with cell counts: activation of wild-type C/EBPα-ER induced a 38% increase in G1 phase cells and a 75% decrease in S phase cells, whereas activation of S21D- or S21A C/EBPα-ER caused a more modest increase in G1 phase cells (5% and 14%, respectively) and a less pronounced decrease in S phase cells (35% and 20%, respectively) (Fig. 4B).

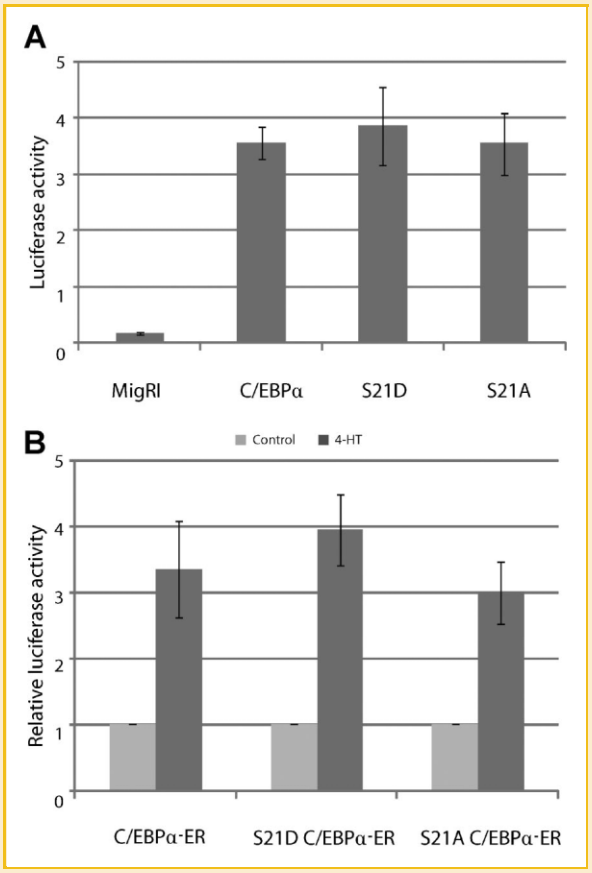

Fig. 3. Effect of wild-type and S21 mutant C/EBPα on the G-CSFR promoter. A: Histogram shows luciferase activity in 293T cells co-transfected with pTK-G-CSFR-luciferase and C/EBPα-HA plasmids or the MigRI empty vector. Results (three independent experiments) are expressed as -fold activation over that in empty vector-transfected cells after normalization for Renilla luciferase activity and are reported as the means of triplicate determinations; error bars represent the SD of the mean. B: Histogram shows luciferase activity in C/EBPα-ER K562 cells nucleofected with pTK-G-CSFR-luciferase after treatment (8 h) with 4-HT. Results (three independent experiments) are expressed as -fold activation over that of untreated cells after normalization for Renilla luciferase activity and are reported as the means of triplicate determinations; error bars represent the SD of the mean.

The differences in the anti-proliferative effects of wild-type and serine 21 mutant C/EBPα were also assessed by performing the BrdU labeling assay in 4-HT-treated C/EBPα-ER K562 cells. Compared to untreated cells, activation of wild-type C/EBPα induced approximately a 72% decrease in the percentage of S phase cells; by contrast, S21D or S21A C/EBPα-ER were less effective (approximately 30% and 38%, respectively) (Fig. 4C).

We also assessed the effects of wild-type and mutant C/EBPα on colony formation of K562 cells. Activation of wild-type C/EBPα-ER markedly suppressed K562 colony formation (approximately 76% inhibition compared to control); S21D C/EBPα-ER and S21A C/EBPα-ER also suppressed K562 colony formation but the effects were less striking (56% and 50% inhibition, respectively, compared to controls) (Fig. 4D).

REDUCED REPRESSION OF E2F ACTIVITY BY S21 C/EBPα MUTANTS

Previous studies have shown that the N-terminus-truncated Δ1-70 C/EBPα mutant did not inhibit E2F-dependent transcription activity in transiently transfected human fibroblast and adipocytic cell lines and in murine myeloid precursor cells [Porse et al., 2001; Ferrari-Amorotti et al., 2010]. Because S21 is located in this region, we undertook experiments to assess how effectively the S21 C/EBPα mutants suppress E2F-dependent transactivation using a reporter plasmid in which expression of the luciferase gene is driven by a minimal promoter containing six E2F binding sites [Helin et al., 1992]. C/EBPα-ER K562 cells were transfected with the luciferase reporter plasmid and 48 h later wild-type or S21 mutant C/EBPα-ER was activated by treatment with 250 nM 4-HT. Eight hours later, cells extracts were assayed for luciferase activity.

Activation of wild-type C/EBPα-ER markedly suppressed endogenous E2F-dependent transactivation, whereas the inhibitory effect of S21D or S21A C/EBPα-ER was more modest (Fig. 5A). Conceivably, these results may reflect decreased interaction of mutant C/EBPα with members of the E2F family. Thus, co-immunoprecipitation experiments of C/EBPα and E2F proteins were carried out in 4-HT-treated K562 cells expressing wild-type or mutant C/EBPα. As shown in Figure 5B, E2F-1, E2F-2, and E2F-3 were all expressed in K562 cells; however, C/EBPα did interact with E2F-2 and E2F-3 but not with E2F-1. As indicated by densitometric analysis, less E2F-2 and E2F-3 was immunoprecipitated by S21D

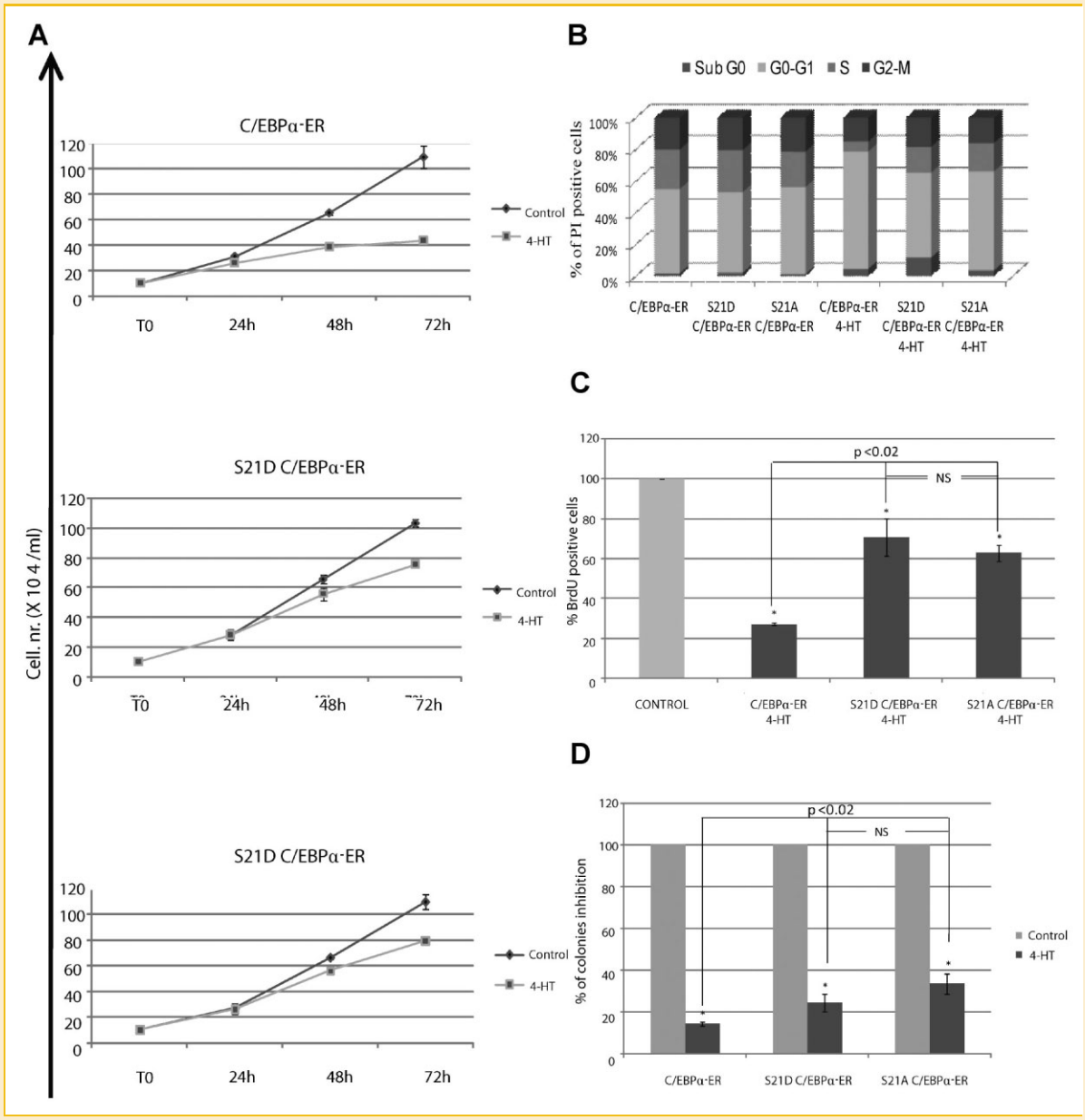

Fig. 4. Effects of wild-type and S21 mutant C/EBPα on the proliferation of K562 cells. Cell counts (A) and cell cycle distribution (B) of 4-HT-treated (48 h) C/EBPα-ER K562 cells; values represent the mean + SD of three independent experiments; (C) percentage of BrdU-labeled cells 48 h after 4-HT treatment of C/EBPα-ER K562 cells; values (mean + SD of three independent experiments) represent the % decrease of BrdU-positive cells in 4-HT-treated versus untreated cells taken as 100%; $^{*}P < 0.02$ relative to untreated cells; statistical significance was calculated using unpaired, two-tailed Student's *t*-test; (D) histogram shows methylcellulose colony formation of 4-HT-treated C/EBPα-ER K562 cells. Colonies were scored 6 days after seeding 5×10^2 cells/plate; values (mean ± SD of three independent experiments performed in duplicate) are expressed as the percentage of colonies from 4-HT-treated cells compared to the corresponding untreated cells taken as 100%. $^{*}P < 0.05$ relative to control; NS indicates that the difference between 4-HT treated S21D C/EBPα-ER and S21A C/EBPα-ER K562 cells is not significant; statistical significance was calculated using unpaired, two-tailed Student's *t*-test.

C/EBPα than by wild-type C/EBPα, while less E2F-2 appears to be in complex with S21A C/EBPα than with wild-type C/EBPα (Fig. 5C).

DISCUSSION

In this study, we investigated the role of serine 21 phosphorylation for the biological effects of C/EBPα in hematopoietic cells transformed by the p210BCR/ABL oncogene. Although C/EBPα activity is often impaired in myeloid leukemia cells by mutations and by transcriptional and post-transcriptional mechanisms which cause a decrease in protein levels [Tenen, 2003; Nerlov, 2004], there is also evidence for the involvement of post-translational modifications. For example, MAP kinase-mediated serine 21 phosphorylation suppresses the differentiation-inducing effect of C/EBPα in myeloid cells transformed by the oncogenic FLT3-ITD

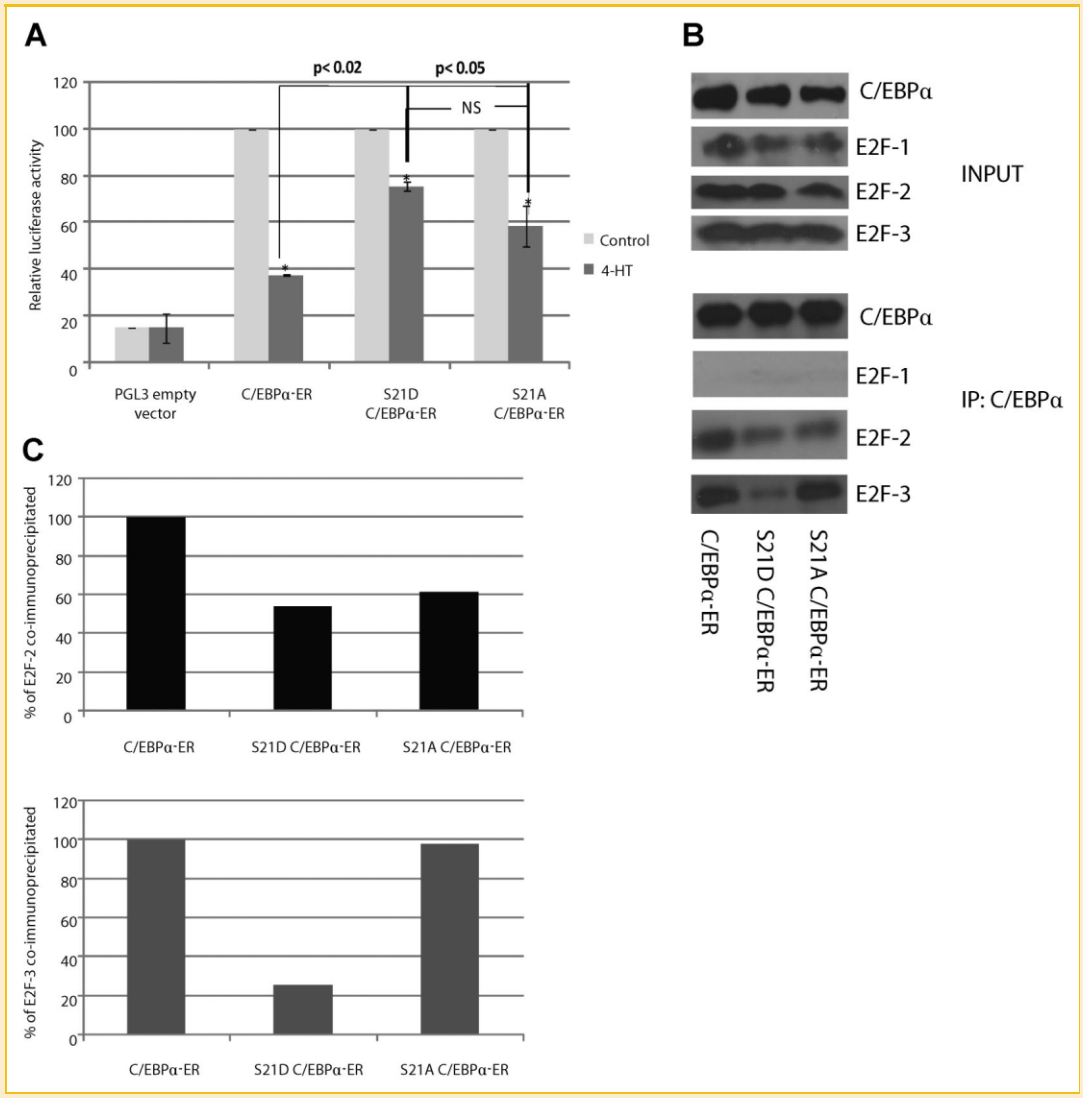

Fig. 5. Effect of wild-type and serine 21 mutant C/EBPα on E2F-dependent transactivation. A: Histogram shows luciferase activity in C/EBPα-ER K562 cells transfected with the E2F-responsive E2F6-TATA-LUC reporter and the Renilla control plasmid after treatment (12 h) with 4-HT. Results (three independent experiments) are expressed as -fold activation over that in empty vector-transfected cells after normalization for Renilla luciferase activity and are reported as the means of triplicate determinations; error bars represent the SD of the mean; *P* values indicate statistical significance of the differences in E2F-dependent luciferase activity in 4-HT-treated wild-type and mutant C/EBPα-ER K562 cells; NS indicates that the difference in luciferase activity between 4-HT treated S21D C/EBPα-ER and S21A C/EBPα-ER K562 cells is not significant; statistical significance was calculated using unpaired, two-tailed Student's *t*-test; $^{*}P < 0.05$ relative to control; (B, top) Western blot shows C/EBPα and E2F expression in whole cell lysates from K562 cells ectopically expressing wild-type or mutant C/EBPα-ER; (B, bottom) Western blot shows E2F-1, -2, or -3 expression in anti-C/EBPα immunoprecipitates from 4-HT-treated (12 h) K562 cells ectopically expressing wild-type or mutant C/EBPα (representative of two experiments). (C) Quantification of co-immunoprecipitated E2F-2 and E2F-3 normalized to total C/EBPα.

tyrosine kinase receptor [Radomska et al., 2006]. The mechanisms whereby this phosphorylation inactivates the differentiation-inducing effects of C/EBPα remain unclear but may depend on changes in protein interactions necessary for the ability of C/EBPα to promote myeloid differentiation [Ross et al., 2004].

Since there are many similarities in the signal transduction pathways activated by oncogenic tyrosine kinases in myeloid cells [Choudhary et al., 2005; Ren, 2005], we asked whether serine 21 of C/EBPα can be also phosphorylated in a MAP kinase-dependent manner in p210BCR/ABL-transformed cells and whether it modulates the biological effects of C/EBPα. Indeed, in K562 cells ectopically expressing C/EBPα phosphorylation of serine 21 was completely suppressed by inhibition of MAP kinase activity, suggesting that, in these cells, activation of this pathway is the predominant, if not the only, mechanism for serine 21 phosphorylation. After 48 h of 4-HT treatment, a time point at which K562 cells show advanced differentiation by morphology and expression of differentiation markers [Ferrari-Amorotti et al., 2010], levels of serine 21 phosphorylation barely changed (Fig. 1B), suggesting that it does not hinder the differentiation-inducing activity of C/EBPα. Phosphorylation of serine 21 is still detectable 72 h after 4-HT treatment, a time point at which granulocytic differentiation of

K562 cells is complete [Ferrari-Amorotti et al., 2010]. Consistent with these findings, the phosphomimetic S21D C/EBPα was almost as effective as wild-type C/EBPα in inducing morphological and molecular differentiation of K562 cells and in transactivating the C/EBPα-regulated G-CSFR promoter. By contrast, the non-phosphorylatable S21A mutant, which should have been a more potent inducer of differentiation if loss of serine 21 phosphorylation was required for C/EBPα-induced differentiation, was clearly less effective in inducing morphological and molecular differentiation of K562 cells, although it was essentially undistinguishable from wild-type and S21D C/EBPα in transactivating the G-CSFR promoter.

Wild-type and S21D C/EBPα were more clearly different in their anti-proliferative effects; wild-type C/EBPα suppressed proliferation and colony formation of K562 cells more efficiently than S21D C/EBPα, an effect that reflects the reduced capability of S21D C/EBPα to block G1/S phase transition as indicated by the more modest changes that it induced in the frequency of G1 and S phase cells (Fig. 4). Since the cell cycle inhibitory effects of C/EBPα depend, in part, on its ability to block the activity of E2F [Porse et al., 2001; D'Alo' et al., 2003], we reasoned that S21D C/EBPα may be less effective than wild-type C/EBPα in suppressing E2F activity. Indeed, S21D C/EBPα did not suppress endogenous E2F activity as effectively as wild-type C/EBPα (Fig. 5A). Previous studies have shown that the N-terminal region of C/EBPα is required for suppressing E2F-dependent transactivation [Porse et al., 2001; D'Alo' et al., 2003]; however, there is little information on the mechanisms of E2F repression by the C/EBPα N-terminus and whether specific residues within the first 70 amino acids are required for this effect. The issue of the interaction of Δ(1-70)C/EBPα with E2F proteins was addressed by D'Alo' et al. [2003]; they showed that, in contrast to mutants of the C-terminal basic region, Δ(1-70)C/EBPα still bound to E2F-4. However, it is unclear whether this interaction was quantitatively similar to that of the wild-type protein; moreover, since E2F-4 forms primarily repressive complexes with all members of the RB family [Chen et al., 2009], its interaction with C/EBPα may not be biologically relevant for the cell cycle inhibitory effects of C/EBPα which, instead, are expected to involve inhibition of proliferation-stimulatory E2F proteins. We found that S21D C/EBPα bound less E2F-2 and E2F-3 proteins than wild-type C/EBPα in anti-C/EBPα immunoprecipitates from K562 cells (Fig. 5B), possibly explaining its reduced proliferation inhibitory effects (Fig. 4). However, this may be only a partial explanation for the lower proliferation-inhibitory effects of S21D C/EBPα since S21A C/EBPα appears to bind E2F-3 as efficiently as wild-type C/EBPα and yet did not inhibit K562 cell proliferation and E2F-dependent transactivation as effectively as wild-type C/EBPα.

In a previous study [Keeshan et al., 2003], we showed that induction of myeloid differentiation by C/EBPα requires DNA binding-dependent transcription activation. Among the C/EBPα-regulated genes, some encode for markers and/or effectors of myeloid differentiation (i.e., the G-CSF receptor) [Zhang et al., 1998], while others encode for proteins that may limit the proliferative potential of early progenitors (i.e., Gfi-1) [Lidonnici et al., 2010]. It is likely that the proliferation-inhibitory and the differentiation-inducing functions of C/EBPα need to be finely coordinated for balancing its biologic effects.

The findings of this study are consistent with this hypothesis; we cannot exclude that, in p210BCR/ABL-transformed cells, MAP kinase-dependent phosphorylation of serine 21 C/EBPα affects simultaneously differentiation-induction and proliferation-inhibitory activities of C/EBPα. However, since phosphomimetic S21D C/EBPα shows only a modest defect in promoting differentiation and the non-phosphorylatable S21A mutant is even less effective, serine 21 phosphorylation may exemplify a post-translational modification which causes a modest perturbation of C/EBPα-regulated myeloid differentiation as consequence of a more pronounced impairment of its proliferation inhibitory activity.

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REFERENCES

Chang JS, Santhanam R, Trotta R, Neviani P, Eiring AM, Briercheck E. 2007. High levels of the BCR/ABL oncoprotein are required for the MAPK-hnRNP-E2 dependent suppression of C/EBPalpha-driven myeloid differentiation. Blood 110:994–1003.

Chen HZ, Tsai SY, Leone G. 2009. Emerging roles of E2Fs in cancer: An exit from cell cycle control. Nat Rev Cancer 9:785–797.

Choudhary C, Muller-Tidow C, Berdel WE, Serve H. 2005. Signal transduction of oncogenic Flt3. Int J Hematol 82:93–99.

D'Alo' F, Johansen LM, Nelson EA, Radomska HS, Evans EK, Zhang P. 2003. The amino terminal and E2F interaction domains are critical for C/EBP alpha-mediated induction of granulopoietic development of hematopoietic cells. Blood 102:3163–3171.

Ferrari-Amorotti G, Keeshan K, Zattoni M, Guerzoni C, Iotti G, Cattelani S. 2006. Leukemogenesis induced by wild-type and STI571-resistant BCR/ABL is potently suppressed by C/EBPalpha. Blood 108:1353–1362.

Ferrari-Amorotti G, Mariani SA, Novi C, Cattelani S, Pecorari L, Corradini F. 2010. The biological effects of C/EBPalpha in K562 cells depend on the potency of the N-terminal regulatory region, not on specificity of the DNA binding domain. J Biol Chem 285:30837–30850.

Geest CR, Buitenhuis M, Laarhoven AG, Bierings MB, Bruin MC, Vellenga E. 2009. p38 MAP kinase inhibits neutrophil development through phosphorylation of C/EBPalpha on serine 21. Stem Cells 27:2271–2282.

Helbling D, Mueller BU, Timchenko NA, Hagemeijer A, Jotterand M, Meyer-Monard S. 2004. The leukemic fusion gene AML1-MDS1-EVI1 suppresses CEBPA in acute myeloid leukemia by activation of Calreticulin. Proc Natl Acad Sci 101:13312–13317.

Helbling D, Mueller BU, Timchenko NA, Schardt J, Eyer M, Betts DR. 2005. CBFB-SMMHC is correlated with increased calreticulin expression and suppresses the granulocytic differentiation factor CEBPA in AML with inv(16). Blood 106:1369–1375.

Helin K, Lees JA, Vidal M, Dyson N, Harlow E, Fattaey A. 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell 70:337–350.

Hendricks-Taylor LR, Darlington GL. 1995. Inhibition of cell proliferation by C/EBP alpha occurs in many cell types, does not require the presence of p53 or Rb, and is not affected by large T-antigen. Nucleic Acids Res 23:4726–4733.

Keeshan K, Santilli G, Corradini, F, Perrotti D, Calabretta B. 2003. Transcription activation function of C/EBPalpha is required for induction of granulocytic differentiation. Blood 102:1267–1275.

Keeshan K, He Y, Wouters BJ, Shestova O, Xu L, Sai H. 2006. Tribbles homolog 2 inactivates C/EBPalpha and causes acute myelogenous leukemia. Cancer Cell 10:401–411.

Landschulz WH, Johnson PF, McKnight SL. 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science 243:1681–1688.

Lidonnici MR, Audia A, Soliera AR, Prisco M, Ferrari-Amorotti G, Waldron T. 2010. Expression of the transcriptional repressor Gfi-1 is regulated by C/EBP{alpha} and is involved in its proliferation and colony formation-inhibitory effects in p210BCR/ABL-expressing cells. Cancer Res 70:7949–7959.

Miyamoto T, Iwasaki H, Reizis B, Ye M, Graf T, Weissman IL. 2002. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. Dev Cell 3:137–147.

Nerlov C. 2004. C/EBPalpha mutations in acute myeloid leukaemias. Nat Rev Cancer 4:394–400.

Pabst T, Mueller BU, Harakawa N, Schoch C, Haferlach T, Behre G. 2001. AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia. Nat Med 7:444–451.

Pedersen TA, Kowenz-Leutz E, Leutz A, Nerlov C. 2001. Cooperation between C/EBPalpha TBP/TFIIB and SWI/SNF recruiting domains is required for adipocyte differentiation. Genes Dev 15:3208–3216.

Perrotti D, Cesi V, Trotta R, Guerzoni C, Santilli G, Campbell K. 2002. BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2. Nat Genet 30:48–58.

Porse BT, Pedersen TA, Xu X, Lindberg B, Wewer UM, Friis-Hansen L. 2001. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. Cell 107:247–258.

Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. 1998. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. Mol Cell Biol 18:4301–4314.

Radomska HS, Bassères D, Zheng R, Zhang P, Dayaram T, Yamamoto Y. 2006. Block of C/EBP alpha function by phosphorylation in acute myeloid leukemia with FLT3 activating mutations. J Exp Med 203:371–381.

Ren R. 2005. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. Nat Rev Cancer 5:172–183.

Ross SE, Radomska HS, Wu B, Zhang P, Winnay JN, Bajnok L. 2004. Phosphorylation of C/EBPalpha inhibits granulopoiesis. Mol Cell Biol 24: 675–686.

Scott LM, Civin CI, Rorth P, Friedman AD. 1992. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood 80:1725–1735.

Slomiany BA, D'Arigo KL, Kelly MM, Kurtz DT. 2000. C/EBPalpha inhibits cell growth via direct repression of E2F-DP-mediated transcription. Mol Cell Biol 20:5986–5997.

Tenen DG. 2003. Disruption of differentiation in human cancer: AML shows the way. Nat Rev Cancer 3:89–101.

Timchenko NA, Wilde M, Nakanishi M, Smith JR, Darlington GJ. 1996. CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. Genes Dev 10:804–815.

Umek RM, Friedman AD, McKnight SL. 1991. CCAAT-enhancer binding protein: A component of a differentiation switch. Science 251:288–292.

Wang ND, Finegold MJ, Bradley A. 1995. Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269:1108–1112.

Wang H, Iakova P, Wilde M. 2001. C/EBPalpha arrests cell proliferation through direct inhibition of Cdk2 and Cdk4. Mol Cell 8:817–828.

Westendorf JJ, Yamamoto CM, Lenny N, Downing JR, Selsted ME, Hiebert, SW. 1998. The t(8;21) fusion product, AML-1-ETO, associates with C/EBP-alpha, inhibits C/EBP-alpha-dependent transcription, and blocks granulocytic differentiation. Mol Cell Biol 18:322–333.

Zhang P, Iwama A, Datta MW, Darlington GJ, Link DC, Tenen DG. 1998. Upregulation of interleukin 6 and granulocyte colony-stimulating factor receptors by transcription factor CCAAT enhancer binding protein alpha (C/EBP alpha) is critical for granulopoiesis. J Exp Med 188:1173–1184.

Zhang, P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, Owens BM. 2004. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. Immunity 21:853–863.