From the Department of Medicine III, Grosshadern Hospital
Ludwig-Maximilians-University, Munich
Chair: **Prof. Dr. med. Wolfgang Hiddemann**

Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248

Thesis
Submitted for the award of Ph.D degree in Human Biology
At the Faculty of Medicine
Ludwig-Maximilians-University, Munich

Submitted by
Sheo Mohan Singh

From
Gonda, India

2003

With permission from the Faculty of Medicine
University of Munich

Supervisor/Examiner:	**Prof. Dr. W. Hiddemann**
Second Examiner:	**Prof. Dr. G.W. Bornkamm**
Co-Examiners:	**Prof. Dr. R. Lamerz** **Prof. Dr. Dr. h.c. W. Schramm** **Prof. Dr. B. Emmerich**
Co-Supervisor:	**Dr. Gerhard Behre**
Dean:	**Prof. Dr. Dr. h.c. K. Peter**
Date of Submission:	**07 June, 2002**
Date of Oral Exam:	**22 July, 2003**

TABLE OF CONTENTS PAGE NO.

To my wife Poonam,

Who had the patience to live 7000 miles away from me for 3 long years…

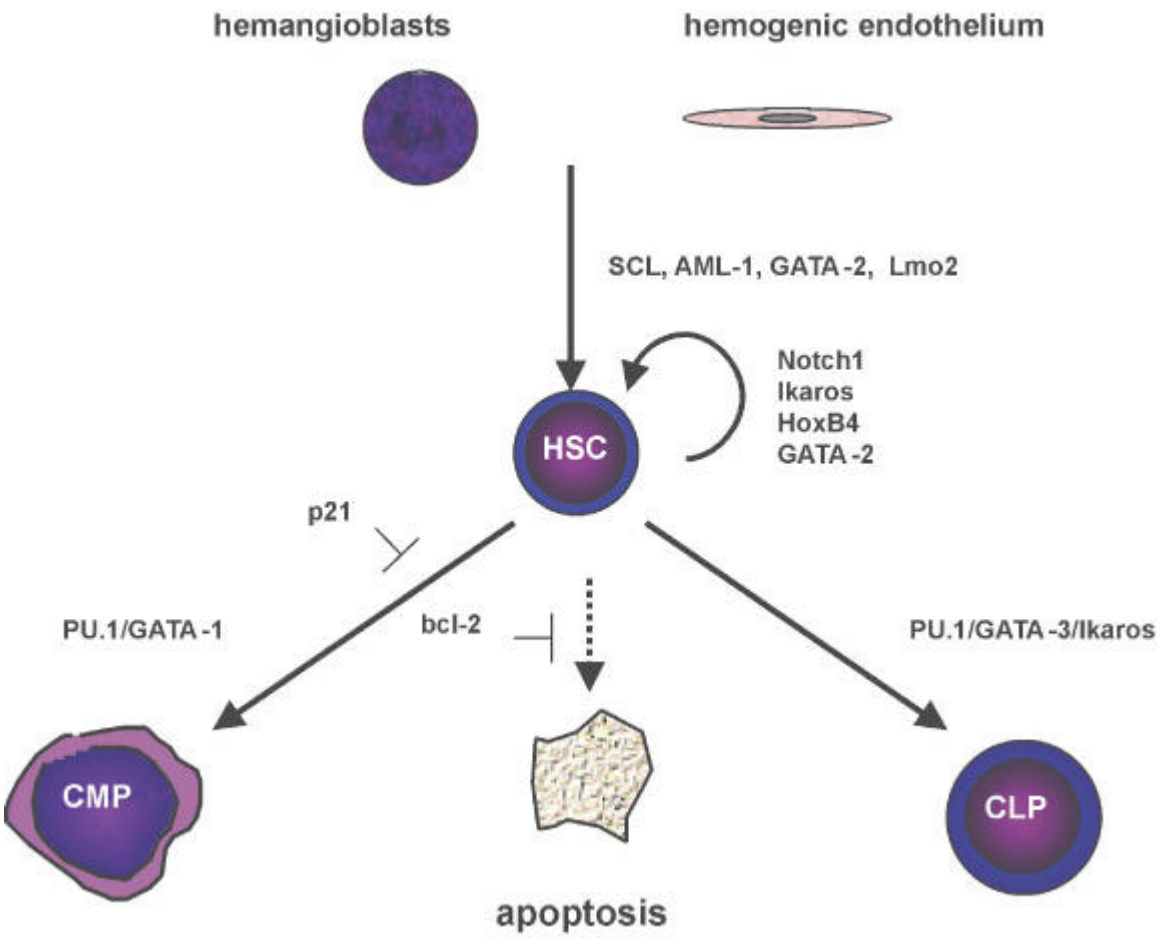
1. INTRODUCTION

1.1 Role of C/EBPa in myelopoiesis

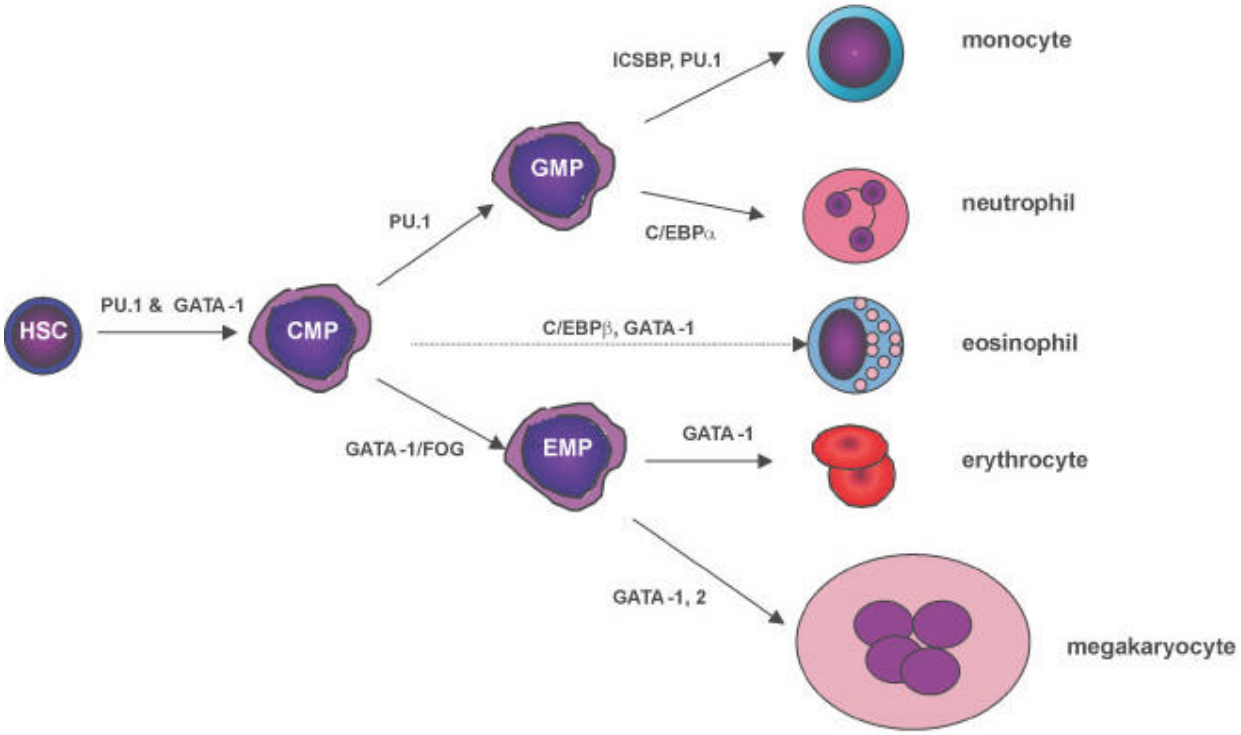
The past two decades have witnessed significant advances in our understanding of the cellular physiology and molecular regulation of hematopoiesis and myelopoiesis. The transcription factors involved in hematopoiesis and myelopoiesis are depicted in **models 1 and 2.**(Zhu and Emerson, 2002)

C/EBPα is one of the most important transcription factors which are involved in granulocytic differentiation. It belongs to CCAAT/enhancer binding proteins (C/EBPs) family of transcription factors that all contain a highly conserved basic-leucine zipper domain at the carboxyl terminus, which is involved in dimerisation and DNA binding. The genes for six C/EBP members have been cloned to date from several species and have been given systematic nomenclature in which members are designated as C/EBP followed by a Greek letter indicating the chronological order of their discovery (C/EBPα-ζ).(Akira et al., 1990; Poli et al., 1990; Descombes et al., 1990; Chang

et al., 1990; Roman et al., 1990; Cao et al., 1991; Williams et al., 1991; Ron and Habener, 1992; Ramji and Foka, 2002)



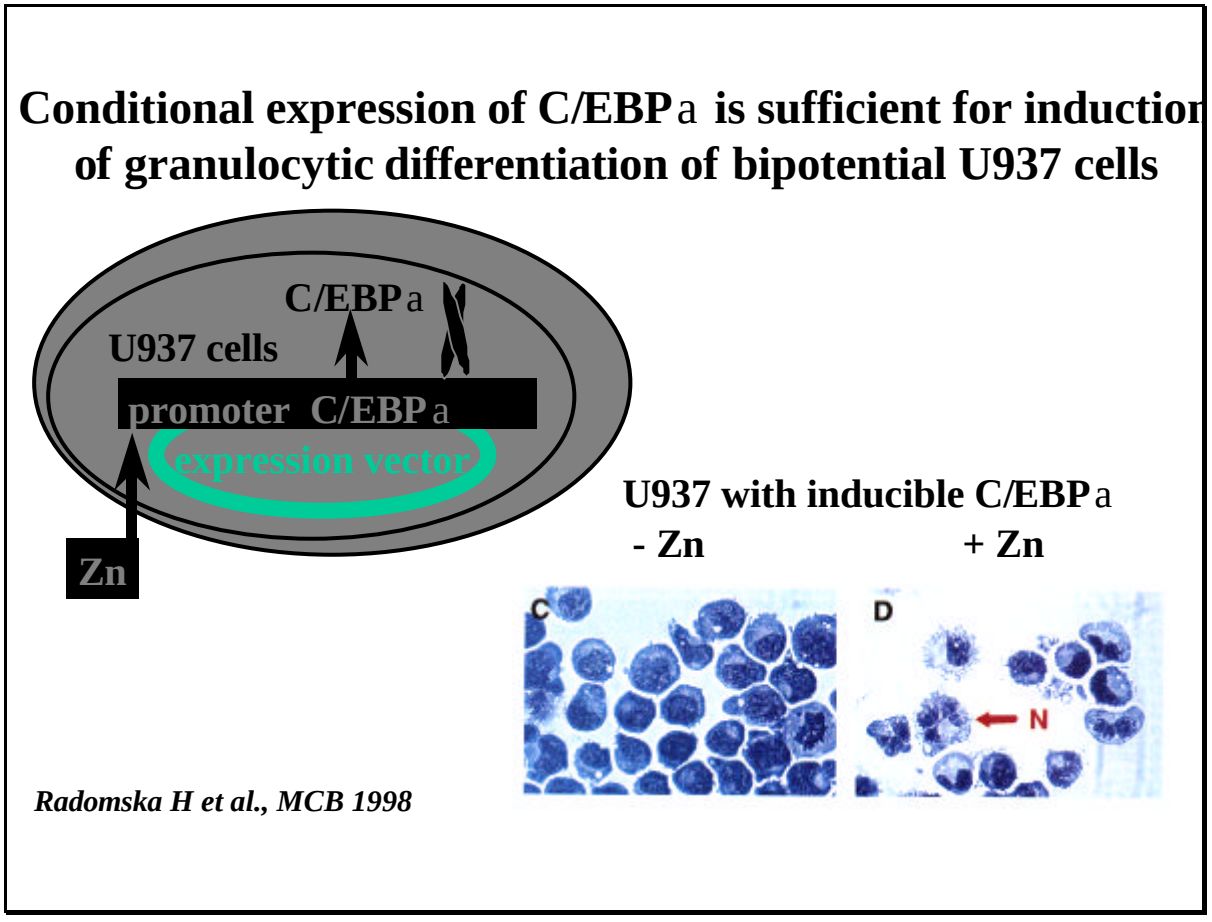
Model 1: Transcriptional regulation of early hematopoietic stem cell development. HSCs, as one progeny of hemangioblasts or hemogenic endothelium, are faced with the cell fate choice either to self-renew or to differentiate into committed common lymphoid or common myeloid hematopoietic precursors. The transcription factors involved in each development direction are depicted.



Model 2: Transcriptional regulation of common myeloid precursor (CMP) commitment. CMPs differentiate into either common precursors for granulocytic and monocytic lineages (GMPs) or common precursors for both erythroid and megakaryocytic lineages (EMPs). A separate, possible, pathway leading to eosinophils is depicted by dotted line. Dual expression of PU.1 and GATA-1 leads HSCs to CMPs, but then dominant expression of PU.1 is restricted to GMPs, while unopposed GATA-1 expression directs differentiation to EMPs.

The transcription factor C/EBPα plays a pivotal role during differentiation in various cell types, including adipocytes and hepatocytes, lung and ovary cells.(Cao et al., 1991; Lin and Lane, 1994; Scott et al., 1992; Flodby et al., 1996; Zhang et al., 1997; Radomska et al., 1998; Umek et al., 1991) In hematopoiesis, C/EBPα is expressed in myeloid cells.(Scott et al., 1992; Radomska et al., 1998) It has been previously demonstrated that the expression of

C/EBPα correlates with the commitment of multipotential precursors to the myeloid lineage, and is specifically upregulated during neutrophilic differentiation.(Scott et al., 1992; Radomska et al., 1998) Furthermore, conditional expression of C/EBPα is sufficient to induce neutrophilic differentiation (Radomska et al., 1998; Wang et al., 1999) and can block the monocytic differentiation program in bipotential myeloid precursors (**model 3**).(Radomska et al., 1998)



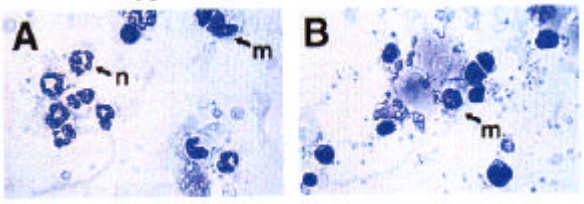
Model 3: Overexpression of C/EBPα leads to granulocytic differentiation.

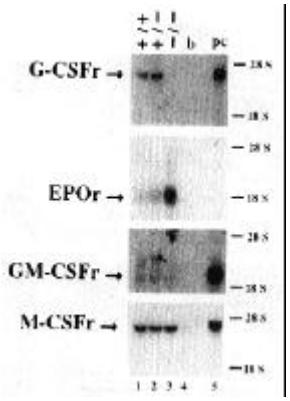
In addition, it has been reported that mice with targeted disruption of C/EBPα gene demonstrate a selective block in differentiation of neutrophils while all the other blood cell types are present in normal proportions. C/EBPα knockout mice do not express granulocyte colony-stimulating factor receptor (GCSFr) that is a critical target gene of C/EBPα. As a result, multipotential myeloid progenitors from the mutant fetal liver are unable to respond to granulocyte colony-stimulating factor (GCSF) signaling, although they are capable of forming granulocyte-macrophage and macrophage colonies in methylcellulose in response to other growth factors (**model 4**).(Zhang et al., 1997)

Absence of neutrophil development and G-CSF receptor expression in C/EBPa deficient mice

C/EBPa

+/- -/-





Zhang DE et al., PNAS 1997

Model 4: Knockout mice of C/EBPα lack neutrophils and GCSFr expression.

Some critical target genes of C/EBPα have been identified and studied which are summarized in the following paragraph. C/EBPα protein activates the CD14 promoter and mediates TGF-β signaling during monocyte development. CD14 is a monocyte/macrophage differentiation marker that is strongly upregulated during monocyte cell differentiation. When myelomonoblastic U937 cells are treated with vitamin D3 and TGF-β, they differentiate toward monocytic cells.

And there is a specific increase in the DNA binding and the expression of C/EBPα and C/EBPβ during U937 monocytic cell differentiation.(Zhang et al., 1997)

C/EBPα, -β, and –δ are also regulated during granulocyte lineage specification. Ford *et al.* have shown that C/EBPα exists as multiple phosphorylated forms in the nucleus of both multipotential and granulocyte-committed hematopoietic progenitor cells. C/EBPβ is unphosphorylated and cytoplasmically localized in multipotential cells but exist as a phosphorylated nuclear enhancer-binding activity in granulocyte-committed cells. GCSF-induced granulocytic differentiation of multipotential progenitor cells results in activation of C/EBPδ expression and functional recruitment of C/EBPδ and C/EBPβ to the nucleus. These results suggest that the C-EBP family members are critical regulators of myeloperoxidase gene expression and are consistent with a model in which a temporal exchange of C/EBP isoforms at the myeloperoxidase enhancer mediates the transition from a primed state in multipotential cells to a transcriptionally active configuration in promyelocytes.(Ford et al., 1996)

Another target gene of C/EBPα in granulopoiesis is c-Myc. Johansen *et al.* identified c-Myc as a C/EBPα negatively regulated gene. They mapped an E2F binding site in the c-Myc promoter as the cis-acting element critical for C/EBPα negative regulation. The identification of c-Myc as a C/EBPα target gene is interesting, as it has been previously shown that down-regulation of c-Myc can induce myeloid differentiation. In this study they show that stable expression of c-Myc from an exogenous promoter not responsive to C/EBPα-mediated down-regulation forces myeloblasts to remain in an undifferentiated state. Therefore, C/EBPα negative regulation of c-Myc is critical for allowing early myeloid precursors to enter a differentiation pathway. This is the first report to demonstrate that C/EBPα directly affects the level of c-Myc expression and, thus, the decision of myeloid blasts to enter into the granulocytic differentiation pathway.(Johansen et al., 2001)

In 32D cl3 myeloblasts cell line the activation of C/EBPα-ER construct by estradiol was sufficient to induce terminal granulocytic differentiation and a G1 cell cycle arrest despite the continued presence of IL-3. bcr-abl^{p210} prevented 32D cl3 cell differentiation, including

myeloperoxidase (MPO) RNA induction. Inhibition of cell growth by C/EBPαWT-ER occurred even in 32D cl3 cells expressing bcr-abl^{p210} or in Ba/F3 B-lymphoid cells, without induction of differentiation. Cell cycle arrest was associated with elevated p27^{Kip1} levels. PU.1 protein and mRNA levels were increased within 4hours of C/EBPαWT-ER activation, in 32D cl3, 32D-bcr-abl^{p210}, or Ba/F3 cells, and induction of PU.1 mRNA occurred even in the presence of cycloheximide, suggesting that induction of endogenous PU.1 RNA by C/EBPαWT-ER results from direct transcriptional activation. However, activation of PU.1-ER(T) in 32D cl3 cells induced MPO RNA but not cell cycle arrest or terminal differentiation. Thus, in 32D cl3 myeloblasts, C/EBPα acts independent of G-CSF signals, directly upstream of PU.1, and upstream of p27^{Kip1} and additional factors to limit proliferation and induce granulocytic differentiation.(Wang et al., 1999)

In a recent study it has been shown that C/EBPs are required for granulopoiesis independent of their induction of the GCSFr.(Wang Qf and Friedman, 2002)

Induction of granulocytic differentiation by C/EBPα seems to be by 2 pathways. In a cell line derived from the fetal liver of C/EBPα-

deficient animals it has been shown that conditional expression of C/EBPα induces the C/EBP family members C/EBPβ and C/EBPε and subsequently granulocytic differentiation. Similar results were obtained when C/EBPα$^{-/-}$ cells were stimulated with the cytokines interleukin-3 and granulocyte-macrophage colony-stimulating factor, but not with all-trans retinoic acid, supporting a model of at least 2 pathways leading to the differentiation of myeloid progenitors to granulocytes and implicating induction of other C/EBP family members in granulopoiesis.(Zhang et al., 2002)

C/EBP family members are also involved in protein-protein interaction and thereby regulate myeloid specific promoters. C/EBPα and GCSFr signals cooperate to induce the myeloperoxidase and neutrophil elastase genes(Wang et al., 2001), C/EBP and AML1 synergistically activate the macrophage colony-stimulating factor receptor (MCSFr) promoter(Zhang et al., 1996), C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter(Oelgeschlager et al., 1996), PU.1 and C/EBPα regulate the granulocyte colony-stimulating factor receptor (GCSFr) promoter in myeloid cells(Smith et al., 1996), and transcriptional coactivator ASC-

2 functionally interact with C/EBPα in granulocytic differentiation of HL60 promyelocytic cells.(Hong et al., 2001)

1.2 Disruption of C/EBPa activity and expression in acute myeloid leukemia

We recently reported that dominant-negative mutations of C/EBPα are found in patients with acute myeloid leukemia of subtypes M1 and M2. Heterozygous mutations were found in *CEBPA* gene in ten patients with acute myeloid leukemia (AML), five mutations in the amino terminus truncated the full-length protein, but did not affect a 30-kD protein initiated further downstream. The mutant proteins block wild-type C/EBPα DNA binding and transactivation of granulocyte target genes in a dominant-negative manner, and fails to induce granulocytic differentiation. It is the first report of *CEBPA* gene mutations in human neoplasia, and such mutations are likely to induce the differentiation block found in AML.(Pabst et al., 2001a)

Furthermore, AML1-ETO associates with C/EBPα, inhibits C/EBPα dependent transcription of myeloid cell specific rat defensin NP-3 promoter, and blocks granulocytic differentiation.(Westendorf et al., 1998) We also demonstrated that the leukemic fusion protein AML1-

ETO, found in patients with acute myeloid leukemia with translocation t(8;21), downregulates both C/EBPα expression and function in primary AML patient samples.(Pabst et al., 2001b)

The hallmark of acute promyelocytic leukemia (APL) is the translocation t(15;17) resulting in PML-RARα fusion protein and a block in promyelocytic differentiation. In primary human APL cells PML-RARα physically interacts with C/EBPα at diagnosis and relapse, but not after All-Trans-Retinoic-Acid (ATRA)-induced remission. Similar results are observed in primary leukemic cells from transgenic mouse models of APL along with marked reduction of C/EBPα DNA binding. Conditional expression of PML-RARα abrogates C/EBPα DNA binding, transactivation, and differentiation of myeloid cell lines in an ATRA-reversible manner. These studies implicate disruption of C/EBPα DNA binding and function by physical interaction with PML-RARα as an additional mechanism contributing to the block in differentiation following expression of the fusion protein in APL (Tracey Lodie and Dan Tenen, pers. communication).

These studies point to the crucial role of C/EBPα in both normal myeloid differentiation and leukemogenesis. However, how the transcriptional activity of C/EBPα is regulated both in normal myelopoiesis as well as in leukemogenesis, is not fully understood.

In a recent review on transcriptional regulation of granulocyte and monocyte development, Friedman, A.D. has set the perspective for the future research in this field. Many questions remain unanswered regarding the transcriptional regulation of granulocyte and monocyte development. With respect to the cellular basis for initiating these lineages: What are the relative contributions of granulocyte/monocyte and B-cell/monocyte progenitors to mature blood elements; do some granulocyte or monocyte progenitors develop directly from pluripotent stem cells; how irreversible are commitment decisions? With respect to gene regulation: Are there additional important transcriptional regulators of myeloid genes remaining to be uncovered via detailed investigation of promoters and distal enhancers; are there lineage-restricted co-activators or co-repressors which participate in lineage specification? With respect to key transcription factors, further clarification of the regulatory network is needed: Which factor or

factors specify each lineage and at what levels of expression; can family members act redundantly in this regard; what additional cooperative mechanisms operate among transcriptional regulators; what roles do cytokine receptor signaling and transcription factor modifications play in each commitment decision and in each step of lineage progression? The answers to these questions will provide general lessons in developmental biology and insights into leukemogenesis and will enable applications in clinical hematology, oncology, and gene therapy.(Friedman, 2002)

1.3 Ras signaling in leukemia and myeloid differentiation

The Ras family of proteins are GTP-dependent molecular switches that are essential for cell growth and differentiation.(Mccormick, 1995; Gutkind, 1998) Ras exerts its effect on cell growth mainly via ETS (Wasylyk et al., 1994) and AP-1 (Johnson et al., 1996) transcription factors. For example, cells with a null mutation in the c-jun gene and expressing oncogenic Ras lack many characteristics of Ras transformation,(Johnson et al., 1993) and dominant negative mutants of ETS-1, ETS-2, or PU.1 containing only the DNA binding domain inhibit Ras activation of transcription and revert Ras-transformed

cells.(Wasylyk et al., 1994) In particular, Ras has been demonstrated to play an important role in myeloid differentiation. Macrophage differentiation and M-CSF-dependent survival are altered in transgenic mice that express dominant suppressors of Ras signaling,(Jin et al., 1995) while a number of hematopoietic cell lines undergo spontaneous myeloid differentiation in response to expression of activated Ras.(Hibi et al., 1993; Maher et al., 1996) In addition, M-CSF, granulocyte-macrophage colony-stimulating factor, or interleukin-3-induced monocytopoiesis of CD34+ cells is inhibited by N-Ras antisense oligonucleotides.(Skorski et al., 1992)

1.4 Aim of this research

Since Ras signaling and the transcription factor C/EBPα both play important roles in myeloid differentiation, we wanted to know how C/EBPα is activated during granulocytic differentiation? We hypothesized that Ras might be involved in the activation of C/EBPα. Experiments were designed to test if Ras changes the DNA binding, expression level or post-translational modification of C/EBPα? After finding that Ras phosphorylates C/EBPα, we designed experiments to map the site of phosphorylation in C/EBPα and to point mutate this site to check the relevance of this phosphorylation. Then we wanted to discover the biological relevance of C/EBPα phosphorylation in myeloid cells by retroviral transduction of C/EBPα WT and C/EBPαS248A mutant and look for its effect on granulocytic differentiation.

Here we demonstrate data which suggest a model where Ras signaling phosphorylates C/EBPα on serine 248 of the transactivation domain resulting in an enhancement of the ability of C/EBPα to transactivate the G-CSF receptor promoter, which contributes to the induction of granulocytic differentiation.

2. MATERIALS AND METHODS

2.1 Cell lines and cell culture

Human kidney 293T cells and human kidney 293E1A cells (kindly provided by John Blenis, Harvard Medical School, Boston, MA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone). 32D cl3 cells were maintained in phenol-red free Iscove's Modified Dulbecco's Medium with 10% heat-inactivated fetal bovine serum (HI-FBS), 1 ng/ml IL-3 (R&D Systems, Minneapolis, MN), and penicillin-streptomycin. φCRE cells were maintained in Dulbecco's Modified Eagle's Medium with 10% heat-inactivated calf serum and pen/strep. pBabePuro-C/EBPα(S248A)-ER was introduced into φCRE cells using lipofectamine (Gibco, BRL, Gaithersburg, MD), and a pool of resistant cells was isolated using 2 μg/ml puromycin. 32D cl3 cells were co-cultured in the presence of 4 μg/ml Polybrene with φCRE-S248A cells which had been irradiated to 3000 cGy. After 48 hrs, the 32D cl3 cells were placed in 96 well dishes with 2 μg/ml puromycin, and individual subclones were isolated. Estradiol was employed at 1 μM. Morphology was assessed by Wright's-Giemsa staining of

cytospins. pBabePuro-C/EBPα(S248A)-ER was constructed by ligating an *Mlu*I/*Nco*I segment containing the mutant serine in place of the identical fragment in pBabePuro-C/EBPαWT-ER. Total cellular extracts were subjected to Western blotting using antiserum recognizing human ERα (Santa Cruz Biotechnology, San Diego, CA) or murine actin (Sigma, St. Louis, MO).

2.2 Reporter constructs and expression plasmids

The human G-CSF receptor promoter ranging from bp -74 to +67 with respect to the major transcription start site (Smith et al., 1996) was subcloned in the firefly luciferase vector pXP2.(Nordeen, 1988) pTK driven by 2 C/EBP sites (p(C/EBP)2TK) is a dimer of the C/EBP site from the granulocyte colony-stimulating factor receptor promoter from bp -57 to bp –37 with respect to the major transcription start site,(Smith et al., 1996) subcloned into pTK81luc, a pXP2-based luciferase construct with a TATA box only as a minimal promoter.(Nordeen, 1988) As an internal control plasmid for co-transfection assays, the pRL-null construct driving a *Renilla* luciferase gene (Promega, Madison, WI) was used as described before.(Behre et al., 1997; Behre et al., 1999; Zhang et al., 1999) The expression

plasmids pMSV-C/EBPα (rat), various deletion mutants of C/EBPα in pMSV, pMSV-C/EBP β, pMSV-C/EBPδ, GAL4-C/EBPα area 1-9, GAL4-C/EBPα area 1-3, and GAL4-C/EBPα area 4-9 and a minimal promoter with GAL4 DNA binding sites were described previously.(Friedman and McKnight, 1990; Oelgeschlager et al., 1996) Human activated pMT3-Ha-Ras(L61) was kindly provided by Larry Feig (Tufts University, Boston, MA).(Feig and Cooper, 1988; Feig, 1988)

To introduce a serine to alanine point mutation at serine 248 of rat C/EBPα we performed PCR site-directed mutagenesis. pcDNA3-C/EBPα was digested with NotI and the resulting small fragment of C/EBPα coding sequence was used as template DNA for all PCR reactions. Four primers were synthesized to amplify two internal fragments from wild type C/EBPα: sense primer A (bp 693-716) and antisense primer B (bp 763-740) were used to amplify fragment AB (size 70 bp), sense primer C (bp 740-763) and antisense primer D (bp 860-837) were used to amplify fragment CD (size 120 bp). The point mutation at bp 742 was introduced in primer B (from A to C) and primer C (from T to G) which overlap with each other (bp 740-763),

so the resulting fragments will have the mutation incorporated during the PCR amplification. The fragments AB and CD were purified from agarose gels using a Gel Extraction kit (Qiagen). These 2 fragments were joined by elongation reaction using primers A and D to create a single fragment AD (size 167 bp) which would have the serine 248 (TCG) mutated to alanine (GCG). This fragment was then digested with SfiI and XcmI and subcloned into SfiI/XcmI digested wild type pcDNA3-C/EBPα. The point mutation was confirmed by DNA sequencing and was used in subsequent experiments.

2.3 Transient transfections using LipofectAMINE Plus and reporter assays for firefly and *Renilla* luciferase

293T cells or 293E1A cells were transfected using LipofectAMINE Plus (Life Technologies) as described by the manufacturer. Firefly luciferase activities from the constructs pG-CSFR, pXP2, pGal4-DBD, pTK and p(C/EBP)2TK and *Renilla* luciferase activity from the internal control plasmid pRL-null were determined 24 h after the initiation of the transfection protocols using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized to the *Renilla* luciferase values of pRL-null. Results are given as means ±

S.E.M. of at least six independent experiments. The following DNA concentrations of the reporter constructs and expression plasmids were used for LipofectAMINE Plus transfections: 0.1 µg of pG-CSFR, pXP2, pGal4-DBD, pTK and p(C/EBP)2TK; 0.01 µg of the internal control plasmid pRL-null; 0.1 µg of the expression plasmids for C/EBPα and C/EBPα mutants and for Ras(L61), and the same concentrations of the empty expression vectors were used as controls, respectively. In the transfections without cotransfection of Ras, the empty vector pMT3 was included instead of pMT3-Ras. pRL-null was chosen as internal control plasmid, because it was not transactivated by Ras in 293T cells.(Behre et al., 1999; Behre et al., 1997)

2.4 Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed as described previously.(Behre et al., 1999; Zhang et al., 1999) As a positive control for C/EBPα binding, a double-stranded C/EBPα probe from the human G-CSF receptor promoter (bp -57 to bp –37 with respect to the major transcription start site, as described before in Fig. 9 of reference (Smith et al., 1996) OligoA: AAG GTG TTG CAA TCC

CCA GC and OligoB: GCT GGG GAT TGC AAC ACC TT) was labeled with Klenow polymerase and [^{32}P]dCTP (NEN Life Science Products) and incubated with 0.1 µg/µl of double-stranded poly(dI-dC) (Sigma) with 1 µl of *in vitro* translated C/EBPα. In some experiments, a 100-fold molar excess of the C/EBP probe was added as specific unlabeled competitor. C/EBPα antibody (14AA) was used for Gel Supershift (Cat # SC61 X, Santa Cruz Biotechnology).

2.5 *In vivo* labeling and phosphopeptide mapping

To detect changes in the phosphorylation pattern of C/EBPα upon stimulation with activated Ras *in vivo*, 0.5 µg of pcDNA3-C/EBPα or pcDNA3-C/EBPαS248A either with or without 0.25 µg of activated Ras(L61), was transfected into 293T cells using LipofectAMINE Plus (Life Technologies). 3 h after transfection, cells were starved in serum-free Dulbecco's modified Eagle's medium. After 18 h, cells were placed into serum-free and phosphate-free Dulbecco's modified Eagle's medium (Life Technologies) for 30 min before they were metabolically labeled with [^{32}P]orthophosphate (2.5 mCi/ml). After 4 h, cells were lysed with radioimmunoprecipitation assay buffer containing 1%

Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 5 mM EDTA, and 50 mM Tris (pH 8.0) and supplemented with aprotinin, phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, antipain, and chymostatin as protease inhibitors (Sigma) and sodium pyrophosphate, sodium fluoride, and sodium vanadate as phosphatase inhibitors (Sigma). In parallel plates, 0.3 µg of the G-CSF receptor promoter was co-transfected in 293T cells, and luciferase activities were determined to ensure that Ras signaling enhances the transactivation function of C/EBPα in the particular experiment used in *in vivo* labeling and subsequent phosphopeptide mapping.

C/EBPα was isolated by immunoprecipitation from the lysates using a rabbit polyclonal antibody against C/EBPα (Santa Cruz), washed four times with radioimmunoprecipitation assay buffer, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose (Bio-Rad) for phosphopeptide mapping. After transfer, the C/EBPα protein bands was excised. To determine the phosphorylated protein residues of C/EBPα, C/EBPα protein bands were digested with 1-chloro-3-tosylamido-7-amino-2-heptanone-treated chymotrypsin (Worthington) and endoproteinase Glu-C (V8 protease) (Boehringer Mannheim) and

processed for phosphopeptide mapping as described previously.(Boyle et al., 1991)

2.6 Western blot assay

24 h after the start of transfection, cells were lysed with radioimmunoprecipitation assay buffer. Equal amounts of total protein were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membrane (Millipore). Membranes were incubated with anti-C/EBPα antibody (Santa Cruz Biotechnology) or β-tubulin antibody as an internal control (catalog no. 1111876; Boehringer Mannheim) for 60 min and then with Protein A-horseradish peroxidase conjugate (Amersham) for 45 min. Signals were detected with ECL Western blotting detection reagents (Amersham). In parallel plates, the G-CSF receptor promoter construct was co-transfected, and luciferase activities were determined to ensure that Ras enhances the transactivation function of C/EBPα in the particular experiment used for Western blot analysis of C/EBPα expression and that the transfection efficacy was the same (less than 10% difference between plates) in the particular experiment. Differences in protein expression were quantitated by ImageQuant software (Molecular Dynamics).

C/EBPα antibody (14AA, SC61) was used for Western Blot and immunoprecipitation (Santa Cruz Biotechnology).

3. RESULTS

3.1 Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor promoter

Since Ras and the regulation of the G-CSF receptor by C/EBPα are both important for myeloid development, we asked whether activated Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor promoter. 293T cells were transfected with a plasmid containing the human G-CSF receptor promoter (Smith et al., 1996) cloned upstream of the luciferase reporter gene along with expression plasmids for C/EBPα and Ras, and reporter gene expression was determined 24 h post-transfection. Transfection of a Ras expression construct significantly enhanced the ability of C/EBPα to transactivate the G-CSF receptor promoter (Fig. 1A). The same effect was demonstrated in myeloid U937 cells as well (Fig. 1D). Western blot analysis for C/EBPα expression demonstrated that activated Ras did not change the expression level of cotransfected C/EBPα (Fig. 1B). Thus, Ras signaling directly acts on the transactivation capacity of C/EBPα. The cooperation of activated Ras with C/EBPα is therefore important for G-CSF receptor promoter activity.

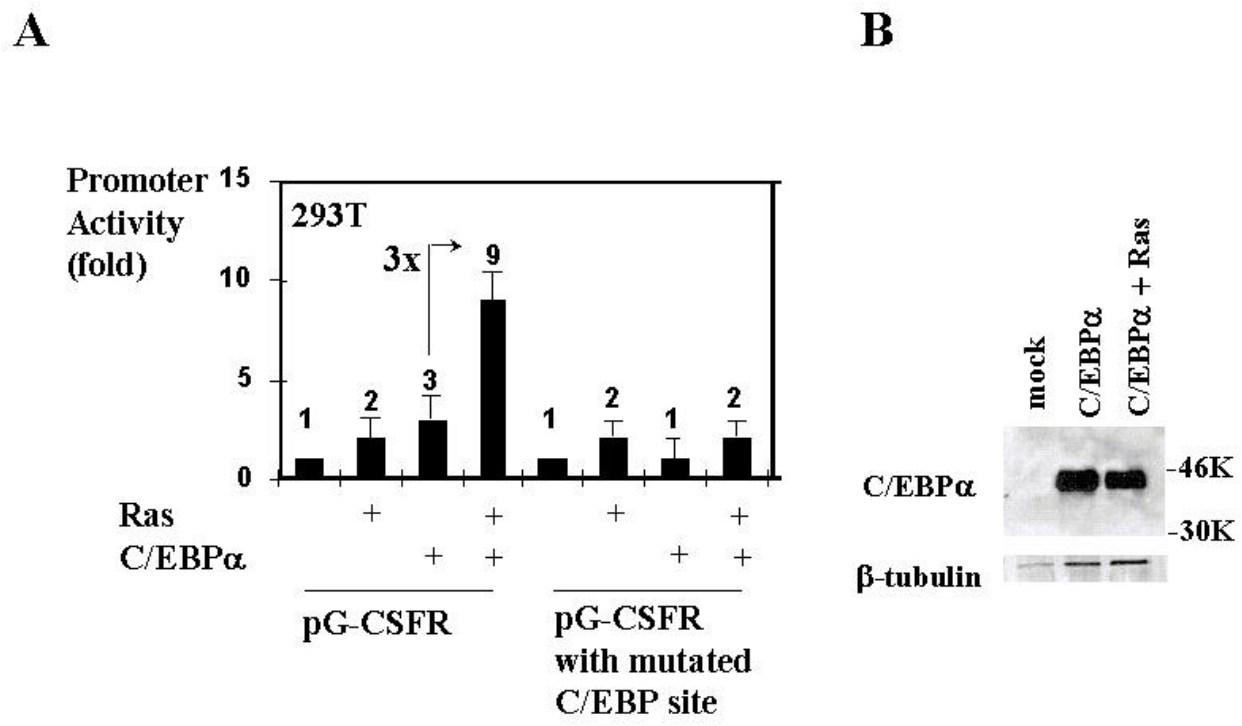


Fig. 1. Ras signaling enhances the transcriptional activity of C/EBPa **. A)** Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor promoter. 293T cells were transfected with the human G-CSF receptor promoter or the human G-CSF receptor promoter with mutated C/EBP sites and with the expression plasmids pMSV-C/EBPα (or empty vector pMSV) and pMT3-Ha-Ras(L61) (or empty vector pMT3). Luciferase activities were determined 24 h after transient transfection with LipofectAMINE Plus and normalized to the activities of the internal control plasmid pRL-null. **B)** Western blot analysis for C/EBPα expression (and β-tubulin expression as loading control) for the experiment shown in Fig. 1A.

3.2 Ras enhances the ability of C/EBPa, but not of C/EBP b or C/EBPd to transactivate a minimal TK promoter driven by C/EBP DNA binding sites

We next asked the following questions: (*a*) whether the binding of C/EBPα to DNA was necessary for its activation by Ras and (*b*) whether a C/EBPα binding site alone was sufficient for the Ras-enhanced C/EBPα activation. We observed enhanced C/EBPα transactivation mediated by activated Ras using a reporter construct containing two C/EBPα binding sites cloned upstream of a minimal TK promoter (p(C/EBP)TK) (Fig. 1C). In control experiments, no effect of Ras on C/EBPα activity was observed when the minimal TK promoter without C/EBPα binding sites was used (Fig. 1C). These data indicate that C/EBPα binding to DNA is necessary for its activation by Ras signaling and that C/EBPα binding sites are sufficient to mediate this effect. Interestingly, the activation of C/EBPα by Ras was specific for C/EBPα, because C/EBP β und C/EBPδ were not activated functionally by Ras signaling in this assay (Fig. 1C).

C

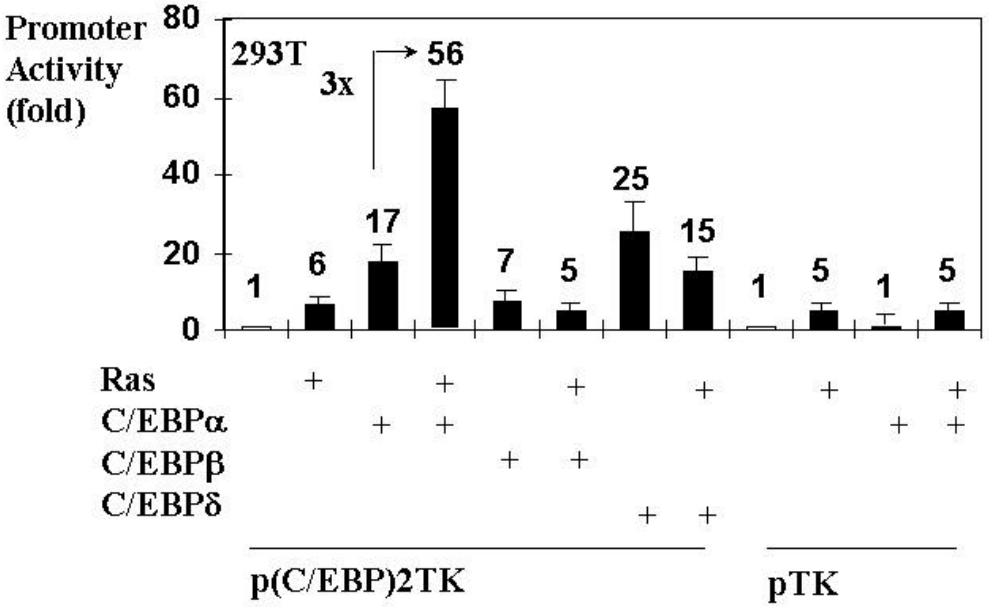


Fig. 1. C) Ras enhances the ability of C/EBPa **, but not of C/EBP** b **or C/EBP**d **to transactivate a minimal TK promoter driven by C/EBP DNA binding sites (p(C/EBP)2TK).** 293T cells were transfected with p(C/EBP)TK or a minimal TK promoter without C/EBP sites (pTK) and with the expression plasmids pMSV-C/EBPα, pMSV-C/EBP β, pMSV-C/EBPδ, and/or pMT3-Ha-Ras(L61) or the respective empty vectors. Luciferase activities were determined 24 h after transient transfection with LipofectAMINE Plus.

D

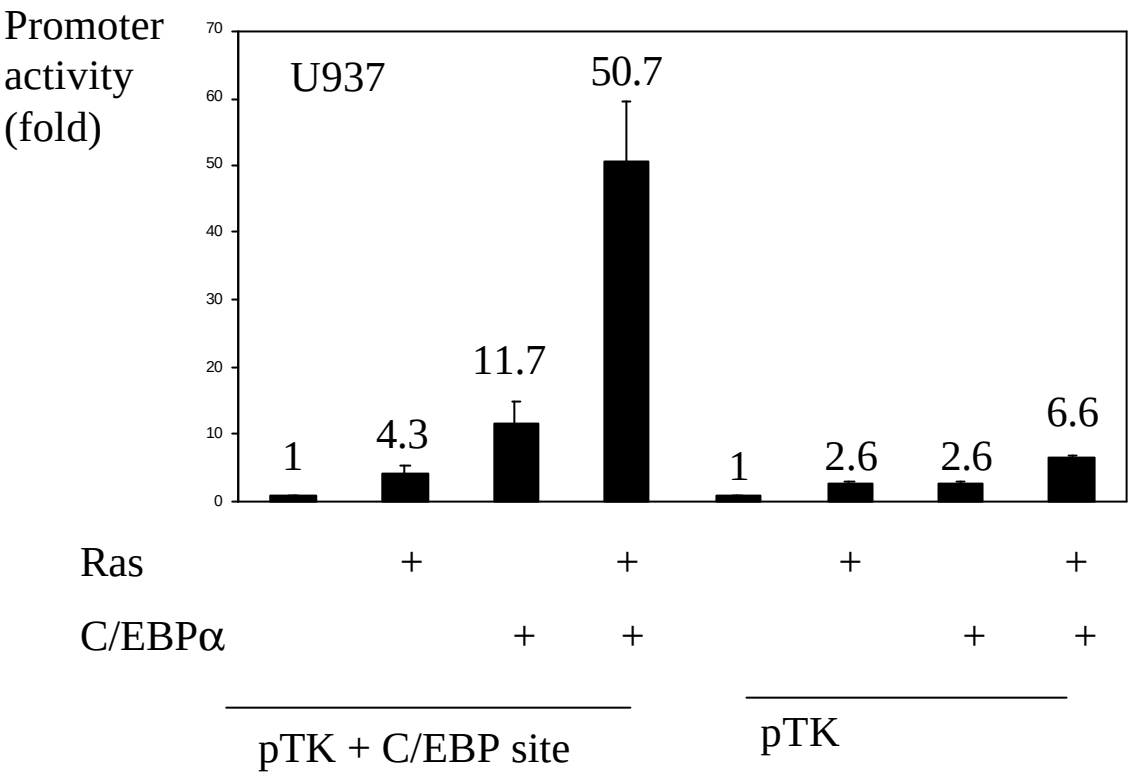


Fig. 1. D) Ras enhances the ability of C/EBPα to transactivate a minimal TK promoter driven by C/EBP DNA binding sites (p(C/EBP)2TK) in myeloid U937 cells.

3.3 Ras enhances the transactivation function of a fusion protein containing a Gal4 DNA binding domain and a discrete region of the C/EBPα transactivation domain

To map the domain of C/EBPα activated by Ras signaling, we used a minimal promoter with GAL4 DNA binding sites and the expression plasmids GAL4-C/EBPα area 1-9, GAL4-C/EBPα area 1-3, and GAL4-C/EBPα area 4-9, using the previously described nomenclature (Smith et al., 1996) to refer to the areas of the C/EBPα transactivation domain (Fig. 2A and 2B). Ras signaling activated a fusion protein containing the GAL4-DNA binding domain and the transactivation domains of C/EBPα (areas 1 to 9). Areas 1-3 of C/EBPα were not activated by Ras, whereas areas 4-9 were still activated by Ras. These data indicate that Ras signaling might act on areas 4-9 of the transactivation domain of C/EBPα.

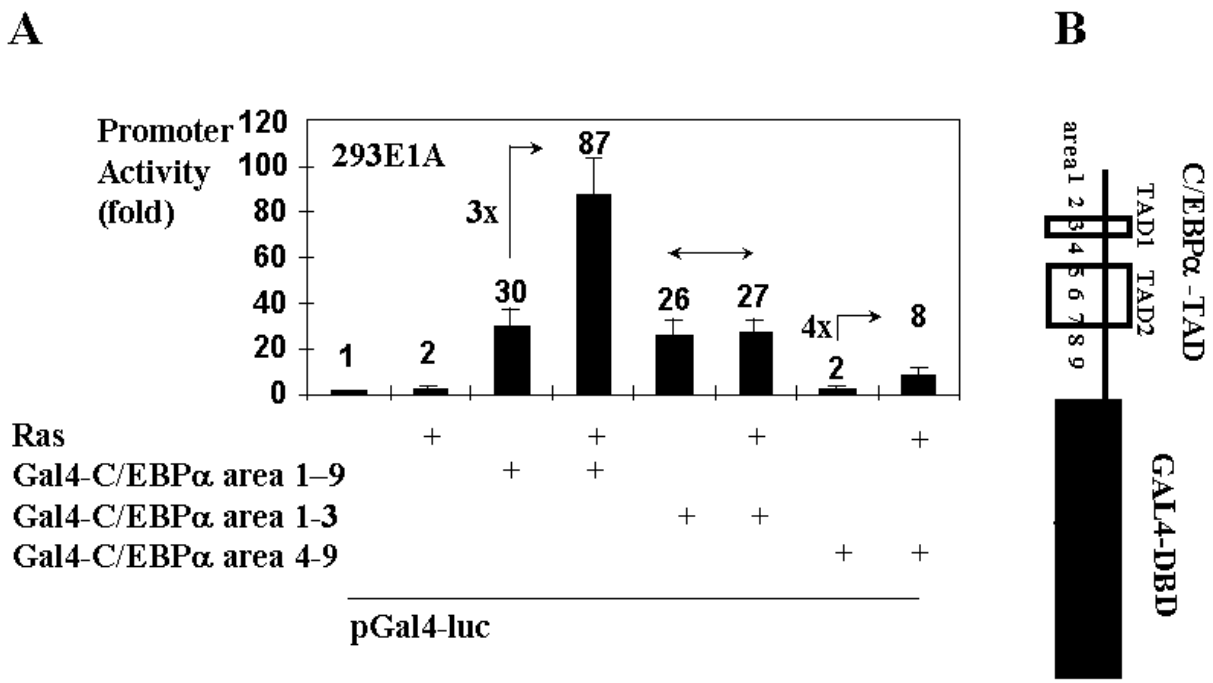


Fig. 2. Ras signaling activates C/EBPa **via the C/EBP**a **transactivation domain.** **A)** Ras enhances the transactivation function of a fusion protein containing a Gal4 DNA binding domain and areas 4-9 of the C/EBPα transactivation domain. 293E1A cells were transfected with a minimal promoter driven by GAL4 DNA binding sites and the expression plasmids GAL4-C/EBPα area 1-9, GAL4-C/EBPα area 1-3, GAL4-C/EBPα area 4-9, and/or pMT3-Ha-Ras(L61). **B)** Model of the construct GAL4-C/EBPα area 1-9 used in Fig. 2A. The transactivation domain of C/EBPα (area 1 to 9) is fused to the DNA binding domain of GAL4.

3.4 Ras does not change DNA binding of C/EBPα

To further elucidate the mechanism by which Ras augments the transcriptional activity of C/EBPα, we performed experiments to determine whether the activation of C/EBPα by Ras was partly due to an increase of C/EBPα DNA binding. An electrophoretic mobility-shift assay was performed using a bp -57 to bp -37 G-CSF receptor promoter fragment containing its C/EBPα binding site.(Smith et al., 1996) A positive control, *in vitro* translated C/EBPα, specifically bound to this probe as expected (Fig. 2C). Nuclear extracts from 293T cells transfected with the expression plasmid of C/EBPα with or without cotransfection of Ras bound to the oligo as well, and most importantly, Ras signaling did not change the DNA binding of C/EBPα. To rule out different C/EBPα protein contents of the nuclear extracts used, we performed a Western blot analysis for C/EBPα expression (Fig. 2D) which showed equal loading of C/EBPα. These results indicate that binding of C/EBPα to the G-CSF receptor promoter DNA is not enhanced by Ras signaling.

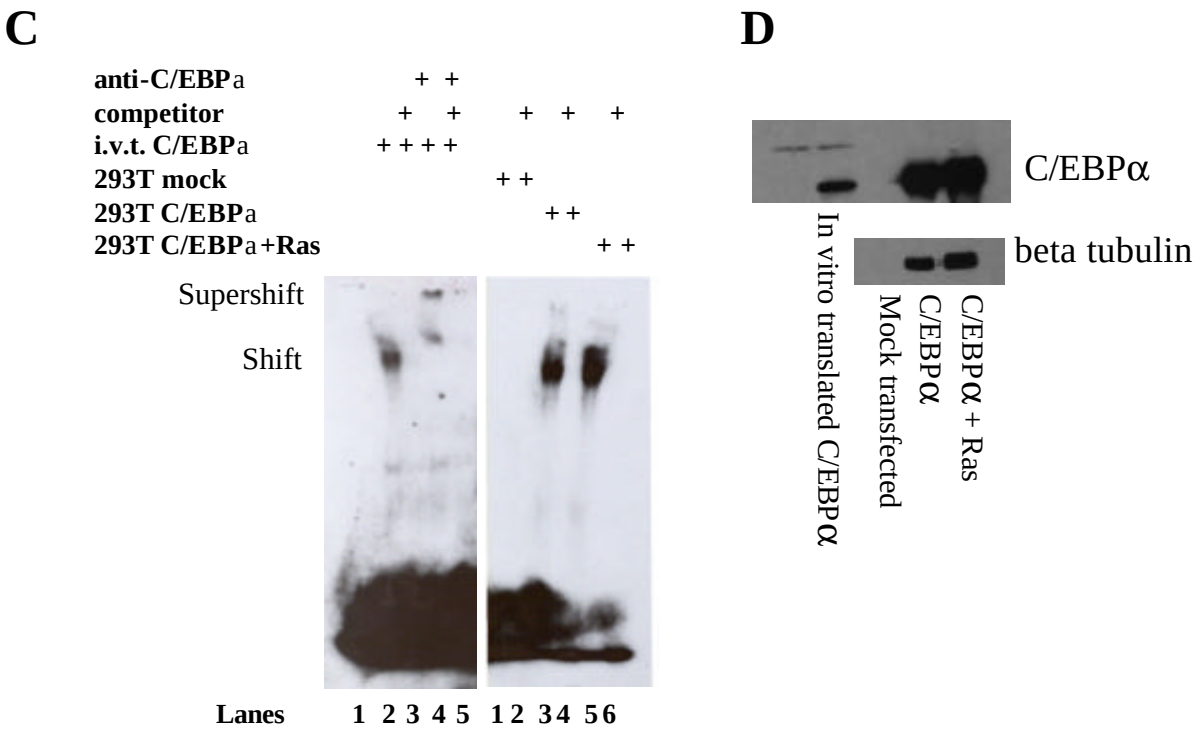


Fig. 2. C) Ras does not change DNA binding of C/EBPa **.** A C/EBPα probe from the human G-CSF receptor promoter (bp -57 to bp –37 with respect to the major transcription start site, as described before in Fig. 9 of reference(Smith et al., 1996)) was used in an electrophoretic mobility shift assay of C/EBPα DNA binding. As controls, probes were incubated with no added protein, 1 µl of *in vitro* translated C/EBPα), *in vitro* translated C/EBPα plus a 100-fold molar excess of self-unlabeled competitor, *in vitro* translated C/EBPα plus an antibody against C/EBPα for supershift (Santa Cruz), or *in vitro* translated C/EBPα plus a 100-fold molar excess of self-unlabeled competitor plus an antibody against C/EBPα for supershift (Santa Cruz). On the right site, with nuclear extracts from 293T cells transfected with the expression plasmids pMSV-C/EBPα and pMT3-Ha-Ras(L61). **D)** Western blot analysis for C/EBPα expression (and β-tubulin expression as loading control) for the experiment shown in Fig. 2C.

3.5 Ras activates C/EBPa via area 9 of the C/EBPa TAD

To further bcalize the site of C/EBPα activated by Ras signaling and to fine map this site, we used various deletion mutants of C/EBPα (Fig. 3A, 3B). C/EBPαΔ4-9 was minimally activated and C/EBPαΔ9 was not activated by Ras. All other mutants were activated at least 2-fold by Ras. These studies demonstrate that Ras acts on area 9 of C/EBPα.

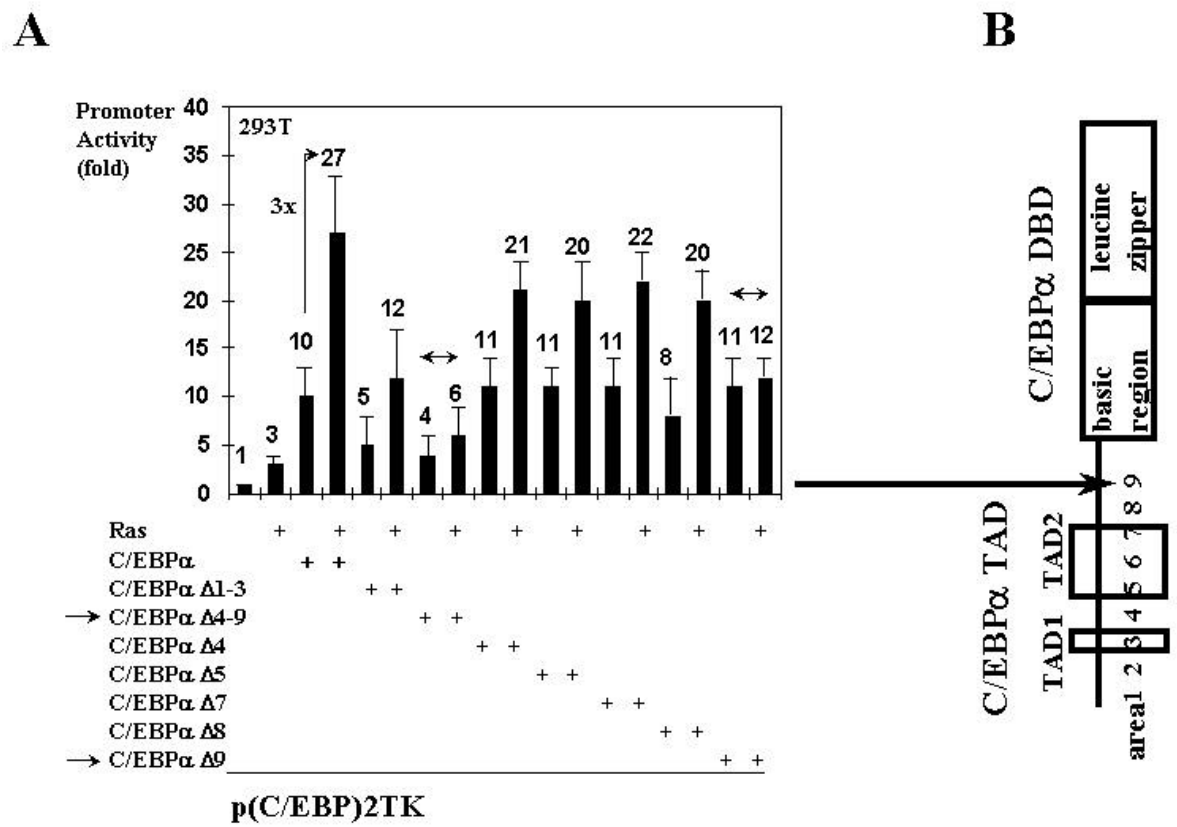


Fig. 3. Ras signaling requires serine 248 of C/EBPa **to activate C/EBP**a **. A)** Ras activates C/EBPα via area 9 of the C/EBPα transactivation domain. 293T cells were transfected with p(C/EBP)2TK and the expression plasmids pMSV-C/EBPα or various deletions mutants of C/EBPα and/or pMT3-Ha-Ras(L61). **B)** Model of C/EBPα, with areas 1-9 containing the first and second transactivation domain (TAD1 and TAD2) and the DNA binding domain of C/EBPα with the basic region and leucine zipper region.

3.6 Ras activates C/EBPα wild type but not a S248A mutant

Because Ras acts on area 9 of the transactivation domain of C/EBPα, we focused on this area for generating C/EBPα point mutants. Area 9 of C/EBPα is depicted in detail in Figure 4B. There is only one potential phosphorylation consensus site in area 9, at serine 248. We generated a point mutant of C/EBPα at serine 248 by mutation to alanine (C/EBPαS248A). C/EBPα wild type and C/EBPαS248A both transactivated the G-CSF receptor promoter 20 fold. While Ras signaling enhanced wild type C/EBPα transactivation capacity from 20 fold to 60 fold, Ras only enhanced the transactivation capacity of the S248A mutant of C/EBPα from 20 to 29 fold (Fig. 3C). These results suggest that Ras signaling might act on serine 248 of the C/EBPα transactivation domain to enhance C/EBPα transcriptional activity.

C

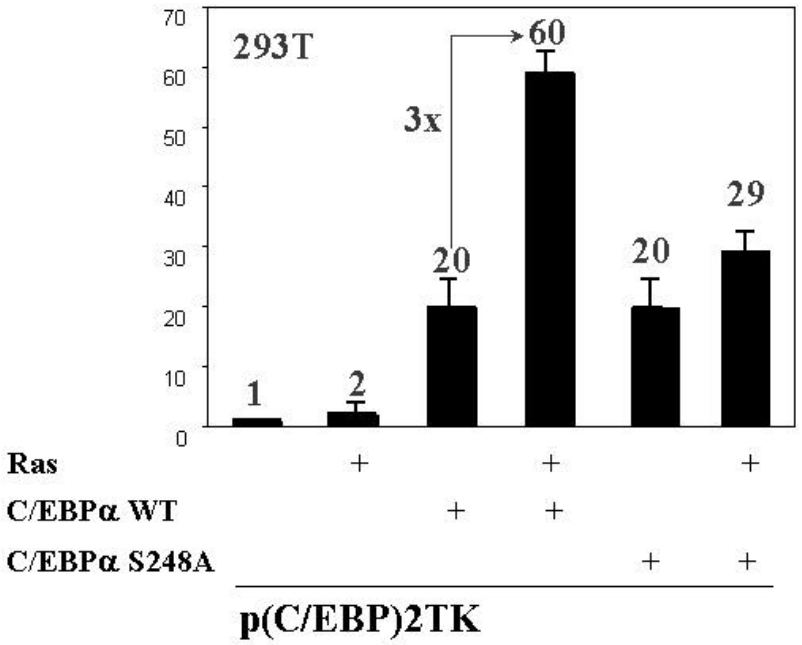


Fig. 3. C) Ras activates C/EBPα wild type, but not a serine 248 to alanine mutant of C/EBPα. 293T cells were transfected with p(C/EBP)2TK and the expression plasmids pcDNA3-C/EBPα or pcDNA3-C/EBPαS248A and/or pMT3-Ha-Ras(L61). Luciferase activities were determined 24 h after transient transfection with LipofectAMINE Plus and normalized to the activities of the internal control plasmid pRL-null.

3.7 A PKC inhibitor blocks the activation of C/EBPa by Ras

Because serine 248 is a potential protein kinase C (PKC) consensus site (Fig. 4B), we asked whether a PKC inhibitor (Staurosporine at 20 nM final concentration) could block the activation of C/EBPα by Ras signaling. In fact, pretreatment of 293T cells with Staurosporine abolished the activation of C/EBPα by Ras signaling (Fig. 4A). These results support the hypothesis that the PKC pathway could be involved in the Ras mediated activation of C/EBPα.

A

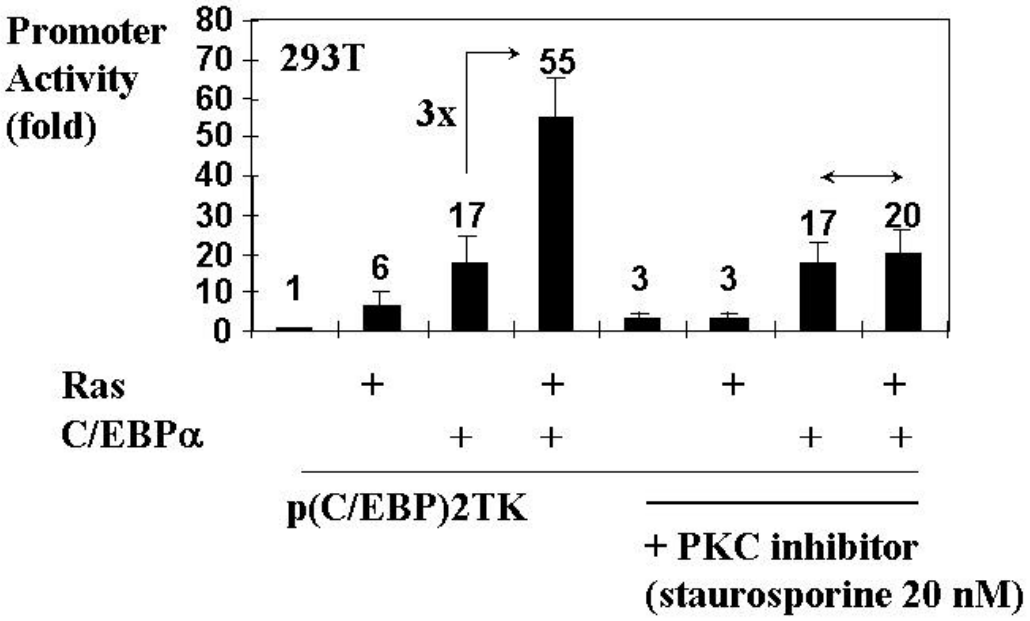


Fig. 4. Activation of C/EBPa **by Ras signaling is PKC dependent. A)** A PKC inhibitor blocks the activation of C/EBPα by Ras. 293T cells were transfected with p(C/EBP)2TK and the expression plasmids pMSV-C/EBPα and/or pMT3-Ha-Ras(L61). Cells were pretreated with a PKC inhibitor (staurosporine at 20 nM) or the vehicle. Luciferase activities were determined 24 h after transient transfection with LipofectAMINE Plus and normalized to the activities of the internal control plasmid pRL-null.

B

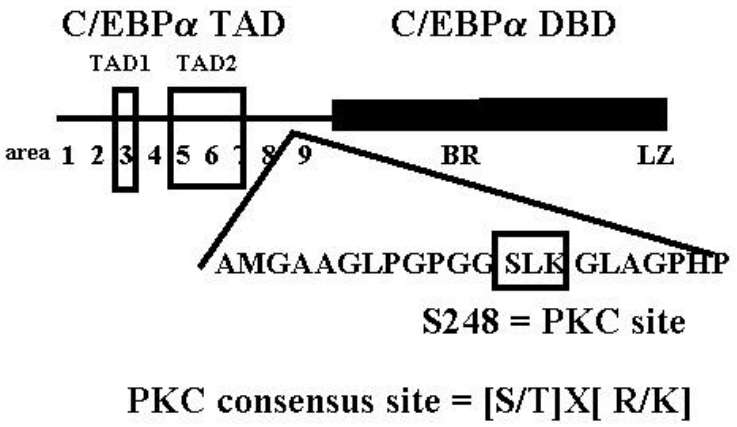


Fig. 4. B) Model of C/EBPα, with areas 1-9 containing the first and second transactivation domain (TAD1 and TAD2) and the DNA binding domain of C/EBPα with the basic region and leucine zipper region. Area 9 is depicted in detail. The amino acid sequence of area 9 contains only one potential phosphorylation site, serine 248, which represents a PKC consensus site.

3.8 Ras phosphorylates C/EBPα at S248 *in vivo*

Because our data suggest that Ras activates C/EBPα via phosphorylation on serine 248, we wanted to prove that Ras signaling, in fact, phosphorylates C/EBPα on serine 248 *in vivo*. 293T cells were transfected with p(C/EBP)2TK and with the expression plasmids for C/EBPα or C/EBPαS248A and/or activated Ras. Phosphopeptide mapping of cells transfected with C/EBPα, C/EBPαS248A, C/EBPα plus Ras, and C/EBPαS248A plus Ras was performed. Ras signaling (Fig. 5C) enhanced a phosphorylation spot in the C/EBPα pattern (Fig. 5A) which was not visible in C/EBPαS248A (Fig. 5B) or C/EBPαS248A plus cotransfected Ras (Fig. 5D). Thus, we conclude that Ras signaling phosphorylates C/EBPα on serine 248 *in vivo*.

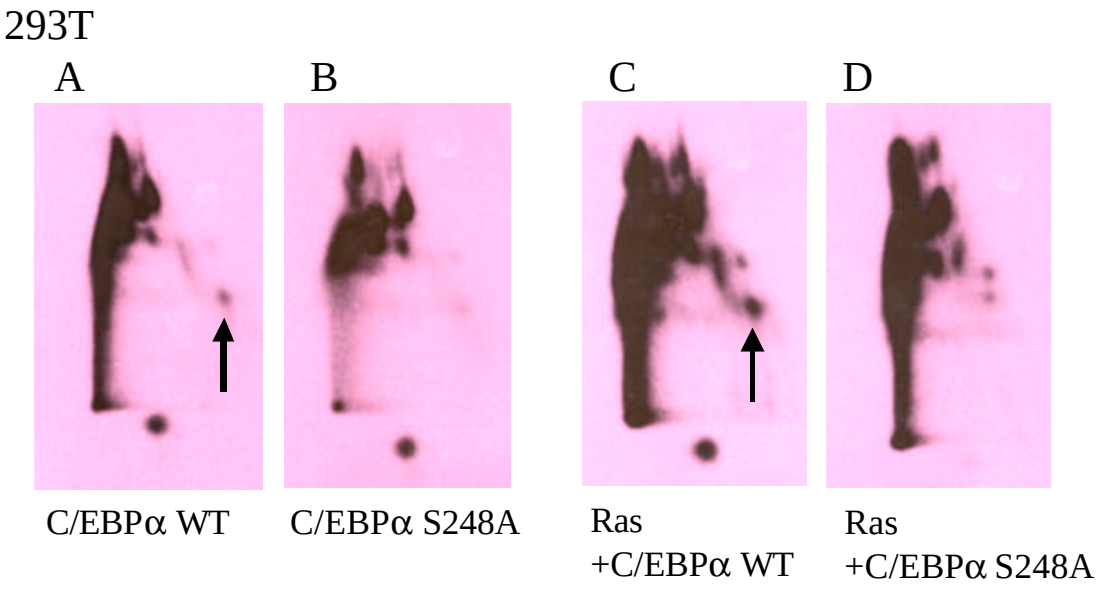


Fig. 5. Ras signaling phosphorylates C/EBPa **at serine 248 *in vivo*.** 293T cells were transfected with p(C/EBP)2TK and the expression plasmids pcDNA3-C/EBPα or pcDNA3-C/EBPαS248A and/or pMT3-Ha-Ras(L61). Phosphopeptide mapping of C/EBPα **(A)**, C/EBPαS248A **(B)**, C/EBPα plus Ras **(C)**, and C/EBPαS248A plus Ras **(D)** was performed. The arrow indicates a phosphopeptide present in C/EBPα WT cells and enhanced by Ras, but not present in C/EBPα S248A cells, even after Ras activation.

E

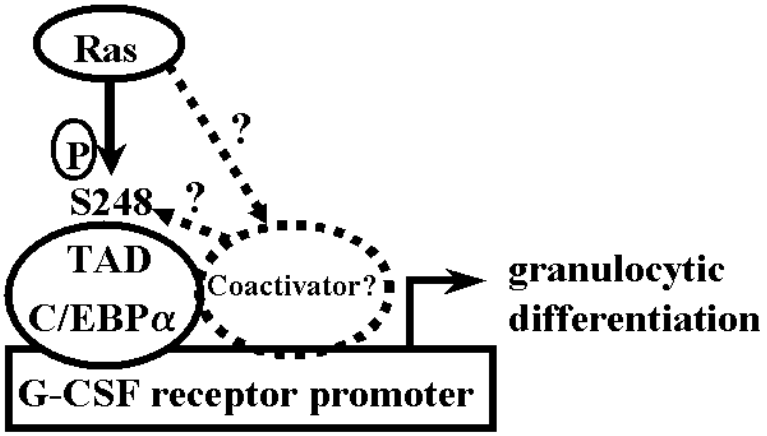


Fig. 5. E) Model of how Ras enhances the ability of C/EBPa **to transactivate the G-CSF receptor promoter by phosphorylation on serine 248**. Our data suggest a model in which Ras signaling phosphorylates C/EBPα on serine 248 of the transactivation domain resulting in an enhancement of the ability of C/EBPα to transactivate the G-CSF receptor promoter. We hypothesize that partly this enhancement could be mediated by recruitment of a co-activator (see discussion). Our data suggest that Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248.

3.9 Mutation of serine 248 to alanine obviates the ability of C/EBPα to induce differentiation

To determine the biological importance of serine 248, we linked C/EBPα(S248A) to the human Estradiol Receptor (ERα) ligand-binding domain and isolated 32D cl3 cell lines expressing this protein. Two subclones with protein expression as high as that we previously achieved for C/EBPαWT-ER were obtained (Fig. 6A). In 32D-C/EBPα(S248A)-ER-1 cells, the full-length species reproducibly migrated faster than that found for clone 2 cells or for cells expressing the wild-type protein. Smaller species, likely representing degradation products, and a larger species (arrow), likely representing a dimer formed via the leucine zipper, also ran faster than comparable bands in the other lanes. Perhaps this difference in migration represents altered phosphorylation at serine 248 or at another residue. It is not clear why this faster migration was only seen with one subclone, but may represent heterogeneity in the 32D cl3 cells themselves.

To assess induction of differentiation by C/EBPα(S248A)-ER, each of the cell lines was placed in estradiol. Morphologically, after 3 days the majority of 32D-C/EBPα(WT)-ER cells had differentiated to

neutrophils (Fig. 6C, right panels), whereas little morphologic change was seen with 32D-C/EBPα(S248A)-ER-1 cells and clone 2 developed primary granules without nuclear maturation (Fig 6C, left and middle panels). To further assess differentiation, RNAs prepared daily from cultures exposed to estradiol were subjected to Northern blotting for MPO, LF, and b-actin (Fig. 6B). As reported previously, C/EBPαWT-ER rapidly induced MPO and induced LF after 48 hrs. In contrast, in 32D-C/EBPα(S248A)-ER-1 cells, neither MPO nor LF were induced, and in clone 2 cells MPO was induced in a delayed fashion and LF was not induced. Mutation of serine 248 to alanine did not prevent the growth inhibitory properties of C/EBPαWT-ER in 32D cl3 cells (data not shown).

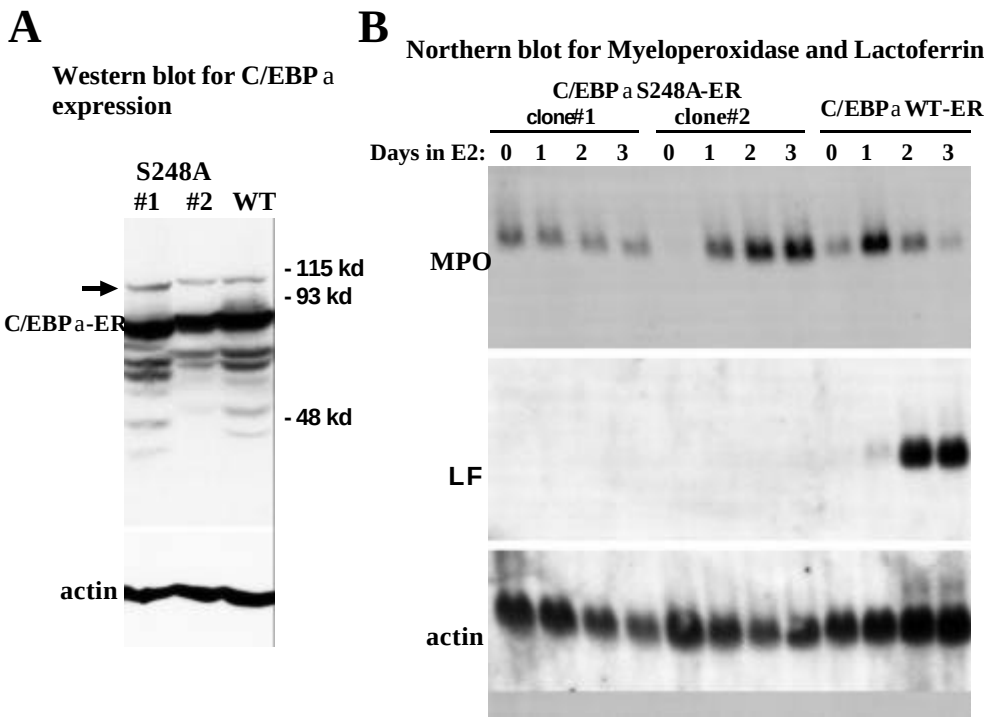


Fig. 6. Mutation of serine 248 to alanine obviates the ability of C/EBPa **to induce differentiation. A)** To determine the biological importance of serine 248, we linked C/EBPα(S248A) to the human Estradiol Receptor (ERα) ligand-binding domain and isolated 32D cl3 cell lines expressing this protein. Two subclones with protein expression as high as that we previously achieved for C/EBPαWT-ER were obtained. **B)** To further assess differentiation, RNAs prepared daily from cultures exposed to estradiol were subjected to Northern blotting for MPO, LF, and b-actin. As reported previously, C/EBPαWT-ER rapidly induced MPO and induced LF after 48 hrs. In contrast, in 32D-C/EBPα(S248A)-ER-1 cells, neither MPO nor LF were induced, and in clone 2 cells MPO was induced in a delayed fashion and LF was not induced.

C

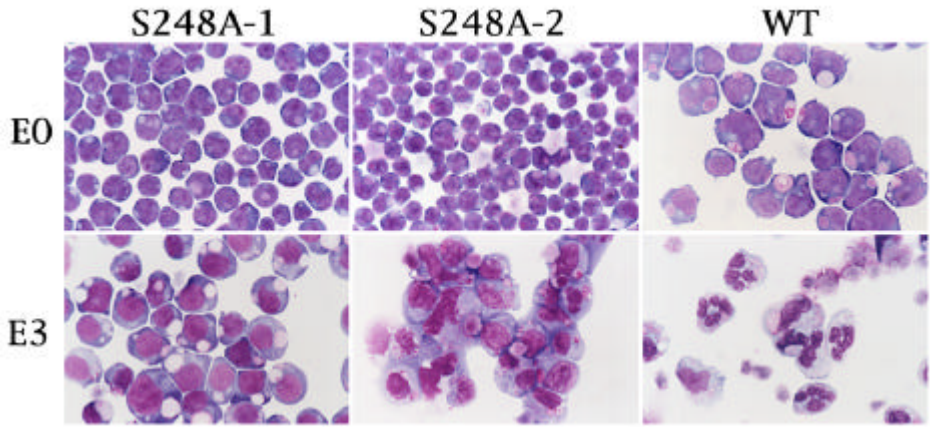


Fig. 6. C) To assess induction of differentiation by C/EBPα(S248A)-ER, each of the cell lines was placed in estradiol. Morphologically, after 3 days the majority of 32D-C/EBPα(WT)-ER cells had differentiated to neutrophils (right panels), whereas little morphologic change was seen with 32D-C/EBPα(S248A)-ER-1 cells and clone 2 developed primary granules without nuclear maturation (left and middle panels).

4. DISCUSSION

As Ras signaling and the transcription factor C/EBPα play important roles in myeloid differentiation, we hypothesized that Ras might be involved in the activation of C/EBPα. Here we demonstrate that Ras signaling phosphorylates C/EBPα on serine 248 of the transactivation domain resulting in an enhancement of its transactivation capacity.

These data link the Ras pathway, which is involved in myelopoiesis (Crespo and Leon, 2000) and acute myeloid leukemia(Schaich et al., 2001; Zuber et al., 2000) to the differentiation inducing C/EBPα pathway. Thus, activated Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor promoter and a minimal TK promoter containing C/EBP DNA binding sites from 10 fold to 40 fold (Fig. 1). The major role of phosphorylation sites in the activation and deactivation of C/EBP family members has already been described by other groups. Ford *et al.* suggested that C/EBPα exists in multiple phosphorylated forms in the nucleus of both multipotential and granulocytic-committed cells. (Ford et al., 1996) Furthermore, it has been shown that C/EBPβ is expressed in both phosphorylated and

unphosphorylated forms in blasts from patients with acute myeloid leukemia.(Iida et al., 2000) In terms of C/EBPβ, a regulation by the Ras signal transduction pathway and Ras-induced phosphorylation of C/EBPβ on threonine-235 is already known.(Nakajima et al., 1993) Furthermore, the MAP kinases ERK1 and ERK2 have been reported to mediate interferon-gamma responsiveness of C/EBPβ.(Hυ ετ αλ., 2001)

Regarding C/EBPα, Mahoney *et al.* reported that phosphorylation of C/EBPα *in vitro* by protein kinase C attenuates its site-selective DNA binding.(Mahoney et al., 1992) Subsequently, Pan *et al.* could identify a specific increase in DNA binding and the expression of C/EBPα and C/EBPβ during U937 monocytic cell differentiation, and related this to the transforming growth factor β signaling pathway.(Pan et al., 1999) Our data do not relate the effect of Ras signaling to the DNA binding domain or DNA binding capacity of C/EBPα. Ras signaling acts on the C/EBPα transactivation domain, because it enhances 4 fold the transactivation function of a fusion protein containing a Gal4 DNA binding domain and the C/EBPα transactivation domain (Fig. 2A), and

does not change the C/EBPα DNA binding capacity (Fig. 2C). In fact, Ras acts on serine 248 of the C/EBPα transactivation domain, because it does not enhance the 10-fold transactivation exhibited by a C/EBPα mutant with a deletion of area 9 (Fig. 3A) or a serine 248 to alanine point mutant in area 9 (Fig. 3C). Thus, we conclude that the Ras pathway acts on the transactivation functions of C/EBPα, via serine 248.

Ross *et al.* reported recently on other important phosphorylation sites in the transactivation domain of C/EBPα.(Ross et al., 1999; Ross et al., 2000) The insulin pathway activates GSK3 kinase which in turns phosphorylates C/EBPα on threonines 222 and 226.(Ross et al., 1999) This activation of C/EBPα is blocked by the Wnt signaling pathway.(Ross et al., 2000) Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of the adipogenic transcription factors C/EBPα and peroxisome proliferator- activated receptor gamma (PPARγ). When Wnt signaling in preadipocytes is prevented by overexpression of axin or dominant-negative TCF4, these cells differentiate into adipocytes.(Ross et al., 2000) Our data in 293T cells point to serine 248 as the major Ras-dependent

phosphorylation site of C/EBPα. Serine 248 of C/EBPα is a PKC consensus site, and a PKC inhibitor blocks the activation of C/EBPα by Ras (Fig. 4A). However, staurosporine is not completely specific for PKC. Therefore, we have not excluded other kinases being responsible for the activation of C/EBPα by Ras.

The serine to alanine mutant of C/EBPα did not completely eliminate the activation of C/EBPα by Ras (Fig. 3C) whereas deletion of region 9 did (Fig. 3A). Perhaps a transcriptional co-activator binds optimally to C/EBPα when serine 248 is phosphorylated (Fig. 5E), but can still bind weakly to the S248A mutant. In this model the effect of Ras on C/EBPα transactivation is at least partly indirect, via a co-activator. This model is supported by the ability of Ras to induce basal activity of p(C/EBP)TK; on the other hand our observation that Ras increases phosphorylation of a C/EBPα peptide, but not of the same peptide from C/EBPαS248A suggests a more direct effect on C/EBPα by Ras (Fig. 5E). Because Ross *et al.* had reported that serine 230 was also phosphorylated by insulin signaling *in vivo*, but not by GSK3,(Ross et al., 1999; Ross et al., 2000) it is also possible that phosphorylation of serine 230 might account for additional effects of Ras on C/EBPα.

However, we could not observe any loss of enhancement of transactivation capacity of C/EBPα by Ras signaling using the serine 230 to alanine mutant of C/EBPα (data not shown, mutants kindly provided by Dr. MacDougald, Ann Arbor). However, the very likely additional Ras-dependent phosphorylation sites in C/EBPα besides serine 248 (Fig. 5A-D) still need to be mapped and to be tested functionally. Our current studies to identify interacting proteins and interacting phosphoproteins of C/EBPα using proteomics techniques (2D-gel electrophoresis of C/EBPα interacting proteins and phosphoproteins and identification of those proteins by mass spectrometry) might also contribute to answer the question whether the effect of Ras signaling on serine 248 of C/EBPα requires a co-activator binding to serine 248 or not.

Mutation of serine 248 to alanine had marked biologic consequences in 32D cl3 cells, obviating the ability of C/EBPα to induce differentiation (Fig. 6). Whether this represents a defect in Ras-dependent phosphorylation of this serine and consequent alteration of trans-activating properties in myeloid cells or whether it represents a more indirect effect in which a Ras-dependent pathway interacts with serine

248 or with another site in C/EBPα affected by mutation of serine 248 to alanine remains to be determined.

Further studies need to address the biological significance of serine 248 of C/EBPα by testing the serine to alanine 248 C/EBPα mutant in biological assays of C/EBPα function such as in rescue assays in C/EBPα knock-out mice. Of note, G-CSF, but not IL-3, signals cooperate with C/EBPα to induce myeloid genes in Ba/F3 lymphoid cells.(Wang et al., 2001) Because impairment of C/EBPα function can contribute to the pathogenesis of acute myeloid leukemia,(Pabst et al., 2001a; Pabst et al., 2001b) it needs to be addressed whether serine 248 of C/EBPα is mutated in patients with acute myeloid leukemia and/or whether inactivating Ras mutations in acute myeloid leukemia might lead to a loss of C/EBPα function.

In a nutshell, our data suggest so far that Ras signaling phosphorylates C/EBPα on serine 248 of the transactivation domain resulting in an enhancement of the ability of C/EBPα to transactivate the G-CSF receptor promoter. Furthermore, our data suggest a model where Ras

signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248.

5. SUMMARY

The transcription factor C/EBPα regulates early steps of normal granulocyte differentiation since mice with a disruption of the C/EBPα gene do not express detectable levels of the G-CSF receptor and produce no neutrophils. We have recently shown that C/EBPα function is also impaired in acute myeloid leukemias. However, how the transcriptional activity of C/EBPα is regulated both in myelopoiesis and leukemogenesis, is not fully understood. The current study demonstrates that activated Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor promoter and a minimal promoter containing only C/EBP DNA binding sites. Ras signaling activates C/EBPα via the transactivation domain, because it enhances the transactivation function of a fusion protein containing a Gal4 DNA binding domain and the C/EBPα transactivation domain, and does not change C/EBPα DNA binding. Ras acts on serine 248 of the C/EBPα transactivation domain, as it does not enhance the transactivation function of a C/EBPα serine 248 to alanine point mutant. Interestingly, serine 248 of C/EBPα is a PKC consensus site, and a PKC inhibitor blocks the activation of C/EBPα by Ras. Ras signaling

phosphorylates C/EBPα on serine 248 *in vivo*. Finally, mutation of serine 248 to alanine obviates the ability of C/EBPα to induce granulocytic differentiation. These data suggest a model where Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248.

6. ZUSAMMENFASSUNG

Der Transkriptionsfaktor C/EBPα reguliert frühe Schritte in der Differenzierung hämatopoetischer Vorläuferzellen zu Granulozyten. Bei C/EBPα *knockout* Mäusen ist der G-CSF-Rezeptor nicht nachweisbar. Diese Mäuse haben auch keine neutrophilen Granulozyten. Die Störung der Funktion von C/EBPα bei Patienten mit akuten myeloischen Leukämien ist bekannt. Es ist jedoch bislang weder die Regulation der Effekte des Proteins C/EBPα auf die Myelopoese noch auf die Leukämogenese zufriedenstellend beschrieben worden. Die vorliegende Arbeit zeigt, daß aktiviertes Ras die Fähigkeit des Transkriptionsfaktors C/EBPα, den Promoter des G-CSF Rezeptors sowie einen Promoter, der nur die DNA bindenden Stellen enthält, zu transaktivieren, verstärkt. Die Ras-Signaltransduktion ändert die Bindung von C/EBPα an die DNA nicht und verstärkt die Fähigkeit der Transaktivierung der beschriebenen Promotoren durch ein Fusionsprotein aus einer Gal4 DNA bindenden Domäne und der Transaktivierungsdomäne von C/EBPα. Also verstärkt Ras die transkriptionelle Aktivität von C/EBPα in seiner Transaktivierungsdomäne. In der vorliegenden Arbeit wurde die

Aminosäure, an der Ras C/EBPα aktiviert, bestimmt. Eine Punktmutation, die die Aminosäure Serin 248 in C/EBPα zu Alanin mutierte, führte zu einem Verlust der beschriebenen verstärkenden Funktion des Protoonkogens Ras auf die transkriptionelle Aktivität von C/EBPα. Die Aminosäure Serin 248 stellt eine *PKC-Konsensus-Stelle* dar. Ein Inhibitor der Proteinkinase C konnte die durch Ras induzierte Aktivierung von C/EBPα hemmen. Ras-Signaltransduktion führt in vivo zu Phosphorylierung von C/EBPα. Ferner führte die Mutation von Serin 248 zu Alanin zu einem Verlust der Fähigkeit C/EBPαs, Differenzierung von Vorläuferzellen zu Granulozyten zu induzieren. Die vorliegenden Daten unterstützen ein Modell, in dem Ras-Signaltransduktion die Kapazität C/EBPαs, die Differenzierung zu der granulozytischen Reihe zu induzieren, durch Phosphorylierung an Serin 248 verstärkt.

7. REFERENCES

Akira,S., Isshiki,H., Sugita,T., Tanabe,O., Kinoshita,S., Nishio,Y., Nakajima,T., Hirano,T., and Kishimoto,T. (1990)
A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J. 9 , 1897-1906*.

Behre,G., Smith,L.T., and Tenen,D.G. (1997)
Use of a Renilla luciferase vector as a control for studies of Ras transactivation. *Blood 90, 158b*.

Behre,G., Whitmarsh,A.J., Coghlan,M.P., Hoang,T., Carpenter,C.L., Zhang,D.-E., Davis,R.J., and Tenen,D.G. (1999)
c-Jun is a JNK independent coactivator of the PU.1 transcription factor. *Journal of Biological Chemistry 274, 4939-4946.*

Boyle,W.J., van,d.G., and Hunter,T. (1991)
Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol. 201:110-49, 110-149.*

Cao,Z., Umek,R.M., and McKnight,S.L. (1991)
Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev. 5, 1538-1552*.

Chang,C.J., Chen,T.T., Lei,H.Y., Chen,D.S., and Lee,S.C. (1990)
Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. *Mol. Cell Biol. 10, 6642-6653*.

Crespo,P. and Leon,J. (2000)

Ras proteins in the control of the cell cycle and cell differentiation. *Cell Mol. Life Sci. 57 , 1613-1636.*

Descombes,P., Chojkier,M., Lichtsteiner,S., Falvey,E., and Schibler,U. (1990)

LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev. 4, 1541-1551.*

Feig,L.A. (1988)

Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell. Biol. 8, 3235-3243.*

Feig,L.A. and Cooper,G.M. (1988)

Relationship among guanine nucleotide exchange, GTP hydrolysis, and transforming potential of mutated ras proteins. *Mol. Cell. Biol. 8, 2472-2478.*

Flodby,P., Barlow,C., Kylefjord,H., Ahrlund-Richter,L., and Xanthopoulos,K.G. (1996)

Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein α. *J Biol. Chem. 271, 24753-24760.*

Ford,A.M., Bennett,C.A., Healy,L.E., Towatari,M., Greaves,M.F., and Enver,T. (1996)

Regulation of the myeloperoxidase enhancer binding proteins PU.1, CEBPα, β, and δ during granulocytic-lineage specification. *Proc. Natl. Acad. Sci. USA 93, 10838-10843.*

Friedman,A.D. (2002)

Transcriptional regulation of granulocyte and monocyte development. *Oncogene 21, 3377-3390*.

Friedman,A.D. and McKnight,S.L. (1990)

Identification of two polypeptide segments of CCAAT/enhancer- binding protein required for transcriptional activation of the serum albumin gene. *Genes Dev. 4, 1416-1426*.

Gutkind,J.S. (1998)

The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol. Chem. 273, 1839-1842*.

Hibi,S., Lohler,J., Friel,J., Stocking,C., and Ostertag,W. (1993)

Induction of monocytic differentiation and tumorigenicity by v- Ha-ras in differentiation arrested hematopoietic cells. *Blood 81, 1841-1848*.

Hong,S., Lee,M.Y., and Cheong,J. (2001)

Functional interaction of transcriptional coactivator ASC-2 and C/EBPalpha in granulocyte differentiation of HL-60 promyelocytic cell. *Biochem. Biophys. Res. Commun. 282, 1257-1262*.

Hu,J., Roy,S.K., Shapiro,P.S., Rodig,S.R., Reddy,S.P., Platanias,L.C., Schreiber,R.D., and Kalvakolanu,D.V. (2001)

ERK1 and ERK2 Activate CCAAAT/Enhancer-binding Protein-beta - dependent Gene Transcription in Response to Interferon-gamma. *J. Biol. Chem. 276, 287-297*.

Iida,H., Towatari,M., Iida,M., Tanimoto,M., Kodera,Y., Ford,A.M., and Saito,H. (2000)

Protein expression and constitutive phosphorylation of hematopoietic transcription factors PU.1 and C/EBP beta in acute myeloid leukemia blasts. *Int. J. Hematol. 71, 153-158.*

Jin,D.I., Jameson,S.B., Reddy,M.A., Schenkman,D., and Ostrowski,M.C. (1995)

Alterations in differentiation and behavior of monocytic phagocytes in transgenic mice that express dominant suppressors of ras signaling. *Mol. Cell. Biol. 15, 693-703.*

Johansen,L.M., Iwama,A., Lodie,T.A., Sasaki,K., Felsher,D.W., Golub,T.R., and Tenen,D.G. (2001)

c-Myc is a critical target for c/EBPalpha in granulopoiesis. *Mol. Cell Biol. 21, 3789-3806.*

Johnson,R., Spiegelman,B., Hanahan,D., and Wisdom,R. (1996)

Cellular transformation and malignancy induced by ras require c- jun. *Mol Cell Biol 16, 4504-4511.*

Johnson,R.S., van Lingen,B., Papaioannou,V.E., and Spiegelman,B.M. (1993)

A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev. 7, 1309-1317.*

Lin,F.T. and Lane,M.D. (1994)

CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc. Natl. Acad. Sci. USA 91, 8757-8761*.

Maher,J., Baker,D., Dibb,N., and Roberts,I. (1996)

Mutant ras promotes haemopoietic cell proliferation or differentiation in a cell-specific manner. *Leukemia 10, 83-90*.

Mahoney,C.W., Shuman,J., McKnight,S.L., Chen,H.C., and Huang,K.P. (1992)

Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. *J Biol Chem. 267, 19396-19403*.

Mccormick,F. (1995)

Ras-related proteins in signal transduction and growth control. *Mol. Reprod. Dev. 42, 500-506*.

Nakajima,T., Kinoshita,S., Sasagawa,T., Sasaki,K., Naruto,M., Kishimoto,T., and Akira,S. (1993)

Phosphorylation at threonine-235 by a ras-dependent mitogen- activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. USA 90, 2207-2211*.

Nordeen,S.K. (1988)

Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques 6, 454-458*.

Oelgeschlager,M., Nuchprayoon,I., Luscher,B., and Friedman,A.D. (1996)

C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. *Mol. Cell. Biol. 16, 4717-4725*.

Pabst,T., Mueller,B.U., Harakawa,N., Schoch,C., Haferlach,T., Behre,G., Hiddemann,W., Zhang,D.E., and Tenen,D.G. (2001b)

AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia. *Nat. Med. 7, 444-451*.

Pabst,T., Mueller,B.U., Zhang,P., Radomska,H.S., Narravula,S., Schnittger,S., Behre,G., Hiddemann,W., and Tenen,D.G. (2001a).

Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat. Genet. 27, 263-270*.

Pan,Z., Hetherington,C.J., and Zhang,D.E. (1999)

CCAAT/enhancer-binding protein activates the CD14 promoter and mediates transforming growth factor beta signaling in monocyte development. *J. Biol. Chem. 274, 23242-23248*.

Poli,V., Mancini,F.P., and Cortese,R. (1990)

IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell 63, 643-653*.

Radomska,H.S., Huettner,C.S., Zhang,P., Cheng,T., Scadden,D.T., and Tenen,D.G. (1998)

CCAAT/Enhancer binding protein α is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol. Cell. Biol. 18, 4301-4309.*

Ramji,D.P. and Foka,P. (2002)

CCAAT/Enhancer Binding Proteins: Structure, Function and Regulation. *Biochem. J. in press.*

Roman,C., Platero,J.S., Shuman,J., and Calame,K. (1990)

Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. *Genes Dev. 4, 1404-1415.*

Ron,D. and Habener,J.F. (1992)

CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev. 6, 439-453.*

Ross,S.E., Erickson,R.L., Hemati,N., and MacDougald,O.A. (1999)

Glycogen synthase kinase 3 is an insulin-regulated C/EBPalpha kinase. *Mol. Cell Biol. 19, 8433-8441.*

Ross,S.E., Hemati,N., Longo,K.A., Bennett,C.N., Lucas,P.C., Erickson,R.L., and MacDougald,O.A. (2000)

Inhibition of adipogenesis by Wnt signaling. *Science 289, 950-953.*

Schaich,M., Ritter,M., Illmer,T., Lisske,P., Thiede,C., Schakel,U., Mohr,B., Ehninger,G., and Neubauer,A. (2001)

Mutations in ras proto-oncogenes are associated with lower mdr1 gene expression in adult acute myeloid leukaemia. *Br. J. Haematol. 112, 300-307.*

Scott,L.M., Civin,C.I., Rorth,P., and Friedman,A.D. (1992)

A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. *Blood 80, 1725-1735*.

Skorski,T., Szczylik,C., Ratajczak,M.Z., Malaguarnera,L., Gewirtz,A.M., and Calabretta,B. (1992)

Growth factor-dependent inhibition of normal hematopoiesis by N-ras antisense oligodeoxynucleotides. *J Exp. Med. 175, 743-750.*

Smith,L.T., Hohaus,S., Gonzalez,D.A., Dziennis,S.E., and Tenen,D.G. (1996)

PU.1 (Spi-1) and C/EBPα regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood 88, 1234-1247.*

Umek,R.M., Friedman,A.D., and McKnight,S.L. (1991)

CCAAT-enhancer binding protein: a component of a differentiation switch. *Science 251, 288-292.*

Wang Qf,Q.F. and Friedman,A.D. (2002)

CCAAT/enhancer-binding proteins are required for granulopoiesis independent of their induction of the granulocyte colony-stimulating factor receptor. *Blood 99, 2776-2785*.

Wang,W., Wang,X., Ward,A.C., Touw,I.P., and Friedman,A.D. (2001)
C/EBPalpha and G-CSF receptor signals cooperate to induce the myeloperoxidase and neutrophil elastase genes. *Leukemia 15, 779-786.*

Wang,X., Scott,E., Sawyers,C.L., and Friedman,A.D. (1999)
C/EBPalpha bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. *Blood 94, 560-571.*

Wasylyk,C., Maira,S.M., Sobieszczuk,P., and Wasylyk,B. (1994)
Reversion of Ras transformed cells by Ets transdominant mutants. *Oncogene 9, 3665-3673.*

Westendorf,J.J., Yamamoto,C.M., Lenny,N., Downing,J.R., Selsted,M.E., and Hiebert,S.W. (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.*

Williams,S.C., Cantwell,C.A., and Johnson,P.F. (1991)
A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes Dev. 5, 1553-1567.*

Zhang,D.-E., Hetherington,C.J., Meyers,S., Rhoades,K.L., Larson,C.J., Chen,H.M., Hiebert,S.W., and Tenen,D.G. (1996)
CCAAT enhancer binding protein (C/EBP) and AML1 (CBFα2) synergistically activate the M-CSF receptor promoter. *Mol. Cell. Biol. 16, 1231-1240.*

Zhang,D.-E., Zhang,P., Wang,N.D., Hetherington,C.J., Darlington,G.J., and Tenen,D.G. (1997)

Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein α-deficient mice. *Proc. Natl. Acad. Sci. USA 94, 569-574*.

Zhang,P., Behre,G., Pan J, Iwama,A., Wara-aswapati,N., Radomska,H.S., Auron,P.E., Tenen,D.G., and Sun,Z. (1999)

Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1 function. *Proc Natl Acad Sci U S A 96, 8705-8710*.

Zhang,P., Nelson,E., Radomska,H.S., Iwasaki-Arai,J., Akashi,K., Friedman,A.D., and Tenen,D.G. (2002)

Induction of granulocytic differentiation by 2 pathways. *Blood 99, 4406-4412*.

Zhu,J. and Emerson,S.G. (2002)

Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene 21, 3295-3313*.

Zuber,J., Tchernitsa,O.I., Hinzmann,B., Schmitz,A.C., Grips,M., Hellriegel,M., Sers,C., Rosenthal,A., and Schafer,R. (2000)

A genome-wide survey of RAS transformation targets. *Nat. Genet. 24, 144-152*.

8. ACKNOWLEDGEMENTS

I am very grateful to **Prof. Wolfgang Hiddemann** and **Dr. Gerhard Behre** for providing the opportunity to work in their research lab at Med III, Klinikum Großhadern, LMU, Munich.

I thank **Prof. Alan Friedman** and **Dr. H. Liu** of Johns Hopkins Oncology Center, Baltimore, Maryland for doing the retroviral transduction assays during the course of this work.

My colleagues (Arun, Abdul, Annika, Venkat, Rajani, Janki, Max, Michi, Deepak, Alex, Nicolei, Matthias, Roman and Mulu to name a few of them) have been very helpful in the lab and during scientific discussions.

I must thank Julius, Sabrina, Manu and Brian for taking me out in Munich beer gardens and pubs and for showing me that another world also exists away from the lab.

And finally, this work was not possible without the support and encouragement of my wife Poonam, who had the patience to live 7000 miles away from me for 3 years.

9. *Curriculum Vitae*

Name: Sheo Mohan Singh

Date of Birth: 01 July 1971

Nationality: Indian

Postal Address: Pfingstrosenstr. 64
App. 3/107
81377 Munich
Germany
Tel: 0049 89 7094 2964 (home), 0049 179 5249050 (mobile)

Email: **sheomohan@yahoo.com**

Research Experience:

Since June/2002 –
Postdoc., Department of Medicine III, Grosshadern Hospital, University of Munich and GSF Hämatologikum, KKG Leukemia, Munich, Germany.
Projects: 1. Target proteins of C/EBPα wild type and C/EBPα S248A mutant by proteomics.
2. Proteomics of AML1-ETO target proteins in t(8;21) myeloid leukemia.

Feb/1999 – May/2002
Ph.D., *Summa cum laude,* Department of Medicine III, Grosshadern Hospital, University of Munich and GSF Hämatologikum, KKG Leukemia, Munich, Germany.
Thesis title: Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248.

March/1998 – Sept/1998
Senior Research Fellow, National Research Centre on Plant Biotechnology, IARI Campus, New Delhi, Worked on DBT project "Genetic engineering of Pigeonpea (*Cajanus cajan* L.) for insect resistance".

Aug/1997 – Feb/1998
Research Student, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, Worked on "Molecular biology of *Bacillus thuringiensis*".

Education:

Oct/1994 – Oct/1995
Master of Science in Biotechnology, University of Kent at Canterbury, U.K.
Thesis Title: Biochemical characterization of ectomycorrhizal fungi.

Aug/1989 – July/1994
B.Sc. (Ag. & A.H.), First Class, Overall Grade Point 9.02 out of 10.00
C.S.A. University of Agriculture & Technology, Kanpur, India

Membership in Professional Societies:

1. American Society of Hematology (ASH), USA
2. German Society of Hematology and Oncology (DGHO), Germany
3. Society for Biochemistry and Molecular Biology (GBM), Germany
4. The Biochemical Society, United Kingdom
5. Association of Microbiologists of India (Life member)

Talks:

1. Annual Meeting, American Society of Hematology (ASH), 6-10 December 2002, Philadelphia, USA
   Title: Proteomics of AML1-ETO target proteins in t(8;21) myeloid leukemia

2. Annual meeting, German Society of Hematology and Oncology, 27-30 October 2002, Munich, Germany
   Title: Proteomics of AML1-ETO target proteins in t(8;21) myeloid leukemia

3. 2nd Harvard/Munich AML Workshop, March 10-13, 2000, Eibsee, Germany
   Title: Ras signaling towards C/EBPa.

4. Annual meeting, German Society of Hematology and Oncology, 3 – 6 October 1999, Jena, Germany
   Title: Ras enhances the ability of C/EBPa to transactivate G-CSF receptor promoter by phosphorylating at S248 of C/EBPa transactivation domain.

List of publications:

1. **Singh,S.M.**, Meisel,A., Kohlmann,A., Zhang,D.E., Haferlach,T., Tenen,D.G., Hiddemann,W., Behre,G. **(2002)**. Proteomics of AML1-ETO target proteins in t(8;21) myeloid leukemia. *Abstract 309 in Blood 100(11):84a, Talk at ASH 2002*

2. **Singh,S.M.***, **Behre,G.***, Liu,H., Bortolin,L.T., Christopeit,M., Radomska,H.S., Rangatia,J., Hiddemann,W., Friedman,A.D., Tenen,D.G. **(2002)**. Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248. *Journal of Biological Chemistry*, *277*:26293-26299, ***Dual First Authors.**

3. Behre,G., Reddy,V.A., Tenen,D.G., Hiddemann,W., Peer Zada, A.A., **Singh,S.M. (2002)**. Proteomic analysis of transcription factor interactions in myeloid stem cell development and leukemia. *Expert Opinion on Therapeutic Targets, 6*:491-495

4. Peer Zada,A.A., **Singh,S.M.**, Reddy,V.A., Meisel,A., Elsässer,A., Haferlach,T., Tenen,D.G., Hiddemann,W., Behre,G. **(2003)**. CD44 ligation inhibits proliferation in acute myeloid leukemia cells by downregulating c-Jun expression and blocking cell cycle. *Oncogene 22(15):2296-308*

5. Vangala,R.K., Neumann,M.S., Rangatia,J., **Singh,S.M.**, Tenen,D.G., Hiddemann,W., Behre,G. **(2003)**. The myeloid master regulator PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. *Blood, 101:270-277*

6. Rangatia,J., Vangala,R.K., **Singh,S.M.**, Peer Zada,A.A., Elsässer,A., Kohlmann,A., Haferlach,T., Tenen,D.G., Hiddemann,W., Behre,G. **(2003)**. Eleveated c-Jun expression in acute myeloid leukemias inhibits C/EBPα DNA binding via lucine zipper domain interaction. *Oncogene 22(30):4760-4*

7. Singh, D.M., Sonia, Sahoo, L., **Singh, S.M.,** and Jaiwal, P.K. **(1998)**. Biotechnological approaches for the improvement of Pigeonpea (*Cajanus cajan* (L.) Millsp.). In *Recent Advances in Biotechnology* (Ed. P.C. Trivedi), Jaipur, India

8. **Singh, S.M. (1997)**. Degradation of polychlorinated biphenyls (PCBs) by isolates of ectomycorrhizal fungi *Pisolithus arhizus*. *Indian Journal of Microbiology* **37** (March 1997): 47-48

9. **Singh, S.M.** and Jefferies, P. **(1997)**. Carbon and nitrogen nutrition in ectomycorrhizal fungi *Pisolithus arhizus*. *Indian Journal of Microbiology* **37** (June 1997): 69-72

10. **Singh, S.M.** and Mukerji, K.G. **(1996)**. Shape of biotechnology to come. *The Botanica* **46**: 13-17

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 29, Issue of July 19, pp. 26293–26299, 2002

Printed in U.S.A.

Ras Signaling Enhances the Activity of C/EBPα to Induce Granulocytic Differentiation by Phosphorylation of Serine 248*

Received for publication, March 8, 2002, and in revised form, April 23, 2002
Published, JBC Papers in Press, April 26, 2002, DOI 10.1074/jbc.M202301200

Gerhard Behre‡§¶, Sheo M. Singh‡§, Huaitian Liu‖, Laura T. Bortolin, Max Christopeit‡, Hanna S. Radomska‡‡, Janki Rangatia‡, Wolfgang Hiddemann‡, Alan D. Friedman‖§§, and Daniel G. Tenen‡‡**

*From the ‡Department of Internal Medicine III, University Hospital Grosshadern, Ludwig-Maximilians-University Munich and GSF-National Research Center for Environment and Health, D-81377 Munich, Germany, ‖Johns Hopkins Oncology Center, Baltimore, Maryland 21287-5001, **Massachusetts Institute of Technology/Lincoln Laboratory, Lexington, Massachusetts 02420-9185, and ‡‡Harvard Institutes of Medicine, Harvard Medical School, Boston, Massachusetts 02115*

The transcription factor C/EBPα regulates early steps of normal granulocyte differentiation since mice with a disruption of the C/EBPα gene do not express detectable levels of the granulocyte colony-stimulating factor receptor and produce no neutrophils. We have recently shown that C/EBPα function is also impaired in acute myeloid leukemias. However, how the transcriptional activity of C/EBPα is regulated both in myelopoiesis and leukemogenesis is not fully understood. The current study demonstrates that activated Ras enhances the ability of C/EBPα to transactivate the granulocyte colony-stimulating factor receptor promoter and a minimal promoter containing only C/EBP DNA binding sites. Ras signaling activates C/EBPα via the transactivation domain because it enhances the transactivation function of a fusion protein containing a Gal4 DNA binding domain and the C/EBPα transactivation domain and does not change C/EBPα DNA binding. Ras acts on serine 248 of the C/EBPα transactivation domain, because it does not enhance the transactivation function of a C/EBPα serine 248 to alanine point mutant. Interestingly, serine 248 of C/EBPα is a protein kinase C (PKC) consensus site, and a PKC inhibitor blocks the activation of C/EBPα by Ras. Ras signaling leads to phosphorylation of C/EBPα *in vivo*. Finally, mutation of serine 248 to alanine obviates the ability of C/EBPα to induce granulocytic differentiation. These data suggest a model where Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248.

The transcription factor C/EBPα plays a pivotal role during differentiation in various cell types, including adipocytes and hepatocytes and lung and ovary cells (1–7). In hematopoiesis, C/EBPα is expressed in myeloid cells (3, 6). We have previously demonstrated that the expression of C/EBPα correlates with the commitment of multipotential precursors to the myeloid lineage and is specifically up-regulated during neutrophilic differentiation (3, 6). Furthermore, conditional expression of C/EBPα is sufficient to induce neutrophilic differentiation (6) and can block the monocytic differentiation program by inactivating transcription factor PU.1 (8). In addition, no mature neutrophils are observed in C/EBPα knockout mice, whereas all the other blood cell types are present in normal proportions (5). One mechanism of how C/EBPα may inhibit cell growth might be the direct repression of E2F-DP-mediated transcription (9).

We recently reported that dominant-negative mutations of C/EBPα are found in patients with acute myeloid leukemia (AML) of subtypes M1 and M2 (10). Furthermore, we also demonstrated that the leukemic fusion protein AML1-ETO, found in patients with acute myeloid leukemia with translocation t (8, 21), down-regulates both C/EBPα expression and function in primary AML patient samples (11). These studies point to the crucial role of C/EBPα in both normal myeloid differentiation and leukemogenesis. However, how the transcriptional activity of C/EBPα is regulated both in normal myelopoiesis as well as in leukemogenesis is not fully understood.

The Ras family of proteins are GTP-dependent molecular switches that are essential for cell growth and differentiation (12, 13). Ras exerts its effect on cell growth mainly via ETS (14) and AP-1 (15) transcription factors. For example, cells with a null mutation in the c-*jun* gene and expressing oncogenic Ras lack many characteristics of Ras transformation (15), and dominant negative mutants of ETS-1, ETS-2, or PU.1 containing only the DNA binding domain inhibit Ras activation of transcription and revert Ras-transformed cells (14). In particular, Ras has been demonstrated to play an important role in myeloid differentiation. Macrophage differentiation and macrophage-colony-stimulating factor-dependent survival are altered in transgenic mice that express dominant suppressors of Ras signaling (16), whereas a number of hematopoietic cell lines undergo spontaneous myeloid differentiation in response to expression of activated Ras (17, 18). In addition, macrophage-colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, or interleukin-3-induced monocytopoiesis of CD34+ cells is inhibited by N-Ras antisense oligonucleotides (19).

Because Ras signaling and the transcription factor C/EBPα both play important roles in myeloid differentiation, we hypothesized that Ras might be involved in the activation of C/EBPα. Here we demonstrate data that suggest a model where Ras signaling phosphorylates C/EBPα on serine 248 of

* This work was supported by Deutsche Forschungsgemeinschaft Grant 2042/2-1 (to G. B.) and National Institutes of Health Grants CA 72009 and HL 56745 (to D. G. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally.

¶ To whom correspondence should be addressed: Dept. of Internal Medicine III, University Hospital Grosshadern, Ludwig-Maximilians-University Munich, Marchioninistrasse 15, D-81377 Munich, Germany. Tel.: 49-89-7095-118; Fax: 49-89-7095-5550; E-mail: gerdbehre@aol.com.

§§ A Leukemia and Lymphoma Scholar.

the transactivation domain, resulting in an enhancement of the ability of C/EBPα to transactivate the G-CSF[1] receptor promoter, which contributes to the induction of granulocytic differentiation.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Human kidney 293T cells and human kidney 293E1A cells (kindly provided by John Blenis, Harvard Medical School, Boston, MA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone). 32D cl3 cells were maintained in phenol-red free Iscove's modified Dulbecco's medium with 10% heat-inactivated fetal bovine serum, 1 ng/ml interleukin-3 (R&D Systems, Minneapolis, MN), and penicillin-streptomycin. φCRE cells were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated calf serum and penicillin-streptomycin. pBabePuro-C/EBPα(S248A)-ER was introduced into φCRE cells using LipofectAMINE (Invitrogen), and a pool of resistant cells was isolated using 2 μg/ml puromycin. 32D cl3 cells were co-cultured in the presence of 4 μg/ml Polybrene with φCRE-S248A cells that had been irradiated. After 48 h, the 32D cl3 cells were placed in 96-well dishes with 2 μg/ml puromycin, and individual subclones were isolated. Estradiol was employed at 1 μM. Morphology was assessed by Wright's-Giemsa staining of cytospins. pBabePuro-C/EBPα(S48A)-ER was constructed by ligating an *Mlu*I/*Nco*I segment containing the mutant serine in place of the identical fragment in pBabePuro-C/EBPαWT-ER. Total cellular extracts were subjected to Western blotting using antiserum recognizing human ERα (Santa Cruz Biotechnology, San Diego, CA) or murine actin (Sigma).

Reporter Constructs and Expression Plasmids—The human G-CSF receptor promoter ranging from bp −74 to +67 with respect to the major transcription start site (20) was subcloned in the firefly luciferase vector pXP2 (21). pTK driven by 2 C/EBP sites (p(C/EBP)2TK) is a dimer of the C/EBP site from the granulocyte colony-stimulating factor receptor promoter from bp −57 to bp −37 with respect to the major transcription start site (20), subcloned into pTK81luc, a pXP2-based luciferase construct with a TATA box only as a minimal promoter (21). As an internal control plasmid for co-transfection assays, the pRL-null construct driving a *Renilla* luciferase gene (Promega, Madison, WI) was used as described before (22–24). The expression plasmids pMSV-C/EBPα (rat), various deletions mutants of C/EBPα in pMSV, pMSV-C/EBPβ, pMSV-C/EBPδ, GAL4-C/EBPα area 1–9, GAL4-C/EBPα area 1–3, and GAL4-C/EBPα area 4–9, and a minimal promoter with GAL4 DNA binding sites were described previously (25, 26). Human activated pMT3-Ha-Ras(L61) was kindly provided by Larry Feig (Tufts University, Boston, MA) (27, 28). A serine to alanine point mutation at serine 248 of rat C/EBPα was introduced by site-directed mutagenesis.

Transient Transfections Using LipofectAMINE Plus and Reporter Assays for Firefly and Renilla Luciferase—293T cells or 293E1A cells were transfected using LipofectAMINE Plus (Invitrogen) as described by the manufacturer. Firefly luciferase activities from the constructs pG-CSFR, pXP2, pGal4-DBD, pTK, and p(C/EBP)2TK and *Renilla* luciferase activity from the internal control plasmid pRL-null were determined 24 h after the initiation of the transfection protocols using the dual-luciferase reporter assay system (Promega). Firefly luciferase activities were normalized to the *Renilla* luciferase values of pRL-null. Results are given as the means ± S.E. of at least six independent experiments. The DNA concentrations of the reporter constructs and expression plasmids used for LipofectAMINE Plus transfections were 0.1 μg of pG-CSFR, pXP2, pGal4-DBD, pTK, and p(C/EBP)2TK, 0.01 μg of the internal control plasmid pRL-null, 0.1 μg of the expression plasmids for C/EBPα and C/EBPα mutants and for Ras(L61); the same concentrations of the empty expression vectors were used as controls, respectively. In the transfections without cotransfection of Ras, the empty vector pMT3 was included instead of pMT3-Ras. pRL-null was chosen as the internal control plasmid, because it was not transactivated by Ras in 293T cells (22, 23).

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays were performed as described previously (22, 24). As a positive control for C/EBPα binding, a double-stranded C/EBPα probe from the human G-CSF receptor promoter (bp −57 to −37 with respect to the major transcription start site, as described before in Fig. 9 of Ref. 20; OligoA (AAG GTG TTG CAA TCC CCA GC) and OligoB (GCT GGG GAT TGC AAC ACC TT)) was labeled with Klenow polymerase and [^{32}P]dCTP (PerkinElmer Life Sciences) and incubated with 0.1 μg/μl double-stranded poly(dI-dC) (Sigma) with 1 μl of *in vitro* translated C/EBPα. In some experiments, a 100-fold molar excess of the C/EBP probe was added as specific unlabeled competitor. C/EBPα antibody (14AA) was used for gel supershift (Cat #SC61 X, Santa Cruz Biotechnology).

In Vivo Labeling and Phosphopeptide Mapping—To detect changes in the phosphorylation pattern of C/EBPα upon stimulation with activated Ras *in vivo*, 0.5 μg of pcDNA3-C/EBPα or pcDNA3-C/EBPαS248A either with or without 0.25 μg of activated Ras(L61), was transfected into 293T cells using LipofectAMINE Plus (Invitrogen). 3 h after transfection, cells were starved in serum-free Dulbecco's modified Eagle's medium. After 18 h, cells were placed into serum-free and phosphate-free Dulbecco's modified Eagle's medium (Invitrogen) for 30 min before they were metabolically labeled with [^{32}P]orthophosphate (2.5 mCi/ml). After 4 h, cells were lysed with radioimmunoprecipitation assay buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 5 mM EDTA, and 50 mM Tris (pH 8.0) and supplemented with aprotinin, phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, antipain, and chymostatin as protease inhibitors (Sigma) and sodium pyrophosphate, sodium fluoride, and sodium vanadate as phosphatase inhibitors (Sigma). In parallel plates, 0.3 μg of the G-CSF receptor promoter was co-transfected in 293T cells, and luciferase activities were determined to ensure that Ras signaling enhances the transactivation function of C/EBPα in the particular experiment used in *in vivo* labeling and subsequent phosphopeptide mapping.

C/EBPα was isolated by immunoprecipitation from the lysates using a rabbit polyclonal antibody against C/EBPα (Santa Cruz), washed four times with radioimmunoprecipitation assay buffer, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose (Bio-Rad) for phosphopeptide mapping. After transfer, the C/EBPα protein bands was excised. To determine the phosphorylated protein residues of C/EBPα, C/EBPα protein bands were digested with 1-chloro-3-tosylamido-7-amino-2-heptanone-treated chymotrypsin (Worthington) and endoproteinase Glu-C (V8 protease) (Roche Molecular Biochemicals) and processed for phosphopeptide mapping as described previously (29).

Western Blot—24 h after the start of transfection, cells were lysed with radioimmunoprecipitation assay buffer. Equal amounts of total protein were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membrane (Millipore). Membranes were incubated with anti-C/EBPα antibody (Santa Cruz Biotechnology) or β-tubulin antibody as an internal control (catalog #1111876; Roche Molecular Biochemicals) for 60 min and then with protein A-horseradish peroxidase conjugate (Amersham Biosciences) for 45 min. Signals were detected with ECL Western blotting detection reagents (Amersham Biosciences). In parallel plates, the G-CSF receptor promoter construct was co-transfected, and luciferase activities were determined to ensure that Ras enhances the transactivation function of C/EBPα in the particular experiment used for Western blot analysis of C/EBPα expression and that the transfection efficacy was the same (less than 10% difference between plates) in the particular experiment. Differences in protein expression were quantitated by ImageQuant software (Molecular Dynamics). C/EBPα antibody (14AA, SC61) was used for Western blot and immunoprecipitation (Santa Cruz Biotechnology).

RESULTS

Ras Enhances the Ability of C/EBPα to Transactivate the G-CSF Receptor Promoter—Because Ras and the regulation of the G-CSF receptor by C/EBPα are both important for myeloid development, we asked whether activated Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor promoter. 293T cells were transfected with a plasmid containing the human G-CSF receptor promoter (20) cloned upstream of the luciferase reporter gene along with expression plasmids for C/EBPα and Ras, and reporter gene expression was determined 24 h post-transfection. Transfection of a Ras expression construct significantly enhanced the ability of C/EBPα to transactivate the G-CSF receptor promoter (Fig. 1*A*). The same effect was demonstrated in myeloid U937 cells as well (Fig. 1*D*). Western blot analysis for C/EBPα expression demonstrated that activated Ras did not change the expression level of co-transfected C/EBPα (Fig. 1*B*). Thus, Ras signaling directly acts on the transactivation capacity of C/EBPα. The cooperation of

[1] The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; TK, thymidine kinase; PKC, protein kinase C: MPO, myeloperoxidase; LF, lactoferrin.

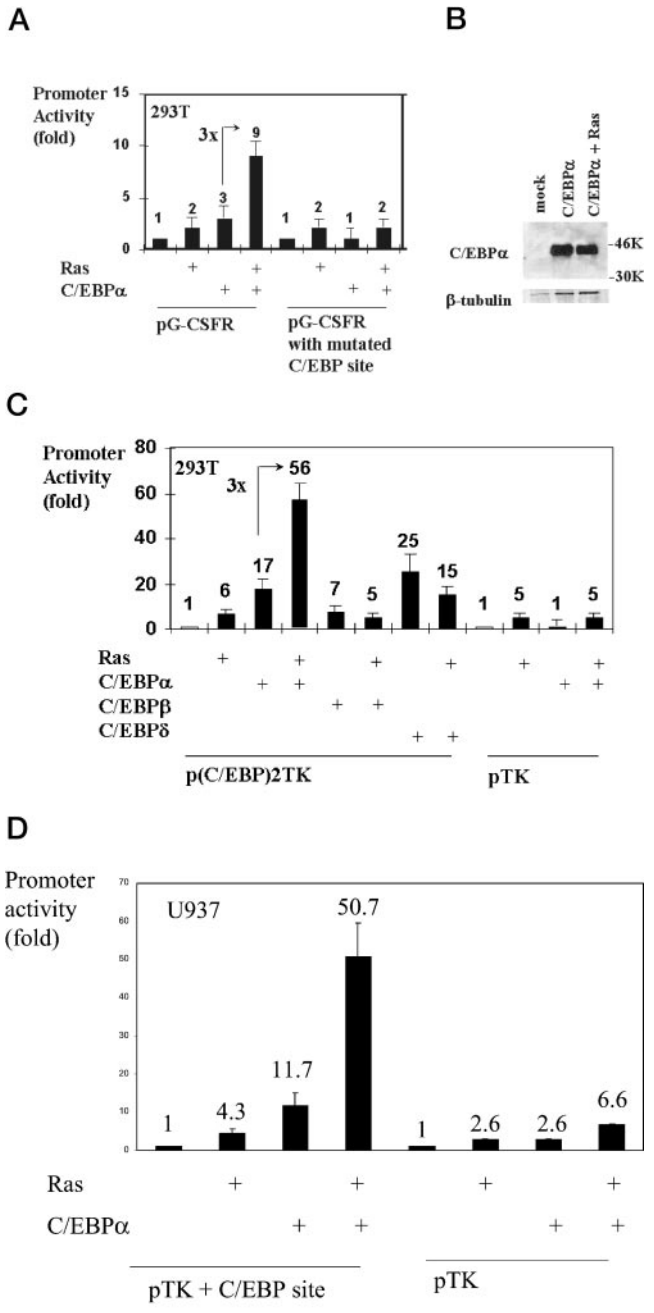


FIG. 1. **Ras signaling enhances the transcriptional activity of C/EBPα.** *A*, Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor (*CSFR*) promoter. 293T cells were transfected with the human G-CSF receptor promoter or the human G-CSF receptor promoter with mutated C/EBP sites and with the expression plasmids pMSV-C/EBPα (or empty vector pMSV) and pMT3-Ha-Ras(L61) (or empty vector pMT3). Luciferase activities were determined 24 h after transient transfection with LipofectAMINE Plus and normalized to the activities of the internal control plasmid pRL-null. *B*, Western blot analysis for C/EBPα expression (and β-tubulin expression as loading control) for the experiment shown in *A*. *C*, Ras enhances the ability of C/EBPα, but not of C/EBP β or C/EBPδ to transactivate a minimal TK promoter driven by C/EBP DNA binding sites (p(C/EBP)2TK). 293T cells were transfected with p(C/EBP)TK or a minimal TK promoter without C/EBP sites (pTK) and with the expression plasmids pMSV-C/EBPα, pMSV-C/EBP β, pMSV-C/EBPδ, and/or pMT3-Ha-Ras(L61) or the respective empty vectors. *D*, Ras enhances the ability of C/EBPα to transactivate a minimal TK promoter driven by C/EBP DNA binding sites (p(C/EBP)2TK) in myeloid U937 cells.

activated Ras with C/EBPα is therefore important for G-CSF receptor promoter activity.

Ras Enhances the Ability of C/EBPα but Not of C/EBP β or C/EBPδ to Transactivate a Minimal TK Promoter Driven by C/EBP DNA Binding Sites—We next asked the questions of (*a*) whether the binding of C/EBPα to DNA was necessary for its activation by Ras and (*b*) whether a C/EBPα binding site alone was sufficient for the Ras-enhanced C/EBPα activation. We observed enhanced C/EBPα transactivation mediated by activated Ras using a reporter construct containing two C/EBPα binding sites cloned upstream of a minimal TK promoter (p(C/EBP)TK) (Fig. 1*C*). In control experiments, no effect of Ras on C/EBPα activity was observed when the minimal TK promoter without C/EBPα binding sites was used (Fig. 1*C*). These data indicate that C/EBPα binding to DNA is necessary for its activation by Ras signaling and that C/EBPα binding sites are sufficient to mediate this effect. Interestingly, the activation of C/EBPα by Ras was specific for C/EBPα, because C/EBPβ and C/EBPδ were not activated functionally by Ras signaling in this assay (Fig. 1*C*).

Ras Enhances the Transactivation Function of a Fusion Protein Containing a Gal4 DNA Binding Domain and a Discrete Region of the C/EBPα Transactivation Domain—To map the domain of C/EBPα activated by Ras signaling, we used a minimal promoter with GAL4 DNA binding sites and the expression plasmids GAL4-C/EBPα area 1–9, GAL4-C/EBPα area 1–3, and GAL4-C/EBPα area 4–9 using the previously described nomenclature (25) to refer to the areas of the C/EBPα transactivation domain (Fig. 2, *A* and *B*). Ras signaling activated a fusion protein containing the GAL4-DNA binding domain and the transactivation domains of C/EBPα (areas 1–9). Areas 1–3 of C/EBPα were not activated by Ras, whereas areas 4–9 were still activated by Ras. These data indicate that Ras signaling might act on areas 4–9 of the transactivation domain of C/EBPα.

Ras Does Not Change DNA Binding of C/EBPα—To further elucidate the mechanism by which Ras augments the transcriptional activity of C/EBPα, we performed experiments to determine whether the activation of C/EBPα by Ras was partly due to an increase of C/EBPα DNA binding. An electrophoretic mobility shift assay was performed using a bp −57 to −37 G-CSF receptor promoter fragment containing its C/EBPα binding site (20). A positive control, *in vitro* translated C/EBPα, specifically bound to this probe as expected (Fig. 2*C*). Nuclear extracts from 293T cells transfected with the expression plasmid of C/EBPα with or without cotransfection of Ras bound to the oligo as well, and most importantly, Ras signaling did not change the DNA binding of C/EBPα. To rule out different C/EBPα protein contents of the nuclear extracts used, we performed a Western blot analysis for C/EBPα expression (Fig. 2*D*), which showed equal loading of C/EBPα. These results indicate that binding of C/EBPα to the G-CSF receptor promoter DNA is not enhanced by Ras signaling.

Ras Activates C/EBPα via Area 9 of the C/EBPα TAD—To further localize the site of C/EBPα activated by Ras signaling and to fine-map this site, we used various deletion mutants of C/EBPα (Fig. 3, *A* and *B*). C/EBPαΔ4–9 was minimally activated, and C/EBPαΔ9 was not activated by Ras. All other mutants were activated at least 2-fold by Ras. These studies demonstrate that Ras acts on area 9 of C/EBPα.

Ras Activates C/EBPα Wild Type but Not a S248A Mutant—Because Ras acts on area 9 of the transactivation domain of C/EBPα, we focused on this area for generating C/EBPα point mutants. Area 9 of C/EBPα is depicted in detail in Fig. 4*B*. There is only one potential phosphorylation consensus site in area 9, at serine 248. We generated a point mutant of C/EBPα at serine 248 by mutation to alanine (C/EBPαS248A). C/EBPα wild type and C/EBPαS248A both transactivated the G-CSF receptor promoter 20-fold. Although Ras signaling enhanced

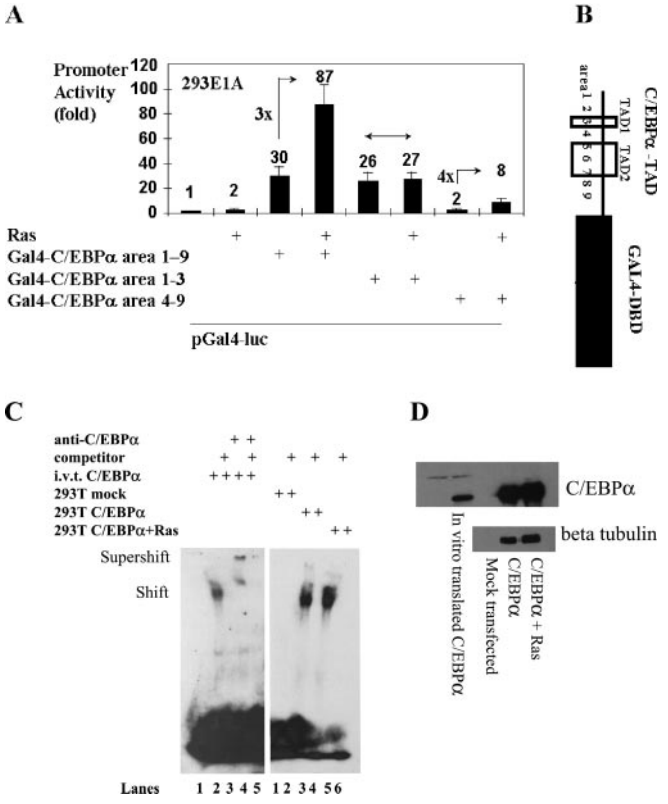


FIG. 2. **Ras signaling activates C/EBPα via the C/EBPα transactivation domain.** *A*, Ras enhances the transactivation function of a fusion protein containing a Gal4 DNA binding domain and areas 4–9 of the C/EBPα transactivation domain. 293E1A cells were transfected with a minimal promoter driven by GAL4 DNA binding sites and the expression plasmids GAL4-C/EBPα area 1–9, GAL4-C/EBPα area 1–3, GAL4-C/EBPα area 4–9, and/or pMT3-Ha-Ras(L61). *B*, model of the construct GAL4-C/EBPα area 1–9 used in *A*. The transactivation domain of C/EBPα (area 1–9) is fused to the DNA binding domain of GAL4. *C*, Ras does not change DNA binding of C/EBPα. A C/EBPα probe from the human G-CSF receptor promoter (bp −57 to −37 with respect to the major transcription start site, as described before in Fig. 9 of Ref. 20) was used in an electrophoretic mobility shift assay of C/EBPα DNA binding. As controls, probes were incubated with no added protein, 1 μl of *in vitro* translated (*i.v.t.*) C/EBPα, *in vitro* translated C/EBPα plus a 100-fold molar excess of self-unlabeled competitor, *in vitro* translated C/EBPα plus an antibody against C/EBPα for supershift (Santa Cruz), or *in vitro* translated C/EBPα plus a 100-fold molar excess of self-unlabeled competitor plus an antibody against C/EBPα for supershift. *Right panel*, as described for the *left panel*, with nuclear extracts from 293T cells transfected with the expression plasmids pMSV-C/EBPα and pMT3-Ha-Ras(L61). *D*, Western blot analysis for C/EBPα expression (and β-tubulin expression as loading control) for the experiment shown in *C*.

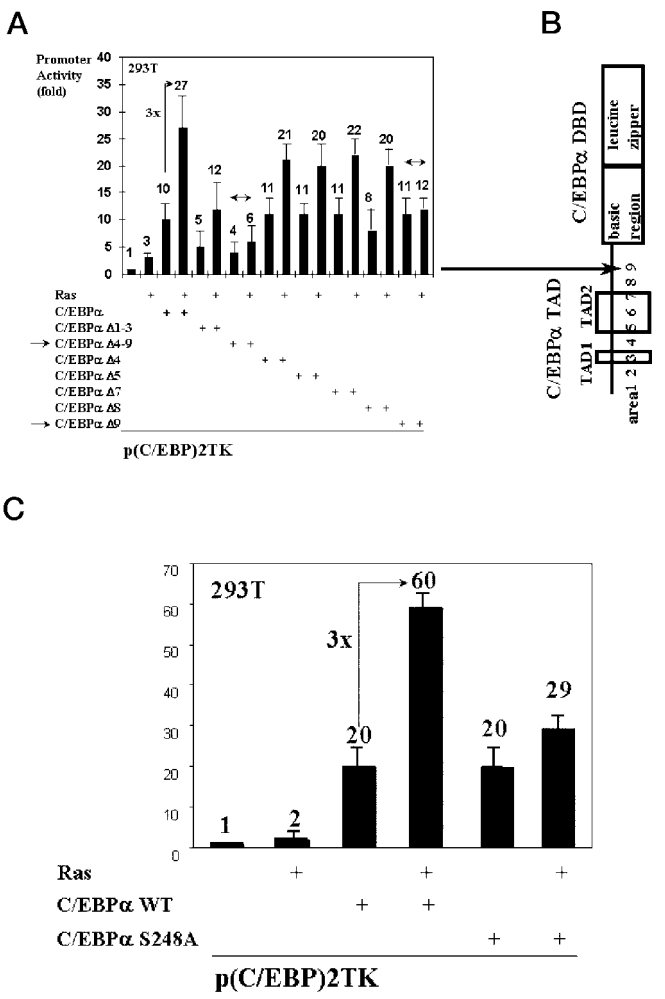


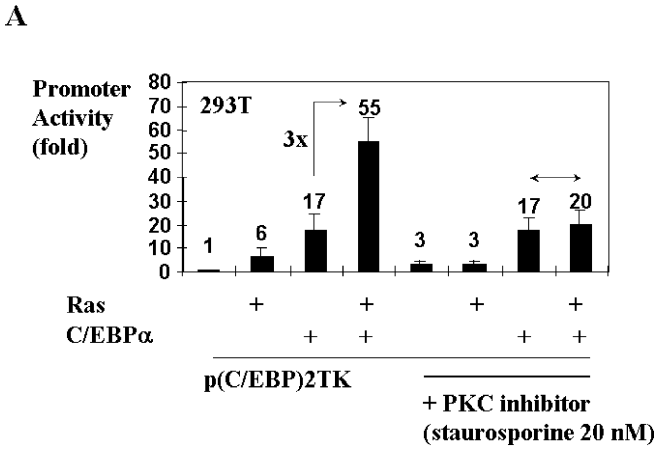
FIG. 3. **Ras signaling requires serine 248 of C/EBPα to activate C/EBPα.** *A*, Ras activates C/EBPα via area 9 of the C/EBPα transactivation domain. 293T cells were transfected with p(C/EBP)2TK and the expression plasmids pMSV-C/EBPα or various deletions mutants of C/EBPα and/or pMT3-Ha-Ras(L61). *B*, model of C/EBPα, with areas 1–9 containing the first and second transactivation domain (TAD1 and TAD2) and the DNA binding domain (*DBD*) of C/EBPα with the basic region and leucine zipper region. *C*, Ras activates C/EBPα wild type but not a serine 248 to alanine mutant of C/EBPα. 293T cells were transfected with p(C/EBP)2TK and the expression plasmids pcDNA3-C/EBPα or pcDNA3-C/EBPαS248A and/or pMT3-Ha-Ras(L61) *WT*, wild type.

wild type C/EBPα transactivation capacity from 20- to 60-fold, Ras only enhanced the transactivation capacity of the S248A mutant of C/EBPα from 20- to 29-fold (Fig. 3*C*). These results suggest that Ras signaling might act on serine 248 of the C/EBPα transactivation domain to enhance C/EBPα transcriptional activity.

A Protein Kinase C (PKC) Inhibitor Blocks the Activation of C/EBPα by Ras—Because serine 248 is a potential PKC consensus site (Fig. 4*B*), we asked whether a PKC inhibitor (staurosporine at 20 nM final concentration) could block the activation of C/EBPα by Ras signaling. In fact, pretreatment of 293T cells with staurosporine abolished the activation of C/EBPα by Ras signaling (Fig. 4*A*). These results support the hypothesis that the PKC pathway could be involved in the Ras-mediated activation of C/EBPα.

Ras Phosphorylates C/EBPα in Vivo—Because our data suggest that Ras activates C/EBPα via phosphorylation on serine 248, we wanted to prove that Ras signaling in fact phosphorylates C/EBPα on serine 248 *in vivo*. 293T cells were transfected with p(C/EBP)2TK and with the expression plasmids for C/EBPα or C/EBPαS248A and/or activated Ras. Phosphopeptide mapping of cells transfected with C/EBPα, C/EBPαS248A, C/EBPα plus Ras, and C/EBPαS248A plus Ras was performed. Ras signaling (Fig. 5*C*) enhanced a phosphorylation spot in the C/EBPα pattern (Fig. 5*A*) that was not visible in C/EBPαS248A (Fig. 5*B*) or C/EBPαS248A plus cotransfected Ras (Fig. 5*D*). Thus, we conclude that Ras signaling phosphorylates C/EBPα on serine 248 *in vivo*.

Mutation of Serine 248 to Alanine Obviates the Ability of C/EBPα to Induce Differentiation—To determine the biological importance of serine 248, we linked C/EBPα(S248A) to the human estradiol receptor (ERα) ligand binding domain and isolated 32D cl3 cell lines expressing this protein. Two subclones with protein expression as high as that we previously achieved for C/EBPαWT-ER were obtained (Fig. 6*A*). In 32D-C/EBPα(S248A)-ER-1 cells, the full-length species reproducibly migrated faster than that found for clone 2 cells or for cells expressing the wild



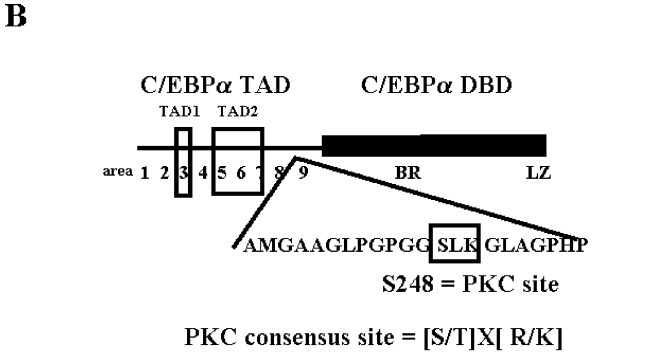
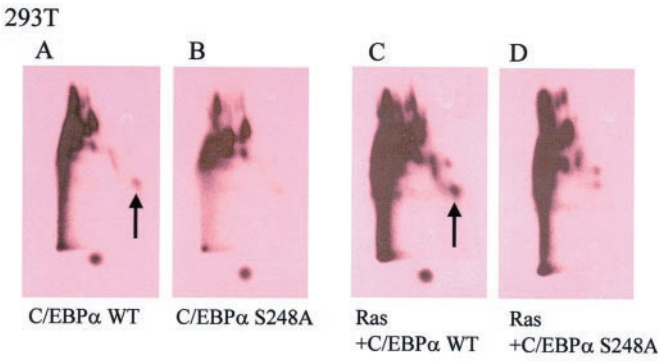


FIG. 4. **Activation of C/EBPα by Ras signaling is PKC-dependent.** *A*, a PKC inhibitor blocks the activation of C/EBPα by Ras. 293T cells were transfected with p(C/EBP)2TK and the expression plasmids pMSV-C/EBPα and/or pMT3-Ha-Ras(L61). Cells were pretreated with a PKC inhibitor (staurosporine at 20 nM) or the vehicle. Luciferase activities were determined 24 h after transient transfection with LipofectAMINE Plus and normalized to the activities of the internal control plasmid pRL-null. *B*, model of C/EBPα, with areas 1–9 containing the first and second transactivation domain (TAD1 and TAD2) and the DNA binding domain (*DBD*) of C/EBPα with the basic region and leucine zipper region. Area 9 is depicted in detail. The amino acid sequence of area 9 contains only one potential phosphorylation site, serine 248, which represents a PKC consensus site.



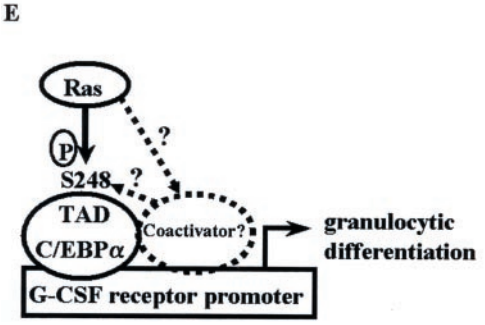


FIG. 5. **Ras signaling phosphorylates C/EBPα at serine 248 *in vivo*.** 293T cells were transfected with p(C/EBP)2TK and the expression plasmids pcDNA3-C/EBPα or pcDNA3-C/EBPαS248A and/or pMT3-Ha-Ras(L61). Phosphopeptide mapping of C/EBPα (*A*), C/EBPαS248A (*B*), C/EBPα plus Ras (*C*), and C/EBPαS248A plus Ras (*D*) was performed. The *arrow* indicates a phosphopeptide present in C/EBPα wild type (*WT*) cells and enhanced by Ras but not present in C/EBPα S248A cells, even after Ras activation. *E*, model of how Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor promoter by phosphorylation on serine 248. Our data suggest a model in which Ras signaling phosphorylates C/EBPα on serine 248 of the transactivation domain (*TAD*), resulting in an enhancement of the ability of C/EBPα to transactivate the G-CSF receptor promoter. We hypothesize that this enhancement could partly be mediated by recruitment of a co-activator (see "Discussion"). Our data suggest that Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248.

type protein. Smaller species, likely representing degradation products, and a larger species (Fig. 6*A*, *arrow*), likely representing a dimer formed via the leucine zipper, also ran faster than comparable bands in the other lanes. Perhaps this difference in migration represents altered phosphorylation at serine 248 or at another residue. It is not clear why this faster migration was only seen with one subclone but may represent heterogeneity in the 32D cl3 cells themselves.

To assess induction of differentiation by C/EBPα(S248A)-ER, each of the cell lines was placed in estradiol. Morphologically, after 3 days, the majority of 32D-C/EBPα(WT)-ER cells had differentiated to neutrophils (Fig. 6*B*, *left panels*), whereas little morphologic change was seen with 32D-C/EBPα(S248A)-ER-1 cells and clone 2 developed primary granules without nuclear maturation (Fig 6*B*, *middle* and *right panels*). To further assess differentiation, RNAs prepared daily from cultures exposed to estradiol were subjected to Northern blotting for MPO, LF, and β-actin (Fig. 6*C*). As reported previously, C/EBPαWT-ER rapidly induced MPO and induced LF after 48 h. In contrast, in 32D-C/EBPα(S248A)-ER-1 cells, neither MPO nor LF were induced, and in clone 2 cells, MPO was induced in a delayed fashion and LF was not induced. Mutation of serine 248 to alanine did not prevent the growth inhibitory properties of C/EBPαWT-ER in 32D cl3 cells (data not shown).

DISCUSSION

Because Ras signaling and the transcription factor C/EBPα play important roles in myeloid differentiation, we hypothesized that Ras might be involved in the activation of C/EBPα. Here we demonstrate that Ras signaling phosphorylates C/EBPα on serine 248 of the transactivation domain, resulting in an enhancement of its transactivation capacity.

These data link the Ras pathway, which is involved in myelopoiesis (30) and acute myeloid leukemia (31, 32), to the differentiation-inducing C/EBPα pathway. Thus, activated Ras enhances the ability of C/EBPα to transactivate the G-CSF receptor promoter and a minimal TK promoter containing C/EBP DNA binding sites from 10- to 40-fold (Fig. 1). The major role of phosphorylation sites in the activation and deactivation of C/EBP family members has already been described by other groups. Ford *et al.* (33) suggest that C/EBPα exists in multiple phosphorylated forms in the nucleus of both multipotential and granulocytic-committed cells (33). Furthermore, it has been shown that C/EBPβ is expressed in both phosphorylated and unphosphorylated forms in blasts from patients with acute myeloid leukemia (34). In terms of C/EBPβ, a regulation by the Ras signal transduction pathway and Ras-induced phosphorylation of C/EBPβ on threonine-235 is already known (35). Furthermore, the mitogen-activated protein kinases ERK1 and ERK2 have been reported to mediate interferon-γ responsiveness of C/EBPβ (36).

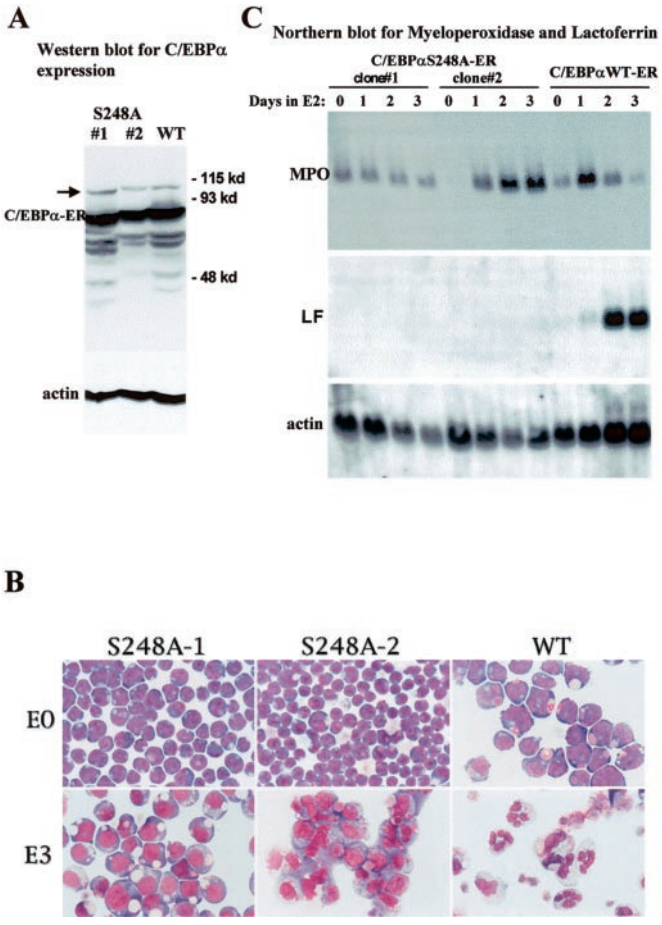


FIG. 6. **Mutation of serine 248 to alanine obviates the ability of C/EBPα to induce differentiation.** To determine the biological importance of serine 248, we linked C/EBPα(S248A) to the human estradiol receptor (ERα) ligand binding domain and isolated 32D cl3 cell lines expressing this protein. Two subclones with protein expression as high as that we previously achieved for C/EBPαWT-ER were obtained (*A*). To assess induction of differentiation by C/EBPα(S248A)-ER, each of the cell lines was placed in estradiol. Morphologically, after 3 days the majority of 32D-C/EBPα(WT)-ER cells had differentiated to neutrophils (*B*, *left panels*), whereas little morphologic change was seen with 32D-C/EBPα(S248A)-ER-1 cells, and clone 2 developed primary granules without nuclear maturation (*B*, *middle* and *right panels*). To further assess differentiation, RNAs prepared daily from cultures exposed to estradiol were subjected to Northern blotting for MPO, LF, and β-actin (*C*). As reported previously, C/EBPαWT-ER rapidly induced MPO and induced LF after 48 h. In contrast, in 32D-C/EBPα(S248A)-ER-1 cells, neither MPO nor LF were induced, and in clone 2 cells, MPO was induced in a delayed fashion and LF was not induced. *WT*, wild type.

Regarding C/EBPα, Mahoney *et al.* (37) report that phosphorylation of C/EBPα *in vitro* by protein kinase C attenuates its site-selective DNA binding . Subsequently, Pan *et al.* (38) could identify a specific increase in DNA binding and the expression of C/EBPα and C/EBPβ during U937 monocytic cell differentiation and related this to the transforming growth factor β-signaling pathway. Our data do not relate the effect of Ras signaling to the DNA binding domain or DNA binding capacity of C/EBPα. Ras signaling acts on the C/EBPα transactivation domain, because it enhances 4-fold the transactivation function of a fusion protein containing a Gal4 DNA binding domain and the C/EBPα transactivation domain (Fig. 2*A*) and does not change the C/EBPα DNA binding capacity (Fig. 2*C*). In fact, Ras acts on serine 248 of the C/EBPα transactivation domain, because it does not enhance the 10-fold transactivation exhibited by a C/EBPα mutant with a deletion of area 9 (Fig. 3*A*) or a serine 248 to alanine point mutant in area 9 (Fig. 3*C*). Thus, we conclude that the Ras pathway acts on the transactivation functions of C/EBPα via serine 248.

Ross *et al.* (39, 40) report on other important phosphorylation sites in the transactivation domain of C/EBPα. Thus, GSK3 kinase phosphorylates C/EBPα on threonines 222 and 226 (39). Furthermore, Wnt signaling maintains preadipocytes in an undifferentiated state and the adipogenic transcription factors C/EBPα and peroxisome proliferator-activated receptor γ are not expressed. When Wnt signaling in preadipocytes is prevented by overexpression of axin or dominant-negative TCF4, these cells differentiate into adipocytes (40). Our data in 293T cells point to serine 248 as the major Ras-dependent phosphorylation site of C/EBPα. Serine 248 of C/EBPα is a PKC consensus site, and a PKC inhibitor blocks the activation of C/EBPα by Ras (Fig. 4*A*). However, staurosporine is not completely specific for PKC. Therefore, we have not excluded other kinases as responsible for the activation of C/EBPα by Ras.

The serine to alanine mutant of C/EBPα did not completely eliminate the activation of C/EBPα by Ras (Fig. 3*C*), whereas deletion of region 9 did (Fig. 3*A*). Perhaps a transcriptional co-activator binds optimally to C/EBPα when serine 248 is phosphorylated (Fig. 5*E*) but can still bind weakly to the S248A mutant. In this model the effect of Ras on C/EBPα transactivation is at least partly indirect via a co-activator. This model is supported by the ability of Ras to induce basal activity of p(C/EBP)TK; on the other hand, our observation that Ras increases phosphorylation of a C/EBPα peptide but not of the same peptide from C/EBPαS248A suggests a more direct effect on C/EBPα by Ras (Fig. 5*E*). It is also possible that phosphorylation of serine 230 (39) might account for additional effects of Ras on C/EBPα. However, we could not observe any loss of enhancement of transactivation capacity of C/EBPα by Ras signaling using the serine 230 to alanine mutant of C/EBPα (data not shown; the mutants were kindly provided by Dr. MacDougald, Ann Arbor, MI). However, the very likely additional Ras-dependent phosphorylation sites in C/EBPα besides serine 248 (Fig. 5, *A–D*) still need to be mapped and to be tested functionally. Our current studies to identify interacting proteins of C/EBPα using proteomics techniques (two-dimensional gel electrophoresis and mass spectrometry) might also contribute to answering the question of whether the effect of Ras signaling on serine 248 of C/EBPα requires a co-activator binding to serine 248 or not.

Mutation of serine 248 to alanine had marked biologic consequences in 32D cl3 cells, obviating the ability of C/EBPα to induce differentiation (Fig. 6). Whether this represents a defect in Ras-dependent phosphorylation of this serine and consequent alteration of trans-activating properties in myeloid cells or whether it represents a more indirect effect in which a Ras-dependent pathway interacts with serine 248 or with another site in C/EBPα affected by mutation of serine 248 to alanine remains to be determined.

Further studies need to address the biological significance of serine 248 of C/EBPα by testing the serine to alanine 248 C/EBPα mutant in biological assays of C/EBPα function such as in rescue assays in C/EBPα knock-out mice. Of note, G-CSF, but not interleukin-3, signals cooperate with C/EBPα to induce myeloid genes in Ba/F3 lymphoid cells (41). Because impairment of C/EBPα function can contribute to the pathogenesis of acute myeloid leukemia (10, 11), whether serine 248 of C/EBPα is mutated in patients with acute myeloid leukemia and/or whether inactivating Ras mutations in acute myeloid leukemia might lead to a loss of C/EBPα function needs to be addressed.

In summary, our data suggest that Ras signaling phosphorylates C/EBPα on serine 248 of the transactivation domain, resulting in an enhancement of the ability of C/EBPα to trans-

activate the G-CSF receptor promoter. Furthermore, our data suggest a model where Ras signaling enhances the activity of C/EBPα to induce granulocytic differentiation by phosphorylation of serine 248.

REFERENCES

1. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) *Genes Dev.* **5,** 1538–1552
2. Lin, F. T., and Lane, M. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8757–8761
3. Scott, L. M., Civin, C. I., Rorth, P., and Friedman, A. D. (1992) *Blood* **80,** 1725–1735
4. Flodby, P., Barlow, C., Kylefjord, H., Ahrlund-Richter, L., and Xanthopoulos, K. G. (1996) *J. Biol. Chem.* **271,** 24753–24760
5. Zhang, D.-E., Zhang, P., Wang, N. D., Hetherington, C. J., Darlington, G. J., and Tenen, D. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 569–574
6. Radomska, H. S., Huettner, C. S., Zhang, P., Cheng, T., Scadden, D. T., and Tenen, D. G. (1998) *Mol. Cell. Biol.* **18,** 4301–4309
7. Umek, R. M., Friedman, A. D., and McKnight, S. L. (1991) *Science* **251,** 288–292
8. Reddy, V. A., Iwama, A., Iotzova, G., Schulz, M., Elsasser, A., Vangala, R. K., Tenen, D. G., Hiddemann, W., and Behre, G. (2002) *Blood*, in press
9. Slomiany, B. A., D'Arigo, K. L., Kelly, M. M., and Kurtz, D. T. (2000) *Mol. Cell. Biol.* **20,** 5986–5997
10. Pabst, T., Mueller, B. U., Zhang, P., Radomska, H. S., Narravula, S., Schnittger, S., Behre, G., Hiddemann, W., and Tenen, D. G. (2001) *Nat. Genet.* **27,** 263–270
11. Pabst, T., Mueller, B. U., Harakawa, N., Schoch C, Haferlach, T., Behre, G., Hiddemann, W., Zhang, D.-E., and Tenen, D. G. (2001) *Nat. Med.* **7,** 444–451
12. McCormick, F. (1995) *Mol. Reprod. Dev.* **42,** 500–506
13. Gutkind, J. S. (1998) *J. Biol. Chem.* **273,** 1839–1842
14. Wasylyk, C., Maira, S. M., Sobieszczuk, P., and Wasylyk, B. (1994) *Oncogene* **9,** 3665–3673
15. Johnson, R., Spiegelman, B., Hanahan, D., and Wisdom, R. (1996) *Mol. Cell. Biol.* **16,** 4504–4511
16. Jin, D. I., Jameson, S. B., Reddy, M. A., Schenkman, D., and Ostrowski, M. C. (1995) *Mol. Cell. Biol.* **15,** 693–703
17. Hibi, S., Lohler, J., Friel, J., Stocking, C., and Ostertag, W. (1993) *Blood* **81,** 1841–1848
18. Maher, J., Baker, D., Dibb, N., and Roberts, I. (1996) *Leukemia* (*Baltimore*) **10,** 83–90
19. Skorski, T., Szczylik, C., Ratajczak, M. Z., Malaguarnera, L., Gewirtz, A. M., and Calabretta, B. (1992) *J. Exp. Med.* **175,** 743–750
20. Smith, L. T., Hohaus, S., Gonzalez, D. A., Dziennis, S. E., and Tenen, D. G. (1996) *Blood* **88,** 1234–1247
21. Nordeen, S. K. (1988) *Biotechniques* **6,** 454–458
22. Behre, G., Whitmarsh, A. J., Coghlan, M. P., Hoang, T., Carpenter, C. L., Zhang, D.-E., Davis, R. J., and Tenen, D. G. (1999) *J. Biol. Chem.* **274,** 4939–4946
23. Behre, G., Smith, L. T., and Tenen, D. G. (1999) *Biotechniques* **26,** 24–26
24. Zhang, P., Behre, G., Pan J, Iwama, A., Wara-aswapati, N., Radomska, H. S., Auron, P. E., Tenen, D. G., and Sun, Z. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 8705–8710
25. Friedman, A. D., and McKnight, S. L. (1990) *Genes Dev.* **4,** 1416–1426
26. Oelgeschlager, M., Nuchprayoon, I., Luscher, B., and Friedman, A. D. (1996) *Mol. Cell. Biol.* **16,** 4717–4725
27. Feig, L. A., and Cooper, G. M. (1988) *Mol. Cell. Biol.* **8,** 2472–2478
28. Feig, L. A. (1988) *Mol. Cell. Biol.* **8,** 3235–3243
29. Boyle, W. J., Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **201,** 110–149
30. Crespo, P., and Leon, J. (2000) *Cell. Mol. Life Sci.* **57,** 1613–1636
31. Schaich, M., Ritter, M., Illmer, T., Lisske, P., Thiede, C., Schakel, U., Mohr, B., Ehninger, G., and Neubauer, A. (2001) *Br. J. Haematol.* **112,** 300–307
32. Zuber, J., Tchernitsa, O. I., Hinzmann, B., Schmitz, A. C., Grips, M., Hellriegel, M., Sers, C., Rosenthal, A., and Schafer, R. (2000) *Nat. Genet.* **24,** 144–152
33. Ford, A. M., Bennett, C. A., Healy, L. E., Towatari, M., Greaves, M. F., and Enver, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10838–10843
34. Iida, H., Towatari, M., Iida, M., Tanimoto, M., Kodera, Y., Ford, A. M., and Saito, H. (2000) *Int. J. Hematol.* **71,** 153–158
35. Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T., and Akira, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2207–2211
36. Hu, J., Roy, S. K., Shapiro, P. S., Rodig, S. R., Reddy, S. P., Platanias, L. C., Schreiber, R. D., and Kalvakolanu, D. V. (2001) *J. Biol. Chem.* **276,** 287–297
37. Mahoney, C. W., Shuman, J., McKnight, S. L., Chen, H. C., and Huang, K. P. (1992) *J. Biol. Chem.* **267,** 19396–19403
38. Pan, Z., Hetherington, C. J., and Zhang, D. E. (1999) *J. Biol. Chem.* **274,** 23242–23248
39. Ross, S. E., Erickson, R. L., Hemati, N., and MacDougald, O. A. (1999) *Mol. Cell. Biol.* **19,** 8433–8441
40. Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., and MacDougald, O. A. (2000) *Science* **289,** 950–953
41. Wang, W., Wang, X., Ward AC, Touw, I. P., and Friedman, A. D. (2001) *Leukemia* (*Baltimore*) **15,** 779–786

Expert Opinion

Oncologic

Proteomic analysis of transcription factor interactions in myeloid stem cell development and leukaemia

Gerhard Behre, Venkateshwar A Reddy, Daniel G Tenen, Wolfgang Hiddemann, Abdul A Peer Zada, Sheo M Singh
Department of Internal Medicine III, University Hospital Grosshadern, Ludwig-Maximilians-University Munich, Germany

Recent results indicate that interactions of transcription factors with other nuclear proteins play an important role in stem cell development, lineage commitment and differentiation in the haematopoietic system, and the pathogenesis of myeloid leukaemias. High-throughput proteomics by mass spectrometric analysis of gel-separated proteins can identify multi-protein complexes and changes in the expression of multiple proteins simultaneously. This review describes an application of proteomic methods (2D gel electrophoresis (GE) and mass spectrometry (MS)), which can be used to identify regulated protein targets of transcription factors important in myeloid differentiation and leukaemia. This global high-throughput functional proteomics approach could lead to new insights into the network of protein–protein interactions and target proteins involved in myeloid stem cell development and leukaemia as well as provide new targets for rational pathogenesis-based therapies of leukaemia and cancer.

Keywords: cancer, leukaemia, myeloid, proteomics, stem cell, transcription factor

*Expert Opin. Ther. Targets (2002) **6**(4):491-495*

1. Introduction

Recent data from different laboratories indicate that interactions of transcription factors with other nuclear proteins plays not only an important role in lineage commitment and differentiation in the haematopoietic system but also in the pathogenesis of acute myeloid leukaemia (AML) [1-10]. AML is characterised by a block in myeloid differentiation and altered proliferation and cell death [11]. Genetic approaches have already identified many cytogenetic abnormalities with corresponding fusion genes or mutations in AML [12]. However, little is known on how these genetic aberrations encoding fusion proteins lead to the biology of AML. Functional genomics aims to identify the target genes of fusion proteins in AML. One of those approaches is functional proteomics, which aims to identify the targets of a fusion protein on a protein, rather than mRNA, level [13-16].

In this respect the generation and use of faithful mouse models of various leukaemias has been instrumental, not only in unravelling the pathogenetic mechanisms of these diseases, but also in developing novel therapeutic targets that are currently being tested in patients [17]. Progress in understanding the biology and mechanisms of disease can be enhanced by the proteomic analysis of leukaemia. Since the term 'proteome' was introduced in 1995 [18], proteomic analysis has gained considerable interest and is presently used as a tool in drug discovery in various contexts, including [19]:

Ashley Publications
www.ashley-pub.com

 ISSN 1472-8222

- determining biochemical processes involved in disease
- monitoring cellular processes
- characterising protein expression levels

Proteomic analysis would further elucidate the underlying protein networks involved in disease pathogenesis. Proteomics can provide details of:

- changes in protein expression
- differential modifications involved in signalling
- interacting network groups of leukaemic fusion proteins and transcription factors important in myeloid differentiation

This review describes an application of 2D gel electrophoresis (GE) and mass spectrometry (MS) which has potential in the identification of regulated protein targets (interacting proteins and proteins altered in expression) of leukaemic fusion proteins and transcription factors important in myeloid differentiation. It also demonstrates the utility of this discovery-based global strategy in elucidating novel pathways.

2. Review

The transcription factors C/EBPα and PU.1 are responsible for the normal myeloid differentiation of stem cells to monocytes and granulocytes, a process which is blocked in AML (**Figure 1**) [1,2]. Recent results indicate that interactions of transcription factors with other nuclear proteins play an important role not only in normal differentiation of the haematopoietic system, but also in the pathogenesis of myeloid leukaemias (**Figure 1**) [3-10]. In contrast to PU.1, the transcription factor C/EBPα is crucial for the differentiation of granulocytes. Data indicates that C/EBPα is activated by the Ras signalling pathway via phosphorylation on ser248 [10]. Dominant negative mutations of C/EBPα have been found in some patients with AML [8] but not in those with the t(8;21) translocation. However, AML1-ETO positive AML blasts had undetectable C/EBPα protein levels. AML1-ETO can bind to C/EBPα, suppress its transcriptional activity and thereby interfere with the autoregulation of the C/EBPα promoter, leading to suppressed C/EBPα expression [9]. In summary, protein–protein interactions of transcription factors are important for normal myeloid development as well as leukaemogenesis.

High-throughput proteomics by mass spectrometric study of gel separated proteins can identify multi-protein complexes and changes in the expression of multiple proteins simultaneously [13-16]. Changes in the post-translational modifications of proteins leading to differential signalling patterns could be determined by this proteomic approach. The authors suggest that the functional studies on transcription factor interactions described above should be extended to a whole proteome (proteomic) approach to transcription factor interactions to identify all protein expression changes caused by the overexpression of a transcription factor or a leukaemic fusion protein such as AML1-ETO, in a cell line overexpressing AML1-

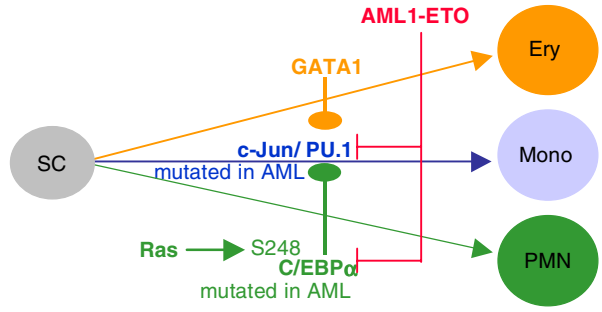


Figure 1. PU.1 and C/EBPα transcription factor interactions in myeloid differentiation and leukaemia. The transcription factor PU.1 (which drives monocytic differentiation and can be mutated in AML [3]) is activated by the co-activator c-Jun [4], a protein-protein interaction which can be disturbed by GATA-1 [5] (leading to erythrocytic differentiation), by C/EBPα [6] (resulting in granulocytic differentiation) or the leukemic fusion protein AML1-ETO [7] (contributing to AML).
AML: Acute myeloid leukaemia; C/EBPα: CCAAT/enhancer binding protein alpha; PMN: Polymorphonucleocyte; SC: Stem cell.

ETO (target proteins) and to determine all interacting proteins of AML1-ETO (the AML1-ETO multiprotein complex), and to characterize those target and interacting proteins of AML1-ETO functionally (functional proteomics).

Proteome analysis has a number of advantages over RNA expression studies using filter arrays or cDNA chips [14-16]. Protein and mRNA levels are only indirectly related and show a correlation coefficient of < 0.5 in a number of studies. Since the proteome reflects the sum of all levels of regulation including transcriptional, translational and post-translational regulation (protein modification), it represents the best biochemical description of a cell or specific condition of a cell (e.g., plus/minus AML1-ETO expression). Moreover, by using ^{32}P-labelled samples, it is possible to identify proteins that are differentially phosphorylated between wild type cells and cells overexpressing the protein of interest (in this case AML1-ETO).

The proteomic studies of the authors use a Zn-inducible AML1-ETO-U937 cell line as model system (**Figure 2**). AML1-ETO is expressed under the control of the metallothionin promoter and AML1-ETO expression can be switched on by adding Zn to the culture medium. Upon Zn-induction of inducible AML1-ETO expression in U937 cells (or any other protein of interest), cells are labelled with ^{35}S-methionine and/or ^{32}P. ^{35}S-labelling is useful for quantification of spot intensity and ^{32}P-labelling is performed to identify phosphorylation changes in proteins.

After 6 hours, protein lysates are prepared for further proteome analysis. Induction or repression of protein expression is determined by proteome analysis combining 2D gel electrophoresis, MS and protein and expressed sequence tag (EST) database searches. The 2D system used consists of immobi-

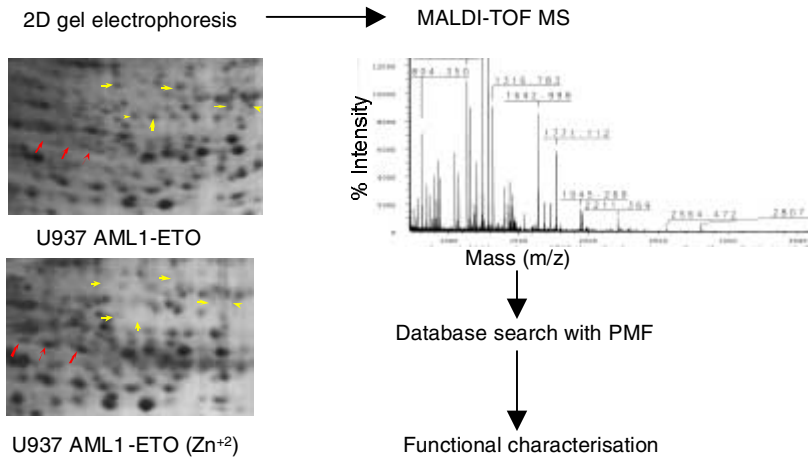


Figure 2. Proteomic analysis of transcription factor interactions: 2DGE and MALDI-TOF MS.
AML: Acute myeloid leukaemia; MALDI-TOF: Matrix assisted laser desorption/ionisation-time-of-flight; MS: Mass spectrometry; PMF: Peptide mass fingerprint.

lised pH gradients first in 1D and then SDS-PAGE in 2D. To cover a wide range of proteins and obtain a resolution sufficiently high enough to detect low abundance proteins, six different gels in 2D are run of each sample: pH 3 – 10, 4 – 7 or 6 – 11 for isoelectric focusing in 1D with 14% and 7% SDS-PAGE gels in 2D. Proteins are detected by silver staining and autoradiography. Proteins with an isoelectric point between pH 3 and pH 10 and ranging in size from 8 kDa to ~ 100 kDa can be reliably analysed. To exclude gel to gel differences, each sample type has to be repeated at least 3 times.

Differential patterns of proteins obtained from 2D gels can be analysed by Proteomweaver software, a new 2DGE image analysis software developed by Definiens AG based on cognition network technology originally used in satellite image classification. This software detects spots in a fully automated manner with high precision and speed in a context-sensitive way. Manual adjustments, such as background subtraction or parameter settings, are unnecessary – even normalisation is fully automated. Also spot matching can be performed without manual interference. The results of Proteomweaver can be investigated with advanced statistics tools. The most interesting spots in terms of expression levels change simultaneously with high statistical significance and can be displayed and/or saved easily. Proteomweaver software seems to be faster, more precise and easier to use than to older software available on the market.

After the determination of reproducible protein spots upregulated or downregulated by AML1-ETO, these proteins are identified by MS (MALDI-TOF, tandem MS (MS/MS)) (**Figure 2**). For relatively abundant proteins, it is enough to scale up the sample load in the analytical gel system and stain proteins by coomassie blue to obtain enough material for further analysis. For less abundant proteins, narrow pH gradients (covering 1 – 2 pH units in 20 cm) are used in 1D allowing higher loading of the gels. If the amount of the protein of interest is too low to be detected by coomassie blue, the cells can be labelled metabolically with ^{35}S-methionine to detect proteins by autoradiography and material from several gels run in parallel can be combined. Finally, low abundant proteins can be detected by MS/MS, rather than MALDI-TOF MS.

In-gel digestion of proteins with trypsin ensures that there is almost no loss of protein and the resulting eluted peptides are analysed directly by MALDI-TOF MS, which generates peptide mass fingerprints. This enables known proteins in DNA or protein databases to be identified with the appropriate search programs (e.g., PeptIdent, PeptideSearch, MOWSE). From small amounts of starting material (< 0.1 pMol), a dual capacity MALDI-TOF MS instrument can identify a mass range of 50 – 10000 amu, effective flight path = 2.3 M, as well as > 500000 amu, effective flight path = 1.0 M. The current protocol allows up to 30 proteins to be processed within three working days. In the few cases where the protein is not present in EST databases, *de novo* sequencing of several peptides per protein has to be performed. For peptides of low concentration not identified via the current annotated databases after MALDI-TOF analysis, a high sensitivity liquid chromatography ion-trap MS/MS system would be used.

Currently, there are some limitations to the very powerful MALDI-TOF technology. Firstly, the database must contain adequate stretches of protein sequence to enable comparison with the experimentally derived mass map derived from the unknown protein. This is not yet the case for the genomic database due to incomplete coding information for all genes [13,20,21]. For mass maps not matched in databases, other MS technologies, such as use of the MS/MS proposed, can be used to reanalyse and identify these unknown proteins present in the fusion protein complex. The use of MS/MS allows

microsequencing by causing predictable fragmentations of amino acid bands in peptides, allowing the determination of individual mass differences in the mass spectrum, which then can be used to infer at which position an amino acid was lost from the peptide under analysis. The resulting MS/MS data can be interpreted using programmes, such as SE-QUEST, which compare the experimental data obtained with predicted spectra from database sequences [22].

Due to the progress of the human genome project, EST clones are already available for most of the proteins identified using the method described. For those not yet available as ESTs, the genes can be cloned with the aid of synthetic degenerated deoxyoligonucleotides deduced from peptide sequences. For proteins with known sequences, clones can be retrieved from the laboratories that cloned the genes.

After target proteins of AML1-ETO (or of any other protein of interest) have been identified in myeloid cells, whether the promoters of these genes have functional AML1B protein binding sites can be investigated. A search for AML1B consensus DNA binding sites in the respective promoters can be performed using transfac software [101] and by performing gel shift assays with *in vitro* translated AML1B [1,2]. Luciferase assays of promoter constructs with mutated AML1B sites (which are generated by polymerase chain reaction mutagenesis) can be performed in myeloid U937 or nonmyeloid CV-1 cells [1,2]. Finally, a gene whose expression is downregulated by AML1-ETO can be subcloned into the Zn-inducible vector pPC18 to generate stable cell lines and to ask whether inducible overexpression of this protein can induce myeloid differentiation. This would indicate that the downregulation of this protein by AML1-ETO might have a biological significance.

To determine a multi-protein complex (e.g., for AML1-ETO), proteomic studies can be performed as outlined above for target proteins. However, different starting material is used for 2DGE and subsequent MS. AML1-ETO is expressed as a fusion protein with a cleavable affinity tag, glutathion-S-transferase (GST) to identify interacting proteins. In this case, AML1-ETO protein is immobilised onto agarose beads using a GST tag. Nuclear cell extracts are incubated with the beads and the beads are then washed extensively. Thrombin is used to cleave between the GST and the AML1-ETO protein, resulting in the elution of all proteins that are specifically bound to AML1-ETO. The advantage of this method is that proteins that are nonspecifically bound to the matrix or the tag itself are not eluted. The eluted proteins are resolved by 1D or 2DGE and compared to GST alone. The bands or spots corresponding to proteins specifically bound to tagged proteins are excised and analysed by MS as described for target proteins. Interacting proteins of AML1-ETO can be characterised for functional relevance by performing GST pull-down assays and luciferase assays of AML1-ETO responsive promoters (such as the Bcl-2 promoter or a minimal thymidine kinase promoter driven by AML1B binding sites only).

3. Conclusion and expert opinion

Functional proteomics, the large-scale analysis of proteins and their interactions, will contribute greatly to the understanding of gene and protein function in the postgenomic era. The global high-throughput functional proteomics approach described will likely lead to new insights into the network of protein–protein interactions and target proteins involved in myelopoiesis and the development of leukaemia as well as provide new targets for rational pathogenesis-based therapies of leukaemia and cancer.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. BEHRE G, ZHANG P, ZHANG D-E, TENEN DG: Analysis of the modulation of transcriptional activity in myelopoiesis and leukemogenesis. *Methods* (1999) **17**:231-237.
2. BEHRE G, SMITH LT, TENEN DG: Use of a Renilla luciferase vector as a control for studies of Ras transactivation. *Biotechniques* (1999) **26**:24-26.
3. MUELLER BU, PABST T, OSATO M *et al.*: Heterozygous PU.1 mutations are associated with acute myeloid leukaemia. *Blood* (2002) In press.
4. BEHRE G, WHITMARSH AJ, COGHLAN MP *et al.*: c-Jun is a JNK independent co-activator of the PU.1 transcription factor. *J. Biol. Chem.* (1999) **274**:4939-4946.
5. ZHANG P, BEHRE G, PAN J *et al.*: Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1 function. *Proc. Natl Acad. Sci. USA* (1999) **96**:8705-8710.
6. REDDY VA, IWAMA A, IOTZOVA G *et al.*: Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. *Blood* (2002) **100**:483-490.
7. VANGALA R, NEUMANN SM, RANGATIA J *et al.*: The myeloid master regulator transcription factor PU.1 is inactivated by AML-ETO in t(8;21) myeloid leukemia. (2002) Submitted for publication.
8. PABST T, MUELLER BU, ZHANG P *et al.*: Dominant negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukaemia. *Nat. Gen.* (2001) **27**:263-270.
9. PABST T, MUELLER BU, SCHOCH C *et al.*: AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukaemia. *Nat. Med.* (2001) **7**:444-451.
10. BEHRE G, SINGH SM, LIU H, BORTOLIN LT *et al.*: Ras signalling enhances the activity of c/ebpα to induce granulocytic differentiation by phosphorylation of serine 248. *J. Biol. Chem.* (2002) **277**:26293-26299.
11. TENEN DG, HROMAS R, LICHT JD, ZHANG D-E: Transcription factors, normal myeloid development and leukaemia. *Blood* (1999) **90**:489-519.

•• **Most useful review on the role of transcription factors in myeloid development and leukaemia.**

12. LOWENBERG B, DOWNING JR, BURNETT A: Acute myeloid leukaemia. *N. Engl. J. Med.* (1999) **341**:1051-1062.

13. PANDEY A, MANN M: Proteomics to study genes and genomes. *Nature* (2000) **405**:837-846.

• **Most useful review and introduction into proteomics.**

14. ABBOTT A: A postgenomic challenge: learning to read patterns of protein synthesis. *Nature* (1999) **402**:713-720.

15. BANKS RE, DUNN MJ, HOCHSTRASSER DF *et al.*: Proteomics: new perspectives, new biomedical opportunities. *Lancet* (2000) **356**:1749-1756.

16. ALAIYA AA, FRANZEN B, AUER G, LINDER S: Cancer proteomics: from identification of novel markers to creation of artifical learning models for tumour classification. *Electrophoresis* (2000) **21**:1210-1217.

17. PANDOLFI PP: *In vivo* analysis of the molecular genetics of acute promyelocytic leukaemia. *Oncogene* (2001) **20**:5726-5735.

18. WASINGER VC, CORDWELL SJ, CERPA-POLJAK A *et al.*: Progress with gene-product mapping of the mollicutes: *Mycoplasma genitalium. Electrophoresis* (1995) **16**:1090-1094.

19. LARSEN MR, LARSEN PM, FEY SJ, ROEPSTORFF P: Characterization of differently processed forms of enolase 2 from Saccharomyces cerevisiae by two-dimensional gel electrophoresis and mass spectrometry. *Electrophoresis* (2001) **22**:566-575.

20. YATES JR: Mass spectrometry. From genomics to proteomics. *Trends Genet.* (2000) **16**:5-8.

21. MANN M, HENDRICKSON RC, PANDEY A: Analysis of proteins and proteomes by mass spectrometry. *Ann. Rev. Biochem.* (2001) **70**:437-473.

22. YATES JR 3RD, MCCORMACK AL, LINK AJ, SCHIELTZ D, ENG J, HAYS L: Future prospects for the analysis of complex biological systems using microcolumn liquid chromatography-electrospray tandem mass spectrometry. *Analyst* (1996) **121**:65R-76R.

Website

101. http://bioinformatic.weizmann.ac.il/transfac

Affiliation

Gerhard Behre[1,†], Venkateshwar A Reddy[1], Daniel G Tenen[2], Wolfgang Hiddemann[1], Abdul A Peer Zada[1], Sheo M Singh[1]
[†]Author for correspondence
[1]Department of Internal Medicine III, University Hospital Grosshadern, Ludwig-Maximilians-University Munich and GSF-National Research Center for Environment and Health, Marchioninistr. 15, Munich, 81377, Germany
[2]Harvard Institutes of Medicine, Harvard Medical School, Boston, MA 02115, USA
Tel: +49 89 7095 118; Fax: +49 89 7095 5550; E.mail: gerdbehre@aol.com

Oncogene (2003) 22, 2296–2308

www.nature.com/onc

Downregulation of c-Jun expression and cell cycle regulatory molecules in acute myeloid leukemia cells upon CD44 ligation

Abdul A Peer Zada[1], Sheo M Singh[1], Venkateshwar A Reddy[1], Annika Elsässer[1], Alexander Meisel[1], Torsten Haferlach[1], Daniel G Tenen[2], Wolfgang Hiddemann[1] and Gerhard Behre*[,1]

[1]*Medicine III at University of Munich Hospital Grosshadern and GSF-Hematologikum, Marchioninistr.15 Munich D 81377, Germany;* [2]*Harvard Institutes of Medicine, Harvard Medical School, Boston MA 02115, USA*

In the present study, we investigated the mechanism of CD44 ligation with the anti-CD44 monoclonal antibody A3D8 to inhibit the proliferation of human acute myeloid leukemia (AML) cells. The effects of A3D8 on myeloid cells were associated with specific disruption of cell cycle events and induction of G0/G1 arrest. Induction of G0/G1 arrest was accompanied by an increase in the expression of p21, attenuation of pRb phosphorylation and associated with decreased Cdk2 and Cdk4 kinase activities. Since c-Jun is an important regulator of proliferation and cell cycle progression, we analysed its role in A3D8-mediated growth arrest. We observed that A3D8 treatment of AML patient blasts and HL60/U937 cells led to the downregulation of c-Jun expression at mRNA and protein level. Transient transfection studies showed the inhibition of c-*jun* promoter activity by A3D8, involving both AP-1 sites. Furthermore, A3D8 treatment caused a decrease in JNK protein expression and a decrease in the level of phosphorylated c-Jun. Ectopic overexpression of c-Jun in HL60 cells was able to induce proliferation and prevent the antiproliferative effects of A3D8. In summary, these data identify an important functional role of c-Jun in the induction of cell cycle arrest and proliferation arrest of myeloid leukemia cells because of the ligation of the cell surface adhesion receptor CD44 by anti-CD44 antibody. Moreover, targeting of G1 regulatory proteins and the resulting induction of G1 arrest by A3D8 may provide new insights into antiproliferative and differentiation therapy of AML.
Oncogene (2003) **22,** 2296–2308. doi:10.1038/sj.onc.1206393

Keywords: CD44; c-Jun; AML; differentiation; proliferation; cell cycle

Introduction

Acute myeloid leukemia (AML) is characterized by a block in differentiation (Tenen *et al*., 1997). The proliferative activity of leukemic blasts is maintained leading to the accumulation of malignant cells and a suppression of normal hematopoiesis (Tenen *et al*., 1997; Lowenberg *et al*., 1999). In experimental systems, and in specific clinical settings such as in acute promyelocytic leukemia, the differentiation block can be overcome and leukemic cells can terminally differentiate after exposure to either chemical agents (Kantarjian, 1999) or by use of monoclonal antibodies (both conjugated and unconjugated) that target antigens on leukemic blasts (Sievers, 2000; Waxman, 2000). Among the various antigens that could serve as targets of antibody-mediated differentiation, the adhesion receptors, such as CD44, deserve considerable attention in view of their role in transmitting signals that can modulate cell proliferation, survival and differentiation as well as their prevalence among leukemic cells (Reuss-Borst *et al*., 1992; Liesveld *et al*., 1994; Moll *et al*., 1998; Verfaillie, 1998).

A compelling body of evidence suggests outside-in signaling through CD44 (Sanford *et al*., 1998; Lowell and Berton, 1999) when ligated with its natural ligand, hyaluronic acid (Aruffo *et al*., 1990; Miyake *et al*., 1990) or with specific monoclonal antibodies (MoAbs). Recent reports have suggested that ligation of CD44 by anti-CD44 MoAbs (e.g. A3D8) has antiproliferative and differentiation-inducing effects on myeloid cell lines and AML patient blasts in all AML subtypes (Allouche *et al*., 2000; Charrad *et al*., 1999). However, the underlying molecular mechanisms remain largely unknown. In this study, we assessed the ability of the anti-CD44 MoAb A3D8 to modulate the expression of c-Jun and cell cycle regulatory molecules in the human myeloid leukemia cell lines HL60 and U937. We focused our attention on c-Jun, since it is implicated in the regulation of cell proliferation and cell cycle progression (Kovary and Bravo, 1991; Smith and Prochownik, 1992; Behre *et al*., 1999; Schreiber *et al*., 1999; Wisdom *et al*., 1999; Bakiri *et al*., 2000; Shaulian and Karin, 2001). Although the role c-Jun in myeloid cells has been largely studied with respect to differentiation (Behre *et al*., 2002; Rangatia *et al*., 2002; Vangala *et al*., 2002), the role of c-Jun in A3D8-mediated growth arrest in human myeloid leukemia cells has not been analysed before. Moreover, at present there are only a few data regarding the influence of c-Jun on cell proliferation and cell cycle progression in myeloid cells. Consequently, it would be of interest to investigate the role of c-Jun and cell cycle regulatory molecules in parallel to further strengthening

*Correspondence: G Behre; E-mail: gerdbehre@aol.com
Received 29 July 2002; revised 14 January 2003; accepted 16 January 2003

our knowledge on this ubiquitous transcription factor. Our recent data (Elsässer *et al*., 2002; Rangatia *et al*., 2002) suggests that c-Jun expression might be a key event in the decision of a myeloid cell to differentiate or to proliferate. A mechanistic link between c-Jun and cell cycle in fibroblasts has been reported (Hilberg and Wagner, 1992; Milde-Langosch *et al*., 2000).

The cell cycle machinery as such, is a highly coordinated process in which cyclins, cyclin-dependent kinases (Cdks) and their inhibitors (CDKIs) are differentially regulated (Muller *et al*., 1993; Matsushime *et al*., 1994; Sherr, 1994; Gitig and Koff, 2000). Each cyclin can associate with one or more of the Cdk family, of which at least Cdk2 and Cdk4 kinase activities have been shown to operate in the G1 phase (Bates *et al*., 1994; Meyerson and Harlow, 1994). The activity of G1 cyclin–Cdk complexes is regulated, at least in part, by CDKIs. Evidence is accumulating that CDKIs are targets of extracellular and intracellular signals that regulate cell growth and differentiation (Harper *et al*., 1993a, b; Hilberg *et al*., 1993; Nead *et al*., 1998; Nishitani *et al*., 1999; Steinman *et al*., 1994). Based on these data and in the present study, we provide evidence that growth inhibition by CD44 ligation with A3D8 in myeloid leukemia cells is mediated through its effect on c-Jun expression. This is the first report providing a link between c-Jun and cell cycle in CD44-mediated growth arrest of myeloid leukemia cells.

Results

CD44 ligation inhibits the proliferation and induces terminal differentiation of myeloid leukemia cells

Treatment of HL60 (myeloblastic) and U937 (monoblastic) cells with the anti-CD44 MoAb antibody A3D8 for the time points as indicated in figure legends resulted in a dramatic decrease of proliferation (Figure 1). We performed the nonradioactive quantification of cell proliferation and cell viability (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay) for investigating the proliferation state of HL60 (Figure 1a) and U937 cells (Figure 1c). The decreased proliferation of these myeloid cells also correlated with decreased CD71 (transferrin receptor) expression (Figure 1b, d). CD71 is known to be a proliferation marker (Theil, 1990; Kuhn, 1994; Trayner *et al*., 1998). Upon CD44 ligation by A3D8 in HL60 and U937 cells, we observed a drastic decrease in CD71 expression (47 and 7%, respectively) compared to the controls. It is important to note that CD71 expression was higher in HL60 cells (>90%, Figure 1b, upper left panel) as compared to U937 cells (<20%, Figure 1d, upper left panel). No inhibitory effect was observed with the isotype-matched MoAb control. Our results are in support of the recent observation that A3D8 has antiproliferative effect on myeloid cell lines (Charrad *et al*., 2002).

To rule out the possibility of a cytotoxic effect of A3D8, we performed differentiation studies. Treatment of human myeloid HL60 and U937 cells with

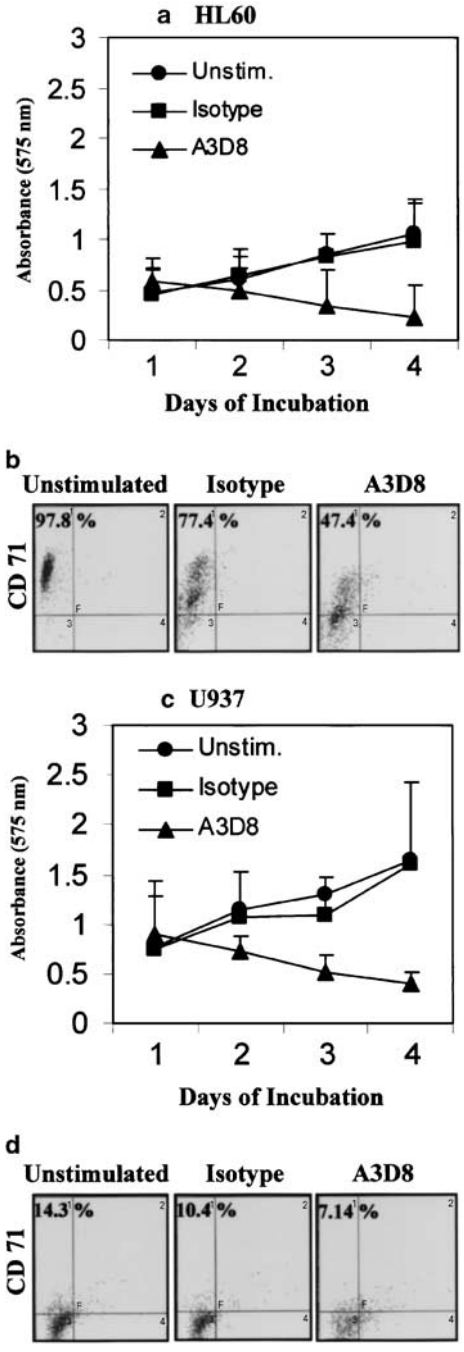


Figure 1 Decreased proliferation in myeloid leukemia cells upon CD44 ligation. Effect of anti-CD44 MoAb (A3D8) on cellular proliferation of HL60 and U937 myeloid leukemia cells as assessed by MTT and FACS analysis. (**a**, **c**) MTT assay: cells were cultured in 96-well plates with or without A3D8 and isotype control antibody (20 μg/ml) for 1–4 days and then MTT incorporation was measured. The absorbance at 575 nm (OD575), which is an estimate of the proliferation state of cells, was measured using an Elisa plate reader. Results are shown as mean$\pm$s.d. of three independent experiments, each experiment in triplicate. (**b**, **d**) FACS analysis: cells were cultured 1×10^5/200 μl/well for 36 h in the presence or absence of A3D8 (20 μg/ml). They were then analysed for their surface CD71 (transferrin receptor) expression and the results were presented as scatter diagrams. The percentage values (upper left panel) in each scatter diagram represents the percentage positive cells of the marker (i.e. cells on upper left quadrant)

A3D8-induced striking changes in the morphology of these cells characteristic of terminal differentiation (Figure 2a, c). For example, A3D8-treated cells showed decreased nucleus: cytoplasm ratios, segmented nuclei, few nucleoli and chromatin condensation. The effects like the formation of aggregates in culture and adherence became visible only after 12–18 h of A3D8 treatment. We also analysed the expression of the cell differentiation marker CD11b in HL60 and U937 cells (Figure 2b, d) and observed that its expression was increased in both cell lines after CD44 ligation. The expression of CD11b increased to ~31% after A3D8 treatment compared to unstimulated (~7%) and isotype control (~9.6%) in HL60 cells (Figure 2b, upper left panels). Corresponding to this, the *D*-value (calculated as described in the Materials and methods) was found to be −57 after A3D8 treatment in HL60 cells compared to +30 for the control (Table 1). In U937 cells, the expression of CD11b increased to ~22% after A3D8 treatment compared to unstimulated (~6%) and isotype control (~7%) (Figure 2d, upper left panels). Corresponding to this, the *D*-value was found to be −78 after A3D8 treatment in U937 cells compared to +30 for the control (Table 1).

CD44 ligation with A3D8 induces a G0/G1 arrest in myeloid leukemia cell lines

Cell cycle arrest is a common feature of cells undergoing terminal differentiation and defective proliferation. Based on the growth inhibitory and differentiation-inducing effects of A3D8 on myeloid leukemia cell lines, we investigated their cell cycle progression in response to A3D8. The DNA content analysis showed that the myeloid cells underwent a G0/G1 arrest (Figure 3). Surprisingly, we observed a change in cell cycle distribution at 6 h of A3D8 treatment in HL60 cells as compared to untreated cells or cells treated with the isotype antibody. The proportion of cells in G0/G1 phase increased from 54% (controls) to 67% in A3D8-treated cells after 24 h (Figure 3a). This was mirrored by a decrease in the proportion of cells in the S

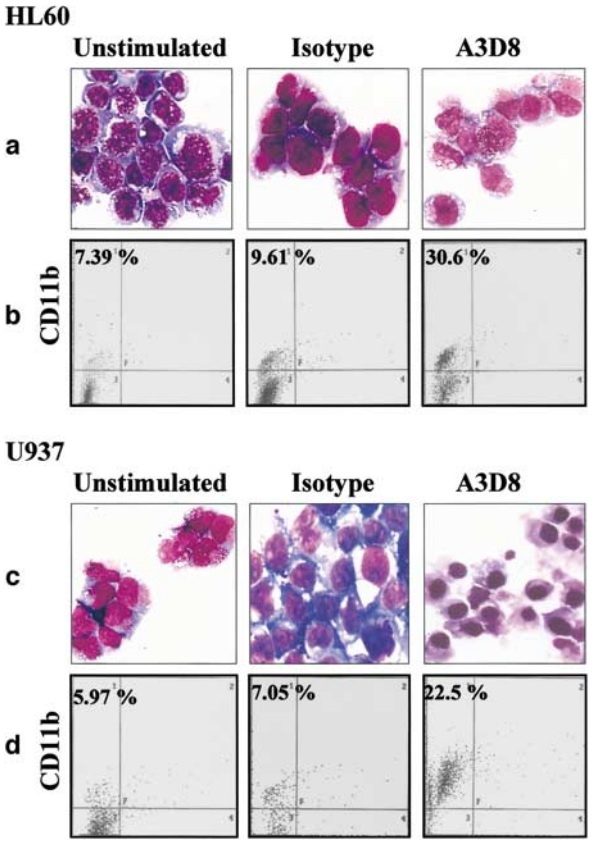


Figure 2 Differentiation induction in myeloid leukemia cells upon CD44 ligation. Morphological analysis of HL60 and U937 myeloid leukemia cells: Cytospin preparations of cells stained with May–Grünwald–Giemsa after *in vitro* treatment for 36 h with medium alone, with isotype antibody (20 μg/ml) and A3D8 (20 μg/ml). (**a**, **c**) Representative data from HL60 and U937 cells, respectively. (**b**, **d**) Changes in the differentiation marker (CD11b) in HL60 and U937 cells before and after CD44 ligation with A3D8, respectively. Cells were cultured 1×10^5/200 μl/well for 24–36 h in the presence or absence of A3D8 (20 μg/ml). They were then analysed for their surface CD11b expression and the results were presented as scatter diagrams. The percentage values (upper left quadrant) in each scatter diagram represents the percentage positive cells of the marker (i.e. cells on upper left quadrant)

Table 1 CD44 ligation decreases proliferation and induces differentiation in myeloid leukemia cells

Marker	*MFI ratio*			*D-value (%)*		
	Unstimulated	*Isotype*	*A3D8*	*Unstimulated*	*Isotype*	*A3D8*
HL 60						
CD11b	1.11	1.03	1.41	30.00	+23.00	−57.00
CD71	4.47	3.10	1.35	30.00	+25.00	+12.00
U937						
CD11b	1.07	1.02	1.32	30.00	+20.00	−78.00
CD71	1.00	1.03	0.98	30.00	−36.00	+24.00

(+) Indicates decreased expression, (−) indicates increased expression.
Expression of CD71 and CD11b in HL60 and U937 cells in response to inducers with their MFI ratio and *D*-values, calculated as: MFI ratio of unstimulated sample−MFI ratio of induced sample/MFI of unstimulated sample) × 100.
The *D*-value for unstimulated control was arbitrarily chosen 30. The (−) sign indicates more MFI ratio of induced sample compared to unstimulated sample and hence, increased expression of the marker

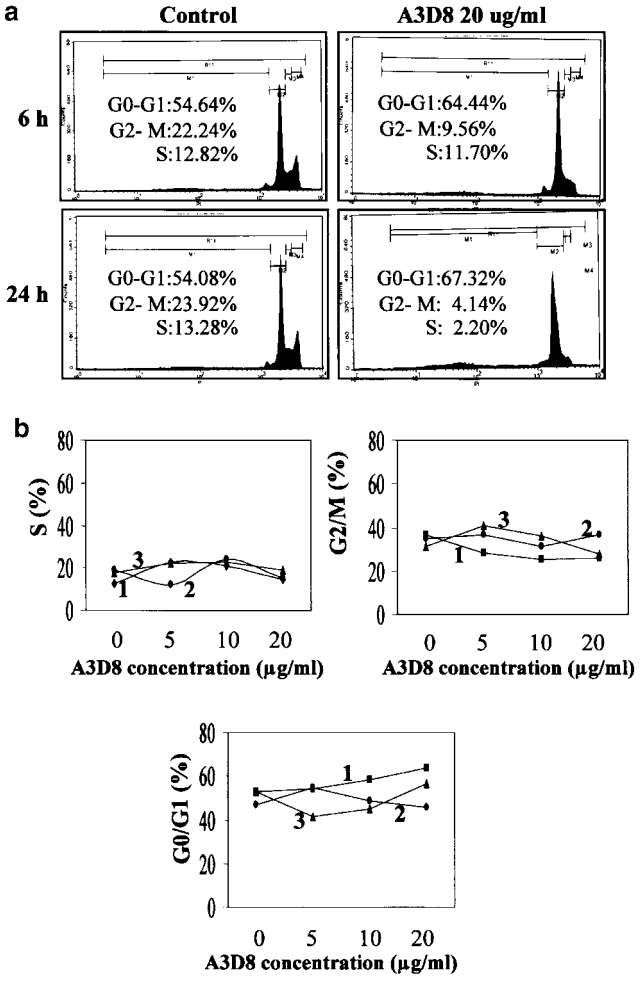


Figure 3 CD44 ligation arrests myeloid leukemia cells in the G1 phase of cell cycle. The figure represents cell cycle distribution of HL60 cells before and after A3D8 treatment for different time points. At the indicated time, cells were washed with PBS, and stained for DNA with propidium iodide as described in Materials and methods. Cell cycle distribution was then determined by FACS analysis. (**a**) Cell cycle distribution at indicated times of treatment with A3D8. (**b**) Cell cycle distribution showing different phases in a dose response of A3D8. Lines: **1**, A particular phase at 6 h of A3D8 treatment. **2**, At 24 h A3D8 treatment and **3**, at 36 h of A3D8 treatment

and G2 phase from 13% (controls) to 2% in A3D8-treated cells and from 23% (controls) to 4% in A3D8-treated cells, respectively. The effect of A3D8 on cell cycle was dose dependent (Figure 3b). In accordance with the induction of terminal differentiation by A3D8, we observed 14–27% of dead cells after 36 h. These data suggest that the growth inhibitory effect of A3D8 on myeloid cells is, in part, because of its effect on cell cycle progression.

CD44 ligation with A3D8 induces the expression of p21 and downregulates the expression of major G1 regulatory proteins

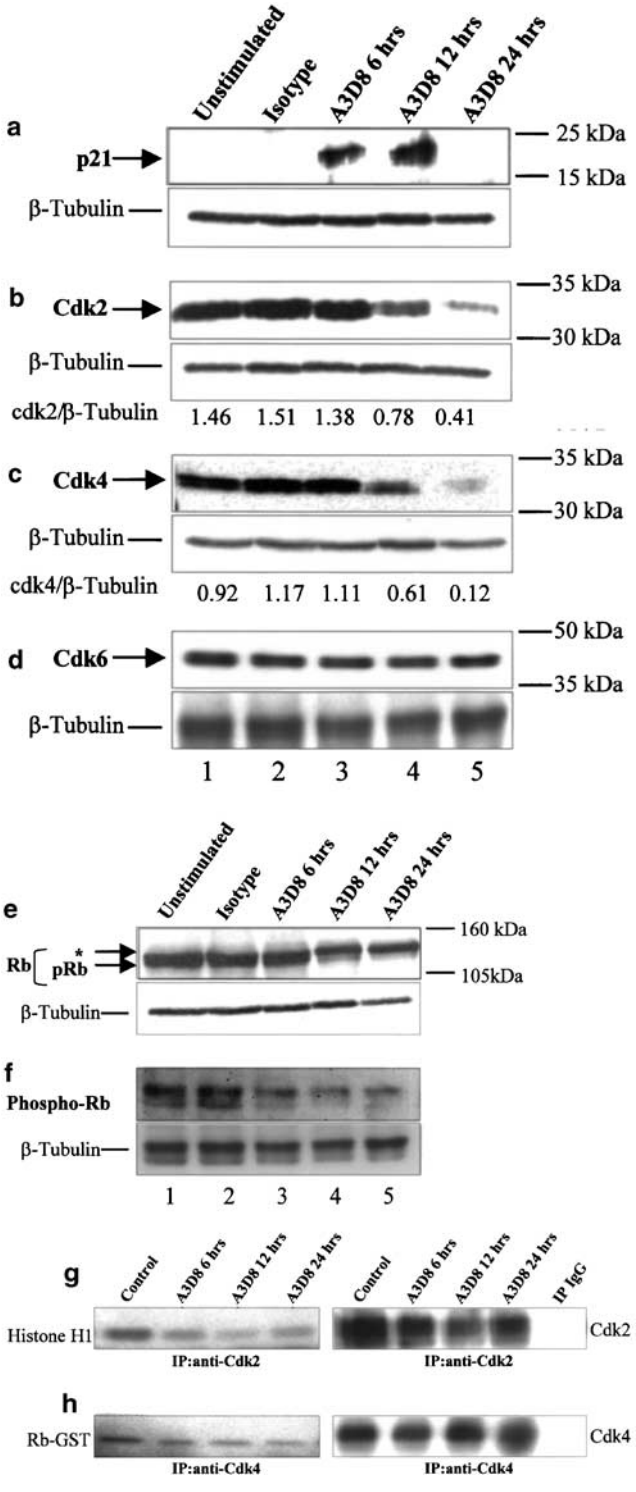
Based on the effects of A3D8 on G1 phase accumulation, we hypothesized the role of major G1 regulatory proteins. We examined the effect of A3D8 on p21, pRb, cyclin D1, cyclin D2, Cdk2, Cdk4 and Cdk6 protein expression. Our results show that A3D8 treatment of HL60 cells caused marked upregulation of p21 protein expression after 6 h (Figure 4a, lane 3). The increased p21 protein level persisted for 12 h and was undetectable thereafter. The p21 level was undetectable in untreated or isotype-treated cells (Figure 4a, lanes 1 and 2). Since HL60 cells are p53 negative because of homozygous deletions (Steinman *et al*., 1998), it is conceivable that p21 induction by A3D8 is p53 independent. Our results also show that treatment of HL60 cells with A3D8 for 12 and 24 h markedly decreases the expression of pRb (Figure 4e). It is important to note the presence of a slow migrating band (upper band) and a faster migrating band (lower band) when the blot was probed with anti-Rb antibody. The upper band corresponds to the hyperphosphorylated (*) form, while the lower band corresponds to the hypophosphorylated form (Slack *et al*., 1993; Savatier *et al*., 1994). Inhibition of pRb correlated with decreased levels of Cdk2 and Cdk4 (Figure 4b, c). There was no effect on Cdk6 expression (Figure 4d), a little downregulation effect on cyclin D1 expression and no effect on the expression on cyclin D2 (data not shown).

CD44 ligation with A3D8 inhibits Cdk4 and Cdk2 activities

Cdk4 and Cdk2 kinase activities have been shown to operate in the G1 phase. G0/G1 arrest by A3D8 led us to analyse the kinase activities associated with these Cdks. Antibodies against Cdk4 and Cdk2 were used to perform immunocomplex kinase assays using recombinant Rb fusion protein and purified histone H1 protein as substrates, respectively. Consistent with its effect on cell cycle progression, A3D8 treatment inhibited Cdk4 and Cdk2 kinase activities (Figure 4g, h). The densitometry analysis showed that A3D8 treatment after 24 h caused greater than fourfold inhibition of Cdk4 kinase activity. Interestingly, Cdk2 kinase activity showed similar results. To normalize for the immunoprecipitation (IP) efficiency, a Western blot for respective Cdks was also performed after IP. IgG served as IP control. The results (Figure 4g, h) clearly show the specificity of our kinase reaction and that Cdk2 and Cdk4 were not degraded during the kinase reaction. It is important to note here that it might seem surprising to correlate Cdk activity with Rb phosphorylation after A3D8 treatment (Figure 4e) in which Rb IF8 (anti-mouse, sc-102) was used. However, this is not the case. Western blot of the lysates when probed with phospho-specific Rb antibody (pRb Ser-780, sc-12901, Santa Cruz) gave the expected results. Our results show that treatment of the cells with A3D8 led to decreased Rb phosphorylation (Figure 4f), thereby correlating with decreased Cdk activity. The difference in the two results could thus, be attributed to antibody specificities. These data suggest that induction of G0/G1 arrest by A3D8 in myeloid cells may involve p21 induction and/or inhibition of Cdk activity.

*CD44 ligation downregulates c-*jun *mRNA and c-Jun protein expression*

The AP-1 transcription factor c-Jun functions as a proliferation-promoting gene and is involved in cell cycle progression. Consequently, the expression of c-Jun would be expected to change in response to decreased proliferation and cell cycle arrest of HL60 and U937 cells upon CD44 ligation with A3D8. Our results show that there is a drastic decrease in c-*jun* mRNA expression (Figure 5a, b) upon A3D8 treatment of the myeloid cells, c-*jun* expression was also downregulated in AML patient blasts after 6 and 12 h of A3D8 treatment *in vitro* (Figure 5c). We also observed a dramatic decrease in c-Jun protein expression upon CD44 ligation (Figure 5d,e). It is important to note that there is a higher basal level of c-Jun expression in HL60 cells than in U937 cells (Figure 5d, e, unstimulated lanes). The amount of protein loaded in U937 blot (Figure 5e) was at least three times that of HL60 cells (Figure 5d). To rule out a general toxic effect, we show that the expression of c-Fos (Figure 5f) is not altered in a similar fashion. These data suggest that the downregulation of c-Jun contributes to A3D8-mediated growth arrest in myeloid cells.



*CD44 ligation downregulates human c-*jun *promoter activity via AP-1 sites*

To elucidate the molecular mechanisms underlying the downregulation of c-Jun expression by CD44 ligation, we performed promoter studies. U937 and HL60 cells were transiently transfected with different c-*jun* promoter/luciferase constructs and then subjected to A3D8 treatment. Our results show that the full-length c-*jun* promoter (bp −1780/+731) activity was downregulated 14-fold after A3D8 treatment (Figure 6). It was not a vector effect since A3D8 had no effect on pGL3, in which c-*jun* promoter constructs were subcloned. As a positive control for our promoter studies, we also show that TPA increases the c-*jun* promoter activity (data not shown). To map site(s) in the c-*jun* promoter responsible for the downregulation, a series of c-*jun* promoter deletion mutant-luciferase gene chimeric plasmids with variable ends (from bp −1780 to −63), (Wei *et al*., 1998) was also transiently transfected into the cells. The promoter activity of each construct (without any treatment) with respect to the full-length promoter activity is shown (Table 2). The results show that the downregulation of c-*jun* promoter activity is lost after deletion of the region between bp −1780 and −63 (with −63/+731 construct), where two AP-1 sites (bp −64 and −182) are located (Figure 6a, c). The presence of the two AP-1 sites in the c-*jun* promoter, a proximal (bp −64) and a distal (bp −182)

Figure 4 CD44 ligation induces the expression of p21 and downregulates the expression of major cell cycle regulatory proteins as well as inhibits Cdk activity. The figure shows immunoblot analysis from whole cell lysates of HL60 cells (p53 negative), for (**a**) p21, a cyclin-dependent kinase inhibitor probed with anti-p21 antibody (SC); (**b**) Cdk2; (**c**) Cdk4; (**d**) Cdk6; (**e**) pRb; (**f**) phospho-Rb. Lanes: 1, 2, Unstimulated and isotype control, 3–5, A3D8 stimulated (6, 12, 24 h, respectively). The numbers underneath the blot indicate protein/respective β-tubulin ratios after densitometric analysis (Aida 2.1 software program). (**g** and **h**) *In vitro* kinase assay for Cdk2 and Cdk4, respectively: HL60 cells were treated with 20 μg/ml A3D8 or isotype antibody for different time points. Whole cell lysates were then prepared and immunoprecipitated with Cdk2 (**g**) and Cdk4 (**h**) antibodies as described under Materials and methods section. Histone H1 was used as substrate for Cdk2- and Rb-fusion protein as substrate for Cdk4 in the *in vitro* kinase assay. Also shown (in the right panel) is a Western blot of Cdk2 and Cdk4 after IP of the respective kinases

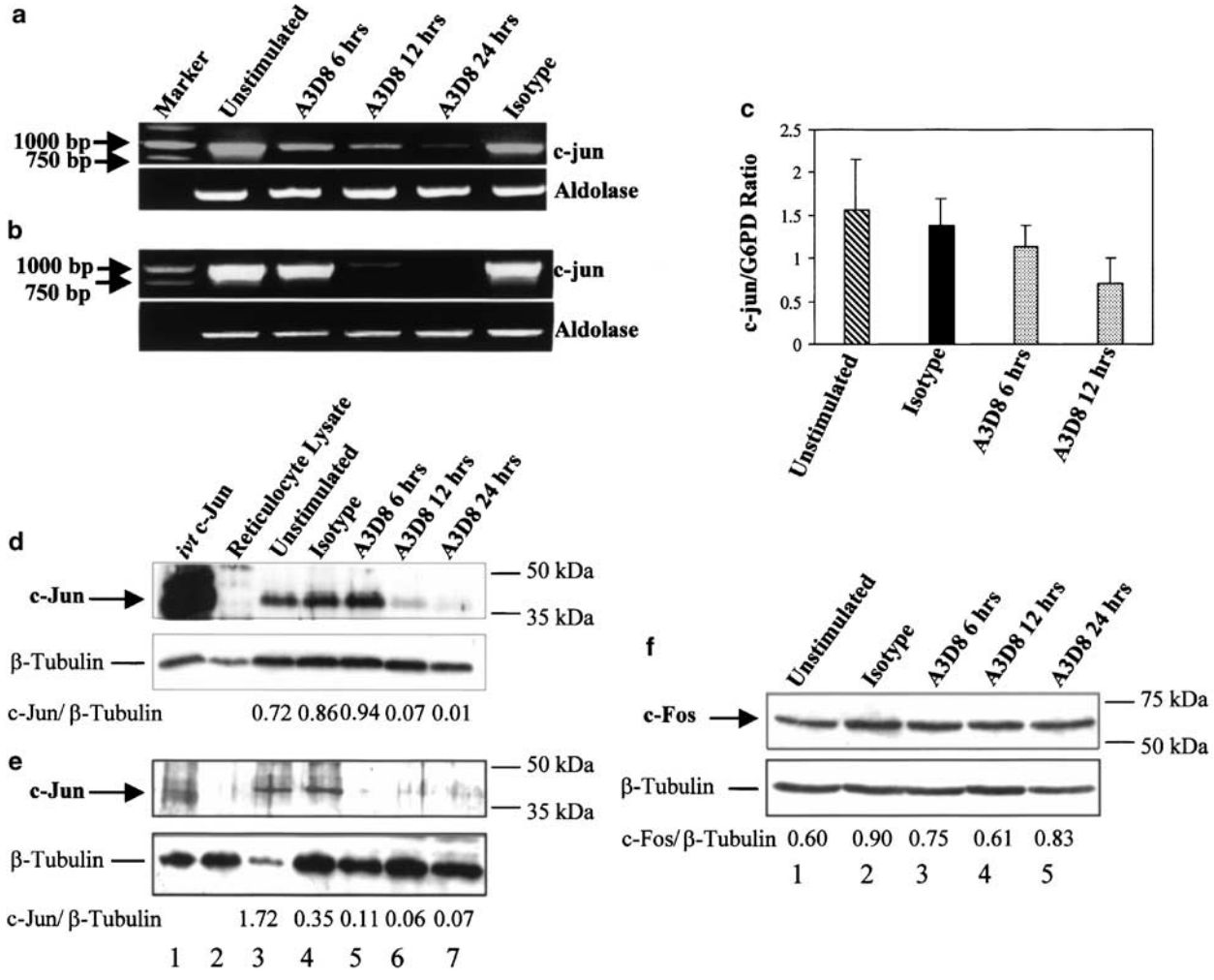


Figure 5 CD44 ligation downregulates c-*jun* mRNA and protein expression in myeloid leukemia cells and AML patient samples. Negative gel image showing c-*jun* mRNA transcript amplified with specific c-*jun* primers and immunoblot analysis of whole cell extract probed with anti-c-*Jun* and anti-c-Fos antibodies (Santa Cruz Biotechnologies) was performed as described in Materials and methods. *β*-tubulin served as loading control. (**a**) HL60 cells: Lanes: 1, Marker, 2, unstimulated control, 3–5, A3D8 stimulated (6, 12, 24 h, respectively), 6, isotype control, (**b**) U937 cells: Lanes: 1, Marker, 2, unstimulated control, 3–5, A3D8 stimulated (6, 12, 24 h, respectively), 6, isotype control. (**c**) In AML patient samples the expression of c-*jun* was measured by quantitative Real-time PCR. The bars represent the mean ratio of c-*jun* to G6PD of four AML patient samples. (**d**) c-Jun expression from whole cell lysates of HL60 cells and (**e**) data from U937 cells. Lanes: 1, *In vitro*-translated c-Jun, 2, reticulocyte lysate, 3–4, unstimulated and isotype control, 5–7, A3D8 stimulated (6, 12, 24 h, respectively). (**f**) Immunoblot analysis for c-Fos from whole cell lysates of HL60 cells. Lanes: 1, 2, Unstimulated and isotype control, 3–5, A3D8 stimulated (6, 12, 24 h, respectively). The numbers underneath the blot indicate the c-Jun/*β*-tubulin ratios after densitometric analysis (Aida 2.1 software program)

one led us to further map the AP-1 site responsible for the downregulation. Upon deletion of the proximal (delpAP-1) or the distal (deldAP-1) AP-1 sites in the c-*jun* promoter, we observed a similar downregulating effect, while the effect was lost upon deletion of both AP-1 sites (Figure 6b). The loss of repression effect cannot be a simple activation because the promoter activity in the presence of A3D8 is the same as that of the promoter alone (Figure 6b, last two bars), although in the presence of A3D8 antibody the activity of the promoter with two mutant AP-1 sites has a higher activity than either of the singly mutated constructs (Figure 6b, compare bars with A3D8 treatment). These results show that both AP-1 sites are important for the downregulation of c-*jun* promoter activity upon CD44 ligation with A3D8.

A3D8 treatment decreases c-Jun phosphorylation and JNK expression

The decreased transactivation property of c-Jun by A3D8 could be mediated through a change in the phosphorylation status of c-Jun. To investigate if A3D8 treatment of the cells caused decreased c-Jun phosphorylation, we performed immunoblot analyses of cell lysates from HL60 cells using a phospho-specific (Ser63) anti-c-Jun antibody (Figure 7). No phosphorylated c-Jun was detected at 12 or 24 h after A3D8 treatment (Figure 7, lanes 4 and 5), although c-Jun phosphorylation was detected at 6 h and in the controls (Figure 7, lanes 1–3). Furthermore, our results showed that A3D8 treatment drastically decreased the expression of JNK1, ahead of

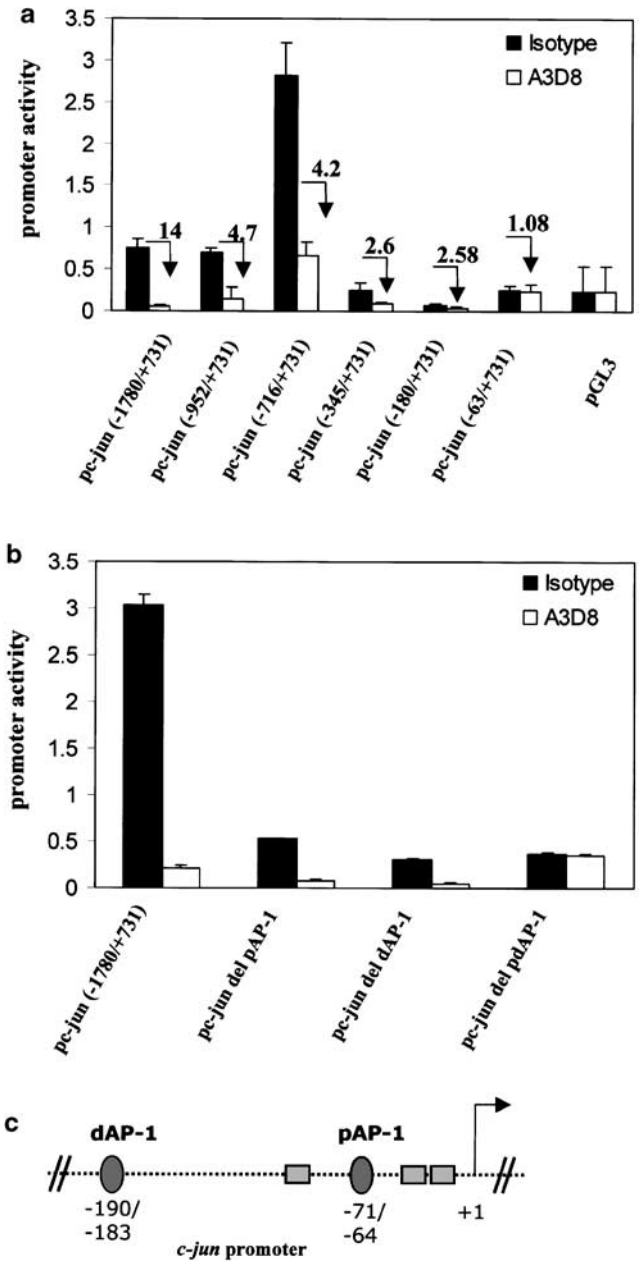


Figure 6 CD44 ligation downregulates c-*jun* promoter activity via AP-1 sites. The figure represents a series of c-*jun* promoter deletion mutants-luciferase gene chimeric plasmids with variable ends (from bp −1780 to −63) (Wei *et al*., 1998). Each construct was transiently transfected into HL60 and U937 cells. Transfected cells were then treated with A3D8, 6 h before measurement of luciferase activity. Promoter activity is normalized for transfection efficiency by dividing firefly luciferase activity by renilla luciferase activity of a cotransfected reporter plasmid pRL-0. Results are presented as mean ± s.d. of at least three independent experiments. pc-*jun* represents the promoter constructs and pGL3 is the vector in which the promoter constructs were subcloned. (**a**) Deletion analysis of c-*jun* promoter. (**b**) Mutagenesis analysis of the AP-1 sites in the c-*jun* promoter. (**c**) A model of c-*jun* promoter showing AP-1 sites

decreased c-Jun phosphorylation (Figure 7b). The effect seems to be JNK-specific since only an insignificant effect on ERK1 expression could be detected (Figure 7c). These data suggest that inhibition of *c*-Jun expression by A3D8 results from inhibition of c-Jun phosphorylation via the JNK pathway. Taken together, our data indicate that inhibition of c-Jun/AP-1 activity may be the mechanism by which A3D8 inhibits the proliferation and causes cell cycle arrest in myeloid cells.

Overexpression of c-Jun in HL60 cells overcomes the proliferation-inhibiting effects of A3D8

To further characterize the role of c-Jun biologically, we overexpressed c-Jun in HL60 cells to investigate if the effects of proliferation inhibition and differentiation induction by A3D8 can be overcome. HL60 cells were transfected with pMV7-c-*jun* retroviral construct as described in the Materials and methods section. After selection of the cells in G418, they were kept in G418-free media with and without A3D8. We observed that the expression of CD71 was markedly increased in pMV7-c-*jun*-transfected cells as compared to vector alone (Figure 8a). Moreover, c-Jun overexpressing HL60 cells showed increased cell numbers (Trypan blue cell counting) as compared to cells containing vector alone over a period of 3 days (Figure 8b). We could clearly observe a slow growth in vector (pMV7)-transfected cells under constant selection pressure, whereas pMV7-c-*jun*-transfected cells showed a higher growth rate under similar conditions. As a more direct measure of cell proliferation, we also performed bromodeoxyuridine (BrdU) incorporation assay. Our results clearly show that pMV7-c-*jun*-transfected cells incorporate more BrdU than pMV7-transfected cells and hence, the former have more proliferation potential than the latter (Figure 8c). After 24 h post-transfection, the percentage of pMV7-c-*jun*-transfected cells showed $>50\%$ BrdU incorporation as compared to $<10\%$ in the control. It is important to mention here that c-Jun overexpression in HL60 cells did not lead to any cell death as determined by propidium iodide staining (data not shown). Thus, c-Jun expression and cell proliferation in fact, do correlate in myeloid leukemia HL60 cells. The expression of c-Jun was 25-fold higher in pMV7-c-*jun*-transfected cells as compared to the controls (Figure 8d, e). Furthermore, we observed that A3D8 treatment of untransfected HL60 cells and the cells transfected with vector alone (pMV7) caused decreased proliferation and hence, decreased CD71 expression as compared to the isotype control (Figures 1 and 8e, panels i, ii). On the contrary, in HL60 cells overexpressing c-Jun (pMV7-c-*jun*), A3D8 treatment did not lead to any changes in CD71 expression as compared to the isotype control (Figure 8e, panel iii). A similar pattern was observed with CD11b expression (Figure 8f), although the differentiation-inducing ability of c-Jun in c-Jun over-expressing HL60 cells is not of the same extent as the proliferation-inducing ability. These results clearly indicate that downregulation of the proliferation promoting transcription factor c-Jun is a prerequisite for A3D8-mediated proliferation-inhibition in our settings.

Table 2 Activity of different c-*jun* promoter/luciferase constructs

DNA constructs (promoter/LUC)	*Basal promoter activity (% of full-length c-jun)*
−1780----------//----------//---------+731	100
−952----------//----------//-----+731	115±17
−716-----//----------//-----+731	483±39
−345-----//----------//-----+731	48±12
−180----------//-----+731	14±4
−63----------//-----+731	45±9

The values represent the promoter activity of different c-*jun* promoter constructs used in this study with respect to the full-length c-*jun* promoter (−1780/+731) chosen as 100. The transfection efficiency was corrected by dividing the promoter luciferase activity by renilla luciferase activity

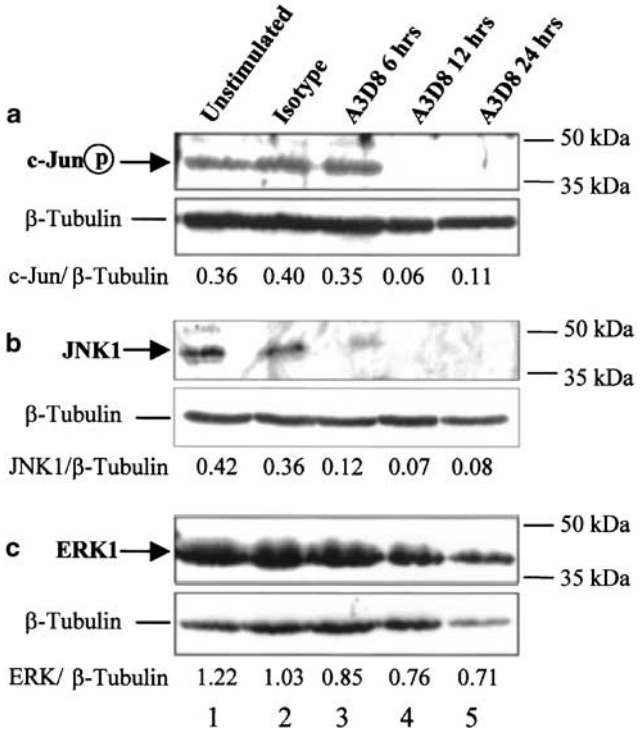


Figure 7 CD44 ligation decreases c-Jun phosphorylation and JNK expression. The figure represents immunoblot analysis of whole cell extract of HL60 cells probed with anti-phospho c-Jun (Ser 63), anti-JNK1 and anti-ERK1 antibodies (Santa Cruz Biotechnologies). (**a**) Phospho c-Jun, (**b**) JNK1, and (**c**) ERK1. Lanes: 1, 2, Unstimulated and isotype control, 3–5, A3D8 stimulated (6, 12, 24 h, respectively). The numbers underneath the blot indicate the protein/respective β-tubulin ratios after densitometric analysis (Aida 2.1 software program)

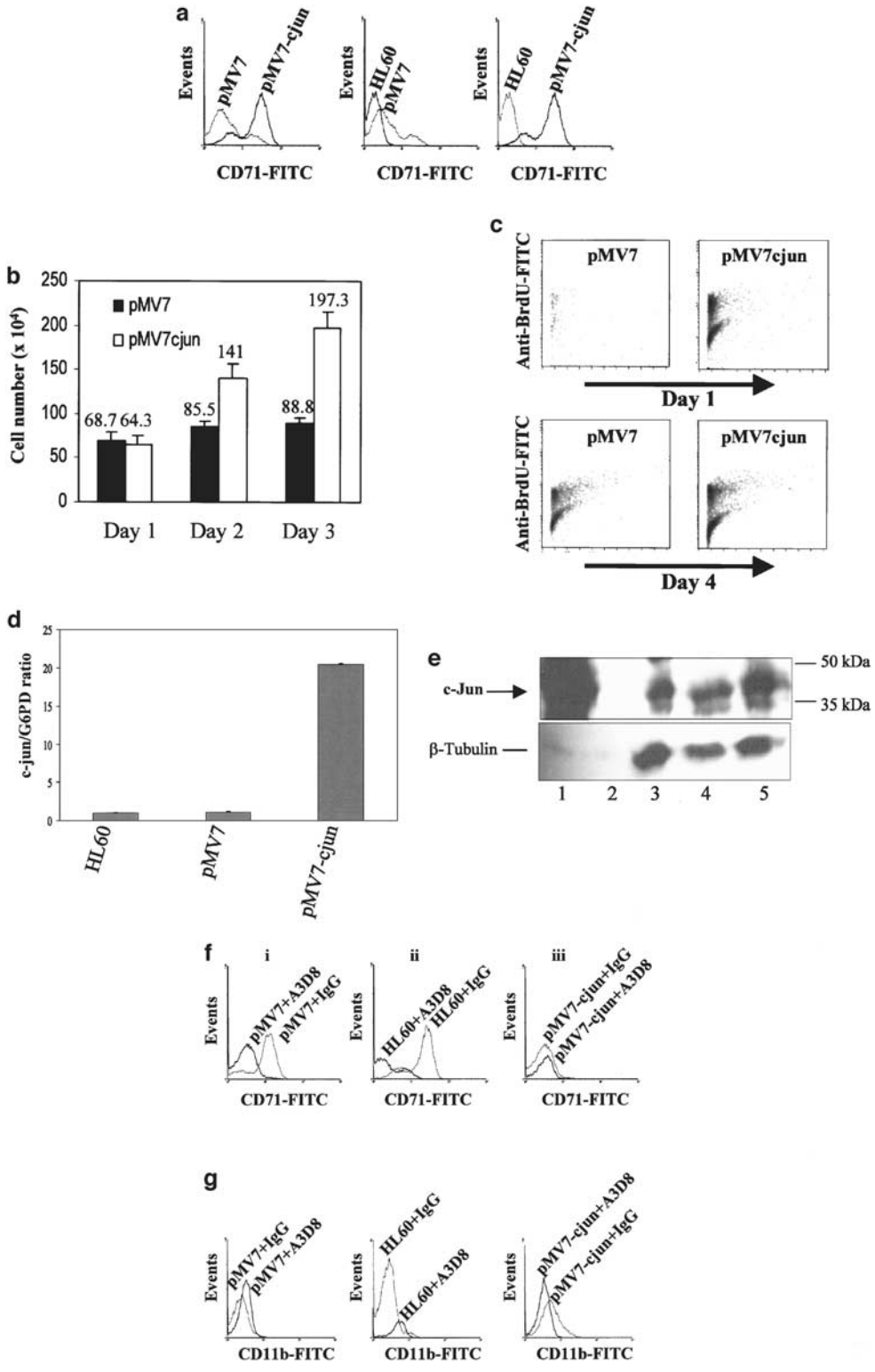
Discussion

In the present study, we investigated the effect of CD44 ligation by anti-CD44 MoAb A3D8 on the proliferation of myeloid leukemia cells and the underlying molecular mechanisms involved. Our results demonstrate that ligation of CD44 with A3D8 causes growth arrest and induces terminal differentiation of human myeloid leukemia cell lines HL60 and U937 through the downregulation of c-Jun expression via AP-1 sites (Figures 5 and 6). Furthermore, decreased JNK expression and a consequent decrease in c-Jun phosphorylation may be involved in A3D8-mediated downregulation of the c-Jun promoter activity (Figure 7). The downregulation of c-Jun expression is a prerequisite for growth inhibitory effects of A3D8 since overexpression of c-Jun is able to prevent A3D8-mediated effects (Figure 8). We also show an induction of G0/G1 arrest by A3D8 (Figure 3), which is accompanied by induction of p21 and inhibition of pRb, Cdk2 and Cdk4 protein expression and Cdk activity (Figure 4). Although our results show c-Jun inactivation as an important mechanism of A3D8-mediated growth arrest, we do not rule out other mechanisms by which A3D8 can block proliferation of myeloid leukemia cells.

Our results revealed that CD44 ligation with A3D8 decreases the proliferation and leads to the induction of terminal differentiation of HL60 and U937 cells (Figures 1 and 2). These results are consistent with previous reports (Charrad *et al*., 2002). A G0/G1 arrest upon A3D8 treatment of HL60 cells (Figure 3) could be explained considering that an exit from the cell cycle is a prerequisite for growth arrest and cell differentiation. Consistent with this and other reports (Danova *et al*., 1990; Hunter and Pines, 1994; Horiguchi-Yamada *et al*., 1994; Jiang *et al*., 1994; Liebermann *et al*., 1995; Steinman *et al*., 1998) A3D8 treatment led to the induction of p21 and inhibition of Cdk4 and Cdk2 kinase activities (Figure 4). Thus, increase of p21 and/or the decrease of Cdk2 and Cdk4 expression in HL60 upon A3D8 treatment are sufficient to inhibit kinase activity required for G0/G1 progression into the S phase. In correlation with decreased Cdk activity, the phosphorylation of Rb was also decreased upon A3D8 treatment suggesting a role of Cdk/Rb pathway in cell cycle arrest by CD44 signaling (Figure 4f).

CD44 ligation with A3D8 markedly downregulated the expression of c-Jun both at mRNA and protein level (Figure 5). The downregulation of c-Jun could be expected because of the decreased proliferation of these cells. The downregulation of c-*jun* upon A3D8 treatment was also observed in AML patient blasts (Figure 5). However, the effect of CD44 ligation on c-*jun* expression in AML patients is less prominent than in myeloid cell lines. This could be because of the difference in cytogenetic set up in different AML patients. Moreover, the requirement of c-Jun could differ in normal versus transformed cells, in tumor cells of different cell lineages, or in cells having undergone transformation via different mechanisms.

To rule out the downregulating effects on c-Jun as a consequence of A3D8 toxicity, we also show that A3D8 treatment does not downregulate the expression of c-Fos nonspecifically. Transient transfections of c-*jun* promoter/luciferase constructs in myeloid cells revealed that both AP-1 sites are responsible for the downregulation of c-*jun* promoter activity by CD44 signaling (Figure 6). Our results also show that the expression of JNK is decreased upon A3D8 treatment, with a consequent decrease in the level of c-Jun phosphorylation, which is consistent with published reports (Figure 7) (Binetruy *et al*., 1991; Minden *et al*., 1994;



Smeal *et al*., 1991). We used JNK1 since it has been shown to preferentially bind c-Jun and phosphorylate c-Jun (Kallunki *et al*., 1994). The role of other JNKs remains to be determined. The effect of A3D8 is JNK-specific because ERK expression is not changed under similar conditions. It is important to note here that c-Fos expression did not change upon A3D8 treatment (Figure 5f). Fos is known to be regulated by the ERK pathway (Chou *et al*., 1992; Deng and Karin, 1994). The decreased JNK expression and hence, c-Jun phosphorylation could in turn be because of the positive effect of A3D8 treatment on p21 protein level (Figure 4a). It has been reported that interaction of p21 with JNK inhibits JNK activity (Shim *et al*., 1996; Patel *et al*., 1998). Thus, it is conceivable that the inhibition of JNK by p21 may be the mechanism for the downregulation of c-Jun phosphorylation and hence, the transactivation potential of c-*jun*.

A3D8-mediated proliferation-inhibition in HL60 cells was prevented by c-Jun overexpression (Figure 8e), suggesting that c-Jun/AP-1 activity is one of the molecular targets downstream of CD44 signaling. BrdU incorporation, CD71 expression (Theil, 1990; Kuhn, 1994; Trayner *et al*., 1998) and Trypan blue cell counting were used as a measure of cell proliferation and clearly demonstrate that c-Jun in fact, increases the proliferation of myeloid cells. c-Jun overexpression had little effect on CD11b expression (Figure 8f). In the context of the role of c-Jun in myeloid cell differentiation, this might seem surprising. However, the role of c-Jun in differentiation could be time limiting. It is important to mention here that the downregulation of c-Jun expression upon CD44 ligation is in accordance with our recent findings in which the transcription factor C/EBPα was shown to downregulate c-Jun expression to induce myeloid differentiation (Rangatia *et al*., 2002). In summary, it is thus, conceivable to propose a model (Figure 9) which shows that the downregulation of c-Jun expression along a pathway which involves inhibition of JNK by p21 resulting in decreased c-Jun phosphorylation and hence, decreased promoter activity concomitant with decreased cell cycle proteins may be an important mechanism for regulating CD44-mediated differentiation-induction and proliferation-arrest with A3D8. Our results provide a framework for further investigations and suggest that clarification of the mechanisms of regulation may reveal novel targets for antiproliferative and/or differentiation therapy in AML.

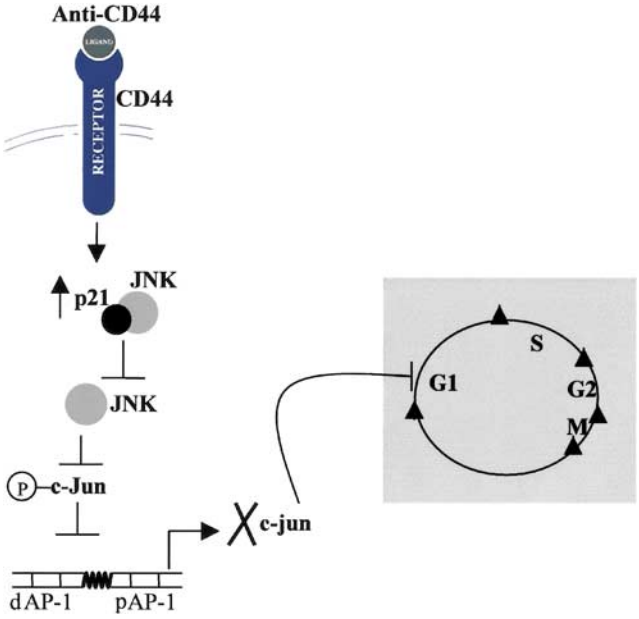


Figure 9 Model of how CD44 ligation inhibits proliferation and blocks cell cycle of myeloid leukemia cells by downregulating c-Jun expression. The figure shows a possible mechanism for the growth inhibitory effect of anti-CD44 antibody A3D8 in myeloid cells. The growth arrest involves decreased c-Jun expression along a pathway, which involves inhibition of JNK by p21 resulting in decreased c-Jun phosphorylation and hence decreased promoter activity concomitant with decreased cell cycle proteins

Materials and methods

AML patient samples

Peripheral blood or bone marrow specimens containing at least 80% leukemic blasts were collected on the occasion of initial diagnosis. The fresh samples were seeded at 1×10^6 cells/ml and stimulated with 20 μg/ml A3D8. RNA isolation was performed using RNeasy Mini kit (Qiagen) at time points indicated in the figure legends and real-time PCR was performed for c-*jun* expression as described below. Control samples were either unstimulated or stimulated with isotype matched antibody (IgG1, Sigma and/or J173, Immunotech). Cytospin was also performed to investigate the differentiation inducing ability of A3D8 as previously reported (Charrad *et al*., 1999).

Cell lines, antibodies and treatments

Human myeloid cell lines HL60 and U937 were obtained from DMSZ (Germany) and cultured in RPMI-1640 medium (PAN

Figure 8 Ectopic overexpression of c-Jun in HL60 cells increases their proliferation and prevents A3D8-mediated downregulation of proliferation. The figure represents an overlay of different peaks from FACS analysis for CD71 and CD11b expression of HL60 cells. (**a**) Overexpression of c-Jun in HL60 cells increases their proliferation. HL60 cells were electroporated with pMV7-c-*jun* and pMV7 and selected in G418. The cells were then analysed for CD71 expression as described in Materials and methods. In addition to empty vector control, untransfected HL60 cells also served as control. The overlay of the peaks was carried out with WinMDI 2.8 software program. (**b**) Trypan blue cell counting of cells transfected with pMV7 and pMV7-c-*jun* when the cells were under selection pressure. (**c**) pMV7 and pMV7-c-*jun*-transfected cells were incubated with BrdU for 1 h and subsequently stained for BrdU-incorporation as described. The figure represents a scatter plot at days 1 and 4 post-transfection. (**d**) This figure represents Real time PCR for c-*jun* expression in c-Jun overexpressing HL60 cells. The bars represent c-*jun*/G6PD ratio of untransfected HL60 cells, transfected with empty vector, pMV7 and with pMV7-c-*jun*. (**e**) This represents Western blot analysis for c-Jun expression in c-Jun overexpressing HL60 cells. Lanes: 1, *In vitro*-translated c-Jun, 2, reticulocyte lysate, 3, untransfected HL60 cells, 4, pMV7-transfected cells and 5, pMV7-c-*jun*-transfected cells. (**f**, **g**) Overexpression of c-Jun in HL60 cells prevents A3D8-mediated growth inhibition and differentiation induction. The figure represents overlay from FACS analysis for CD71 and CD11b expression in c-Jun overexpressing HL60 cells before and after treatment with A3D8. After selection in G418 for 1 week, HL60 cells were stimulated with A3D8 at final concentration of 20 μg/ml for further 36 h and the cells were analysed for CD71 and CD11b expression by flow cytometry

Biotech GMBH) supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 10% FBS (GIBCO-BRL, Eragny, France) at 37°C with 5% CO_2 enrichment. The cells were suspended at 3×10^5 cells/ml in 96-well tissue culture plates (Nunc) with 200 μl/well. Monoclonal anti-CD44 antibody, A3D8 (Sigma) was added at the final concentration of 20 μg/ml. All the control samples were either untreated or treated with 20 μg/ml isotype-matched antibody (IgG1, Sigma or J173, Immunotech). For direct immunofluorescence staining, fluorescein isothiocyanate (FITC) MoAbs to CD11b (FITC, immunotech, IgG1) and CD71 (FITC, DAKO, IgG1) were used.

Proliferation assay

To assess the proliferation state of cells after various treatments, MTT proliferation assay kit (Boehringer Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, cells were incubated with or without A3D8 for 1–4 days in 96-well plates. A volume of 10 μl MTT labeling reagent (5 mg/ml) was added every day to each well and the plates were incubated at 37°C for 4 h. The resulting formazon crystals were solubilized by adding 100 μl of solubilization buffer (10% SDS in 0.01 M HCl) per well and the plates were incubated at 37°C overnight. The absorbance of the formazon measured at 575 nm was used to account for the proliferation state of cells. Trypan blue cell exclusion was also used to assess the cell viability and the cell number.

Morphological analysis of cellular differentiation

After various cell treatments, HL60 and U937 cells were cytocentrifuged on glass slides using a cytospin device (Shandon-Elliot, Paris, France). May–Grünwald–Giemsa-stained cytospin smears were then analysed under light microscopy. The analysis was performed on 200–300 cells.

Cell cycle analysis and flow cytometry

To investigate the surface expression of myeloid differentiation and proliferation markers, FACS analysis was performed. HL60 and U937 cells (3×10^5 cells/ml) were stimulated for 36 h with A3D8 (20 μg/ml) and then stained with FITC or PE labeled antibodies at a concentration of 10 μg/ml at 4°C for 30 min and washed twice with FACS buffer (PBS, 3% FCS, 0.01% NaN_3). Fluorescence was then analysed on Coulter EPICS XL/XL-MCL SystemII Software. Data were collected after 5000 cell analysis (per sample) and the results shown as scatter diagrams or expressed as *D*-value which is calculated as

$$\text{Mean fluorescence intensity (MFI) ratio} = \text{Mean of sample stained with MoAb/mean of isotype control MoAb}$$

Then, the percentage of difference between MFI ratio of sample incubated with or without A3D8 was calculated, giving a *D*-value (Pisani *et al*., 1997) for each sample. The *D*-value for unstimulated control was arbitrarily chosen and that of stimulated sample expressed relative to it. Negative *D*-values indicate more MFI of stimulated sample than unstimulated and hence, implies increased expression of the particular marker. Similarly, positive *D*-values imply decreased expression of the marker. For the cell cycle analysis, cells with or without A3D8 treatment were centrifuged at 1500 r.p.m. for 3 min, washed with PBS and then the DNA was stained with 100 μg/ml propidium iodide for 30 min at 4°C protected from light. The cells were then analysed with the FACScan (Beckton-Dickinson) for different cell populations.

RNA isolation and semiquantitative RT–PCR

Total RNA was isolated from 7×10^5 cells before and after treatment with A3D8 (20 μg/ml) and IgG1 (20 μg/ml) using RNeasy Mini kit (Qiagen). RNA of 100 ng was used for first-strand cDNA synthesis in a 20 μl reaction with 10 × RT buffer, dNTP (5 mM), RNasin (1 U/μl), oligo dT (1 μM) and the reaction was incubated at 37°C for 90 min. Equal amounts of cDNA were taken for c-*jun* PCR amplification using a Qiagen kit. Aldolase was used as an internal control. Primers used for PCR amplification were for c-*jun* (Gene Bank Acc. no. J04111): forward primer 5′-ACTGCAAAGA TGGAAAC-GAC-3′ (bp 1264–1283) and reverse primer 5′-AAAATGTTT GCAACTGCTGC-3′ (bp 2235–2254); and for aldolase: forward primer 5′-AGCTGTCTG ACATCG CTCACCG-3′ and reverse primer 5′-CACATA CTGG CAGCGC TTCAAG-3′. The PCR cycling program consisted of 30 cycles of 94°C for 2 min, 55°C for 1 min and 72°C for 80 s using DNA thermal cycler (Perkin-Elmer). PCR products of ~971 bp (c-*jun*) and ~580 bp (Aldolase) were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining with UV irradiation.

Quantitative Real-time PCR in AML patient samples

Real-time PCR for c-*jun* and for the housekeeping gene glucose-6-phosphate dehydrogenase (G6PD) was performed using the Light Cycler Technology (Roche Diagnostics, Mannheim, Germany). For amplification of G6PD, primers were used according to Emig *et al*. (1999): G6PD-1 forward primer 5′-CCGGATCGACCACTACCTGGGCAAG-3′ and G6PD-2 reverse primer 5′-GTTCCCCACGTACTGGC-CCAGGACCA-3′. c-*jun* was amplified using the primer set according to Kiaris *et al* (1999). c-*jun*-1 forward primer 5′-GCATGAGGAACC GCATCGCTGCCTCCAAGT-3′ and c-*jun*-2 reverse primer 5′-GCGACCAAGTCCTT CCCACTCGTGCACACT-3′. G6PD plasmid: pGdBBX, kindly provided by A Hochhaus, University of Mannheim was serially diluted to 10 000, 1000 and 100 fg and used as a standard curve. The procedure for Real-time PCR was essentially the same as described previously (Reddy *et al*., 2002).

Immunoblot analysis

Total cellular protein was extracted from HL60 and U937 cells before and after A3D8 treatment and subjected to electrophoresis on 10% SDS–PAGE gels. The Western blotting procedure was performed and the blots detected with the ECL system as described previously (Behre *et al*., 1999). Anti-β-tubulin antibody (Boehringer, Mannheim) was used as internal loading control on the same blot after stripping. Immunoblot analysis was performed for c-Jun (anti-rabbit, sc-45), c-Fos (anti-rabbit polyclonal, sc-52), JunB (anti-mouse monoclonal IgG1, sc-8051), Cdk2 (anti-rabbit, sc-163), Cdk4 (anti-rabbit, sc-260), Cdk6 (anti-rabbit, sc-177), cyclin D1 (anti-rabbit, sc-718), p21 (anti-mouse, sc-817), pRb (anti-mouse monoclonal, sc-102) and phospho-specific Rb (anti-goat, pRb-Ser780, sc-12901). The other antibodies used for the Immunoblot analysis were JNK1 (anti-rabbit, sc-474), ERK1 (anti-rabbit, sc-94 Santa Cruz), phospho-c-Jun (anti-mouse monoclonal, sc-822 Santa Cruz) and β-tubulin (anti-rabbit, sc-9104). All the blots were quantified by densitometry (Aida 2.1 software program) and the results reported in relation to β-tubulin. Comparison of the

differences in expression was done between isotype control and A3D8-treated samples. Unstimulated samples served as a reference for the basal level of expression.

Immunocomplex kinase assay

After the described treatments, HL60 cells were washed with cold PBS and RIPA lysates prepared at different time points. Lysates were collected by centrifugation for 30 min and protein concentrations were quantified by the Bradford assay (Bio-Rad Laboratories, Germany). Protein of 200 μg was incubated with 2 μg of anti-Cdk2 or anti-Cdk4 antibody at 4°C for 2 h with rotation. Protein A agarose beads (20 μl) were then added and the incubation continued for another 2 h. Immunocomplex beads were washed twice with PBS buffer and three times with kinase buffer (150 mM NaCl; 1 mM EDTA; 50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; and 10 mM DTT). Kinase activity was assayed by incubating the beads at 37°C for 30 min with 25 μl kinase buffer, 3 μg histone H1 (Upsdate, Germany; Cdk2) or Rb-fusion protein (Santa Cruz; Cdk4), 10 μM ATP, and 4 μCi [γ-32P]ATP (3000 Ci/mmol). Samples were then boiled for 5 min in 2 × sample buffer, electrophoresed through a 12% SDS–PAGE, dried, and phosphorylated histone H1 and Rb proteins were visualized by autoradiography and quantified by Aida 2.1 software program.

Reporter constructs and transient transfections

The human full-length c-*jun* promoter plus 731 bp of downstream sequences (−1780/+731) subcloned into the firefly luciferase vector pGL3, and deletion mutants in the proximal and distal AP-1 sites of the same construct used in this study were kindly provided by Wayne Vedeckis (Wei *et al*., 1998). As an internal control plasmid for cotransfection assays, the pRL-0 construct driving the Renilla luciferase gene (Promega) was used as described (Behre *et al*., 1999). Transient transfections were performed with these constructs by using Effectene transfection reagent (Qiagen) in myeloid cells. Effectene allows transfections in the absence of serum, which was important to rule out any serum-induced fluctuations in c-*jun* promoter activity. The cells were transiently transfected with 1 μg c-*jun* promoter/luciferase constructs and pRL-0 plasmid per well of the six-well plates. At 18 h after transfection, A3D8 was added to the wells to a final concentration of 20 μg/ml for additional 6 h. Promoter activities were determined by measuring the luciferase activity with the Dual Luciferase Assay System (Promega). Firefly luciferase activities of different c-*jun* promoter constructs in pGL3 were normalized to the Renilla luciferase values of pRL-0 (Behre *et al*., 1999). Results are given as mean ± s.d. of at least three different independent experiments.

Stable cell lines overexpressing c-Jun and BrdU incorporation

To generate cell lines overexpressing c-Jun, the retrovirus-derived cDNA expression vector was used for the study. This vector, designated pMV7-c-*jun* was kindly provided by Dr Yaniv. The vectors are described elsewhere (Kirschmeier *et al*., 1988). pMV7-c-*jun* and the empty vector pMV7 (lacking the c-Jun cDNA insert) were transfected into HL60 cells by electroporation (300 V/975 μF). After 24–48 h, the cells were transferred into selective medium containing 1 μg/ml G418 and analysed for CD71 expression and BrdU incorporation by flow cytometry. Trypan blue cell counting and PI staining was also performed at different time points. In addition to CD71 expression and cell counting, BrdU, a thymidine analogue which gets incorporated into cellular DNA was also used as a measure of cell proliferation in c-Jun overexpressing HL60 cells. The assay was performed by using *in situ cell proliferation kit, FLUOS* (Roche, Mannheim, Germany, cat. no. 1810740) according to the manufacturer's instructions. Briefly, after transfer into selection medium, HL60 cells (3×10^5 cells/ml previously transfected with pMV7 and pMV7-c-*jun*) were incubated with 10 μM BrdU for 1 h at 37°C in 5% CO_2. The cells were then fixed in 70% ethanol in 50 mM glycine buffer, pH 2.0, washed in PBS, acid denatured (4 M HCl) and incubated with anti-BrdU-FLUOS antibody solution for 45 min at 37°C in 5% CO_2. The cells were then analysed by flow cytometry and the data presented as scatter diagrams. In a similar experiment, after 1 week in the selection medium, the cells transfected with pMV7 or pMV7-c-*jun* were then stimulated with A3D8 or IgG with a final concentration of 20 μg/ml. At 36 h after treatment with A3D8, the cells were analysed for CD11b and CD71 expression. The expression of c-Jun in c-Jun overexpressing HL60 cells was measured by Real-time PCR and Western blot analysis.

Acknowledgements
We thank Dr Dirk Eick and Alloy Sheppers for valuable comments and useful discussions, Christian Kurzreder, Doreen Westhaus and Vijay Rawat for help with flow cytometry experiments and Susanne Schnittger for providing AML patient samples. This work was supported by a DFG (German research foundation) grant given to GB (2042/2-1).

References

Allouche M, Charrad RS, Bettaieb A, Greenland C, Grignon C and Smadja-Joffe F. (2000). *Blood*, **96,** 1187–1190.

Aruffo A, Stamenkovic I, Melnick M, Underhill CB and Seed B. (1990). *Cell*, **61,** 1303–1313.

Bakiri L, Lallemand D, Bossy-Wetzel E, Yaniv M. (2000). *EMBO J.*, **2,** 2056–2068.

Bates S, Parry D, Bonetta L, Vousden K, Dickson C and Peters G. (1994). *Oncogene*, **9,** 1633–1640.

Behre G, Singh SM, Liu H, Bortolin LT, Christopeit M, Radomska HS, Rangatia J, Hiddemann W, Friedman AD and Tenen DG. (2002). *J. Biol. Chem.*, **277,** 26293–26299.

Behre G, Smith LT and Tenen DG. (1999). *Biotechniques*, **26,** 24–26.

Behre G, Whitmarsh AJ, Coghlan MP, Hoang T, Carpenter CL, Zhang DE, Davis RJ and Tenen DG. (1999). *J. Biol. Chem.*, **274,** 4939–4946.

Behre G, Zhang P, Zhang DE and Tenen DG. (1999). *Methods*, **17,** 231–237.

Binetruy B, Smeal T and Karin M. (1991). *Nature*, **351,** 122–127.

Charrad RS, Gadhoum Z, Qi J, Glachant A, Allouche M, Jasmine C, Chomienne C and Smadja-Joffe F. (2002). *Blood*, **1,** 290–299.

Charrad RS, Li Y, Delpech B, Balitrand N, Clay D, Jasmin C, Chomienne C and Smadja-Joffe F. (1999). *Nat. Med.*, **5,** 669–676.

Chou SY, Baichwal V and Ferrell Jr JE. (1992). *Mol. Biol. Cell.*, **3,** 1117–1130.
Danova M, Giordano M, Mazzini G and Riccardi A. (1990). *Leukemia Res.*, **14,** 417–422.
Deng T and Karin M. (1994). *Nature*, **371,** 171–175.
Elsässer A, Franzen M, Reddy V and Behre G . (2002). *ASH* [Abstract]. **100,** 200.
Emig M, Saussele S, Wittor H, Weisser A, Reiter A, Willer A, Berger U, Hehlmann R, Cross NC and Hochhaus A. (1999). *Leukemia*, **13,** 1825–1832.
Gitig DM and Koff A. (2000). *Methods Mol. Biol.*, **142,** 109–123.
Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ. (1993a). *Cell*, **75,** 805–816.
Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ. (1993b). *Cell*, **19,** 805–816.
Hilberg F, Aguzzi A, Howells N and Wagner. (1993). *Nature*, **365,** 179–181.
Hilberg F and Wagner EF. (1992). *Oncogene*, **7,** 2371–2380.
Horiguchi-Yamada J, Yamada H, Nakada S, Ochi K and Nemoto T. (1994). *Mol. Cell. Biochem.*, **132,** 31–37.
Hunter T and Pines J. (1994). *Cell*, **79,** 573–582.
Jiang H, Lin J, Su ZZ, Collart FR, Huberman E and Fisher PB. (1994). *Oncogene*, **9,** 3397–3406.
Kallunki T, Su B and Tsigelny I. (1994). *Genes Dev.*, **8,** 2996–3007.
Kantarjian H. (1999). *Semin. Hematol.*, **36,** 16–25.
Kiaris H, Schally AV, Sun B, Armatis P and Groot K. (1999). *Oncogene*, **18,** 7168–7173.
Kirschmeier PT, Housey GM, Johnson MD, Perkins AS and Weinstein IB. (1988). *DNA*, **7,** 219–225.
Kovary K and Bravo R. (1991). *Mol. Cell. Biol.*, **11,** 4466–4472.
Kuhn LC. (1994). *Bailliere's Clin. Haematol.*, **7,** 763–785.
Liesveld JL, Dipersio JF and Abboud CN. (1994). *Leukemia Lymphoma*, **14,** 19–28.
Liebermann DA, Hoffman B and Steinman RA. (1995). *Oncogene*, **11,** 199–210.
Lowell C-A and Berton G. (1999). *J. Leukoc. Biol.*, **65,** 313–320.
Lowenberg B, Downing JR and Burnett A. (1999). *N. Engl. J. Med.*, **341,** 1051–1062.
Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ and Kato JY. (1994). *Mol. Cell. Biol.*, **14,** 2066–2076.
Meyerson M and Harlow E. (1994). *Mol. Cell. Biol.*, **14,** 2077–2086.
Milde-Langosch K, Bamberger AM, Methner C, Rieck G and Loning T. (2000). *Int. J. Cancer*, **87,** 468–472.
Minden A, Lin A, Smeal T, Derijard B, Cobb M, Davis R and Karin M. (1994). *Mol. Cell. Biol.*, **14,** 6683–6688.
Miyake K, Underhill CB, Lesley J and Kincade PW. (1990). *J. Exp. Med.*, **172,** 69–75.
Moll J, Khaldoyanidi S, Sleeman JP, Achtnich M, Preuss I, Ponta H and Herrlich P. (1998). *J. Clin. Invest.*, **102,** 1024–1034.
Muller R, Mumberg D and Lucibello FC. (1993). *Biochim. Biophys. Acta*, **1155,** 151–179.
Nead MA, Baglia LA, Antinore MJ, Ludlow JW and McCance DJ. (1998). *EMBO J.*, **17,** 2342–2352.
Nishitani J, Nishinaka T, Cheng CH, Rong W, Yokoyama KK and Chiu R. (1999). *J. Biol. Chem.*, **274,** 5454–5461.
Patel R, Bartosch B and Blank JL. (1998). *J. Cell Sci.*, **111,** 2247–2255.
Pisani F, Del Poeta G, Aronica G, Venditti A, Caravita T and Amadori S. (1997). *Ann. Hematol.*, **75,** 145–147.
Rangatia JS, Vangala RK, Treiber N, Zhang P, Radomska HS, Tenen DG, Hiddemann W and Behre G. (2002). *Mol. Cell. Biol.*, **22,** 8681–8785.
Reuss-Borst MA, Buhring HJ, Klein G and Muller CA. (1992). *Ann. Hematol.*, **65,** 169–174.
Reddy V, Iwama A, Iotzova G, Schulz M, Elsasser A, Vangala RK, Tenen DG, Hiddemann W and Behre G. (2002). *Blood*, **100,** 483–490.
Sanford J, Shattil, Hirokazu, Kashiwagi and Pampori N. (1998). *Blood*, **91,** 2645–2657.
Savatier P, Huang S, Szekely L, Wiman KG and Samarut J. (1994). *Oncogene*, **9,** 809–818.
Schreiber M, Kolbus A, Piu F, Szabowski A, Mohle-Steinlein U, Tian J, Karin M, Angel P and Wagner EF. (1999). *Genes Dev.*, **13,** 607–619.
Shaulian E and Karin M. (2001). *Oncogene*, **20,** 2390–2400.
Sherr CJ. (1994). *Stem Cells*, **1,** 47–55.
Shim J, Lee H, Park J, Kim H and Choi EJ. (1996). *Nature*, **381,** 804–806.
Sievers EL. (2000). *Curr. Opin. Oncol.*, **12,** 30–35.
Slack RS, Hamel PA, Bladon TS, Gill RM and McBurney MW. (1993). *Oncogene*, **8,** 1585–1591.
Smeal T, Binetruy B, Mercola DA, Birrer M and Karin M. (1991). *Nature*, **354,** 494–496.
Smith MJ and Prochownik EV. (1992). *Blood*, **79,** 2107 2115.
Steinman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA and El-Houseini ME. (1994). *Oncogene*, **9,** 3389–3396.
Steinman RA, Jianping H, Beatrice Y, Julie PG, Ball ED and Nguyen A. (1998). *Blood*, **91,** 4531–4542.
Tenen DG, Hromas R, Licht JD and Zhang DE. (1997). *Blood*, **90,** 489–519.
Theil EC. (1990). *J. Biol. Chem.*, **265,** 4771–4774.
Trayner ID, Bustorff T, Etches AE, Mufti GJ, Foss Y and Farzaneh F. (1998). *Leukemia Res.*, **22,** 537–547.
Vangala RK, Heiss-Neumann MS, Rangatia JS, Singh SM, Schoch C, Tenen DG, Hiddemann W and Behre G. (2002). *Blood*, **101,** 270–277.
Verfaillie CM. (1998). *Blood*, **92,** 2609–2612.
Waxman S. (2000). *Leukemia*, **14,** 491–496.
Wei P, Inamdar N and Vedeckis WV. (1998). *Mol. Endocrinol.*, **12,** 1322–1333.
Wisdom R, Johnson RS and Moore C. (1999). *EMBO J.*, **18,** 188–197.

Oncogene (2003) 22, 4760–4764

www.nature.com/onc

Elevated c-Jun expression in acute myeloid leukemias inhibits C/EBPα DNA binding via leucine zipper domain interaction

Janki Rangatia[1], Rajani K Vangala[1], Sheo M Singh[1], Abdul A Peer Zada[1], Annika Elsässer[1], Alexander Kohlmann[1], Torsten Haferlach[1], Daniel G Tenen[2], Wolfgang Hiddemann[1], Gerhard Behre*[,1]

[1]*Medicine III at University of Munich Hospital Grosshadern and GSF Research Center, Munich, Germany;* [2]*Harvard Institutes of Medicine, Harvard Medical School, Boston, MA, USA*

Transcription factor C/EBPα induces normal myeloid differentiation, inactivation of C/EBPα leads to a differentiation block in acute myeloid leukemias (AML), and overexpression of C/EBPα results in AML growth arrest and differentiation. Recent reports suggest that C/EBPα is activated or inactivated via protein–protein interactions. We previously reported that C/EBPα needs to inactivate the proto-oncogene c-Jun via leucine zipper domain interaction in order to induce granulocytic differentiation. We, therefore, hypothesized that c-Jun expression might be elevated in AML and subsequently inactivate C/EBPα. In fact, compared to normal bone marrow mononuclear cells, c-Jun expression is increased in AML patient samples (Affymetrix expression microarray analysis, $n = 166$). c-Jun binds to C/EBPα via the leucine zipper domains and prevents C/EBPα from DNA binding. Inactivation of C/EBPα by c-Jun is necessary for c-Jun to induce proliferation because c-Jun-induced proliferation can be prevented by ectopic overexpression of C/EBPα. The dominant-negative 30-kDa C/EBPα protein, found in AML, fails to downregulate c-Jun mRNA expression in AML patient samples. Thus, our data suggest a model for AML in which c-Jun promotes proliferation and prevents differentiation by inhibiting C/EBPα DNA binding via leucine zipper domain interaction. It might depend on the expression levels of C/EBPα and c-Jun, if inhibition of C/EBPα by c-Jun or if inhibition of c-Jun by C/EBPα is more predominant: proliferation versus differentiation; AML versus normal myeloid development.

Oncogene (2003) **22,** 4760–4764. doi:10.1038/sj.onc.1206664

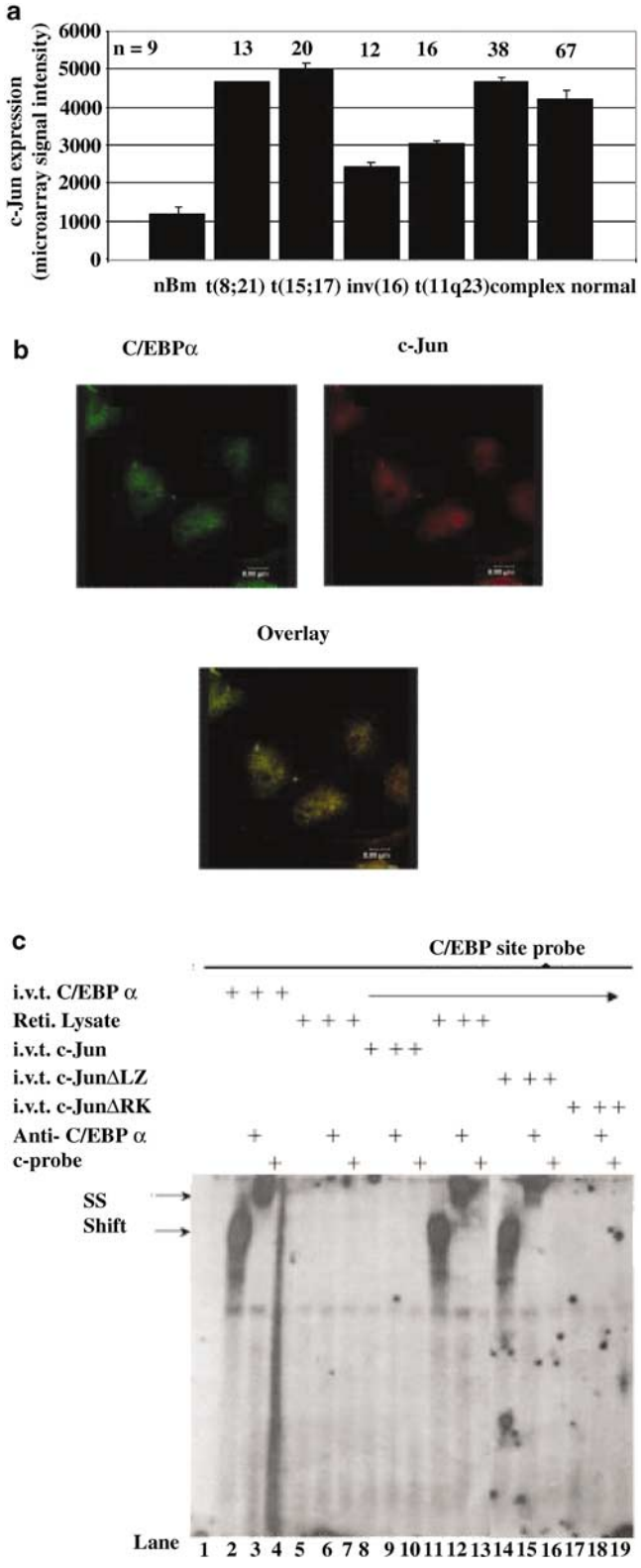
Keywords: C/EBPα; c-Jun; AML

Acute myeloid leukemias (AML) are characterized by the outgrowth of immature myeloid cells which are blocked in differentiation (Tenen, 2003). Recently, a concept is arising in which for AML to develop the activity of the transcription factors C/EBPα and PU.1, which are both pivotal for normal myeloid differentiation, must be impaired by either mutations (Pabst *et al*., 2001b; Mueller *et al*., 2002) or by antagonistic protein–protein interactions (Pabst *et al*., 2001a; Vangala *et al*., 2003). Thus, we previously reported that C/EBPα (Pabst *et al*., 2001b) and PU.1 (Mueller *et al*., 2002) are mutated in AML, and that AML1-ETO in t(8;21)-AML binds to C/EBPα (Pabst *et al*., 2001b) and PU.1 (Vangala *et al*., 2003) and blocks their activity via direct protein–protein interactions. Furthermore, the fusion protein BCR-ABL in chronic myeloid leukemia (Perrotti *et al*., 2002; Schuster *et al*., 2003) and AML-specific Flt3 mutations (Mizuki *et al*., 2002) downregulate C/EBPα expression. Thus, inactivation of C/EBPα and PU.1 by direct protein–protein interactions is an arising new mechanism in the pathogenesis of myeloid leukemias. Most interestingly, protein–protein interactions are not only responsible for inactivation of C/EBPα in AML, but also C/EBPα itself seems to function via protein–protein interactions (McKnight, 2001). Thus, C/EBPα arrests cell proliferation through direct inhibition of Cdk2 and Cdk4 (Wang *et al*., 2001, 2002), E2F repression by C/EBPα is required for adipogenesis and granulopoiesis (Porse *et al*., 2001), and C/EBPα binds to and leads to phosphorylation of p300 (Schwartz *et al*., 2003).

The proto-oncogene c-Jun, a member of the AP-1 transcription factor family, can induce proliferation by repression of tumor suppressors, as well as upregulation of positive cell cycle regulators, and c-Jun expression is elevated in many types of cancers (Shaulian and Karin, 2002). In contrast, the transcription factor C/EBPα is necessary for normal myeloid differentiation (Zhang *et al*., 1997; Porse *et al*., 2001), inactivation of C/EBPα can lead to myeloid leukemia (Pabst *et al*., 2001a,b; Perrotti *et al*., 2002; Schuster *et al*., 2002), and overexpression of C/EBPα in AML leads to growth arrest and partial differentiation (Radomska *et al*., 1998; Truong *et al*., 2003). We previously reported that C/EBPα is functionally activated by the Ras signal transduction pathway via phosphorylation on serine 248 (Behre *et al*., 2002). After being activated, C/EBPα inactivates the transcription factor PU.1 via direct protein–protein interaction to prevent dendritic and

*Correspondence: G Behre, Medicine III at University of Munich Hospital Grosshadern, Marchioninistr. 15, D-81377 Munich, Germany; E-mail: gerdbehre@aol.com
Received 11 January 2003; revised 27 March 2003; accepted 27 March 2003

monocytic cell differentiation (Iwama *et al*., 2002; Reddy *et al*., 2002). Furthermore, C/EBPα needs to inactivate the proto-oncogene c-Jun via leucine zipper domain interaction in order to prevent proliferation and to induce granulocytic differentiation (Rangatia *et al*., 2002). We therefore hypothesized that c-Jun expression might be elevated in AML and inactivate C/EBPα.

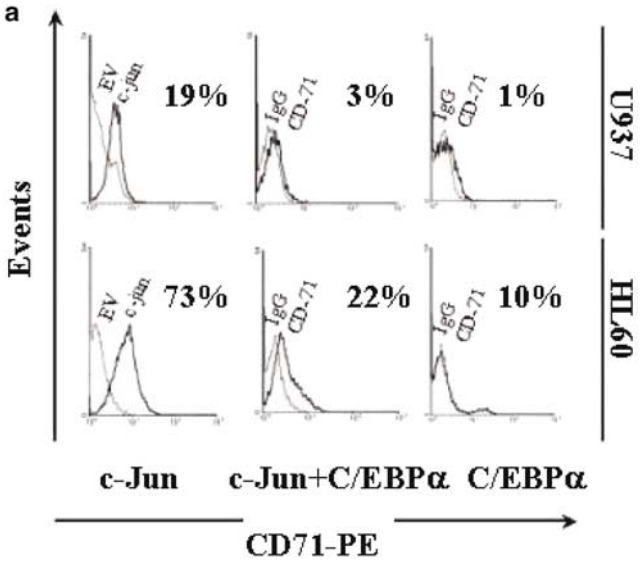


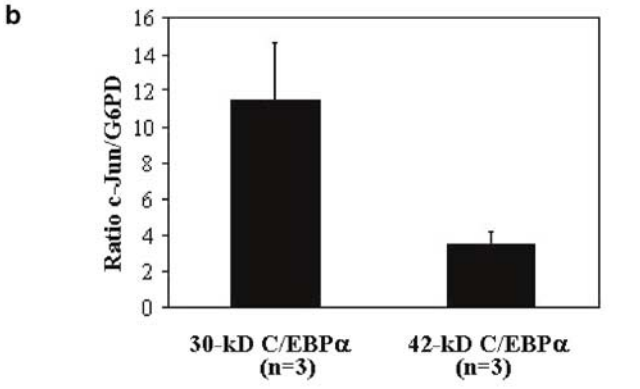
Elevated c-Jun expression in AML

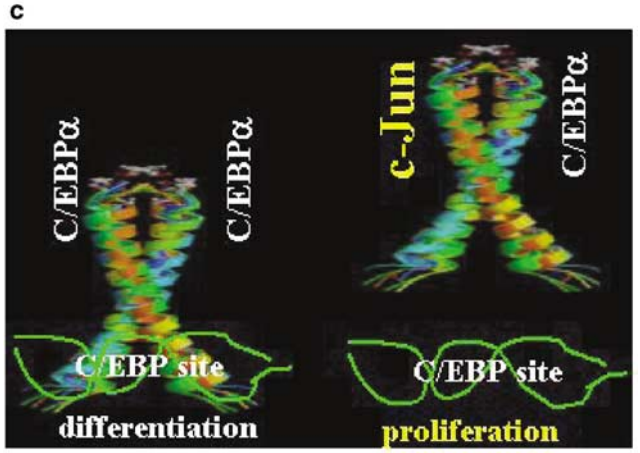
The proto-oncogene c-Jun can induce proliferation (Peer Zada *et al*., 2003) and c-Jun expression is elevated in many types of neoplasia (Shaulian and Karin, 2002). The leukemic fusion proteins BCR-ABL (Burgess *et al*., 1998) and AML1-ETO (Elsaesser *et al*., 2003) upregulate c-Jun expression, and differentiation induction of AML blasts with antibodies inducing CD44-signaling downregulates c-Jun expression (Peer Zada *et al*., 2003). Furthermore, we previously reported that C/EBPα needs to downregulate c-Jun expression in order to induce granulocytic differentiation (Rangatia *et al*., 2002). We therefore hypothesized that c-Jun expression might be elevated in AML. Total RNA from AML patient samples was isolated, and gene expression analysis was performed (Affymetrix HG-U133A microarray). As seen in Figure 1a, c-Jun, c-Jun mRNA expression level in normal bone marrow mononuclear cells from healthy volunteers is lower in comparison with c-Jun expression

Figure 1 Elevated c-Jun expression in AML; c-Jun inhibits C/EBPα DNA binding via leucine zipper domain interaction. (**a**) c-Jun mRNA expression is increased in AML patient samples ($n=166$) in comparison with normal bone marrow (nBM) mononuclear cells ($n=9$). Total RNA was isolated and processed as described before (Schoch *et al*., 2002). Standard Affymetrix software (Microarray Suite, Version 5.0) and the HG-U133A set of normalization controls were used for data analysis (mask file online available, www.affymetrix.com). As recommended by the manufacturer, 100 human maintenance genes served as a tool to normalize and scale the data prior to performing data comparisons. Expression signal intensities are given as absolute numbers (Schoch *et al*., 2002). The error bars indicate the s.e.m. *n* indicates the number of patient samples analysed in each subgroup. AML patient samples included translocation t(8;21), t(15;17), t(11q23)/MLL, inv(16) positive cases, as well as cases with complex aberrant and normal karyotypes. (**b**) C/EBPα and c-Jun colocalize in the nucleus: Confocal image of c-Jun and C/EBPα cotransfected HeLa cells. FITC and Cy3 staining show nuclear localization of C/EBPα and c-Jun, respectively. Overlay of the confocal images in the right panel indicates the colocalization of these two proteins. HeLa cells were plated on glass coverslips and transfected with the respective DNA plasmids using a lipofectamine transfection protocol. At 24-h post transfection, the cells were washed in PBS and fixed with 3% paraformaldehyde for 10 min followed by incubation in 2% glycine for 15 min. Cells were permeablized with 0.2% Triton X-100 for 10 min. Cells were blocked in 0.2% BSA for 15 min followed by incubation in the primary antibody (1 : 100 dilution) for 1 h. At 1 : 100 dilution for FITC and 1 : 500 dilution for Cy3 was used for secondary antibody. After incubation of the cells with secondary antibody for 1 h, cells were washed, dried and mounted on coverslip. Fluorescence for the respective secondary antibody was observed using Leica confocal microscope. (**c**) c-Jun inhibits C/EBPα DNA binding via the c-Jun leucine zipper domain. Electromobility shift assays (EMSA) were performed as described earlier (Rangatia *et al*., 2002; Vangala *et al*., 2003). A G-CSF receptor C/EBP consensus site oligo was used as the probe for C/EBPα (Rangatia *et al*., 2002). *In vitro* translated C/EBPα, c-Jun, c-JunΔRK (deletion of the DNA binding domain), and c-JunΔLZ (deletion of the leucine zipper dimerization domain) were generated using Promega in vitro translation kit (Behre *et al*., 1999; Rangatia *et al*., 2002). C/EBPα (SC-61X) antibody and normal rabbit IgG (SC-2027) from Santa Cruz were used for supershift experiments

in AML patient samples. Various subtypes of AML are depicted with the patient number analysed ($n=166$). These data suggest that c-Jun expression is elevated in AML. Whereas cytogenetics accomplished the subclassification of AML in many subgroups, many of those genetic events might target common mechanisms to induce proliferation and block differentiation, such as inducing c-Jun expression as potential common theme in AML.







c-Jun inhibits C/EBPα DNA binding via the c-Jun leucine zipper domain

Since C/EBPα is inactivated by BCR-ABL (Perrotti *et al*., 2002; Schuster *et al*., 2003), AML1-ETO (Pabst *et al*., 2001a), PML-RARα (Truong *et al*., 2003), and through mutation (Pabst *et al*., 2001b), thus being a common target in myeloid leukemias, we hypothesized that elevated c-Jun expression in AML might inactivate C/EBPα function. We have earlier shown using co-immunoprecipitation and GST pulldown assays that C/EBPα and c-Jun interact through their leucine zipper domains (Rangatia *et al*., 2002). Moreover, we could demonstrate that C/EBPα can prevent c-Jun from binding to an AP-1 site through this leucine zipper domain interaction (Rangatia *et al*., 2002). Finally, we could demonstrate that c-Jun and C/EBPα colocalize in the nucleus (Figure 1b). Therefore, we speculated that c-Jun binds to C/EBPα with a leucine zipper domain interaction and pulls C/EBPα away from C/EBP sites in target promoters. In fact, EMSA experiments using a G-

Figure 2 Inactivation of C/EBPα by c-Jun is necessary for c-Jun to induce proliferation in AML; 30-kDa C/EBPα protein in AML fails to downregulate c-Jun mRNA expression. (**a**) c-Jun-induced proliferation in AML cell lines is inhibited by ectopic overexpression of C/EBPα U937 and HL60 cells were transduced with pMV7-neo, pMV7-neo-c-jun and pMSCV-C/EBPα-IRES-GFP vectors. FACS analysis for CD71-PE (clone M-A712, BD Biosciences) and its isotype control IgG2aκ PE (clone G155-178, BD Biosciences) was performed as described before (Rangatia *et al*., 2002; Vangala *et al*., 2003). (**b**) 30-kDa C/EBPα protein in AML fails to downregulate c-Jun mRNA expression. Real-time PCR reactions for c-Jun and G6PD from AML FAB M2 patient samples without translocation t(8;21) and with or without reported 30-kDa C/EBPα mutations were carried out. The error bars indicate the s.e.m. from three experiments. *n* indicates the number of patient samples analysed in each subgroup. Real-time quantitative PCR was performed using the light cycler technology (Roche Diagnostic) as described before (Vangala *et al*., 2003). The primers for c-Jun: forward 5′GCA TGA GGA AAC GCA TCG CTG CCT CCA AGT 3′ and reverse 5′GCG ACC AAG TCC TTC CCA CTC GTG CAC ACT 3′. G6PD primers were forward 5′CCG GAT CGA CCA CTA CCT GGG CAA C 3′ and reverse 5′GTT CCC CAC GTA CTG GCC CAG GAC CA 3′. In all, 35 cycles of c-Jun and G6PD amplification conditions were: 95°C for 10 min; 95°C for 0.5 s; 64°C for 10 s; 72°C for 25 s. Running the samples on 1.2% agarose gel, the PCR fragment size of 400 bps for c-Jun and 340 bps for G6PD were observed. (**c**) Model for AML in which c-Jun promotes proliferation and prevents differentiation by inhibiting C/EBPα DNA binding via leucine zipper domain interaction. c-Jun expression is increased in AML patient samples. c-Jun binds to C/EBPα via the leucine zipper domains and prevents C/EBPα from DNA binding. Inactivation of C/EBPα by c-Jun is necessary for c-Jun to induce proliferation because c-Jun-induced proliferation can be prevented by ectopic overexpression of C/EBPα. The dominant-negative 30-kDa C/EBPα protein, found in AML, fails to downregulate c-Jun mRNA expression in AML patient samples. Thus, our data suggest a model for AML in which c-Jun promotes proliferation and prevents differentiation by inhibiting C/EBPα DNA binding via leucine zipper domain interaction. It might depend on the expression levels of C/EBPα and c-Jun, if inhibition of C/EBPα by c-Jun or if inhibition of c-Jun by C/EBPα is more predominant: proliferation versus differentiation; AML versus normal myeloid development

CSF receptor C/EBP binding site oligo and *in vitro* translated C/EBPα, c-Jun, c-JunΔLZ and c-JunΔRK demonstrate that wild-type c-Jun as well as c-JunΔRK (basic domain mutant) prevent C/EBPα from binding to the DNA, whereas c-JunΔLZ (leucine zipper domain mutant) does not prevent C/EBPα DNA binding (Figure 1c). The inactivation of C/EBPα by c-Jun might depend on very few amino acids of C/EBPα targeted by interaction with c-Jun, because it has been recently reported that DNA binding of C/EBPα primarily depends on Arg289 and its interacting partners Tyr285 and Asn293 (Miller *et al*., 2003). In conclusion, c-Jun binds to C/EBPα via the leucine zipper domains and prevents C/EBPα DNA binding. In one model, it could simply depend on the relative concentrations of c-Jun and C/EBPα, whether the C/EBPα–c-Jun leucine zipper domain interaction leads to granulocytic differentiation or to a block in differentiation. Interestingly, competing concentration-dependent protein–protein interactions have not only been described before in the case of AML, such as AML1-ETO–PU.1 interaction (Vangala *et al*., 2003), AML1-ETO–C/EBPα interaction (Pabst *et al*., 2001a), or PML-RARα–C/EBPα interaction (Truong *et al*., 2003), but also as a driving force in the context of lineage commitment and normal differentiation, such as PU.1–c-Jun interaction (Behre *et al*., 1999), PU.1–C/EBPα interaction (Reddy *et al*., 2002), or GATA1–PU.1 interaction (Zhang *et al*., 1999). Agonistic and antagonistic protein–protein interactions, therefore, might play a major role in stem cell development and leukemogenesis.

Inactivation of C/EBPα by c-Jun is necessary for c-Jun to induce proliferation in AML

As shown before, c-Jun is highly expressed in AML and inactivates C/EBPα via leucine zipper domain interaction. We, therefore, asked the question whether inactivation of C/EBPα by c-Jun is absolutely necessary for c-Jun to induce proliferation in AML, or whether inactivation of C/EBPα by c-Jun is a mechanistically interesting, but biologically not important phenomenon. In fact, overexpression of c-Jun induced proliferation in AML cell lines, measured by Trypan blue cell counting (Peer Zada *et al*., 2003), brdU assay (Peer Zada *et al*., 2003), and CD71 FACS analysis (Figure 2a). Most importantly, ectopic overexpression of C/EBPα prevented c-Jun-induced proliferation (Figure 2a). These data confirm that C/EBPα function needs to be downregulated by c-Jun in order for c-Jun to be able to induce proliferation. It might depend on the expression levels of C/EBPα and c-Jun, if inhibition of C/EBPα by c-Jun or if inhibition of c-Jun by C/EBPα is more predominant: proliferation versus differentiation.

30-KDa C/EBPα protein in AML fails to downregulate c-Jun MRNA expression

C/EBPα mutants, mostly encoding a 30-kDa form that acts dominant negative to wild-type 42-kDa C/EBPα on the second allele, have been described before in around 13% of AML (Pabst *et al*. , 2001b; Gombart *et al*., 2002; Preudhomme *et al*., 2002; Waalwijk Van Doorn-Khosravani *et al*., 2002). Interestingly, in AML FAB M2 patient samples with the 30-kDa C/EBPα protein, c-Jun mRNA expression measured by real-time PCR is much higher than in AML patient samples of the FAB M2 subtype without C/EBPα mutants and normal karyotype (Figure 2b). This is expected because intact and functional C/EBPα downregulates c-Jun expression as we have described before (Rangatia *et al*., 2002). One mechanism for the upregulation of c-Jun expression in AML might be the downregulation of C/EBPα activity by the dominant-negative 30-kDa C/EBPα protein found in AML. Once upregulated, c-Jun further downregulates C/EBPα activity by inhibiting C/EBPα DNA binding as shown in the present report. Thereby, c-Jun is, in fact, downstream of C/EBPα (Rangatia *et al*., 2002), but also upstream of C/EBPα, as shown here. This way in AML a deadly inactivation circle for C/EBPα might be happening. In summary (Figure 2c), our data suggest a model for AML in which c-Jun promotes proliferation and prevents differentiation by inhibiting C/EBPα DNA binding via leucine zipper domain interaction.

References

Behre G, Singh SM, Liu H, Bortolin LT, Christopeit M, Radomska HS, Rangatia J, Hiddemann W, Friedman AD and Tenen DG. (2002). *J. Biol. Chem.,* **277,** 26293–26299.

Behre G, Whitmarsh AJ, Coghlan MP, Hoang T, Carpenter CL, Zhang DE, Davis RJ and Tenen DG. (1999). *J. Biol. Chem.,* **274,** 4939–4946.

Burgess GS, Williamson EA, Cripe LD, Litz-Jackson S, Bhatt JA, Stanley K, Stewart MJ, Kraft AS, Nakshatri H and Boswell HS. (1998). *Blood,* **92,** 2450–2460.

Elsaesser A, Franzen M, Kohlmann A, Weisser M, Schnittger S, Schoch C, Reddy V, Burel S, Zhang DE, Ueffing M, Tenen DG, Hiddemann W and Behre G. (2003). *Oncogene*, in press.

Gombart AF, Hofmann WK, Kawano S, Takeuchi S, Krug U, Kwok SH, Larsen RJ, Asou H, Miller CW, Hoelzer D and Koeffler HP. (2002). *Blood,* **99,** 1332–1340.

Iwama A, Osawa M, Hirasawa R, Uchiyama N, Kaneko S, Onodera M, Shibuya K, Shibuya A, Vinson C, Tenen DG and Nakauchi H. (2002). *J. Exp. Med.,* **195,** 547–558.

McKnight SL. (2001). *Cell,* **107,** 259–261.

Miller M, Shuman JD, Sebastian T, Dauter Z and Johnson PF. (2003). *J. Biol. Chem.*, 2003 Feb 10 [epub ahead of print].

Mizuki M, Schwaeble J, Steur C, Choudhary C, Agrawal S, Sargin B, Steffen B, Matsumura I, Kanakura Y, Boehmer FD, Mueller-Tidow C, Berdel WE and Serve H. (2002). *Blood* 2002 Dec 5 [epub ahead of print].

Mueller BU, Pabst T, Osato M, Asou N, Johansen LM, Minden MD, Behre G, Hiddemann W, Ito Y and Tenen DG. (2002). *Blood,* **100,** 998–1007.

Pabst T, Mueller BU, Harakawa N, Schoch C, Haferlach T, Behre G, Hiddemann W, Zhang DE and Tenen DG. (2001a). *Nat. Med.,* **7,** 444–451.

Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, Behre G, Hiddemann W and Tenen DG. (2001b). *Nat. Genet.,* **27,** 263–270.

Peer Zada AA, Singh SM, Meisel A, Reddy VA, Elsaesser A, Haferlach T, Tenen DG, Hiddemann W and Behre G. (2003). *Oncogene*, **22,** 2296–2308.

Perrotti D, Cesi V, Trotta R, Guerzoni C, Santilli G, Campbell K, Iervolino A, Condorelli F, Gambacorti-Passerini C, Caligiuri MA and Calabretta B. (2002). *Nat. Genet.,* **30,** 48–58.

Porse BT, Pedersen TA, Xu X, Lindberg B, Wewer UM, Friis-Hansen L and Nerlov C. (2001). *Cell,* **107,** 247–258.

Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S, Thomas X, Raffoux E, Lamandin C, Castaigne S, Fenaux P and Dombret H. (2002). *Blood,* **100,** 2717–2723.

Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT and Tenen DG. (1998). *Mol. Cell. Biol.,* **18,** 4301–4314.

Rangatia J, Vangala RK, Treiber N, Zhang P, Radomska H, Tenen DG, Hiddemann W and Behre G. (2002). *Mol. Cell. Biol.,* **22,** 8681–8694.

Reddy VA, Iwama A, Iotzova G, Schulz M, Elsasser A, Vangala RK, Tenen DG, Hiddemann W and Behre G. (2002). *Blood,* **100,** 483–490.

Schoch C, Kohlmann A, Schnittger S, Brors B, Dugas M, Mergenthaler S, Kern W, Hiddemann W, Eils R and Haferlach T. (2002). *Proc. Natl. Acad. Sci. USA,* **99,** 10008–10013.

Schuster C, Forster K, Dierks H, Elsaesser A, Behre G, Simon N, Danhauser-Riedl S, Hallek M and Warmuth M. (2003). *Blood,* **101,** 655–663.

Schwartz C, Beck K, Mink S, Schmolke M, Budde B, Wenning D and Klempnauer KH. (2003). *EMBO J.,* **22,** 882–892.

Shaulian E and Karin M. (2002). *Nat. Cell Biol.,* **4,** E131–E136.

Tenen DG. (2003). *Nat. Rev. Cancer,* **3,** 89–101.

Truong BT, Lee YJ, Lodie TA, Park DJ, Perrotti D, Watanabe N, Koeffler HP, Nakajima H, Tenen DG and Kogan SC. (2003). *Blood,* **101,** 1141–1148.

Vangala RK, Heiss-Neumann MS, Rangatia JS, Singh SM, Schoch C, Tenen DG, Hiddemann W and Behre G. (2003). *Blood,* **101,** 270–277.

Waalwijk Van Doorn-Khosravani SB, Erpelinck C, Meijer J, Van Oosterhoud S, Van Putten WL, Valk PJ, Beverloo HB, Tenen DG, Lowenberg B and Delwel R. (2002). *Hematol. J.,* **3,** 324.

Wang H, Goode T, Iakova P, Albrecht JH and Timchenko NA. (2002). *EMBO J.,* **21,** 930–941.

Wang H, Iakova P, Wilde M, Welm A, Goode T, Roesler WJ and Timchenko NA. (2001). *Mol. Cell,* **8,** 817–828.

Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ and Tenen DG. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 569–574.

Zhang P, Behre G, Pan J, Iwama A, Wara-Aswapati N, Radomska HS, Auron PE, Tenen DG and Sun Z. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 8705–8710.

tapraid4/h8-bloo/h8-bloo/h80103/h83591d03a | mazzam | S=11 | 10/16/02 | 13:21 | Art: 2002-04-1288

DOI: 10.1182/blood-2002-04-1288 Collection Codes: 00,00

NEOPLASIA

The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia

Rajani K. Vangala, Marion S. Heiss-Neumann, Janki S. Rangatia, Sheo M. Singh, Claudia Schoch, Daniel G. Tenen, Wolfgang Hiddemann, and Gerhard Behre

The transcription factor PU.1 plays a pivotal role in normal myeloid differentiation. PU.1$^{-/-}$ mice exhibit a complete block in myeloid differentiation. Heterozygous PU.1 mutations were reported in some patients with acute myeloid leukemia (AML), but not in AML with translocation t(8;21), which gives rise to the fusion gene *AML1-ETO*. Here we report a negative functional impact of AML1-ETO on the transcriptional activity of PU.1. AML1-ETO physically binds to PU.1 in t(8;21)$^{+}$ Kasumi-1 cells. AML1-ETO binds to the $\beta_3\beta_4$ region in the DNA-binding domain of PU.1 and displaces the coactivator c-Jun from PU.1, thus down-regulating the transcriptional activity of PU.1. This physical interaction of AML1-ETO and PU.1 did not abolish the DNA-binding capacity of PU.1. AML1-ETO down-regulates the transactivation capacity of PU.1 in myeloid U937 cells, and the expression levels of PU.1 target genes in AML French-American-British (FAB) subtype M2 patients with t(8;21) were lower than in patients without t(8;21). Conditional expression of AML1-ETO causes proliferation in mouse bone marrow cells and inhibits antiproliferative function of PU.1. Overexpression of PU.1, however, differentiates AML1-ETO–expressing Kasumi-1 cells to the monocytic lineage. Thus, the function of PU.1 is down-regulated by AML1-ETO in t(8;21) myeloid leukemia, whereas overexpression of PU.1 restores normal differentiation. (Blood. 2003;101:000-000)

Introduction

The Ets family of transcription factors plays a key role in the growth, survival, differentiation, and activation of hematopoietic cells. This family of proteins is characterized by presence of an 85-amino acid,[1] winged helix-turn-helix DNA-binding domain. PU.1 is one of the most important Ets transcription factors.[2] Its expression is limited to hematopoietic cells, including primitive CD34^{+} cells, macrophages, B lymphocytes, neutrophils, mast cells, and early erythroblasts.[2,3] In vitro studies suggest that PU.1 regulates the activity of a number of myeloid- and lymphoid-specific promoters and enhancers.[4-10] PU.1 is a key transcription factor for normal myeloid development as demonstrated by a complete block of myeloid development in PU.1$^{-/-}$ mice.[11,12] Fetal or newborn PU.1$^{-/-}$ mice have no detectable monocytes/macrophages or neutrophils.[11,12] We have recently shown that PU.1 is mutated in patients with acute myeloid leukemia (AML).[13] These studies all point to the crucial role of PU.1 in both normal myeloid differentiation and leukemogenesis.

AML1 is a member of the *Runt*-like transcription factors (*Runx*-1, -2, and -3) named after the *Runt* protein that regulates segmentation during *Drosophila* embryogenesis.[14-16] AML1 appears to act as an "organizing" factor for many promoters and enhancers by interacting with various coactivators and DNA-binding transcription factors.[17-22] The *AML1* gene is one of the most frequently translocated or mutated genes in human cancer.[23-25] The t(8;21)(q22;q22) translocation fuses residues 1-177 of *AML1* (including the DNA-binding domain) to nearly all of *ETO* (also known as CBF2T1).[26] ETO is the human homolog of *Drosophila* NERVY protein.[27-29] The t(8;21) belongs to the most common chromosomal abnormalities in AML, accounting for 10% of all AML cases and 40% of the AML French-American-British (FAB) M2 phenotype.[30-33] AML1 activates transcription from enhancer core motifs (TGT/cGGY), which are present in a number of genes relevant to myeloid development, including the macrophage colony-stimulating factor (M-CSF) receptor, granulocyte-macrophage colony-stimulating factor (GM-CSF), myeloperoxidase, and neutrophil elastase.[34-39] Like AML1, AML1-ETO can act as a transcriptional activator,[40-43] but it is also a transcriptional repressor in other contexts.[44] Only one allele of AML1 is altered in leukemia cells expressing t(8;21), and AML1-ETO can efficiently repress AML1-dependent transcriptional activation. Therefore, AML1-ETO has been postulated to act as a dominant inhibitor of AML1 function.[34,37,44]

Recently, we have shown that AML1-ETO blocks CCAAT enhancer-binding protein (C/EBPα)–dependent activation of its own promoter thus blocking normal granulocytic differentiation of myeloid cells.[36] Furthermore, AML1-ETO was shown to repress AML1 and MEF-2–dependent gene activation.[45] In our earlier studies we demonstrated that c-Jun, a member of AP-1 transcription factor family, can interact with PU.1 at the $\beta_3\beta_4$ domain in PU.1 and coactivate the transcriptional activity of PU.1.[46] Here we show that AML1-ETO blocks the transcriptional activity of PU.1 by displacing its coactivator c-Jun.

From the Department of Internal Medicine III, University Hospital Grosshadern, Ludwig-Maximilians-University Munich and GSF-National Research Center for Environment and Health, Munich, Germany; and Harvard Institutes of Medicine, Harvard Medical School, Boston, MA.

Submitted April 30, 2002; accepted July 30, 2002. Prepublished online as *Blood* First Edition Paper, August 29, 2002; DOI 10.1182/blood-2002-04-1288.

Supported by a DFG (German research foundation) grant to G.B. (no. 2042/2-1).

Reprints: Gerhard Behre, Department of Internal Medicine III, University Hospital Grosshadern, Ludwig-Maximilians-University Munich, Marchioninistr 15, D-81377 Munich, Germany; e-mail: gerdbehre@aol.com.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

tapraid4/h8-bloo/h8-bloo/h80103/h83591d03a | mazzam | S=11 | 10/16/02 | 13:21 | Art: 2002-04-1288

Materials and methods

Cell lines and cell culture

Human kidney 293T, mouse embryonal carcinoma F9, and ecotrophic Phoenix cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco,) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% Penstrep (all from Gibco). Human monoblastic U937 cells and t(8;21)$^+$ Kasumi-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS (both from Gibco).

Bone marrow cells were isolated from the femurs of Balb/C mice. The femurs were removed and stripped of the soft tissue and crushed to release cells within marrow cavity. The red blood cells were lysed with a 0.15-mol/L solution of ammonium chloride. The pelleted cells were subjected to low-density mononuclear cell separation by incubating with density gradient (Histopaque 1083; Sigma, St Louis, MO) for 10 minutes and centrifuged at 600 rpm for 30 minutes, washed twice in phosphate-buffered saline (PBS), followed by culturing in Iscove modified Dulbecco medium (IMDM; Stem Cell Technologies,) supplemented with 10% FBS (Stem Cell Technologies), 50 ng/mL stem cell factor (R & D Systems, Minneapolis, MN), 50 ng/mL interleukin 6 (IL-6; R & D Systems), and 50 ng/mL Flt-3 ligand (Flt-3L; R & D Systems).

Coimmunoprecipitation assay

Kasumi-1 cells (2×10^7) were lysed and 200 μg protein was used to perform immunoprecipitation as mentioned by Mao et al.[45] The following antibodies were used: rabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA; catalog no. sc2027), goat IgG (Santa Cruz Biotechnologies; catalog no. 2028), anti-AML1 antibody (Calbiochem, ; catalog no. PC284), anti-PU.1 (Santa Cruz Biotechnologies; catalog no. sc352), and protein-A agarose beads (Santa Cruz Biotechnologies; catalog no. sc2001).

Western blot

After plating in 100-mm plates, 293T cells were transfected using the LipofectAMINE Plus kit (Gibco) as per the manufacturer's protocol. At 24 hours after transfection, cells were harvested and lysed in RIPA lysis buffer, and immunoblot for PU.1 was performed with 100 μg protein as described earlier.[46-48] To generate protein lysates, 1×10^6 F9, Kasumi-1, or 293T cells were lysed and nuclear extracts were prepared and immunoblot was performed with 100 μg protein for c-Jun (Santa Cruz Biotechnologies; catalog no. sc54). Mouse bone marrow cells transduced with PU.1, AML1-ETO, or respective empty vectors were similarly lysed (RIPA lysis) and 100 μg protein was used for immunoblot analysis for PU.1 and AML1-ETO (anti-ETO antibody, Santa Cruz Biotechnologies; catalog no. sc9737). Mouse monoclonal anti–β-tubulin purchased from Roche (; catalog no. 1111876) was used for immunoblot assay as internal control. Protein A-peroxidase–conjugated for antirabbit (Amersham Pharmacia, ; catalog no. NA 9120), or antigoat peroxidase-conjugated immunoglobulins (Dako, ; code no. p0449) were used as secondary antibodies.

Reporter constructs and expression plasmids

The human monocyte–specific M-CSF receptor promoter with or without AML1-binding site, p(PU.1)4TK, and p(mutPU.1)4TK (PU.1-binding sites and mutated PU.1-binding sites subcloned into pTK61luciferase) were described earlier.[46] As an internal control plasmid for transient transfection assay, we used the pRL-null construct driving a Renilla luciferase gene (Promega, Madison, WI).[49] Other vectors used were pECE-PU.1-murine, pcDNA.1-PU.1, pGEX-2TK-PU.1 or $\beta_3\beta_4$, pS3H-c-Jun, and pSP6-c-Jun, as described previously.[46,50] AML1B-pCMV5 and CBFβ-pCMV5 were described earlier.[42] AML1-ETO-pcDNA3 was constructed by enzymatic digestion of AML1-ETO-pCMV5[42] with *Xba*I and subcloning the resulting 2258-bp fragment into the *Xba*I site of pcDNA3 plasmid (Invitrogen,).

Transfection assays

Transient transfections in 293T or F9 cells were carried out with LipofectAMINE transfection kit (Gibco) in 24-well plates as described earlier.[46,47,49] U937 cells were transiently transfected by electroporation in RPMI medium at 980 μF and 280 V. *Firefly* luciferase activities from the constructs M-CSF receptor promoter luciferase, pXP2, p(PU.1)4TK, p(mutPU.1)4TK, and *Renilla* luciferase activity from internal control plasmid pRL-null were determined 24 hours after transfection using Dual Luciferase Reporter Assay System (Promega). Results are given as means + SEMs from at least 3 independent experiments.

Protein interaction assays

c-Jun and AML1-ETO were transcribed in vitro and translated in the presence of [^{35}S]-methionine (Amersham Pharmacia,) using the T7/SP6-coupled reticulocyte system (Promega) in accordance with the manufacturer's instruction. Glutathione-S-transferase (GST) precipitation assays were performed as described earlier.[46,48]

EMSA

γ^{32}P-adenosine triphosphate (ATP; Amersham Pharmacia)–labeled double-stranded oligonucleotides of PU.1 DNA-binding site[51] and AML1-binding site[52] for electrophoretic mobility shift assay (EMSA) were prepared. The assay was performed with in vitro–translated proteins as mentioned earlier.[11,47] For supershift experiments 3 μL of either anti-PU.1 or anti-ETO antibodies were added to the reaction mixture.

Retroviral transduction assay

Ecotrophic Phoenix cells (5×10^6) were plated in 10-cm plates and transfected with 5 μg PINCO-GFP, PINCO-AML1-ETO-GFP, pGsam-PU.1-ires-NGFR, or pGsam-ires-NGFR vectors using LipofectAMINE transfection kit (Gibco). At 24 hours after transfection, the transfection medium was replaced with IMDM (supplemented with 10% FBS, 50 ng/mL stem cell factor, 50 ng/mL IL-6, and 50 ng/mL Flt-3L) for collection of the virus particles. After the viral particle production, freshly isolated mouse bone marrow cells were incubated with viral medium on fibronectin-coated plates and centrifuged for 30 minutes at 1000*g* (this step was repeated every 12 hours).[53] At 60 hours after first transduction, NGFR$^+$ or enhanced green fluorescence protein-positive (EGFP$^+$) cells were isolated by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson,). To detect the expression of tNGFR on the cell surface, cells were stained with mouse antihuman NGFR (Chemicon, ; catalog no. MAB5246) followed by phycoerythrin (PE)–conjugated rabbit antimouse immunoglobulins (mouse IgG RPE; Dako; catalog no. R0439). Then, 1×10^4 transduced cells sorted for NGFR positivity were plated in 1.2 mL mouse colony-forming medium (Stem Cell Technologies). After 3, 6, and 12 days of plating live cells were counted by trypan blue staining.

Patient material and FACS analysis

Bone marrow cells from AML-M2 patients with or without t(8;21) were obtained after informed consent was given by the patients. Mononuclear cells were isolated from the bone marrow by density gradient centrifugation with Histopaque (Sigma). FACS analysis was performed with CD11b (Pharmingen, ; catalog no. 555388), CD14 (Pharmingen; catalog no. 555397), and CD64 (Pharmingen; catalog no. 555527).

Transfection of Kasumi-1 cells and FACS analysis

Kasumi-1 cells were electroporated as mentioned above with pGsam-PU.1-ires-NGFR or pGsam-ires-NGFR vectors and sorted 24 hours after transfection for NGFR positivity by FACS (with anti-NGFR antibody from Chemicon, catalog no. MAB5246, and mouse IgG RPE from Dako, catalog no. R0439). Five days after sorting for NGFR expression, morphologic changes were observed by Wright-Giemsa staining of cells. Then, 1×10^6 NGFR$^+$ Kasumi-1 cells were incubated with 10 μL recombinant PE-conjugated mouse monoclonal CD11b (Pharmingen; catalog no. 555388) or fluorescein isothiocyanate (FITC)–conjugated mouse monoclonal CD14 (Pharmingen; catalog no. 555397) in 100 μL PBS for 60 minutes on ice, washed in PBS followed by analysis on a FACScan flow cytometer (Becton Dickinson) using Cellquest software. The cells were also analyzed for the

isotype controls, PE-conjugated mouse IgG1κ (Pharmingen, catalog no. 554680) for CD11b-PE– and CD11b-FITC–conjugated mouse IgG1κ (Pharmingen; catalog no. 555748) for CD14-FITC. At 24 hours after transfection, 5×10^4 $NGFR^+$ cells were plated in a 6-well plate and passaged with fresh medium every 24 hours. Cell count for live cells was performed by trypan blue staining every 24 hours.

Results

AML1-ETO interacts with PU.1 in vivo and inhibits its transcriptional activity

To determine whether PU.1 interacts with AML1-ETO, coimmunoprecipitation assays were performed in Kasumi-1 cells, a human cell line containing t(8;21). PU.1 coprecipitated with both AML1 and ETO antibodies but not with IgG control, suggesting that PU.1 interacts with AML1-ETO in vivo (Figure 1A). A similar experiment was performed using a PU.1-specific antibody: AML1-ETO coprecipitated with PU.1, but not with rabbit IgG control (Figure 1A).

To investigate the functional impact of this in vivo interaction, we performed transient transfection assays in 293T cells. An M-CSF receptor promoter luciferase reporter construct, which was transactivated 12-fold by PU.1 and 28-fold by PU.1/c-Jun, is completely down-regulated by AML1-ETO (Figure 1B). AML1-ETO had no effects on serum response element (pSRE)/Ras activity nor on the empty vector (pXP2) as negative controls. The expression levels of cotransfected PU.1 did not change in the presence of AML1-ETO indicating that the transactivating capacity, but not the expression of cotransfected PU.1, was down-regulated (Figure 1B).

AML1 does not affect transactivation capacity of PU.1 or PU.1/c-Jun

The M-CSF receptor promoter has adjacent AML1- and PU.1-binding sites.[45] AML1-ETO retains the 177 N-terminus amino acids of AML1, suggesting that AML1 might also have an influence on transactivation of PU.1 or PU.1/c-Jun. Therefore, we addressed if AML1 had any functional impact on the transactivation capacity of PU.1 or PU.1/c-Jun using a promoter containing only PU.1-binding sites (p(PU.1)4TK). Transient transfection assays in 293T cells were performed with p(PU.1)4TK and expression plasmids of PU.1, c-Jun, AML1, and core-binding factor β (CBFβ). Results (Figure 1C) show that AML1 did not affect the PU.1 or PU.1/c-Jun transactivation capacity. In the same experiment AML1 could transactivate the M-CSF receptor promoter 4-fold in the presence of CBFβ (data not shown). AML1, PU.1, c-Jun, and CBFβ had no effects on control vectors (p(mutPU.1)4TK and pXP2) in the experiments above (data not shown).

AML1-ETO inhibits the coactivation of PU.1 by c-Jun

We have earlier shown that c-Jun can coactivate transactivation of PU.1 in a JNK-independent manner.[46] PU.1 induced strong transactivation of p(PU.1)4TK in 293T cells (Figure 1C). This is possibly due to high expression of its coactivator c-Jun in these cells. Immunoblot assay for c-Jun indicated that 293T cells have high amounts of c-Jun (Figure 2A, lane 1) comparable to Kasumi-1 cells (Figure 2A, lane 3). However, F9 cells had no detectable c-Jun protein (Figure 2A, lane 2). Therefore, further experiments were carried out in F9 cells, which served as a model cell line for understanding how AML1-ETO might interfere with the capacity of c-Jun in coactivating PU.1. PU.1/c-Jun could transactivate p(PU.1)4TK (Figure 2B) and also the M-CSF receptor promoter (Figure 2C) in F9 cells as described earlier.[46] In the presence of AML1-ETO, the capacity of PU.1/c-Jun in transactivating the target promoters (Figure 2B,C) was down-regulated. c-Jun up-regulated the p(PU.1)4TK promoter in 293T cells (Figure 1B) and F9 cells (Figure 2B,C), which might be due to presence of noncanonical sites in the promoter construct or unknown factors in these 2 cell lines collaborating with c-Jun. A similar effect was also reported earlier.[48] However, this does not influence the final conclusion.

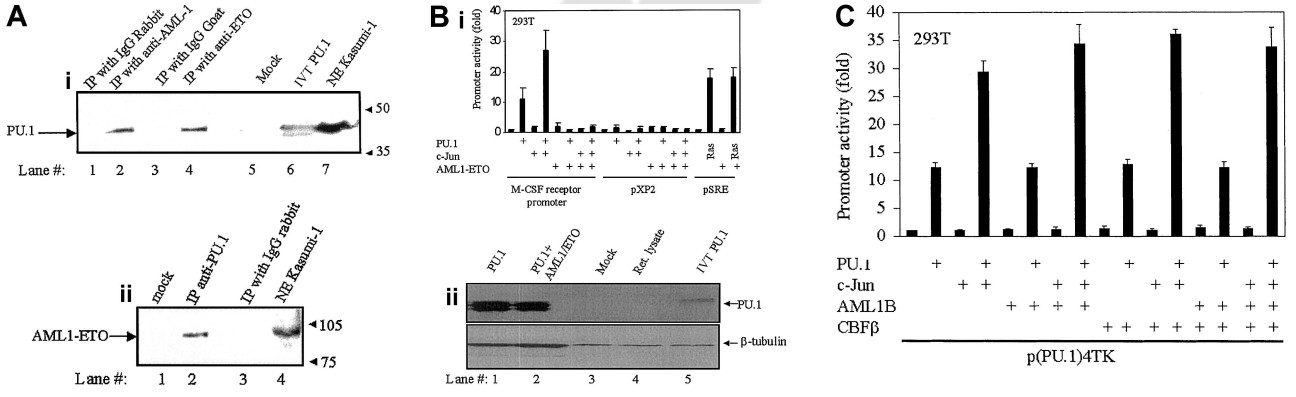


Figure 1. AML1-ETO binds to PU.1 and down-regulates transactivation capacity of PU.1. (A) AML1-ETO binds to PU.1 in vivo. (i) Kasumi-1 cell nuclear extracts (200 μg) were immunoprecipitated with rabbit IgG (lane 1), anti-AML1 antibody (lane 2), goat IgG (lane 3), or anti-ETO antibody (lane 4). The immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) along with in vitro translated PU.1 (lane 6) and nuclear extracts (NE; lane 7) and further subjected to immunoblotting with PU.1 antibody. (ii) Kasumi-1 nuclear extracts were immunoprecipitated with anti-PU.1 (lane 2) or IgG (lane 3) and subjected to SDS-PAGE along with nuclear extracts of Kasumi-1 cells (lane 4) and blotted with anti-ETO antibody. (B) AML1-ETO inhibits transactivation capacity of PU.1. (i) 293T cells were transiently transfected with human monocyte-specific M-CSF receptor promoter or promoterless vector pXP2 or pSRE (serum response element) and with expression plasmids of PU.1 (100 ng), c-Jun (50 ng), AML1-ETO (20 ng), and activated Ras (50 ng). Promoter activities (fold) were determined 24 hours after transfection and normalized to the activities of the internal control plasmid pRL0. (ii) AML1-ETO does not change the expression of cotransfected PU.1. The 293T cells were transfected as above and whole cell lysates were subjected to SDS-PAGE followed by immunoblot assay with PU.1-specific antibody. (C) AML1 does not affect transactivation capacity of PU.1. The 293T cells transfected with p(PU.1)4TK-luc and expression plasmids of PU.1 (100 ng), c-Jun (50 ng), AML1 (50 ng), or CBFβ (50 ng), PU.1, c-Jun, AML1, and CBFβ had no effects on negative control p(mut.PU.1)4TK (data not shown).

tapraid4/h8-bloo/h8-bloo/h80103/h83591d03a | mazzam | S=11 | 10/16/02 | 13:21 | Art: 2002-04-1288

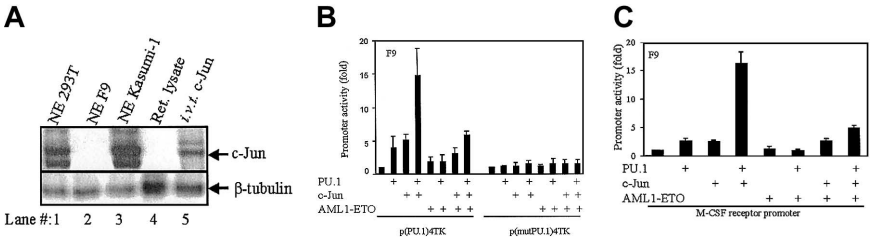


Figure 2. AML1-ETO inhibits coactivation of PU.1 by c-Jun. (A) F9 cells do not express c-Jun. Nuclear extracts (100 μg) of 293T, F9, and Kasumi-1 cells along with in vitro–translated c-Jun were subjected to SDS-PAGE and immunoblotted for c-Jun. (B) AML1-ETO inhibits PU.1/c-Jun transactivation capacity. F9 cells were transfected with p(PU.1)4TK, a minimal TK promoter driven by PU.1 DNA-binding sites only or control vector p(mut.PU.1)4TK along with expression plasmids of PU.1 (100 ng), c-Jun (50 ng), and AML1-ETO (20 ng). (C) AML1-ETO down-regulates the PU.1-regulated M-CSF receptor promoter activity by inhibiting PU.1/c-Jun function. F9 cells were transfected with M-CSF receptor promoter and PU.1 (100 ng), c-Jun (50 ng), and AML1-ETO (20 ng). PU.1, c-Jun, and AML1-ETO had no effects on control vector pXP2 (data not shown).

AML1-ETO displaces c-Jun by binding to the $\beta_3\beta_4$ region in PU.1

The results in F9 cells (Figure 2B,C) suggest that AML1-ETO interferes with coactivation of PU.1 by c-Jun. To investigate this, in vitro protein-protein interaction assays were performed. c-Jun and AML1-ETO bind to the full-length PU.1 fused to GST (Figure 3A). c-Jun was shown to interact at the $\beta_3\beta_4$ region of the DNA-binding domain of PU.1.[48] Therefore, we performed protein-protein interaction assays using GST-$\beta_3\beta_4$ and found that AML1-ETO also binds to GST-$\beta_3\beta_4$ (Figure 3B). In competitive protein-protein interaction assays on increasing the AML1-ETO protein, c-Jun protein bound to GST-$\beta_3\beta_4$ was reduced (Figure 3B). These results indicate that AML1-ETO competes c-Jun away from binding to the $\beta_3\beta_4$ domain of PU.1. Thus, the c-Jun coactivation function of PU.1 is down-regulated and this in turn down-regulates transcriptional activity of PU.1.

AML1-ETO does not change the DNA binding of PU.1

The protein-protein interactions described above demonstrate that AML1-ETO directly interacts with PU.1. The physical interaction of AML1-ETO/PU.1 might down-regulate the DNA-binding capacity of PU.1. To address this possibility, we performed an EMSA using in vitro–translated PU.1 and AML1-ETO and oligonucleotide probes having respective DNA-binding sequences.[51,52] In vitro–translated PU.1 binds specifically to the PU.1-binding oligonucleotide (Figure 4). Even in presence of AML1-ETO, no change of DNA binding of PU.1 was observed (Figure 4), indicating that AML1-ETO blocks the transactivation capacity, but not DNA binding of PU.1. In the same experiment in vitro–translated AML1-ETO was found to bind to the AML1 probe (data not shown).

AML1-ETO down-regulates PU.1 transcriptional activity in myeloid cells

All the above transfections were performed in nonmyeloid 293T or F9 cells. We asked whether the same effects were also observed in myeloid cells. Therefore, we performed transient transfection assays in myelomonocytic U937 cells. U937 cells were transfected with wild type M-CSF receptor promoter, M-CSF receptor promoter without AML1-binding site, minimal promoter having PU.1-binding sites (p(PU.1)4TK), minimal promoter with mutated PU.1-binding sites (p(mutPU.1)4TK) as control, and empty vector

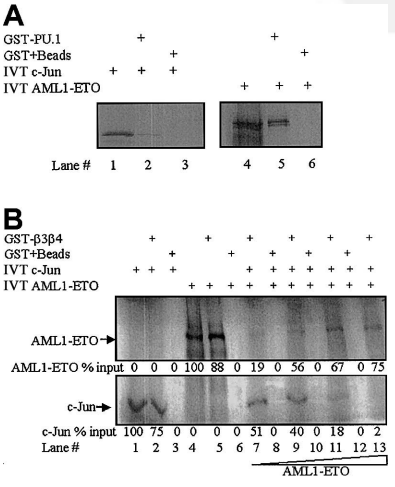


Figure 3. AML1-ETO displaces the coactivator c-Jun from PU.1 by binding to the $\beta_3\beta_4$ region of PU.1. (A) AML1-ETO physically binds to PU.1 in vitro. GST pull-down assay was performed using [^{35}S]-methionine–labeled in vitro–translated c-Jun (lane 1) or AML1-ETO (lane 4) incubated with equal amounts of bacterially expressed GST-PU.1 (lanes 2 and 5) or GST plus beads (lanes 3 and 6). GST-PU.1 or GST was recovered using glutathione-agarose beads and separated by SDS-PAGE prior to autoradiography. (B) AML1-ETO displaces c-Jun from binding to the $\beta_3\beta_4$ domain of PU.1. Saturating amounts of in vitro–translated c-Jun (20 μL) were incubated with GST-$\beta_3\beta_4$ and increasing amounts (from lanes 7-13) of in vitro–translated AML1-ETO (7.5-12.5 μL) were incubated. Densitometric quantification was also performed (given as percent input of the proteins).

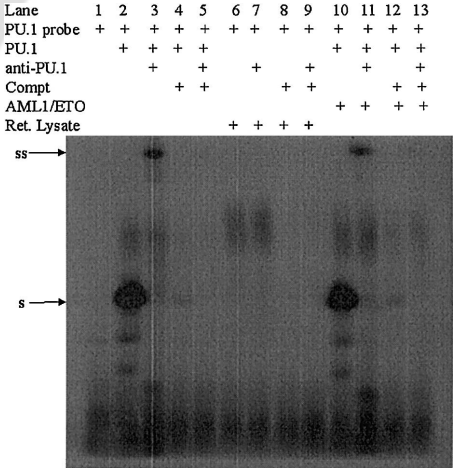


Figure 4. AML1-ETO does not change the DNA binding of PU.1. The PU.1-binding sequence from the CD11b promoter was chosen and labeled with γ^{32}p-dATP (lane 1), incubated with in vitro–translated PU.1 (lane 2), or in vitro–translated PU.1 and anti-PU.1 antibody (lane 3). As a competitor, unlabeled probe was used in 100 molar excess with (lane 5) and without (lane 4) anti-PU.1 antibody. To investigate if this binding and supershift is specific for PU.1, similar experiments were performed with rabbit reticulocyte lysate (lanes 6-9). In presence of AML1-ETO, PU.1 still binds to its DNA (lane 10) and supershifts with anti-PU.1 antibody (lane 11). To the left of blots, ss indicates supershift; s, shift.

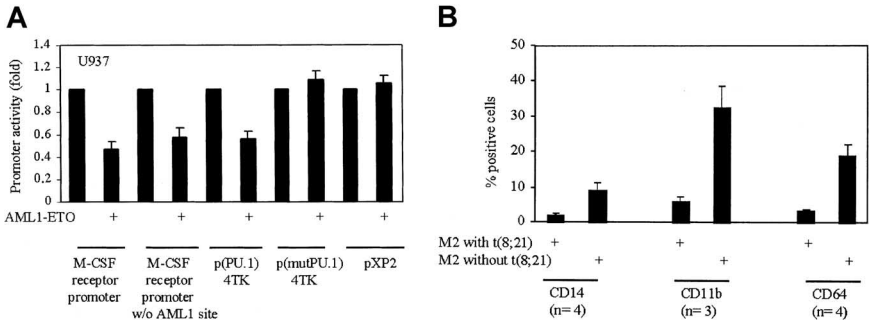


Figure 5. AML1-ETO down-regulates transactivation capacity of PU.1 in myeloid cells and the expression of the PU.1 target genes in AML patients with t(8;21). (A) AML1-ETO down-regulates transactivation of PU.1 in myeloid cells. U937 cells were electroporated with wild-type M-CSF receptor promoter, M-CSF receptor promoter without (w/o) AML1-binding site, p(PU.1)4TK, p(mutPU.1)4TK, or pXP2 with and without AML1-ETO. (B) Low expression of PU.1 target genes in patients with t(8;21). AML patients (n = number of patients) with t(8;21) have less positive cells for cell surface markers regulated by PU.1 as compared to patients without t(8;21). CD14 and CD64 promoters have PU.1-binding sites, but no putative C/EPBα-, AML1-, or MEF-binding sites.

with or without AML1-ETO expression plasmid. We observed that all the promoters were down-regulated by AML1-ETO without any effect on the empty vectors (Figure 5A). These data confirm that AML1-ETO down-regulates the transcriptional activity of PU.1 in myeloid cells also. U937 cells express high levels of PU.1 and C/EBPα; therefore AML1-ETO might not only down-regulate PU.1 but also C/EBPα. Therefore only 50% down-regulation of the promoters transfected into U937 cells could be seen. Furthermore, there might be proteins in myeloid cells (in contrast to 293T or F9 cells) that might interfere with the capacity of AML1-ETO to block PU.1 function.

Low expression of PU.1 target genes in patients with t(8;21)

To further understand if the down-regulation of the PU.1/c-Jun transactivation capacity by AML1-ETO leads to down-regulation of the PU.1 target genes, we performed FACS analysis of PU.1 target cell surface markers.[7,51] In AML-M2 patients with t(8;21), CD14, CD11b, and CD64 were 4.6-, 5.4-, and 5.8-fold less expressed in comparison to patients with normal M2 karyotype (Figure 5B). Regulation of CD11b promoter by PU.1 has been shown[51] and further analysis (by TRANSFAC analysis to identify potential transcription factor-binding sites in a promoter) of the promoter revealed potential AML1-binding sites were present (data not shown). Down-regulation of CD11b might also be due to down-regulation of AML1 in addition to PU.1's transactivation capacity by AML1-ETO. Similar analysis of CD14 and CD64 promoters showed (data not shown) that these gene promoters have PU.1-binding sites but no C/EBPα-, AML1-, or MEF-binding sites. Therefore, CD14 and CD64 down-regulation could be due to specific down-regulation of PU.1's transactivation capacity by AML1-ETO in these patients.

AML1-ETO causes proliferation of mouse bone marrow cells by inhibiting PU.1

To investigate the functional consequences of AML1-ETO down-regulating the transactivation capacity of PU.1, we transduced mouse bone marrow cells with PU.1 (pGsam-NGFR-PU.1) and AML1-ETO (PINCO-AML1-ETO-GFP). The cells transduced with AML1-ETO rapidly increased in number over 12 days, as did the cells overexpressed with AML1-ETO and PU.1 (Figure 6A). The cells transduced with PU.1 showed no increase in cell number (Figure 6A). Furthermore, transduction of AML1-ETO blocks PU.1-induced monocytic differentiation in mouse bone marrow cells (data not shown). The expression of transduced genes is shown in Figure 6, panels B and C. Densitometric quantification of the PU.1 protein expression in the same experiment revealed down-regulation of endogenous PU.1 expression on overexpression of AML1-ETO (Figure 6B). This could be due to AML1-ETO preventing the autoregulation of PU.1.[54] The expression of AML1-ETO was also quantified (data not shown).

Overexpression of PU.1 initiates differentiation in t(8;21)$^+$ Kasumi-1 cells

Our data so far shows that AML1-ETO interacts with PU.1 at the $\beta_3\beta_4$ region in the DNA-binding domain of PU.1 and displaces c-Jun from binding and coactivating PU.1 (Figures 2 and 3). Moreover, overexpression of AML1-ETO down-regulated the PU.1 expression in mouse bone marrow cells (Figure 6B). It is important to note that Kasumi-1 cells shows high levels of c-Jun protein expression (Figure 2A). Hence, we asked whether overexpression of PU.1 could overcome the functional block of PU.1 by AML1-ETO. Transient overexpression of PU.1 (pGsam-NGFR-PU.1) in t(8;21)–bearing Kasumi-1 cells was performed. FACS

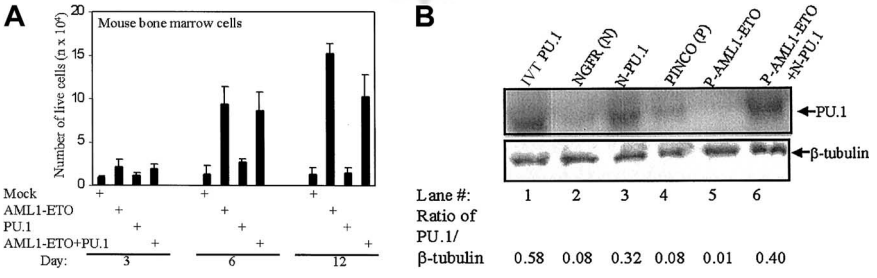


Figure 6. The antiproliferative effect of PU.1 is down-regulated by AML1-ETO in mouse bone marrow cells. (A) AML1-ETO causes proliferation in mouse bone marrow cells. Live transduced mouse bone marrow cells with PU.1, AML1-ETO, or PU.1 and AML1-ETO were counted on days 3, 6, and 12 after trypan blue staining. Because both the empty vectors gave the same cell count, only one vector (PINCO) has been represented as mock. (B) Expression of PU.1 in mouse bone marrow cells. The cells of the transduction described in the legend to Panel A were lysed and immunoblot assays were performed for PU.1 and β-tubulin. NGFR (N; lysate of empty vector of PU.1), N-PU.1 (NGFR-PU.1–transduced cells), PINCO (P; lysate of empty vector of AML1-ETO–transduced cells), P-AML1-ETO (lysate of PINCO-AML1-ETO–transduced cells), and P-AML1-ETO+N-PU.1 (lysate of PINCO-AML1-ETO– and NGFR-PU.1–transduced cells) were analyzed. The ratio of PU.1/β-tubulin was calculated after densitometric quantification of the bands.

sorting (for NGFR) of the transfected cells showed the PU.1 expression, which was further shown by immunoblot analysis of sorted cells for PU.1 expression (Figure 7B). Fourfold overexpression was observed after transfection (Figure 7B).

Five days after transfection of PU.1, morphologic changes (Figure 7A) were observed by Wright-Giemsa staining of cells. PU.1-transfected cells differentiated to the monocyte like cells, whereas the empty vector (pGsam-NGFR) transfected cells showed no morphologic change. The PU.1-transfected Kasumi-1 cells also showed an increase in cell surface markers CD11b (Figure 7C; marker for myeloid differentiation) and CD14 (Figure 7D; marker for the monocytic lineage). At 24 hours after transfection, the NGFR-sorted cells were further plated and counted for live cells every 24 hours. In PU.1-transfected cells a decrease in cell number was observed (Figure 7E).

Discussion

The importance of PU.1 in myeloid differentiation is well established. Recently we have reported that PU.1 is mutated in patients with AML[13] similar to C/EBPα,[55] suggesting that PU.1 also plays a major role in leukemogenesis. However, PU.1 was not found to be mutated in AML patients with t(8;21), which suggests that distinct pathways of inactivation of PU.1 might be occurring in t(8;21) leukemia. We show here that PU.1 plays a major role in leukemogenesis in t(8;21) leukemia because it interacts with fusion protein AML1-ETO (Figure 1A). We have previously reported a similar phenomenon for C/EBPα,[36] an important transcription factor in granulocytic differentiation. The physical interaction of PU.1 and AML1-ETO results in inactivation of the transactivation activity of PU.1 by displacing PU.1's coactivator c-Jun (Figures 1B, 2B, 2C, and 5A). AML1B was shown to interact with PU.1 and synergize on M-CSF receptor promoter.[10,56] In contrast, AML1B does not influence PU.1's transactivation capacity on a promoter driven by PU.1-binding sites only (Figure 1C). These data taken together suggest that AML1B and PU.1 synergy is possible in the promoters having the respective binding sites in near proximity for physical interaction, like the M-CSF receptor promoter.

We observed that AML1-ETO down-regulates the transcriptional activity of PU.1 in myeloid cells (Figure 5A) and physically interacts at the $\beta_3\beta_4$ region in the DNA-binding domain of PU.1 (Figure 3B). Our earlier data show that c-Jun, an AP-1 transcription factor complex member, binds to the $\beta_3\beta_4$ region and coactivates PU.1 in a JNK-independent manner.[46] The competitive protein-protein interaction experiments with in vitro–translated proteins indicate that AML1-ETO disrupted PU.1/c-Jun interaction in a

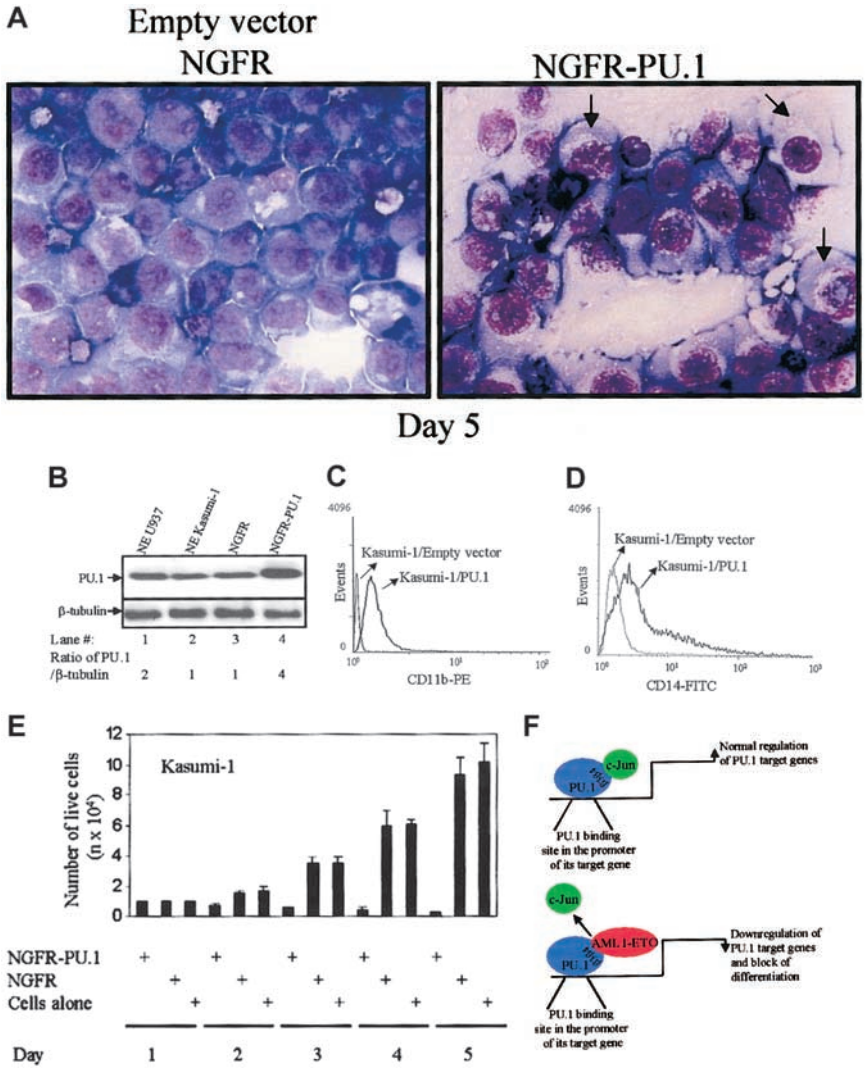


Figure 7. Transient overexpression of PU.1 induces differentiation toward the monocytic lineage in AML1-ETO⁺ Kasumi-1 cells. (A) PU.1 induces differentiation in t(8;21)⁺ Kasumi-1 cells. Kasumi-1 cells were transiently transfected with PU.1 (pGsam-PU.1-ires-NGFR) or the empty vector (pGsam-ires-NGFR) and morphologic changes were observed on day 5. Arrows indicate the differentiating cells. (B) PU.1 overexpression in Kasumi-1 cells. Western blot shows PU.1 expression and β-tubulin in transfected Kasumi-1 cells after day 5. (C) PU.1 induces CD11b expression in Kasumi-1 cells. FACS analysis was performed for the cell surface expression of CD11b in Kasumi-1 cells transfected with empty vector or PU.1. (D) PU.1 induces CD14 expression in Kasumi-1 cells. In the same experiment FACS analysis was performed for the cell surface expression of CD14 in Kasumi-1 cells transfected with empty vector or PU.1. (E) Kasumi-1 cell number decreases in PU.1-transfected cells. The transfected cells described in the legend to Panel D were counted by trypan blue staining on days 1, 2, 3, 4, and 5 after transfection. (F) Model of AML1-ETO blocking PU.1 function. Model is of AML1-ETO interacting with PU.1 and displacing its coactivator c-Jun. This down-regulation of the PU.1 transcriptional activity by AML1-ETO results in down-regulation of PU.1 target genes important for myeloid differentiation.

competitive manner (Figure 3B), thus blocking c-Jun from coactivating PU.1. We have described a similar mechanism for GATA-1[50] and C/EBPα.[48] The role of c-Jun in myeloid differentiation was shown to be rather important, because it could enhance the extent of differentiation in U937 cells.[57] In other studies AP-1 and C/EBPβ were shown to cooperate in regulation of common target genes, including the human TSG-6,[58] collagenase-1 gene,[59] and tumor necrosis factor α (TNF-α).[60] These data suggest that the capacity of c-Jun to coactivate PU.1 is also a very important mechanism, which is down-regulated by AML1-ETO.

We observed that physical interaction between AML1-ETO and PU.1 did not abolish the DNA- binding capacity of PU.1 (Figure 4A), although AML1-ETO interacted with the PU.1 DNA-binding domain. Interestingly, in PU.1's crystal structure,[1] the $\beta_3\beta_4$ domain does not interact with DNA, but is exposed to the solvent. This structural ability allows PU.1 to retain its DNA binding though being functionally repressed. We show here that the normal interaction between coactivators and transcription factors are altered in presence of AML1-ETO, which could be one of the important mechanisms for disrupted myelopoiesis in t(8;21)$^+$ leukemia (Figure 7F).

AML1B and AML1-ETO have been shown to transactivate the M-CSF receptor,[42] suggesting that interaction between AML1B and AML1-ETO could be important for leukemogenesis. To investigate the importance of AML1-ETO/PU.1 interaction in leukemogenesis, transactivation, proliferation, and differentiation assays were performed in cells expressing wild-type AML1B protein. In the presence of AML1-ETO, the M-CSF receptor promoter was down-regulated and similarly the AML1 site mutated M-CSF receptor promoter and minimal promoter containing only PU.1-binding sites in U937 cells (Figure 5A). This could be explained by a dual function of AML1-ETO in regulation of the M-CSF receptor expression. During normal myeloid differentiation, M-CSF receptor expression is required for G_1-to-S phase transition, which could be down-regulated by AML1-ETO through the functional interaction with PU.1, and then AML1-ETO cooperates with AML1B to up-regulate the M-CSF receptor expression for transformation and proliferation of abnormal progenitor cells.

In patients with t(8;21), expression of the cell surface markers CD11b, CD14, and CD64 was less in comparison to patients without t(8;21) (Figure 5B). CD14 and CD64 promoters have putative PU.1 binding sites but not AML1-, C/EBPα-, or MEF-binding sites suggesting that down-regulation of the function of PU.1 by AML1-ETO could possibly be an important step in progression toward leukemia. CD11b, another marker for differentiation, was also less expressed in patients with t(8;21) in comparison to patients without t(8;21) (Figure 5B). CD11b is regulated by PU.1 and its promoter contains putative binding sites of AML1. In this case AML1-ETO interaction and down-regulation of important myeloid transcription factors like PU.1 and AML1 could explain the lower CD11b expression. The phenotype of PU.1$^{-/-}$ mice suggests that PU.1 is critical for myeloid differentiation and development.[4,11,12] Interaction of PU.1 with AML1-ETO and subsequent suppression of PU.1 target genes (Figure 5B) might contribute to the phenotypic changes seen in t(8;21). Furthermore, PU.1 is a self-regulatory protein[54] and AML1-ETO overexpression in mouse bone marrow cells down-regulated the endogenous PU.1 expression (Figure 6B).

Because AML1$^{-/-}$ mice lack PU.1 expression,[61] AML1-ETO could down-regulate PU.1 expression through repressing AML1 function. However, in a diseased condition or in presence of AML1-ETO, like in Kasumi-1 cells, the expression of AML1 and PU.1 genes was still observed. Furthermore, AML1 does not have a down-regulatory effect on PU.1 (Figure 1C). Therefore, the presence of AML1-ETO does not completely repress the expression levels of these genes, but may block their functions by protein-protein interactions. To analyze the functional impact of AML1-ETO on PU.1 in the presence of wild-type AML1B protein, we performed experiments in cells expressing endogenous AML1B protein. Overexpression of PU.1 in mouse bone marrow cells leads to a block in proliferation, but in presence of AML1-ETO this function of PU.1 was abrogated (Figure 6A). Recently, it was shown that AML1-ETO expression in human progenitor cells leads to expansion of human hematopoietic stem cells.[62] Therefore, the block of differentiation and increase in abnormal proliferation of hematopoietic stem cells could be due to down-regulation of the activity of PU.1 in t(8;21) leukemia.

Overexpression of PU.1 in t(8;21)$^+$ Kasumi-1 cells differentiates them toward the monocytic lineage (Figure 7). Morphologically, cells did not appear to be terminally differentiated even though the cell surface markers CD11b and CD14 were increased in expression. It has been earlier shown that short-term activation of PU.1 in multipotent hematopoietic cells leads to immature eosinophils.[63] However, stable overexpression of PU.1 could lead to myeloid lineage in hematopoietic progenitor cells.[63] Therefore, higher and stable expression of PU.1 in Kasumi-1 cells might be needed to terminally differentiate toward the monocytic lineage. The cell number of PU.1-transfected Kasumi-1 cells decreased over a course of time (Figure 7E) showing that PU.1 functions as an antiproliferative factor on overexpression in Kasumi-1 cells. However, this mechanism needs to be further elucidated. Our data suggest that the ectopic expression of PU.1 in Kasumi-1 cells overcomes the functional block by AML1-ETO. PU.1 and C/EBPα are important factors for myeloid differentiation and AML1-ETO down-regulating these 2 factors could be an important step toward leukemia. This also suggests the possibility of using these 2 factors independently or in combination for therapy of t(8;21) myeloid leukemias.

Acknowledgments

We thank Dr Atsushi Iwama, University of Tsukuba, Japan for providing pGsam-PU.1-ires-NGFR and PGsam-ires-NGFR retroviral vectors.

AQ: 12

References

1. Kodandapani R, Pio F, Ni CZ, et al. A new pattern for helix-turn-helix recognition revealed by PU.1 ETS-domain-DNA complex. Nature. 1996;380: 456-460.
2. Tenen DG, Hromas R, Licht, JD, Zhang DE. Transcription factors, normal myeloid development and leukemia. Blood. 1997;90:489-499.
3. Chen H, Zhang P, Voso MT, et al. Neutrophils and monocytes express high levels of PU.1 (Spi-1) but not Spi-B. Blood. 1995;85:2918-2928.
4. Anderson KL, Perkin H, Surth CD, et al. Transcription factor PU.1 is necessary for development of thymic and myeloid progenitor-derived dendritic cells. J Immunol. 2000;164:1855-1861.
5. Behre G, Zhang P, Zhang DE, Tenen DG. Analysis of the modulation of transcriptional activity in leukemogenesis. Methods. 1999;17:231-237.
6. Hohaus S, Petrovick MS, Voso MT, et al. PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. Mol Cell Biol. 1995; 15:5830-5845.
7. Olweus J, Thompson PA, Lund-Johansen F.

Granulocytic and monocytic differentiation of CD34hi cells is associated with distinct changes in the expression of the PU.1 regulated molecules, CD64 and macrophage colony-stimulating factor receptor. Blood. 1996;88:3741-3754.

8. Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG. PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. Blood. 1996;88:1234-1247.
9. Zhang DE, Hetherington CJ, Chen H, Tenen DG. The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. Mol Cell Biol. 1994;14:373-381.
10. Zhang DE, Hohaus S, Voso MT, et al. Function of PU.1 (Spi-1), C/EBP, and AML1 in early myelopoiesis regulation of multiple myeloid CSF receptor promoters. Curr Top Microbiol Immunol. 1996;211:137-147.
11. McKercher SR, Torbett BE, Anderson KL, et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J. 1996;15:5647-5658.
12. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science. 1994;265:1573-1577.
13. Mueller BU, Pabst T, Osato M, et al. Heterozygous PU.1 mutations are associated with acute myeloid leukemia. Blood. In press.

AQ: 2

14. Crute BE, Lewis AF, Wu Z, Bushweller JH, Speck NA. Biochemical and biophysical properties of core binding factors 2 (AML1) DNA binding domain. J Biol Chem. 1996;271:26251-26260.
15. Daga A, Tighe JE, Calabi F. Leukemia/*Drosophila* homology. Nature. 1992;356:484-489.
16. Golling G, Li L, Pepling M, Stebbins M, Gergen JP. *Drosophila* homologous of the proto-oncogene product PEBP2/CBF beta regulate the DNA binding properties of the *Runt*. Mol Cell Biol. 1996;16:932-942.
17. Hernandez-Munian C, Krangel MS. c-Myb and core binding factor PEBP2 display functional synergy but bind independently to adjacent sites in the T-cell receptor enhancer. Mol Cell Biol. 1995;15:3090-3099.
18. Look AT. Oncogenic transcription factors in human acute myeloid leukemias. Science. 1997;278:1059-1067.
19. Ness SA, Kowenz-Leutz E, Casini T, Graf T, Leutz A. Myb and MF-M: combinatorial activators of myeloid genes in heterologous cell types. Genes Dev. 1993;7:749-759.
20. Ogawa E, Inuzuka M, Maruyama M, et al. Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of novel *Drosophila* runt-related DNA binding protein PEBP2. Virology. 1993;194:314-331.
21. Sun W, O'Connell M, Speck NA. Characterization of a protein that binds multiple sequences in mammalian type C retrovirus enhancers. J Virol. 1993;67:1986-1995.
22. Wang S, Wang Q, Crute BE, et al. Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol Cell Biol. 1993;13:3329-3332.
23. Nucifora G, Birn DJ, Erickson P, et al. Detection of DNA rearrangements in the AML1 and ETO loci and an AML1-ETO fusion mRNA in patients with t(8;21) acute myeloid leukemia. Blood. 81;1993:883-888.
24. Osato M, Asou N, Abdalla E, et al. Biallelic and heterozygous point mutations in the Runt domain of the AML1/PEBP2αB gene associated with myeloblastic leukemias. Blood. 1999;93:1817-1824.
25. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukemia. Nat Genet. 1999;23:166-175.
26. Era T, Asou N, Kunisada T, et al. Identification of two transcripts of AML1-ETO-fused gene in t(8;21) leukemic cells and expression of wild-type ETO gene in hematopoietic cells. Genes Chromosomes Cancer. 1995;13:25-33.
27. Erickson P Gao J, Chang KS, et al. Identification of break point in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1-ETO, with similarity to *Drosophila* segmentation gene, runt. Blood. 1992;80:1825-1831.
28. Miyoshi H, Kozu T, Shimizu K, et al. The t(8;21) translocation in acute myeloid leukemia results in production of an AML1-MTG8 fusion transcript. EMBO J. 1993;12:2715-2721.
29. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell. 1996;84:321-330.
30. Bitter MA, Le Beau MM, Rowley JD, et al. Associations between morphology, karyotype, and clinical features in myeloid leukemias. Hum Pathol. 1987;18:211-225.
31. Downing JR. The AML1-ETO chimeric transcription factor in acute myeloid leukemia: biology and clinical significance. Br J Haematol. 1999;106:296-308.
32. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999;30:1051-1061.
33. Miyoshi H, Shimizu K, Kazu T, Maseki N, Kaneko Y, Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc. Natl. Acad. Sci. U S A. 1991;88:10431-10434.
34. Frank R, Zhang J, Uchida H, Meyers S, Hiebert SW, Nimer SD. The AML1-ETO protein blocks transactivation of GM-CSF promoter by AML1B. Oncogene. 1995;11:2667-2674.
35. Nuchprayoon L, Meyers S, Scott LM, Suzow J, Hiebert S, Friedman AD. PEBP2/CBF, the murine homolog of human myeloid AML1 and PEBP2β/CBFβ protooncoproteins, regulates the murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells. Mol Cell Biol. 1994;14:5558-5568.
36. Pabst T, Mueller BU, Schoch C, et al. AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia. Nat Med. 2001;7:444-451.
37. Yergeau DA, Hetherington CJ, Wang Q, et al. Embryonic lethality and impairment of hematopoiesis in mice heterozygous for AML1-ETO fusion gene. Nat Genet. 1997;15:303-306.
38. Zhang DE, Fujioka K, Hetherington CJ, et al. Identification of a region which directs the monocytic activity of the colony-stimulating factor 1 (macrophage colony-stimulating factor) receptor promoter and binds PEBP2/CBF (AML1). Mol Cell Biol. 1994;14:8085-8095.
39. Zhang DE, Hetherington CJ, Meyers S, et al. CCAAT enhancer binding protein (C/EBP) and AML1 (CBFalpha2) synergistically activates the macrophage colony stimulating factor receptor promoter. Mol Cell Biol. 1996;16:1231-1240.
40. Frank RC, Sun X, Berguido FJ, Jakubowaik A, Nimer SD. The t(8;21) fusion protein, AML1-ETO transforms NIH3T3 cells and activates AP-1. Oncogene. 1999;18:1701-1710.
41. Klampfer L, Zhang J, Zelenetz AO, Uchida H, Nimer SD. The AML1-ETO fusion protein activates transcription of BCL-2. Proc Natl Acad Sci U S A. 1996;93:14059-14064.
42. Rhoades KL, Hetherington CJ, Rowley JD, et al. Synergistic up-regulation of the myeloid-specific promoter for macrophage colony-stimulating factor receptor by AML1 and the t(8;21)fusion protein may contribute to leukemogenesis. Proc Natl Acad Sci U S A. 1996;93:11895-11900.
43. Rhoades KL, Heterington CJ, Harakawa N, et al. Analysis of the role of AML1-ETO in leukemogenesis using inducible transgenic mouse model. Blood. 2000;96:2108-2115.
44. Meyers S, Lenny N, Hiebert SW. The t(8;21) fusion protein interferes with AML1B-dependent transcriptional activity. Mol Cell Biol. 1995;15:1974-1982.
45. Mao, S., Franks RC, Zhang J, Miyazaki Y, Nimer SD. Functional and physical interactions between AML1 proteins and ETS protein: MEF; implications for pathogenesis of t(8;21) positive leukemias. Mol Cell Biol. 1999;19:3635-3644.
46. Behre G, Whitmarsh AJ, Coghlan MP, et al. c-Jun is a JNK-independent coactivator of PU.1 transcription factor. J Biol Chem. 1999;274:4939-4946.
47. Behre G, Singh SM, Liu H, et al. Ras signaling enhances the activity of C/EBPalpha to induce granulocytic differentiation by phosphorylation of serine 248. J Biol Chem. 2002;277:26293-26299.
48. Reddy V, Iwama A, Iotzava G, et al. The granulocytic inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. Blood. 2002;100:483-490.
49. Behre G, Smith LT, Tenen DG. Use of a promoterless Renilla luciferase vectors as an internal control plasmid for transient co-transfection assays of Ras-mediated transcription activation. Biotechniques. 1999;26:24-26.
50. Zhang.P., Behre G, Pan J, et al. Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1 function. Proc Natl Acad Sci U S A. 1999;96:8705-8710.
51. Pahl HL, Scheibe RJ, Zhang DE, et al. The proto-oncogene PU.1 regulates expression of the myeloid-specific CD11b promoter. J Biol Chem. 1993;268:5014-5020.
52. Meyers S, Downing JR, Hiebert SW. Identification of AML-1 and t(8;21) translocation protein (AML1-ETO) as sequence-specific DNA-binding proteins: the runt homology domain is required for DNA binding and protein-protein interactions. Mol Cell Biol. 1993;13:6336-6345.
53. Grignani F, Kinsella T, Mencarelli A, Gergen JP. High efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res. 1998;58:14-19.
54. Chen H, Ray D, Zhang P, et al. PU.1 (Spi-1) autoregulates its expression in myeloid cells. Oncogene. 1995;11:1549-1560.
55. Pabst T, Mueller BU, Zhang P, et al. Dominant negative mutations of C/EBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet. 2001;27:263-270.
56. Petrovick MS, Hiebert SW, Friedman AD, et al. Multiple functional domains of AML1: PU.1 and C/EBPalpha synergize with different regions of AML1. Mol Cell Biol. 1998;18:3915-3925.
57. Szabo E, Francis J, Birrer MJ. Alterations in differentiation and apoptosis induced by bufalin in c-Jun overexpressing U937 cells. Int J Oncol. 1998;12:403-409.
58. Klampfer L, Lee TH, Hsu W, Vilcek J, Chen-Kiang S. NF-IL6 and AP-1 cooperatively modulate the activation of TSG-6 gene by tumor necrosis factor alpha and interleukin-1. Mol Cell Biol. 1994;14:6561-6569.
59. Doyle GAR, Pierce RA, Parks WC. Transcriptional induction of collagenase-1 in differentiated monocyte-like U937 cells is regulated by AP-1 and upstream C/EBPbeta site. J Biol Chem. 1997;272:11840-11849.
60. Zagariya A, Mungre S, Lovis R, et al. Tumor necrosis factor alpha gene regulation: enhancement of C/EBPbeta-induced activation by c-Jun. Mol Cell Biol. 1998;18:2815-2824.
61. Okada H, Watanabe T, Niki M, et al. AML1(−/−) embryos do not express certain hematopoiesis-related gene transcripts including those of the PU.1 gene. Oncogene. 1998;17:2287-2293.
62. Mulloy JC, Cammenga J, MacKenzie KL, et al. The AML1-ETO fusion protein promotes the expansion of human stem cells. Blood. 2002;99:15-23.
63. Nerlov C, Graf T. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. Genes Dev. 1998;12:2403-2412.