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C/EBPα or C/EBPα oncoproteins regulate the intrinsic and extrinsic apoptotic pathways by direct interaction with NF-κB p50 bound to the bcl-2 and FLIP gene promoters

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Abstract

CCAAT/enhancer binding protein α (C/EBPα) is mutated in 10% of acute myeloid leukemias, resulting in either a truncated protein or an altered leucine zipper (C/EBPαLZ) that prevents DNA-binding. C/EBPα induces bcl-2 in cooperation with NF-κB p50 to inhibit apoptosis. We now demostrate that C/EBPα or a C/EBPαLZ oncoprotein bind the bcl-2 P2 promoter in chromatin immunoprecipitation assays and induce the promoter dependent on the integrity of a κB site. C/EBPα expressed as a transgene in B cells binds and activates the bcl-2 promoter, but not in *nfkb1*−/− mice lacking NF-κB p50. Bcl-2 is central to the intrinsic apoptotic pathway, while FLICE inhibitory protein (FLIP) modulates caspase-8, the initiator caspase of the extrinsic pathway. C/EBPα and C/EBPαLZ also bind the FLIP promoter and induce its expression dependent upon NF-κB p50. Moreover, induction of FLIP by C/EBPα protects splenocytes from Fas ligand-induced apoptosis, but only if p50 is present. We also demonstrate direct interaction between bacterially produced C/EBPα and NF-κB p50, mediated by the C/EBPα basic region. These findings indicate that C/EBPα or its oncoproteins activate the bcl-2 and FLIP genes by tethering to their promoters via bound NF-κB p50. Targeting their interaction may favor apoptosis of transformed cells.

Keywords

C/EBPα; NF-κB; bcl-2; FLIP; apoptosis

Introduction

CCAAT/enhancer binding protein α (C/EBPα) is the founding member of a family of transcription factors having a C-terminal amphipathic, leucine-rich dimerization domain (leucine zipper, LZ), a basic amino acid-rich DNA-binding domain (basic region, BR) and N-terminal trans-activation domains (TADs).1–3 The full-length protein is 42 kD, and initiation of translation from an internal AUG results in a truncated, 30 kD protein (C/

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EBPαp30) lacking one TAD. Within hematopoiesis, C/EBPα is critical for myeloid differentiation4–8 and is mutated in 5–15% of acute myeloid leukemia (AML) patients with normal cytogenetics.9–13 The AML-associated mutations can be grouped into two clusters. Changes in the N-terminal region lead to expression of C/EBPαp30, which has a dominant negative effect and altered DNA binding.11,14 Mutations in the C-terminal region (C/EBPαLZ) are typically in-frame insertions or deletions, interrupting the α-helical conformation of the LZ domain and preventing dimerization and thereby also DNA binding, as C/EBPα binds DNA as an obligatory dimer.10,11 Interestingly, C/EBPα null mutations have not been described in AML, suggesting that expression of the mutated isoforms are of functional importance to the malignant cell. In addition to mutations in the *CEBPA* gene, the products of common AML related translocations interfere with C/EBPα expression. AML1-ETO binds and represses the *CEBPA* promoter, FLT3-ITD signaling reduces *CEBPA* mRNA expression, and bcr-abl inhibits *CEBPA* translation.15–17

Dysregulation of transcription factors in leukemia is common.18 These altered proteins often contribute to leukemogenesis through blockade of differentiation and stimulation of proliferation. Uniquely, C/EBPα protects hematopoietic cells from apoptosis by inducing bcl-2, potentially in cooperation with the p50 subunit of Nuclear Factor-κB (NF-κB).19 The AML-associated C/EBPα mutants, including C/EBPαLZ variants that cannot bind DNA, retain the ability to induce bcl-2.19 Dysregulated apoptosis is an important feature of the malignant phenotype in AML, contributing to the high rate of therapy failure.20–23 The bax/bcl-2 ratio impacts the prognosis of patients with AML independent of cytogenetics, and the leukemic cells are resistant to Fas mediated apoptosis.24 However, the underlying mechanisms that allow many AMLs to resist apoptosis remain uncertain. Here we show that C/EBPα or a C/EBPαLZ oncogenic variant interact directly with NF-κB p50 to bind and activate the endogenous bcl-2 promoter. Interaction with NF-kB p50 is shown to be essential for activation of bcl-2 by C/EBPα. In addition, C/EBPα or its C/EBPαLZ mutant also bind and activate the FLIP promoter dependent on the presence of NF-κB p50, and induction of FLIP by C/EBPα reduces apoptosis and cell death induced by Fas ligand, indicating that C/EBPα and NF-κB p50 cooperate to regulate both the extrinsic and intrinsic apoptosis pathways. We also demonstrate using purified proteins that C/EBPα and NF-κB contact each other directly. The potential for targeting interaction of these proteins as an approach to therapy for AML or other malignancies will be discussed.

Materials and Methods

Cells, plasmids and transient transfection

Ba/F3 cells19 were cultured in RPMI 1640 with 10% heat inactivated fetal bovine serum (HI-FBS) and 1 ng/mL IL-3 (Peprotech, Rocky Hill, NJ, USA). HF-1 cells25 were maintained in Iscove's modified Dulbecco's medium, 10% HI-FBS and 2.5 ng/mL GM-CSF (Peprotech). F9 and 293T cells were grown in DMEM with 10% HI-FBS. C/EBPα or the human AML derived C/EBPαLZ mutant F3901 (ΔK312)10 were cloned into the MTCB6 plasmid downstream of the metallothionein (MT) promoter. *Sca*I linearized plasmid DNAs were electroporated into Ba/F3 cells, and stably expressing cells were selected using 1.2 mg/mL G-418. Expression from the MT promoter was induced by culturing cells with 100

μmol/L zinc chloride and estradiol was used at 1 μmol/L. CMV expression vectors encoding C/EBPα, C/EBPαGZ,26 NF-κB p50 and NF-κB p65 were previously described.19 CMV-C/EBPαbZIP encodes the COOH-terminal amino acid residues 273–359 of rat C/EBPα. The bcl-2 P2-LUC reporter (containing bps −1278/+1) and its −170 bp NF-κB binding site mutant were previously described.27 F9 cells were transfected in 60 mm dish using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Each plate was transfected with 1.5 μg of the reporter plasmid, with 100 ng control CMV vector or CMV-C/EBP plasmids, and 5 ng CMV-β-galactosidase as an internal control. Luciferase and β-galactosidase activities were assayed 48 hours after the transfection.

Mice

H2K-C/EBPα-Eμ transgenic (αTG) mice in which the rat C/EBPα is expressed from the H2K promoter upstream of the immunoglobulin heavy chain enhancer were previously described.19 The transgene was detected by PCR of tail DNA using the following primers:

αTG-F: 5'-CCATCTCCACAGTTTCACTTCTGC;

αTG-R: 5'-CCGCGGCTCCGCCTCGTAGAAGTCG.

Nfkb1−/− mice28 or the B6;129PF1 strain matched control (Jackson Laboratories Bar Harbor, ME, USA) were genotyped by PCR using the following primers:

nfkb1-common: 5'-GCAAACCTGGGAATACTTCATGTGACTAAG;

nfkb1-WT: 5'- ATAGGCAAGGTCAGAATGCACCAGAAGTCC;

nfkb1-KO: 5'- AAATGTGTCAGTTTCATAGCCTGAAGAACG.

Bone marrow cells were extracted from the hind limbs. Single cell suspensions of splenocytes were obtained from wild-type (WT), WT;αTG, *nfkb1−/−*, and *nfkb1−/−*;αTG mice using a cell strainer, and the cells were cultured in RPMI 1640 with 10% HI-FBS and 50 nmol/L β-mercaptoethanol. Red cells were removed using an Accu-Prep gradient (Accurate Chemicals, Westbury, New York, USA). For Fas-mediated apoptosis, cells were cultured in RPMI1640, 10% HI-FBS and 50 μg/mL lipopolysaccharide (List Biological Laboratories, Campbell, CA, USA) for 30 hrs.29 The splenocytes were then exposed to soluble Fas ligand (FasL) (Alexis Biochemicals, San Diego, CA, USA) for 16 hrs. Cell viability and apoptosis were assessed by staining with APC-conjugated annexin V and propidium iodide (PI) followed by analysis using a fluorescence-activated cell sorter (FACS).

Western blotting, co-immunoprecipitation, and gel shift assay

Western blot and co-immunoprecipitation (co-ip) were performed as previously described19. For co-ip, 293T cells in 100 mm dishes were transfected using Lipofectamine 2000. Bacterially produced proteins were coincubated at 4°C for an hour in 150 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 0.5% Triton X-100, 1 mmol/L EDTA and than subjected to immunoprecipitation as described.19 The following antibodies were used: C/EBPα (14AA), bcl-2 (N19), NF-κB p50 (NLS), and NF-κB p65 (C20), mouse monoclonal anti-β-tubulin (D-10), and mouse monoclonal NF-κB p50 (E10; Santa Cruz Biotechnology, Santa Cruz,

CA, USA), mouse anti-C/EBPα (MA1-825, Affinity Bioreagents, Golden CO, USA), rabbit anti C/EBPα COOH-terminal.5 Bands were quantified using NIH ImageJ software. Purified C/EBPα protein was subjected to gel shift analysis as described.30 The sequence of the sense strand C/EBPα(NE) oligonucleotide with 4 bp overhang was: 5'-TCGAGGCCAGGATGGGGCAATACAACCCG. The κB oligonucleotide with 1 bp overhang was: 5'-GGGGGAATCCCC.

Chromatin immunoprecipitation

Ten million Ba/F3, HF-1, or mouse whole bone marrow cells were used in each chromatin immunoprecipitation (ChIP) reaction. Cells were incubated with 1% formaldehyde at 37°C for 10 minutes, and the reaction was quenched with glycine at a final concentration of 0.125 M. Cells were washed twice with ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA) and resuspended in 1 ml ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, 1x protease inhibitors). Lysates were sonicated on ice using a Branson Sonifier 250, and the chromatin was sheared to 500–1000 bp fragments. Lysates were centrifuged for 10 min at 16000 × g to remove insoluble cell debris, and ChIP dilution buffer (167 mM NaCl, 16.7 mM Tris pH 8.0, 1.1% Triton X-100, 0.01% SDS, 1.2 mM EDTA, 1x protease inhibitors) was added to a final volume of 4 mL. Lysates were precleared with 65 μL blocked protein-A/G sepharose beads (Upstate Biotechnologies, Lake Placid, NY, USA) for 1 hour at 4°C. They were briefly centrifuged, and input was obtained before incubating the resulting supernatant with the antisera against C/EBPα, C/EBPβ, NF-κB p50, NF-κB p65 or rabbit IgG (Santa Cruz) overnight at 4°C with rocking. Sixty microliters of blocked protein-A/G sepharose beads were added, followed by incubation at 4°C for 2 hours with gentle agitation. The beads were precipitated by centrifugation, and the bead-bound complexes were washed for 5 min in each of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% IGEPAL CA-630, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.0) and two washes in TE (10 mM Tris pH 8.0, 1 mM EDTA). DNA-protein complexes were eluted from beads using 200 μL of 1% SDS, 0.1 M $NaHCO_3$ at room temperature. Cross-links were reversed by adding 10 μL of 4 M NaCl to the eluates and incubation at 65°C overnight. Each sample was treated with RNAse and Proteinase K, and DNA was isolated with UltraClean DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA, USA). Precipitation of promoters of interest was detected by PCR, and the PCR products were resolved on agarose gels and visualized with ethidium bromide. The following primers were used: P1-F: 5'- CTGGAGGTCTGAAGCGGTC; P1-R: 5'-AGTCCAGCATTTGCAGAAGTC; P2-F: 5'- CATTGGTACCTGCAGCTTC; P2-R: 5'-CTGTGACAGCTTATAATGTATG; FLIP-F: 5'- CGCCCGGTAGTGTCTCTATT; FLIP-R: 5'- CTCGTCCAGTCTCCATCCAT; NE-F: 5'-ATGGATGATGCTGAAATGGAG; NE-R: 5'-CTCACCACCCAGGAACAATG.

Reverse transcriptase and quantitative PCR

Total cellular RNAs were extracted using the RNeasy kit (Qiagen Sciences, MD, USA) according to the manufacturer's protocol, and first strand cDNA was synthesized from 1 μg

of RNA using the ImProm II Reverse Transcriptase System (Promega, Madison, WI, USA). Quantitative real time PCR was performed using the iCycler iQ Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, Ca, USA) with a total reaction volume of 25 μL per reaction using the iQ SYBR Green Supermix (Bio-Rad). Each sample was assayed in triplicate, and mouse ribosomal protein S16 (mS16) transcript levels were used to normalize between samples. Standard curves were constructed for each assay to ensure high amplification efficiencies and comparability across all assays. The comparative C_t method was employed for quantification of the transcript expression levels. Triplicate C_t values were generated for all assays, and $2^{-\Delta\Delta Ct}$ values were then calculated. qRT-PCR primers were: FLIP-T: 5'- GGATACATCGTTCTGATCTAAG; FLIP-B: 5'-GTCTATTCTGTGGATGTTCTTC; Bcl2-F: 5'-GGATTGTGGCCTTCTTTGAG; Bcl2-R: 5'-GATGCCGGTTCAGGTACTC; NE-T: 5'-GAACGGTCTAAATTTCCGGTCA; NE-B: AAGGTCTGTCGAGTGCGCTC; mS16-T: 5'- CTTGGAGGCTTCATCCACAT; mS16-B: 5'- ATATTCGGGTCCGTGTGAAG.

Bacterially expressed proteins

The full length rat C/EBPα DNA was cloned as an *Nde*I/*Xho*I fragment into the pET28a(+) vector (EMD Biosciences, Gibbstown, NJ, USA), resulting in an N-terminus linker containing 6 histidine residues. The NF-κB p50 Rel homology domain (RHD) corresponding to residues 39–366 was cloned as a *Xma*I/*Xho*I fragment into a modified version of the pET22b(+) vector (EMD Biosciences). The resulting construct included an N-terminal 8 histidine residue repeat. Codon-adjusted *E coli* bacteria Rosetta2-DE(3) (EMD Biosciences) were transformed, grown, and induced with IPTG at final concentration of 100 μM. For C/EBPα purification, the bacteria were lysed (50 mM Tris-Cl, 200 mM NaCl, pH 7.3, 5 mM β-mercaptoethanol, 2–3 mg Lysozyme, 1 mM PEFA, Protease Inhibitor Cocktail [Sigma]), sonicated, and centrifuged at 34,000 g for 1 hour. The supernatant was loaded on equilibrated Hi-Trap Ni-NTA columns (GE Healthcare, Piscataway, NJ, USA). After washing with buffer A (50 mM Tris-Cl, 200 mM NaCl, pH 7.3, 5 mM β-mercaptoethanol) the proteins were eluted with 50, 100, 200, 500 mM immidazole using a step-gradient method. Fractions containing C/EBPα were pooled, unfolded for 1 hour in 37°C using urea (final concentration 8M) and DTT (final concentration 1 mM), centrifuged at 15,000 g for 30 minutes and stored at −80°C. The optimal refolding conditions were determined using the QuickFold Protein Refolding Kit (Athena Environmental Sciences, Baltimore, MD, USA). After thawing, the purified C/EBPα was refolded using a buffer containing 50 mM Tris-Cl, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA , 0.5% Triton X-100, 1 mM DTT, pH 8.5. Bacteria expressing the NF-κB p50 RHD were lysed (25 mM Tris-Cl, 50 mM NaCl, 5 mM β-mercaptoethanol, 2.5 mg lysozyme, 1 mM PEFA, pH 7.5, Protease Inhibitor Cocktail [Sigma]), sonicated on ice and centrifuged at 34,000 × g for 1 hour. The supernatant was loaded on equilibrated Hi-Trap Ni-NTA columns and subsequently washed in buffer containing 25 mM Tris-Cl, 50 mM NaCl, 5 mM β-mercaptoethanol, pH 7.5. RHD was eluted with an immidazole gradient as described above, and fractions containing RHD were pooled, dialyzed against 25 mM Tris-Cl, pH 7.5, 50 mM NaCl and stored at −80° C.

Statistics

Statistical comparisons were via the student's t test.

Results

C/EBPα binds the endogenous bcl-2 promoter

We employed chromatin immunoprecipitation (ChIP) to demonstrate binding of C/EBPα to the endogenous bcl-2 promoter. HF-1 is a myeloid cell line expressing C/EBPα. Unstimulated cell lysates were subjected to immunoprecipitation with antisera against C/EBPα, C/EBPβ, NF-κB p50, NF-κB p65, or IgG control. The precipitated DNA was purified, and amplification with primers directed at the bcl-2 P2 promoter demonstrates binding of each of these endogenous proteins to the bcl-2 P2 promoter (Figure 1a). Similar findings were obtained in two additional independent experiments (not shown).

To investigate the binding of a C/EBPαLZ leukemic isoform, we generated Ba/F3 cell lines that express wild type C/EBPα or the AML patient-derived leucine zipper mutant F3901 under the control of the inducible metallothionein promoter (MT-C/EBPα and MT-F3901, respectively). These cell lines express the C/EBPα isoform at similar levels, and C/EBPα is not expressed in parental Ba/F3 (Figure 1b). Following culture with zinc chloride for 16 hours to induce the MT promoter, cells were subjected to ChIP analysis using antiserum specific for C/EBPα. Both C/EBPα isoforms interacted with the endogenous bcl-2 promoter, and F3901 did so despite its inability to bind DNA directly (Figure 1c). No interaction was seen when parental Ba/F3 cells exposed to zinc chloride were analyzed similarly. Neutrophil elastase (NE) is a marker of early granulocytic myeloid differentiation. Remarkably, wild-type C/EBPα but not F3901 bind the NE promoter, indicating that LZ oncoproteins expressed in AML bind a specific subset of genes, thereby retaining the anti-apoptotic effect of C/EBPα but not its ability to induce differentiation.

C/EBPα and C/EBPα oncoproteins preferentially bind and activate the P2 bcl-2 promoter

The bcl-2 gene is expressed from two promoters, P1 and P2 (Figure 2a), with P1 being more active. Ba/F3-αER cells express a C/EBPα-estrogen receptor (ER) fusion protein that is inactive in the absence of estradiol. Withdrawal of Ba/F3 or Ba/F3-αER cells from IL-3 leads to rapid onset of apoptosis; however, this effect is delayed by activation of C/EBPα-ER, dependent upon induction of bcl-2 expression.19 Bcl-2 protein expression was evident by 16 hrs in this earlier study, but RNA expression was not examined. Total cellular RNAs prepared from Ba/F3-αER cells withdrawn from IL-3 in the absence or presence of estradiol were evaluated for bcl-2 mRNA expression by Northern blotting (Figure 2b). Although expressed at low levels, P2 mRNA was induced within 4 hrs, whereas transcription from the P1 promoter remained constant. To confirm the results of the Northern blot, we designed sets of PCR primers corresponding to the P1 or P2 promoters. Ba/F3 MT-C/EBPα or MT-F3901 cells were cultured with zinc chloride, and total cellular RNAs were prepared 16 hours after withdrawal from IL-3. First strand cDNA was analyzed by quantitative PCR. Each sample was assayed in triplicate in each experiment, and mS16 transcript levels were used as an internal control to normalize between samples. Compared with parental Ba/F3 cells, in four different RNA preparations, C/EBPα or F3901 induced P2 transcripts 8.2 or 5.4 fold on average, while P1 transcript levels increased only 1.5 or 2.1 fold (Figure 2c).

In addition, Ba/F3 MT-F3901 or Ba/F3-C/EBPα cells were induced with zinc chloride for 16 hours and subjected to ChIP analysis. In concordance with the RNA analyses, PCR amplification demonstrates binding of F3901 or C/EBPα and NF-κB p50 to the endogenous P2 but not the P1 promoter (Figure 2d). Together, these findings indicate that C/EBPα and its leukemia-associated LZ mutant bind and induce transcription from the endogenous P2 promoter of the bcl-2 gene.

NF-κB p50 is necessary for C/EBPα to bind and activate the bcl-2 promoter

We have previously shown that C/EBPα and its leukemia-related isoforms synergize with NF-κB p50 to activate the bcl-2 promoter. We now set out to test the hypothesis that absence of p50 would diminish bcl-2 induction by C/EBPα. Splenocytes from H2K-C/EBPα-Eμ transgenic (αTG) mice are resistant to radiation induced apoptosis and have increased bcl-2 compared to control littermates when subjected to apoptotic stress19. *Nfkb1*−/− mice and strain matched wild-type controls were bred with αTG mice to generate *nfkb1*−/−;αTG and matched WT;αTG mice. Single cell suspensions of splenocytes from *nfkb1*−/−, *nfkb1*−/−;αTG, WT, and WT;αTG mice were exposed to 200 cGy. Total cell lysates obtained 7 and 24 hours later were analyzed by Western blotting for bcl-2 (Figure 3a). As seen previously,19 bcl-2 levels were increased 1.5-fold by C/EBPα in wild-type NF-κB p50 cells (compare lanes 9 and 12). In contrast, the C/EBPα transgene failed to induce bcl-2 in the *nfkb1*−/− splenocytes 24 hours after radiation (compare lanes 3 and 6). Thus, NF-κB p50 is required for the induction of bcl-2 by C/EBPα.

We next evaluated the importance of NF-κB p50 for binding of C/EBPα to the bcl-2 promoter. Bone marrow cells were employed in this experiment as their use offers two advantages over splenocytes. First, they allow evaluation of binding of C/EBPα at physiologic levels, and second they provide a cellular context where C/EBPα is expected to occupy additional genetic targets associated with myeloid differentiation. Total bone marrow cells were subjected to ChIP analysis (Figure 3b). In the absence of NF-κB p50, C/EBPα only minimally bound the endogenous bcl-2 promoter compared to the strong interaction evident in WT bone marrow cells. On the other hand, NF-κB p50 was not necessary for the binding of C/EBPα to the myeloid neutrophil elastase (NE) promoter. In addition, we assessed bcl-2 mRNA expression in wild-type versus *nfkb1*−/− marrow mononuclear cells and observed a significant, 4-fold reduction in bcl-2 RNA expression in the absence of NF-κB p50, whereas NE mRNA levels were not significantly altered (Figure 3c). Together, these finding lend further support to the conclusion that a subset of C/EBPα genetic targets exist in which binding to the promoter and activation of transcription depend on cooperation with NF-κB p50.

C/EBPα and C/EBPαLZ mutants synergize with NF-κB p50 to trans-activate the bcl-2 promoter in the F9 cell line, which has only low-level endogenous NF-κB proteins19. The P2 promoter contains a κB binding site at −170.27 F9 cells were co-transfected with P2-LUC or P2-LUCmκB, harboring point mutations in the −170 κB site, and with CMV, CMV-C/EBPα or CMVC/EBPαF3901. Fold-activation was determined relative to the empty CMV vector, and an activation ratio for P2-LUC versus P2-LUCmκB was also calculated for each isoform (Figure 3d). Mutating the κB site significantly reduced activation by C/EBPα or its

F3901 variant. These findings indicate that activation of the bcl-2 P2 promoter by C/EBPα depends in part on the integrity of the κB site at −170 and supports the idea that C/EBPα or its non-DNA-binding F3901 variant tether to NF-κB p50 bound to this κB site to activate transcription from the bcl-2 P2 promoter. Residual 5-fold activation of P2-LUCmκB by C/EBPα or F3901 suggests the presence of an additional functional κB site in the 1.3 kb P2 promoter.

C/EBPα binds the FLIP promoter and induces FLIP transcription via NF-κB p50

A microarray screen using mRNA from Ba/F3 F3901-ER cells identified FLICE inhibitory protein (FLIP) as a potential C/EBPα or F3901 genetic target. FLIP modulates death receptor activation of caspase 8, the initiator caspase of the extrinsic apoptotic pathway. To validate this finding, parental Ba/F3 cells and MT-C/EBPα or MT-F3901 subclones were induced with zinc chloride for 16 hours followed by IL-3 withdrawal for an additional 16 hours in the presence of zinc. Total cellular RNAs were extracted, and FLIP expression was analyzed by quantitative RT-PCR. Expression was normalized to mS16, encoding a large ribosomal subunit. Compared to parental cells, FLIP was induced 6.5 fold by C/EBPα and 10.5 fold by C/EBPαF3901 (Figure 4a), on average from 3 independent RNA preparations. ChIP analysis using similar cells shows binding of C/EBPα or F3901 to the endogenous FLIP promoter, with no interaction evident in parental cells (Figure 4b). Similar ChIP data was obtained in an independent, second experiment with Ba/F3 cell lines (not shown). To determine whether absence of NF-κB p50 reduces the ability of C/EBPα to induce FLIP expression, we carried out ChIP using WT;αTG and *nfkb1*−/−;αTG splenocytes. Splenocytes were employed as lymphoid cells are expected to express increased FLIP compared to myeloid cells. Interaction of the C/EBPα transgene with the FLIP promoter was reduced 8-fold in the absence of NF-κB p50 (Figure 4c), whereas no interaction was evident in wild-type marrow cells (not shown). To determine whether induction of FLIP by C/EBPα protects cells from death via the extrinsic apoptotic pathway, splenocytes from WT, WT;αTG, *nfkb1*−/−, and *nfkb1*−/−;αTG mice were exposed to LPS followed by FasL (Figure 4d). LPS induces Fas expression, allowing induction of apoptosis in response to FasL.29 These results demonstrate that expression of exogenous C/EBPα in lymphoid cells significantly protects splenocytes from FasL-induced apoptosis and that protection from Fas-mediated apoptosis by C/EBPα requires the presence of NF-κB p50.

C/EBPα and NF-κB p50 interact directly

Exogenous or endogenous C/EBPα or C/EBPα oncoproteins present in mammalian cell extracts interact with endogenous NF-κB p5019. To exclude bridging by an additional protein and to further validate drug targetting efforts by demonstrating that this interaction is direct, we expressed histidine tagged full length C/EBPα and the NF-κB p50 Rel homology domain (RHD) in bacteria. The purified proteins were eluted form the Hi-Trap Ni-NTA columns, and fractions containing maximal C/EBPα or NF-κB p50 RHD, as judged by SDS-PAGE, were pooled (Figure 5a). Purified p50 RHD was soluble and bound a cognate κB site in gel shift analysis (data not shown). In contrast, recombinant C/EBPα formed insoluble inclusion bodies due to misfolding. The aggregated protein was solubilized by denaturation in 8M urea with 1 mM DTT. To determine the conditions required for the recovery of a soluble, active protein we screened a commercially available panel of buffer/detergent

mixtures for their ability to allow the denatured C/EBPα to refold to an active form, as judged by its ability to bind its cognate DNA sequence (Figure 5b). The buffer containing 50 mM Tris-Cl, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, pH 8.5 (lane 13) was chosen for subsequent experiments. Of note, presence of 0.5% Triton X-100 was the only feature common to all the buffers that yielded active protein.

Purified, refolded C/EBPα and NF-κB p50 RHD were mixed and subjected to co-immunoprecipitation using either NF-κB p50 or C/EBPα antisera (Figure 5c, left panels). This assay reproducibly demonstrated interaction between these bacterially generated proteins. When the co-immunoprecipation procedure was performed using purified C/EBPα alone with NF-κB p50 antisera or purified RHD alone with C/EBPα antisera, no non-specific interaction of these proteins with the agarose beads was evident (Figure 5c, right panels). Addition of oligonucleotides containing the C/EBPα(NE) site, a κB site, or both oligonucleotides did not interfere with this interaction (Figure 5d). These data demonstrate that C/EBPα and NF-κB p50 directly interact in the absence or in presence of their cognate DNAs.

The basic region of C/EBPα is sufficient for the interaction with NF-κB p50

Point mutation R303G in the BR of C/EBPα prevents its interaction with NF-κB p5019. However, it is not clear if other domains of C/EBPα are required for this interaction. To demonstrate that the BR is sufficient for this interaction, we co-expressed the C/EBPα bZIP domain with NF-κB p50 or NF-κB p65 in 293T cells. The bZIP domain includes C-terminal residues 273–359 that contain only the BR and adjacent LZ. Cell lysates were immunoprecipitated with rabbit NF-κB p50 or NF-κB p65 antisera followed by Western blot analysis using rabbit antisera specific for the C-terminus of C/EBPα. The bZIP domain of C/EBPα was sufficient for interaction with either NF-κB p50 or p65 (Figure 6a). A similar pattern was obtained when this experiment was repeated using extracts from 293T cells transfected with C/EBPα(bZIP) alone (data not shown), reflecting interaction with endogenous NF-κB p50 and p65.

To further define the essential domain required for the C/EBPα:NF-κB p50 interaction we used C/EBPαGZ, an isoform in which the majority of the C/EBPα LZ is replaced by the GCN4 LZ starting at residue 31526. 293T cells were co-transfected with C/EBPα or C/EBPα-GZ and NF-κB p50 or p65, and lysates obtained two days later were subjected to co-immunoprecipitation (Figure 6b, left and right panels). Replacement of the C/EBPα LZ with that of the yeast transcriptional activator GCN4 did not prevent interaction with NF-κB p50 or NF-κB p65, although affinity for p50 was diminished. When the co-immunoprecipation procedure was performed after transfection with NF-κB p50 or p65 alone, no non-specific interactions with the agarose beads were evident (Figure 6b, center panel). Together, these data indicate that 42 amino acids (residues 273–315) that encompass the C/EBPα BR and the first α-helix of its LZ are important for its interaction with NF-κB family members.

Discussion

The key conclusions of this study are that C/EBPα binds and activates the endogenous *bcl-2* or *FLIP* promoters via direct interaction of the C/EBPα BR with NF-κB p50. A C/EBPαLZ

myeloid oncoprotein that cannot interact with DNA also binds and activates the endogenous *bcl-2* and *FLIP* promoters. These findings have important implications for the role of C/EBPα and other C/EBPs in malignant transformation and for the potential utility of targeting the C/EBP:NF-κB interaction to favor apoptosis of malignant cells.

Previously, we found that C/EBPα inhibits apoptosis of Ba/F3 cells upon IL-3 withdrawal and induces endogenous bcl-2 protein in Ba/F3 cells or murine splenocytes; in addition, mutation of the C/EBPα BR prevented trans-activation of the bcl-2 P2 promoter in cooperation with NF-κB p50 and obviated interaction of C/EBPα with NF-κB p50 but not p65 in co-ip experiments using extracts from transfected 293T cells. However, several issues were left unsettled.19 First, the bcl-2 gene has two promoters, both of which have κB sites, and it was unclear whether one, the other, or both was the key target of the C/EBPα:NF-κB p50 complex. We, now demonstrate that C/EBPα or the F3901 C/EBPαLZ oncoprotein specifically induces transcription from the bcl-2 P2 promoter and that these activities are greatly diminished upon mutation of the −170 bp κB site. Second, although C/EBPα induces bcl-2 RNA expression and activates the bcl-2 P2-LUC reporter, it was uncertain whether induction of bcl-2 was via direct interaction with the endogenous gene. We now demonstrate using ChIP that C/EBPα, F3901, and NF-κB p50 each localize to the endogenous bcl-2 P2 promoter. Third, although we had correlated loss of C/EBPα interaction with NF-κB p50 and inability to induce bcl-2, we had not proven that interaction with NF-κB p50 is actually required. Now, we find that C/EBPα does not induce endogenous bcl-2 expression or bind the endogenous bcl-2 P2 promoter in cells derived from *nfkb1*−/− mice lacking NF-κB p50. Fourth, although C/EBPα and NF-κB interact when expressed in 293T cells, we had not eliminated the possibility of a bridging protein. Now we demonstrate direct interaction using purified proteins expressed in bacteria. Fifth, although we had implicated the C/EBPα BR as being required for interaction with NF-κB p50 by showing that BR point mutations prevent interaction, we had not assessed whether the BR was sufficient. We now demonstrate that the bZIP domain alone interacts with NF-κB p50. The bZIP domain only includes the BR and LZ. When we replaced the C/EBPα LZ with the homologous region from GCN4, interaction with NF-κB p50 was maintained, although reduced. Together, these data suggest the BR largely accounts for the contact between C/EBPα and NF-κB p50, although further mutagenic studies and perhaps co-crystallization are required to completely map the C/EBPα BR or LZ amino acids that contribute to affinity for NF-κB p50. Interestingly, the bZIP domain also interacted with NF-κB p65, and substitution of the GCN4 LZ in C/EBPα did not reduce affinity for p65, suggesting that NF-κB p65 also predominantly contacts C/EBPα via its BR.

Finally, we envision that C/EBPα cooperates with NF-κB p50 to activate additional genes to regulate apoptosis or other pathways and that induction of at least a subset of these requires tethering of C/EBPα to NF-κB p50 bound to DNA, as we surmise occurs on the bcl-2 P2 promoter. We now provide evidence indicating that the FLIP gene falls into this category: Not only C/EBPα but also the F3901 C/EBPαLZ oncoprotein, that cannot bind DNA but retains the ability to bind NF-κB p50, strongly induces FLIP mRNA in Ba/F3 cells; both C/EBPα and F3901 interact with the endogenous FLIP promoter, as does NF-κB p50; and interaction of C/EBPα with the FLIP promoter in the ChIP assay is markedly reduced in

cells from *nfkb1*−/− mice. In addition, just as we previously found that C/EBPα inhibits apoptosis via the intrinisic pathway upon cytokine withdrawal, we now find that C/EBPα also reduces apoptosis via the extrinsic pathway induced by Fas-FasL interaction.

The bZIP domains of C/EBPβ and C/EBPδ are highly similar to the C/EBPα bZIP domain, and their BR segments are virtually identical. In fact, C/EBPβ also inhibits apoptosis of Ba/F3 cells withdrawn from IL-3 (I.P.P. and A.D.F., unpublished). As C/EBPβ is widely expressed, we suggest that many cell types, and their malignant counterparts, express bcl-2, FLIP and other genes due to C/EBP:NF-κB cooperation. NF-κB most commonly exists as p65:p50 or p50:p50 dimers in cells. Each of these complexes includes an NF-κB p50 subunit available to interact with the C/EBP BR, and the p65 subunit also interacts with C/EBPα or C/EBPβ, though potentially with reduced affinity.19,31,32 A subset of C/EBP:NF-κB target genes are regulated by binding of both a C/EBP and an NF-κB dimer to separate *cis* DNA elements in their regulatory regions, whereas an additional subset of genes bind NF-κB which then potentially attract one or more C/EBP family member to the promoter via protein:protein interaction, expanding the repertoire of C/EBP target genes. Of note, F3901 retains the ability to induce bcl-2 and FLIP but cannot activate the NE gene, whose promoter does not bind NF-κB p50, indicating a mechanism that allows this C/EBPα oncoprotein to inhibit apoptosis without inducing myeloid differentiation. In fact, *CEBPA* LZ mutation reduces expression of wild-type C/EBPα, thereby interfering with normal differentiation.

Besides genes that regulate apoptosis, C/EBP and NF-κB cooperatively bind and activate multiple genes that contribute to inflammation, including those encoding IL-6, IL-8, G-CSF, serum amyloid, ICAM-1, superoxide dismutase, and Mediterranean fever promoter.4 Notably, the C/EBPβ bZIP domain alone increases the activity of the IL-6 promoter and does so only if the adjacent NF-κB binding site is intact.33 We propose that direct interaction of C/EBP family members with NF-κB dimers inhibits both the intrinsic and extrinsic apoptosis pathways via induction of bcl-2, FLIP, and other genetic targets and is also important for induction of inflammation. Although we did not detect interaction of C/EBPα with the FLIP promoter in normal marrow mononuclear cells, this does not preclude regulation of FLIP by C/EBPα in a small subset of these cells. In fact, FLIP was detected at high levels in human $CD34^+$ stem cells, which represent <1% of marrow cells, but not in $CD34^-$ cells, and the immature $CD34^+$ cells are resistant to Fas-mediated killing.34

Increased expression of bcl-2 family members or FLIP contributes to resistance of leukemias and lymphomas to apoptosis.20–24,35–37 In addition, elevated FLIP is detected in prostate cancers, and activation of the NF-κB pathway is a common feature of breast cancers, which also often express a truncated C/EBPβ isoform.38–40 A small molecule that blocks the C/EBP:NF-κB interaction might favor cell death in these and other malignancies and may also reduce inflammation that produces growth and angiogenic factors that themselves contribute to the survival of solid tumors.41

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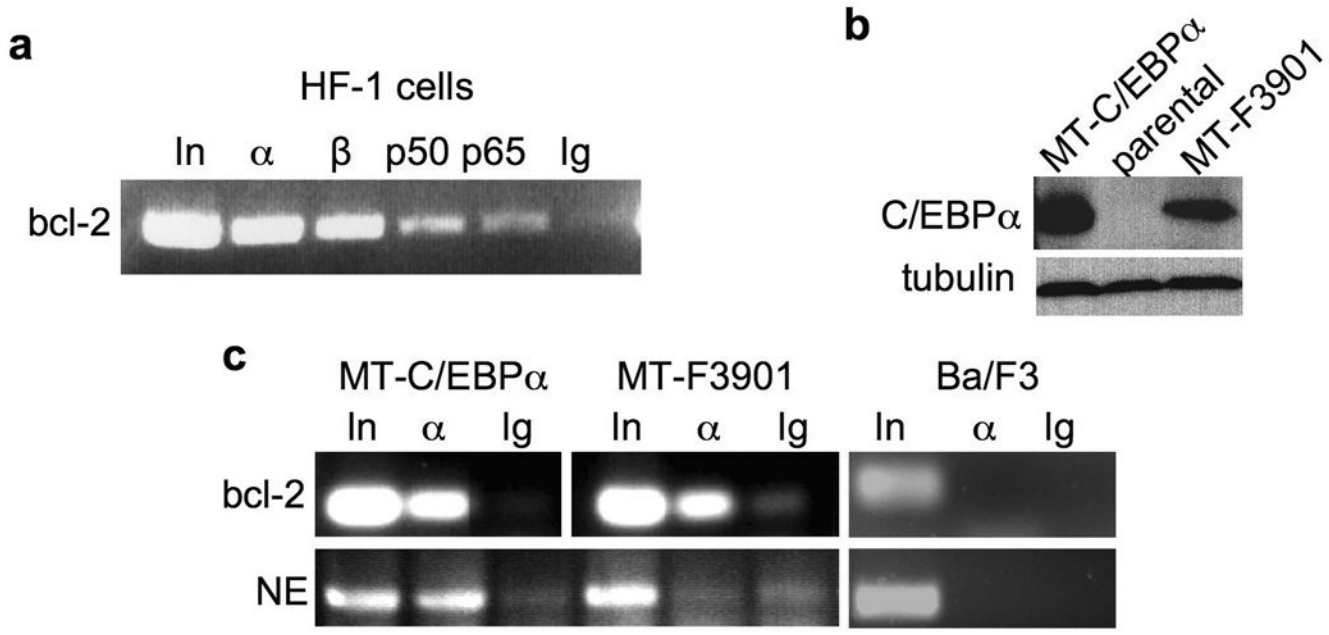

Figure 1. C/EBPα and a C/EBPαLZ oncoprotein bind the endogenous bcl-2 promoter

(**a**) HF-1 cells were subjected to ChIP analysis using antisera against C/EBPα (α), C/EBPβ (β), NF-κB p50, NF-κB p65, or IgG control. PCR products were subjected to agarose gel electropheresis and visualized using ethidium bromide. In – input, 1% of DNA used for ChIP. (**b**) Total cellular proteins from Ba/F3 lines expressing the indicated MT-C/EBPα isoform or from parental cells were subjected to Western blotting using C/EBPα and β-tubulin antisera. (**c**) Ba/F3 cells expressing MT-C/EBPα or MT-F3901, or parental Ba/F3 cells, were cultured with zinc chloride for 16 hours and subjected to ChIP analysis. After immunoprecipitation with antiserum against C/EBPα (α) or rabbit IgG, the precipitated DNAs were subjected to PCR for the bcl-2 or neutrophil elastase (NE) promoters, as indicated. Data representative of 3 independent experiments is shown.

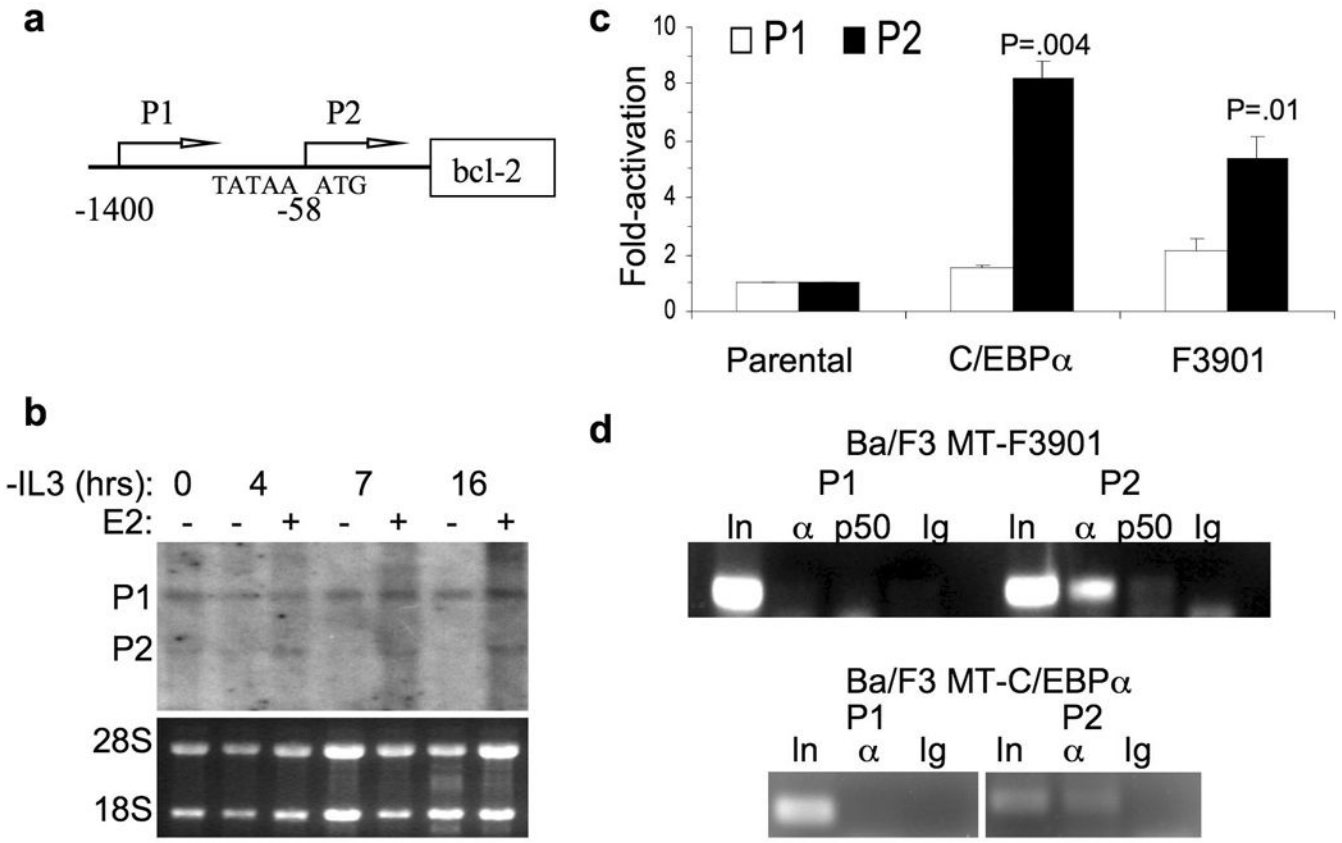

Figure 2. C/EBPα preferentially binds and activates the endogenous bcl-2 gene P2 promoter

(**a**) Diagram of the human bcl-2 gene. P2 transcription initiates at −58 relative to the initial ATG codon, and P1 initiates at several sites in the vicinity of −1400. The P2, but not the P1, promoter contains a TATAA box (at −88). (**b**) Ba/F3-C/EBPα-ER cells withdrawn from IL-3 were cultured with (+) or without (−) estradiol (E2). The expression of bcl-2 mRNA at the indicated time points was assessed by Northern blot analysis (top). The positions of P1 and P2 bcl-2 transcripts are indicated and RNA loading was assessed by ethidium bromide staining of the 28S and 18S ribosomal RNAs (bottom). (**c**) Ba/F3 MT-C/EBPα, MT-F3901, or parental Ba/F3 cells were cultured with zinc for 16 hours and then withdrawn from IL-3. RNA was extracted 24 hours after IL-3 removal. The levels of P1 and P2 transcripts were analyzed by quantitative RT-PCR and normalized to mS16 expression. Fold-activation relative to the parental Ba/F3 cells is presented. (**d**) Ba/F3 MT-F3901 or MT-C/EBPα cells were treated with zinc for 16 hours and subjected to ChIP using antisera against C/EBPα (α), NF-κB p50, or normal rabbit IgG and primers specific for the P1 or P2 bcl-2 promoters. Data representative of two independent experiments is shown.

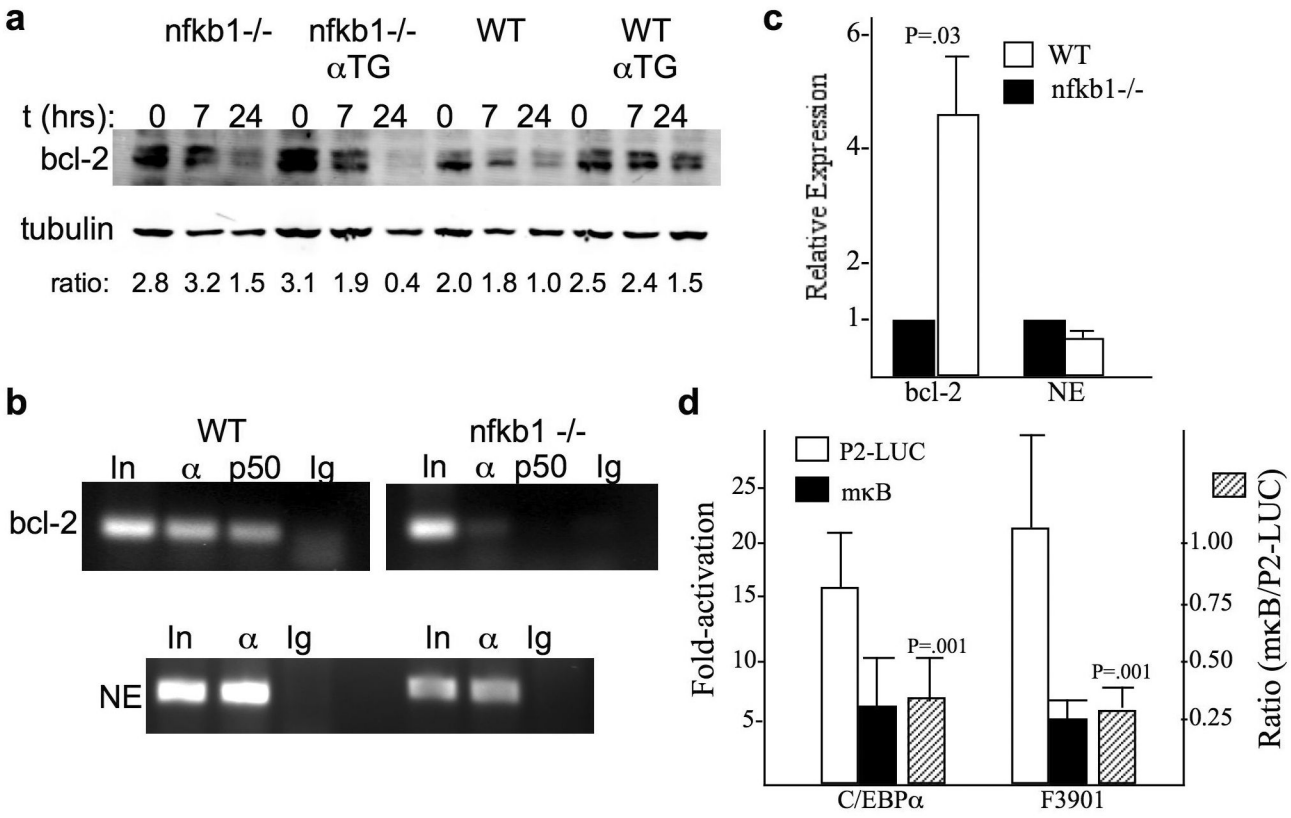

Figure 3. C/EBPα and a C/EBPαLZ oncoprotein depend on NF-κB p50 for binding to the bcl-2 promoter

(**a**) Splenocytes from mice with the indicated genotype were exposed to 200 cGy and cultured for 0, 7, or 24 hours. Total cellular proteins extracts were obtained and subjected to Western blotting for bcl-2 and β-tubulin. The ratio of bcl-2:tubulin in each sample is shown. (**b**) Total bone marrow cells extracted from *nfkb1*−/− or wild-type control mice were subjected to ChIP using C/EBPα (α) or NF-κB p50 (p50) antisera or IgG control and primers specific for the bcl-2 P2 or neutrophil elastase (NE) promoters. (**c**) RNA isolated from total bone marrow cells from the hind limbs of age, sex, and strain-matched *nfkb1*−/− or wild-type (WT) mice were subjected to quantitative RT-PCR analysis of bcl-2 or NE expression, normalized to mS16. Relative mRNA expression between WT and *nfkb1*−/− mice is shown, with expression in *nfkb1*−/− marrow set to 1. Data from four comparisons are shown. (**d**) F9 cells were transiently co-transfected with 1.5 μg of P2-LUC or its variant harboring clustered point mutations in the −170 κB site (mκB), with 100 ng of CMV, CMV-C/EBPα, or CMV-F3901, and 5 with ng of CMV-β-galactosidase as an internal control. Activation of the wild type and mutant promoters by C/EBPα or F3901 was analyzed 48 after transfection. Fold-activation compared to the empty CMV vector was determined after adjustment for β-galactosidase activity. The mean of four independent experiments is presented. Also shown is the average ratio of P2-LUC:P2-LUCmκB induction by C/EBPα or F3901. The p-values shown compare these induction ratios to the null hypothesis value of 1.0.

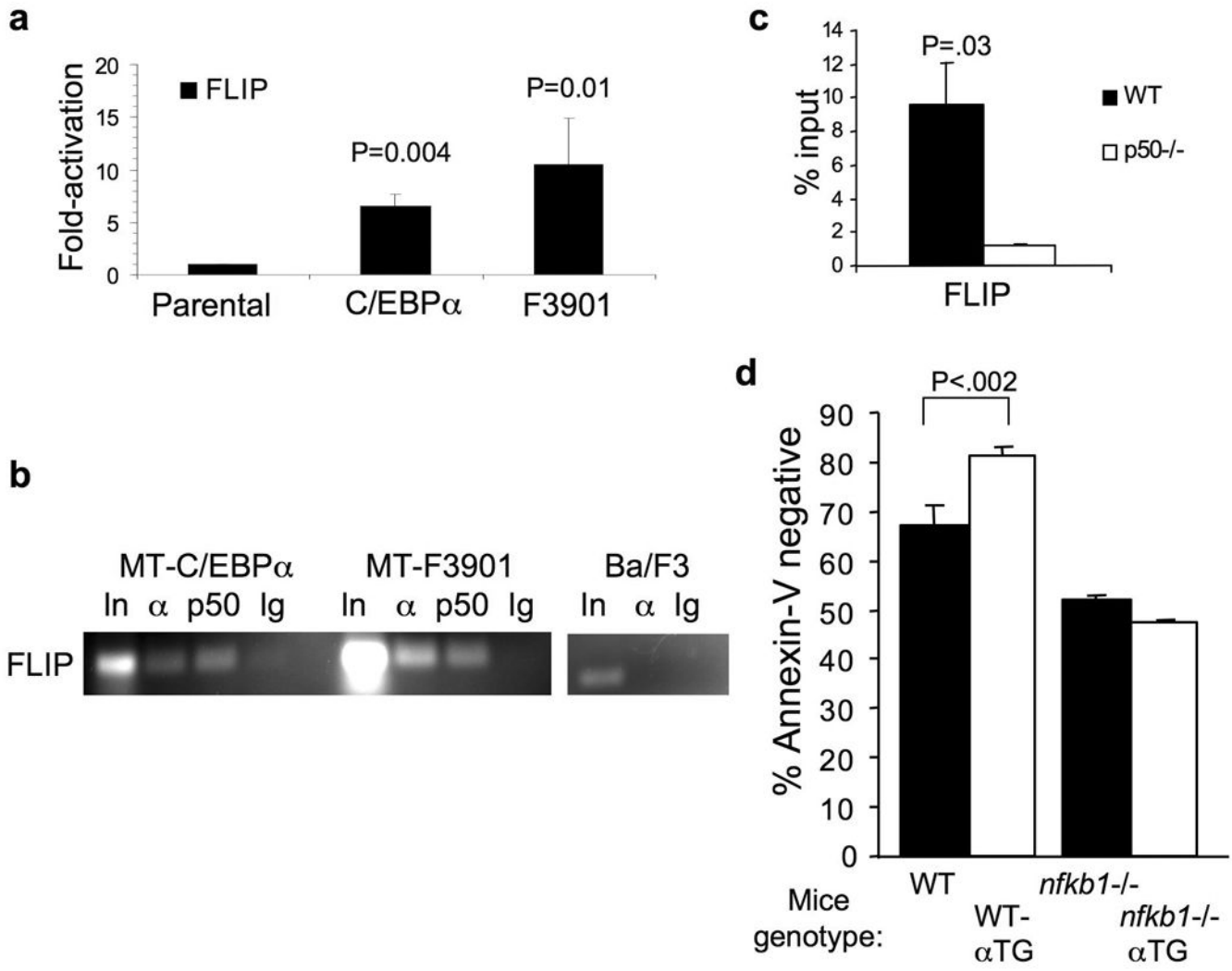

Figure 4. C/EBPα induces FLIP expression and binds its endogenous promoter

(**a**) Parental Ba/F3 cells and lines expressing C/EBPα or C/EBPαF3901 from the MT promoter were cultured in zinc chloride for 16 hours and then withdrawn from IL-3. RNA was extracted 16 hours after IL-3 removal. FLIP transcripts were measured using quantitative RT-PCR and expressed as fold-activation compared with parental cells. (**b**) Ba/F3 MT-C/EBPα or MT-C/EBPαF3901 or parental Ba/F3 cells were cultured with zinc for 16 hours and subjected to ChIP using C/EBPα (α) or NF-κB p50 (p50) antisera or control rabbit IgG and primers corresponding to the FLIP promoter. Data representative of two independent experiments is shown. (**c**) Splenocytes from H2K-C/EBPα-Eμ transgenic (αTG) or *nfkb1*−/−;αTG mice were subjected to ChIP using C/EBPα antiserum. Shown is the ratio of signal detected in the immunoprecipitate compared with input in three repetitions. (**d**) Single cell suspensions of splenocytes were obtained from mice with the indicated genotypes, stimulated with LPS for 30 hours and cultured with 200 ng/mL of soluble FasL for 16 hours. The cells were then stained with APC-Annexin V and PI. Shown

are the percent of cells that were Annexin V-negative, all of which excluded PI (mean and SD from three experiments).

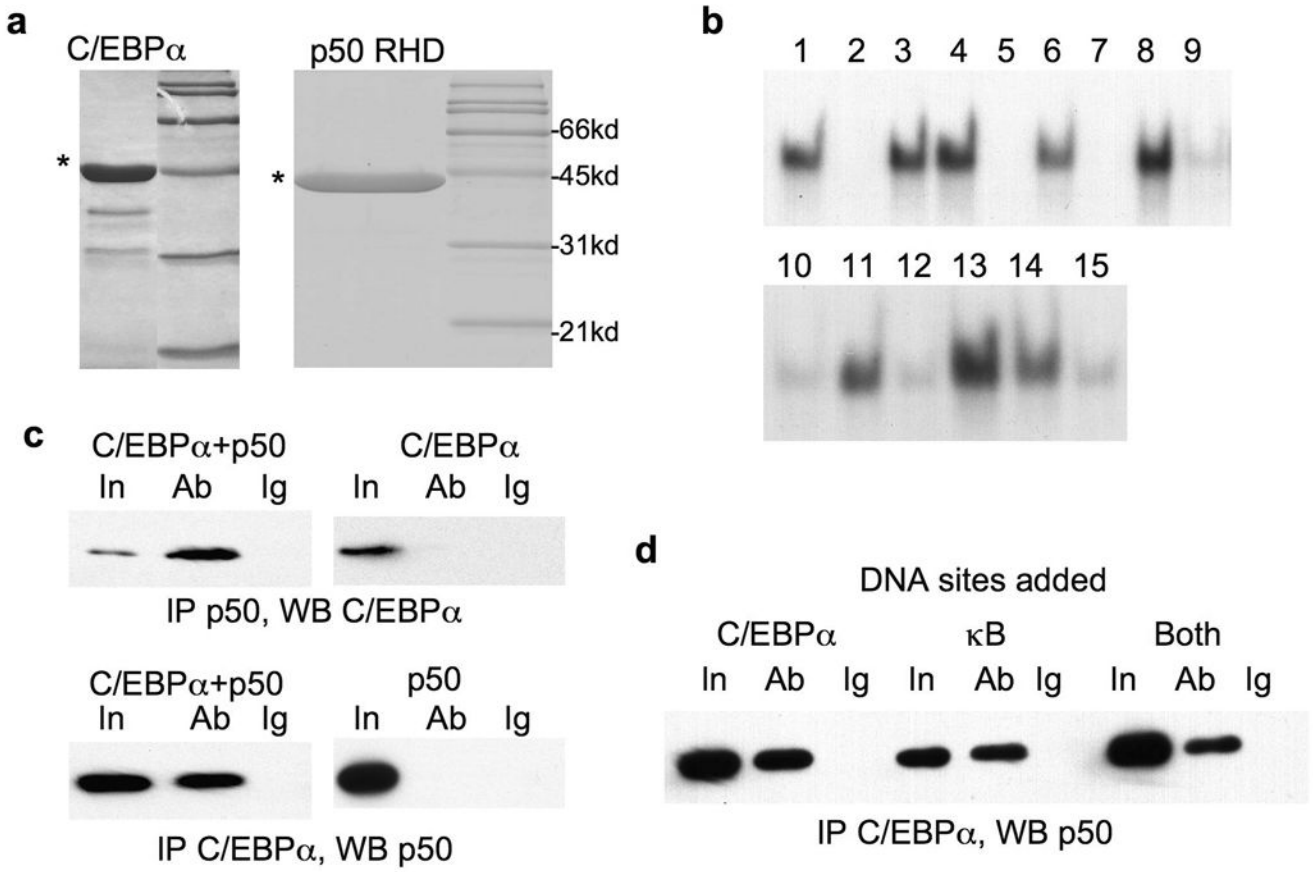

Figure 5. C/EBPα binds NF-κB p50 directly

(**a**) Histidine tagged C/EBPα and the Rel homology domain (RHD) of NF-κB p50 were expressed in *E. coli*, purified, resolved on an acrylamide gel, and stained with Coomassie blue. The positions of the molecular weight markers are indicated. (**b**) Refolding of purified C/EBPα in 15 different solutions, containing a range of salt, pH, and detergents, was examined. Activities of the renatured proteins were assessed by their ability to bind a consensus C/EBPα DNA sequence from the NE promoter in a gel shift assay. The lane numbers correspond to that of each buffer (QuickFold Protein Refolding Kit). A complete list of the buffers is available at http://athenaes.com/osc/QuickFoldAppMan.php. (**c**) Purified C/EBPα and NF-κB p50 RHD were co-incubated, subjected to immunoprecipitation (IP) with C/EBPα or p50 antisera, as indicated, followed by Western blot (WB) analysis with the reciprocal antibody (left panels). Purified C/EBPα alone was subjected to IP with p50 antisera, and purified RHD alone was subjected to IP with C/EBPα antisera as additional controls (right panels). (**d**) Co-immunoprecipitation was assessed after coincubation of purified C/EBPα and NF-κB p50 with double stranded oligonucleotides containing consensus DNA binding sites for C/EBPα or p50 (κB), or with both oligonucleotides.

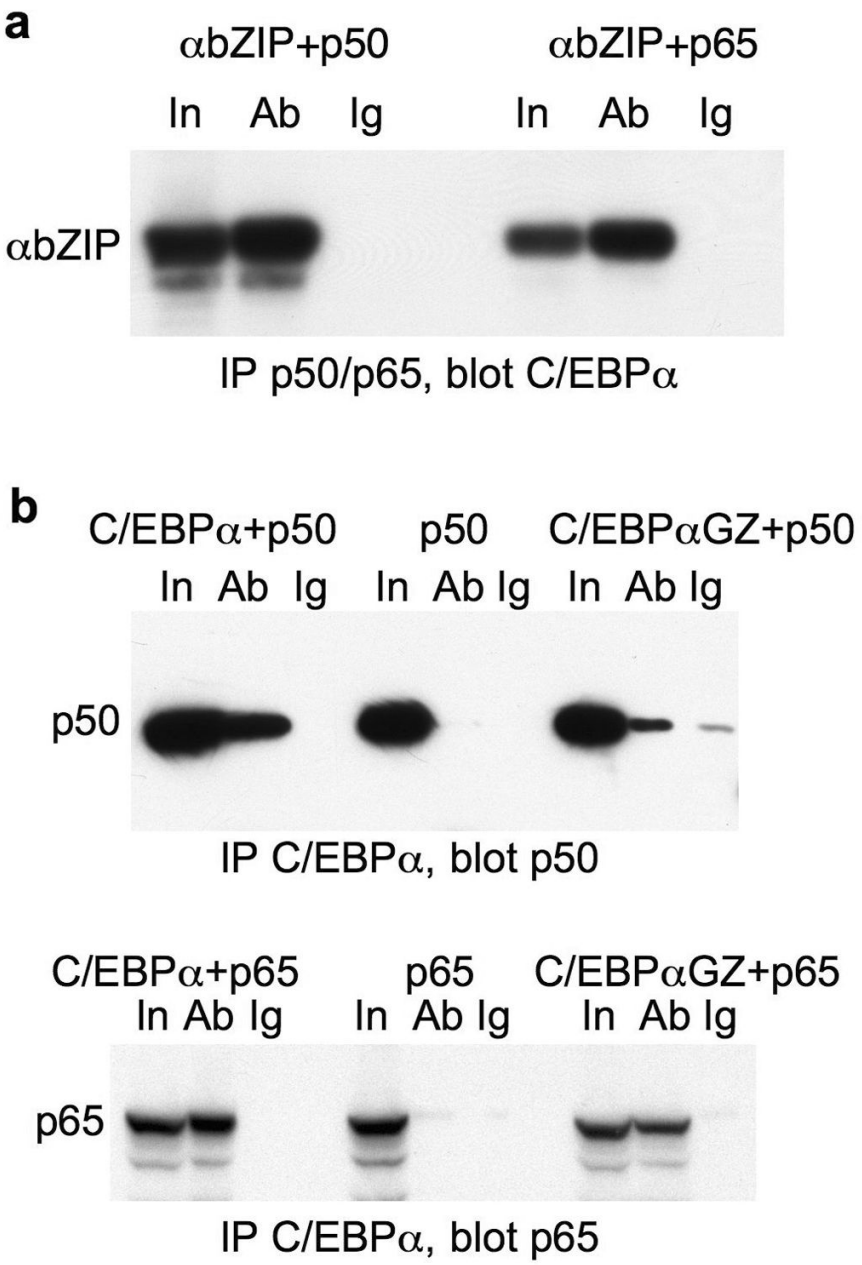

Figure 6. The C/EBPα basic region mediates its interaction with NF-κB p50 or p65

(**a**) 293T cells in 100 mm dishes were cotransfected with 2 μg CMV-C/EBPαbZIP and 2 μg of either CMV-NFκB p50 or CMV-NF-κB p65. Two days later, cells extracts were immunoprecipitated with p50 or p65 rabbit antisera (Ab) or rabbit IgG control (Ig) and immunoblotted with rabbit antiserum raised against the C/EBPα COOH-terminal sequence. (**b**) Extracts from cells transfected with CMV-C/EBPα or CMV-C/EBPαGZ and either CMV-NF-κB p50 or CMV-NF-κB p65 were immunoprecipitated with C/EBPα antiserum or rabbit IgG and immunoblotted with monoclonal p50 or p65 antibodies, as indicated (right

and left panels). Extracts from cells transfected with p50 or p65 alone were assayed similarly as additional controls (center panel).