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C/EBPα, C/EBPα Oncoproteins, or C/EBPβ Preferentially Bind NF-κB p50 Compared with p65 Focusing Therapeutic Targeting on the C/EBP:p50 Interaction

Julia E. Dooher[1], **Ido Paz-Priel**[1], **Simone Houng**[1], **Albert S. Baldwin Jr.**[2], and **Alan D. Friedman**[1]

[1]Division of Pediatric Oncology, Johns Hopkins University, Baltimore, Maryland, USA

[2]Lineberger Comprehensive Cancer Center and Department of Biology, University of North Carolina, Chapel Hill, NC, USA

Abstract

Canonical NF-κB activation signals stimulate nuclear translocation of p50:p65, replacing inhibitory p50:p50 with activating complexes on chromatin. C/EBP interaction with p50 homodimers provides an alternative pathway for NF-κB target gene activation, and interaction with p50:p65 may enhance gene activation. We previously found that C/EBPα cooperates with p50 but not p65 to induce *Bcl-2* transcription and that C/EBPα induces *Nfkb1/p50* but not *RelA/p65* transcription. Using p50 and p65 variants containing the FLAG epitope at their N- or C-termini, we now demonstrate that C/EBPα, C/EBPα myeloid oncoproteins, or the LAP1, LAP2, or LIP isoforms of C/EBPβ have markedly higher affinity for p50 in comparison to p65. Deletion of the p65 trans-activation domain did not increase p65 affinity for C/EBPs, suggesting that unique residues in p50 account for specificity, and clustered mutation of HSDL in the "p50 insert" lacking in p65 weakens interaction. Also, in contrast to *Nfkb1* gene deletion, absence of the *RelA* gene does not reduce *Bcl-2* or *Cebpa* RNA in unstimulated cells or prevent interaction of C/EBPα with the *Bcl-2* promoter. Saturating mutagenesis of the C/EBPα basic region identifies R300 and nearby residues, identical in C/EBPβ, as critical for interaction with p50. These findings support the conclusion that C/EBPs activate NF-κB target genes via contact with p50 even in the absence of canonical NF-κB activation and indicate that targeting C/EBP:p50 rather than C/EBP:p65 interaction in the nucleus will prove effective for inflammatory or malignant conditions, alone or synergistically with agents acting in the cytoplasm to reduce canonical NF-κB activation.

Keywords

NF-κB; C/EBP; inflammation; apoptosis; leukemia

Introduction

NF-κB is implicated in the regulation of genes encoding inflammatory cytokines such as IL-2, IL-6, IL-8, and GM-CSF, or regulators of apoptosis, such as bcl-2 and bcl-xL (1, 2). Five NF-κB family members exist in mammalian cells: c-Rel, Rel A/p65, Rel B, NF-κB1/p50 and NF-κB2/p52. Each possesses an approximately 300 amino acid Rel homology

Corresponding author: Alan D. Friedman, Johns Hopkins University, Division of Pediatric Oncology, CRB I, Rm. 253, 1650 Orleans St., Baltimore, MD 21231, USA. Phone: 410-955-2095; Fax 410-955-8897. afriedm2@jhmi.edu.

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domain (RHD) that mediates dimerization and DNA binding. The p50:p65 and p50:p50 complexes are the most common subunit combinations found in most cell types. p65 has a C-terminal trans-activation domain (TAD) lacking in p50, allowing the p50:p65 heterodimer to activate genes in association with co-activators such as p300, whereas p50 homodimers often repress transcription via interaction with HDAC1 (3, 4). p65 is sequestered in the cytoplasm by a series of IκB proteins. These bind the C-terminal segment of the RHD that contains the nuclear localization sequence. Canonical activation of p65, by a variety of stimuli, depends on phosphorylation of IκB by IκB kinases (IKKs) followed by its ubiquitination and proteosomal degradation (5). Notably, p50:p50 binds DNA even in unstimulated cells, reflecting the finding that IκB has approximately 60-fold lower affinity for p50 compared with p65 (3, 6).

We have provided evidence indicating that C/EBPα, C/EBPα variants associated with acute myeloid leukemia (AML), or C/EBPβ cooperate with NF-κB p50 to activate the murine *Bcl-2* and *Flip* genes to inhibit apoptosis via the intrinsic and extrinsic pathways (7, 8). Importantly, this cooperative gene activation does not require DNA-binding by the C/EBP proteins. Approximately 10% of human AML cases harbor point mutations in the *CEBPA* genes. One allele often harbors an N-terminal mutation leading to expression of a truncated p30 isoform that retains an intact DNA-binding basic region (BR)-leucine zipper (LZ) or bZIP domain that acts as a dominant-negative protein due to its weakened TAD, whereas the other allele in the same patient often harbors an in-frame insertion or deletion in the LZ that prevents dimerization or DNA-binding. These C/EBPαLZ variants retain the ability to activate transcription and inhibit apoptosis in cooperation with NF-κB p50 (7, 8). The finding that *CEBPA* leucine zipper mutations associated with human AML are always in-frame and so retain the ability to induce *Bcl-2, Mcl-1*, and *Flip* expression provides a powerful biological "experiment of nature" indicating the potential importance of C/EBP:NF-κB interaction in cancer.

Several lines of evidence suggest that interaction with p50 rather than p65 is most critical for transcriptional synergy between C/EBP proteins and NF-κB complexes. C/EBPα or a C/EBPαLZ oncoprotein each cooperate with p50 but not p65 for human *BCL-2* promoter activation; C/EBPα co-immunoprecipitates with endogenous p50 more effectively than endogenous p65 in two myeloid leukemia cell lines; mutation of the C/EBPα BR weakens interaction with p50 but not p65 and prevents endogenous bcl-2 gene induction and inhibition of apoptosis; and germ line deletion of the *Nfkb1* gene encoding the p50 subunit diminishes bcl-2 expression and chromatin immunoprecipitation (ChIP) of endogenous C/EBPα on the *Bcl-2* promoter (7, 8). Moreover, C/EBPs displace HDAC1 or HDAC3 from p50 bound to the *Flip* promoter to enable gene activation (9).

Herein we demonstrate that C/EBP proteins preferentially interact with NF-κB p50 compared with p65 using N- or C-terminally FLAG tagged p50 or p65 in co-immunoprecipitation (co-IP) reactions to uncover a consistently striking difference in affinity. Deletion of the p65 TAD did not increase affinity for C/EBPs, suggesting that amino acid differences in the p50 versus p65 RHD accounts for the increased affinity of p50 for C/EBP proteins. In addition, germ line deletion of floxed *RelA/p65* alleles did not reduce *Bcl-2* expression or interaction of C/EBPα with the *Bcl-2* promoter, in contrast to findings with *Nfkb1/p50* gene deletion. These observations support the idea that C/EBPs can induce NF-κB target genes even in the absence of canonical p65 activation via interaction with otherwise repressive p50:p50 complexes on chromatin. In addition, we have localized key residues in the C/EBPα BR required for interaction with p50 as well as a cluster of residues in p50 but lacking in p65 that contribute to interaction with C/EBPα. The implications of these findings for regulation of NF-κB target genes in normal or malignant cells and for the

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development of inhibitors of C/EBP:NF-κB interaction to treat AML and additional malignancies will be discussed.

Materials and Methods

Plasmids

The cDNAs encoding C/EBPα, C/EBPβ, CMV-C/EBPαp30, or four C/EBPαLZ variants were inserted downstream of the CMV promoter in pGEM/CMV. CMV-C/EBPαbZIP and CMV-p50 were previously described (7, 10); CMV-p65, CMV-C-p50, and CMV-C-p65, the latter two expressing p50 or p65 with an in-frame C-terminal FLAG tag, were obtained commercially (Addgene), and CMV-N-p50 or CMV-N-p65 were generated by ligating the p50 or p65 cDNAs, with the endogenous initiating methionine deleted, downstream of an initiating methionine, the FLAG epitope, and a *Cla*I site encoding isoleucine-aspartic acid (M-DYKDDDDK-ID). CMV-N-p50Δ356 and CMV-N-p65Δ297 were generated by PCR from the full-length proteins with addition of a stop codon. To generate C/EBPα BR mutants while avoiding PCR of the highly GC-rich C/EBPα cDNA, the codons for amino acids 270–271 were first replaced with a *Kpn*I site via site-directed mutagenesis, changing glycine-alanine to glycine-threonine. DNA encoding the 87 residue bZIP domain and subsequent stop codon, with a 5′ *Kpn*I site and a 3′ *Xho*I site, were then synthesized (Blue Heron) and ligated downstream of the introduced *Kpn*I site, and the resulting variant cDNAs were positioned downstream of the CMV promoter in pGEM/CMV. CMV-p50 was subjected to site-directed mutagenesis to generate clustered point mutants or to replace deleted segments with a *Kpn*I site. All DNA constructs were confirmed by DNA sequencing.

Cell culture, co-immunoprecipitation, and Western blotting

293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). Subconfluent 293T cells in 100 mm dishes were transiently transfected with 5 μg CMV expression vectors and 12 μL Lipofectamine 2000 (Invitrogen) per the manufacturer’s instructions. Cell extracts were prepared two days later by washing twice with phosphate-buffered saline (PBS), followed by rotation at 4°C for 20 minutes with 1000 μL of 0.5% Triton X-100, 150 mmol NaCl, 1 mmol/L EDTA, 20 mmol Tris (pH 7.5), 1 mmol/L phenylmethylsulfonyl fluoride, and a cocktail of protease inhibitors (Sigma). The lysates were then briefly sonicated and clarified at 14,000 × g for 10 minutes, and the supernatants were precleared with 50 μL of 50% protein A-Sepharose. Supernatants expressing C/EBP proteins (500 μL) and NF-κB proteins were combined and 1% was saved as input. NF-κB lysate volumes were chosen to approximately match NF-κB protein inputs based on FLAG Westerns. Total volume of each C/EBP:NF-κB mixture was brought to 1000 μL using mock lysate, and this solution was then incubated with 1.2 μg primary antiserum or rabbit Ig together with 30 μL protein A-Sepharose overnight at 4°C. For FLAG IP, 30 μL agarose beads conjugated to M2-anti-FLAG monoclonal antibody or to mouse Ig as a control (Sigma) were employed. The beads were then washed thrice with lysis buffer, and the samples were eluted in Laemmli sample buffer and subjected to Western blotting as described (11). Antibodies employed were C/EBPα (14AA) for IP, C/EBPα (C-18) for Western blotting, C/EBPβ (C19), or NF-κB p50 (NLS) from Santa Cruz Biotechnology, and anti-FLAG M2 antibody or anti-β-actin AC-15 from Sigma. All co-IP results shown are representative of at least duplicate experiments.

Gel shift analysis and reporter assay

Nuclear extracts were prepared from 293T cells transfected 2 days earlier with CMV expression vectors and subjected to gel shift analysis using annealed, double-stranded oligonucleotides containing either a consensus κB site or a C/EBP binding site from the neutrophil elastase promoter, as described (12). The sense strands of the gel shift probes

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containing 4 bp 5′ overhangs, with binding sites underlined were:κB: 5′-GTACGTAGGGGACTTTCCGAGCTCGAGATCCTATG and NE-C/EBP: 5′-TCGAGGCCAGGATGGGGCAATACAACCCG. Plasmid $(\kappa B)_5$-LUC, containing five κB sites, 5′-GGGACTTTCC, upstream of a TATA box, initiation site, and the luciferase cDNA (PathDetect System, Agilent Technologies), was transiently transfected into 293T cells along with CMV vectors expressing NF-κB subunits and CMV-βGal as internal control. Luciferase and β-galactosidase activities were assessed two days later, as described (13).

Murine marrow isolation, RNA analysis, and chromatin immunoprecipitation

C57Bl/6 mice harboring floxed p65 alleles (14) were bred with C57Bl/6 Mx1-Cre mice (Jackson Laboratories) to generate p65(f/f);Mx1-Cre mice or p65(f/f) littermates. These mice were injected with 400 μg pIpC intraperitoneally every other day for 7 injections, and five weeks after the first injection bone marrow cells were collected from the femurs by flushing with phosphate-buffered saline. After red cell lysis with NH_4Cl, cells were placed in Laemmli sample buffer for Western blotting or total cellular RNA was obtained using the Nucleospin RNA II kit, in the presence of RNase-free DNase (Macherey-Nagel). First strand cDNA was generated from 1 μg RNA using the ImProm II Reverse Transcriptase System (Promega) with random hexamer primers. Quantitative real time PCR was performed using the iQ SYBR Green Supermix and the iCycler iQ Real Time PCR Detection System (Bio-Rad Laboratories). Each sample was assayed in triplicate, and each experiment was repeated at least three times. Amplification of mouse large ribosomal subunit (mS16) RNA was used as a reference to standardize between samples. cDNA generated without reverse transcriptase did not yield detectable products. Primers employed were:

Bcl2-F: 5 ′-GGATTGTGGCCTTCTTTGAG; Bcl2-R: 5 ′-GATGCCGGTTCAGGTACTC; CEBPA-F: TGGATAAGAACAGCAACGAG; CEBPA-R: 5′-TCACTGGTCAACTCCAACAC; p65-F: 5′-CTCATCCACATGAACTTGTGG p65-R: 5′-GCTTCTTCACACACTGGATC mS16-F: 5′-CTTGGAGGCTTCATCCACAT; mS16-R: 5′-ATATTCGGGTCCGTGTGAAG. In addition, 7×10^6 marrow mononuclear cells were subjected to ChIP using C/EBPα or p65 antisera or rabbit Ig followed by real time PCR for the *Bcl-2* P2 promoter, as described (8), or for Bcl-2 intron 5 as a control, using the following primers:

P2-F: CATTGGTACCTGCAGCTTC; P2-R: 5′-CTGTGACAGCTTATAATGTATG; Intron 5-F: 5′-CCTAGACCTGAATTCAGT; Intron 5-R: 5′-CTGAGGGTTCCCTCTGAAG.

Results

C/EBPα and C/EBPβ have increased affinity for NF-κB p50 compared with p65

To enable direct comparison of C/EBP affinity for NF-κB p50 versus p65 we used p50 and p65 variants containing the FLAG epitope at their N- or C-termini, as diagrammed (Fig. 1A, top panel). Use of the FLAG epitope allows uniform immunoprecipitation (IP) and direct control of p50 and p65 inputs into co-IP reactions. Use of the FLAG antibody for IP also minimizes antibody contact with residues in p50 or p65 that may interact with C/EBP proteins, and interference by the antibody is further alleviated if consistent results are obtained with use of both N- and C-terminally tagged p50 in comparison with similarly tagged p65. CMV expression vectors, adjusted based on preliminary experiments to allow similar levels of NF-κB protein expression, were transiently transfected into separate dishes of 293T cells, and expression of N-p50, N-p65, C-p50, and C-p65, representing N- or C-terminally FLAG-tagged p50 or p65, were compared by Western blotting using the FLAG antibody (Fig. 1A, bottom panel). N-p50 was expressed at a similar level as N-p65, while C-p50 was expressed at approximately a 2-fold lower level than C-p65 in these extracts.

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Adjustment in NF-κB lysate volumes were then made so that approximately matched amounts of these NF-κB isoforms were mixed with lysate containing exogenously expressed C/EBPα, and IP was carried out with FLAG antibody followed by Western blotting for C/EBPα (Fig. 1B, top panel). While both N-p50 and C-p50 readily interacted with C/EBPα, with signal in the antibody (Ab) lane comparable to 1% input, no interaction with p65 was evident on this exposure, though weak interaction was evident on longer exposures (not shown). In addition, a reciprocal IP was conducted with the same protein mixtures, with IP conducted with C/EBPα antiserum followed by Western blotting with FLAG antibody (Fig. 1B, bottom panel). In the input lanes, N-p50 was expressed at a mildly higher level than N-p65, but IP of C/EBPα as a percentage of input was much higher for N-p50. C-p50 was expressed at a lower level than C-p65 in the input lanes, and yet in both absolute terms and as a percentage of input, IP of C/EBPα was much higher with C-p50. Of note, we detect strong co-IP between p50 and N-p65 expressed under similar conditions (9). Thus, as assessed using four different reaction conditions, namely use of N- or C-terminally tagged NF-κB subunits and use of FLAG or C/EBPα antibody for IP, affinity of C/EBPα for NF-κB p50 was much higher than for p65.

We found that C/EBPα harboring clustered mutations in its BR has greatly reduced affinity for p50 (7), and the C/EBPα and C/EBPβ BRs are highly conserved (29/35 residues identical). We therefore also compared the affinity of C/EBPβ for N- or C-terminally FLAG-tagged p50 or p65. C/EBPβ is expressed in three alternate translational forms termed LAP1, LAP2, and LIP, 296, 275, and 145 residues respectively, each of which retain the C-terminal bZIP domain. IP with FLAG antibody followed by Western blotting for C/EBPβ demonstrates that each C/EBPβ isoform has much increased affinity for N-p50 or C-p50 compared with N-p65 or C-p65 (Fig. 1C, upper panels). Electrophoresis on a 12% rather than a 10% acrylamide gel and for a longer time period better resolved the closely migrating LAP1 and LAP2 isoforms and confirmed that these as well as the LIP isoform of C/EBPβ bind N-p50 with substantially higher affinity than N-p65 (Suppl. Fig. 1A). Also, reciprocal IP with C/EBPβ antiserum followed by Western blotting using FLAG antibody provided additional results consistent with the conclusion that C/EBPβ, like C/EBPα, has much higher affinity for NF-κB p50 compared with NF-κB p65 (Fig. 1C, lower panels).

Although it is unlikely that both an N- and C-terminal FLAG epitope would directly interfere with C/EBP binding or indirectly disrupt the structure of p65, we carried out gel shift reactions using untagged p65, N-p65, or C-p65 and a radio-labeled consensus κB site (Fig. 2A, lanes 1, 2, 7, and 8) as well as untagged p50, N-p50, or C-p50 (Fig. 2A, lanes 4, 5, 9, and 10). Although there was some degradation of C-p65, the FLAG tag did not prevent proper folding, dimerization, and DNA-binding when placed at the N- or C-terminus of p65 or p50. In addition, untagged p65, N-p65, and C-p65 were compared for their ability to activate a reporter containing five consensus κB sites linked to a minimal promoter and the luciferase reporter, with CMV-βGal serving as an internal control plasmid. Relative to p50 plus empty CMV vector, addition of untagged p65, N-p65, or C-p65 increased reporter activity similarly, by 16- to 24-fold (Fig. 2B). Together these data indicate that addition of the FLAG epitope to the N- or the C-terminus of p65 did not alter p65 protein folding to enable dimerization, DNA-binding, and trans-activation.

C/EBPα AML oncoproteins have increased affinity for NF-κB p50 compared with p65

We also compared the affinity of NF-κB p50 versus p65 for the two categories of C/EBPα AML oncoproteins. First, the N-terminally truncated C/EBPα p30 protein, lacking residues 1–117 of the 359 residue C/EBPα, was mixed with N-p50, N-p65, C-p50, or C-p65 and subjected to IP with FLAG antibody followed by Western blotting for C/EBPα p30 (Fig. 3A). Regardless of whether the FLAG epitope was C- or N-terminal, C/EBPα p30 demonstrated higher affinity for NF-κB p50 compared with p65. Second, we used a similar

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approach to compare the affinity of four different, patient-derived C/EBPαLZ oncoproteins for p50 versus p65 (Fig. 3B and 3C). These C/EBPα variants, designated 3901, 3820, J3, and K6, had previously been evaluated for their ability to induce bcl-2 protein expression and to inhibit apoptosis in Ba/F3 cells, with K6 showing much less activity in these assays compared to the other three oncoproteins (7). Correlating with these prior functional findings, 3901, 3820, and J3 each bound p50 with significantly higher affinity that p65, whereas the K6 C/EBPαLZ oncoprotein had much reduced affinity for NF-κB p50 in comparison to the other three C/EBPαLZ oncoproteins.

Removal of the p65 TAD does not increase its interaction with C/EBPα or C/EBPβ

Increased affinity of C/EBPα for NF-κB p50 compared with p65 may result either from amino acid differences between their respective N-terminal Rel homology domains (RHDs) or from interference by the C-terminal p65 TAD. To evaluate these two possibilities, we generated a truncation mutant of N-p65 designated N-p65Δ297 lacking its 254 residue TAD but retaining is entire RHD as well as 17 more N-terminal residues. For comparison, we developed an analogous truncation mutant of p50, N-p50Δ356, lacking 77 C-terminal residues. A diagram of these truncated proteins and their wild-type, N-terminally FLAG-tagged counterparts is shown (Fig. 4A, top). Both N-p65Δ297 and N-p50Δ356 bound a consensus κB site in gel shift analysis using nuclear extracts from 293T cells expressing these proteins (Fig. 2A, lanes 3 and 6). These four proteins were expressed in 293T cells using equivalent amount of CMV expression vectors, and total cellular extracts were prepared for co-IP with C/EBPα. Western blot analysis of lysates expressing the NF-κB proteins indicated that N-p65Δ297 was expressed 2- to 3-fold higher than p65, whereas N-p50Δ356 was expressed approximately 2-fold lower level than p50 (Fig. 4A, bottom). Co-IP was then conducted, adjusting extract volumes to approximately equalize NF-κB input and using C/EBPα or C/EBPβ extracts isolated from separate dishes of 293T cells to ensure equal C/EBP input to each co-IP (Figs. 4B and 4C). Removal of the p65 TAD did not increase interaction of p65 with C/EBPα or C/EBPβ, whereas truncation of p50 to the homologous location did not significantly alter its interaction with either C/EBP protein. These data suggest that the p65 TAD does not sterically hinder contact of C/EBPα with the p65 N-terminal region but that amino acid differences between the N-terminal segments of p50 and p65, mainly consisting of their RHDs, account for reduced affinity of p65 for C/EBP proteins.

Identification of a cluster of residues unique to NF-κB p50 required for C/EBPα binding

The p50 and p65 RHDs are highly homologous and can be almost entirely co-aligned with the exception that the p50 RHD contains a 30 amino acid segment referred to as the “p50 insert”, mainly comprising a loop between two α-helices, α1 and α2 (Fig. 5A). As a first step towards localizing p50 residues that contact C/EBPα, we sought to determine whether the “p50 insert” contributes to C/EBPα:p50 interaction using three deletion and two clustered point mutant variants, as diagramed. The deletion variants proved unstable and could not be further evaluated. Mutant 1, with HSDL (residues 170–173) changed to AAAA, interacted much more weakly than wild-type p50 with C/EBPα in a co-IP reaction despite its expression at levels exceeding p50, whereas mutant 2 retained nearly full affinity for C/EBPα (Fig. 5B). Mutants 1 and 2 each bound a radio-labelled κB site with affinity similar to wild-type p50 (Fig. 5C), indicating that these p50 variants fold properly, allowing them to homodimerize and bind DNA. These data indicate that residues HSDL within the loop region of the “p50 insert” and absent in NF-κB p65 contribute to interaction of NF-κB p50 with C/EBPα.

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Identification of key residues in the C/EBPα basic region required for NF-κB p50 binding

The C/EBPα bZIP domain is sufficient for interaction with p50 (8, 15). We compared full-length, 359 residue C/EBPα and its 89 residue, C-terminal bZIP domain (αbZIP) for strength of interaction with N-p50 by co-IP followed by Western blotting using a C-terminal C/EBPα antiserum (Fig. 6A). Relative to input samples, interaction of p50 with αbZIP was not weaker but in fact stronger than with the full-length protein, consistent with the idea that all relevant protein contacts are made with the bZIP domain. In addition, we compared the affinity of full-length C/EBPα or αbZIP for N-p65 (Suppl. Fig. 1B). These data demonstrate that deletion of residues 1–269 of C/EBPα did not increase p65 affinity, arguing against steric hindrance due to contact between the p65 TAD and this N-terminal region of C/EBPα. An 88 residue, C-terminal bZIP domain derived from C/EBPβ also bound N-p50 with increased affinity compared with N-p65 (Suppl. Fig. 1C).

The C/EBPα LZ domain is not essential for binding to p50 as swapping the GCN4 LZ for the C/EBPα LZ in the context of full-length protein only mildly reduces C/EBPα:p50 interaction (8). In addition, as shown herein, inserting 8 or 5 residues between the BR and LZ protein domains, as occurs in C/EBPαLZ oncoproteins 3820 or J3, does not substantially alter interaction with p50. This is particularly noteworthy as the bZIP domain is a continuous α-helix with a 7 amino acid helical repeat, and so insertion of 8 or 5 residues would not only distance the BR from the LZ, but would also rotate them relative to each other. Mutant 3901 also retains p50 binding despite deletion of 1 residue between the BR and LZ. As a step towards designing a small molecule that might interfere with C/EBPα:p50 interaction, we therefore sought to further define the residues within the C/EBPα BR that contact p50. The amino acid sequence of the BR, a diagram of 18 mutant BR variants evaluated, and a summary of the effect of these alterations on interaction with p50, as assessed by co-IP, and on DNA-binding, as measured by electrophoretic mobility shift assay using a radio-labelled C/EBP-binding site, is shown (Fig. 6B). With the exception of the C/EBPα(BR3) and C/EBPα(R300G) variants, all indicated residues in each mutant were changed to alanine. The *abcdefg* position of each residue in the α-helix is shown, as is a wheel diagram of the α-helix. Mutants A (*deg*), B (*be*), C (*gad*), and D (*abe*) were designed so that the altered residues would be adjacent on the cylindrical surface of the α-helix. Mutant E (*degbe*) is a combination of mutants A and B, mutant F (*deggad*) is a combination of mutants A and C, and mutant L (*begad*) is a combination of mutants B and C. Representative co-IP and DNA-binding data are shown for wild-type C/EBPα and for each mutant variant (Fig. 6C and 6D and Suppl. Fig. 2).

C/EBPα(BR3) contains four altered amino acids and was previously found not to induce bcl-2expression or bind p50; and in that same study individual mutation of each of these four residues uncovered R300G as the alteration critical for bcl-2 induction and prolonged Ba/F3 survival upon IL-3 withdrawal (7). Consistent with these functional data, we now find that R300G does not bind p50. As glycine disrupts α-helical structure, the R300G mutation might have disrupted distant folding. We therefore also generated C/EBPα(R300A) and find that as with R300G binding of p50 to this variant is lost. Saturating mutagenesis of the remainder of the BR identified a six amino acid cluster, RERNNI, common to mutant G and mutant H, required for interaction with p50. Mutant H was divided into mutants H1 (*dea*) and H2 (*fgb*), each containing non-overlapping, triple alanine mutations derived from RERNNI chosen to be on the same surface of the α-helix. The H1 and H2 variants each had reduced affinity for p50, in comparison with wild-type C/EBPα. Finally, although mutants A, B, C, or L (B+C) bound p50 well, the combination of A with B (mutant E) or to an even greater extent A with C (mutant F) led to a significant, reproducible reduction in p50 interaction.

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None of the C/EBPα variants with significantly reduced affinity for p50 (R300G, R300A, or mutants E, F, G, H, H1, or H2) retained DNA-binding activity for a consensus C/EBP site, suggesting that the same surface of the BR α-helix that contacts DNA also binds p50. In the nuclear extracts used for gel shift analysis, mutants A, E, H, and H1 were expressed at somewhat lower levels than the other variants as assessed by Western blot analysis; nevertheless, no evidence of DNA-binding by these variants was evident even upon prolonged exposure of the gel shift autoradiographs (not shown).

Deletion of the gene encoding p65 does not reduce expression of C/EBPα or bcl-2

Mice lacking both copies of the *Nfkb1* gene encoding NF-κB p50 are viable, and their marrow mononuclear cells have 3-fold reduction in C/EBPα protein and RNA levels, reduced binding of p50 and C/EBPα to the *Bcl-2* promoter, and 4-fold reduced *Bcl-2* RNA expression (8, 16). Of note, mutation of a κB site located at -170 bp in the *Bcl-2* P2 promoter reduces trans-activation by C/EBPα or by the 3901 C/EBPαLZ variant that cannot directly bind DNA (8). Homozygous deletion of the *RelA/p65* gene encoding NF-κB p65 is embryonic lethal (17). We bred mice containing loxP sites surrounding exons 5–8 of *RelA/p65*, encoding the majority of the RHD (14), with Mx1-Cre transgenic mice to generate p65(f/f);Mx1-Cre mice or control p65(f/f) littermates. Intraperitoneal injection of these mice with pIpC to induce Cre recombinase expression led to efficient loss of p65 protein and RNA from bone marrow mononuclear cells without reduction in C/EBPα protein or RNA or reduction in *Bcl-2* RNA in unstimulated cells (Fig. 7A and 7B). In addition, homozygous deletion of the *RelA/p65* gene markedly reduced binding of p65 to the *Bcl-2* P2 promoter without affecting C/EBPα binding, as assessed by ChIP (Fig. 7C). Providing a negative control, no binding of these factors was seen within intron 5 of *Bcl-2*. These data and our prior findings are consistent with the idea that p50 but not p65 is required for regulation of *Cebpa* gene transcription and that interaction of C/EBPα with chromatin-bound p50 but not p65 is required for activation of the gene encoding bcl-2.

Discussion

NF-κB is a central mediator of the inflammatory response, both within the tumor microenvironment and in tumor cells, and induces expression of growth factors and angiogenic factors that contribute to cancer induction and progression. In addition, NF-κB activates multiple anti-apoptotic genes that may contribute further to malignant progression. Activation of NF-κB is common in acute myeloid leukemia (18), several lymphoma subsets (19–21), breast cancer (22, 23); colorectal cancer (24); prostate cancer (25, 26), ovarian cancer (27), malignant gliomas (28), and additional malignancies. Antagonism of receptors such as Her2 that mediate NF-κB activation or direct targeting of cytoplasmic IKKα or IKKβ offer means to inhibit NF-κB activation in cancer (29, 30). As an additional, potentially synergistic, therapeutic approach we envision inhibition of NF-κB in the nucleus. In particular, we have defined an alternative pathway for induction of NF-κB target genes in which C/EBP proteins displace HDACs and potentially other co-repressors from NF-κB p50:p50 homodimers (7–9). In addition, interaction of C/EBPs with the p50:p65 complex may further induce NF-κB target genes. As p50 has much lower affinity for IκB than p65, p50:p50 homodimers exist in the nucleus of cells that lack canonical NF-κB activation (3). Induction or activation of C/EBP proteins, as may occur during malignant progression, may enable their interaction with p50:p50 homodimers or p50:p65 heterodimers on chromatin. For example, N- or C-terminal C/EBPα mutants found in a subset of acute myeloid leukemia cases cannot bind DNA and so are defective in differentiation induction, but retain the ability to induce NF-κB target genes via interaction with p50 (7–9). In addition, C/EBPβ is induced by NPM-ALK in anaplastic large cell lymphomas (31), directly activates bcl-2 transcription in multiple myeloma (32), and transforms normal mammary epithelium (33),

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and C/EBPα or C/EBPβ are induced or activated by TNF-α, AKT, or MAP kinases in hepatocellular, prostate, or breast carcinoma cells, where they potentially cooperate with NF-κB to inhibit apoptosis (34–37).

We previously found that NF-κB p50 but not p65 cooperates with C/EBPα to induce *Bcl-2* gene transcription to inhibit apoptosis and that C/EBPα activates the promoter of the *Nfkb1/p50* gene in cooperation with p50 but does not induce *RelA/p65* RNA expression (7, 9). The present study demonstrates that C/EBPα, C/EBPαp30 or C/EBPαLZ oncoproteins, or the LAP1, LAP2, or LIP isoforms of C/EBPβ bind NF-κB p50 with substantially greater affinity than NF-κB p65, thereby further focusing translational efforts on inhibition of the C/EBP:p50 rather than the C/EBP:p65 interaction. Use of FLAG-tagged p50 or p65 allowed their inputs to co-IP reactions to be monitored and approximately equalized, and expression of both N- and C-terminally tagged p50 or p65 proteins allowed us to control for potential interference by the FLAG epitope.

Underscoring the idea that C/EBPα interaction with p50 is critical for its anti-apoptotic effect, C/EBPα(K6), the one C/EBPαLZ oncoprotein of four studied herein that bound p50 poorly, is the only one that does not induce bcl-2 or substantially inhibit apoptosis in Ba/F3 cells withdrawn from IL-3 (7). The present study further emphasizes the biologic importance of C/EBP:p50 as compared to the C/EBP:p65 interaction by demonstrating that homozygous deletion of the *RelA/p65* gene in adult mice does not reduce bcl-2 or C/EBPα expression in bone marrow mononuclear cells or prevent endogenous C/EBPα interaction with the *Bcl-2* promoter, in striking contrast to the effect of *Nfkb1/p50* gene deletion (8, 16). Also, C/EBPα stimulates *Nfkb1/p50* but not *RelA/p65* gene transcription, p50 binds and activates the *Cebpa* promoter, and mice lacking p50 have substantially reduced marrow C/EBPα protein and RNA levels (16), establishing a positive feedback loop mediating expression of these two proteins.

Elevated affinity of C/EBPα or C/EBPβ for p50 might have reflected interference by the p65 TAD. However, deletion of this C-terminal domain did not increase affinity of either C/EBP for p65. We therefore conclude that differences in the N-terminal domains of p50 and p65, which largely comprise their RHDs, accounts for stronger interaction of p50 with C/EBPs. Of note, the RHD is sufficient for interaction with the bZIP domain of C/EBPα or CEBPβ (15). Although approximately 1/3 of the residues in the p50 and p65 RHDs are identical when aligned, and while there are additional conservative matches, many residues differ substantially. Of note, the p50 C-terminal truncation variant studied herein, N-p50Δ356, is 59 residues longer than N-p65Δ297, which was deleted to a homologous position, in part because the p50 RHD contains 30 consecutive internal residues known as the “p50 insert” lacking in the p65 RHD. p50 also has 21 residues N-terminal to the RHD lacking in p65 and several additional internal residues. We find that mutation of four residues, HSDL, within the loop region of the “p50 insert” strongly weakens interaction with C/EBPα. Future studies will focus on further delineating the p50 residues that mediate C/EBP:p50 interaction utilizing both genetic and structural approaches.

In addition, we have further localized residues in C/EBPα that contact p50. First, we demonstrate that the C/EBPα bZIP domain has similar or perhaps greater affinity for p50 than does full-length C/EBPα, consistent with earlier work showing that introduction of four clustered point mutations in the BR of C/EBPα, the BR3 mutant, prevents p50 binding (7). When these four residues are mutated individually, only R300G reduces bcl-2 induction and protection from apoptosis upon IL-3 withdrawal (7). Correlating with these findings, we now show that R300G does not bind p50 and that R300A, avoiding disruption of the α-helix by glycine, also does not bind p50. The bZIP domain consists of the BR and adjacent LZ. Two lines of evidence suggest that the LZ does not make significant contacts contributing to

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affinity of C/EBPα for p50. First, swapping the yeast GCN4 LZ only mildly reduces C/EBPα:p50 interaction (8). Second, the 3820 and J3 C/EBPα oncoproteins contain insertions of 8 or 5 residues between the BR and LZ, thereby distancing these domains, and yet retain strong affinity for p50. We therefore conducted scanning alanine mutagenesis of the C/EBPα BR in the context of full-length C/EBPα. In addition to R300, we found that residues 289–294, RERNNI, contribute to p50 binding and that residues surrounding R300 also participate in C/EBPα interaction with p50. Residues VRS of mutant A are particularly implicated by the finding that mutant combinations A+B or A+C, but not B+C, reduced p50 interaction. These data will assist interpretation of future protein biochemical studies, such as NMR or co-crystallization, designed to identify C/EBPα:p50 contacts.

Of note, RERNNI, R300, and residues surrounding R300 are identical in the BRs of C/EBPβ and C/EBPδ. C/EBPα BR residues that contact DNA in the reported co-crystal structure (38) are indicated in Fig. 6B, where B denotes contact with bases in the major groove and P denotes contact with the phosphate backbone. The C/EBPα BR α-helix contacts specific DNA bases via residues on its *cgda* surface and also contacts phosphate residues via positions *e* and *f*. Mutants BR3, R300G, R300A, A, E, F, G, H, H1, and H2 each alter residues that contact DNA bases, thereby preventing DNA-binding. C/EBPα BR variants that disrupt p50 binding also prevent C/EBPα DNA-binding, suggesting that interaction of wild-type C/EBP protein with p50 may interfere with C/EBP DNA-binding. However, it remains possible that residues such as R300 can contact DNA and p50 simultaneously as we previously found that neither a κB nor a C/EBP DNA-binding site prevents C/EBPα:p50 co-IP, although this experiment may be complicated by rapid DNA on/off kinetics (8). For a subset of genes, C/EBPs may bind DNA and then interact with nearby, DNA-bound p50 to synergistically stimulate gene activation, but C/EBPs may more commonly tether to DNA via p50:p50 homodimers or p50:p65 heterodimers to mediate cooperative activation of NF-κB target genes. In regulating *Nfkb1* promoter activity C/EBPα or CEBPα oncoproteins tether to p50 bound to either of two κB sites to activate transcription without themselves directly binding DNA (9), and there may be additional genes activated by C/EBPs through a similar mechanism. On the other hand, activation of the C-reactive protein (CRP) gene promoter involves synergistic C/EBPβ and p50 DNA-binding, potentially mediated by direct protein interaction, to overlapping κB and C/EBP DNA elements (39). Identifying genes whose expression is mediated by C/EBP:p50 interaction and demonstrating their relevance to human malignant transformation would further validate the utility of targeting the C/EBP:p50 interaction and is an additional subject of our ongoing investigations.

In summary, we find that C/EBP proteins have substantially higher affinity for NF-κB p50 compared with p65, and that p65 gene deletion does not reduce marrow C/EBPα protein or RNA or *Bcl-2* RNA expression in contrast to prior findings with p50 gene deletion, providing further support for our proposal that interaction of C/EBPs with p50:p50 or p50:p65 NF-κB complexes provides an alternative means to activate NF-κB target genes, even in cells lacking canonical NF-κB activation. In addition, these findings focus translational efforts on targeting the C/EBP:p50 interaction, and data herein contribute to such efforts by localizing interacting residues within the C/EBPα basic region and p50 and by demonstrating that differences between the p50 and p65 N-terminal regions rather than the presence of the p65 TAD contributes to their substantially different affinities for C/EBP proteins.

Supplementary Material

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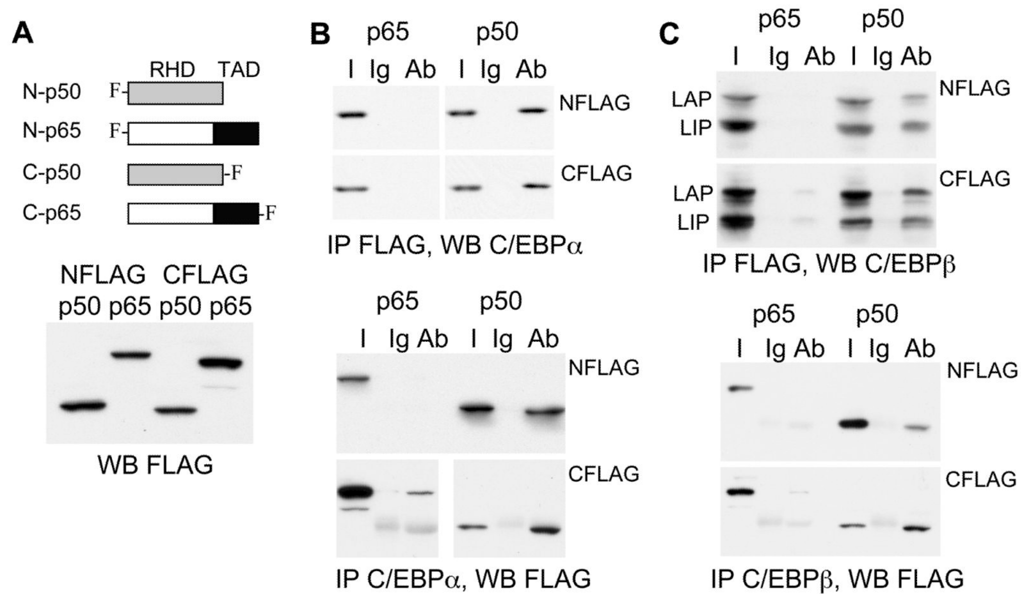

FIGURE 1.

NF-κB p50 has higher affinity than p65 for C/EBPα or C/EBPβ. A, Diagram of Nor C-terminal FLAG-tagged p50 or p65 (top). RHD – Rel homology domain, TAD – trans-activation domain. Total cellular proteins from 293T cells transfected with 1–5 μg of CMV vectors expressing these four proteins were subjected to Western blotting using FLAG antibody (bottom). DNA amounts were chosen to allow expression of similar amounts of each NF-κB protein. B, 293T extracts expressing similar levels of N-terminally FLAG tagged (NFLAG) p50 or p65 or C-terminally tagged (CFLAG) p50 or p65 were combined with equal volumes of C/EBPα expressed in a separate lysate and subjected to immunoprecipitation (IP) with FLAG antibody or mouse Ig control followed by Western blotting (WB) for C/EBPα (top panels) or to IP using C/EBPα antiserum or rabbit Ig followed by WB with FLAG antibody (bottom panels). I – 1% input, Ig – control IP, Ab – antibody IP. C, Experiments were conducted as in part B, using extracts expressing C/EBPβ rather than C/EBPα. Positions of the long LAP1 and LAP2 (LAP) and short (LIP) C/EBPβ isoforms resulting from alternative translation initiation sites are shown.

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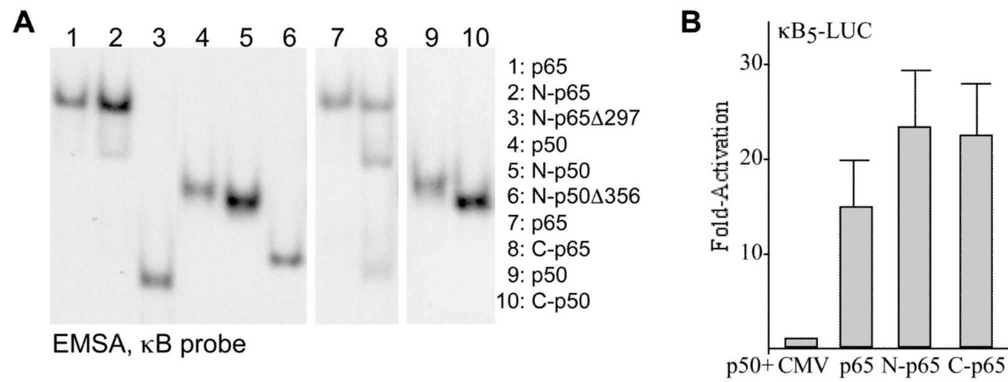

FIGURE 2.

DNA-binding and trans-activation by modified p50 and p65 proteins. A, 12 μg of 293T nuclear extracts expressing the indicated p50 or p65 proteins after transient transfection were subjected to electrophoretic mobility shift assay (EMSA) with 1 ng of a radio-labelled oligonucleotide containing a consensus κB site. B, 293T cells in 60 mm dishes were transiently transfected with 500 ng $(\kappa B)_5$-LUC, 1 ng CMV-βGal, 10 ng CMV-p50, and 100 ng of empty CMV vector or CMV vectors encoding p65, N-p65 or C-p65. Luciferase and β-galactosidase activities were determined two days later. Shown are mean and S.E. of fold-activation from three determinations by p65, N-p65, or C-p65 relative to empty CMV vector, the activity of which was set to 1.0 in each experiment.

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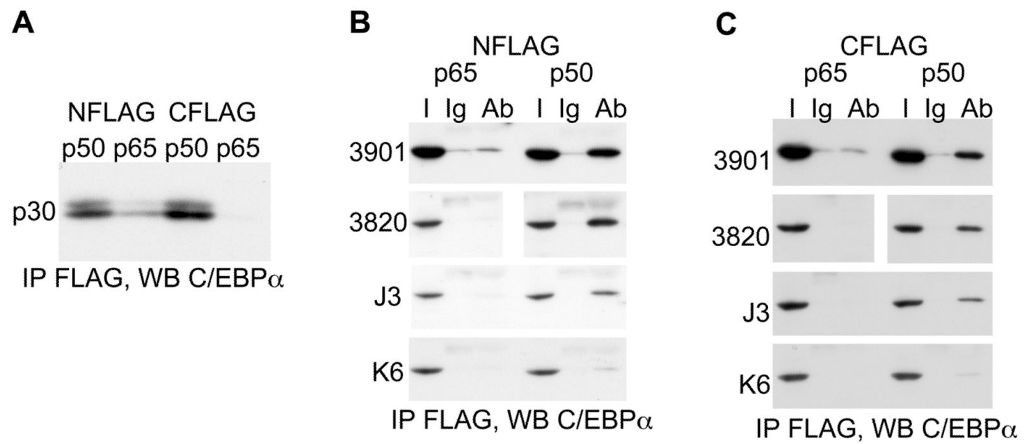

FIGURE 3.

NF-κB p50 has higher affinity than p65 for C/EBPαp30 or C/EBPαLZ myeloid oncoproteins. A, 293T extracts expressing matched levels of N-terminally FLAG tagged (NFLAG) p50 or p65 or C-terminally tagged (CFLAG) p50 or p65 were combined with equal volumes of a separate extract expressing C/EBPαp30 and subjected to immunoprecipitation with FLAG antibody or Ig control followed by Western blotting for C/EBPα. B, Extracts expressing matched levels of NFLAG p50 or p65 were combined with equal volumes of extracts expressing one of 4 patient-derived C/EBPαLZ variants (3901, 3820, J3, or K6) followed by IP with FLAG antibody or Ig control and WB for C/EBPα. C, Similar analysis was conducted using CFLAG p50 or p65.

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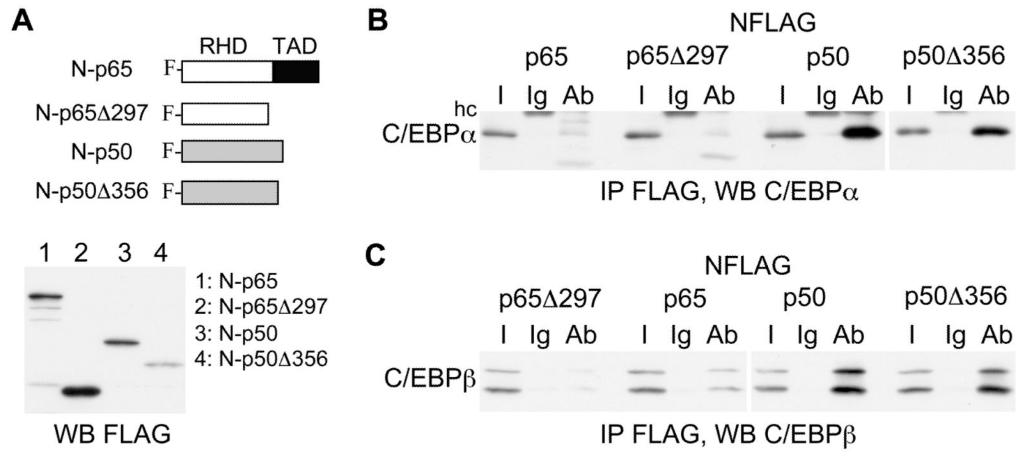

FIGURE 4.

Deletion of the p65 TAD does not increase its affinity for C/EBPα or C/EBPβ. A, Diagram of N-terminally FLAG tagged p65, p65Δ297, p50, and p50Δ356 (top). Total cellular proteins from 293T cells transfected with CMV vectors expressing these four proteins were subjected to Western blotting using FLAG antibody (bottom). B, Extracts expressing matched levels of these proteins were combined with equal volumes of an extract expressing C/EBPα, followed by IP with FLAG antibody or Ig and Western blotting for C/EBPα. The locations of C/EBPα and of non-specific heavy chain cross-reactivity are shown (hc). C, Similar analysis was conducted using C/EBPβ, the upper band representing the LAP isoforms and the lower band LIP. Both panels in B or C are from the same autoradiograph exposure.

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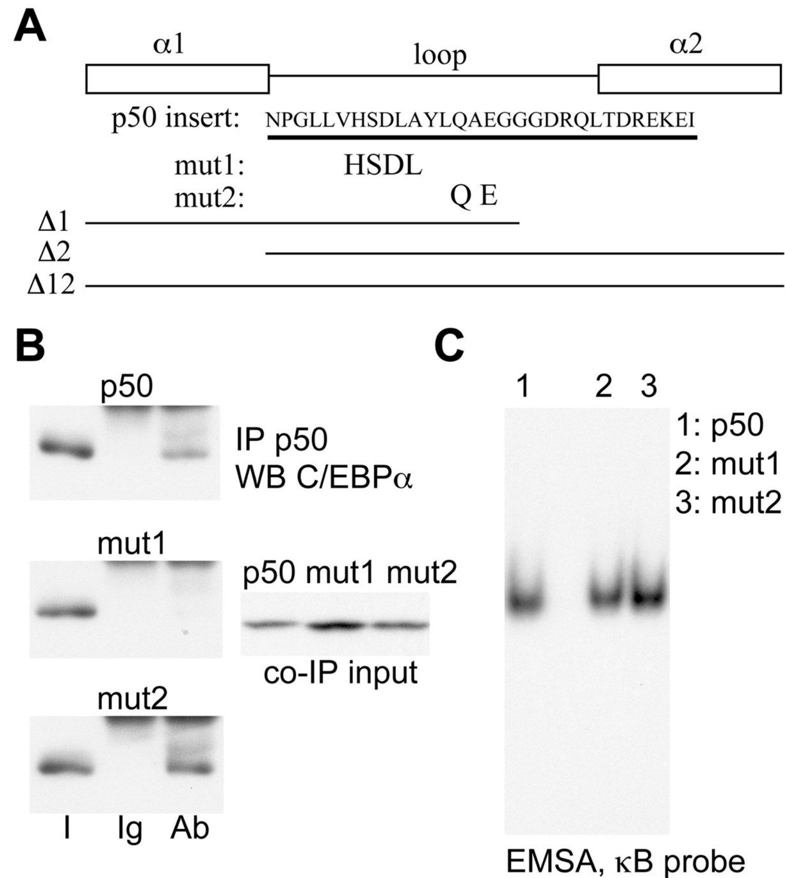

FIGURE 5.

A cluster of residues in the "p50 insert" is required for interaction with C/EBPα. A, Diagram of NF-κB p50 amino acids 147–202, including α-helices α1 and α2 and the intervening loop. Residues corresponding to the "p50 insert" present in NF-κB p50 but not p65 are shown as are the locations of two clustered point mutants (mut1 and mut2), in which indicated residues were changed to alanine, and three deletion mutants (Δ1, Δ2, and Δ12), in which the bars indicate deleted segments. Δ1 lacks residues 147–180, Δ2 residues 162–203, and Δ12 residues 147–203. B, Wild-type p50 or its mut1 or mut2 variants were subjected IP with p50 antiserum followed by Western blotting (WB) with C/EBPα antiserum (left panels). Also shown are the relative expression of p50, mut1, and mut2 in the input lysates (right panel). The three deletion variants failed to express in 293T cells and could not be analyzed similarly. C, NF-κB p50 and its mut1 or mut2 variants were assessed for homodimerization and DNA binding to a κB site using the electrophoretic mobility shift assay (EMSA).

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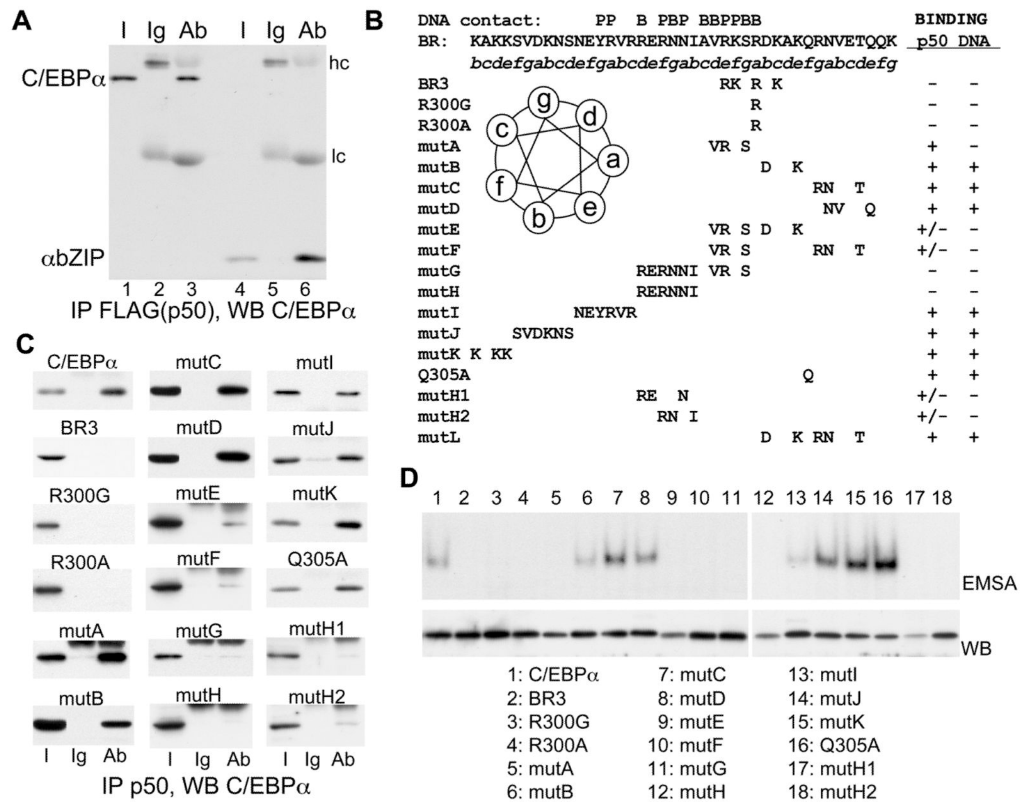

FIGURE 6.

Saturating C/EBPα BR mutagenesis to identify residues required for interaction with p50. A, 293T extracts expressing C/EBPα (lanes 1–3) or the bZIP domain of C/EBPα (αbZIP, lanes 4–6) were incubated with equal volumes of an extract expressing N-p50, followed by immunoprecipitation using FLAG antibody or Ig control and Western blotting using an antiserum recognizing the C-terminus of C/EBPα. B, Sequence of the C/EBPα BR, diagram of BR mutants analyzed for interaction with p50 or DNA, and summary of the experimental findings. In each variant, indicated residues were changed to alanine. "B" denotes residues contacting DNA bases and "P" denotes residues contacting DNA phosphates in the co-crystal structure. *abcdefg* indicates the position of each residue in the BR α-helical structure, a diagram of which is shown as an insert. C, 293T extract expressing NF-κB p50 was combined with extracts expressing C/EBPα or the indicated variants, followed by IP with p50 antiserum and subsequent Western blotting for C/EBPα. D, 12 μg of 293T nuclear extracts expressing C/EBPα or the indicated variants were subjected to EMSA using 1 ng of a radio-labelled oligonucleotide containing a C/EBP-binding site (top). The nuclear extracts were subjected to Western blotting (WB) using C/EBPα antiserum (bottom).

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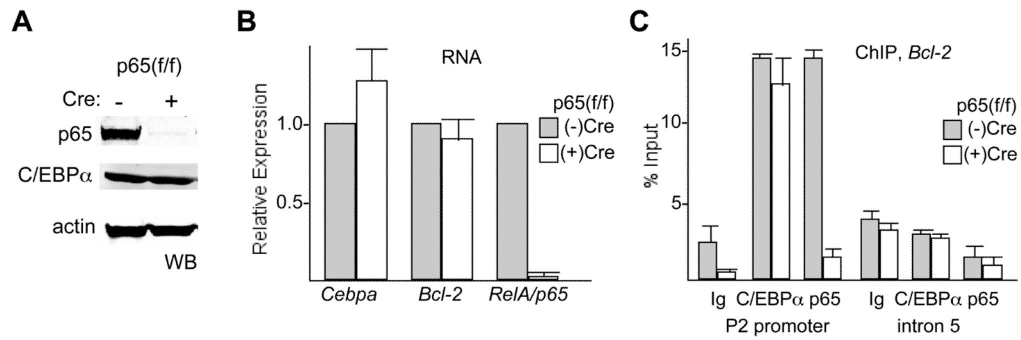

FIGURE 7.

Homozygous deletion of the *RelA/p65* gene does not affect C/EBPα or bcl-2 expression in marrow cells. A, Total cellular proteins isolated from the marrow of p65(f/f) or p65(f/f);Mx1-Cre mice exposed to pIpC to delete the floxed alleles were subjected to Western blotting for p65, C/EBPα, or β-actin. B, Total cellular RNA isolated from these cells was subjected to real time RT-PCR analysis for *Cebpa*, *Bcl-2*, or *RelA/p65* RNAs. The level of expression of each RNA isolated from mice lacking Mx1-Cre was set to 1.0, and relative expression of each RNA from p65(f/f);Mx1-Cre mice are shown as the mean and S.E. from three separate mice in each group. C, Marrow cells from p65(f/f) or p65(f/f):Mx1-Cre mice exposed to pIpC were subjected to ChIP using C/EBPα or p65 antiserum or rabbit Ig, followed by PCR analysis for binding to the *Bcl-2* P2 promoter or intron 5. Percent input is shown for each condition as the mean and S.E. from three separate ChIP assays.