CCAAT/Enhancer Binding Protein \( \alpha \) (C/EBP\( \alpha \)) and C/EBP\( \alpha \) Myeloid Oncoproteins Induce Bcl-2 via Interaction of Their Basic Regions with Nuclear Factor-\( \kappa \)B p50

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Abstract
The CEBPA gene is mutated in 10% of acute myeloid leukemia (AML) cases. We find that CEBPA and Bcl-2 RNA levels correlate highly in low-risk human AMLs, suggesting that inhibition of apoptosis via induction of bcl-2 by CCAAT/enhancer binding protein \( \alpha \) (C/EBP\( \alpha \)) or its mutant variants contributes to transformation. C/EBP\( \alpha \)p30, lacking a NH2-terminal transactivation domain, or C/EBP\( \alpha \)LZ, carrying in-frame mutations in the leucine zipper that prevent DNA binding, induced bcl-2 in hematopoietic cell lines, and C/EBP\( \alpha \) induced bcl-2 in normal murine myeloid progenitors and in the splenocytes of H2K-C/EBP\( \alpha \)-Eμ transgenic mice. C/EBP\( \kappa \) protected Ba/F3 cells from apoptosis on interleukin-3 withdrawal but not if bcl-2 was knocked down. Remarkably, C/EBP\( \kappa \)LZ oncoproteins activated the bcl-2 P2 promoter despite lack of DNA binding, and C/EBP\( \kappa \)p30 also activated the promoter. C/EBP\( \alpha \) and the C/EBP\( \alpha \) oncoproteins cooperated with nuclear factor-\( \kappa \)B (NF-\( \kappa \)B) p50, but not p65, to induce bcl-2 transcription. Endogenous C/EBP\( \alpha \) preferentially coimmunoprecipitated with p50 versus p65 in myeloid cell extracts. Mutation of residues 287 to 302 in the C/EBP\( \alpha \) basic region prevented induction of endogenous bcl-2 or the bcl-2 promoter and interaction with p50 but not p65. These findings suggest that C/EBP\( \alpha \) or its mutant variants tether to a subset of NF-\( \kappa \)B target genes, including Bcl-2, via p50 to facilitate gene activation and offer an explanation for preferential in-frame rather than out-of-frame mutation of the leucine zipper with sparing of the basic region in C/EBP\( \kappa \)LZ oncoproteins. Targeting interaction between C/EBP\( \kappa \) basic region and NF-\( \kappa \)B p50 may contribute to the therapy of AML and other malignancies expressing C/EBPs. (Mol Cancer Res 2005;3(10):585–96)

Introduction
CCAAT/enhancer binding protein \( \alpha \) (C/EBP\( \alpha \)) binds DNA as an obligate dimer, with dimerization mediated by its COOH-terminal leucine zipper domain and DNA contact mediated by the adjacent basic region (1). Once bound to DNA, C/EBP\( \alpha \) activates transcription via its two NH2-terminal transactivation domains (2). C/EBP\( \alpha \) is expressed in multiple cell types, including adipocytes, hepatocytes, pneumocytes, and smooth muscle cells, and C/EBP\( \beta \) and C/EBP\( \delta \) are even more widely expressed (3-6). Within hematopoiesis, C/EBP\( \alpha \), C/EBP\( \beta \), and C/EBP\( \delta \) are present predominantly in granulocytic and monocytic myeloid cells (7).

One or both copies of the CEBPA gene are mutated in 10% of acute myeloid leukemia (AML) cases (8, 9). The majority are French-American-British M1 or M2, with a predominance of immature granulocytic blasts. In \( \sim 60\% \) of alleles, mutations near the NH2 terminus lead to loss of translation of the full-length 42-kDa isoform and to increased expression of a truncated C/EBPp30 from an internal ATG. C/EBPp30 lacks a transactivation domain and acts as a dominant negative by upbringing with wild-type C/EBP\( \alpha \) and by virtue of its reduced DNA affinity for a subset of C/EBP consensus sites (8, 10). Thirty-five percent of mutant CEBPA alleles harbor in-frame insertions, deletions, or point mutations within or just NH2 terminal to the first \( \alpha \)-helix of the leucine zipper, preventing DNA binding and transactivation (9, 11, 12). We designate these as C/EBP\( \alpha \)LZ oncoproteins. Wild-type C/EBP\( \alpha \) is down-regulated in additional AML cases: AML1-ETO binds and represses the CEBPA promoter, flt3ITD signaling reduces CEBPA mRNA expression, and Bcr-Abl inhibits CEBPA translation (13-15). C/EBP\( \alpha \) contributes to myeloid differentiation by activating lineage-specific genes, such as those encoding neutrophil elastase and the granulocyte colony-stimulating factor receptor (16, 17). In addition, C/EBP\( \alpha \) inhibits cell cycle progression both via transactivation of the p21\( \text{waf}1/cip1 \) promoter and via direct interaction with cyclin-dependent kinase 2/4, E2F1, or the SWI/SNF complex.
(18-22). Reduced expression or activity of C/EBPα may therefore contribute to myeloid transformation by inhibiting differentiation and by stimulating proliferation. Herein, we provide evidence that, in addition, C/EBPα oncoproteins and residual wild-type C/EBPα potentially contribute to leukemogenesis by inhibiting apoptosis.

C/EBPα increases expression of endogenous bcl-2 mRNA and protein in the DHL-4 t(14;18) lymphoma cell line, in which the bcl-2 gene is fused to the immunoglobulin heavy chain promoter. In addition, C/EBPα or nuclear factor-κB (NF-κB) individually bind and transactivate the bcl-2 P2 promoter (23-25), and the basic region-leucine zipper domain of C/EBPβ interacts with the Rel domain of NF-κB proteins (26). We now show that C/EBPα or C/EBPβ myeloid oncoproteins cooperate with NF-κB p50 to induce bcl-2, inhibit apoptosis, and identify residues in the C/EBPα basic region required for interaction with NF-κB p50 but not p65. Of note, NF-κB is constitutively activated in AML stem cells (27). In addition, cooperation of C/EBPα or C/EBPβ with NF-κB induces multiple genes involved in the inflammatory response, a process defective in C/EBPα(−/−) mice (28) and emerging as relevant to transformation of a variety of cell types, as will be discussed. Targeting interaction between the C/EBPα basic region and NF-κB p50 may thus be useful for the therapy of a variety of malignancies expressing C/EBP family members and NF-κB.

Results

C/EBPα and Bcl-2 mRNA Expression Correlates in Low-Risk Human AML

We examined the relationship between CEBPA and Bcl-2 mRNA expression using publicly available microarray data from two independent studies that examined gene expression patterns in AML (29, 30), one using two-channel cDNA arrays to measure expression from 101 AML cases (32 low risk, 47 intermediate risk, 13 high risk, and 9 other; as defined in Materials and Methods) and the second using Affymetrix (Santa Clara, CA) oligonucleotide arrays with suitable data available from 236 patients (59 low, 118 intermediate risk, 26 high risk, and 33 other). Correlation estimates (denoted by r) and their significance within each risk groups in each study are shown (Fig. 1). A highly significant linear correlation was observed between CEBPA and Bcl-2 expression in low-risk patients in either study. The r (95% confidence interval) were 0.50 (0.10-0.73) and 0.42 (0.18-0.61), with P = 0.003 and 0.001, whereas this strong correlation was not seen in intermediate- or high-risk patients. The significance of the observed correlation between CEBPA and Bcl-2 expression is further evident from our finding that, of 1,000 randomly sampled gene pairs, only 3.2% in the first study and 2.7% in the second have r values greater than that observed between CEBPA and Bcl-2. Four patients in the first study and 16 in the second had +8 karyotype, and 17 in the second study had 11q23 karyotypes; these patients were in the “other” risk group, for which no correlation was observed between CEBPA and Bcl-2 expression (data not shown).

When the low-risk patients were further examined by individual karyotypes, CEBPA levels were on average lower in AMLs associated with t(8;21) versus inv(16) (P < 0.05) and levels in inv(16) cases were on average lower than those associated with t(15;17) (P < 0.05). The smaller study showed a similar trend, with the average CEBPA level associated with t(8;21) being lower than with t(15;17) (P < 0.05). This observation is consistent with the prior finding that AMLs with t(8;21) generally express lower levels of bcl-2 compared with other AMLs (31) and thus supports the biological relevance of the correlation between CEBPA and Bcl-2. There were very few cases to attempt to correlate CEBPA and Bcl-2 levels within each karyotype.

The observed lack of correlation between CEBPA and Bcl-2 expression in the intermediate- and high-risk patients may reflect the presence of additional mutations that inhibit apoptosis. The larger study identified 14 patients with CEBPA mutations. Their positions, mainly among the intermediate-risk samples, are highlighted in red (Fig. 1). The average CEBPA level in cases harboring a mutant allele was significantly higher than those only having wild-type alleles among intermediate-risk patients (P = 0.0002).

C/EBPα Inhibits Apoptosis of Hematopoietic Cells

As bcl-2 protects cytokine-dependent hematopoietic cell lines from cell death on cytokine withdrawal (32), we evaluated the effect of exogenous C/EBPα in this experimental paradigm. Ba/F3 is a widely studied immature murine hematopoietic cell line with surface markers consistent with a pro-B lymphoid phenotype but dependent on interleukin (IL)-3 for survival and proliferation, a property of the myeloid lineage. Ba/F3 cells lack endogenous C/EBPα. Activation of C/EBPα-estrogen receptor (ER) with estradiol in Ba/F3 cells leads to their G1-S cell cycle arrest in IL-3 (33). When Ba/F3-C/EBPαER(−/−) cells are removed from IL-3 in the absence of estradiol, only 30% of cells are viable at 24 hours and >98% of the cells are dead by 48 hours. However, in the presence of estradiol, the cells proliferate by 1.4-fold during the first day and then cease proliferating, with 37% of cells still viable at 72 hours (Fig. 2A, top). In contrast, control Ba/F3-Puro cells were not protected by estradiol. C/EBPα-ER also protected an additional IL-3-dependent pro-B-cell line (FL5.12; data not shown), two cytokine-dependent myeloid cell lines (HF1 and 32DCl3), and an immortalized C/EBPα(−/−) myeloid cell line from cell death (Fig. 2A, bottom). The data for the myeloid lines are presented as a ratio of cells surviving in the presence of estradiol to those surviving in its absence, but the temporal pattern of survival was similar to that shown for Ba/F3 cells. To confirm that C/EBPα inhibits cytokine withdrawal–induced apoptosis, Ba/F3-αER cells removed from IL-3 and cultured for 24 hours in the absence or presence of estradiol were stained with Annexin V-phycocerythrin (Fig. 2B). The proportion of cells staining with Annexin V was reduced by >2-fold by activation of C/EBPα-ER.

In addition to cytokine withdrawal, DNA damage and activation of death receptors also induce apoptosis. Induction of p53 by ionizing radiation (IR) induces a G1 cell cycle arrest in Ba/F3 cells cultured in IL-3 and accelerates apoptosis in its absence (34). Activation of C/EBPα-ER in Ba/F3 cells withdrawn from IL-3 and exposed to 600 cGy IR slowed cell death during the first 24 hours, but all cells were dead by day 2 (Fig. 2C, left). Ba/F3 cells are resistant to tumor necrosis factor-α (TNF-α)–induced cell death, but FL5.12 cells undergo...
TNF-mediated apoptosis if sensitized using low-dose cycloheximide (35). Exposure of FL5.12-αER cells, cultured in IL-3, to cycloheximide or TNF alone for 24 hours did not alter viability. However, exposure to cycloheximide and TNF together reduced survival by 4-fold, and this effect was not altered by activation of C/EBPα-ER with estradiol (Fig. 2C, right). Thus, we show for the first time that C/EBPα inhibits cell death induced by cytokine withdrawal or DNA damage but not apoptosis induced via the TNF death receptor.

C/EBPα Induces Endogenous Bcl-2 Expression

Ba/F3-αER cells withdrawn from IL-3 ± estradiol were evaluated for the expression of bcl-2, bcl-xL, bim, JunB, c-Jun, C/EBPβ, C/EBPα, and actin by Western blotting (Fig. 3A). Levels of the proapoptotic BH3-only bcl-2 family member bim were evaluated because of its role in cytokine withdrawal–induced apoptosis (36). Bcl-2 levels were elevated by several-fold by C/EBPα-ER at 16 and 24 hours, whereas the other proteins were largely unaffected. Induction of C/EBPα from the zinc-regulated metallothionein promoter also induced endogenous bcl-2 (data not shown), indicating that the ER segment was not required. Of note, bim levels increased in the absence of IL-3, as expected, irrespective of exposure to estradiol. Signal transducers and activators of transcription 3 and 5, Akt, and phosphatidylinositol 3-kinase levels were also unaffected (data not shown). C/EBPβ protects myc/raf-transformed macrophages from apoptosis via induction of insulin-like growth factor-I, whereas C/EBPα and C/EBPδ are ineffective (37). We did not detect induction of insulin-like growth factor-I RNA by C/EBPα-ER in Ba/F3 cells, and exogenous insulin-like growth factor-I did not induce bcl-2 or protect Ba/F3 cells from apoptosis after IL-3 withdrawal (data not shown). Induction of p53 by IR is evident by 1.5 hours in Ba/F3 cells (34, 38). Activation of C/EBPα-ER in Ba/F3-αER cells withdrawn from IL-3 and exposed to 600 cGy IR did not prevent induction of p53 or its target, p21 (Fig. 3B).

Bcl-2 induction was also evident in HFI-αER or 32Dcl3-αER lines, by as early as 7 hours (Fig. 3C, left), in NIH 3T3 cells cultured in 10% or 0.1% serum (Fig. 3D, left) and in Ba/F3 cells cultured in IL-3 (data not shown). In HFI and 32Dcl3 cells, bcl-2 levels fell on IL-3 withdrawal, whereas C/EBPα-ER maintained these levels at or above the baseline present in IL-3. C/EBPα-ER also induced bcl-2 in C/EBPα(-/-) myeloid cells, and Northern blotting or real-time reverse transcription-PCR analysis showed that C/EBPα-ER induced the bcl-2 transcript regulated by the P2 promoter without affecting the P1 transcript (data not shown), consistent with the finding that C/EBPα specifically activates the P2 promoter in transient assay (25). Exogenous bcl-2 prolongs the survival of 32Dcl3 or FL5.12 cells after IL-3 withdrawal to an extent similar to that observed on activation C/EBPα-ER (32). C/EBPβ-ER or C/EBPδ-ER each induced bcl-2 and inhibited Ba/F3 cell death without inducing detectable C/EBPα (data not shown). However, induction of bcl-2 by C/EBPα-ER was not due to increased levels of endogenous C/EBPβ or C/EBPδ (Fig. 3A).

To determine whether induction of bcl-2 is necessary for protection from apoptosis by C/EBPα, Ba/F3-C/EBPαER cells were stably transduced with bcl-2 or scrambled short hairpin RNAs (shRNA). The bcl-2 shRNA reduced bcl-2 protein levels, relative to β-actin, in IL-3 or 24 hours after removal of IL-3 and obviated protection from apoptosis on IL-3 withdrawal (Fig. 3E).

To evaluate induction of bcl-2 by C/EBPα in normal cells, murine marrow cells cultured in IL-3, IL-6, and stem cell factor
were transduced with pBabePuro-C/EBPα-ER, selected in puromycin, and subjected to lineage depletion. The resulting myeloblasts were then cultured for 24 hours, and viable cell counts were enumerated daily. The percent of cells surviving on subsequent days, relative to the number on day 0, is shown. The indicated HF1, 32Dcl3, or C/EBPα(-/-) myeloid cell lines were treated similarly. A survival ratio, the number of viable cells in the +E2 culture divided by the number in the −E2 culture, is shown. Columns, mean of two determinations; bars, SE. B. Ba/F3-αER cells on day 0 or 24 hours after IL-3 withdrawal were stained with Annexin V-phycoerythrin. C. Ba/F3-αER cells were withdrawn from IL-3 and exposed to 600 cGy IR on day 0 and then cultured for 24 hours after IL-3 withdrawal ± estradiol were stained with Annexin V-phycoerythrin. FL5.12-αER cells were exposed to 5 or 50 ng/mL TNF-α with or without cycloheximide (CHX) ± estradiol. The proportion surviving at 24 hours is shown (right). Columns, mean of two determinations; bars, SE.

FIGURE 2. C/EBPα inhibits apoptosis induced by cytokine withdrawal or irradiation but not death receptor activation. A. Ba/F3 pro-B cells expressing C/EBPα-ER (αER) or the puromycin control (puro) were withdrawn from IL-3 (-IL3) on day 0 in the absence or presence of estradiol (E2), and viable cell counts were enumerated daily. The percent of cells surviving on subsequent days, relative to the number on day 0, is shown. The indicated HF1, 32Dcl3, or C/EBPα(-/-) myeloid cell lines were treated similarly. A survival ratio, the number of viable cells in the +E2 culture divided by the number in the −E2 culture, is shown. Columns, mean of two determinations; bars, SE. B. Ba/F3-αER cells on day 0 or 24 hours after IL-3 withdrawal were stained with Annexin V-phycoerythrin. C. Ba/F3-αER cells were withdrawn from IL-3 and exposed to 600 cGy IR on day 0 and then cultured for 24 hours after IL-3 withdrawal ± estradiol were stained with Annexin V-phycoerythrin. FL5.12-αER cells were exposed to 5 or 50 ng/mL TNF-α with or without cycloheximide (CHX) ± estradiol. The proportion surviving at 24 hours is shown (right). Columns, mean of two determinations; bars, SE.

were transduced with pBabePuro-C/EBPα-ER, selected in puromycin, and subjected to lineage depletion. The resulting myeloblasts were then cultured ± estradiol for 24 hours, and bcl-2 levels were assessed by Western blotting (Fig. 3C, right). Activation of C/EBPα-ER induced bcl-2 by ~3-fold relative to β-actin. In addition, we developed a line of transgenic mice expressing C/EBPα, without the ER segment, using the widely active H2Kb promoter and the lymphoid-specific Eμ enhancer. Expression of C/EBPα was readily detected in splenocytes and thymocytes of transgenic mice but not in those derived from control littermates (Fig. 4A). Fluorescence-activated cell sorting analysis indicated that the spleens from both C/EBPα transgenic...
and control littermates contained ~65% B220+CD19+ B cells, 25% TCR+ T cells, and 10% CD11b+ macrophages (data not shown). Splenocytes exposed to 100 cGy IR immediately after being placed in culture underwent apoptotic cell death more rapidly than unirradiated cells (data not shown). Baseline bcl-2 levels did not differ between unirradiated transgenic or control splenocytes, but the presence of exogenous C/EBPα maintained bcl-2 expression levels, which otherwise fell after exposure to 100 or 300 cGy (Fig. 4B).

In summary, these observations in hematopoietic and non-hematopoietic cell lines and in normal myeloid and lymphoid cells show that C/EBPα has the capacity to induce the normal, endogenous bcl-2 gene.

Induction of Bcl-2 by C/EBPα Does Not Require DNA Binding

To localize the regions of C/EBPα critical for induction of bcl-2, Ba/F3 lines expressing a series of C/EBPα-ER mutants, at similar levels, were generated. Mutant C/EBPαs employed herein are diagrammed (Fig. 5A). BRM2 (I294A, R297A) has reduced ability to bind E2F1 and slow cell cycle progression; BR3 (R297G, K298T, R300G, K302N) is the initial C/EBPα basic region mutant studied and does not bind DNA; L12V (L317V, L324V) does not dimerize or bind DNA due to mutation of the first two leucines of the leucine zipper to valine; GZ has the GCN4 leucine zipper in place of the C/EBPα leucine zipper beginning at leucine 2; Δ3-8 lacks the two C/EBPα transactivation domains; p30 is the NH2-terminally truncated isoform of C/EBPα expressed in AML cases due to NH2-terminal mutations; and F3901, F3820, J3, and K6 are patient-derived C/EBPαLZ mutants (1, 2, 8, 9, 21, 39). BRM2, BR3, L12V, GZ, and Δ3-8 were expressed in Ba/F3 cells, as ER fusions, at levels similar to C/EBPα-ER, and p30-ER was expressed at a reduced level (Fig. 5B). Each of these Ba/F3 populations was evaluated for survival in the absence of IL-3 and for bcl-2 expression ± estradiol (Fig. 5C and D). Survival ratios are again shown to simplify presentation, although low numbers of surviving cells on days 2 and 3 in the absence of estradiol lead to significant variation of this measure between experiments in some instances. BR3 reproducibly increased survival by only 2- to 3-fold, whereas the other C/EBPα variants stimulated survival by at least 7-fold on days 2 and 3. The large majority of cells were dead by day 4 (data not shown). Strikingly, bcl-2 was induced by each of the variants, except BR3, and bcl-2 was not induced by estradiol treatment of Ba/F3-Puro cells (Fig. 5D).

To further pinpoint the amino acid residues critical for bcl-2 induction, we generated Ba/F3 lines expressing C/EBPα-ER variants carrying mutations in single amino acids within the basic region, corresponding to the four mutations present in BR3 (Fig. 5A). R297G, K298T, R300G, and K302N were expressed at levels similar to C/EBPα-ER and the BR3 variant.

![FIGURE 3.](image-url)

C/EBPα Oncoproteins Induce Bcl-2 via NF-κB p50

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C/EBPα induces bcl-2 in normal splenocytes. A, Total cellular proteins from spleen and thymus cells from H2K-C/EBPα-E3 transgenic (+) mice or control littermates (−) were subjected to Western blotting for C/EBPα and actin. B, Splenocytes from transgenic or control mice were exposed to 0, 100, or 300 cGy IR and then cultured for 0, 7, or 24 hours. Total cellular proteins were isolated and subjected to Western blotting for bcl-2 and actin. The ratio of bcl-2 to actin is shown below each lane.

The basic region mutants were also expressed transiently in 293T cells, without a linked ER segment, and evaluated for their ability to bind a strong C/EBP-binding site from the neutrophil elastase promoter (Fig. 6B, left). R297G and K298T bound DNA as well as wild-type, R300G did not bind DNA, and K302N had increased affinity for DNA. A critical role for R300 in DNA binding is consistent with the co-crystal structure of the C/EBPα basic region-leucine zipper domain and a consensus binding site, as its side chain hydrogen bonds to guanine residues on each strand (40). These Ba/F3 lines were evaluated for their survival in the absence of IL-3 and expression of bcl-2 ± estradiol (Fig. 6C and E). R300G only mildly increased survival and did not induce bcl-2, as seen with BR3, whereas the other single basic region mutants retained these activities. K302N was more effective than R297G or K298T. Note that the apparent increase in bcl-2 at 24 hours −E2 for R300G reflects increased loading compared with time 0. Mutation of R300G may prevent both DNA binding and tethering of C/EBPα to the bcl-2 promoter via another protein.

To assess the role of proper positioning of the basic region relative to the leucine zipper, Ba/F3 lines expressing wild-type human C/EBPα-ER and four different C/EBPαLZ mutant variants derived from patients were generated (Fig. 5A). The C/EBPαLZ mutants were expressed at levels similar to or higher than wild-type human C/EBPα-ER relative to β-actin (Fig. 6A, right lanes). Three of the C/EBPαLZ mutants were chosen because they differ in the number of residues added or deleted in the first α-helix of the leucine zipper, −1, +8, +5 for F3901, F3820, and J3, respectively. As there are seven residues per turn of the α-helix, the basic region and leucine zipper will be out of phase in these mutants. K6 carries a single alteration, R to P, in the fork region between the basic region and leucine zipper. As proline disrupts the α-helix, the relative position of the basic region and leucine zipper would likely again be altered. F3901, F3820, and J3 do not bind the C/EBPα consensus site, as seen previously (9), and K6 also does not bind DNA (Fig. 6B, right). F3901, F3820, and J3 each increased survival and bcl-2 levels to an extent similar to wild-type human C/EBPα-ER, whereas K6 was less effective (Fig. 6D and F). As K6 retains an intact leucine zipper, it may have a greater ability to dominantly inhibit endogenous C/EBPα and C/EBPβ. The results with L12V and the four C/EBPαLZ mutants clearly indicate that DNA binding is not required for bcl-2 gene induction.

C/EBPα Oncoproteins Activate the bcl-2 Promoter via Interaction with NF-κB p50

Transactivation of P2(−1278)-LUC, containing bp −1278/+1 from the human bcl-2 P2 promoter, by C/EBPα, the BR3 basic region mutant, and the p30 or F3901 C/EBPαLZ oncoproteins was assessed in NIH 3T3 cells (Fig. 7A). C/EBPα induced P2(−1278)-LUC by 6-fold, p30 by 5-fold, and F3901 by 18-fold relative to the empty vector, whereas BR3 was ineffective. This pattern fits that seen with activation of the endogenous bcl-2 gene by stably expressed C/EBPα-ER, BR3-ER, p30-ER, and F3901-ER and suggests that the C/EBPαLZ oncoprotein activates the promoter via a DNA binding–independent mechanism.

To directly assess cooperation between C/EBPα or its derivatives and NF-κB, we used F9 cells, which have low levels of endogenous NF-κB (26). Cytomegalovirus (CMV)-C/EBPα induced the P2 promoter by 8-fold, CMV-NF-κB p50 reduced its activity by 2-fold, and together they synergistically activated the promoter by 17-fold (Fig. 7B). In contrast to p50, CMV-NF-κB p65 induced the P2 promoter by 7-fold, and coexpression of C/EBPα reduced this activation. Reducing the amount of C/EBPα and p65 expression vectors transfected (to 5 and 2 ng) also did not show cooperativity (data not shown). F3901 also synergistically activated the bcl-2 P2 promoter in cooperation with p50, whereas the BR3 variant did not (Fig. 7B). C/EBPαp30 cooperate with NF-κB p50 as well, but less effectively than C/EBPα or F3901, perhaps due to its lack of a transactivation domain.

To evaluate the ability of C/EBPα and its mutant variants to interact with p50, these proteins were coexpressed by transient transfection in 293T cells. Cell lysates were subjected to immunoprecipitation with a rabbit C/EBPα antiserum followed by Western blotting with a mouse anti-p50 antibody (Fig. 8A, top). Use of antibodies from rabbit and mouse avoids detection of the immunoglobulin heavy chain. C/EBPα, L12V, F3901,
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and p30 each interacted with p50, whereas BR3 did not, although BR3 was expressed at an equivalent level (data not shown). This pattern of interaction with p50 was reproducible in a second experiment and correlates strikingly with the ability of these variants to induce endogenous bcl-2 and to transactivate the bcl-2 promoter in NIH 3T3 cells. In a separate set of transfections, we found that C/EBPα, BR3, and L12V immunoprecipitated with C/EBPα antiserum each interacted with p65 in 293T cells (Fig. 8A, bottom). Cell lysates were also subjected to immunoprecipitation with p50 or p65 rabbit antisera followed by Western blotting using C/EBPα antibody (Fig. 8B). Strikingly, this reciprocal experiment, which was reproducible on three occasions, confirmed the conclusion that the BR3 mutations prevent interaction with p50 but not p65 and that C/EBPα and the F3901 C/EBPα/LZ oncoprotein retain the ability to interact with p50 and p65. p30 could not be evaluated in this experiment because the epitope for the C/EBPα monoclonal antibody is located at its NH2 terminus.

Finally, we sought to assess interaction of endogenous C/EBPα with endogenous p50 or p65 in myeloid cells. Using extracts from the human U937 and HL60 cell lines, which express C/EBPα at higher levels than are present in 32Dcl3 cells (7), we found that C/EBPα preferentially coimmunoprecipitated with NF-κB p50 (Fig. 8C).

Discussion

Major conclusions of this study are that CEBPA levels correlate with Bcl-2 in a cytogenetically defined subset of AML cases, that C/EBPα induces the endogenous bcl-2 gene in a variety of lineages, that C/EBPα has the capacity to inhibit apoptosis of hematopoietic cells under stress conditions, that induction of bcl-2 is necessary to inhibit apoptosis of Ba/F3 cells withdrawn from IL-3, that induction of bcl-2 transcription occurs cooperatively with NF-κB p50 but not p65, that C/EBPα interacts preferentially with endogenous p50, that interaction with p50 maps to a cluster of residues in the C/EBPα basic region, and that the C/EBPα/LZ oncoproteins retain the ability to interact with p50 and induce bcl-2 despite their inability to bind DNA. Perhaps most importantly, this study (a) suggests that C/EBPα and C/EBPα oncoproteins may contribute to transformation by inducing apoptosis and (b) provides new insights into the C/EBPα/NF-κB protein-protein interaction that may serve as a useful target for inducing apoptosis and inhibiting inflammation in cancer and other diseases.

C/EBPα is a key mediator of granulocytic differentiation and has an antiproliferative effect in myeloid progenitors and even in pluripotent hematopoietic stem cells (33, 41, 42). Therefore, it is perhaps not surprising that C/EBPα activity is reduced through one of several mechanisms in the majority of AML cases. Our results now suggest, however, that residual levels of wild-type C/EBPα as well as mutant isoforms contribute to transformation by induction of bcl-2 and inhibition of apoptosis. The correlation between wild-type CEBPA and Bcl-2 expression in low-risk human AML cases in two independent studies is striking. The generally lower levels of CEBPA among t(8;21) patients may reflect the ability of AML1-ETO to potently repress the CEBPA promoter (13), and the intermediate levels in inv(16) patients may reflect a more
modest effect of CBFβ-SMMHC on the same promoter. Lack of correlation between CEBPA and Bcl-2 in intermediate- and high-risk patients may indicate that many of these leukemias do not rely predominantly on bcl-2 for inhibition of apoptosis. Although we show lack of effect of exogenous C/EBPa on several other proteins known to regulate apoptosis and find that bcl-2 shRNA prevented C/EBPa from slowing Ba/F3 apoptosis, in future studies, we will use microarray approaches in transduced myeloid progenitors to systematically search for additional relevant C/EBPa targets. It is interesting that CEBPA levels in mutant cases were generally higher than CEBPA levels in other intermediate-risk AMLs. Their reduced ability to block differentiation and cell proliferation may allow them to achieve higher levels to maximize induction of bcl-2 and possibly other genetic targets. The ability of the C/EBPaLZ oncoproteins to induce the endogenous bcl-2 gene despite their inability to dimerize and bind DNA provides an explanation for the selective advantage of in-frame rather than out-of-frame alterations in the leucine zipper during leukemogenesis. To our knowledge, gene activation by C/EPBs independent of DNA binding is without precedent. Of note, the basic DNA contact domain of C/EBPa is rarely mutated in AML, although this would also prevent DNA binding and activation of granulocytic genes. Our finding that integrity of the basic region is required for interaction with NF-κB and for bcl-2 induction provides a rationale for this phenomenon and for the lack of null mutations in CEBPA in AML cases. Development of leukemia models requiring C/EBPaLZ oncoproteins will enable evaluation of the role of direct interaction with NF-κB during leukemogenesis.

C/EBPa was shown to induce endogenous bcl-2 in a lymphoma cell line harboring t(14;18) (ref. 25). This translocation fuses the immunoglobulin heavy chain promoter upstream of the bcl-2 P1 and P2 promoters and activates the P2 promoter (25). We now show that C/EBPa induces endogenous bcl-2 expression in myeloid and lymphoid cell lines and normal cells harboring an unaltered bcl-2 gene. In addition, we show that transactivation of the P2 promoter by C/EBPa can even occur independent of DNA binding in cooperation with NF-κB during leukemogenesis.

The Rel domains of NF-κB p50 or p65 directly interact with the C/EBPaβ basic region-leucine zipper domain (26, 43, 44), and C/EBPaβ and NF-κB cooperatively bind and activate the IL-6, IL-8, granulocyte colony-stimulating factor, serum amyloid, intercellular adhesion molecule-1, superoxide dismutase, and Mediterranean fever promoters (45-51). These prior studies

FIGURE 6. C/EBPaLZ oncoproteins inhibit apoptosis and induce bcl-2, whereas the single amino acid basic region mutant R300G does not. A, Total cellular proteins from Ba/F3 lines expressing the indicated C/EBPaLZ-ER fusion variants were subjected to Western blotting using ER and actin antibodies. B, Nuclear extracts (10 μg) from 293T cells transiently transfected with plasmids expressing the indicated C/EBPs, without a linked ER segment, were subjected to gel shift analysis using a consensus C/EBP-binding site derived from the neutrophil elastase promoter. C and D, Survival ratios on day 2 and 3 are shown for each Ba/F3 line withdrawn from IL-3 ± estradiol. Columns, mean of two determinations; bars, SE. E and F, Total cellular proteins isolated from each line 0 or 24 hours after IL-3 withdrawal ± estradiol were analyzed for bcl-2 and actin expression by Western blotting.
strongly implicate a role for C/EBP-NF-κB cooperation in the regulation of a variety of genes involved in the inflammatory response. Of note, the acute-phase response is defective in C/EBPα(−/−) mice, and inflammation is emerging as a key mediator of tumor progression via induction of cytokines, such as IL-6 and vascular endothelial growth factor, which stimulate survival and angiogenesis (28, 52-56). Moreover, constitutive NF-κB activation is common in AML stem cells, and combining an anthracycline with a NF-κB inhibitor leads to apoptosis of leukemic stem cells while sparing normal hematopoietic stem cells (27, 57). Perhaps C/EBPα activity recently detected in hematopoietic stem cells contributes to their survival in cooperation with NF-κB (42).

We have made several contributions to our understanding of C/EBP-NF-κB interaction. We show that C/EBPα, like C/EBPβ, interacts with the NF-κB p50 and p65 subunits and provide the first demonstration that an endogenous C/EBP interacts with endogenous NF-κB. Second, we have localized interaction with p50 to residues 297 to 302 in the rat C/EBPα basic region, a segment identical in human C/EBPα, in C/EBPβ, and in C/EBPδ. R300G also prevented induction of bcl-2, but, as glycine disrupts local α-helical structure, additional point mutations in this region will need to be generated to pinpoint the residues that contact NF-κB p50. Alteration of combinations of residues, excluding R300, may be required to eliminate interaction. A specific effect of the BR3 mutations on interaction with p50 compared with p65 was evident when either C/EBPα or NF-κB antibodies were used for immunoprecipitation, strengthening the conclusion that p50 and p65 contact C/EBPα in different manners. Perhaps C/EBPα interacts with a region conserved in p50 and p65 and with an additional region in p50 via the basic region. Consistent with this idea, we find that C/EBPα preferentially binds endogenous p50 compared with p65, suggesting also that C/EBPα preferentially binds p50-p50 homodimers compared with p50-p65 heterodimers. Importantly, the presence of p50-p50 homodimers correlates with bcl-2 expression in lymphoma cell lines, and exogenous p50 stimulated nuclear runoff transcription from a linearized, exogenous bcl-2 promoter template (58). Future experiments will use highly purified proteins to assess relative affinities and to further map interaction surfaces. We will also explore the range of genes activated cooperatively by these proteins and the specific role of p50 (versus p52, p65, c-Rel, and RelB) in their induction. This analysis may be complicated by the ability of C/EBPα and C/EBPα oncoproteins to bind both p50 and p65 and by compensatory cross-regulation within the NF-κB regulatory network. For example, p52 is increased in the absence of p50, c-Rel is increased in the absence of p65, and IκBα and IκBβ are reduced in the absence of p65 (59).

The role of C/EBPα-NF-κB interaction during normal hematopoiesis remains an open question that likely awaits the generation of mice in which a C/EBPα basic region variant, which does not bind NF-κB p50 but still binds E2F and DNA, is identified and introduced into the genome by homologous recombination. Nevertheless, our findings suggest that an agent that prevents C/EBP-NF-κB p50 interaction may be useful in the therapy of a variety of malignancies and inflammatory diseases. As IκB-B binds p50 with 30-fold reduced affinity compared with p65 (60), agents that target IκB may not inhibit genes regulated cooperatively by C/EBPs and p50 as effectively.

Materials and Methods

Cell Culture, Transduction, and Survival Assays

Ba/F3 cells (61) and FL5.12 cells (30) were maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum (HI-FBS) and 1 ng IL-3/mL (Peprotech, Rocky Hill, NJ). U937 and

![Figure 7](image)

FIGURE 7. C/EBPα and C/EBPα oncoproteins activate the bcl-2 promoter in cooperation with NF-κB p50 and dependent on the C/EBPα basic region. A, NIH 3T3 cells were transiently transfected with 1,500 ng of the indicated reporters, 100 ng CMV or CMV-C/EBP plasmids, and 5 ng CMV-\( \gamma \)-galactosidase. Cell extracts were analyzed 2 days later for luciferase and \( \gamma \)-galactosidase activities. Activity, luciferase/\( \gamma \)-galactosidase, was defined as 1.0 for each reporter transfected with empty CMV vector. Columns, mean fold increase above this baseline (three determinations); bars, SE. B, F9 cells were cotransfected with P2(-1278)-LUC with the indicated amounts of CMV-C/EBP expression vectors, CMV-p50 or CMV-p65, and 5 ng CMV-\( \gamma \)-galactosidase. Fold activation relative to the same quantity of empty CMV vector is shown. Columns, mean of two determinations; bars, SE.
FIGURE 8. Mutation of the C/EBPα basic region prevents interaction with NF-κB p50 but not p65, and C/EBPα preferentially binds endogenous p50. A, The 293T cells in 100-mm dishes were cotransfected with 2 μg CMV-C/EBPα or the indicated mutants and 2 μg pkat2ecopac (65) and 16 μL LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Supernatants collected 2 and 3 days later were adsorbed to retronectin-coated dishes (Takara, Shiga, Japan). Hematopoietic cell lines were then added in the presence of 4 μg/mL polybrene for 2 days. Pools of transduced cells were selected using 2 μg/mL puromycin or 1.2 mg/mL G418 after transduction with vectors expressing shRNAs. Murine marrow mononuclear cells, isolated from C57BL/6 mice treated 6 days earlier with 150 mg/kg 5-fluorouracil i.p. and cultured in Iscove’s modified Dulbecco’s medium with 10% HI-FBS, 10 ng IL-3, 10 ng IL-6, and 50 ng stem cell factor/mL, were transduced and selected similarly. After selection, viable murine marrow cells were isolated using Lympholyte-M gradient centrifugation (Cedarlane Labs, Hornby, Ontario, Canada) and subjected to lineage depletion using an antibody cocktail (B220, CD5, Mac-1, Gr-1, and 7-4) and immunomagnetic beads (Stem Cell Technologies, Vancouver, British Columbia, Canada). To remove cytokines, cells were washed twice with PBS. Viable cell counts were enumerated using trypan blue dye. Assessment of apoptosis by staining with Annexin V conjugated to FITC or phycoerythrin was as described (38). Estradiol was employed at 1 μmol/L from a 1,000× stock, and ethanol was added to control groups.

**Plasmids and Transient Transfection**

pBabePuro-C/EBPα-ER, containing rat C/EBPα fused to the ligand-binding domain of human ERα, and several mutant derivatives (BR3, L12V, BRM2, Δ3-8, GZ, and p30) have been described (10, 33, 66, 67). PCR mutagenesis was employed to generate C/EBPα-ER mutants R297G, K298T, R300G, and K302N, which were confirmed by DNA sequencing. Human C/EBPα and 4 C/EBPα mutants derived from human cases (F3901, F3820, J3, and K6) were ligated in place of the C/EBPα segment in pBabePuro-C/EBPα-ER as NcoI fragments (9). Expression vectors for C/EBPα variants, without the ER segment, were generated by transfer into CMV-C/EBPα (10). For p30, a Kozak’s consensus sequence 5’-GCCGCCACCATGG-3’ was positioned at the italicized initiating ATG. shRNAs were expressed from the U6 promoter in the pRNAT-U6 vector (GeneScript, Piscataway, NJ). The sense strand sequence for the shRNAs were: bcl-2 5’-GATCCGAGGTATGATAACCC-3’ and scrambled 5’-GATCCCGAGGTATGATAACCC-3’. pCMV-β-galactosidase was included as an internal control.

**Western Blotting, Gel Shift, and Coimmunoprecipitation**

Total cellular proteins were subjected to Western blotting as described (38). Antibodies employed were rabbit polyclonal C/EBPα (14AA), C/EBPβ (C19), ERα (HC20), bcl-2 (N19), and β-galactosidase (Promega). With the C/EBPα mutants, 4 μg of total cellular proteins and 1 μg of β-galactosidase were subjected to Western blotting. Expression levels of C/EBPα and β-galactosidase were as described (38). Immunoprecipitations were performed using 1 μg of polyclonal antibody (C/EBPα, BR3, or Rabbit IgG) or 1 μg of monoclonal antibody (GC-1278) and 1 μg of β-galactosidase. Western blotting was as described (38).
bcl-xL (L19), JunB (N17), c-Jun (N), NF-κB p50 (NLS), and NF-κB p65 (C20) and mouse monoclonal NF-κB p50 (E10; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-bim and mouse anti-p53 pAB-240 (BD PharMingen, San Diego, CA), mouse anti-p21/WAF1 (Ab-3; Oncogene Research, Boston, MA), mouse anti-β-actin (AC-15, Sigma, St. Louis, MO), mouse anti-C/EBPα (MA1-825, Affinity Bioreagents, Golden CO), and rabbit anti-C/EBPβ (7). Densitometric analysis was carried out using the NIH Image 1.62 program. Nuclear extracts were prepared from transiently transfected 293T cells and subjected to gel shift assay using a C/EBPα-binding site from the neutrophil elastase promoter as described (16). For coimmunoprecipitation, cell extracts were prepared from transfected 293T cells or from hematopoietic cell lines by washing with TBS [150 mmol/L NaCl, 20 mmol/L Tris (pH 7.5)] followed by incubation at 4°C for 20 minutes with 500 μL of 0.5% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris (pH 7.5), 1 mmol/L phenylmethylsulfonyl fluoride, and a cocktail of peptide protease inhibitors. The lysates were then briefly sonicated and spun at 12,000 × g for 10 minutes. Aliquots were saved as “input” and the rest of the supernatants was precleared by incubation with 50 μL of 50% protein A/G-Sepharose. The supernatants were then incubated with 1 μg primary antibody for 3 hours at 4°C followed by addition of 50 μL protein A/G-Sepharose for 1 hour. The beads were then washed thrice with lysis buffer, and the samples were eluted in 1× Laemmli sample buffer at 95°C.

Transgenic Mice

The rat C/EBPα cDNA was ligated downstream of the H2Kβ promoter and upstream of a β-globin genomic segment providing polyadenylic acid signals and the Eμ immunoglobulin heavy chain enhancer (69) to generate H2K-C/EBPα-Eμ. Transgenic mice were generated in the absence of vector sequences by the Johns Hopkins Transgenic Facility. A founder was identified by Southern analysis of tail DNAs and has been bred for eight generations into the C57BL/6 background. Single-cell suspensions of splenocytes generated using a cell strainer were cultured in RPMI 1640 with 10% HI-FBS and mercaptoethanol.

Microarray Data Analysis

Microarray expression data from human adult AML samples from two studies was obtained from the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/, accession nos. GSE415 and GSE1159; refs. 41, 42). In the first study, 39,711 cDNAs from a two-channel microarray experiment provided information on 6,283 well-measured genes highly variable between samples, and in the second study, 22,283 probe sets on an Affymetrix GeneChips were cultured in RPMI 1640 with 10% HI-FBS and surfactant protein genes in lung cells. Am J Physiol 1995;269:241–7.

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