CCAAT Box Enhancer Binding Protein α (C/EBP-α) Stimulates κB Element-mediated Transcription in Transfected Cells*

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NF-κB proteins regulate the expression of many different genes, including genes encoding cytokines and acuate phase proteins and some viral genes (1–3). The NF-κB family includes p50 (NFκB1), p52 (NFκB2), p65 (RelA), RelB, v-Rel, c-Rel and Drosophila proteins Dorsal and Dif. The N-terminal portion of NF-κB proteins contains a 300-amino acid domain termed the Rel homology region. This domain is responsible for DNA binding and dimerization of NF-κB proteins. Less homology is found within the C-terminal region of NF-κB proteins that is known to serve as the activation domain. In their inactive state NF-κB proteins are usually sequestered in the cytoplasm in a complex with an inhibitory subunit termed I-κB (1). NF-κB proteins are activated by phosphorylation of the I-κB subunit and its subsequent proteasome-driven degradation leading to the release from the NF-κB-I-κB complex (4). NF-κB then translocates into the nucleus and rapidly activates gene expression. Another group of transcription factors important in the regulation of cytokine and acute phase protein genes is the C/EBP family (5–7). A typical structural feature of C/EBP proteins is the presence of a domain comprising a region of basic amino acids and a leucine zipper region (z-ZIP domain) (8, 9). The leucine zipper domain is responsible for dimerization, causing changes in the structural conformation of the protein that allow the binding of the basic region to specific DNA sequences. The C/EBP family includes C/EBP-α, C/EBP-β (also termed IL-6 DBP or LAP; its human analogue is NF-IL6), C/EBP-γ (IgE/EBP-1), C/EBP-δ, and CHOP/GADD53 (7, 10–12).

An important feature of NF-κB and C/EBP proteins is their ability to heterodimerize with each other and with members of other transcription factor families (13–18). NF-κB-C/EBP heterodimers are formed through the interaction of the Rel homology region of NF-κB proteins with leucine zipper domains within b-ZIP regions of C/EBP transcription factors. Heterodimerization is facilitated by the presence of adjacent κB and C/EBP binding sites in several genes encoding cytokines (IL-6, IL-8, G-CSF, neutrophil-activating peptide ENA-78, MGSA/GRO) (16, 19–24), acute phase proteins (angiotensinogen, serum amyloid A protein, α1-acid glycoprotein, TSG-14/PTX-3) (25–28) or adhesion receptors (intercellular adhesion molecule-1) (29). The presence of C/EBP/NF-κB protein heterocomplexes has been demonstrated in intact cells (17, 30). Heterodimerization between NF-κB and C/EBP proteins can lead to both cooperative and antagonistic interactions. For example, in the IL-8 promoter NF-κB and C/EBP proteins can lead to mutually augment their binding to the adjacent κB and C/EBP binding sites, resulting in increased gene expression (20). Similarly, C/EBP-α and C/EBP-β increased NF-κB p50- or p65-driven expression of a SAA3-CAT gene construct (31). On the other hand, it was found that C/EBP can inhibit promoters with κB binding sites. Thus, when NF-κB p65 and C/EBP-β were co-transfected together with an expression vector driven solely by the κB element from the IL-8 gene, C/EBP-β inhibited p65-stimulated transcription (20).

In our present study we show that when overexpressed in human F5-4 fibroblasts, C/EBP-α activates transcription from a CAT reporter construct based on a trimeric repeat of the κB element from the enhancer region of the IL-8 gene (32). How-

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2 The abbreviations used are: C/EBP, CCAAT box enhancer binding protein; IL-8, interleukin-8; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; IL, interleukin; TPA, 12-0-tetradecanoylphorbol-13-acetate; USF, upstream stimulatory factor.
ever, C/EBP-α did not stimulate transcription of the same construct in mouse F9 embryonic teratocarcinoma cells, which fail to express endogenous NF-κB activity (33, 34). Therefore, an interaction of C/EBP-α with NF-κB and/or other transcription factors is likely to be involved in the transcriptional activation of the κB-driven construct in F5-4 cells. Our studies of transcriptional activation were confirmed by electrophoretic mobility shift assays (EMSAs), showing that in the presence of the κB probe nuclear extracts from unstimulated F5-4 cells formed specific complexes with recombinant C/EBP-α protein.

EXPERIMENTAL PROCEDURES

Cytokines, Transcription Factors, and Antibodies—Recombinant human and murine TNF-α were kind gifts from Masafumi Tsujimoto (Suntory Institute for Biomedical Research, Osaka, Japan). Recombinant human IL-1α was a gift from Peter Lomedico and Alvern Stern (Hoffmann-La Roche, Nutley, N.J.). Recombinant murine IL-1β was from Program Resources (National Cancer Institute, Frederick, MD). NF-κB proteins p50 and p65 were a generous gift from J. Ohn Hiscott (Lady Davis Institute for Medical Research, Montreal, Canada). These recombinant proteins were prepared as glutathione-S-transferase fusion proteins, enzymatically cleaved, and purified (35). Recombinant C/EBP-α protein was a generous gift of Steven McKnight (Tularik Inc., South San Francisco, CA) (36). Polyclonal rabbit antibodies specific for NF-κB protein was a generous gift of Steven McKnight (Tularik Inc., South San Francisco, CA). Plasmid pSVK3-IκB-α(−708) was a kind gift from Steven McKnight (Tularik Inc., South San Francisco, CA) (37). The construction of recombinant expression vectors pMSV-C/EBP-α and pMSV-C/EBP-β(−708) gene is TGGAATTTCC as compared with TGGGGATTCC for the HLA B-7 gene. Polyclonal antibody against C/EBP-α was a generous gift from Nancy Rice (NCI-Frederick Cancer Research and Development Center, Frederick, MD). The polyclonal antibody against C/EBP-β was a gift from Steven McKnight (Tularik Inc., South San Francisco, CA).

Plasmid Construction and Oligonucleotides—The construction of reporter plasmids containing three tandemly repeated copies of the NF-κB binding site from the human IL-8 gene (3xNF-κB) or three copies of the NF-κB binding site from the human IL-8 gene is TGGAATTTCC as compared with TGGGGATTCC for the HLA B-7 gene.) Expression vector for p50B (p52) (37) was a gift from Ulrich Siebenlist (Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Expression vectors pMSV-C/EBP-α (39) and pMSV-C/EBP-β (11) were a kind gift from Steven McKnight (Tularik Inc., South San Francisco, CA). Plasmid pSVK3-IκB-α (38) was a kind gift of Steven McKnight (Tularik Inc., South San Francisco, CA) (36). Polyclonal rabbit antibodies specific for NF-κB proteins p50 (number 1141, raised against a peptide that includes the N terminus; number 1613, raised against a peptide that includes the nuclear localization signal), and p65 (number 1226, raised against a peptide from the C terminus) were a generous gift from Nancy Rice (NCI-Frederick Cancer Research and Development Center, Frederick, MD). The polyclonal antibody against C/EBP-β was a gift from Steven McKnight (Tularik Inc., South San Francisco, CA).

RESULTS

Activation of NF-κB-responsive CAT Reporter Construct by C/EBP-α in Human FS-4 Fibroblasts—Previous studies demonstrated that expression of the IL-8 gene is cooperatively regulated by members of two transcription factor families, NF-κB and C/EBP, which bind to two distinct, adjacent cis-acting DNA elements in the IL-8 promoter (16, 19, 20). We have examined the ability of specific polyclonal antibodies to NF-κB and C/EBP families to induce the transcriptional activation of a reporter construct comprising a trimerized NF-κB binding site from the human IL-8 gene linked to the minimal promoter of the IL-8 gene and the CAT gene (3xNF-κB) (32). While co-transfection of FS-4 cells with 3xNF-κB-CAT and the NF-κB p50 expression plasmid stimulated CAT activity less than 5-fold, co-transfection with NF-κB p65 or with both p50 and p65 increased CAT activity approximately 50-fold over control levels (Fig. 1, A and D). Transcription from the 3xNF-κB-κB construct was not activated by co-transfection with C/EBP-β and C/EBP-α expression plasmids, but to our surprise, C/EBP-α produced an approximately 5-fold increase in CAT activity (Fig. 1, B and D). To determine whether activation of the 3xNF-κB-κB construct is κB sequence-specific, we also employed expression construct 3xNF-κB (HLA)CAT, comprising three copies of the κB element from the human HLA B-7 gene that differs from the IL-8 κB sequence in three positions (32). The 3xNF-κB (HLA)CAT construct contains the same minimal promoter from the IL-8 gene as 3xNF-κB-κB. The 3xNF-κB (HLA)CAT construct was less responsive to stimulation by the p65 expression vector than the 3xNF-κB-κB construct containing the κB sequence from the IL-8 gene (Fig. 1C). Unlike
the latter construct, 3xNF-κB(HLA)CAT was not stimulated by co-transfection with the C/EBP-α expression vector, indicating that the sequence specificity of the κB element affects the activation process.

Lack of Demonstrable Cooperative Interactions between C/EBP-α and NF-κB Factors in the Transcriptional Activation of the 3xNF-κB CAT Reporter Gene—To examine a possible cooperation between C/EBP-α and NF-κB proteins in the transcriptional activation of 3xNF-κB CAT seen upon overexpression of C/EBP-α, we co-transfected FS-4 cells with the reporter construct 3xNF-κBCAT and the C/EBP-α vector alone or in combination with vectors encoding different members of the NF-κB family. Transfection of different concentrations of the C/EBP-α vector alone stimulated activation of the CAT construct dose-dependently, up to approximately 8-fold (Fig. 2A). Transfection with 1 μg of the p50 (Fig. 2B) or p65 (Fig. 2C) vector alone stimulated CAT activity only modestly. Co-transfection with different amounts of C/EBP-α and 1 μg of either the p50 or the p52 expression plasmids did not have a major effect on the induction of CAT activity when compared with the results obtained by transfection of C/EBP-α alone (Fig. 2, B and C). Hence, these data did not reveal striking cooperative or antagonistic interactions between p50 or p52 NF-κB transcription factors and C/EBP-α. A possible interaction between C/EBP-α and p65 was examined by co-transfecting into FS-4 cells different doses of the p65 expression vector and a constant dose of the C/EBP-α vector along with 3xNF-κBCAT (Fig. 3). In general, the increase in CAT activity seen after co-transfection with p65 and C/EBP-α was less than the sum of CAT activities produced with either expression vector alone, suggesting some degree of antagonism between C/EBP-α and the p65 NF-κB protein. This apparent antagonism was most pronounced with
C/EBP-α Stimulates κB-mediated Transcription

Fig. 2. Co-transfection of C/EBP-α with NF-κB p50 or p52 proteins does not result in a cooperative stimulation of the 3xNF-κB CAT reporter construct. FS-4 cells were co-transfected with 10 μg of the 3xNF-κB CAT reporter construct and the indicated amounts of expression vector pMSV-C/EBP-α (A), or combinations of different amounts of pMSV-C/EBP-α with 1 μg of pCMV-p50 (B) or pCMV-p52 (C). pCMV4T is the "empty" vector.

0.25 μg of the p65 expression vector.

C/EBP-α Fails to Activate 3xNF-κB CAT in the F9 Embryonic Carcinoma Cell Line—Earlier studies have shown that F9 mouse embryonic carcinoma cells are deficient in NF-κB-dependent functions (33, 42, 43). Thus, this cell line offered an opportunity to determine if C/EBP-α could produce activation of the 3xNF-κB CAT construct in the absence of functional NF-κB proteins. To confirm that F9 cells lack activable NF-κB proteins, we first transfected F9 cells with the 3xNF-κB CAT plasmid and then treated the transfected cultures with different agents known to activate gene transcription via induction of NF-κB. Neither cytokines (TNF or IL-1) nor the phorbol ester TPA induced CAT activity, confirming that F9 cells lack activable NF-κB (Fig. 4). In contrast, treatment with TNF or IL-1 caused a marked stimulation of CAT activity in FS-4 cells transfected with the same 3xNF-κB CAT reporter construct (Ref. 32 and data not shown). We then examined the ability of the expression vectors for C/EBP-α or NF-κB proteins p50, p52, and p65 to activate the 3xNF-κB CAT reporter construct in F9 cells. In contrast to the results obtained in FS-4 cells, C/EBP-α failed to produce any activation of CAT activity (Fig. 5, A and B). p50 or p52 alone, or combinations of C/EBP-α with p50 or p52 also failed to stimulate CAT activity. However, CAT activity was strongly stimulated in F9 cells by co-transfection with p65 (Fig. 5C). Co-transfection of p50 further potentiated the stimulatory effects of p65 on CAT activity (Fig. 5D). The stimulatory effect of p65 alone or of the combination of p65 and p50 was reduced by co-transfecting C/EBP-α, indicating an antagonistic relationship (Fig. 5, C and D).

Effects of IκB on the Activation of 3xNF-κB CAT by p65 or C/EBP-α—The inhibitory protein IκB is known to block NF-κB-regulated gene expression by binding to the NF-κB complex in the cytoplasm, thus preventing nuclear translocation. Overexpression of IκB can inhibit NF-κB-mediated gene activation (34, 39, 44). We compared the effects of IκB overexpression on the stimulation of the 3xNF-κB CAT reporter construct by transfection with either p65 or C/EBP-α in FS-4 cells (Fig. 6). Overexpression of IκB significantly inhibited the stimulation of CAT activity by p65 but had a much less marked influence on the ability of C/EBP-α to activate the 3xNF-κB CAT reporter construct.

Nuclear Protein Binding to NF-κB Site from the IL-8 Promoter—To determine if increased CAT activity in FS-4 cells transfected with 3xNF-κB CAT and C/EBP-α is due to the direct binding of C/EBP-α to the κB DNA element, we examined binding of recombinant C/EBP-α protein by EMSAs with a synthetic oligonucleotide probe containing the same κB DNA sequence from the IL-8 gene as the 3xNF-κB CAT construct. Incubation of recombinant C/EBP-α protein alone with the labeled oligonucleotide probe did not lead to the formation of a detectable complex (Fig. 7A, lane 2). However, FS-4 nuclear extracts mixed with small amounts of recombinant C/EBP-α protein reproducibly gave rise to four distinct bands (marked C1, C2, C3, and C4) that were not formed by the FS-4 nuclear extract in the absence of C/EBP-α protein (Fig. 7A, lanes 1 and 3). Formation of all four complexes was competed by preincubation of the nuclear extracts with an excess of the unlabeled κB oligonucleotide, indicating that these interactions are specific (Fig. 7A, lane 4). The addition of C/EBP-α antibody to the binding reactions resulted in the formation of a very large supershifted complex, with a concomitant disappearance of C1, C2, C3, and C4, suggesting that C/EBP-α is involved in the formation of all four complexes (Fig. 7B). In contrast, nonimmune serum did not interfere with complex formation. We also
attempted to determine whether complexes C1–C4 contained proteins reactive with antibodies against various NF-kB proteins. The addition of two different antibodies to p50 decreased the intensity of complex C4 without a reduction in any of the other three complexes (Fig. 7C). The addition of anti-p65 antibody to the binding reaction did not significantly decrease the intensity of the complexes. In other experiments we found that antibodies to p52, RelB, and c-Rel also failed to reduce the formation of any of the four complexes formed in EMSAs with recombinant C/EBP-α protein, nuclear proteins from unstimulated FS-4 cells, and the NF-κB probe (data not shown).

**DISCUSSION**

In this study we have shown that C/EBP-α expression in human FS-4 fibroblasts stimulates transcriptional activity of the 3xNF-κB CAT reporter construct comprising three tandemly repeated copies of the NF-κB binding site from the human IL-8 gene. The stimulatory effect of C/EBP-α was sequence-specific, because C/EBP-α expression did not activate a similar construct, 3xNF-κB(HLA)CAT, comprising three copies of the NF-κB binding site from the human HLA B-7 gene (Fig. 1). Two lines of evidence indicate that stimulation of the 3xNF-κB CAT construct was not the result of a direct binding of C/EBP-α to the κB sequence. First, C/EBP-α failed to produce transcriptional activation of 3xNF-κB CAT in the murine F9 embryonic carcinoma cell line (Fig. 5) in which the active (nuclear) form of NF-κB is not inducible (33, 42) and which is deficient in some other transcription factors (43, 45). Second, in EMSAs recombinant C/EBP-α protein failed to bind to a probe comprising the same κB binding sites from the IL-8 gene as the 3xNF-κB CAT expression construct (Fig. 7). Our data support the notion that complex formation of C/EBP-α with other protein factor(s), present in FS-4 but not in F9 cells, is necessary for the transcriptional activation of 3xNF-κB CAT. Direct evidence of the formation of specific complexes between recombinant C/EBP-α protein and proteins from the nuclei of unstimulated FS-4 cells was obtained in EMSAs (Fig. 7).

The stimulatory effect on the 3xNF-κB CAT construct was specific for C/EBP-α, because no transcriptional activation was seen on co-transfection with C/EBP-β or -δ (Fig. 1B). C/EBP-α is primarily a regulator of genes involved in energy metabolism (46) and was recently shown to be critical for energy homeo-
C/EBP-α is abundant in the liver and adipose tissue (48). In murine 3T3-L1 preadipocytes and various murine fibroblast lines C/EBP-α is both necessary and sufficient for the induction of adipogenesis (49–51). The role of C/EBP-α in the acute phase response is a complex one. Although C/EBP-α was shown to transactivate the serum amyloid protein A3 gene promoter in hepatoma cells, cytokine treatment led to a down-regulation of C/EBP-α activity with a concomitant increase in C/EBP-β and C/EBP-δ binding activities (27). An inverse correlation between C/EBP-α levels and the acute phase response was also seen with respect to the regulation of the α1-antitrypsin gene (25, 52). Besides the

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**Fig. 5.** Activation of 3×NF-κBmediated Transcription in F9 cells. F9 cells were co-transfected with 10 μg of the 3×NF-κBCAT reporter plasmid and various expression plasmids, as indicated. Numbers in parentheses denote μg of plasmid DNA/culture. Cell lysates were prepared 48 h after transfection and analyzed for CAT activity. A, co-transfections were performed with expression plasmids pMSV-C/EBP-α alone, pCMV-p50 alone, or their combinations as indicated. B, co-transfection with 1 μg of the C/EBP-α expression plasmid and/or indicated amounts of expression plasmid for p52. C, co-transfection with expression plasmids for C/EBP-α and/or p65. D, co-transfection with expression plasmids for C/EBP-α and p50 or p65, and their combinations.

**Fig. 6.** Effect of IκB on NF-κB p65- or C/EBP-α-induced transactivation in FS-4 cells. FS-4 cells were transfected with the 3×NF-κBCAT reporter plasmid (10 μg) and combinations of pCMV-p65 (1 μg) or pMSV-C/EBP-α (1 μg) with 10 μg of expression plasmid pSVK3-IκBα. At 24 h after transfection cell lysates were prepared and analyzed for CAT activity. Control represents CAT activity in cells transfected with the reporter plasmid alone.
liver and adipose tissues, high levels of C/EBP-α were also found in myelomonocytic cells and in granulocytes, but its functional role in these cells has not been analyzed (53).

Transactivation of 3xNF-κB by C/EBP-α was demonstrated in FS-4 fibroblasts but not in F9 embryonic carcinoma cells (Fig. 5). Earlier studies have demonstrated the absence of NF-κB activity in undifferentiated F9 cells, as demonstrated by a lack of nuclear protein binding to κB DNA probes or failure of κB-mediated transcriptional activation (33, 42). F9 cells were also found to be deficient in some other transcription factor activities, e.g., transcriptional activation through the cAMP-response element (CRE) was blocked in F9 cells due to the absence of functional protein kinase A activity (43, 45). We confirmed the absence of activable NF-κB in F9 cells by demonstrating that a variety of stimuli failed to activate the transfected 3xNF-κBACAT construct (Fig. 4). FS-4 and F9 cells also differed in the transactivation of 3xNF-κBACAT by various exogenously provided NF-κB proteins: transfection with p50 or p52 alone activated this construct in FS-4 cells (Figs. 1 and 3), but not in F9 cells (Fig. 5). While p65 was about equally stimulatory in FS-4 and F9 cells, a cooperative effect of p50 with p65 was seen in F9 but not in FS-4 cells (Figs. 2, 3, and 5). In addition, co-transfection of C/EBP-α with p65 seemed to have a stronger inhibitory effect in F9 than in FS-4 cells. Hence, FS-4 and F9 cells differ in the makeup of endogenous factors that can affect the function of exogenously provided NF-κB and C/EBP proteins. Earlier we showed that EMSAs with extracts from unstimulated FS-4 cells gives rise to two NF-κB-specific bands, one composed of p50 homodimers and one comprising p50/p65 heterodimers (32). The pattern seen in Fig. 7 (especially panel A, lane 1) is in agreement with these earlier data.

The most likely interpretation of the observed transcriptional activation of the 3xNF-κBACAT construct by C/EBP-α in FS-4 cells is that C/EBP-α stimulates NF-κB-regulated expression through the formation of a heteromeric complex with nuclear protein factors preexisting in FS-4 cells. Alternatively, transfection with C/EBP-α stimulates de novo synthesis of protein(s) responsible for the transcriptional activation in FS-4 cells, but this notion is inconsistent with results of EMSAs, which show that nuclei of unstimulated FS-4 cells contain proteins capable of forming specific complexes with recombinant C/EBP-α in the presence of the NF-κB probe. One possible candidate for complex formation with C/EBP-α was p65 because a recent report showed that a heterodimer of p65 and C/EBP-δ acts as a potent activator of transcription from both NF-κB and C/EBP sites (17). However, we found no evidence for a role of p65. First, there was only antagonism between p65 and C/EBP-α when the two factors were co-transfected into cells (Figs. 3 and 5). Second, no evidence for the presence of p65 protein in the complexes formed by FS-4 cell nuclear proteins with recombinant C/EBP-α could be obtained in EMSAs with antibody to p65 (Fig. 7C). On the other hand, results obtained with two different antibodies specific for the p50 NF-κB protein suggest the presence of p50 in one of the four complexes formed between recombinant C/EBP-α and nuclear proteins from FS-4.
cells (Fig. 7C). Yet, co-transfection of p50 and C/EBP-α showed no cooperative interaction either in FS-4 (Fig. 2) or in F9 cells (Fig. 5). Failure of antibodies to p52, RelB, and c-Rel (data not shown) to interfere with the appearance of any of the four bands formed between recombinant C/EBP-α and nuclear proteins from unstimulated FS-4 cells suggests that other common NF-κB proteins are probably not present in these complexes. In addition, the failure of transfected IκB to significantly inhibit the stimulatory effect of C/EBP-α (Fig. 6) and the failure of p52 to synergize with C/EBP-α in the activation of 3xNF-κB CAT (Figs. 2 and 5B) also argue against the involvement of other NF-κB proteins.

The fact that four different complexes were formed in EMSAs when recombinant C/EBP-α was mixed with nuclear proteins from FS-4 cells (Fig. 7) suggests the presence of multiple interacting proteins. Besides NF-κB proteins, C/EBP proteins can heterodimerize with a variety of other transcription factors, including c-Fos, c-Jun (13), C/ATF (54), and the glucocorticoid receptor (18). It is conceivable that complex formation between C/EBP-α and these or some other transcription factor(s) would alter the binding specificity of the resulting complex, which then could recognize “nonspecific” sites. This notion finds support in the recent demonstration that the transcription factor CHOP can dimerize with other C/EBP proteins and that the resulting dimers are directed away from classical C/EBP sites recognizing instead other specific DNA binding domains (12). Also possibly related is the reported increase in the binding of upstream stimulatory factor (USF) to the USF element after overexpression of C/EBP-α (but not C/EBP-β), implicated in the autostimulatory effect of C/EBP-α on its own promoter, which lacks any C/EBP binding domain (55). Since there was no accompanying increase in USF synthesis, C/EBP-α is likely to increase USF binding by forming a complex with USF. In a somewhat similar manner, in our system C/EBP-α does not bind to 3xNF-κB CAT, yet it stimulates transcription from this construct, most likely by complexing with, and thereby altering the specificity and binding affinity of, some other nuclear factor(s) present in unstimulated cells. Our findings establish a mechanism whereby C/EBP-α may cause activation of some κB element-containing genes that lack C/EBP binding sites.