C/EBPβ Regulation of the Tumor Necrosis Factor α Gene

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Abstract

Activated macrophages contribute to chronic inflammation by the secretion of cytokines and proteinases. Tumor necrosis factor α (TNFα) is particularly important in this process because of its ability to regulate other inflammatory mediators in an autocrine and paracrine fashion. The mechanism(s) responsible for the cell type–specific regulation of TNFα is not known. We present data to show that the expression of TNFα is regulated by the transcription factor C/EBPβ (NF-IL6). C/EBPβ activated the TNFα gene promoter in cotransfection assays and bound to it at a site which failed to bind the closely related protein C/EBPα. Finally, a dominant-negative version of C/EBPβ blocked TNFα promoter activation in myeloid cells. Our results implicate C/EBPβ as an important regulator of TNFα by myelomonocytic cells. (*J. Clin. Invest.* 1994. 94:1449–1455.) Key words: transcription factor • cytokine • promoter • inflammation

Introduction

Activated macrophages produce an array of inflammatory cytokines including tumor necrosis factor α (TNFα), interleukins-1, -6, and -8, macrophage chemotactic protein-1, and macrophage inflammatory protein 1α (MIP-1α)[1] (1–6). Many of these cytokines are capable of both attraction and activation of additional inflammatory cells and induction of angiogenesis (7). In addition, in chronic inflammation, such as rheumatoid arthritis, macrophages contribute directly to joint destruction by producing proteases, such as collagenase (matrix metalloproteinase 1) and stromelysin (matrix metalloproteinase 3) (8, 9). TNFα may play a crucial role in the inflammatory process, since it is capable of inducing cytokines and matrix metalloproteinases in an autocrine and paracrine fashion (10, 11).

A variety of stimuli can induce cells to produce TNFα, including infection by viruses or other agents, interaction with adhesion molecules, or treatment with phorbol esters, endotoxins, or cytokines such as IL-1 or even TNFα itself (12–14). Examination of the upstream promoter region of the human TNFα gene has revealed both cell type– and stimulus-specific regulatory elements, including potential binding sites for the transcription factors NF-κB and AP-1 (15–22). Although many cell types including lymphocytes, smooth muscle cells, mast cells, and others are capable of TNFα synthesis, activated monocytes/macrophages are the principal source of TNFα (12). Nonetheless, little is known about how the TNFα gene is regulated in a cell type–specific fashion.

C/EBPβ (CCAAT/enhancer binding protein β, also called NF-IL6, NF-M, LAP, IL6-DBP, AGP/EBP, and CRP2) is a member of a family of transcription factors termed bZip proteins which have a basic DNA-binding domain linked to a leucine zipper dimerization motif. It is closely related to the founding member of this family, C/EBPα, which has been shown to be intimately involved in the regulation of adipocyte differentiation (23). Earlier studies identified monocytes and macrophages as the principal source of C/EBPβ (24, 25). Cell type–specific gene regulation by C/EBPβ has been shown to depend on interactions with other transcription factors, such as NF-κB, c-*jun*, the glucocorticoid receptor, and the nuclear oncoprotein v-Myb (26–29). In addition, binding sites for C/EBPβ have been identified in the promoters for a large number of cytokine genes which are expressed in myeloid cells, including the genes for interleukins-1, -6, and -8, MIP-1α, and chicken monocyte growth factor (cMGF) (24, 30–35), suggesting that the factor may be an important activator of inflammation and of growth regulatory genes. Finally, ectopic expression of Myb and C/EBPβ has been shown to induce the expression of myeloid-specific genes in heterologous cell types, implicating C/EBPβ as a key regulator of myeloid cell differentiation (27).

In this paper, we have investigated the relationship between C/EBPβ activity and TNFα expression. We identified a specific binding site for C/EBPβ in the TNFα gene promoter, suggesting that C/EBPβ directly regulates the expression of the cytokine. We tested this in cotransfection assays and found that C/EBPβ, more strongly than C/EBPα, activated a TNFα promoter–reporter construct. Furthermore, ectopic expression of a dominant-negative form of C/EBPβ was able to block activation of the TNFα promoter by endogenous transcription factors. Our results demonstrate that C/EBPβ is an important regulator of the TNFα gene and that it is responsible for the cell type–specific regulation of the TNFα gene in myelomonocytic cells.

Methods

Cell lines. The human myelomonocytic cell lines U937 and THP-1 and the Jurkat human T cell line were obtained from the American Type

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1. *Abbreviations used in this paper:* c, chicken; C/EBP, CCAAT/enhancer binding protein; cMGF, chicken monocyte growth factor; CMV, cytomegalovirus; DN, dominant-negative; EMSA, electrophoretic mobility shift assay; h, human; MIP-1α, macrophage inflammatory protein 1α; r, rat.

Culture Collection (Rockville, MD) and were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine, penicillin, and streptomycin. HD-11, an MC29 virus (v-myc oncogene) transformed chicken macrophage cell line, was maintained in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum, 3% chicken serum, glutamine, and antibiotics as above.

Plasmids. The human C/EBPβ expression vector (hC/EBPβ), which expresses the NF-IL6 cDNA under control of the cytomegalovirus (CMV) promoter (from S. Akira, Osaka University, Japan), has been described (24), as have similar plasmids that express full-length chicken C/EBPβ (cC/EBPβ, also referred to as NF-M) and the 5D229 dominant-negative deletion mutant (27, 31). The latter plasmid expresses a protein having an NH_2-terminal deletion so that only the last 229 amino acids remain. This protein is 92% identical and 99% homologous to its human counterpart, and the COOH-terminal 212 amino acids, representing the DNA binding and leucine zipper domains, are 99% identical. The TNFα promoter–luciferase reporter plasmids have been described (15, 22). The pCDM8 expression vector plasmid was purchased from Invitrogen (San Diego, CA).

Transfections and reporter gene assays. Jurkat T cells were transfected using DEAE-dextran as follows. On the day before transfection, the cells were split to $\sim 0.7 \times 10^6$ cells/ml. For each sample, $\sim 1 \times 10^7$ cells were washed twice, then resuspended in 2 ml STBS (25 mM Tris/HCl, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2PO_4, 0.7 mM $CaCl_2$, and 0.5 mM $MgCl_2$) containing 750 μg/ml DEAE-dextran and a total of 15 μg of CsCl-purified plasmid DNA (specific plus carrier), for 30 min at 37°C, with gentle inversion every 5 min. The cells were then collected by centrifugation, resuspended in fresh medium, and allowed to recover for 3–5 h before treatment with 20 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) as indicated. Cells were harvested 18–24 h later. The U937 cells were transfected by electroporation as described previously (15), using an electroporation apparatus from Invitrogen. DEAE-dextran–mediated transfection of HD-11 cells and luciferase assays have been described (27, 31). All transfection results were normalized for protein content, as determined using an assay reagent from Bio-Rad Laboratories (Richmond, CA). All experiments were performed a minimum of three times, and similar results were obtained using independent preparations of DNA.

Electrophoretic mobility shift assays (EMSAs). Nuclear extracts were obtained from U937, THP-1, and Jurkat cells as described (36). All buffers were supplemented with PMSF (20 μg/μl), aprotinin, pepstatin A, and leupeptin (each 5 μg/ml). For expression in insect cells, the cC/EBPβ CDNA was transferred to the transfer vector pVL1392 and transfected into Sf9 cells along with linearized Baculovirus DNA using a kit from PharMingen (San Diego, CA). The resulting virus was used to infect new Sf9 or High-5 cells, and nuclear extracts were prepared 2 d later as follows. All buffers were chilled and supplemented with protease inhibitors as described above. The cells were collected, washed twice with PBS, and resuspended in Tris-X buffer (20 mM Tris-Cl, pH 7.3, 50 mM KCl, 1 mM EDTA, 1.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$) at a concentration of 1×10^7/ml. The cells were lysed by adding NP-40 to a final concentration of 0.5%, then the nuclei were recovered by centrifugation, resuspended at fourfold higher concentration of Tris-X buffer plus 0.3 M KCl, left 5 min on ice, and then centrifuged again. The supernatant was diluted with three volumes of Tris-X buffer (to make 0.085 M KCl final), and centrifuged again, and the resulting nuclear extract was stored at −80°C. Bacterially expressed, purified rat C/EBPβ (rC/EBPβ) and rat C/EBPα (rC/EBPα) proteins were generously provided by P. Johnson (National Institutes of Health, Bethesda, MD). Bacterially expressed human NF-κB1 (p50) was purchased from Promega Corp. (Madison, WI).

The methods for radiolabeling the double-stranded oligonucleotide probes using Klenow polymerase and [α-^{32}P]dCTP and for performing the EMSAs have been described (27). Where indicated, antibodies or excess unlabeled competitor oligonucleotides were incubated with the proteins or nuclear extracts for 20 min on ice before the addition of the probe. The samples were then incubated for an additional 20 min before electrophoresis on nondenaturing polyacrylamide gels. Antibodies specific for hC/EBPβ, cC/EBPβ, and rC/EBPβ were obtained from S. Akira, A. Leutz, and P. Johnson, respectively.

Synthetic oligonucleotides. The following synthetic oligonucleotides were used to generate the −130/+35 TNFα radiolabeled EMSA probe using the polymerase chain reaction: forward, 5′-GGCCCACTACCG-CTTCCTC-3′; reverse, 5′-CCTCTGCTGTCCTTGCTGA-3′. The following radiolabeled oligonucleotides were used as probes in EMSAs: the −100/−74 C/EBPβ binding site of the TNFα promoter, 5′-TCG-ACATGGGTTTCTCCACCAAGGAAGTTTTC-3′, 3′-GTACCCAA-AGAGGTGGTTCCTTCAAAAGAGCT-5′, the C/EBPβ binding sequence from the IL-6 promoter (24), 5′-TCGAGACATTGCACA-ATCTG-3′, 3′-CTGTAACGTCTTAGACAGCT-5′, and the Ig/HIV promoter binding sequence for NF-κB (37), 5′-TCGAGAAGGGAC-TTTCCGCTGGGGACTTTCCAGGGC-3′, 3′-CTTCCCTGAAAGG-CGACCCCTGAAAGGTCCCGAGCT-5′.

DNaseI footprinting. The −615 TNFα–luciferase plasmid was digested with XhoI, radiolabeled by end-filling using Klenow polymerase and [α-^{32}P]dCTP, and then further digested with HindIII. The resulting fragment containing the TNFα promoter was purified after agarose gel electrophoresis using Geneclean (Bio101) and subjected to DNaseI footprinting as described (38).

Results

C/EBPβ activates the TNFα promoter. The TNFα promoter has been shown to be strongly activated by treatment with PMA (15, 22), an activator of protein kinase C which also activates C/EBPβ activity (31, 39). We used transient transfection assays to determine whether C/EBPβ could cooperate with PMA treatment to activate the TNFα promoter. We chose the Jurkat human T cell line, which normally does not express either C/EBPβ or TNFα, under the conditions used in our assays (data not shown). A series of TNFα promoter–luciferase reporter gene constructs, containing portions of the promoter extending from just downstream to several hundred nucleotides upstream of the transcription start site (22), were transfected into Jurkat cells either with a control plasmid or with a plasmid expressing hC/EBPβ, under the control of the constitutive CMV promoter. Parallel experiments were performed with and without PMA treatment. As shown in Fig. 1, none of the TNFα promoter constructs were expressed at significant levels in the absence of PMA treatment, even in the presence of hC/EBPβ, and none were significantly activated by PMA treatment alone. However, PMA treatment of cells expressing C/EBPβ led to strong activation of all but the shortest promoter constructs tested (−36 bp from the transcription start site), suggesting that PMA and C/EBPβ acted cooperatively to induce TNFα promoter activity. In addition, the data suggest that crucial PMA- and C/EBPβ-responsive elements must lie in the region bounded by positions −95 and −36 in the TNFα promoter. The promoter construct extending only to position −95 was consistently less active than the longer versions (−120 and −295), suggesting that an important regulatory element(s) might lie near the −95 site in the promoter.

C/EBPβ binds a specific site in the TNFα promoter. The experiments described above suggested that C/EBPβ could regulate the TNFα promoter. An EMSA was used to determine whether the promoter contained a bona fide C/EBPβ binding site. Based on our results with the promoter–reporter gene constructs (see above), we generated a radiolabeled fragment of the TNFα promoter bounded by positions −130 to +35 relative to the transcription start site. Fig. 2 *A* shows an EMSA experiment in which such an oligonucleotide was efficiently bound

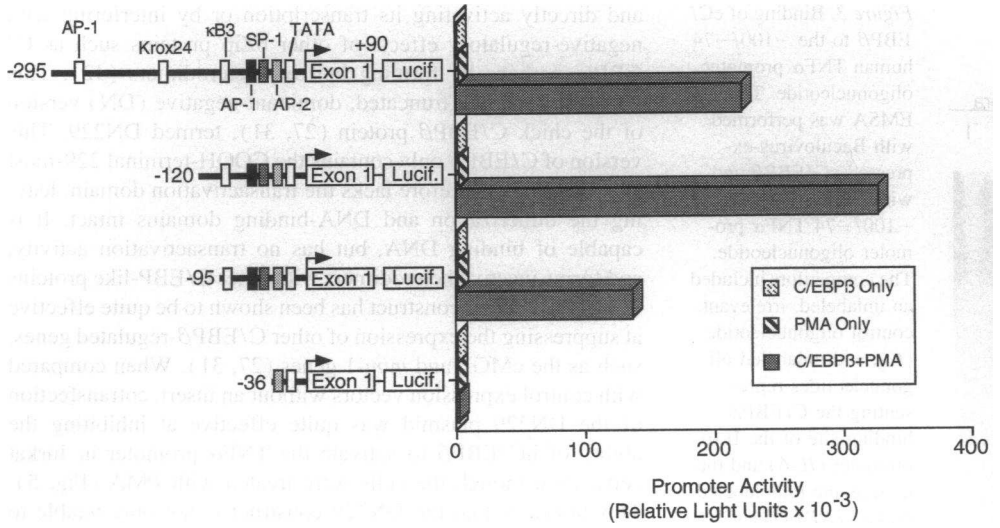

Figure 1. C/EBPβ activates the TNFα promoter. Transfections of Jurkat T cells were performed as described in Methods, using 0.5 μg of the hC/EBPβ plasmid and 3 μg of the indicated TNFα promoter reporter construct. On the left of the bar graphs are diagrammed the TNFα reporter–luciferase constructs used. Potential binding sites for a number of transcription factors are indicated by labeled boxes. The relative light units have been corrected for the total protein in the cell lysates. Similar results have been obtained in > 10 experiments and with different plasmid preparations.

by insect cell–expressed chicken C/EBPβ (cC/EBPβ). Binding was inhibited completely by the addition of excess oligonucleotide containing the C/EBPβ-binding site from the human IL-6 promoter, and the addition of antibodies specific for cC/EBPβ resulted in a "supershift" of the protein–DNA complex (not shown), demonstrating the specificity of the binding reaction. Similar complexes were also formed using nuclear extracts from the human myeloid cell line THP-1 (not shown). These results suggest that C/EBPβ could play a direct role in the regulation of the TNFα promoter.

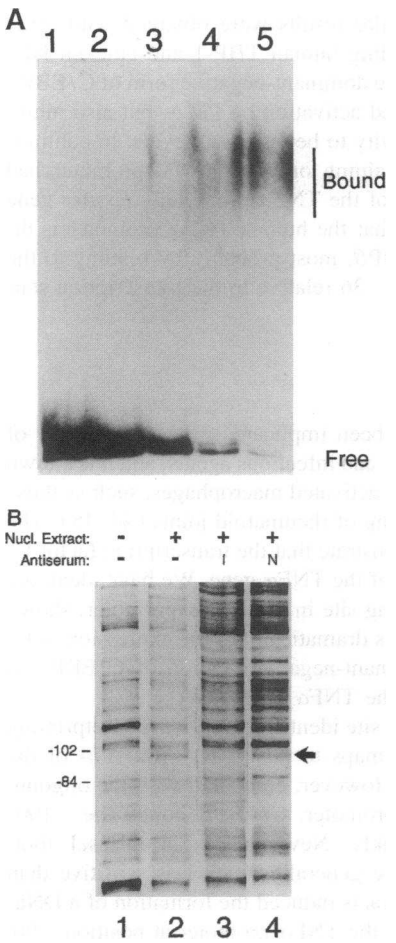

Figure 2. C/EBPβ binds to the human TNFα promoter. (*A*) Binding of C/EBPβ to the −130/+35 TNFα promoter oligonucleotide. Lanes *1–5* represent increasing quantities (0.5–3 μl) of cC/EBPβ, which was expressed in Baculovirus-infected insect cell nuclear extracts. The positions of free and C/EBPβ-bound oligonucleotides are indicated. (*B*) DNaseI footprinting of the TNFα promoter. Binding and footprint reactions are described in Methods. The lanes that received the THP-1 nuclear extracts (*Nucl. Extract*), the immune anti-hC/EBPβ (*I*), or control nonimmune serum (*N*) are indicated at the top of the figure. The −102 and −84 represent the base pair distance 5′ of the transcription start site determined using size standards (not shown). The DNaseI hypersensitive site, located at −90 bp from the transcription start site, is identified by the arrow on the right.

A DNaseI footprint assay was used to more precisely localize the binding sites for C/EBPβ on the TNFα promoter. However, as shown in Fig. 2 *B*, the addition of THP-1 nuclear extracts to an end-labeled fragment of the TNFα promoter resulted in the formation of a new DNaseI hypersensitive site at position −90 relative to the start site of transcription, rather than a discrete protected region. These results suggest that the extracts contained a protein or complex that specifically interacted with this region of the promoter. Addition of antibodies specific for hC/EBPβ, but not nonimmune serum, blocked the hypersensitive site formation, indicating that C/EBPβ, or a protein complex containing C/EBPβ, was responsible for this change in the DNaseI sensitivity of the promoter.

Two potential C/EBPβ-binding sites, in opposite orientations, were noted extending from −97 to −79 bp relative to the transcription start site of the TNFα promoter, which conformed at 9 of 12 positions to the consensus C/EBPβ-binding site, T(T/G)NNGNAA(T/G) (24). As shown in Fig. 3, insect cell–expressed cC/EBPβ protein bound efficiently to a radiolabeled, synthetic 26-mer oligonucleotide containing this site. Again, C/EBPβ binding was inhibited completely when an excess of either unlabeled oligonucleotide used as the probe or one containing a previously identified C/EBPβ-binding site from the human IL-6 gene promoter was included. The complex was not inhibited by an unlabeled NF-κB binding oligonucleotide representing the Ig/HIV promoter (not shown), even though the oligonucleotide used as the probe contained a previously characterized binding site for NF-κB (data not shown; 17). Addition of antibodies specific for cC/EBPβ, but not control antibodies, resulted in a supershift of the protein–DNA complex. Similar results have also been obtained using recombinant, purified rat C/EBPβ (rC/EBPβ) (Fig. 4 *A;* data not shown). Although C/EBPβ bound quite efficiently to the TNFα promoter–derived binding site, the oligonucleotide derived from the IL-6 promoter was a better competitor when the −100/−74 TNFα oligonucleotide was used as probe, suggesting that the binding site in the TNFα promoter was relatively weak. We conclude that C/EBPβ regulates the human TNFα gene pro-

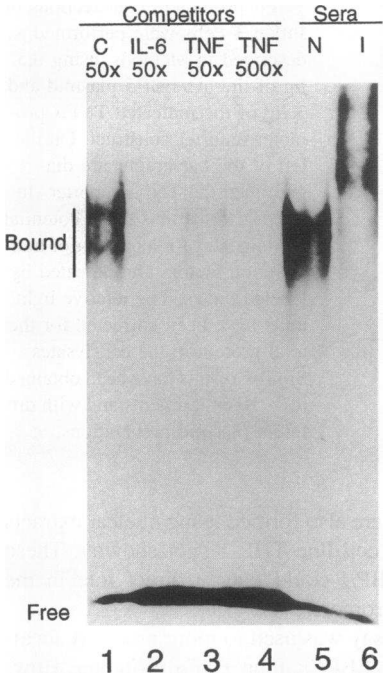

Figure 3. Binding of cC/EBPβ to the $-100/-74$ human TNFα promoter oligonucleotide. The EMSA was performed with Baculovirus-expressed cC/EBPβ and with the radiolabeled $-100/-74$ TNFα promoter oligonucleotide. The competitors included an unlabeled, irrelevant, control oligonucleotide (*C*), and unlabeled oligonucleotides representing the C/EBPβ-binding site of the IL-6 promoter (*IL-6*) and that used as the radiolabeled probe (*TNF*) (lanes *1–4*). These competitors were used at 50- or 500-fold excess as indicated in the figure. Nonimmune (*N*) or immune (*I*), anti-cC/EBPβ, rabbit antisera were included where indicated (lanes *5* and *6*).

moter by binding directly to a specific site and activating its transcription.

Selectivity of binding to $-100/-74$ TNFα oligonucleotide. C/EBPβ and C/EBPα are both expressed in macrophage cell lines and are capable of heterodimerization and of binding to the same oligonucleotides (40–42). We next examined the ability of C/EBPα and C/EBPβ to interact differently with the TNFα promoter. Using an EMSA, purified recombinant C/EBPβ and C/EBPα both bound strongly to the oligonucleotide derived from the IL-6 promoter (Fig. 4 *A*). Binding by both isoforms to the IL-6 probe was inhibited by excess unlabeled IL-6, but not TNFα, promoter oligonucleotide. Similar results have been observed previously with oligonucleotides derived from the cMGF promoter (31, 43). In contrast, only C/EBPβ bound to the $-100/-74$ TNFα oligonucleotide, suggesting that the site was specific for only one member of this highly related family of bZip proteins (Fig. 4 *A*).

A cotransfection assay was used to test whether both bZip proteins could activate the TNFα promoter. Jurkat T cells were cotransfected with TNFα or cMGF promoter–reporter constructs, plus either C/EBPβ or C/EBPα expression vectors. Transfected cells were then treated with PMA. Both transcription factors activated the cMGF promoter to approximately equal levels (Fig. 4 *B*). In contrast, C/EBPβ was a stronger activator of the TNFα promoter than C/EBPα, consistent with its stronger binding in the EMSA. When C/EBPα was coexpressed with C/EBPβ, the activation of the TNFα promoter was comparable with that observed with C/EBPβ alone, suggesting either that C/EBPα/β heterodimers were as active as C/EBPβ homodimers or that C/EBPβ was somehow dominant.

Dominant-negative C/EBPβ inhibits the TNFα promoter. Overexpression of C/EBPβ could affect the regulation of the TNFα promoter in two ways, either by binding to the promoter and directly activating its transcription or by interfering with negative-regulatory effects of other bZip proteins such as C/EBPα, perhaps by forming inactive heterodimers (42). This was tested using a truncated, dominant-negative (DN) version of the chick C/EBPβ protein (27, 31), termed DN229. This version of C/EBPβ only contains the COOH-terminal 229-most amino acids. It therefore lacks the transactivation domain, leaving the dimerization and DNA-binding domains intact. It is capable of binding DNA, but has no transactivation activity, and forms inactive heterodimers with other C/EBP-like proteins (31). The DN229 construct has been shown to be quite effective at suppressing the expression of other C/EBPβ-regulated genes, such as the cMGF and *mim*-1 genes (27, 31). When compared with control expression vectors without an insert, cotransfection of the DN229 plasmid was quite effective at inhibiting the ability of hC/EBPβ to activate the TNFα promoter in Jurkat cells, even though the cells were treated with PMA (Fig. 5). This indicates that the DN229 construct is not only unable to activate the TNFα promoter, but that expression of a truncated form of C/EBPβ blocks activation by the full-length protein.

A similar experiment was performed using the human myelomonocyte cell line U937, which expresses endogenous C/EBPβ protein and secretes a high concentration of TNFα in response to PMA (data not shown) to determine whether ectopically expressed DN229 could likewise block the expression of the TNFα promoter in myeloid cells. Fig. 6 shows that the TNFα promoter construct extending to position -120, the construct that consistently provided the greatest activation, was moderately active when transfected into U937 cells and that its expression was stimulated three- to fivefold when the cells were treated with PMA. (Similar results were obtained with other myeloid cell lines, including human THP-1 and chicken HD-11 cells.) Expression of the dominant-negative form of C/EBPβ (DN229) not only blocked activation by PMA, but also inhibited TNFα promoter activity to below basal levels. In contrast, the control plasmid containing only the CMV promoter had no effect on the activity of the TNFα promoter–reporter gene construct. We conclude that the human TNFα promoter is directly regulated by C/EBPβ, most probably by binding to the region between -95 and -36 relative to the transcription start site.

Discussion

Activation of TNFα has been implicated in a wide variety of responses to inflammatory and infectious agents, and it is known to be highly expressed by activated macrophages, such as those found in the synovial lining of rheumatoid joints (44, 45). The results in this paper demonstrate that the transcription factor C/EBPβ is a key regulator of the TNFα gene. We have identified a specific C/EBPβ-binding site in the TNFα promoter, shown that the promoter responds dramatically to the expression of C/EBPβ, and used a dominant-negative version of C/EBPβ to inhibit the activation of the TNFα promoter.

The C/EBPβ-binding site identified by DNaseI footprinting and EMSA experiments maps to positions $-100/-74$ of the human TNFα promoter. However, compared with the oligonucleotide from the IL-6 promoter, C/EBPβ bound the $-100/-74$ site relatively weakly. Nevertheless, in DNaseI footprinting assays, which are generally much less sensitive than EMSAs, myeloid cell extracts induced the formation of a DNaseI hypersensitive site on the TNFα promoter at position -90,

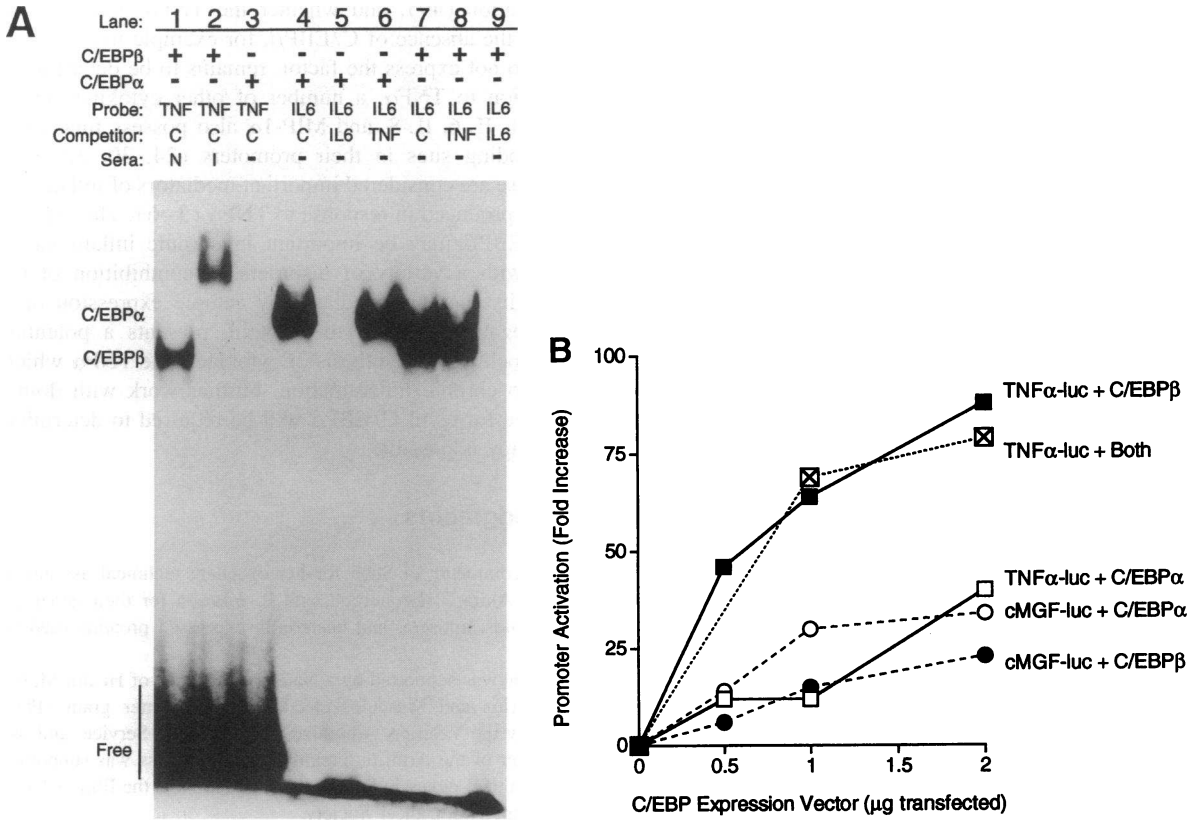

Figure 4. The TNFα promoter is specific for C/EBPβ. (*A*) Selective binding of C/EBPβ, but not C/EBPα, to the −100/−74 TNFα promoter. The EMSA was performed with recombinant, purified rC/EBPα (lanes *3–6*) and rC/EBPβ (lanes *1, 2,* and *7–9*), as indicated at the top of the figure. The radiolabeled probes, which included the C/EBPβ-binding site of the IL-6 promoter (*IL-6*) and the −100/−74 TNFα promoter (*TNF*) oligonucleotides, and the competitor oligonucleotides, used at 100-fold excess, are also indicated at the top of the figure (symbols as in Fig. 3). Nonimmune (*N*) or monospecific, immune anti-rC/EBPβ (*I*) sera were added where indicated. The mobilities of the C/EBPα and C/EBPβ bound and the free, unbound radiolabeled −100/−74 TNFα oligonucleotide are indicated on the left of the figure. (*B*) C/EBPβ preferentially activates the TNFα promoter. Jurkat cells were transfected as described in Methods. They were cotransfected with the TNFα-luciferase (3 μg) or with cMGF-luciferase (4.5 μg) constructs and with the hC/EBPβ or the rC/EBPα expression vectors in the concentrations indicated at the bottom of the figure. Where indicated, both C/EBPα and C/EBPβ were cotransfected at equal concentrations. The fold increase represents the fold increase over baseline induced by PMA. These experiments were repeated twice with similar results.

and this activity was inhibited by C/EBPβ-specific antisera. Thus, in the context of a nuclear extract, the C/EBPβ appears to interact with a relatively high affinity for the promoter, suggesting that the factor may be part of a multiprotein complex which binds the TNFα promoter in myeloid cells.

The TNFα promoter was differentially recognized by C/EBPβ and C/EBPα. Purified recombinant C/EBPβ, but not C/EBPα, bound to the TNFα promoter oligonucleotide in EMSA assays. Consistent with this difference, C/EBPβ was considerably more active than C/EBPα at activating the TNFα promoter construct. As a control, the cMGF promoter, which readily binds both C/EBP proteins, was activated equally well by each. Both C/EBPα and C/EBPβ are expressed in myelomonocytic cell lines, however, C/EBPα decreased while C/EBPβ increased with differentiation and activation (40, 41). These data suggest that the differential binding of the two transcription factors may be important in the regulation of TNFα in monocytes/macrophages where both bZip proteins are expressed.

The importance of the region of the TNFα promoter that contains the C/EBPβ-binding site (at position −90) has also been noted by others. For example, three copies of a 43-nucleotide-long fragment of the promoter spanning this site was sufficient to confer both elevated basal and TNFα-inducible activity to an otherwise minimally active heterologous promoter (21). More recently, a cyclosporin A– and FK506-sensitive factor in the murine T cell line Ar-5 was identified that functions through a portion of the promoter overlapping the C/EBPβ-binding site (17). Of interest, the −90 site is adjacent to a potential binding site for NF-κB (17, 24), which has been shown to interact with C/EBPβ in vitro (26, 29). In addition, both C/EBPβ and NF-κB bound to the IL-6 and the IL-8 gene promoters, and the ratio of the two factors appeared to regulate the activities of those genes (34, 46). Our own preliminary data suggest that overexpression of NF-κB1 or RelA may inhibit C/EBPβ-mediated activation of the TNFα promoter in Jurkat cells (Pope, R., and S. A. Ness, unpublished observations). Further work will be required to determine whether these two transcription factors interact to regulate the TNFα gene.

An interesting feature of TNFα gene regulation in Jurkat cells was its apparent dependence on both C/EBPβ and PMA treatment, which presumably participates by stimulating protein kinase C. PMA treatment has been shown to lead to phosphorylation of C/EBPβ, induce its translocation to the nucleus, and enhance its ability to stimulate transcription (31, 39), sug-

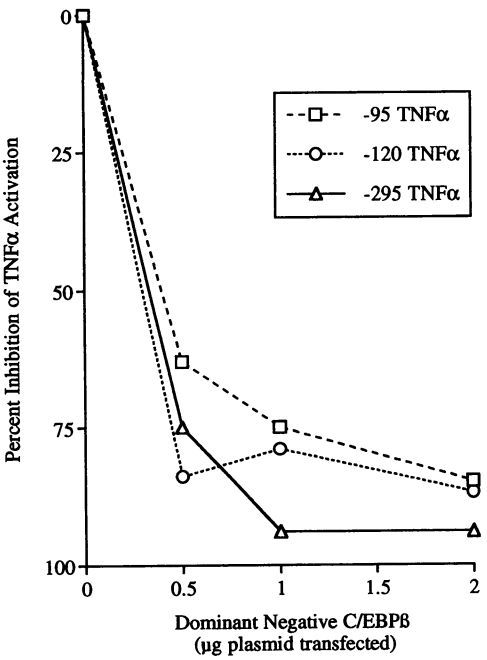

Figure 5. Dominant-negative C/EBPβ inhibits TNFα promoter activation in Jurkat T cells. These experiments were performed as described in Fig. 2 except that in addition to the TNFα reporter (3 μg) and the hC/EBPβ (0.5 μg) expression plasmids, the DN C/EBPβ (DN229) or vector (pCDM8) plasmids were cotransfected in the indicated quantities. Relative light units were corrected for the total protein in the cell lysates. Percent suppression was determined by comparison with the control vector. These experiments were repeated three times with similar results.

gesting that PMA treatment may be required simply to convert the over-expressed C/EBPβ to a more active form. Alternatively, PMA may activate other transcription factors which cooperate with C/EBPβ, such as NF-κB or c-*jun* (47, 48). Several potential PMA-responsive elements have been identified in the TNFα promoter, including binding sites for the transcription factors AP-1 and AP-2 located at positions −63 and −34, respectively (22). Cotransfection with c-*jun* enhanced and mutation of the AP-1–binding site diminished TNFα gene expression, supporting the importance of the AP-1 site (22). Whether or not interaction between C/EBPβ and c-*jun* is important in

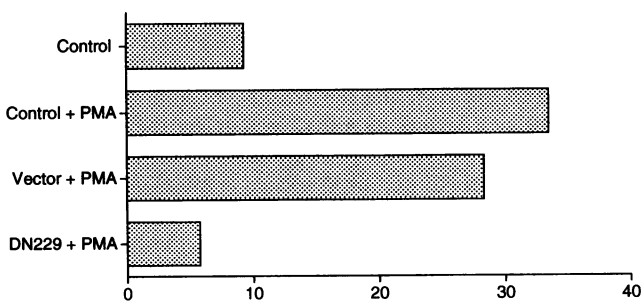

Figure 6. PMA-induced TNFα promoter activation in U937 cells is inhibited by DN C/EBPβ. The transfections were performed by electroporation as described in Methods, using 5 μg of the −120 TNFα reporter construct, and 0.5 μg of the DN C/EBPβ (DN229) or vector (pCDM8). Relative light units were corrected for the total protein in the cell lysates. These experiments were repeated four times with similar results.

TNFα activation (49), and whether the TNFα gene can be activated in the absence of C/EBPβ, for example in other cell types that do not express the factor, remains to be determined.

In addition to TNFα, a number of other cytokine genes, such as IL-1, IL-6, IL-8, and MIP-1α also possess functional C/EBPβ-binding sites in their promoters (24, 30, 32–35). Many of these are considered important mediators of inflammation and are produced in response to TNFα (3–6). The expression of C/EBPβ may be important in chronic inflammation associated with a variety of disorders. The inhibition of C/EBPβ activity, perhaps mediated by ectopic expression of a dominant-negative form of the protein, presents a potential means of blocking the synthesis of cytokines like TNFα which contribute to chronic inflammation. Further work with dominant-negative forms of C/EBPβ will be required to determine if such therapy is possible.

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