The C/EBP bZIP Domain Can Mediate Lipopolysaccharide Induction of the Proinflammatory Cytokines Interleukin-6 and Monocyte Chemoattractant Protein-1*

C/EBP{a, b, and d} are all expressed by bone marrow-derived macrophages. Ectopic expression of any of these transcription factors is sufficient to confer lipopolysaccharide (LPS)-inducible expression of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) to a B lymphoblast cell line, which normally lacks C/EBP factors and does not display LPS induction of proinflammatory cytokines. Thus, the activities of C/EBP{a, b}, and d are redundant in regard to expression of IL-6 and MCP-1. Surprisingly, the bZIP region of C/EBP{b}, which lacks any previously described activation domains, can also confer LPS-inducible expression of IL-6 and MCP-1 in stable transfectants. Transient transfections reveal that the bZIP regions of C/EBP{b, b}, and d are redundant in regard to expression of IL-6 and MCP-1. In addition, C/EBP{a} can activate the IL-6 promoter and augment its induction by LPS. Furthermore, the transdominant inhibitor, LIP, can activate expression from the IL-6 promoter. The ability of the C/EBP{b} bZIP region to activate the IL-6 promoter in transient transfections is completely dependent upon an intact NF-xB-binding site, supporting a model where the bZIP protein primarily functions to augment the activity of NF-xB. Replacement of the leucine zipper of C/EBP{b} with that of GCN4 yields a chimeric protein that can dimerize and specifically bind to a CEBP consensus sequence, but shows a markedly reduced ability to activate IL-6 and MCP-1 expression. These results implicate the leucine zipper domain in some function other than dimerization with known C/EBP family members, and suggest that C/EBP redundancy in regulating cytokine expression may result from their highly related bZIP regions.

CCAT/enhancer-binding protein (C/EBP){a, b, and d} are members of a family of basic region-leucine zipper (bZIP) transcription factors (reviewed in Ref. 1). These proteins dimerize through their leucine zippers and bind to a consensus DNA motif through their adjacent basic regions. C/EBP{b} and C/EBP{d} have both been shown to activate a reporter gene controlled by the IL-6 promoter in transient expression assays (2, 8). We have previously demonstrated that the stable expression of C/EBP{b} in a murine B lymphoblast cell line is sufficient to confer lipopolysaccharide (LPS) inducibility of IL-6 and monocyte chemoattractant protein 1 (MCP-1) expression (9).

There have been some reports demonstrating specificity of a particular C/EBP family member for a given promoter. One example is the rat CYP2D5 gene, which encodes a cytochrome P450 protein. It is transactivated cooperatively by C/EBP{b} and SP1, but not C/EBP{a} (10). The specificity of this cooperativity is determined by both the leucine zipper and activation domains of C/EBP{b} (11). Another case is promoter P1 of furin (12). It is transactivated by C/EBP{b}, but not C/EBP{a} or C/EBP{d}. Recently, it has been reported that C/EBP{d} can transactivate the promoter for nerve growth factor (13). Conversely, C/EBP{a}, b, and d are all functional in a heterologous transgenic rescue assay for a Drosophila C/EBP mutant, slow border cells (14). Our own studies have demonstrated redundancy in the abilities of C/EBP{a}, b, and d to support LPS induction of IL-6 and MCP-1 gene transcription (15). A simple hypothesis for the redundancy of C/EBP{b} that we have observed is that only their highly homologous bZIP regions (16) are required for activation of the IL-6 and MCP-1 genes. Against this hypothesis stand results obtained with a truncated form of C/EBP{b} that initiates at Met-132 and lacks activation domains. This protein, referred to as LIP, could not activate transcription and, in fact, inhibited C/EBP{b}-mediated transcriptional activation of an artificial promoter derived from the DE-I site of the albumin gene (17). In this report, we have unexpectedly found that a truncated form of C/EBP{b} lacking all known activation domains and consisting solely of the C/EBP{b} bZIP region is capable of supporting LPS induction of IL-6 and MCP-1 expression. Furthermore, the transdominant inhibitor LIP, as well as a truncated form of C/EBP{d} and, to a

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† The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; bZIP, basic region-leucine zipper; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; EMSA, electrophoretic mobility shift assay.
less extent, a truncated form of C/EBPα that similarly lack conventional activation domains have transcriptional activity on the IL-6 promoter. The transcriptional activity of both C/EBPβ and the C/EBPβ bZIP region is completely dependent on the presence of an intact NF-κB-binding site in the IL-6 promoter, implying that these proteins primarily act by augmenting NF-κB activity. Replacement of the leucine zipper of C/EBPβ with that of GCN4 yields a chimeric protein that can dimerize and specifically bind to a C/EBP consensus sequence, but has reduced ability to activate IL-6 and MCP-1. Furthermore, the majority of both full-length and truncated C/EBPβ exists as heterodimers with C/EBPγ (Ig/EBP), a C/EBP family member lacking any known activation domains. These results imply that the leucine zipper domain has a function beyond dimerization to known C/EBP family members, and that the basis for C/EBP redundancy in regulating cytokine expression resides in their highly related bZIP regions.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—P388 cells are murine B lymphoblasts (4) (American Type Culture Collection; CCL46). P388-Cβ cells and P388-Neo cells have been described previously by Hu et al. (15). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and 10 μg/ml β-mercaptoethanol. Inductions were conducted with LPS derived from Escherichia coli serotype 055:B5 (Sigma) at 10 μg/ml.

Transfections—Stable transfections were conducted with 2 × 10⁶ cells, 5 μg of DNA, and 10 μl of DMRIE-C reagent (Life Technologies) in 1.2 ml of Opti-MEM I medium (Life Technologies). Cells were incubated in the transfection mixture for 16 h followed by addition of 2.8 ml of standard growth medium. After 24 h, the medium was replaced with standard growth medium supplemented with G418 (Life Technologies) at 0.67 mg/ml.

Transient transfections were conducted with 2 × 10⁶ cells, 4 μg of DNA, and 8 μl of DMRIE-C reagent (Life Technologies) in 1.2 ml of Opti-MEM I medium (Life Technologies). The DNA was comprised of either 1 μg of IL-6 promoter-reporter or albumin DE-I promoter-reporter, 1 μg of SV40 early promoter-reporter, 0.5 μg of C/EBP expression vector, and pMEX plasmid to total 4 μg. Cells were incubated in the transfection mixture for 5 h followed by addition of RPMI 1640 medium supplemented to 15% with fetal calf serum. After 24 h, the medium of certain transfections was supplemented with 10 μg/ml LPS. After 4 h in the presence or absence of LPS, transfected cells were harvested, lysed, and analyzed for luciferase activity by using the Luciferase Reporter Gene Assay Kit (Roche Molecular Biochemicals) and for β-galactosidase activity by using the Luminescent β-Galactosidase Genetic Reporter System II (CLONTECH).

Expression Vectors and Promoter Reporters—For stable transfections, C/EBPα, β, or δ were expressed from pZIP-NEO SVXⅰⅰⅰ constructs (18). C/EBPα sequences were inserted into the BanHI site of the vector. Inserted sequences were transcribed from the Moloney murine leukemia virus promoter and the gene conferring G418-resistance was expressed from a subgenomic splicing product from the same promoter.

For transient transfections, C/EBPα, C/EBPβ, or C/EBPδ were expressed from pMEX (16), which utilizes the Moloney murine sarcoma virus promoter. The construction of C/EBPα deletions and C/EBPβδΔ19 have been described previously (16).

The IL-6 promoter-reporter consists of the murine IL-6 promoter (19) (250 to +1) inserted into the luciferase vector, pXP2 (20). The IL-6 promoter-reporter with a mutated NF-κB-binding site replaces GGAGTTTCCCT with AAGATTTTCCCT. The albumin DE-I promoter-reporter is DEI(1–350)bLUC (21) which is derived from pXP2 (20) and contains four copies of the DE-I element upstream of the albumin minimal promoter. The SV40 early promoter-reporter is a commercial product, ppgal-Control (CLONTECH), where the SV40 early promoter and enhancer sequences are cloned upstream and downstream, respectively, of the β-gal gene.

RNA Isolation and Analysis—Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s directions. RNAs were electrophoresed through 1% agarose/formaldehyde gels. Transcripts to membranes were hybridized and washed to a stringency of 0.1 × SSPE at 65 °C. Hybridization probes were prepared with a random priming kit (Life Technologies) with the incorporation of 5'-[α-32P]dATP (3000 Ci/mmol; NEN Life Science Products Inc.). The IL-6 probe was a 0.65-kilobase murine cDNA (from N. Jenkins and N. Copeland, National Cancer Institute-Frederick Cancer Research and Development Center). The MCP-1 probe was a 0.58-kilobase murine cDNA (22). The glyceraldehyde-3-phosphate dehydrogenase probe was a 1.3-kilobase rat cDNA (23).

Western Analysis—Nuclear extracts were prepared as described below. The extracts (50 μg) were adjusted to 1× Laemmli sample buffer (24) and processed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis. The gel was transferred to a Transron membrane (Schleicher and Schuell), and antigen-antibody complexes were visualized with the Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared as follows. Cells were washed in phosphate-buffered saline and lysed in 15 mM KCl, 10 mM HEPES (pH 7.6), 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000 × g for 20 s at 4 °C. Proteins were extracted from nuclei by incubation at 4 °C with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin). Nuclear debris was pelleted by centrifugation at 14,000 × g for 15 min at 4 °C and the supernatant extracted and collected at −70 °C.

The EMSA probe was a double-stranded oligonucleotide containing an optimal C/EBP-binding site (5′-GATCCAGATATCCTTGAGCCAT-3′) annealed with 5′-AAACGCTGATATCTAGT-3′ labeled with the incorporation of 5′-[α-32P]dATP (3000 Ci/mmol; NEN Life Science Products Inc.) and Klenow DNA polymerase. A probe containing a CTNF/VP-1-binding site (2′5′-GATCCTTTGGCGATCTCCGATATG-3′) annealed with 5′-AAATCGATATCTAGT-3′ was used as a nonspecific competitor in some assays. Underlined sequences correspond to the binding motifs of the specified transcription factors.

DNA binding reactions were performed at room temperature in a 25-μl reaction mixture containing 6 μl of nuclear extract (1 mg/ml in buffer C) and 5 μl of 5× binding buffer (20% (v/v) Ficoll, 50 mM HEPES (pH 7.9), 5 mM EDTA, 5 mM dithiothreitol). The remainder of the reaction mixture contained 1 μg of poly(dI-dC), 1.25 ng of probe, bromophenol blue to a final concentration of 0.06% (v/v), and water to volume. For supershifts, nuclear extracts were preincubated with antibodies for 30 min at 4 °C prior to the binding reaction. Samples were electrophoresed through 5.5% polyacrylamide gels in 1× TBE (90 mM Tris base, 90 mM boric acid, 0.5 mM EDTA) at 160 V.

Antibodies—Rabbit anti-C/EBPα specific to the carboxyl terminus (product C-19) and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Rabbit anti-C/EBPβ specific to the amino terminus has been described (16). Rabbit anti-C/EBPγ (IgG) was prepared against a synthetic peptide corresponding to the carboxyl terminus of C/EBPγ and will be described elsewhere.²

RESULTS

Ectopic Expression of the bZIP Domain of C/EBPβ—We previously found that C/EBPα, β, and δ were all effective in supporting the LPS-induced transcription of the genes encoding IL-6 and MCP-1 (15). To test whether this redundancy was based on the bZIP domain which is highly conserved among these C/EBP family members (16), we examined the expression of a truncated form of C/EBPβ (amino acids 192–276; C/EBPβ192–276) that lacks all conventional activation and regulatory domains and simply consists of the bZIP domain of C/EBPβ (21) (Fig. 1). It was expected that the activity of C/EBPβ192–276 would be similar to that of LIP, which initiates at Met-132 and lacks transcriptional activation domains (17) (Fig. 1).

We performed stable transfections of P388 cells with a murine retroviral vector expressing C/EBPβ192–276 and compared those transfectants to P388-Cβ cells that had been transfected with a vector expressing C/EBPβ, as well as a control population transfected with the same vector lacking an expressed insert (P388-Neo) (9, 15). P388 is a murine B lymphoblastic cell line (26) that lacks C/EBPα, C/EBPβ, C/EBPδ, and C/EBPγ.

² S. Parkin, M. Baer, and P. F. Johnson, unpublished data.
expression and has been a useful system for analyzing C/EBP protein function (9, 15). The population of cells transfected for C/EBPβ192–276 expression was designated P388-Cbp192–276.

EMSA of nuclear extracts from transfected populations, as well as Western blot analyses, verified proper expression of stably transfected C/EBPβ genes. In comparison to nuclear extracts from P388-Neo, nuclear extracts from P388-Cβ and P388-Cbp192–276 yielded supershifted protein-DNA complexes upon incubation with an antibody specific for C/EBPβ (Fig. 2A, lanes 5, 6, and 9). The EMSA species that gave rise to the supershifts were also evident in the samples incubated with normal IgG (Fig. 2A, lanes 4 and 7). As expected, the C/EBPβ-specific EMSA species from P388-Cbp192–276 cells were of greater mobility than those of P388-Cβ cells reflecting their truncated structure. Additionally, the C/EBPβp192–276 EMSA species could only be supershifted with antibody specific to the carboxyl terminus of C/EBPβ (Fig. 2A, lanes 8 and 9), while EMSA species of intact C/EBPβ could be supershifted by both amino and carboxyl terminus specific antibodies (Fig. 2A, lanes 5 and 6).

Western analysis of nuclear extracts from transfected populations examined actual levels of protein expression of C/EBPβ and P388-Cbp192–276 (Fig. 2B). The level of C/EBPβ192–276 expression in transfected cells was much lower than that of C/EBPβ. Interestingly, although the level of protein expression for P388-Cbp192–276 was much lower than that of intact C/EBPβ, its binding activity in the EMSA was quite high. We note that C/EBPβp192–276 has been reported to have enhanced affinity for its binding site (21).

To ensure that the supershifted species observed in Fig. 2A specifically bound to the C/EBP-binding site, competitions were performed with the addition of unlabeled C/EBP-binding site and an unlabeled CTF/NF-1-binding site to supershifted samples (Fig. 2C). All of the supershifted protein-DNA complexes observed upon incubation with C/EBPβ-specific antibody were effectively competed by a 30-fold excess of C/EBP-binding site (Fig. 2C, lanes 3 and 9) while a 100-fold excess of CTF/NF-1-binding site had little effect (Fig. 2C, lanes 6 and 12). The prominent protein-DNA complex not supershifted by specific antibody, but effectively competed by unlabeled C/EBP-binding site, was surmised to be C/EBPγ (Ig/EBP) (Fig. 1), which is highly expressed in immature B cells (27). C/EBPγ has been shown to act as a transdominant inhibitor of C/EBP family transcriptional activators (28).

To verify the expression of C/EBPγ and to ascertain whether C/EBPγ might be forming heterodimers with either C/EBPβ or C/EBPβp192–276, EMSA was performed in the presence of antibodies specific for C/EBPβ and antibodies specific for C/EBPγ (Fig. 3). Nuclear extracts from P388-Neo cells clearly showed a C/EBPγ-DNA complex that could be supershifted with C/EBPγ-specific antibody (Fig. 3, lanes 1 and 2). In nuclear extracts from P388-Cβ cells, a single C/EBPβ-DNA complex of lower mobility than that for C/EBPγ was observed and it could be supershifted both by antibodies specific to C/EBPβ and C/EBPγ (Fig. 3, lanes 4–6). Analysis of extracts from P388-Cbp192–276 cells found two C/EBPβ-DNA complexes, both more rapidly migrating than that for C/EBPγ (Fig. 3, lane 7). The more abundant of the two C/EBPβ-DNA complexes could again be supershifted by both C/EBPβ- and C/EBPγ-specific antibodies (Fig. 3, lanes 8 and 9). Surprisingly, the majority of both C/EBPβ and C/EBPβp192–276 was found to be in heterodimers with C/EBPγ (Fig. 3). Since we had previously shown that ectopic expression of C/EBPβ was sufficient to confer LPS inducibility of IL-6 and MCP-1 to P388 cells (9), it is clear that C/EBPγ is not strongly inhibitory to C/EBPβ activity on the IL-6 promoter.

A Truncated Form of C/EBPβ Lacking Activation Domains Retains the Ability to Support LPS-induced Transcription of IL-6 and MCP-1 Genes—P388-Cβ and P388-Cbp192–276 cells were treated with LPS over a time course of 0, 2, 4, 8, and 24 h, and RNA was isolated. Northern analyses were performed to detect transcripts encoding IL-6 and MCP-1. Unexpectedly, P388-Cbp192–276 cells behaved similarly to P388-Cβ cells in their ability to induce IL-6 and MCP-1 transcription in response to LPS (Fig. 4). While IL-6 and MCP-1 mRNAs were not induced to as high a level with C/EBPβp192–276 as with intact C/EBPβ and the kinetics of their expression were somewhat altered, the activity of a truncated form of C/EBPβ lacking all known activation domains is surprising. The level of C/EBPβp192–276 expression in P388-Cbp192–276 cells is much lower than that of C/EBPβ in P388-Cβ cells (Fig. 2B) making its ability to support expression of IL-6 and MCP-1 all the more remarkable. A second population of P388 cells independently transfected for expression of C/EBPβp192–276 behaved similarly (data not shown).
A Chimeric Form of C/EBP with a Heterologous Leucine Zipper Has No Activity in Stable Transfectants—Since C/EBP_192–276 retains only the DNA-binding and leucine zipper domains of C/EBP yet is still active on the IL-6 promoter, we decided to examine whether the leucine zipper domain contains determinants for activation other than those necessary for homodimerization. To that end, we tested a chimeric C/EBP protein in which the C/EBP leucine zipper was replaced with that of yeast GCN4 (C/EBP:GLZ) (16) by stable expression in P388 cells (P388-CbGLZ) (Fig. 1). C/EBP:GLZ retains wild type amino-terminal activation and regulatory domains as well as the DNA-binding domain, and has previously been shown to activate transcription from an albumin DE-I site-driven reporter (21). We verified the ability of C/EBP:GLZ to activate a DE-I promoter in P388 cells (data not shown). In contrast to that result, P388-CbGLZ cells did not induce IL-6 or MCP-1 mRNA in response to LPS (Fig. 4).

To verify that C/EBP:GLZ was properly expressed in P388-CbGLZ cells, both EMSA and Western blot analyses were performed. While a C/EBP:GLZ protein-DNA complex was not obvious in a simple EMSA, the addition of C/EBP:GLZ-specific antibodies revealed a supershifted protein-DNA complex with amino-terminal specific but not carboxyl terminus-specific antibodies (Fig. 5A, lanes 8 and 9). This result is consistent with replacement of the leucine zipper at the carboxyl terminus of the C/EBP protein. These data also demonstrate that C/EBP:GLZ retains the capacity to dimerize and bind to the optimal C/EBP-binding site. Competition experiments were performed with unlabeled C/EBP binding site and unlabeled CTF/NF-1 binding site to verify the specificity of C/EBP:GLZ binding (Fig. 5C). The supershifted protein-DNA complexes were effectively competed by a 30-fold excess of the C/EBP-binding site (Fig. 5C, lanes 3 and 9), while a 100-fold excess of the CTF/NF-1-binding site had little effect (Fig. 5C, lanes 6 and 12). Western analyses of nuclear extracts from P388-CbGLZ cells and other transfectant populations show that, while not expressed as efficiently as C/EBP, the C/EBP:GLZ protein was expressed at a level comparable to that of C/EBP_192–276.
sites of both factors (29). If this synergy is largely based on the capacity of the bZIP domain by itself to modify NF-κB activity, then the activity of C/EBPβ192–276 on the IL-6 promoter should be dependent upon NF-κB. To test this notion, we repeated the transient transfection of expression vectors for C/EBPβ and C/EBPβ192–276 with an IL-6 promoter-reporter containing a mutated NF-κB-binding site (Fig. 7). Not only was the promoter no longer LPS responsive in the presence of either C/EBPβ and C/EBPβ192–276 expression, but C/EBPβ and C/EBPβ192–276 showed a complete lack of activity in the absence of a functional NF-κB-binding site. Presumably, the activity of C/EBP isoforms on the wild type IL-6 promoter in the absence of LPS stimulation (Figs. 6–8) reflects a low level of constitutive NF-κB activity.

The expression of both C/EBPβ and C/EBPβ192–276 in P388 cells as heterodimers with C/EBPγ (Fig. 3) presents the possibility that C/EBPγ as a heterodimer with other C/EBP family members is a critical factor in activation of the IL-6 promoter. However, co-transfection of an expression vector for C/EBPγ with one for C/EBPβ192–276 did not augment the activity of an expression vector for C/EBPβ192–276 alone and was, in fact, mildly inhibitory both with and without LPS stimulation (data not shown). Thus, it is unlikely that C/EBPγ is responsible for the observed activity.

Other investigators may not have observed significant activity of C/EBPβ192–276 and LIP because they were tested on artificial promoter-reporter constructs based on the DE-1 site of the albumin promoter and were thus solely C/EBP-dependent (17, 21). We performed transient transfections in P388 cells with a multimeric albumin DE-1 site promoter-reporter and found that C/EBPβ192–276 and LIP are, in fact, inactive on the simpler DE-1 promoter, both in the presence and absence of LPS stimulation (data not shown); presumably their activity on the IL-6 promoter is dependent on interactions with other transcription factors including NF-κB that are available on the more complex promoter.

A Truncated Form of C/EBPδ Analogous to C/EBPβ192–276 Is Capable of Activating the IL-6 Promoter.—We previously showed that C/EBPα, β, and δ have virtually redundant activities in regard to the IL-6 promoter (15). These earlier results suggested that whatever structural feature allows activity of C/EBPβ192–276 might be a general feature of C/EBPs. To test this hypothesis, we performed transient transfections with vectors expressing truncated forms of C/EBPα and C/EBPδ (C/EBPδ192–276 and C/EBPδ181–272 Fig. 1) in comparison to intact C/EBPβ in P388 cells (Fig. 8). To more directly examine the contribution of the leucine zipper to C/EBP activity, we also transfected a vector expressing a truncated form of C/EBPδ in which the C/EBPβ leucine zipper was replaced with that of yeast GCN4 (C/EBPδ192–276:GLZ; Fig. 1). The transfections were performed with and without LPS stimulation, and the expression vectors were co-transfected with an IL-6 promoter-luciferase reporter. C/EBPδ192–272 was almost as active as intact C/EBPβ. LPS treatment of C/EBPδ transfectants induced luciferase expression to a mean value of 24-fold over an untreated, reporter-only control, while LPS treatment of C/EBPδ192–272 transfectants induced luciferase expression to 20-fold over the control value. C/EBPδ192–272 transfectants treated with LPS had levels of luciferase expression 7-fold over the control value and LPS treatment by itself without transfection of a C/EBP expression vector induced a mean value of only 2.6-fold over the control value.

We previously showed that LPS stimulation induces NF-κB activity in P388 cells (15). C/EBPβ and NF-κB can synergistically activate the IL-6 promoter requiring the cognate binding
Once again, LPS induction of luciferase expression was observed in either the presence or absence of C/EBP expression, although in the case of C/EBP\(\beta_{192-276}:G_{12}\) this induction is slight at best (1.4-fold). EMSA and Western blot analysis did not detect C/EBP\(\beta_{192-276}:G_{12}\) species (data not shown), so their level of expression relative to
Transcriptional Activation by bZIP Domains

Fig. 6. C/EBPβ\textsubscript{192–276} and LIP, although lacking activation domains, can activate the IL-6 promoter in transient transfections of P388 cells. Transient transfections were carried out in duplicate with and without LPS treatment. Luminometer values were normalized for expression from a co-transfected SV40 early promoter-galactosidase reporter. These values were then normalized to a relative value of 1 for the cells not receiving a C/EBP expression vector and untreated with LPS. The data presented are the mean of three experiments with their standard error.

C/EBPβ could not be assessed. C/EBPα\textsubscript{273–358} expression increases the basal level of IL-6 promoter activity without augmenting LPS induction. This modest level of activation by C/EBPα\textsubscript{273–358} is consistent with the reduced activity of intact C/EBPα observed in LPS inductions of the endogenous IL-6 promoter in stable transfectants (15).

**DISCUSSION**

The data presented in this paper demonstrate that the conventional activation domains of C/EBPβ (21) and C/EBPβ\textsuperscript{3} are dispensable for their roles in the LPS-induced activation of IL-6 and MCP-1 expression. C/EBPβ\textsubscript{192–276}, a truncated form of C/EBPβ lacking its first 191 amino acids, is capable of activating transcription of IL-6 and MCP-1 genes after LPS stimulation of both stably and transiently transfected P388 lymphoblasts. Transient transfections showed that LIP, a form of C/EBPβ lacking its first 131 amino acids, and C/EBP\textsubscript{α181–272}, a truncated form of C/EBPα, were also effective in activating the IL-6 promoter. A truncated form of C/EBPα, C/EBPα\textsubscript{273–358}, also showed modest activity.

The activity of the C/EBP bZIP domains and of LIP was unexpected. LIP has been found to possess little or no transcriptional activity (17, 21, 28, 30). Previous investigators may not have observed this activity because they used different truncations of C/EBPβ, different reporters, and/or different cell types in their transfection systems. Using an embryonic carcinoma cell line, an internally deleted form of C/EBPβ failed to activate the IL-6 promoter (29). This mutant, however, would have retained regulatory sequences that inhibit transactivation potential and mediate cell specificity (21, 31). Others have assayed LIP and C/EBP\textsubscript{β192–276} on promoter-reporter constructs based on the DE-I site of the albumin promoter (17, 21) or other tandem arrangements of C/EBP-binding sites (28, 30), all of which are solely C/EBP-dependent. We also found that these forms of C/EBPβ are inactive on an albumin DE-I site-based promoter in P388 lymphoblasts (data not shown).

Presumably, the activity that we have observed on induction of IL-6 and MCP-1 is dependent on interactions with other transcription factors, such as NF-κB, that bind to these promoters. It is interesting that the majority of both C/EBPβ and C/EBP\textsubscript{β192–276} are present as heterodimers with C/EBPγ (IgE/Eβ) in P388 cells (Fig. 3). Although C/EBPγ has been characterized as a transdominant inhibitor of C/EBP transcriptional activation (30), it does not act in that manner in the context of the IL-6 promoter. On the other hand, C/EBPγ does not appear to be an activator of IL-6 transcription either. Endogenous expression of C/EBPγ at a level affording DNA binding activity similar to that of C/EBPβ and C/EBP\textsubscript{β192–276} (Fig. 3) is not sufficient to allow LPS induction of IL-6 expression.

In addition, co-transfection of a C/EBPγ expression vector with a vector for C/EBP\textsubscript{β192–276} expression was only mildly inhibitory to LPS activation of the IL-6 promoter (data not shown). Since C/EBPγ lacks any known activation domains and is essentially a C/EBP bZIP domain (28), the apparent ability of a C/EBP\textsubscript{β192–276}/C/EBPγ heterodimer to activate the IL-6 pro-

\[^{3}\text{P. F. Johnson, unpublished observations.}\]
Transcriptional Activation by bZIP Domains

There is good evidence that C/EBPβ and NF-κB synergistically activate the IL-6 promoter (29). C/EBPα, β, and δ have all been shown to synergize with NF-κB in activating the IL-8 promoter (32, 33). This synergy may involve direct physical association through their respective bZIP and Rel homology domains (33, 34). Thus, the basis for the ability of truncated C/EBPs to activate the IL-6 promoter may involve the capacity of the bZIP domain alone to synergize with NF-κB. Supporting this notion, we found that an intact NF-κB-binding site is required for C/EBP activity in transient transfections of the IL-6 promoter-reporter (Fig. 7). Furthermore, robust activation of the IL-6 promoter by C/EBPs is only observed under conditions of NF-κB stimulation (9, 15, this paper), and NF-κB stimulation induces NF-κB activity in P388 cells (15). Future experiments will examine the activity of truncated forms of C/EBPβ, as well as point mutants within the bZIP domain, to synergize with NF-κB in activating the IL-6 promoter.

Regardless of the mechanism by which the C/EBP bZIP domain mediates LPS induction of IL-6 and MCP-1 expression, our findings suggest that the basis for C/EBP redundancy in the activation of these genes (15) resides in this well conserved region that is shared by all C/EBP isoforms. Our experiments replacing the leucine zipper of C/EBPβ with that of GCN4 suggest that the critical structural feature for activity of bZIP domains may be further localized to, or at least require, the leucine zipper. While the leucine zipper swap mutant C/EBPβα181–272 can transactivate a DE-I promoter-reporter in P388 cells (data not shown), it shows no activity compared with intact C/EBPβ in the LPS induction of IL-6 and MCP-1. There is some uncertainty in the interpretation of this result because the levels of C/EBPβα181–272 expression are below that for intact C/EBPβ. On the other hand, the level of C/EBPβα273–358 expression is similar to that of C/EBPβα192–276 (Figs. 2B and 5B), which is a far more potent transcriptional activator (Fig. 4). Consistent with these findings, the truncated zipper swap mutant C/EBPβα192–276GLZ displays very little activity in transient expression assays and may even suppress LPS inducibility (Fig. 8). It is possible that the leucine zipper possesses critical determinants for the activity of C/EBPs on the IL-6 promoter in addition to mediating dimerization to known C/EBP family members. For example, the leucine zipper might mediate dimerization to an as yet unknown dimerization partner with inherent activation potential or, as proposed above, it might mediate synergistic interaction with NF-κB. The leucine zippers of C/EBP proteins have previously been implicated in functions beyond dimerization. The leucine zipper of C/EBPα has been shown to mediate cell type specificity of albumin promoter activation (35). Another instance of a non-dimerization function residing in the leucine zipper is that of serine 276 of human C/EBPβ (36). Phosphorylation of this serine residue confers calcium-regulated transcriptional stimulation to a promoter that contains binding sites for C/EBPβ. The experiments reported here show C/EBPα273–358 to have quite modest activity in comparison to the truncated forms of C/EBPβ and C/EBPδ in transient transfections, as well as being the least able to support LPS induction of the IL-6 promoter-reporter. It would be useful to evaluate the activity of a chimeric C/EBPα with the leucine zipper of C/EBPβ. Such a mutant would be expected to have enhanced activity in our model. In future experiments, it will be useful to examine single amino acid substitutions in the leucine zipper not only because they may more sharply delineate critical structural features, but because these altered forms of C/EBPβ may provide levels of expression more comparable to that of intact C/EBPβ and thus allow more direct comparisons of activity.

Interestingly, we have found that the effects of C/EBP expression are more profound in stable than in transient transfection assays. While C/EBP expression activated the IL-6 promoter in both assay systems, the level of IL-6 induction and synergy with LPS stimulation is much more dramatic in stably transfected cells. This is probably not a function of C/EBP overexpression, since EMSA assays of P388 lymphoblasts with stable ectopic C/EBP expression do not reveal abnormally high levels of DNA binding activity in comparison to bone marrow-derived macrophages (15). It seems likely that stable C/EBP transfectants with intact IL-6 promoters at normal diploid abundance in chromatin are a more accurate reflection of C/EBP activity on the IL-6 promoter. The IL-6 promoter would be expected to be in a more repressed basal state in chromatin and more dependent upon the activity of C/EBP to achieve activation. In either system, however, the surprising activity of truncated forms of C/EBP is plainly evident. Finally, our results show a significant capacity for LIP to activate the IL-6 promoter. LIP has previously been proposed to be a transdominant inhibitor of transcription (17). It is proposed that high levels of LIP observed in fetal liver constitute a mechanism for inhibiting the activity of other C/EBP isoforms in hepatocytes that are not yet terminally differentiated. Furthermore, it has been proposed that LIP could block C/EBPβ-mediated inhibition of hepatocyte proliferation (37). The regulation of LIP expression has also been proposed to play
a role in the regulation of lactation-associated genes such as β-casein (38). More recently, LIP expression has been correlated with neoplastic transformation of mammary tissue and has been proposed as a prognostic indicator for breast cancer because of its overexpression in breast tumors that were negative for the estrogen and progesterone receptors (39, 40). The central theme of these models is that the expression of LIP in immature proliferating cells suppresses the activity of C/EBPβ and other isoforms in activating the expression of gene products associated with terminal differentiation. It is clear from the findings reported here that conditions favoring LIP expression would not universally down-regulate C/EBPβ-regulated genes, but would be permissive for the expression of IL-6 and other genes with a similar promoter structure.

Among tissues particularly noted for IL-6 production, high levels of LIP expression have been observed in fetal liver (17), as well as transiently in adult liver following partial hepatectomy (41) or LPS treatment (42). While LIP might repress the activation of C/EBP-dependent gene products that inhibit hepatocyte proliferation, it could at the same time support the activation of C/EBP-dependent gene products that inhibit hepatectomy (41) or LPS treatment (42). While LIP might repress the expression of IL-6 and other genes, but would be permissive for the expression of IL-6 and other genes with a similar promoter structure.

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