C/EBPγ Has a Stimulatory Role on the IL-6 and IL-8 Promoters*

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Hongwei Gao‡, Sara Parkin§, Peter F. Johnson§, and Richard C. Schwartz‡¶

From the ℄Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824-4320 and the ℅Eukaryotic Transcriptional Regulation Section, Regulation of Cell Growth Laboratory, NCI-Frederick, Frederick, Maryland 21702-1201

CCAT/enhancer-binding protein γ (C/EBPγ) is an ubiquitously expressed member of the C/EBP family of transcription factors that has been shown to be an inhibitor of C/EBP transcriptional activators and has been proposed to act as a buffer against C/EBP-mediated activation. We have now unexpectedly found that C/EBPγ dramatically augments the activity of C/EBPβ in lipopolysaccharide induction of the interleukin-6 and interleukin-8 promoters in a B lymphoblast cell line. This activating role for C/EBPγ is promoter-specific, neither being observed in the regulation of a simple C/EBP-dependent promoter nor the TNFα promoter. C/EBPγ activity also shows cell-type specificity with no activity observed in a macrophage cell line. Studies with chimeric C/EBP proteins implicate the formation of a heterodimeric leucine zipper between C/EBPβ and C/EBPγ as the critical structural feature required for C/EBPγ stimulatory activity. These findings suggest a unique role for C/EBPγ in B cell gene regulation and, along with our previous observation of the ability of C/EBP basic region-leucine zipper domains to confer lipopolysaccharide inducibility of interleukin-6, suggest that the C/EBP leucine zipper domain has a role in C/EBP function beyond allowing dimerization between C/EBP family members.

CCAT/enhancer-binding protein (C/EBP)α, β, γ, δ, ε, and ζ comprise a family of basic region-leucine zipper (bZIP) transcription factors (reviewed in Ref. 1). These proteins dimerize through their leucine zippers and bind to DNA through their adjacent basic regions. C/EBPα, β, δ, and ε can activate in vivo transcription from promoters that contain a consensus binding site: 5′-TGT(G)NNNGAA(T/G)-3′ (2). At this time, the reported in vitro binding activities of C/EBPα, β, γ, δ, and ε are nearly identical, but the variety of C/EBP isoforms and their potential for heterodimer formation could provide a large repertoire of transcription factors with complex in vivo regulatory features. C/EBPβ and C/EBPδ have been implicated in the regulation of proinflammatory cytokines as well as other gene products associated with the activation of macrophages and the acute phase inflammatory response (reviewed in Ref. 3). For example, the promoter regions of the genes for interleukin-6 (IL-6), IL-1α, IL-1β, IL-8, tumor necrosis factor α (TNFα), granulocyte-colony stimulating factor, inducible nitric-oxide synthase, lysozyme, hemopexin, haptoglobin, α1-acid glycoprotein, serum amyloid A1, A2, A3, complement C3, and C-reactive protein all contain C/EBP binding motifs (3). Furthermore, C/EBPβ and C/EBPγ have both been shown to activate a reporter gene controlled by the IL-6 promoter in transient expression assays (2, 4). We have previously demonstrated that the stable expression of C/EBPβ, β, δ, and ε in a B lymphoblast cell line is sufficient to confer lipopolysaccharide (LPS) inducibility of IL-6 and monocyte chemoattractant protein 1 (MCP-1) expression (5–7). The basis for this redundancy among C/EBP isoforms lies with the requirement of only the well-conserved C/EBP bZIP domain for this activity (8).

We have found that C/EBPβ is overwhelmingly present as a heterodimer with C/EBPγ in B lymphoblasts dependent upon C/EBPβ for LPS-induced IL-6 expression (8, 9). C/EBPγ is most highly expressed in immature B cells, although its expression is rather ubiquitous (9, 10). Its binding specificity is similar to that of other C/EBP family members (10), but it has a truncated structure. C/EBPγ lacks known activation domains and is essentially a C/EBP bZIP domain (11). Consistent with this structure, it has been shown to inhibit C/EBP transcriptional activators (9, 11) and has been proposed to act as a “buffer” for C/EBP activators (11). In this model, C/EBPγ prevents the activation of C/EBP-dependent gene expression under conditions where the abundance of classical C/EBP activators is low. Activation of C/EBP-dependent genes would occur only when the abundance of C/EBPα, β, δ, and ε exceed a threshold. It has been proposed that the predominance of C/EBPγ over C/EBPβ in early B cells prevents transcription of C/EBP-dependent genes, whereas increased expression of C/EBPβ in mature cells, or in cells stimulated by proinflammatory cytokines or LPS, is permissive for expression (12).

Contrary to the notion of C/EBPγ as an inhibitor, there have been studies suggesting an activation function for C/EBPγ. An activating role for C/EBPγ has been reported in transcription from immunoglobulin heavy chain promoters (13, 14). C/EBPγ has also been implicated in β-globin (15) and pp52 (16) gene expression. Whether C/EBPγ functions as an activator or an inhibitor, both its lack of expression and overexpression have consequences in vivo. C/EBPγ-deficient mice have defects in natural killer cell cytotoxic activity and interferon γ production (17). Moderate erythroid overexpression of C/EBPγ in transgenic mice increases γ-globin expression relative to β-globin, while high level expression blocks erythropoiesis (18).

Our observation that heterodimers between C/EBPβ and C/EBPγ predominate in lymphoblasts dependent upon C/EBPβ for LPS-induced IL-6 expression (8, 9), as well as the widespread occurrence of C/EBPβ:γ heterodimers (9), led us to
further explore the role of C/EBPγ in regulating IL-6 transcription. In this report, we have unexpectedly found that C/EBPγ dramatically augments the activity of C/EBPβ in LPS induction of IL-6 in a B lymphoblast cell line. This activating role for C/EBPγ is promoter-specific, being observed for the IL-6 and IL-8 promoters, but neither for a simple C/EBP-dependent promoter nor the TNFα promoter. C/EBPγ activity also shows cell type-specificity, with stimulatory activity in a B lymphoblast and no effect in a macrophage cell line. Studies with chimeric C/EBP proteins implicated the formation of a heterodimeric leucine zipper between C/EBPβ and C/EBPγ as the critical structural feature required for C/EBPγ stimulatory activity. Our current findings suggest a unique role for C/EBPγ in B cell gene regulation and, along with our previous observation of the ability of C/EBP bZIP domains to confer LPS inducibility of IL-6, suggest that the C/EBP leucine zipper domain has a role in C/EBP function beyond allowing dimerization between C/EBP family members.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—P388 cells are murine B lymphoblasts (19) (American Type Culture Collection; CCL 46). P388-C cells and P388-Neo cells have been described previously by Hu et al. (6). WEHI-231 cells are murine B cells (20) (American Type Culture Collection; CRL 1702). P388D1(II1) cells are macrophages (19) (American Type Culture Collection; TIB 63). P388 cells and their derivatives were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and 50 μg/ml aprotinin (Sigma). P388D1(II1) cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μg/ml aprotinin. WEHI-231 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. All IL-6 inductions were conducted with LPS derived from E. coli 055:B5 (Sigma) added to 10 μg/ml.

Transfections—Transient transfections were conducted with 2 × 10⁶ cells, 4 μg of DNA, and 8 μl of DMRIE-C reagent (Invitrogen) in 1.2 ml of Opti-MEM I medium (Invitrogen). The DNA was comprised of 1 μg of a promoter-reporter, C/EBP expression vector, and pMEX plasmid to total 4 μg. The quantities of C/EBP expression vectors are as indicated in the figure legends. Cells were incubated in the transfection mixture for 5 h followed by the 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 transient transfections, C/EBPβs were expressed from pMEX (21), which utilizes the Moloney murine sarcoma virus promoter. NFκB p65 was expressed form pRc/CMV (Invitrogen), which utilizes the cytomegalovirus promoter (from N. Rice, NCI-Frederick). C/EBPγ-GCN4Lz has been described previously (22). C/EBPγΔNco was constructed by religating pMEX-C/EBPβ after restriction digestion with NcoI. C/EBPγΔPβLz was constructed by introducing an XhoI site at nucleotide position 283 in the C/EBPγ gene by site-directed mutagenesis. The XhoI-HindIII fragment bearing the leucine zipper was removed from this pMEX-C/EBPγ plasmid and replaced with an analogous fragment (nucleotides 703-831) from a rat C/EBPβ vector in which an XhoI site had been inserted between the basic region and leucine zipper. The forms of C/EBPβ and C/EBPγ used in this manuscript are depicted in Fig. 1.

The IL-6 promoter-reporter consists of the murine IL-6 promoter (23) (−250 to +1) inserted into the luciferase vector, pXP2 (24). DE1(−350b)LUC (21) is also derived from pXP2 (24) and contains four copies of the DE1 element upstream of the albumin minimal promoter. The TNFα promoter-reporter contains sequences extending to −1260 of the TNFα promoter inserted into the luciferase vector, pXP1 (23). The IL-8 promoter-reporter contains sequences extending from +44 to +133 inserted into pGL3-basic (Promega) (25–27). The SV40 early promoter-reporter is a commercial product, pSVgal-Control (Clontech), where the SV40 early promoter and enhancer sequences are cloned upstream and downstream, respectively, of the lacZ gene.

RNA Isolation and Analysis—Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s directions. RNAs were electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to high stringency in 40 mM sodium phosphate/1% SDS/1 mM EDTA at 65 °C. Hybridization probes were prepared with a random priming kit (Invitrogen) with the incorporation of 5'-α-32PdATP (3000 Ci/mmol, PerkinElmer Life Sciences). The IL-6 probe was a 0.65 kb murine cDNA (from N. Jenkins and N. Copeland, NCI-Frederick). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1.3 kb rat cDNA (28).

Western Analysis—Nuclear extracts were prepared as described below. The extracts (50 μg) were adjusted to 1× Laemmli sample buffer (29) and processed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis. The gel was transferred to Protran membrane (Schleicher and Schuell), and antigen-antibody complexes were visualized with the Enhanced Chemiluminescence Kit (Amersham Biosciences).

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 μg/ml aprotinin, 0.5% (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, 0.2% (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 extract was collected and stored at −70 °C.

The EMSA probes were double-stranded oligonucleotides containing an optimal C/EBP binding site (5'-GATCCTAGATATCCCTGTAGGCG-CAATAGGCTCAAAAGCTG-3') annealed with 5'-AATTCACGTTTGAGGCTATTTGCGGAAATCTGAT-3'), a murine IL-6 C/EBP binding site (5'-CTAAAAGGAGGCATTTGCATTGTTATGACTATTTAGTAGTTG-3') annealed with 5'-TGAGGACATTTAGTGGATGATTT-3'), and a murine albumin DE1 binding site (5'-TGGACTTAGATTTTGGATG-3') annealed with 5'-TGAGGCCGATTACACATAG-3'). These probes were labeled with the incorporation of 5'-α-32PdATP (3000 Ci/mmol; PerkinElmer Life Sciences) and Klenow DNA polymerase. Underlined sequences correspond to the C/EBP binding motifs.

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% (w/v) Ficoll, 50 mM Hepes pH 7.9, 5 mM EDTA, 5 mM dithiothreitol). The remainder of the reaction mixture contained KCl to a final concentration of 50 mM, Nonidet P-40.
RESULTS

C/EBPβ Heterodimerizes with C/EBPγ in B Cell Lines—In our previous studies, we found C/EBPβ to be predominantly in heterodimers with C/EBPγ in P388 B cells that are dependent upon transfected C/EBPβ expression for LPS induction of IL-6 and MCP-1 (Refs. 8 and 9; Fig. 2A). We also found both C/EBPβ and C/EBPγ to be in heterodimers with C/EBPγ in WEHI-231, a B cell line that has been used in several studies of IL-6 expression (30–33). In these cells, LPS-induced IL-6 expression was associated with induction of C/EBPβ:γ and C/EBPγ heterodimers (Fig. 2, B and C). In order to further test the entry of C/EBPβ into C/EBPβ:γ heterodimers, a C/EBPβ expression vector was transiently transfected into P388 cells over a range of quantities, including those that effectively transactivated the IL-6 promoter following LPS stimulation (see Fig. 3A). EMSA of nuclear extracts of the transfected cells revealed that C/EBPβ:γ heterodimers were the predominant binding species at all quantities tested (Fig. 3C). Apparently, C/EBPβ:γ heterodimers formed at the expense of C/EBPβ homodimers at lower quantities of vector (Fig. 3C; 0.5, 1, 2 μg). C/EBPβ homodimers were observed only at higher vector quantities, where C/EBPγ homodimers were no longer observable (Fig. 3C; 2, 4, 6, 8, 12 μg). The fact that the major C/EBP species observed with LPS-stimulation were C/EBPβ:γ heterodimers is inconsistent with an inhibitory role for C/EBPγ in the LPS induction of IL-6 expression.

C/EBPγ Augments C/EBPβ-stimulated Transcription of the IL-6 Promoter—C/EBPγ by itself is clearly not an activator of the IL-6 promoter because its presence in P388 cells is not sufficient to allow LPS induction of IL-6. However, our observations suggested that C/EBPγ-containing heterodimers might activate the IL-6 promoter in LPS-stimulated cells. To test this notion, we performed transient transfections of increasing quantities of C/EBPβ vector with and without added expression of C/EBPγ (Fig. 3A). C/EBPγ augmented LPS-induced expression from the IL-6 promoter at all quantities of C/EBPβ expression vector used. This is very surprising for a factor generally believed to be a transdominant inhibitor of C/EBP activators (10). If C/EBPγ acted as an inhibitor, C/EBPβ would be expected to induce less luciferase expression in the presence of added C/EBPγ, rather than more luciferase expression. In fact, 0.5 μg of C/EBPβ vector with 0.5 μg of C/EBPγ vector is twice as effective as 1 μg of C/EBPβ vector alone. This is consistent with C/EBPβ:γ heterodimers being more potent activators than C/EBPβ homodimers. Presumably, overexpression of C/EBPγ drives more C/EBPβ into heterodimers than would occur at
endogenous levels of C/EBPγ expression. When EMSA was performed upon nuclear extracts prepared from P388 cells transiently transfected with C/EBPβ expression vector with and without added C/EBPγ expression vector, a higher ratio of C/EBPβ:γ heterodimer to C/EBPβ homodimer is indeed observed in cells transfected with C/EBPγ expression vector (2.2 as opposed to 1.3) (Fig. 3E). To further test the ability of C/EBPγ to promote formation of C/EBPβ:γ heterodimers, a constant quantity of C/EBPβ expression vector was transiently transfected into P388 cells with and without C/EBPγ expression vector over a range of quantities including those that effectively transactivated the IL-6 promoter following LPS stimulation (see Fig. 3A). An EMSA of nuclear extracts of the transfected cells revealed that C/EBPβ:γ heterodimers became apparent and increased in abundance with increasing quantities of C/EBPγ (Fig. 3D).

The stimulatory effects of C/EBPγ were also observed in transient transfections where increasing amounts of C/EBPγ expression vector were added to a constant amount of C/EBPβ expression vector. These transfections were performed with LPS stimulation, and the expression vectors were cotransfected with an IL-6 promoter-reporter. C/EBPγ clearly augmented the ability of C/EBPβ to mediate LPS induction of the IL-6 promoter (Fig. 3B). C/EBPγ activity was observed even when the C/EBPβ vector was transfected at a 8-fold excess over C/EBPβ vector, although C/EBPγ by itself exhibited no activity (data not shown). Our results therefore suggest that C/EBPγ, rather than functioning as an inhibitor to low levels of C/EBPβ activity, actually augments that activity on the IL-6 promoter.

In contrast to the stimulatory effects observed when C/EBPγ was cotransfected with C/EBPβ in LPS-induced IL-6 expression, C/EBPγ actually inhibited the modest activation of the
IL-6 promoter that can be observed by transfection of C/EBPβ without LPS stimulation (Fig. 4). This inhibition was reversed by cotransfection with NF-κB p65, allowing dosage-dependent C/EBPγ stimulatory activity in the absence of LPS stimulation (Fig. 4). The lowest quantity of p65 vector used in the cotransfection (0.05 μg) potentiated robust stimulation by C/EBPγ. These data support the notion that C/EBPγ may play a key role in the synergy between C/EBPβ and NF-κB.

It is possible that the C/EBPγ expressed from our expression vector differed from endogenous C/EBPγ in its ability to stimulate IL-6 transcription. Furthermore, other investigators who found that C/EBPγ acted as an inhibitor of C/EBP transactivation performed their studies in the absence of LPS stimulation. Perhaps, LPS leads to the modification of C/EBPγ into a form capable of transactivation. To test these possibilities, transient transfections were performed with the C/EBPγ expression vector by itself with the IL-6 promoter-reporter. No stimulation of the IL-6 promoter above that induced by LPS stimulation alone was observed over a range of C/EBPγ expression vector amounts comparable to that used in the transient transfections where C/EBPγ stimulatory activity was observed (data not shown). Thus C/EBPγ has no stimulatory activity by itself, even in the presence of LPS treatment.

C/EBPγ Stimulatory Activity Shows Both Promoter and Cell-type Specificity—In order to test whether the presence of a C/EBP binding site is sufficient for the stimulatory activity of C/EBPγ, we performed transient transfections with DE1, a derivative of P388 D1, where a promoter-reporter that contains four copies of a C/EBP binding site tandemly arrayed upstream of the albumin minimal promoter (Fig. 5A). This simple C/EBP reporter failed to show any stimulation by C/EBPγ expression suggesting that a more complex promoter is required for stimulatory activity. We then performed transient transfections with the TNFα and IL-8 promoters (Fig. 5A). These promoters, like IL-6, are in part regulated by NF-κB and C/EBP. The TNFα promoter does not display synergy between NF-κB and C/EBPβ (34), while the IL-8 promoter shows strong synergy between these two factors (35–37). Consistent with a possible role in the synergy between NF-κB and C/EBPβ, C/EBPγ expression had little effect upon the TNFα promoter, but displayed a marked enhancement of the IL-8 promoter than was observed for the IL-6 promoter. In contrast to the promoter specificity observed for C/EBPγ, C/EBPβ was stimulatory for all of the promoters tested (Fig. 5A, compare control cells treated with LPS to cells treated with LPS and cotransfected with C/EBPβ). Furthermore, C/EBPγ stimulatory activity does not appear to be dependent upon differential binding of C/EBPγ to differing C/EBP binding sites. Both the IL-6 and DE1 C/EBP binding motifs bound C/EBPγ-containing species in EMSA performed upon nuclear extracts from P388 cells overexpressing C/EBPβ (Fig. 5B), while neither the TNFα nor the IL-8 C/EBP binding motifs detectably bound any C/EBP species under the same conditions (data not shown). The ability of C/EBPγ to stimulate transcription does not seem to correlate with its avidity for specific C/EBP binding motifs, but rather depends upon more complex aspects of promoter structure such as those that determine synergy between transcription factors. The stimulatory activity of C/EBPγ is thus promoter specific, requires a complex promoter to be observed, and may function in the synergistic activation of promoters by NF-κB and C/EBP family members.

The fact that C/EBPγ is most prominently expressed in cells of the B lymphoid lineage (10) led us to ask if its stimulatory activity was unique to that cell type or could be observed in another cell lineage that displays LPS-inducible IL-6 expresion. To test this, we utilized P388D1(IL1) macrophages. This cell line is actually a derivative of the original P388 B lymphoblast tumor (19). Only a relatively low proportion of C/EBPβ-DNA complexes from these cells are supershifted by anti-C/EBPβ in an EMSA (Ref. 9; data not shown). LPS is a potent inducer of IL-6 expression in this cell line (data not shown). Transient transfections were performed where increasing amounts of C/EBPγ expression vector were added to a constant amount of C/EBPβ expression vector. These transfections were performed with LPS stimulation, and the expression vectors were cotransfected with an IL-6 promoter-reporter. In contrast to P388 B cells where C/EBPγ clearly augmented the ability of C/EBPβ to mediate LPS induction of the IL-6 promoter, C/EBPγ had no effect on C/EBPβ stimulation of LPS-induced IL-6 expression in P388D1(IL1) cells (data not shown). Thus in addition to promoter specificity, the stimulatory activity of C/EBPγ shows cell-type specificity.

C/EBPγ Stimulatory Activity Requires Heterodimerization with C/EBPβ—We next sought to test whether C/EBPγ stimulatory activity in transfections with C/EBPβ requires heterodimer formation between these two proteins. To that end, we performed transient transfections with a chimeric C/EBPβ containing the leucine zipper of yeast GCN4. In comparison to intact C/EBPβ, C/EBPβ-GCN4LZ (Fig. 1) can activate transcription at a reduced level from an albumin DE1 site-driven reporter (22), as well as the IL-6 promoter-reporter in conjunction with LPS treatment (Fig. 6C, see controls), and is unable to heterodimerize with C/EBPγ in vitro or in vivo (9). The heterologous leucine zipper prevents heterodimerization, but allows the chimeric protein to homodimerize. To verify expression, DNA binding, and the heterodimerization properties of C/EBPβ-GCN4LZ, Western blot analysis and EMSA were performed using nuclear extracts of transiently transfected cells.
Fig. 5. C/EBPγ stimulates LPS-induced transcription from the IL-8 promoter but is inactive for the TNFα promoter and a simple C/EBP-driven promoter. A, transient transfections of P388 cells were carried out in duplicate with the microgram quantities of expression vector and LPS treatment as indicated. Luminometer values were normalized as in Fig. 3B. The data presented for the IL-6, TNFα, and DEI (−35αb) promoters are means of three, three, and five experiments, respectively, with S.E. The data for the IL-6 promoter from Fig. 3B are presented for comparison. B, EMSA was performed using nuclear extracts from P388-C8 cells and labeled binding site oligonucleotides corresponding to the C/EBP consensus binding site, the IL-6 promoter C/EBP binding site, and the DEI albumin C/EBP binding site. Binding reactions included normal rabbit IgG (N), carboxyl terminus-specific anti-C/EBPβ (β), or carboxyl terminus-specific anti-U- C/EBPγ (γ). Arrows labeled βγγ, γγ, and βγ indicate the positions of C/EBP-DNA complexes.

(Fig. 6, A and B). Western analysis of nuclear extracts from P388 cells transfected with increasing quantities of C/EBPβ-GCN4LZ expression vector detected increasing quantities of a C/EBP-related protein at the expected molecular mass of ~38 kDa (Fig. 6A). As can be seen in an EMSA of the same nuclear extracts, the overexpression of C/EBPβ-GCN4LZ fails to drive C/EBPγ into heterodimers (Fig. 6B), in contrast to C/EBPβ (Fig. 3C). The major EMSA species associated with transfection of the C/EBPβ-GCN4LZ expression vector could be supershifted with antibody specific to the amino terminus of C/EBPβ, but not with antibody specific to the carboxyl terminus of C/EBPβ as would be expected for replacement of the carboxyl terminus (Fig. 6B). Furthermore, this EMSA species could not be supershifted with antibody specific to the carboxyl terminus of C/EBPγ, indicating a lack of dimerization with C/EBPγ. Transient transfection of increasing amounts of C/EBPγ expression vector with a constant amount of C/EBPβ-GCN4LZ expression vector were carried out in comparison to increasing amounts of C/EBPγ expression vector with a constant amount of C/EBPβ expression vector (Fig. 6C). The ability of C/EBPγ to augment C/EBPβ activity was largely blocked by the GCN4 leucine zipper. This is consistent with C/EBPγ stimulatory activity being dependent on its ability to dimerize with C/EBPβ. The fact that C/EBPβ-GCN4LZ by itself supports LPS induction of the IL-6 promoter indicates that while C/EBPγ can augment C/EBPβ activity, formation of heterodimers containing C/EBPγ is not necessary for C/EBPγ activity on the IL-6 promoter.

C/EBPγ Stimulatory Activity Besides Its Leucine Zipper Domain—We next initiated studies to determine the structural components of C/EBPγ sufficient for its stimulatory activity. A form of C/EBPγ deleted for the region amino-terminal to the bZIP domain (Fig. 1; C/EBPγΔNco) was compared with intact C/EBPγ in the same experimental regime as described for Fig. 3B, where increasing amounts of C/EBP expression vector were added to a constant amount of C/EBPβ expression vector. These transfections were performed with LPS stimulation, and the expression vectors were cotransfected with an IL-6 promoter-reporter. C/EBPγΔNco, although lacking the 57-residue amino terminus, had as much stimulatory activity as wild type C/EBPγ (Fig. 7A). An EMSA species that increased in abundance with increasing quantities of the C/EBPγΔNco vector further indicated successful expression of C/EBPγΔNco (Fig. 7B). Thus, the amino terminus of C/EBPγ is unnecessary for its stimulatory activity.

Since C/EBPγ homodimers by themselves have no stimulatory activity (data not shown) and the ability of C/EBPγ to heterodimerize with C/EBPβ appears to be critical for its stimulatory activity (Fig. 6), we tested whether C/EBPγ activity required the formation of a heterodimeric leucine zipper, a heterodimeric DNA binding domain, or both. To that end, we performed transient transfections with a vector expressing a chimeric C/EBP comprised of a C/EBPγ amino-terminal and basic region, and a C/EBPβ leucine zipper (Fig. 1; C/EBPγβγLZ). As a control for C/EBPγβγLZ expression and DNA binding, Western blot analysis and EMSA were performed using nuclear extracts of cells transiently transfected over a range of quantities of the C/EBPγβγLZ expression vector (Fig. 8, A and B). Western analysis with antibody specific to the carboxyl terminus of C/EBPβ detected increasing quantities of a C/EBP-related protein at the expected molecular mass of ~19 kDa.
FIG. 6. C/EBPγ stimulatory activity is dependent upon the formation of C/EBPβγ heterodimers. The replacement of the C/EBP leucine zipper in C/EBPβ with that of GCN4 blocked C/EBPγ activity. A, a Western blot was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX-C/EBPβ-GCN4LZ. The primary antibody used in the detection of C/EBPβ-GCN4LZ was amino terminus-specific anti-C/EBPβ. An arrow marks the position of C/EBPβ-GCN4LZ. The positions of protein standards are noted. B, EMSA was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX-C/EBPβ-GCN4LZ. The EMSA of the 12-μg transfectants was also performed with binding reactions that included normal rabbit IgG (N), amino terminus-specific anti-C/EBPβ (Nβ), carboxyl terminus-specific anti-C/EBPβ (Cβ), or carboxyl terminus-specific anti-C/EBPγ (Cγ). Arrows labeled β-GCN4LZ, ββ, βγ, and γγ indicate the positions of C/EBP-DNA complexes. Arrows on the right indicate supershifts. The C/EBPβ-GCN4LZ complex is only supershifted by amino terminus-specific anti-C/EBPβ, while the C/EBPβ complexes from P388-Cβ are supershifted by all of the specific antisera. Weak, nonspecific background species co-migrating with C/EBPβ:γ and C/EBPβ:γ are evident in the 0-μg lane. C, transient transfections of P388 cells were carried out in duplicate with the microgram quantities of expression vectors and LPS treatment as indicated. Luminometer values were normalized as in Fig. 3. The data for C/EBPβ-GCN4LZ+γC/EBPγ (beta-GCN4LZ+gamma) are the mean of four experiments ± S.E. The data for C/EBPβ+γC/EBPγ (beta+gamma) from Fig. 3B are presented for comparison.
stimulatory activity of C/EBPγ is not dependent on its formation of heterodimers with C/EBPβ and, indeed, C/EBPβ is largely found in heterodimers with C/EBPγ in P388 B cells that have gained the capacity for LPS-induced IL-6 expression upon transfection of a C/EBPβ expression vector. Surprisingly, the critical structural feature for this stimulatory activity is the formation of a heterodimeric leucine zipper between C/EBPβ and C/EBPγ. C/EBPγ stimulatory activity was found to be promoter-specific with activity seen on IL-6 and IL-8 promoter-reporters, and not on TNFα and albumin DEI promoter-reporters. C/EBPγ stimulatory activity was also found to be cell-type specific, being observed in P388 B cells, but not in their P388D1(IL-1) macrophage derivative.

The stimulatory activity of C/EBPγ was surprising, since it is generally accepted as being an inhibitor of C/EBP transcriptional activators (11, 12). However, the same investigators that first demonstrated the inhibitory activity of C/EBPγ found that immunodepletion of C/EBPγ from an in vitro transcription assay inhibited the activity of the BCL1 immunoglobulin heavy chain and the Rous sarcoma virus promoters (13). Similarly, C/EBPγ synergizes with Stat6 and NF-κB p50/p65 to induce the germline gamma 3-immunoglobulin heavy chain (14). C/EBPγ has also been found to enhance β-globin gene expression in collaboration with CP-1 (15). Another instance of a positive role for C/EBPγ has been found in the expression of pp52, a leukocyte-specific phosphoprotein postulated to regulate cytoskeleton structure (16). Thus, the role of C/EBPγ as a transcriptional activator does not seem unusual. It seems nei-
FIG. 8. The formation of a heterodimeric C/EBPβ-γ leucine zipper is sufficient for the stimulatory activity of C/EBPγ. A, Western blot was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX-C/EBPγ-βLz. The primary antibody used in the detection of C/EBPγ-βLz was carboxyl terminus-specific anti-C/EBPβ. An arrow marks the position of C/EBPγ-βLz. The positions of protein standards are noted. B, EMSA was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX- C/EBPγ-βLz. EMSA of P388 cells and the 12-μg transfectants also was performed with binding reactions that included normal rabbit IgG (N), amino terminus-specific anti-C/EBPβ (Nβ), carboxyl terminus-specific anti-C/EBPβ (Cβ), amino terminus-specific anti-C/EBPγ (Nγ), or carboxyl terminus-specific anti-C/EBPγ (Cγ). Arrows labeled γγ and γγβLz indicate the positions of C/EBP-DNA complexes. Arrows on the right indicate supershifts. The C/EBPγ-βLz complex (similar in mobility to the C/EBPγ complex in P388 cells) is supershifted by carboxyl terminus-specific anti-C/EBPβ, in addition to the C/EBPγ-specific antisera. C, transient transfections were carried out in duplicate with and without 0.5 μg of C/EBPγ vector, with the microgram quantities of C/EBPγ-βLz expression vector and LPS treatment as indicated. Luminometer values were normalized as described in Fig. 3A. The data are the mean of three experiments ± S.E.
Stimulatory activity was seen in P388 B cells, but not in their
IL-6 inhibitory effect is converted to a stimulatory effect by NF-
IL-6 inactivated WEHI 231 cells (Fig. 2). This prediction could be tested in B lineage cells derived from C/EBPγ-deficient mice (17).

C/EBPγ stimulatory activity was observed with the IL-6 and IL-8 promoter-reporters, but not with the TNFα or the DEI promoter-reporters. One distinguishing characteristic of the IL-6 and IL-8 promoters is synergistic regulation by C/EBPβ and NF-κB (35–37). It is tempting to propose a specific role for C/EBPγ in promoting this synergy. While the experiments reported here do not provide a direct demonstration for such a mechanism, the findings that C/EBPγ inhibits C/EBPβ activation of the IL-6 promoter in the absence of LPS and that this inhibitory effect is converted to a stimulatory effect by NF-κB p65 expression (Fig. 4) are consistent with this model. Furthermore, our previous studies found that the activity of C/EBPγ is limited to promoters that exhibit synergy between C/EBPβ and NF-κB. Other promoters for which C/EBPγ stimulatory activity have been suggested, including immunoglobulin heavy chain (13, 14), β-globin (15), and pp52 (16), do not display synergistic regulation by C/EBPβ and NF-κB.

C/EBPγ stimulatory activity displays cell-type specificity. This is also the case for the inhibitory activity of C/EBPγ (9). Stimulatory activity was seen in P388 B cells, but not in their macrophage derivative, P388D1(IL-1) (data not shown). C/EBPγ is normally a minor component of the C/EBP family members expressed in these macrophages, where C/EBPγ forms heterodimers with another as yet unidentified protein (9). Perhaps, C/EBPγ stimulatory activity in P388D1(IL-1) macrophages is precluded by the heterodimerization of C/EBPβ with this other protein. The activity of C/EBPγ in specific cell-types may be dependent upon the availability of an appropriate partner for heterodimerization. Our studies strongly suggest that heterodimerization is critical for stimulatory activity (Fig. 6C).

The promoter and cell-type specificity of C/EBPγ activity lead us to speculate that the ability of C/EBPγ to augment LPS stimulation of IL-6 transcription in B cells may provide a mechanism of autocrine IL-6 production to drive the maturation of B cells, whereas suppressing or having a neutral effect on other inflammatory cytokines such as TNFα. This could be particularly important as a source of IL-6 in a T-independent B cell response. Perhaps C/EBPγ-deficient mice (17) will exhibit slower kinetics in their B cell response to Gram-negative bacteria.

While we have observed C/EBPγ stimulatory activity on both the IL-6 and IL-8 promoters, it is interesting to note that no IL-8 orthologue exists in mouse and rat (38, 39). In humans, however, both IL-6 and IL-8 are autocrine factors in myeloma tumor progression (40, 41). It would be interesting to test whether a functional association exists between C/EBPγ expression and the autocrine production of these cytokines in myelomas.

Although C/EBPγ is most abundantly expressed in immature B cells (10), we have found C/EBPγ;γ and C/EBPγ heterodimers to be the predominant form of C/EBP in LPS-stimulated WEHI 231 cells (Fig. 2C), a relatively mature, surface-IgM expressing B cell. The occurrence of C/EBPβ;γ heterodimers as a major species has also been observed in glioma, mammary tumor, and hepatoma cell lines, as well as in brain, pancreas, and ovary (9). It will be worthwhile to evaluate whether C/EBPγ can stimulate target genes that are known to be positively regulated by C/EBPβ in these cell-types and tissues.

We found that ectopic expression of C/EBPβ in P388 cells led to the formation of C/EBPβ;γ heterodimers at the expense of C/EBPγ homodimers, while C/EBPβ homodimers were observed only at the highest levels of C/EBPβ expression (Fig. 3C). This may indicate a preference for heterodimerization between these C/EBP family members. This result cannot be explained by large pools of either monomeric C/EBPγ or unbound C/EBPβ dimers being available for dimerization with C/EBPβ. If this were the case, C/EBPγ homodimers would not be eliminated as they are by C/EBPβ expression. However, His-tagged recombinant forms of these proteins do not show preferential dimerization (9). It is possible that post-translational modifications of these C/EBP family members regulate their dimerization.

Perhaps, the most surprising result reported here is the ability of a chimeric C/EBP consisting of C/EBPγ with the leucine zipper of C/EBPβ to stimulate the IL-6 promoter in cells that express only endogenous C/EBPγ (Fig. 8). Since C/EBPγ by itself is unable to support LPS induction of the IL-6 promoter (data not shown), this result demonstrates that the formation of a C/EBPβ;γ heterodimeric zipper in the absence of any conventional activation domains to sufficient to support LPS induction of the IL-6 promoter. This is consistent with our earlier finding that expression of the bZIP domains of C/EBPβ, δ, or α was sufficient to confer LPS inducibility to the IL-6 promoter in P388 cells (8). In those studies, we found that the C/EBPβ bZIP domain was largely dimerized with C/EBPγ and that activity required an intact NF-κB binding site. We have now found that C/EBPγ stimulatory activity is observed on two promoters that show synergy between C/EBPβ and NF-κB and that C/EBPγ expression actually becomes inhibitory in the absence of NF-κB expression (Fig. 4). Our findings are consistent with the C/EBP leucine zipper being a critical determinant in facilitating the synergy between NF-κB and C/EBP family members that is observed for several genes encoding cytokines and class I acute phase proteins including IL-6, IL-8, IL-12, granulocyte-colony stimulating factor, IL-1α, serum amyloid A1, A2, A3, and α1-acid glycoprotein (3). Functions other than dimerization have been demonstrated for leucine zipper domains. In the C/EBP family, the leucine zipper of C/EBPα mediates cell-type specificity of albumin promoter activation (42) and phosphorylation of serine 276 in the leucine zipper of human C/EBPβ confers calcium-regulated transcriptional stimulation to a promoter that contains binding sites for C/EBPβ (43). Recently, the leucine zipper of transcription factor v-Myb has been found to regulate the commitment of hematopoietic progenitors (44). Mutation of the leucine zipper can alter the transforming potential of v-Myb from the macrophage lineage to the erythroid and granulocytic lineages. It is tempting to speculate that the leucine zipper of C/EBP family transcription factors interacts differentially with other transcription factors such as NF-κB or with coactivators of transcription in a manner dependent upon leucine zipper dimerization partners.

REFERENCES
1. Johnson, P. F., and Williams, S. C. (1994) in Liver Gene Expression (Yaniv, M., and Tronche, F., eds), pp. 231–256, R. G. Landes Company, Austin, TX
2. Akira, S., Ishikii, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) EMBO J. 9, 1897–1906
3. Poli, V. (1998) J. Biol. Chem. 273, 29279–29282
4. Kinoshita, S., Akira, S., and Kishimoto, T. (1992) Proc. Natl. Acad. Sci. U. S. A.

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5. Bretz, J. D., Williams, S. C., Baer, M., Johnson, P. F., and Schwartz, R. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7306–7311
6. Hu, H. M., Baer, M., Williams, S. C., Johnson, P. F., and Schwartz, R. C. (1998) J. Immunol. 160, 2334–2342
7. Williams, S. C., Du, Y., Schwartz, R. C., Weiler, M., Keller, J. R., and Johnson, P. F. (1998) J. Biol. Chem. 273, 13493–13501
8. Hu, H.-M., Tian, Q., Baer, M., Spooner, C. J., Williams, S. C., Johnson, P. F., and Schwartz, R. C. (2000) J. Biol. Chem. 275, 16373–16381
9. Parkin, S., Baer, M., Copeaud, T. D., Schwartz, R. C., and Johnson, P. F. (2002) J. Biol. Chem. 277, 23563–23572
10. Roman, C., Platero, J. S., Shuman, J., and Calame, K. (1990) Genes Dev. 4, 1494–1415
11. Cooper, C., Henderson, A., Artandi, S., Avitahl, N., and Calame, K. L. (1995) Nucleic Acids Res. 23, 4371–4377
12. Cooper, C. L., Berrier, A. L., Roman, C., and Calame, K. L. (1994) J. Immunol. 153, 5049–5055
13. Cooper, C., Johnson, D., Roman, C., Avitahl, N., Tucker, P., and Calame, K. L. (1992) J. Immunol. 149, 3225–3231
14. Fan, Q., Petit-Preze, C., Stavzev, J., and Hammarstrom, L. (2000) Eur. J. Immunol. 30, 1019–1029
15. Wall, L., Destromaisons, N., Delvoye, N., and Guy, L. G. (1996) J. Biol. Chem. 271, 16477–16484
16. Omori, S. A., Smale, S., O'Shea-Greenfield, A., and Wall, R. (1998) J. Immunol. 159, 1800–1808
17. Kaisho, T., Tsutsui, H., Tanaka, T., Terajima, T., Takeda, K., Kawai, T., Yoshida, N., Nakanishi, K., and Akira, S. (1999) J. Exp. Med. 180, 1573–1581
18. Zafarana, G., Rottier, R., Grosveld, F., and Philipson, S. (2000) EMBO J. 19, 4695–4699
19. Bauer, S. R., Holmes, K. L., Morse III, H. C., and Potter, M. (1986) J. Immunol. 136, 4695–4699
20. Gutman, G. A., Warner, N. L., and Harris, A. W. (1981) Clin. Immunol. Immunopathol. 18, 220–244
21. Williams, S. C., Cantwell, C. A., and Johnson, P. F. (1991) Genes Dev. 5, 1553–1567
22. Williams, S. C., Baer, M., Dilnner, A. J., and Johnson, P. F. (1995) EMBO J. 14, 3170–3183
23. Tanabe, O., Akira, S., Kamiya, T., Weng, G. G., Hirano, T., and Kishimoto, T. (1988) J. Immunol. 141, 3575–3581
24. Nordeen, S. K. (1988) BioTechniques 6, 454–457
25. Okamoto, S., Mukaida, N., Yasumoto, K., Rice, N., Ishikawa, Y., Horiguchi, H., Murakami, S., and Matsuura, K. (1994) J. Biol. Chem. 269, 8582–8589
26. Murayama, T., Ohara, Y., Osuchi, M., Khabar, K. S. A., Higashi, H., Mukaida, N., and Matsuura, K. (1997) J. Virol. 71, 5692–5695
27. Zhang, X., Wang, J. M., Gong, W. H., Mukaida, N., and Young, H. A. (2001) J. Immunol. 166, 7104–7111
28. Fort, P., Marty, L., Piechaczyk, M., El Salrouty, S., Dani, C., Jeanteur, J., and Blanchard, J. M. (1985) Nucleic Acids Res. 13, 1431–1442
29. Laemmli, U. K. (1970) Nature 227, 680–685
30. Hobbs, M. V., McEvilly, R. J., Koch, R. J., Cardenas, G. J., and Noonan, D. J. (1991) Cell. Immunol. 132, 442–450
31. Macfarlane, D. E., and Manzel, L. (1998) J. Immunol. 160, 1122–1131
32. Lee, J. R., and Kornetzky, G. A. (1998) Eur. J. Immunol. 28, 4188–4197
33. Venkataraman, C., Shankar, G., Sen, G., and Bondada, S. (1999) Immunol. Lett. 69, 233–238
34. Liu H., Sidirogoulos, P., Song, G., Pagliari, L. J., Birrer, M. J., Stein, B., Anrather, J., and Pope, R. M. (2000) J. Immunol. 164, 4277–4285
35. Matsuura, T., Fujiwara, K., Nishio, Y., Mukaida, N., Matsuura, K., Kishimoto, T., and Akira, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10193–10197
36. Stein, B., and Baldwin, A. S. (1993) Mol. Cell. Biol. 13, 7191–7198
37. Kunish, C., Lang, R. K., Rosen, C. A., and Shannon, M. F. (1994) J. Immunol. 153, 155–164
38. Huang, S., Paulauskis, J. D., Godleski, J. J., and Kobzik, L. (1992) Am. J. Pathol. 141, 981–988
39. Wuyts, A., Haelens, A., Proost, P., Lenaerts, J. P., Conings, R., Opdenakker, G., and Van Damme, J. (1996) J. Immunol. 157, 1736–1743
40. Treon, S. P., and K. C. Anderson. (1998) Curr. Opin. Hematol. 5, 42–48
41. Shapiro, V. S., Mollenauer, M. N., and Weiss, A. (2001) Blood 98, 187–193
42. Nerlov, C., and Ziff, E. B. (1994) Genes Dev. 8, 350–362
43. Wegner, M., Cao, Z., and Rosenfeld, M. G. (1992) Science 256, 370–373
44. Kunsch, C., Lang, R. K., Rosen, C. A., and Shannon, M. F. (1994) J. Immunol. 153, 153–164
45. Huang, S., Paulauskis, J. D., Godleski, J. J., and Kobzik, L. (1992) Am. J. Pathol. 141, 981–988
46. Wuyts, A., Haelens, A., Proost, P., Lenaerts, J. P., Conings, R., Opdenakker, G., and Van Damme, J. (1996) J. Immunol. 157, 1736–1743
47. Treon, S. P., and K. C. Anderson. (1998) Curr. Opin. Hematol. 5, 42–48
48. Shapiro, V. S., Mollenauer, M. N., and Weiss, A. (2001) Blood 98, 187–193
49. Nerlov, C., and Ziff, E. B. (1994) Genes Dev. 8, 350–362
50. Wegner, M., Cao, Z., and Rosenfeld, M. G. (1992) Science 256, 370–373
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Hongwei Gao, Sara Parkin, Peter F. Johnson and Richard C. Schwartz

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