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ROLE OF C/EBPγ AND C/EBPζ IN REGULATING IL-6 EXPRESSION IN B CELLS

By

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ABSTRACT

ROLE OF C/EBPγ AND C/EBPζ IN REGULATING IL-6 EXPRESSION IN B CELLS

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CCAAT/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-specificity with no activity being 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.

In the second part of my study, I evaluated the roles of C/EBPζ in LPS induction of interleukin-6 in B cells. C/EBPζ was originally identified as a gene induced upon DNA damage and growth arrest. It has been shown to be involved in the cellular response to endoplasmic reticulum stress. Because of sequence divergence from other C/EBP family members in its DNA binding domain and its consequent inability to bind the C/EBP consensus-binding motif, C/EBPζ can act as a dominant negative inhibitor of other C/EBPs. C/EBP transactivators are essential to the expression of many proinflammatory cytokines and acute phase proteins, but a role for C/EBPζ in regulating their expression has not been described. We have found that expression of C/EBPζ is induced in response to LPS treatment of B cells at both the mRNA and protein levels. Correlating with the highest levels of C/EBPζ expression at 48 hours after LPS treatment, both the abundance of C/EBP DNA binding species and IL-6 expression are downregulated. Furthermore, ectopic expression of C/EBPζ inhibited C/EBPβ-dependent IL-6 expression from both the endogenous IL-6 gene and an IL-6 promoter-reporter. These results suggest that C/EBPζ functions as negative regulator of IL-6 expression in B cells and that it contributes to the transitory expression of IL-6 that is observed after LPS treatment.

To my wife, Mei and my daughter, Anqi

&

To my parents, Shuzhen Wei and Deshun Gao, and my sister, Shuangmei

For their deep love and sustained support

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C/EBP	CCAAT/enhancer binding protein
LAP	Liver activating protein
LIP	Liver inhibitory protein
bZIP	Basic region-leucine zipper
IL	Interleukin
CHOP	C/EBP homology protein
GADD153	Growth arrest and DNA damage-inducible gene 153
TNFα	Tumor necrosis factor α
LPS	Lipopolysaccharide
GAPDH	Glyceraldehyde-3-phospahate dehydrogenase
EMSA	Electrophoretic mobility shift assay
Ig/EBP	Immunoglobulin enhancer binding protein
STAT	Signal transducer and activator of transcription

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

1. C/EBP family of transcription factors

CCAAT/enhancer binding proteins (C/EBPs) form a family of transcription factors with structural as well as functional homologies. Over eight C/EBP isoforms encoded by six genes have been found (reviewed by Lekstrom-Himes and Xanthopoulos, 1998). The additional isoforms are produced by translation initiation at different in-frame AUG codons as well as by differential splicing and use of alternative promoters.

All C/EBP family members possess a highly conserved basic region-leucine zipper motif (bZIP). The basic region makes direct contact with DNA and determines sequence-specific binding properties (Agre et al 1989; Johnson et al 1993). The leucine zipper motif mediates dimerization between C/EBP polypeptides, which is required for DNA binding and for transactivation (Landschultz et al 1988). Figure 1 shows the structure of the C/EBPβ bZIP region homodimer bound to DNA. According to a model for DNA binding by bZIP proteins (Vinson et al 1989; Hurst 1995), the dimer forms an inverted Y shaped structure in which each arm of the Y is made of a basic region, which binds to one half of a palindrome recognition sequence in the DNA major groove like a fork or a pair of scissors (Figure 1).

The conserved bZIP domain has several implications. First, all C/EBP isoforms with DNA-binding domains are at least potentially capable of binding to

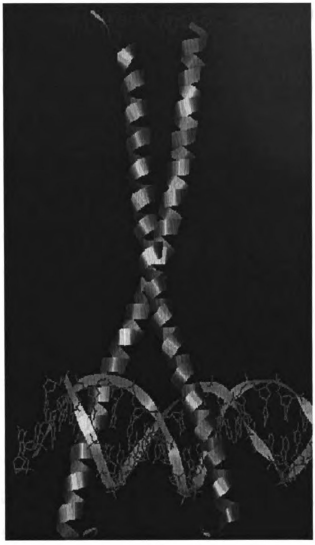

Figure 1. Structure of the C/EBPβ basic region/leucine zipper domain bound to DNA. The two α-helical basic regions (bottom) dimerize through the α-helical leucine zipper domain (top) to form an inverted Y-shaped structure. Each arm of the Y is formed by a single α helix, one from each monomer, which binds to one-half of a palindromic recognition sequence.

a given C/EBP binding site in a promoter, although there is some evidence that phosphorylation of specific residues in the basic region of individual C/EBP isoforms may alter their binding affinity. As a result, different C/EBP isoforms may compete for binding to a cognate DNA sequence. Second, the conserved nature of the leucine zippers makes them compatible, allowing for the formation of heterodimers (Cao et al 1991). Thus, dimerization between isoforms has the potential to increase the variety of transcriptional responses elicited from these factors.

The amino terminus of C/EBPs carries the transactivation domain (Friedman et al 1990; Trautwein et al 1995; Williams et al 1995; Figure 2). The amino acid sequences of the transactivation domains are generally unique for each isoform, although some short, conserved domains have been identified in C/EBPα, C/EBPβ, and C/EBPδ that are critical for the transcriptional activity of these isoforms (Nerlov et al 1995; Figure 2). These transactivation domains act more or less independently of the bZIP domain, displaying similar activity when fused to heterologous DNA-binding domains (Trautwein et al 1995; Williams et al 1995). This modularity has been explored extensively by studying the function of specific domains and amino acid residues in C/EBP proteins.

Since their discovery, the function of the C/EBP family has been investigated in detail and pivotal roles of the proteins have been identified in numerous cellular processes. These include the control of cellular growth and differentiation, immune and inflammatory processes, and various diseases. The

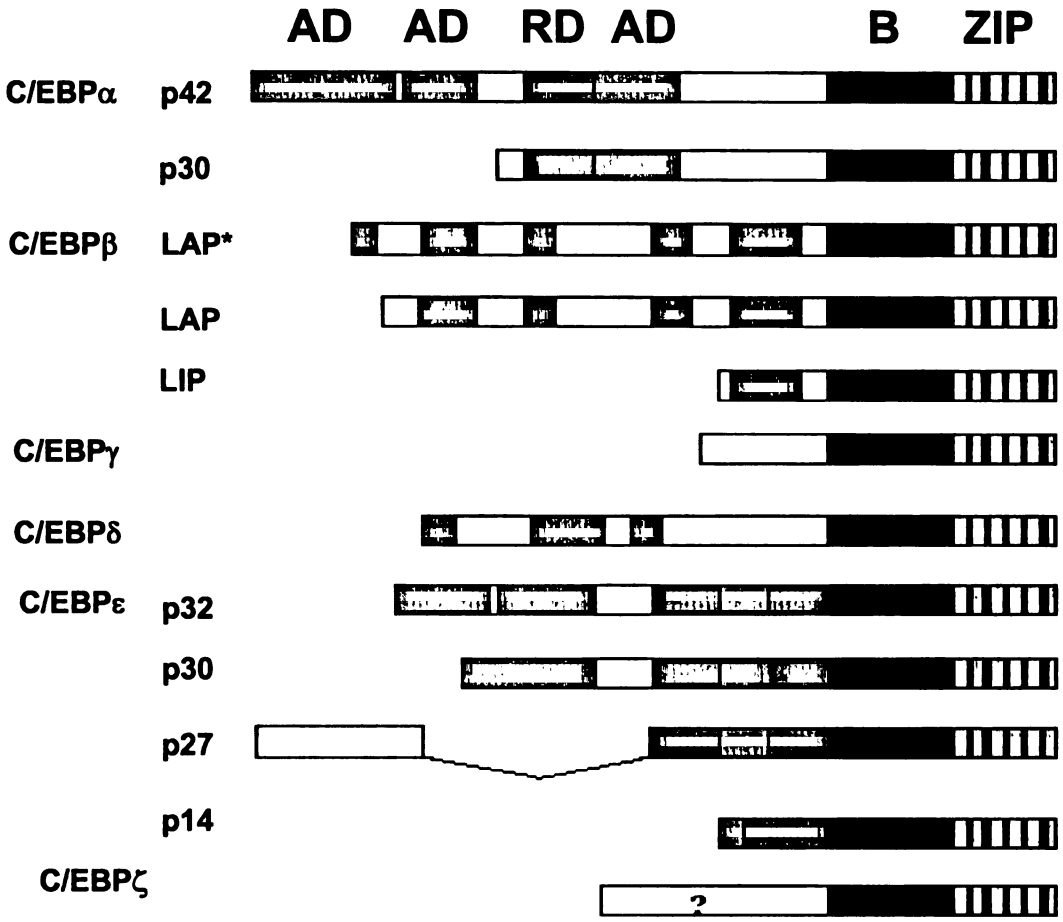

Figure 2. Schematic representation of the C/EBP family members. The leucine zipper is shown in light gray, with black vertical lines indicating the leucine residues, and the basic region is shown in dark gray. The position of the activation domains (AD) and negative regulatory domains (RD) are shown in gray. ? Indicates that the N-terminus of C/EBPζ contains an activation domain although its exact position remains to be determined.

expression of C/EBPs has also been found to change markedly during a number of physiological and pathophysiological conditions through the action of extracellular signals. Here, I will review the structure, function and the regulation of all six primary C/EBP isoforms (C/EBPα, β, δ, γ, ε, and ζ) with emphasis on the dominant negative inhibitors of this family, C/EBPγ and C/EBPζ.

1.1 C/EBPα

C/EBPα is the first cloned member of the C/EBP family and also the bZIP prototype gene. It was isolated from a rat liver cDNA library and originally named C/EBP (Landschulz et al 1988). Later renamed C/EBPα, homologues have now been identified from several different species including mouse (Xanthopoulos et al 1989), human (Antonson et al 1995), chicken (Calkhoven et al 1992) and frogs (Chen et al 1994 and Xu et al 1992). C/EBPα can be translated into multiple proteins with different transactivation potentials (Ossipow et al 1993). All these proteins have the same bZIP domain but different N-terminal amino acid sequences. A ribosome-scanning mechanism that uses different in-frame AUGs to initiate translation from the same mRNA has been suggested to explain the production of multiple C/EBPα proteins (Ossipow et al 1993).

C/EBPα mRNA is most abundant in liver and adipose tissue, but it is also expressed in other tissues (Antonson et al 1995; Birkenmeier et al 1989; Williams et al 1991). Consistent with its tissue distribution, C/EBPα can transactivate the promoters of hepatocyte and adipocyte energy metabolism-related genes such as GLUT4 (Kaestner et al 1990) and PEPCK (Park et al 1990). Therefore, it has

been proposed to be a global regulator of genes involved in energy metabolism (McKnight et al 1989).

C/EBPα clearly functions in other cellular processes, as well. C/EBPα regulates adipocyte differentiation. Inhibition of C/EBPα blocks differentiation while upregulation induces differentiation of adipocytes (Cao et al 1991; Lin et al 1994). C/EBPα also plays a critical role in myelopoiesis. C/EBPα-deficient mice are completely blocked in the development of neutrophils (Zhang et al 1997). Conditional expression of C/EBPα in transfected bipotential cells induces neutrophilic differentiation and blocks monocytic differentiation (Radomska et al 1998).

A number of studies additionally point to a role for C/EBPα in the regulation of cellular growth. Overexpression of a chimeric C/EBPα-estrogen receptor fusion protein can cause cessation of mitotic growth (Umek et al 1991). C/EBPα has been found to interact with several proteins that are involved in the control of cell cycle progression, thereby indicating the existence of multiple pathways through which it mediates growth arrest (McKnight 2001; Timchenko et al 1997; Chen et al 1996; Timchenko et al 1999; Wang et al 2001; Wang et al 2002). For example, C/EBPα-mediated growth arrest is accompanied by increased expression of the cell cycle inhibitor, p21. C/EBPα interacts with and stabilizes the p21 protein (Timchenko et al 1997). Most recently, it was shown that a short region outside of the C/EBPα DNA binding domain interacts directly with the cyclin-dependent kinases Cdk2 and Cdk4, and arrests cell proliferation by inhibiting their activity (Wang et al 2001). In the case of cdk4, it has been further

shown that this interaction leads to proteasome-dependent degradation of the protein (Wang et al 2002).

The lines of research demonstrating an involvement of C/EBPα in the regulation of both growth and differentiation have come together in some recent studies. C/EBP has been shown to repress E2F function in the terminal differentiation of adipocytes and granulocytes (Porse et al 2001). Using standard molecular biological approaches, Tenen's group discovered dominant-negative mutations in the human gene encoding C/EBPα in acute myeloid leukemias that phenotypically resemble cells blocked in differentiation by knockout of the gene encoding C/EBPα (Pabst et al 2001). These results led to the conclusion that C/EBPα is a tumor suppressor gene (McKnight 2001). Collectively, these data lead to a view of C/EBPα as playing a key role in the linked processes of cell growth arrest and terminal differentiation.

1.2 C/EBPβ

C/EBPβ was identified and cloned as NF-IL6, the nuclear factor that bound to the IL-1 response element of the IL-6 gene (Akira et al 1990). Several other names for C/EBPβ homologues cloned from rat, mouse, chicken and aplysia are IL-6-DBP (Poli et al 1990), LAP (Descombes et al 1990), CRP2 (Williams et al 1991), AGP/EBP (Chang et al 1990), NF-M (Katz et al 1993) and ApC/EBP (Alberini et al 1994). C/EBPβ is expressed in several tissues with highest expression in liver and kidney (Williams et al 1991; Cao et al 1991; Descombes et al1990; and Chang et al 1990). While C/EBPβ was originally identified as a

nuclear factor binding to the IL-1 response element of the human IL-6 gene (Akira et al 1990), many studies have now demonstrated that C/EBPβ is responsible for the regulation of genes encoding other proinflammatory cytokines, as well as many acute phase proteins (Akira et al 1992). The gene encoding C/EBPβ can generate two proteins: LAP which acts as an activator and LIP which acts as a repressor. LIP shares the same bZIP domain as LAP, but lacks the N-terminal activation domain (Descombes et al 1991). In contrast to these findings, results from our lab suggest that LIP functions as an activator rather than a repressor of the IL-6 promoter in P388 lymphoblast cells upon LPS and IL-1β treatment. (Hu et al 2000; Spooner et al unpublished data). LIP seems to act either as an inhibitor of C/EBP transcriptional activity or as a transcriptional activator of other genes depending on the promoter and the cell type.

C/EBPβ-deficient mice have been produced. The phenotype of C/EBPβ-deficient mice indicates a potential role in the activation and/or differentiation of macrophages (Tanaka et al 1995). On the other hand, C/EBPβ appears to play an important role in promoting proliferation, and its levels are increased in a number of tumors (Greenbaum et al 1998; Zhu et al 2002; Buck et al 1999; Buck et al 2001). For example, Zhu et al (2002) have shown that C/EBPβ-deficient mice are completely refractory to skin tumour development induced by a variety of carcinogens. In v-Ha-ras transgenic mice, C/EBPβ deficiency results in a significant reduction in tumourogenesis, thereby linking the proto-oncogene ras and C/EBPβ (Zhu et al 2002).

C/EBPβ can be phosphorylated on a number of different residues by several protein kinases, some of which appear to play a role in the regulation of its biological functions. For example, phosphorylation has been demonstrated to alter the intrinsic transactivation ability of C/EBPβ (Wegner et al 1992; Trautwein et al 1993). The phosphorylation status of C/EBPβ can also modulate its ability to bind to DNA. Trautwein et al showed that in vitro phosphorylation of rat C/EBPβ Ser^{240} by PKA or PKC inhibited its DNA-binding activity (Trautwein et al 1994). Phosphorylation of C/EBPβ has also been shown to stimulate translocation of C/EBPβ from the cytosol to the nucleus (Metz et al 1991) and to be required for TGFα-induced hepatocyte proliferation (Buck et al 1999). Recently, it was reported that phosphorylation of mouse C/EBPβ on serine 239 induced its nuclear export, which, in turn, inhibits transcription from the albumin gene upon TNF-α treatment (Buck et al 2001). Furthermore, Buck and colleagues show that RSK-mediated phosphorylation of mouse threonine 217 of C/EBPβ constitutes a critical event allowing stellate cells to evade programmed cell death upon liver injury (Buck et al 2001). They argue that this modification creates a functional XEXD caspase substrate inhibitor, thus suggest that C/EBPβ may play a role other than that of a transcription factor.

1.3 C/EBPδ

C/EBPδ was originally cloned from the rat (Cao et al 1991). It is also termed as CRP3 (Williams et al 1991) and NF-IL6β (Kinoshita et al 1992). C/EBPδ is expressed in many tissues but most highly in the lung (Williams et al 1991 and

Cao et al 1991). C/EBPδ is induced by LPS, IL-1, or IL-6, as with C/EBPβ (Kinoshita et al 1992), suggesting its role in inflammation. Unlike C/EBPβ, the induction of C/EBPδ mainly occurs at the transcriptional level (Ramji et al 1993). C/EBPδ is a stronger transactivator than C/EBPβ, and perhaps acts in a combinatorial or synergistic manner with C/EBPβ to regulate the gene expression involved in the immune and inflammatory responses (Kinoshita et al 1992). So far, although no information about regulation of the acute phase response in C/EBPβ and δ double deficient mice is available, the results from these mice indeed demonstrated that C/EBPβ and C/EBPδ have a synergistic role in terminal adipocyte differentiation in vivo (Tanaka et al 1997).

Like C/EBPβ, C/EBPδ is a phosphoprotein that translocates into the nucleus following threonine phosphorylation of the MAP kinase site. The phosphorylation of C/EBPδ by casein kinase II increases its binding activity, but does not affect binding specificity, although the phosphorylation of C/EBPα and C/EBPβ decreased binding affinity (Osada et al 1996). Studies on the regulation of the α1-acid glycoprotein and the serum amyloid A genes during the acute phase response have shown that dephosphorylation of C/EBPδ results in an inhibition of its DNA binding activity (Ray et al 1994). Additionally, the trans-activation potential of C/EBPδ was also found to be increased when hepatocytes were treated with cellular phosphatase inhibitors, such as okadiac acid and sodium orthovanadate (Ray et al 1994).

1.4 C/EBPε

The complete gene encoding C/EBPε was first cloned from both human (Antonson et al 1996; Chumakov et al 1997) and mouse (Yamanaka et al 1997a) cDNA and genomic libraries. A partial sequence of the rat homologue was cloned from a rat genomic library and was called CRP1 (Williams et al 1991). Unlike other C/EBP members, C/EBPε has a very restricted pattern of expression and is detected in human peripheral blood cells, in the T-cell Jurkat line and in HL60 promyelocytic cells (Antonson et al 1996). Its expression is normally limited to organs of the immune system and bone marrow in humans (Antonson et al. 1996). C/EBPε is upregulated during in vitro granulocytic differentiation of human primary $CD34^+$ cells (Yamanaka et al 1997a). Furthermore, there are functional and maturational defects in the granulocytes of C/EBPε-deficient mice, as well as impaired T-cell proliferation (Yamanaka et al 1997b; Kawano et al 1999). Additionally, macrophage functional maturation and cytokine production are impaired in C/EBPε-deficient mice (Tavor et al 2002). Taken together, these studies implicate C/EBPε as an important transcription factor required for normal function and/or development of granulocytes, macrophages, and T lymphocytes. The human C/EBPε gene is transcribed by two alternative promoters, Pα and Pβ. Alternative use of promoters and differential splicing generates four mRNA isoforms, which encode four proteins of MW 32 kDa, 27 kDa, 20 kDa and 14 kDa. These four proteins contain the identical DNA binding and dimerization domains. However, they differ in the length of their transactivation domains and have differing transcriptional activities (Yamanaka et al 1997).

1.5 C/EBPγ

C/EBPγ is also called Ig/EBP. It was originally isolated from an expression library from murine fibroblasts as a protein binding to the immunoglobulin heavy chain (IgH) enhancer (Roman et al 1990). A human homologue which binds to the PRE-1 enhancer element of the human interleukin-4 promoter was also cloned (Davydov et al 1995). C/EBPγ is most highly expressed in immature B cells, although its expression is rather ubiquitous (Roman et al 1990). C/EBPγ is a short gene containing one intron. It encodes a 16.4-kDa protein, which lacks known activation domains and is essentially a C/EBP bZIP domain (Cooper et al 1995). Consistent with this structure, C/EBPγ has been shown to inhibit C/EBP transcriptional activators and has been proposed to act as a buffer for C/EBP activators. The predominance of C/EBPγ over C/EBPβ in early B cells would prevent transcription of C/EBP-dependent genes, while increased expression of C/EBPβ in mature cells would be permissive for expression (Cooper et al 1995). A fusion protein containing a TFE3 activation domain and the bZIP domain from C/EBPγ can activate transcription through C/EBP sites, providing additional evidence that the inability of C/EBPγ to induce transcription is due to the absence of an activation motif (Artandi et al 1994). Contrary to this notion, C/EBPγ also plays some positive roles in the regulation of gene expression. For example, C/EBPγ was found to synergize with Stat6 and NF- B p50/p65 to induce the germline gamma 3-immunoglobulin promoter in a B cell line (Pan et al 2000). Another instance of a positive role for C/EBPγ is its enhancement of β-globin gene expression in collaboration with CAAT binding protein CP-1 (Wall et al

1996). In addition, C/EBPγ has also been implicated to exert a stimulatory effect in the expression of pp52, a leukocyte-specific phosphoprotein postulated to regulate cytoskeleton structure (Omori et al 1997). Studies using an in vitro transcription system have also shown that nuclear extracts depleted of C/EBPγ have reduced C/EBP site-dependent promoter activity (Cooper et al 1992). Whether C/EBPγ functions as an activator or a repressor, both its lack of expression and overexpression have consequences in vivo. C/EBPγ-deficient mice show a high mortality rate within 48 hours after birth, and have defects in natural killer cell cytotoxic activity and interferon γ production (Kaisho et al 1999). Moderate erythroid overexpression of C/EBPγ in transgenic mice increased γ-globin expression relative to β-globin, while high-level expression blocked erythropoiesis (Zafarana et al 2000). C/EBPγ can form heterodimers with C/EBPβ (Cooper et al 1992; Thomassin et al 1992) and C/EBPα (Roman et al 1990; Thomassin et al 1992), but the function for the heterodimer is not clear. The occurance of C/EBPβ:C/EBPγ heterodimers are certainly widespread, having been observed in glioma, mammary tumor, and hepatoma cell lines, as well as in liver, brain, pancreas, and ovary (Parkin et al 2002). Furthermore, C/EBPγ was reported to form heterodimers with proteins of other leucine-zipper transcription factors such as ATF (Nishizawa et al 1992; Vinson et al 1993). In this way it might promote the binding of other transcriptional activators to DNA. Indeed, it was observed that human C/EBPγ interacts with Fos, a member of the AP-1 family, to form a complex on the positive regulatory element-I site (PRE-I) of interleukin-4 promoter (Davydov et al 1995). We have found that C/EBPγ is

overwhelmingly present as a heterodimer with conventional activating C/EBP isoforms in lymphoblasts dependent upon C/EBPβ, δ or α for IL-6 expression. In these cells, C/EBP γ plays an activating role in LPS induction of IL-6. These results will be described in detail in the second chapter.

1.6 C/EBPζ

C/EBPζ has also been reported as CHOP (Ron et al 1992) and Gadd153 (Luethy et al 1990; Park et al. 1992). Like other C/EBP proteins, C/EBPζ possesses a leucine zipper dimerization domain and DNA-binding region (Ron et al. 1992). C/EBPζ can form heterodimers with other C/EBPs, but two prolines in its DNA-binding region disrupt its helical structure and prevent the dimer from binding to the C/EBP consensus motif (Ron et al. 1992). Thus, C/EBPζ functions as a dominant negative inhibitor of C/EBP transcriptional activator by preventing heterodimer binding to classic C/EBP enhancer sequences. When expressed in cells, C/EBPζ attenuates the ability of other C/EBP proteins to activate promoters containing such sequences (Ron et al 1992). However, recent studies have shown that C/EBPζ-C/EBP heterodimers can activate downstream target genes although their significance remains unknown (Wang et al 1998; Sok et al 1999). For example, the C/EBPζ-C/EBPβ can specifically activate transcription of the murine carbonic anhydrase VI gene through a non-consensus binding site (Sok et al 1999). Furthermore, it was found that C/EBPζ can also interact with members of the AP-1 transcription factor family, JunD, c-Jun, and c-Fos, to activate promoter elements in the somatostatin, JunD, and collagenase genes

(Ubeda et al. 1999). It was also found that C/EBPζ was recruited to the AP-1 complex by a tethering mechanism rather than by direct binding to DNA, implicating a novel mechanism by which C/EBP can regulate gene expression (Ubeda et al 1999).

C/EBPζ was originally identified as a gene induced upon DNA damage and growth arrest (Fornace et al 1988). However, subsequent studies have demonstrated a strong correlation between development of endoplasmic reticulum (ER) stress and induction of C/EBPζ . C/EBPζ expression is coordingly regulated with the ER chaperone BiP (Brewer et al 1997; Wang et al 1996; Halleck et al 1997) and is inducible by agents that either directly (Bartlett et al 1992; Chen et al 1992; Price et al 1992; Halleck et al 1997) or indirectly (Carlson et al 1993; Marten et al 1994; Bruhat et al 1997) lead to impairment of the ER folding environment (ER stress). However, the mechanism by which ER stress leads to C/EBPζ gene expression is not known. On the other hand, ER stress regulates C/EBPζ not only by inducing expression of the gene, but post-translationally at the level of phosphorylation. For example, C/EBPζ protein undergoes stress-inducible phosphorylation by stress-inducible members of the p38-MAP kinase family and this phosphorylation is associated with enhanced transcriptional activation by C/EBPζ (Wang and Ron 1996).

Most evidence supports the notion that C/EBPζ negatively regulates cell growth and, additionally, may induce apoptosis. Overexpression of C/EBPζ can lead to cell cycle arrest and apoptosis (Barone et al 1994; Zhan et al 1994). Disruption of the C/EBPζ gene by a chromosomal translocation t(12:16)(q13:p11)

is associated with human myxoid liposarcoma. This translocation results in a fusion between a novel glycine-rich protein and C/EBPζ (Crozat et al 1993; Rabbitts et al 1993). Mice with a homozygous deletion in the C/EBPζ gene displayed reduced apoptosis in the renal epithelium in response to tunicamycin injection (Zinszner et al 1998). In addition, there is less subsequent tissue regeneration. These results suggest that C/EBPζ may signal death in response to ER stress and that it may also play a role in cellular regeneration. Furthermore, C/EBPζ -deficient mice are defective in the development of an apoptotic response to agents that cause destruction of the pancreatic β cells and thereby cause diabetes (Oyadomari et al 2001; Oyadomari et al 2002). Very recently, research from Dr. Nakshatri's group showed that NF-κB could inhibit C/EBPζ activation in breast cancer cells exposed to nutrient deprived media, tunicamycin (which blocks protein folding in the ER) or calcium ionopore (which depletes calcium stores in ER) (Nozaki et al 2001). These results establish a correlation between repression of pro-apoptotic genes by NF-κB and increased cell survival during ER stress, and also identify a distinct NF-κB regulated cell survival pathway. C/EBPζ has also been shown to play a role in the programmed activation of C/EBPβ during adipogenesis (Tang et al 2000) and to play a role in erythropoiesis (Coutts et al 1999). For example, C/EBPζ transiently interacts with C/EBPβ in growth-arrested preadipocytes, delaying acquisition of DNA-binding activity and activation of the C/EBPα gene until mitotic clonal expansion is underway (Tang et al 2000). In normal hematopoietic cells, the highest levels of C/EBPβ were found in erythroid cells, with levels peaking during terminal

differentiation. Artificial downregulation of C/EBPζ in normal murine bone marrow cells inhibited colony-forming unit-erythroid-derived colony growth in a concentration-dependent manner (Coutts et al 1999). These results strongly suggest that C/EBPζ plays a role during erythroid differentiation.

2. Regulation of Interleukin-6 expression

2.1 Introduction to cytokines

The development of an effective immune response involves T cells, B cells, macrophages, and other hematopoietic cells. The complex interactions among these cells are mediated by a group of proteins designated cytokines to denote their role in cell-to-cell communication. Cytokines are a group of low-molecular-weight regulatory proteins secreted by white blood cells and a variety of other cells in response to a number of inducing stimuli. Cytokines bind to specific receptors on the membrane of target cells, triggering signal-transduction pathways that ultimately alter gene expression in those target cells. Originally, cytokines were thought to function in a cell-specific manner eliciting a limited range of effects. Now, it is known that they function in a pleiotropic manner (Paul 1989; Kishimoto et al 1992) eliciting different biological effects on different target cells. Another feature characteristic of cytokines is that they exert biological activities in a redundant manner. Many cytokines are referred to as interleukins, a name indicating that they are secreted by some leukocytes and act upon other leukocytes. Presently, interleukins 1 through 17 have been identified. Other cytokines are known by common names; these include the interferons and tumor

necrosis factors (TNF). A group of low-molecular-weight cytokines, including interleukin 8, is classified in the chemokine family. Many of these molecules play an important role in the inflammatory response.

Cytokines are proteins or glycoproteins that generally have a molecular mass of less than 30 kDa. Many cytokines belong to a family of structurally related proteins, called the hematopoietins. Although the amino acid sequences of the various hematopoietins differ considerably, all of them have a high degree of α-helical structure and little or no β-sheet structure. Cytokines generally function as intercellular messenger molecules that evoke particular biological activities after binding to a receptor on a responsive target cell. Although a variety of cells can secrete cytokines, the two principal producers in the immune system are the T_H cell and the macrophage. Among the numerous physiologic responses that require cytokine involvement are development of cellular and humoral immune responses, induction of the inflammatory response, regulation of hematopoiesis, control of cellular proliferation and differentiation, and induction of wound healing. Cytokines rarely act alone in vivo. Instead, a cell is exposed to an environment having many cytokines. These cytokines may have synergistic or antagonistic effects. Also, one cytokine can induce the synthesis of another cytokine.

The structures of the receptors for the various cytokines are quite diverse. These receptors belong to five families: class I cytokine receptors family, class II cytokine receptors family, Immunoglobulin superfamily receptors, TNF receptor family and chemokine receptor family.

2.2 Overview of IL-6

IL-6 is produced by many different cell types. The main sources in vivo are stimulated monocytes, fibroblasts, and endothelial cells. Macrophages, T-cells and B-lymphocytes, granulocytes, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cells, and keratinocytes also produce IL-6 after stimulation. Physiological stimuli for the synthesis of IL-6 are IL-1, bacterial endotoxins, TNF, PDGF, and Oncostatin M. Glucocorticoids inhibit the synthesis of IL-6 (Braciak et al 1991), as do IL-4 (Donnelly et al 1993 and Zissel et al 1996) and TGF-beta (Reinhold et al 1994).

IL-6 is a protein of 185 amino acids glycosylated at positions 73 and 172. It is synthesized as a precursor protein of 212 amino acids. Murine and human IL-6 show 65% sequence homology at the DNA level and 42% homology at the protein level (Tanabe et al 1988). IL-6 is a member of a family of cytokines that also includes IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M, and cardiotrophin. All known members of the IL-6 cytokine family induce hepatic expression of acute phase proteins. The IL-6 receptor is expressed on T-cells, mitogen-activated B-cells, peripheral monocytes and some macrophage- and B-cell-derived tumor cells. It is not expressed in resting B-cells but is expressed in resting T-cells. IL-6 receptor-complex-mediated signal transduction involves activation of JAK kinases and the transcription factor Stat3 (Hirano et al 2000). Other signaling pathways including the Ras/MAP kinase (Boulton et al 1994; Zauberman et al 1999) and protein

kinase C (Jain et al 1999) pathways are also induced although their functions are not yet totally understood.

IL-6 is a pleiotropic cytokine. It was originally identified as a factor that induced immunoglobulin production in activated B cells and was initially designated as B cell differentiation factor or B cell stimulatory factor-2. But it now has also been found to exhibit a wide range of biological functions in cells outside the B lymphocyte lineage (Akira et al 1993). It is involved in the regulation of differentiation, proliferation, and survival of many target cells within the hematopoietic lineage, as well as astrocytes and endothelial cells (Hirano 1998). Among the hematopoietic lineages, IL-6 acts on (i) myeloma and plasmacytoma cells to induce proliferation, (ii) hematopoietic progenitors to induce expansion, (iii) megakaryocyte progenitors to induce proliferation and differentiation, (iv) M1 myeloid leukemia cells to stop proliferation and induce macrophage differentiation, and (v) T lymphocytes to induce proliferation and differentiation into cytotoxic T cells. Outside the hematopoietic system, IL-6 functions as a hepatocyte-stimulating factor serving as a major mediator of the acute-phase response. This response is characterized by the synthesis and secretion of acute-phase plasma proteins by the liver, elevated serum glucocorticoid levels, and fever. Furthermore, IL-6 controls bone turnover and is crucial for liver regeneration (Poli et al 1994; Cressman et al 1996). IL-6 exerts its effects in an endocrine, paracrine, and autocrine manner. A detailed list of the pleiotropic functions of IL-6 is given at Table 1.

Table 1. Pleiotropic functions of IL-6

Effect on B cells	Ig production Proliferation of myeloma cells Proliferation of Epstein-Barr virus-infected B cells
Effect on T cells	Proliferation and differentiation of T cells Differentiation of cytotoxic T lymphocytes Induction of IL-2R expression and IL-2 production Augmentation of NK activities
Effect on hematopoietic progenitor cells	Enhancement of multipotential hematopoietic colony formation
Effect on megakaryocytes	Megakaryocyte maturation
Effect on macrophages	Growth inhibition of myeloid leukemic cell lines and induction of their macrophage differentiation
Effect on hepatocytes	Acute-phase protein synthesis
Effect on bone metabolism	Stimulation of osteoclast formation Induction of bone resorption
Effect on blood vessels	Induction of platelet-derived growth factor Proliferation of vascular smooth muscle cells
Effect on heart muscle cells	Negative inotropic effect on heart
Effect on neuronal cells	Neural differentiation of PC12 cells Support of survival of cholinergic neurons Induction of adrenocorticotropic hormone synthesis
Effect on placenta	Secretion of chorionic gonadotropin from trophoblasts

The generation of IL-6-deficient mice has provided clearer insight into the function of IL-6. Their phenotype demonstrates the pivotal role of IL-6 in the acute phase response (Kopf et al 1994; Fattori et al 1994; Xing et al 1998). Furthermore, the mice show major defects in inflammatory and immune responses, exhibiting impaired defense against many types of infections (Fattori et al 1994; Romani et al 1996). Impaired macrophage and neutrophil responses have been demonstrated in IL-6-deficient mice, and several studies describe a shift from T helper cell 1 (Th1) to Th2 responses (Ladel et al 1997; Romani et al 1996; Okuda et al 1999). IL-6-deficient mice are also resistant to experimental autoimmune encephalomyelitis (Okuda et al 1998; Samoilova et al 1998; Mendel et al 1998) and develop milder forms of experimental arthritis (Alonzi et al 1998). These results support the notion that IL-6 functions in autoimmune and chronic inflammatory diseases. Furthermore, IL-6-deficient mice show a reduced production of chemokines and impaired leukocyte accumulation in local inflammatory reactions (Romano et al 1997). Consistent with the established role of IL-6 in plasmacytoma and myeloma growth, IL-6-deficient mice do not develop pristine-oil-induced plasmacytomas (Lattanzio et al 1997). Conversely, the generation of monoclonal transplantable plasmacytomas was observed in transgenic mice overexpressing IL-6 (Suematsu et al 1992). These results demonstrate a critical role for IL-6 in plasmacytoma development.

2.3 Transcriptional regulation

Kishimoto's group isolated the chromosomal genes for both human and murine IL-6 (Tanabe et al 1988; Yasukawa et al 1987). The complete human and mouse IL-6 genes are approximately 5 kb and 7 kb in length respectively, and both consist of five exons and four introns. The genes for human and mouse IL-6 were mapped to chromosomes 7 and 5 respectively. Besides the sequence similarity in the coding region, the 5' flanking region and 3' untranslated region are highly conserved between the human and mouse IL-6 genes. The 3' untranslated region contains the ATTTA sequences which are commonly observed in the 3' untranlated regions of mRNAs for lymphokines, cytokines and protooncogenes, and are thought to be involved in mRNA stability (Shaw et al 1986; Conne et al 2000). The region extending about 350 bp upstream of the transcriptional start site is highly homologous between the human and the mouse IL-6 genes. Five known functional transcriptional control elements are identified within this conserved region of the IL-6 promoter. There are two C/EBP binding sites, one NF-κB binding site, one cAMP response element (CRE) and one AP-1 binding site. Each of these sites contributes differently to IL-6 induction in response to different stimuli and in different cells. For example, LPS and lipoarabinomannan (LAM) from the mycobacterial cell wall potently induce IL-6 gene expression in peripheral blood monocytes (Zhang et al 1994). By deletion analysis and transient transfection assays in the human myelomonocytic leukemia cell line THP-1, both LPS- and LAM-inducible IL-6 promoter activities were localized to a DNA fragment at positions -158 to -49 bp, where two C/EBP and one NF-κB site are located. Site-directed mutagenesis of one or more of

these sites within the IL-6 promoter demonstrated that they all have positive regulatory activity. Deletion of all three sites abolished the inducibility of IL-6 promoter activity by both LPS and LAM, showing that the C/EBP and NF-κB sites mediate IL-6 induction in response to both LPS and LAM. Similarly, Liebermann's group reported that these sites are involved in IL-6 gene activation by prostaglandin E1, its second messenger cAMP, and by LPS in the mouse monocytic cell line PU5-1.8 (Dendorfer et al 1994). Mutations within these regulatory elements (AP-1, CRE, C/EBP, and NF-κB) significantly reduced, but did not completely abrogate, the inducibility by prostaglandin E1 or its second messenger cAMP. However, LPS-induced promoter activity was almost completely abolished by mutations of the NF-κB site. These results suggest that a single regulatory element is crucial for LPS inducibility, whereas prostaglandins and cAMP act through multiple, partially redundant regulatory elements. Thus, the activity of at least four transcription factors is simultaneously required to maximally induce IL-6 gene transcription upon stimulation with either cAMP or LPS, but the contribution of each regulatory element to the transcriptional activation of IL-6 gene appears to vary depending on the stimulus.

Several studies demonstrated that IL-6 gene expression can also be negatively regulated at the transcriptional level. Ray et al showed that the activated glucocorticoid receptor (GR) can bind to the CRE and C/EBP site as well as to the basal transcription regulatory regions (TATA box and RNA start site) in the IL-6 promoter (Ray et al 1990). This binding interfered with the binding of positive-acting inducible and basal transcription factors, resulting in the

highly efficient repression of transcription by dexamethasone. Santhanam et al showed that p53 or RB can also repress the IL-6 promoters in serum-induced HeLa cells, suggesting that p53 and RB may be involved as transcriptional repressors in IL-6 gene expression (Santhanam et al 1991). Although it has been previously shown that the IL-6 κB motif functions as a potent IL-1/tumor necrosis factor-responsive element in nonlymphoid cells, Yamamoto's group found that a lymphoid cell-specific nuclear factor that contains c-Rel but not p50 epitopes, termed IL-6 κB binding factor II, functions as a repressor specific for IL-6 κB-related κB motifs in lymphoid cells (Nakayama et al 1992). More recently, Armanante showed that in an IL-6-non-expressing cell line, IL-6 repression is associated with a distinctive modification of chromatin structure, as suggested by a decreased sensitivity of the IL-6 promoter to DNAase I relative to the IL-6-expressing cells (Armenante et al 1999). Moreover, they showed that in IL-6-non-expressing cells, local chromatin remodelling at the proximal promoter of IL-6 is inhibited by negative regulators, whose binding is suggested by two specific footprints of nuclear factor binding that are not observed in IL-6-expressing cells.

2.4 Post-Transcriptional regulation

Although the regulation of IL-6 gene expression occurs mainly at the transcriptional level, post-transcriptional regulation has also been described (Elias et al 1990; Roger et al 1998; Garcia et al 1999; Winzen et al 1999; Neininger et al 2002). The IL-6 bears an AU-rich sequence in its 3'-UTR, which has been demonstrated to contribute to mRNA stabilization in response to pro-

inflammatory cytokines (Winzen et al 1999). Using a tetracycline-controlled expression system, Winzen et al analyzed the effects of cytokine/stress-induced signaling pathways on the half-life of IL-6 and IL-8 mRNAs (Winzen et al 1999). They found that both transcripts were rapidly degraded in unstimulated HeLa cells, while expression of a constitutively active form of a MAP kinase kinase kinase (MEKK1) markedly stabilized those transcripts as well as reporter RNAs containing the 3'-UTR sequences of IL-6 and IL-8. Furthermore, they found that stabilization was also induced upon activation of p38 MAP kinase by expressing its selective activator MKK6. Correspondingly, a dominant-negative form of p38 MAP kinase interfered with MKK6-induced and IL-1-induced stabilization. Finally, an active form of MK2, a substrate kinase of p38 MAP kinase, induced stabilization, whereas its dominant-negative mutant interfered with MKK6-induced stabilization. Using a human lung-derived epithelial cell line, H292, Roger et al showed that Cycloheximide (CHI), which inhibits protein synthesis by 80%, can cause an 80-fold induction of IL-6 mRNA levels predominantly due to a stabilization of IL-6 mRNA (20-fold) (Roger et al 1998). Employing transient transfection assays, they showed a small positive effect of CHI on transcription mediated by the proximal and the distal C/EBP sites of the IL-6 promoter and paralleled by increased C/EBP DNA-binding activity. However, this effect of CHI on IL-6 gene transcription was transient, supporting the notion that ongoing protein synthesis is required for C/EBP activity. Rather these findings indicate that IL-6 mRNA superinduction is regulated predominantly by modulating the repressive system that ensures a rapid degradation of IL-6 mRNA.

2.5 C/EBPs in regulation of IL-6 expression

C/EBP-binding motifs have been identified in the functional regulatory regions of various genes involved in inflammatory and immunological response including acute phase protein and cytokine genes (Akira et al 1992). These include the genes for IL-6, IL-1β, TNF-α, IL-8, IL-12, IL-4, albumin, α1-acid glycoprotein, lysozyme, myeloperoxidase, inducible nitric oxide synthase, neutrophil elastase, G-CSF, the macrophage, granulocyte, and granulocye-macrophage receptor genes. Among the C/EBP members, C/EBPβ has been assigned a predominant role for the induction of proinflammatory cytokines. Indeed, C/EBPβ (NF-IL6) was originally identified as a DNA-binding protein responsible for IL-1-stimulated IL-6 induction (Isshiki et al 1990). C/EBPβ is expressed at low or undetectable levels in all normal tissues, but it is significantly induced by stimulation with LPS, IL-1, TNF, or IL-6. Its expression is also dramatically induced during the differentiation of macrophages, which are a major source of proinflammatory cytokines upon activation (Scott et al 1992; Natsuka et al 1992). While C/EBPβ is highly expressed in macrophages, it is not expressed in lymphoblasts. Thus, lymphoblasts provide a good model system to study the function of C/EBPβ using ectopic expression. Taking advantage of this, our lab showed that the ectopic expression of C/EBPβ conferred LPS-inducible expression of IL-6 and monocyte chemoattractant protein 1 (MCP-1) to lymphoblasts, which normally do not display the LPS induction of these inflammatory cytokines (Bretz et al 1994). On the other hand, C/EBPβ knockout mice presented normal induction of IL-6 (Tanaka et al 1995; Screpanti et al

1995), suggesting that other C/EBP members could compensated for the lack of C/EBPβ. Indeed, Kishimoto's group showed that C/EBPδ was consistently a stronger transactivator of the human IL-6 promoter than C/EBPβ in transient transfection assays (Kinoshita et al 1992). In addition, they showed that C/EBPδ had a synergistic transcriptional effect with C/EBPβ. Results from our lab have shown that the ectopic expression of either C/EBPα, β, or δ is sufficient to confer the LPS-inducible expression of IL-6 and MCP-1 to lymphoblasts (Hu et al 1998). These results suggest that C/EBPα, β, and δ are redundant in regard to the expression of IL-6. Furthermore, we have shown that C/EBPγ and C/EBPζ also participate in the regulation of IL-6 in lymphoblasts (Chapter 2 and 3).

2.6 Other cooperating transcription factors

A number of different transcription factors such as NF-κB, AP-1, CREB, STATs, PU.1, Myb, and Glucocorticoid receptor have been reported to physically and functionally interact with C/EBP family members, in particular with C/EBPβ. Among those, the interactions with members of the NF-κB family of transcription factors are well studied (Stein et al 1993; LeClair et al 1992). NF-κB was originally characterized as a immunoglobulin enhancer DNA-binding protein. It is a dimer of members of the rel family of proteins (reviewed by Kopp et al 1995 and Verma et al 1995). So far, six members have been identified in this family. They are: c-Rel (v-rel), dorsal, p50 (p105), p52 (p100), RelA (p65) and RelB. Each family member contains an N-terminal 300 amino acid conserved region known as the rel homology domain (RHD). This region is responsible for DNA-

binding, dimerization, and interaction with IκB family members. It also contains a nuclear localization sequence. Although the amino-terminal regions of NF-κB members are highly conserved and perform similar functions, the carboxy terminals of these proteins differ significantly. For example, the p50 protein has very little carboxyl terminal sequence apart from the RHD, and lacks transcriptional activity. RelA harbors transcription-activating domains in its C-terminal portion. Apart from forming homodimers, most NF-κB family members can form heterodimers with each other, but each individual member or heterodimer complex may differ in DNA-binding specificity and transcription activity for a particular κB site (Liou et al 1993). It is now known that NF-κB preexists in the cytoplasm of most cells in an inactive form bound to the inhibitor, IκB. Upon receipt of an appropriate signal, NF-κB is released from IκB and translocates to the nucleus where it can upregulate transcription of specific genes. NF-κB is involved in the regulation of many genes activated during inflammatory, immune and acute phase responses. Binding sites for NF-κB have been identified in the promoter region of several cytokine genes including IL-6 and IL-8, acute phase response genes, and several viral enhancers including HIV-1. For example, by site-directed mutagenesis, it was shown that the NF-κB site (positions -173 to -151) was the key IL-1β and TNF-α responsive element on IL-6 promoter in U-937 monocytic cells and in HeLa cells (Libermann et al 1990). Employing adenoviral-mediated gene delivery of a nondegradable IκBα, Pope's group (Georganas et al 2000) showed that inhibition of NF-κB activation significantly reduced the spontaneous and IL-1β-induced secretion of IL-6 by

rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) and human dermal fibroblasts. In contrast, inhibition of C/EBPβ with a dominant negative version of C/EBP modestly reduced constitutive and IL-1β-induced IL-6 by RA FLS, but not by human dermal fibroblasts. Inhibition of c-Jun/AP-1 with a dominant negative had no effect on the production of IL-6. Fibroblasts in which both NF-κB p50/p65 genes were deleted failed to express IL-6 in response to IL-1. These findings document the dominant role of NF-κB for the regulation of the IL-6 expression by RA FLS. On the other hand, adjacent C/EBP and NF-κB motifs are found in the promoters of many AP class I genes that require both IL-1 and IL-6 for their induction, as well as those of several cytokine genes. These suggest that cooperative interaction between these two families of transcription factors may represent a general mechanism of coordinating transcriptional response ro different stimuli. Indeed, synergistic activation by C/EBP and NF-κB members has been demonstrated for the genes encoding the acute phase proteins serum amyloid A1, A2, A3, α1-acid glycoprotein and C-reactive protein, as well as the cytokines IL-6, IL-8, IL-12, and the G-CSF (Ray et al 1995; Li et al 1992; Betts et al 1993; Agrawal et al 2001; Matsusaka et al 1993; Plevy et al 1997; Dunn et al 1994; Lee et al 1996; and Vietor 1996). In regard to IL-6, it has also been shown that both the C/EBP and NF-κB binding sites are required for synergistic activation (Matsusaka et al 1993). In contrast, C/EBP and NF-κB interactions can also lead to antagonistic effects (Stein et al 1993; Braiser et al 1990). For example, cross-coupling between NF-κB and C/EBPα, C/EBPβ, or C/EBPδ results in the inhibition of a promoter with a κB enhancer motif and the

synergistic stimulation of promoters with C/EBP binding sites (Stein et al 1993). This suggests that distinct mechanisms involving synergism and cooperativity as well as competition between C/EBP and NF-κB may contribute to the regulation of gene expression. They also suggest that promoter architecture and specific cell type are likely to play a major role. Although the mechanisms responsible for cooperative effects have not yet been entirely clarified, it has been shown that productive interaction requires the integrity of both the NF-κB rel homology domain and the C/EBP leucine zipper motif (LeClair et al 1992). Increased affinity of C/EBP and NF-κB for their respective sites has been demonstrated (Ruocco et al 1996; Stein et al 1993), and DNA-protein complexes containing both proteins have been detected using both C/EBP sites and NF-κB sites (Ray et al 1995; Ruocco et al 1996; Vietor et al 1996). Data from our lab have also shown that NF-κB DNA binding activity is induced by LPS stimulation (Hu et al 1998), and that RelA (p65) can replace LPS to cooperate with C/EBPβ in transactivation of IL-6 expression (Gao et al in press). Furthermore, studies in the Schwartz lab found that although C/EBP activity is essential to IL-6 expression, C/EBP activity is dependent upon an intact NF-κB binding site (Hu et al 2000). This suggests that C/EBP stimulatory activity is dependent upon synergy with NF-κB in lymphoblasts. Another example of C/EBP interaction with other transcription factors is that between C/EBP and the glucocorticoid receptor (GR) or the estrogen receptor (ER). It has been shown that glucocorticoid hormones (GHs) and estrogen downregulate the expression of IL-6 and IL-8 by a direct interaction between C/EBP or NF-κB and the GR or ER (Ray et al 1994a,

b; Scheinman et al 1995; Stein et al 1995). It is also demonstrated that the physical and functional interaction depends on the DNA-binding domain of the GR or ER and on the RHD of NF-κB or the bZIP region of C/EBPβ.

Taken together, these results suggest that the combinatorial effects of C/EBPs with various other transcription factors are very important in considering the role of C/EBPs in the regulation of pro-inflammatory cytokine gene expression.

3. Transcription factors of pre-B and B cells

3.1 B cell development

The developmental process that results in generation of functional plasma cells can be divided into three stages: generation of mature, immunocompetent B cells (maturation), activation of mature B cells by interaction with antigen, and differentiation of activated B cells into plasma cells. B-cell maturation, which occurs in the bone marrow, involves an orderly sequence of Immunoglobulin (Ig)-gene rearrangements and progresses in the absence of antigen. This stage is the antigen-independent phase. A mature B cell expressing membrane-bound immunoglobulin (mIgM and mIgD) leaves the bone marrow to enter the blood and lymph. When these naive B cells are activated by interacting with the antigen for which its membrane-bound antibody is specific, they undergo proliferation and differentiation, generating a population of antibody-secreting plasma cells and memory B cells. These two stages are the antigen-dependent phase.

A number of transcription factors that regulate expression of various gene products at different stages of B-cell development have been identified (reviewed by Glimcher and Singh, 1999). Here, I will briefly summarize the functions or status of C/EBPs, NF-κB, and AP-1, which in addition to their roles in B lymphopoiesis, have been shown to regulate the IL-6 transcription.

3.2 C/EBPs

C/EBP binding sites have been shown to be functionally important in Ig heavy chain (IgH) variable region (V_H) promoters (Cooper et al 1992), the IgH intronic enhancer (Tsao et al 1988), the κ intronic enhancer (Sen et al 1986), and the γ 1 germ-line promoter (Xu et al 1992). By using B cell lines at various development stages and normal splenic B cells, Cooper et al showed that expression of C/EBPs is limited and regulated during B cell development (Cooper et al 1994). They found that C/EBPβ and C/EBPγ were the major regulators of C/EBP site-dependent transcriptional activity in B cells. In early B cells, C/EBPγ was predominantly present. C/EBPβ increased in more mature B cells and was induced by LPS activation of splenic B cells although its RNA was virtually undetectable in proB and preB lines. These results suggest that the C/EBP motif functions as an activator site in mature B cells, implying a role in increased Ig expression and regulation of class switching. The significance of high C/EBPγ levels in early B cells where C/EBPβ expression is low has remained a puzzle. The ability of C/EBPγ to inhibit C/EBPβ activation of artificial promoters in transient transfections suggested that it may act as a buffer to C/EBPβ activity in

immature cells (Cooper et al 1994 and Cooper et al 1995). Our results showed that C/EBPγ can augment C/EBPβ stimulatory activity in the LPS induction of IL-6 expression in P388 lymphoblast cells. IL-6 is essential for the B cell differentiation and C/EBPγ may participate in autocrine IL-6 production. This may be particularly important in the absence of T cell help in T-independent responses to gram-negative bacteria. The notion that C/EBPβ and γ may play more of a role in B cell maturation than in the function of mature cells is supported by the work of Sun and co-workers, who reported that C/EBPβ is a component of the two major pro-B-cell-specific enhancer (PBE)-binding complex of the Id1 gene, a gene which encodes a protein that acts as a negative regulator in early-B-cell differentiation by antagonizing the function of the basic helix-loop-helix transcription factors (Saisanit and Sun, 1997). In contrast to the situation in pro-B cells, they found that C/EBPβ is bound to C/EBPζ and thus inactivated in mature B cells. This suggests that C/EBP proteins, by forming a pro-B-cell-specific active complex or a mature-B-cell-specific inactive complex, may play an important role in the regulation of B-cell development. More evidence for the function of C/EBPβ in B cell development comes from the analysis of knock-out mice. The C/EBPβ-/- mice show a lymphoproliferative disorder, similar to Castleman's Disease, where high circulating levels of IL-6 lead to expansion of mature germinal center B cells and splenomegaly and peripheral lymph node enlargement (Screpanti et al 1995). At the same time there is impaired B cell differentiation with impaired expansion of bone marrow B lymphocytes and reduced proliferative responsiveness of B-cell precursors to IL-7 (Chen et al

1997). Thus, while C/EBPβ may have a positive role in activated B cells where its expression is highest, it clearly also has a critical function in B lymphopoiesis.

NF-κB

B cell differentiation is dependent upon the programmed expression of Ig heavy (μ) and light chain loci (κ or λ) (eviewed by Gorman and Alt 1998). This developmental program is regulated primarily at the level of gene expression by the action of transcription factors, including NF-κB. In most cells including pre-B cells, NF-κB is maintained in an inactive form bound to the inhibitor, IκB, in cytoplasm. In contrast, mature B cells constitutively express nuclear NF-κB, primarily in the form of a c-Rel/p50 heterodimer (Liou et al 1994; Grumont et al 1994). This constitutive NF-κB activity is believed to play a critical role in the development of B lymphocytes because it controls stage-specific expression of genes such as Igκ (Sen et al 1986; Scherer et al 1996), Oct-2 (Bendall et al 1997) and c-Rel itself (Grumont et al 1993). In addition, NF-κB activity has been implicated in promoting the survival of splenic B cells (Bendall et al 1999; Wu et al 1996), as well as the capacity of B cells to proliferate (Kontgen et al 1995; Sha et al 1995). Despite these findings, the biochemical mechanisms that lead to constitutive NF-κB activation in mature B cells remain largely undefined. Several mechanisms have been suggested, including the enhanced degradation of IκBα and/or IκBβ by the proteasome pathway (Schauer et al 1998; Kistler et al 1998), and resynthesis of a new hypophosphorylated IκBβ which facilitate transport of a portion of NF-κB to the nucleus in a manner that protects it from cytosolic IκBα

(Phillips et al 1997). Recently, a calcium-dependent protease calpain mechanism has been suggested to cause IκBα degradation (Fields et al 2000). Analyses of B cells derived from different knock-out animals reveal that NF-κB is a mediator in numerous pathways that regulate B cell activation and proliferation, including those pathways responding to llipopolysacharride (LPS), CD40 ligand, and antigen-receptor cross-linking; it is also indispensable in isotype switching (Reviewed by Gerondakis et al 2000).

3.4 AP-1

AP-1 is a collection of sequence-specific transcriptional activators composed of members of the Jun and Fos families (Reviewed by Shaulian and Karin 2001). So far, seven members of AP-1 have been isolated. They are: c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and Jun D. Like C/EBPs, AP-1 members also belong to the bZIP superfamily of DNA binding proteins. Fos and Jun proteins can form heterodimers, but only Jun proteins can form homodimers (Angel et al 1991). Since its discovery, the AP-1 family has been demonstrated to be induced by a diverse range of stimuli and to play important roles in many cellular processes, such as cell proliferation, cell transformation, oncogenesis, and cell differentiation (Angel et al 1991; Shaulian and Karin 2001). AP-1 has also been shown to be an important regulator of nuclear gene expression in leukocytes (reviewed by Foletta et al 1998). In B cells, AP-1 complexes have been shown to transactivate the kappa light chain promoter (Schanke et al 1994) and the Ig heavy chain gene (Grant et al 1995), suggesting that AP-1 is involved in immunoglobulin production

and class switching (Ruther et al 1988; Grant et al 1995). AP-1 was also shown to play a role in B cell development by using bone marrow cells from two different transgenic mice carrying exogenous c-fos genes controlled by either the promoter of the H-2Kb gene (H2-c-fos) or the interferon alpha/beta (IFN)-inducible Mx gene (Mx-c-fosD) (Imoto et al 1996). Imoto et al found that development of B lineage cells was retarded in bone marrow cell cultures from H2-c-fos mice. Although B lineage cells developed normally in bone marrow cell cultures from Mx-c-fosD mice in the absence of IFN stimulation, their development was completely blocked in the Mx-c-fosD culture when transgenic c-fos was induced in BM cells by IFN stimulation. Furthermore, IL-7-dependent proliferation of B lineage cells in Mx-c-fosD bone marrow cultures was also suppressed by the induction of c-Fos. These results suggest that the c-Fos plays a role as a negative regulator in the early B cell development. Beyond these studies, the role of AP-1 in B cell development and activation remains to be determined.

4. Objectives for this thesis

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 by Poli 1998). We have previously demonstrated that the stable expression of C/EBPα, 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 (Bretz et al 1994; Hu et al 1998; Williams et al 1998). We also 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 (Hu et al 2000). These observations as well as the widespread occurrence of C/EBPβ:γ heterodimers (Parkin et al 2002) led us to explore further the role of C/EBPγ in regulating IL-6 transcription. This part of the study is described in Chapter 2.

C/EBPζ was originally identified as a gene induced upon DNA damage and growth arrest. It has been shown to be involved in the cellular response to endoplasmic reticulum stress. Because of sequence divergence from other C/EBP family members in its DNA binding domain and its consequent inability to bind the C/EBP consensus-binding motif, C/EBPζ can act as a dominant negative inhibitor of other C/EBPs. C/EBP transactivators are essential to the expression of many proinflammatory cytokines and acute phase proteins, but a role for C/EBPζ in regulating their expression has not been described. We have found that expression of C/EBPζ is induced in response to LPS treatment of B cells at both the mRNA and protein levels. Correlating with the highest levels of C/EBPζ expression at 48 hours after LPS treatment, both the abundance of C/EBP DNA binding species and IL-6 expression are downregulated. These results suggest that C/EBPζ functions as negative regulator of IL-6 expression in B cells. This question was addressed in Chapter 3.

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CHAPTER 2

C/EBPγ HAS A STIMULATORY ROLE ON THE INTERLEUKIN-6 AND INTERLEUKIN-8 PROMOTERS

ABSTRACT

CCAAT/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-specificity with no activity being 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.

INTRODUCTION

CCAAT/enhancer binding protein (C/EBP) α, β, γ, δ, ε, and ζ comprise a family of basic region-leucine zipper (bZIP) transcription factors (reviewed by Johnson et al 1994). 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'-T(T/G)NNGNAA(T/G)-3' (Akira etal 1990). At this time, the reported in vitro functions of C/EBPα, β, 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 by Poli 1998). 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, α_1-acid glycoprotein, serum amyloid A1, A2, A3, complement C3 and C-reactive protein all contain C/EBP binding motifs (Poli 1998). Furthermore, C/EBPβ and C/EBPδ have both been shown to activate a reporter gene controlled by the IL-6 promoter in transient expression assays (Akira et al 1990; Kinoshita et al 1992). We have previously demonstrated that the stable expression of C/EBPα, 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 (Bretz et al 1994; Hu et al 1998; Williams et al 1998). 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 (Hu et al 2000).

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 (Hu et al 2000). C/EBPγ is most highly expressed in immature B cells, although its expression is rather ubiquitous (Roman et al 1990). Its binding specificity is similar to that of other C/EBP family members (Roman et al 1990), but it has a truncated structure. C/EBPγ lacks known activation domains and is essentially a C/EBP bZIP domain (Cooper et al 1995). Consistent with this structure, it has been shown to inhibit C/EBP transcriptional activators and has been proposed to act as a "buffer" for C/EBP activators. C/EBPγ would prevent 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/EBP α, β, δ, and ε exceeded a threshold. It has been proposed that the predominance of C/EBPγ over C/EBPβ in early B cells would prevent transcription of C/EBP-dependent genes, while increased expression of C/EBPβ in mature cells, or in cells stimulated by LPS or proinflammatory cytokines, would be permissive for expression (Cooper et al 1994).

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 (Cooper et al 1992; Pan et al 2000). C/EBPγ has also been implicated in β-globin (Wall et al 1996) and pp52 (Omori et al 1998) 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 (Kaisho et al 1999). Moderate erythroid overexpression of C/EBPγ in transgenic mice increases γ-globin expression relative to β-globin, while high-level expression blocks erythropoiesis (Zafarana et al 2000).

Our observation that heterodimers between C/EBPβ and C/EBPγ predominate in lymphoblasts dependent upon C/EBPβ for LPS-induced IL-6 expression (Hu et al 2000), as well as the widespread occurrence of C/EBPβ:γ heterodimers (Parkin et al, 2002), 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.

MATERIALS AND METHODS

Cells and cell culture-P388 are murine B lymphoblasts (Bauer et al 1986) (American Type Culture Collection (ATCC); CCL 46). P388-Cβ cells and P388-Neo cells have been described previously by Hu et al. (Hu et al 1998). WEHI-231 are murine B cells (Gutman et al 1981) (ATCC; CRL 1702). P388D1(IL1) are macrophages (Bauer et al 1986) (ATCC; TIB 63). P388 cells and their derivatives were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and 50 μM β-mercaptoethanol. WEHI-231 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol. P388D1(IL1) cells, IC21 cells and ANA-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. All IL-6 inductions were conducted with LPS derived from Escherichia coli serotype 055:B5 (Sigma) added to 10 μg/ml.

Transfections-Stable transductions of G418-resistant vectors encoding C/EBPγ-β_{LZ} were carried out by retroviral infection. Retrovirus stocks were prepared by transient expression in 293T cells. 3 μg of constructs in the retroviral expression vector pSV(X)Neo were cotransfected with 3 μg pMOV-ψ (Mann et al 1983), which is a packaging construct. Transfections were performed on 60cm plates using DMRIE-C (Life Technologies) on 80% confluent 293 T cells. Virus was harvested 60 hours post-transfection by centrifuging the supernatants at 1500 rpm for 5 min and then filtering the clear supernatants

through 0.45-μM-pore-size filters. Retroviral infections were performed by the addition of 3ml virus stock Viral stock to $2x10^6$ cells in the presence of 8 μg/ml polybrene (Sigma). The cells were then incubated at 37°C for 3 hours during which time the cells were resuspended every 30 min. Then the cells were resuspended in normal medium. After 24 hours, the cells were split to four 60 cm plates and neomycin (Sigma) was added into the media at a final concentration of 670 μg/ml for about 7 days.

Transient transfections were conducted with $2x10^6$ 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 1.0μg 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) 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/EBPs were expressed from pMEX (Williams et al 1991), which utilizes the Moloney murine sarcoma virus promoter. P65 was expressed from pRc/CMV

(Invitrogen), which utilizes the cytomegalovirus promoter (from N. Rice, National Cancer Institute-Frederick). C/EBPβ-GCN4$_{LZ}$ has been described previously (Williams et al 1995). C/EBPγ-ΔNco was constructed by religating pMEX-C/EBPγ after restriction digestion with Nco I. C/EBPγ-β$_{LZ}$ 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 (nt 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 project are depicted in Figure 1.

The IL-6 promoter-reporter consists of the murine IL-6 promoter (Tanabe et al 1988) (-250 to +1) inserted into the luciferase vector, pXP2 (Nordeen 1988). DEI$_4$(-35alb)LUC (Williams et al 1991) is also derived from pXP2 (Nordeen 1988) and contains four copies of the DEI 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 (Tanabe 1988). The IL-8 promoter-reporter contains sequences extending from +44 to –133 inserted into pGL3-basic (Promega)(Okamoto et al 1994; Murayama et al 1997; Zhang et al 2001). The SV40 early promoter-reporter is a commercial product, pβgal-Control (Clontech), where the SV40 early promoter and enhancer sequences are cloned upstream and downstream, respectively, of the lacZ gene.

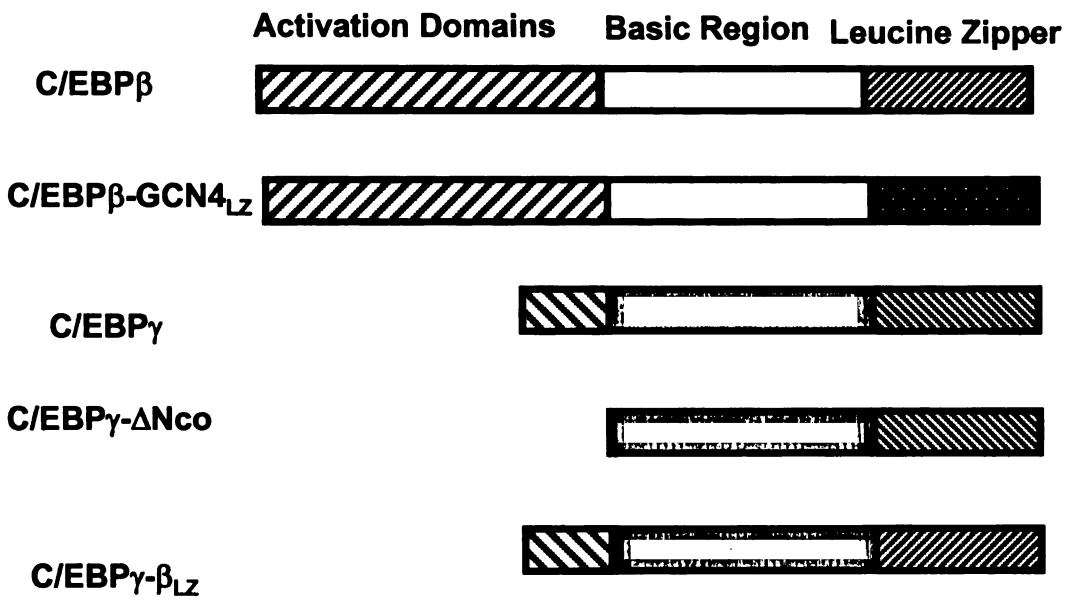


Figure 1. Diagram of the major C/EBP isoforms and mutants used in this chapter.

RNA isolation and analysis-Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's directions. RNA's were electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to high stringency in 40 mM sodium phosphate/1% SDS/1mM EDTA at 65°C. Hybridization probes were prepared with a random priming kit (Life Technologies) with the incorporation of 5'-[-^{32}P]dATP (3000 Ci/mmol; DuPont-New England Nuclear). The C/EBPγ probe consisted of the murine C/EBPγ coding sequence (Cooper et al 1995). The IL-6 probe was a 0.65 kb murine cDNA (from N. Jenkins and N. Copeland, National Cancer Institute-Frederick). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1.3 kb rat cDNA (Fort et al 1985).

Western analysis-Nuclear extracts were prepared as described below. The extracts (60 μg) were adjusted to 1x Laemmli sample buffer (Laemmli 1970) and processed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis. Separated proteins were transferred to Protran membrane (Schleicher and Schuell), and antigen-antibody complexes were visualized with the Enhanced Chemiluminescence Kit (Amersham).

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 $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% [vol/vol] NP-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 13,000 rpm for 60 sec at 4°C. Proteins were extracted from nuclei by incubation at 4°C for 20 min with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 25% [vol/vol] 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 13,000 rpm for 30 min at 4°C and the supernatant extract was collected and stored at -80°C.

The EMSA probes were double-stranded oligonucleotides containing an optimal C/EBP binding site (5'-GATCCTAGATATCCCTGATTGCGCAATAGGCTCAAAGCTG-3' annealed with 5'-AATTCAGCTTTGAGCCTATTGCGCAATCAGGGATATCTAG-3'), a murine IL-6 C/EBP binding site (5'-CTAAACGACGTCACATTGTGCAATCTTAATAAGGTT-3' annealed with 5'-TGGAAACCTTATTAAGATTGCACAATGTGACGTCGT-3'), and a murine albumin DEI binding site (5'-TCGACTATGATTTTGTAATGGGGC-3' annealed with 5'-TCGAGCCCCATTACAAAATCATAG-3'). These probes were labeled with the incorporation of 5'-[-32P]dATP (3000 Ci/mmol; DuPont-New England Nuclear) 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 6.0μg of nuclear extract (1mg/ml in buffer C)] and 5 μl of 5x binding buffer (20% [wt/vol] Ficoll, 50 mM HEPES [pH 7.9], 5mM EDTA, 5

mM dithiothreitol). The remainder of the reaction mixture contained 1 μg poly(dI-dC), 200 pg of probe (unless otherwise noted), bromophenol blue to a final concentration of 0.06% [wt/vol], and water to volume. For supershifts, nuclear extracts were preincubated with antibodies for 20 min at 4°C prior to the binding reaction. Samples were electrophoresed through 5.5% polyacrylamide gels in 1x TBE (90 mM Tris base, 90 mM boric acid, 0.5 mM EDTA) at 160 V.

Antibodies-Rabbit antibodies specific to the carboxyl terminus of C/EBPγ and the amino terminus of C/EBPγ were prepared against synthetic peptides corresponding to these sequences (Parkin et al 2002). Rabbit anti-C/EBPα (14AA), rabbit anti-C/EBPβ specific to the carboxyl terminus (C-19), rabbit anti-C/EBPδ (C-22), rabbit anti-C/EBPε (C-22) and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Rabbit anti-C/EBPβ specific to the amino terminus has been described (Williams et al 1991).

RESULTS

C/EBPβ heterodimerizes with C/EBPγ in B cell lines-Previous work in our lab has shown that stable expression of C/EBPβ, C/EBPα, or C/EBPδ in P388 murine B lymphoblasts confers the ability to induce IL-6 expression with LPS (Bretz et al 1994; Hu et al 1998). Furthermore, we also found the leucine zipper possesses critical determinants for the activity of C/EBPs on the IL-6 promoter in addition to mediating dimerization to known positive effectors of the C/EBP family (Hu et al 2000). In the course of verifying the DNA binding activity of C/EBPβ in the P388-Cβ cell line (P388 cell stably transfected by C/EBPβ), we surprisingly found the majority of C/EBPβ to be in heterodimers with C/EBPγ (Fig. 2). We have since analyzed nuclear extracts from P388 cells stably transfected for expression for C/EBPα (P388-Cα), C/EBPβ (P388-Cβ), C/EBPδ (P388-Cδ) and found the majority of all three isoforms to be in heterodimers with C/EBPγ. As shown in Fig. 2, the heterodimer:DNA complexes are supershifted by C/EBPα, β, and δ-specific antibodies, as well as by C/EBPγ-specific antibody. A gel shift complex formed with each extract that migrates even more rapidly than the C/EBP heterodimers was also supershifted by C/EBPγ-specific antibody, suggesting that this species is a C/EBPγ homodimer. In addition, we also performed an electrophoretic mobility shift assay (EMSA) using nuclear extract from P388 cells stably transduced for both C/EBPβ and C/EBPδ expression. Consistently, we found that most of C/EBPβ and C/EBPδ were in the form of heterodimers with C/EBPγ (Fig. 3).

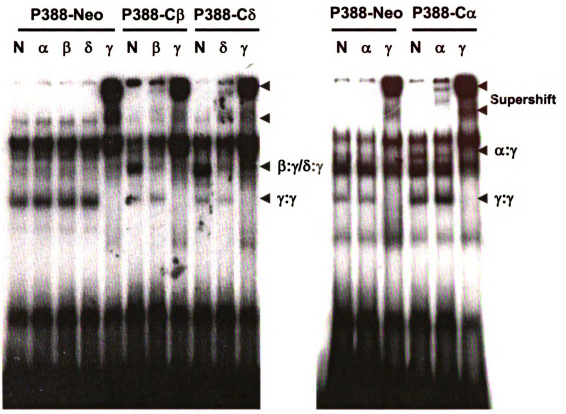


Figure 2. Ectopically expressed C/EBPα, β and δ predominantly form heterodimers with C/EBPγ. EMSA was performed using nuclear extracts of P388-Neo, P388-Cα, P388-Cβ, and P388-Cδ cells. Binding reactions included normal rabbit IgG (N), carboxyl-terminus-specific anti-C/EBPα (α), anti-C/EBPβ (β), anti-C/EBPδ (δ), or anti-C/EBPγ (γ). Arrows labeled α:γ, β:γ, δ:γ and γ:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right indicate supershifts. The major C/EBPα, β or δ complex is supershifted by both C/EBPα, β, or δ-specific and C/EBPγ-specific antibodies.

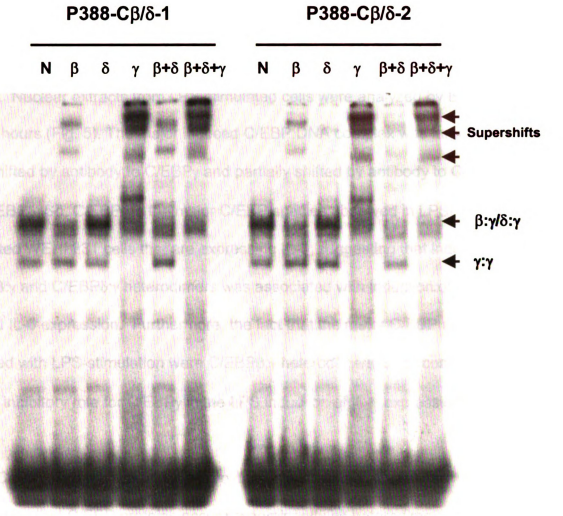


Figure 3. Ectopically expressed C/EBPβ and δ predominantly form heterodimers with C/EBPγ. EMSA was performed using nuclear extracts of P388-Cβ/δ cells. Binding reactions included normal rabbit IgG (N), carboxyl-terminus–specific anti-C/EBPβ (β), anti-C/EBPδ (δ), or anti-C/EBPγ (γ). Arrows on the right indicate the positions of C/EBP:DNA complexes and supershifts. The major C/EBPβ and δ complex are shifted by both C/EBPβ, δ-specific and C/EBPγ-specific antibodies.

Next, we sought to extend our findings to a B cell line commonly used in studies of IL-6 expression, WEHI 231 (Hobbs et al 1991; Macfarlane et al 1998; Lee et al 1998; Venkataraman et al 1999). Treatment of WEHI 231 cells with LPS induced IL-6 mRNA by 2 hours and expression increased through 24 hours (Fig. 4). Nuclear extracts from LPS stimulated cells were analyzed by EMSA at 0 and 24 hours (Fig. 5). The major induced C/EBP:DNA complex was largely supershifted by antibody to C/EBPγ and partially shifted by antibody to C/EBPβ and C/EBPδ. So, C/EBPγ is the major C/EBP species observed in LPS-stimulated WEHI 231 cells that are expressing IL-6, suggesting that induction of C/EBPβ:γ and C/EBPδ:γ heterodimers was associated with induction of LPS-induced IL-6 expression. Furthermore, the fact that the major C/EBP species observed with LPS-stimulation were C/EBPβ:γ heterodimers is not consistent with an inhibitory role for C/EBPγ in the LPS induction of IL-6 expression.

C/EBPγ is the preferential heterodimeric partner of C/EBPβ-The observation that C/EBP proteins occur predominantly as heterodimers with C/EBPγ and not as homodimers suggested that they might preferentially associate with C/EBPγ. In order to 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 with LPS stimulation (see Fig. 9). EMSA of nuclear extracts of the transfected cells revealed that C/EBPβ:γ heterodimers were the predominant binding species at all quantities tested (Fig. 6). Apparently, C/EBPβ:γ

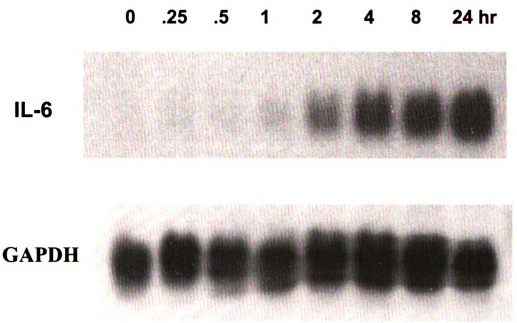
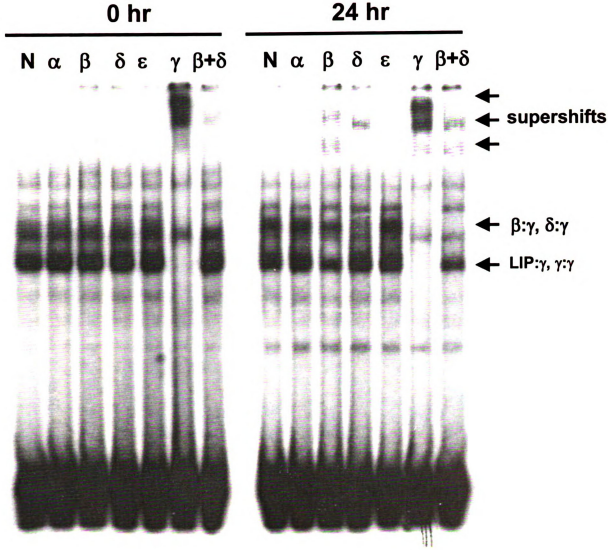


Figure 4. A northern blot of RNA samples isolated from a time course of LPS treatment upon WEHI 231 B cells was successively hybridized for IL-6 and GAPDH

Figure 5. EMSA was performed using nuclear extracts of WEHI 231 cells that were untreated or LPS-treated for 24 hours. Binding reactions included normal rabbit IgG (N), anti-C/EBPα(α), carboxyl-terminus–specific anti-C/EBPβ (β), anti-C/EBPδ (δ), anti-C/EBPε (ε), carboxyl-terminus–specific anti-C/EBPγ (γ), or anti-C/EBPβ and anti-C/EBPδ (β+δ). Arrows labeled β:γ, δ:γ, LIP: γ and γ:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right indicate supershifts. The C/EBPβ and LIP (a truncated form of C/EBPβ consisting of amino acids 132-276) complexes are supershifted by both C/EBPβ-specific and C/EBPγ-specific antibodies. The C/EBPδ complex is supershifted by both C/EBPδ-specific and C/EBPγ-specific antibodies.


0 hr
24 hr
N α β δ ε γ β+δ
N α β δ ε γ β+δ
supershifts
β:γ, δ:γ
LIP:γ, γ:γ

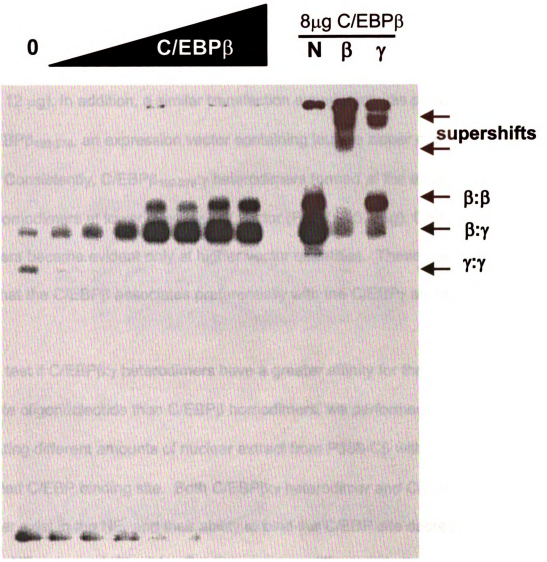


Figure 6. C/EBPβ: γ heterodimers are detected in preference to C/EBPβ and C/EBPγ homodimers. EMSA was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX-C/EBPβ. The EMSA of the 12 μg transfectants also was performed with binding reactions that included normal rabbit IgG (N), carboxyl-terminus–specific anti-C/EBPβ (β), or carboxyl-terminus–specific anti-C/EBPγ (γ). Arrows labeled β:β, β:γ, and γ:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right also indicate supershifts. The C/EBPβ:β complex is supershifted by only C/EBPβ-specific antibodies, the C/EBPγ:γ complex by only C/EBPγ-specific antibodies, and the C/EBPβ:γ complex by both C/EBPβ-specific and C/EBPγ-specific antibodies. A weak, non-specific background species co-migrating with C/EBPβ:γ is evident in the 0 μg lane.

heterodimers formed at the expense of C/EBPγ homodimers at lower quantities of vector (Fig. 6; 0.5, 1, 2 μg). C/EBPβ homodimers were observed only at higher vector quantities, where C/EBPγ homodimers were no longer observable (Fig. 6; 2, 4, 6, 8, 12 μg). In addition, a similar transfection experiment was performed by using C/EBP$\beta_{192\text{-}276}$, an expression vector containing leucine zipper domain of C/EBPβ. Consistently, C/EBP$\beta_{192\text{-}276}$:γ heterodimers formed at the expense of C/EBPγ homodimers at lower quantities of vector (Fig. 7; 0.5, 1μg). C/EBP$\beta_{192\text{-}276}$ homodimers became evident only at higher vector quantities. These results suggest that the C/EBPβ associates preferentially with the C/EBPγ as compared to itself.

To test if C/EBPβ:γ heterodimers have a greater affinity for the C/EBP binding site oligonucleotide than C/EBPβ homodimers, we performed an EMSA by incubating different amounts of nuclear extract from P388-Cβ with a radiolabeled C/EBP binding site. Both C/EBPβ:γ heterodimer and C/EBPβ homodimer exist in the NE, and their ability to bind the C/EBP site decreased when less NE was used (Fig. 8A). But there was no difference in the relative abundance of C/EBPβ:γ heterodimer and C/EBPβ homodimer shift species with decreasing NE (Fig. 8B), suggesting that C/EBPγ being a preferred heterodimerization partner for C/EBPβ rather than enhanced binding affinity of the heterodimer.

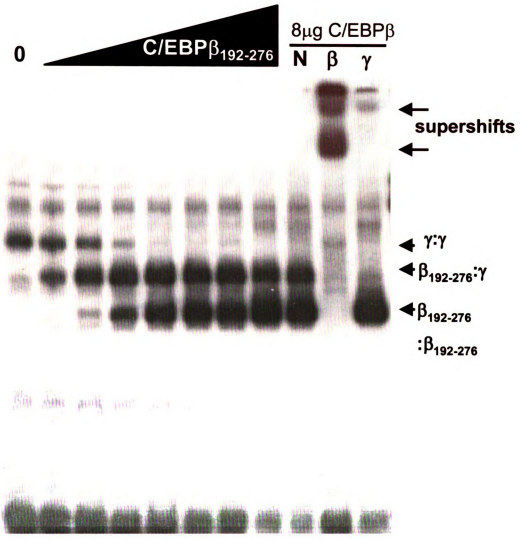


Figure 7. C/EBPβ$_{192-276}$: γ heterodimers are detected in preference to C/EBPβ$_{192-276}$ and C/EBPγ homodimers. EMSA was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX-C/EBPβ$_{192-276}$. The EMSA of the 12 μg transfectants also was performed with binding reactions that included normal rabbit IgG (N), carboxyl-terminus–specific anti-C/EBPβ (β), or carboxyl-terminus–specific anti-C/EBPγ (γ). Arrows labeled β$_{192-276}$:β$_{192-276}$, β$_{192-276}$:γ, and γ:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right also indicate supershifts. The C/EBPβ$_{192-276}$:β$_{192-276}$ complex is supershifted by only C/EBPβ-specific antibodies, the C/EBPγ:γ complex by only C/EBPγ-specific antibodies, and the C/EBPβ$_{192-276}$:γ complex by both C/EBPβ-specific and C/EBPγ-specific antibodies. A weak, non-specific background species co-migrating with C/EBPβ$_{192-276}$:γ is evident in the 0 μg lane.

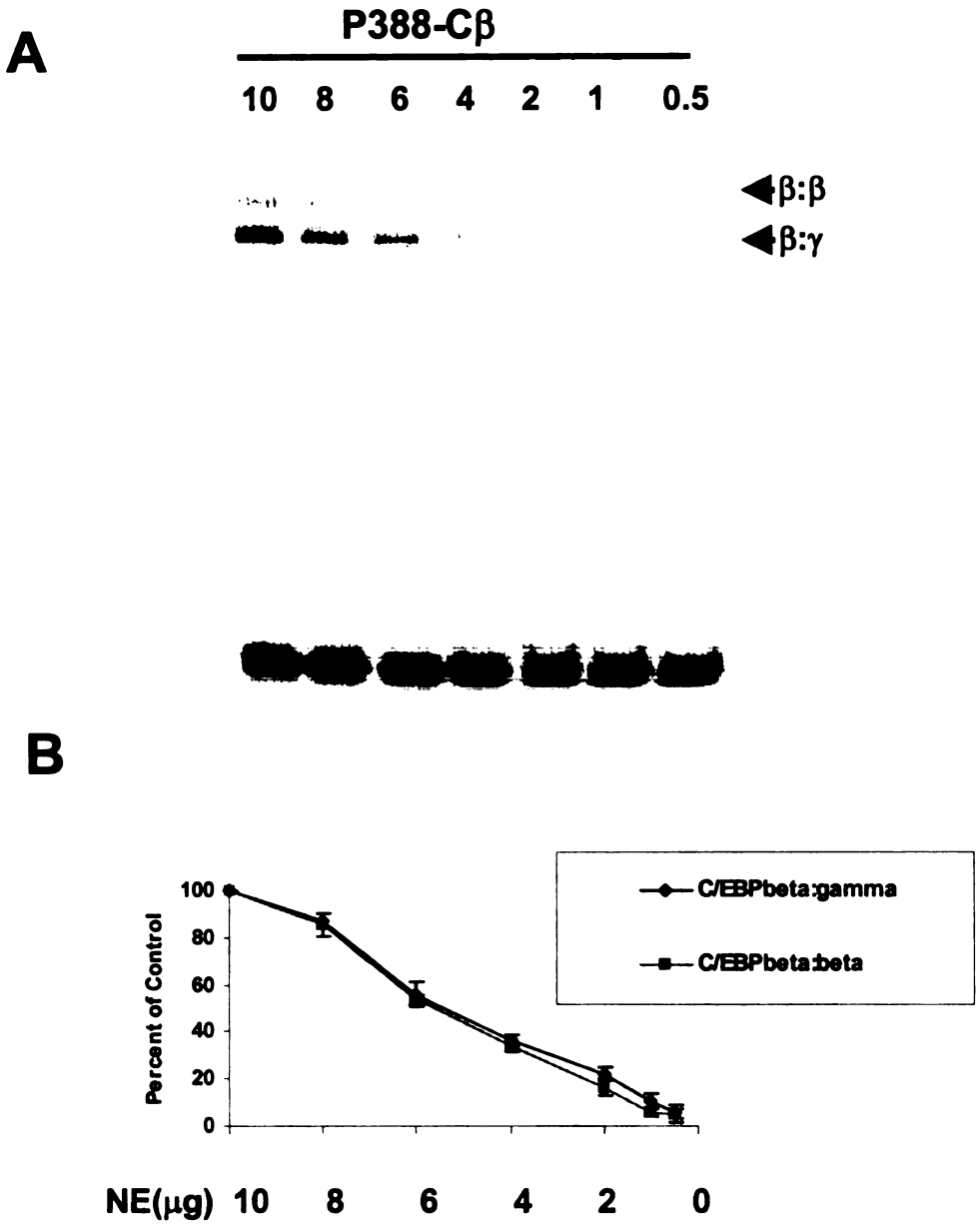


Figure 8. C/EBPβ:β and C/EBPβ:γ have similar affinity for the C/EBP binding site in the IL-6 promoter. A. EMSA was performed using decreasing quantities (10, 8, 6, 4, 2, 1 and 0.5 μg) of nuclear extracts of P388-Cβ and and a labeled oligonucleotide corresponding to the IL-6 promoter C/EBP binding site. Arrows labeled β:β and β:γ indicate the positions of C/EBP:DNA complexes. **B.** The radioactivity associated with C/EBPβ homodimers and C/EBPβ:γ heterodimers were quantitated using a Storm PhosphorImager (Molecular Dynamics) when decreasing quantities of nuclear extracts were used. The change of C/EBPβ:γ and C/EBP β:β binding to IL-6 promoter C/EBP binding site are shown.

C/EBPγ augments C/EBPβ-stimulated transcription of the IL-6 promoter-

C/EBPγ by itself is certainly 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. 9). 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 (Cooper et al 1995). 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 effective 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. 10). To further test the

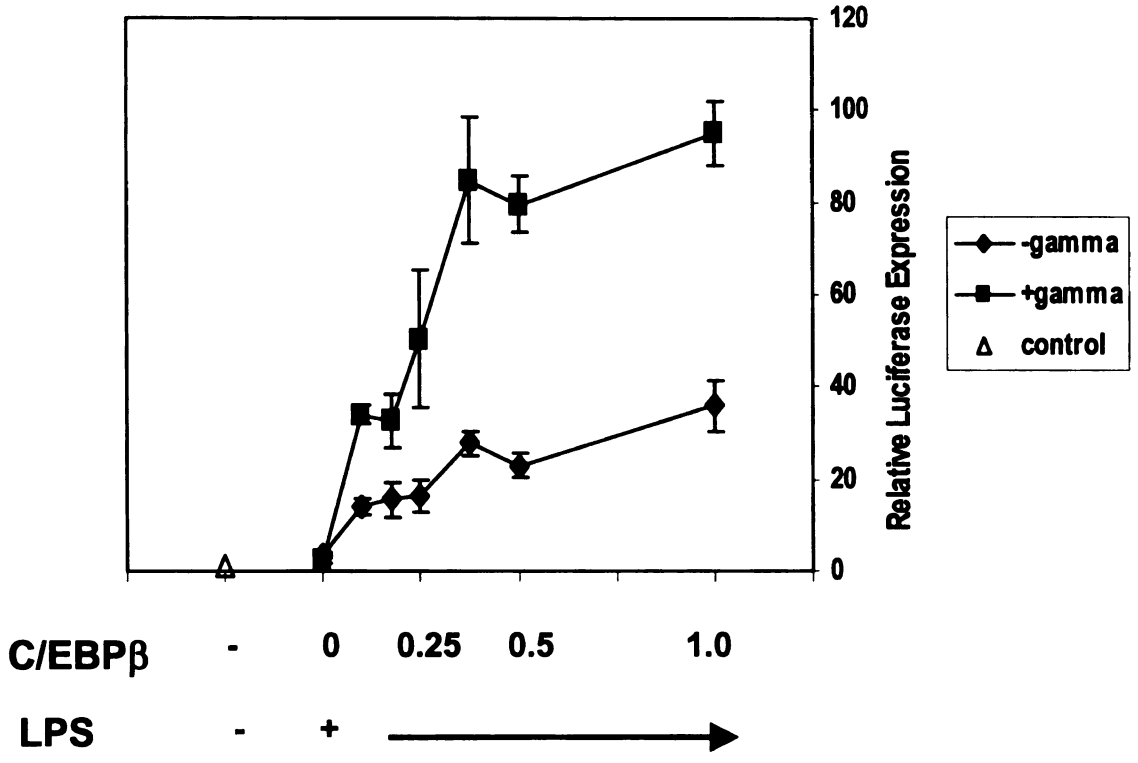
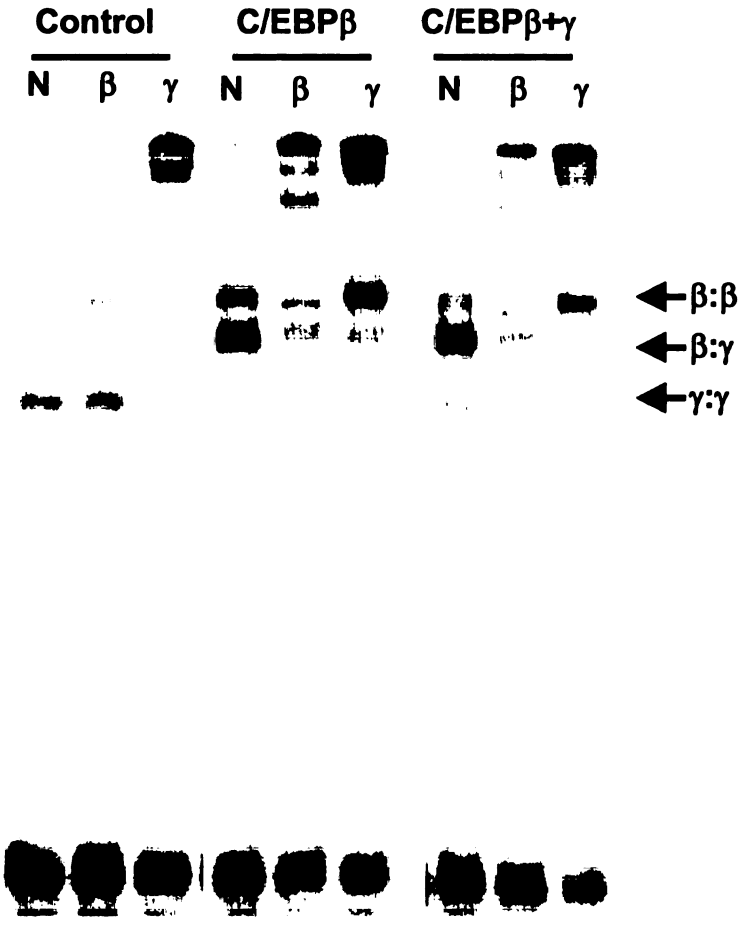


Figure 9. C/EBPβ is a more potent activator of LPS-induced IL-6 transcription under conditions of added C/EBPγ expression. Transfections were carried out in duplicate with (+gamma) and without (-gamma) 0.5 μg C/EBPγ vector, with the μg quantities of C/EBPβ vector and LPS treatment as indicated. Luminometer values were normalized for expression from a co-transfected SV40 early promoter β-galactosidase-reporter. These values were then normalized to a relative value of 1 for cells receiving neither a C/EBP expression vector nor LPS. The data presented are the mean of 3 experiments with their standard error.



Ratio of C/EBPβ:γ to C/EBPβ:β: 1.3 2.2

Figure 10. C/EBPγ drives C/EBPβ into C/EBPβ:γ heterodimers. EMSA was performed using nuclear extracts of P388 cells transiently transfected pMEX control vector, 2 μg pMEX-C/EBPβ or 2 μg pMEX-C/EBPβ plus 0.5 μg pMEX-C/EBPγ. Arrows labeled β:β, β:γ, and γ:γ indicate the positions of C/EBP:DNA complexes. The radioactivity associated with C/EBPβ homodimers and C/EBPβ:γ heterodimers was quantitated using a Storm PhosphorImager (Molecular Dynamics) and the ratio of C/EBPβ:γ to C/EBP β:βis shown.

ability of C/EBPγ to promote formation of C/EBPβ:γ heterodimers, a constant quantity of C/EBPβ expression vector and over a range of quantities of the C/EBPγ expression vector including those that effectively transactivated the IL-6 promoter following LPS stimulation was transfected into P388 cells (Fig. 9). 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. 11).

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. 12). C/EBPγ activity was observed even when the C/EBPγ vector was transfected at a 8-fold excess over C/EBPβ vector, although C/EBPγ by itself exhibited no activity (see Fig. 15). Our results therefore suggest that C/EBPγ, rather than functioning as an inhibitor to lower 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 limited activation of the IL-6 promoter that can be observed in transfection of C/EBPβ alone (Fig. 13A). This inhibition was reversed by

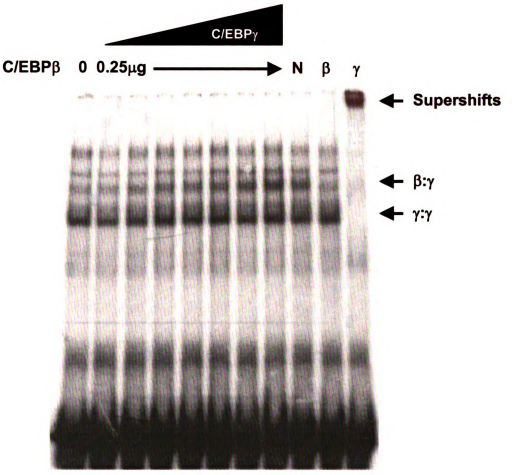


Figure 11. C/EBPγ promotes formation of C/EBPβ:γ heterodimers. EMSA was performed using nuclear extracts of P388 cells transiently transfected pMEX control vector, 0.25μg pMEX-C/EBPβ, and 0.25μg pMEX-C/EBPβ with increasing quantities (0, 0.1,0.25, 0.5, 1, 2, and 4 μg) of pMEX-C/EBPγ. The EMSA of the 4 μg pMEX-C/EBPγ transfectants also was performed with binding reactions that included normal rabbit IgG (N), carboxyl-terminus-specific anti-C/EBPβ (β), or carboxyl-terminus-specific anti-C/EBPγ (γ). Arrows labeled β:β and β:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right also indicate supershifts. The C/EBPγ:γ complex is supershifted by only C/EBPγ-specific antibody and the C/EBPβ:γ complex by both C/EBPβ-specific and C/EBPγ-specific antibodies. Two unidentified slower migrating species that are not modulated by transfection and are reactive with C/EBPγ-specific antibody are evident in control and experimental lanes.

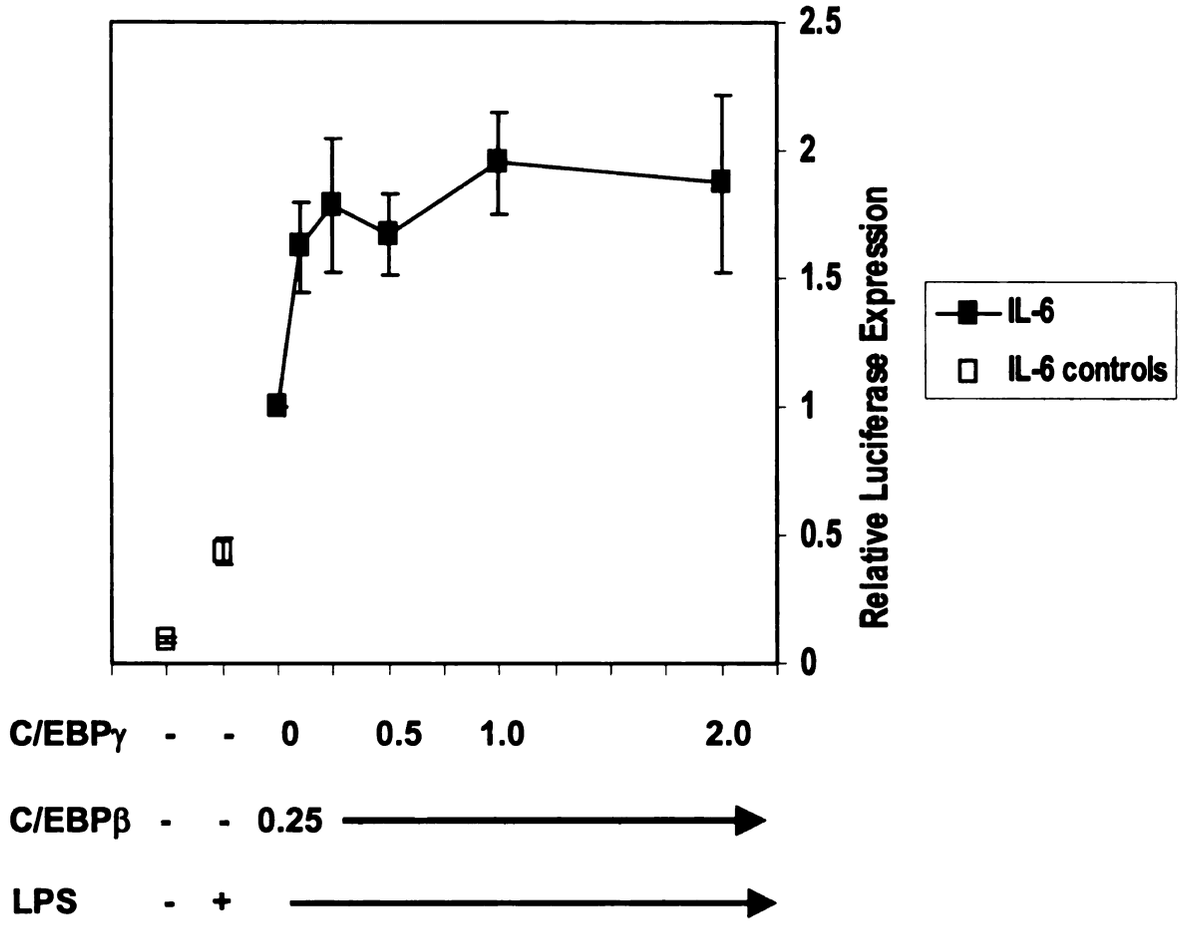


Figure 12. C/EBPγ stimulates LPS-induced IL-6 transcription when expressed with C/EBPβ. Transient transfections of P388 cells were carried out in duplicate with the μg quantities of expression vectors and LPS treatment as indicated. Luminometer values were normalized for expression from a cotransfected SV40 early promoter-β-galactosidase reporter. These values were then normalized to a relative value of 1 for the cells receiving C/EBPβ expression vector and treated with LPS. The data presented are the mean of 7 experiments with their standard error.

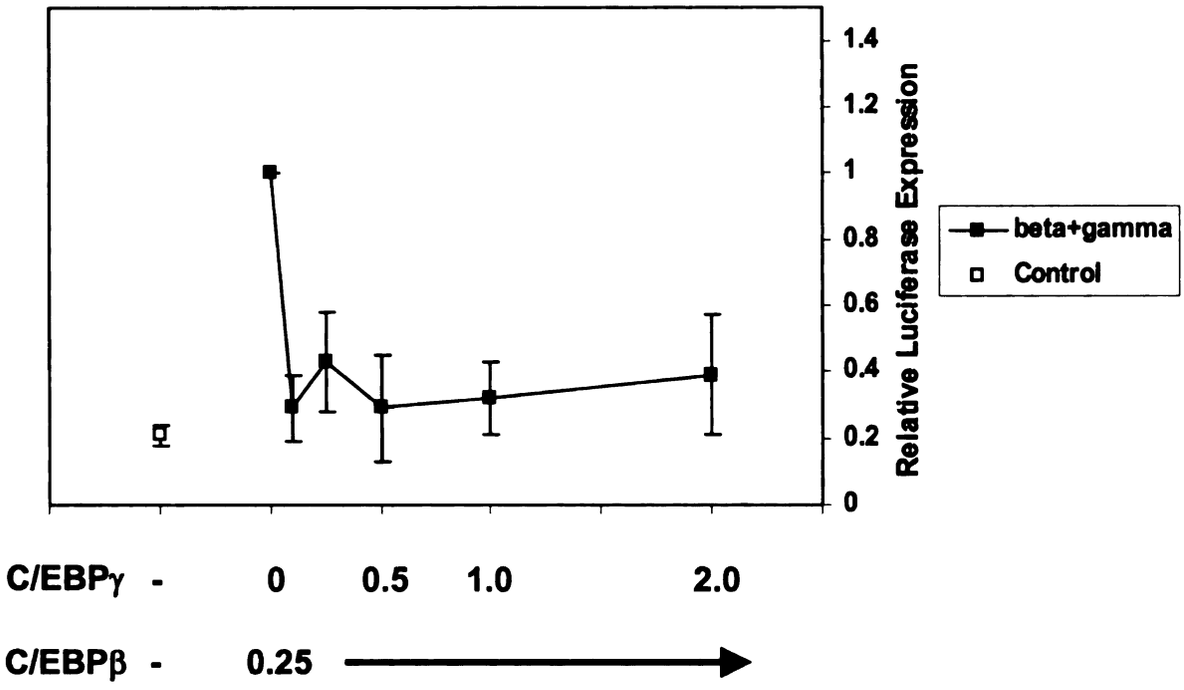


Figure 13. C/EBPγ inhibits C/EBPβ-induced IL-6 transcription in the absence of LPS treatment, while that inhibition is reversed by NF-κB p65 expression. A. Transient transfections of P388 cells were carried out in duplicate with the μg quantities of expression vectors as indicated. Luminometer values were normalized as in Figure 12, except final values were normalized to a relative value of 1 for the cells receiving C/EBPβ expression vector alone. The data presented are the means of 3 experiments with standard error.

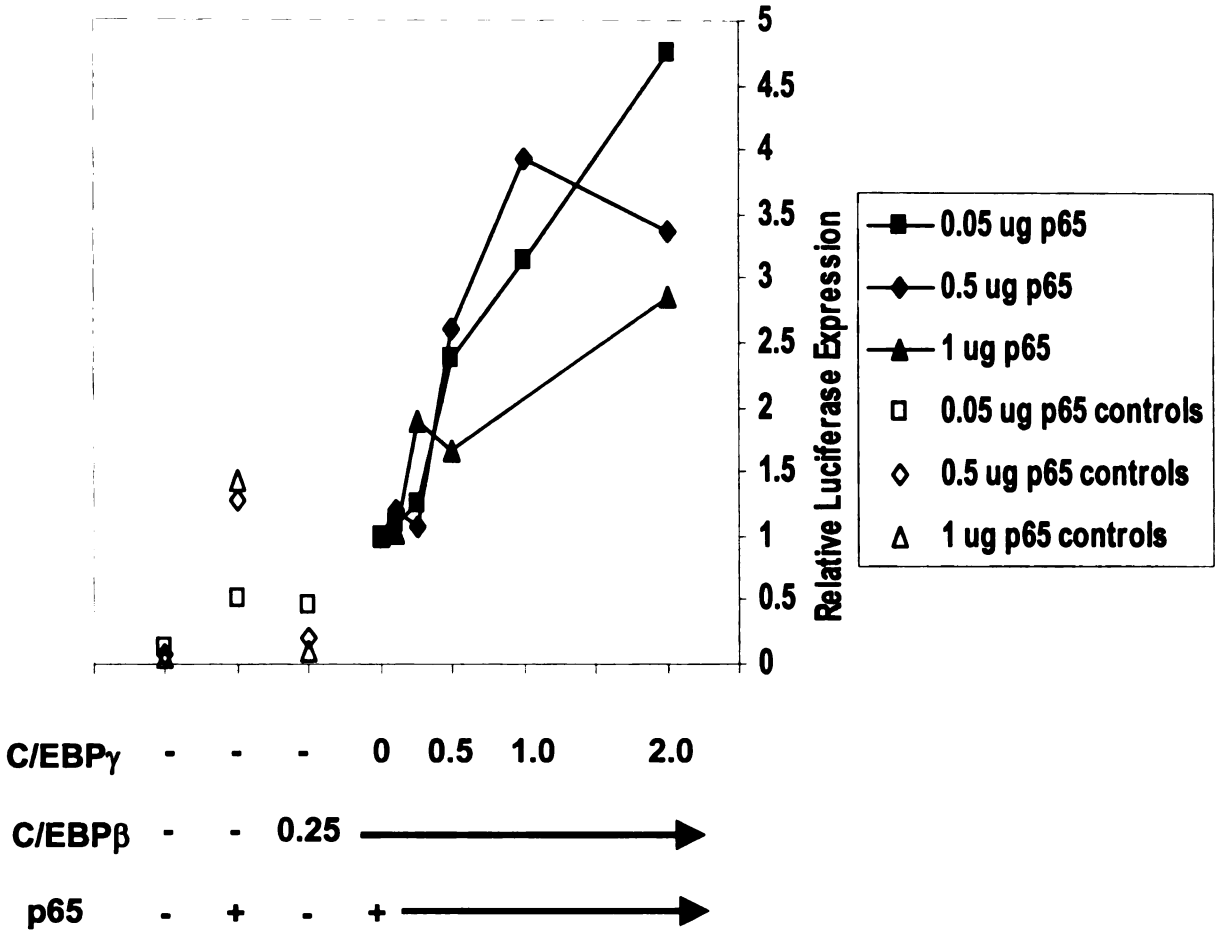


Figure 13. C/EBPγ inhibits C/EBPβ-induced IL-6 transcription in the absence of LPS treatment, while that inhibition is reversed by NF-κB p65 expression. B. Transient transfections of P388 cells were carried out in duplicate with the μg quantities of expression vectors as indicated. Luminometer values were normalized as in Figure 12, except final values were normalized to a relative value of 1 for the cells receiving both C/EBPβ and NF-κB p65 expression vectors. The data are derived from one experiment carried out at various doses of p65 vector.

cotransfection with NF-κB p65, allowing dosage-dependent C/EBPγ stimulatory activity in the absence of LPS stimulation (Fig. 13B). 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. Indeed, NF-κB was also activated upon LPS treatment of P388 cells. As shown in Figure 14A, when P388 cells were treated with LPS over a time course, NF-κB binding to the IL-6 promoter NF-κB motif was detected at as early as one hour LPS treatment with increased binding through 24 hours. To test which NF-κB member is activated in this B cell by LPS, a supershift experiment was performed with different NF-κB antibodies. As shown in Figure 14B, p50:p65 heterodimers were the major species induced; p50:p50 homodimers and p50:c-Rel heterodimers' DNA binding were also induced. Thus, NF-κB was translocated to the nucleus upon LPS treatment and was available to support the expression of IL-6 together with C/EBPs.

It is possible that the C/EBPγ expressed from our expression vector differed from endogenous C/EBPγ in its ability to stimulated 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 (Fig. 15). No stimulation of the IL-6 promoter above that induced by LPS stimulation

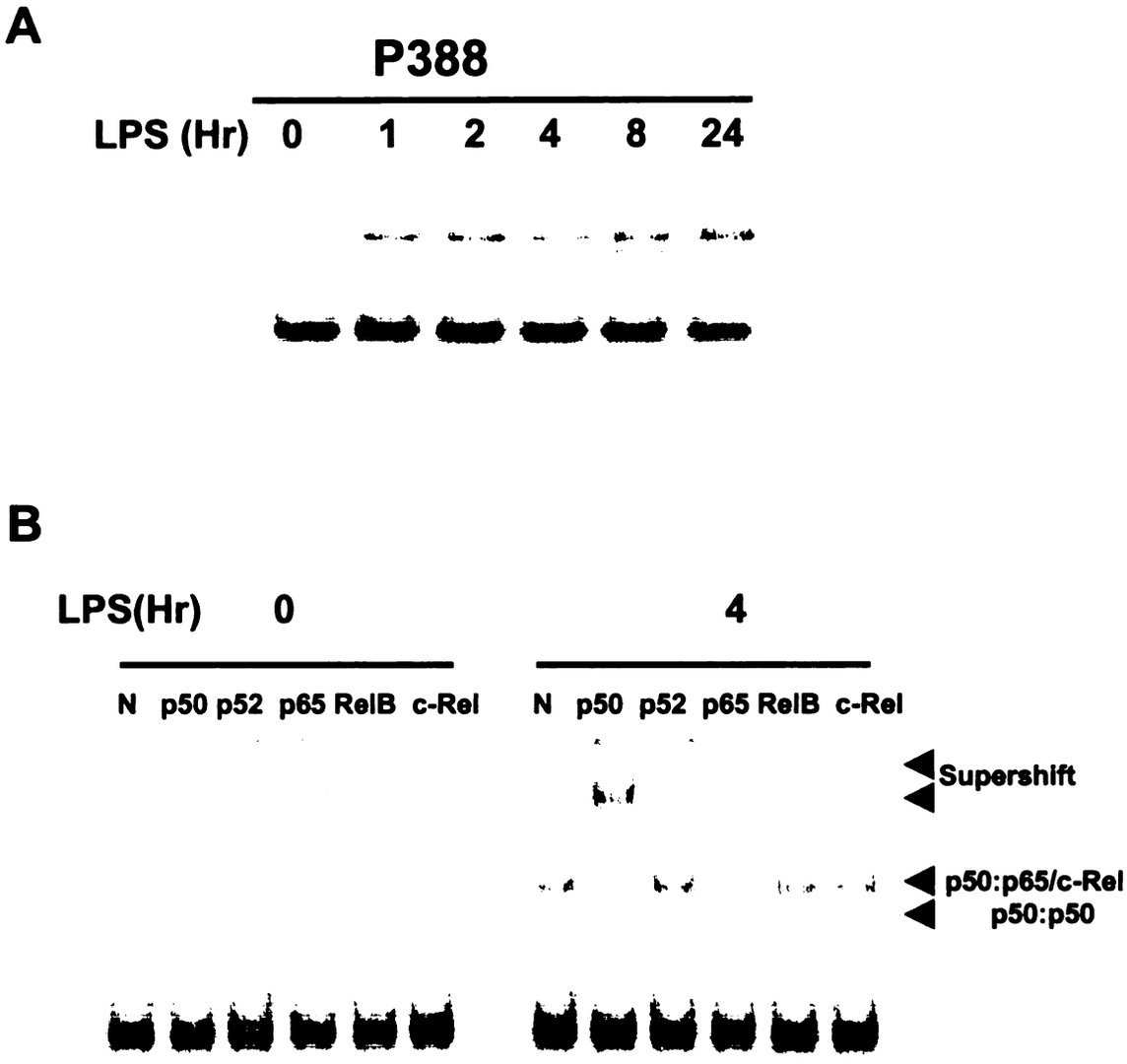


Figure 14. LPS induces NF-κB DNA binding of IL-6 promoter in P388 cells. A. EMSA was performed using nuclear extracts of P388 cells that were untreated or LPS-treated over a time course as shown and a labeled oligonucleotide corresponding to the IL-6 promoter NF-κB binding site. **B.** Nuclear extract of untreated P388 cells or LPS-treated P388 cells were incubated with normal rabbit IgG (N), anti-p50, anti-p52, anti-p65, anti-RelB, or anti-c-Rel. Arrows labeled p50:p65/c-Rel and p50:p50 indicate the positions of NF-κB:DNA complexes. Arrowheads on the right indicate supershifts. The NF-κB complexes are supershifted by p50, p65 and c-Rel-specific antibodies.

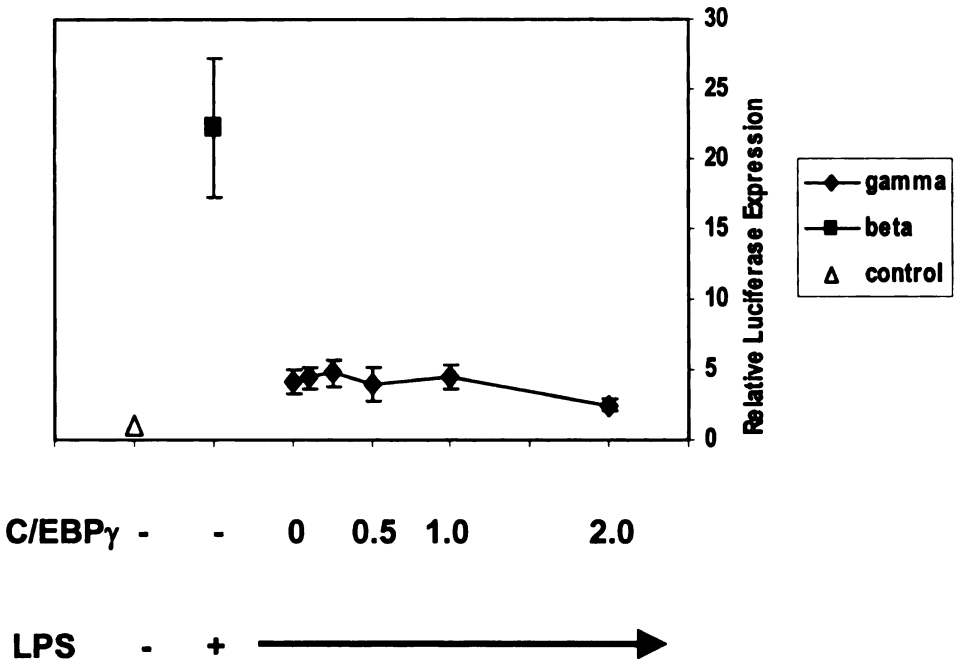


Figure 15. C/EBPγ by itself has no stimulatory activity upon the IL-6 promoter. Transient transfections were carried out in duplicate with increasing quantities of C/EBPγ expression vector as indicated with LPS stimulation. Transient transfection with 0.25 μg C/EBPβ expression vector served as a positive control for transactivation. Luminometer values were normalized as described in Figure 9. The data are the mean of 3 experiments with standard error.

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. 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 DEI_4(-35alb)LUC, a promoter-reporter that contains four copies of a C/EBP binding site tandemly arrayed upstream of the albumin minimal promoter (Fig.16). 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. 16). 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 (Liu et al 2000), while the IL-8 promoter shows strong synergy between these two factors (Matsuaka et al 1993; Stein et al 1993; Kunsch et al 1994). 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 even more stimulation 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. 16, compare control cells treated with LPS to cells treated with LPS and cotransfected with C/EBPβ). Furthermore, C/EBPγ stimulatory

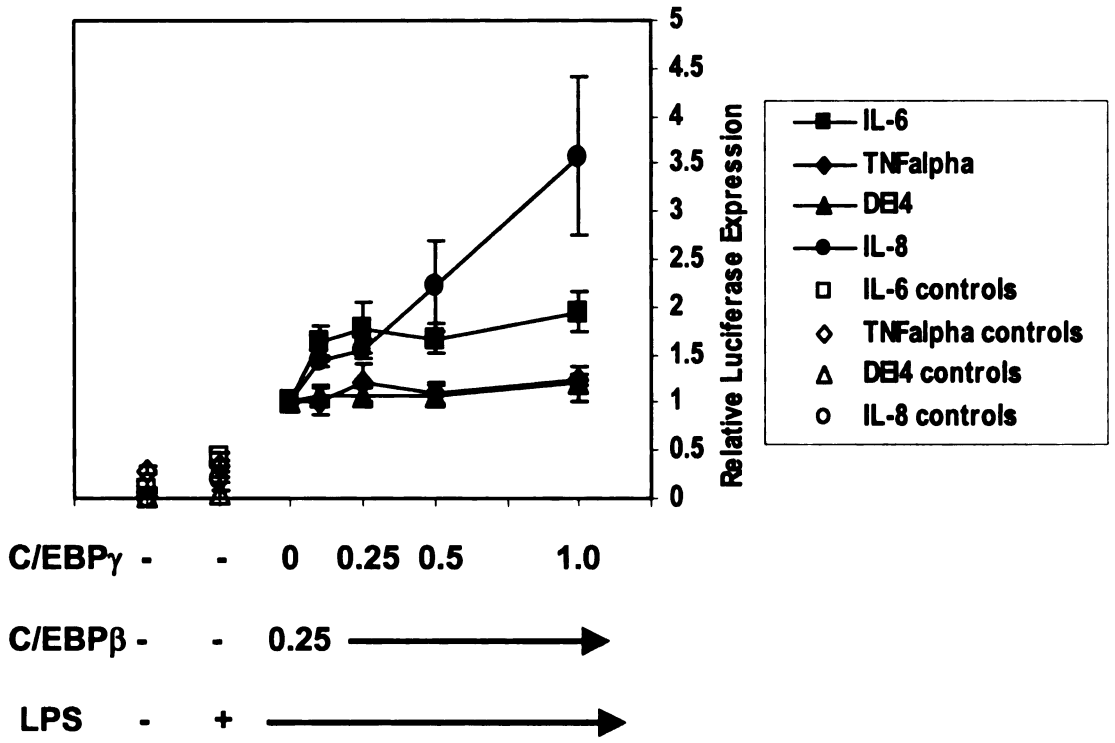


Figure 16. C/EBPγ stimulates LPS-induced transcription from the IL-8 promoter, but is inactive for the TNFα promoter and a simple C/EBP-driven promoter. Transient transfections of P388 cells were carried out in duplicate with the μg quantities of expression vector and LPS treatment as indicated. Luminometer values were normalized as in Fig. 5. The data presented for the IL-8, TNFα, and DEI4(-35alb) promoters are means of 3, 3, and 5 experiments, respectively, with standard error. The data for the IL-6 promoter from Figure 12 are presented for comparison.

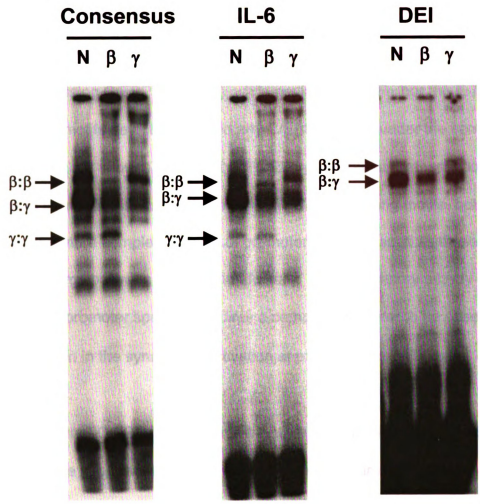
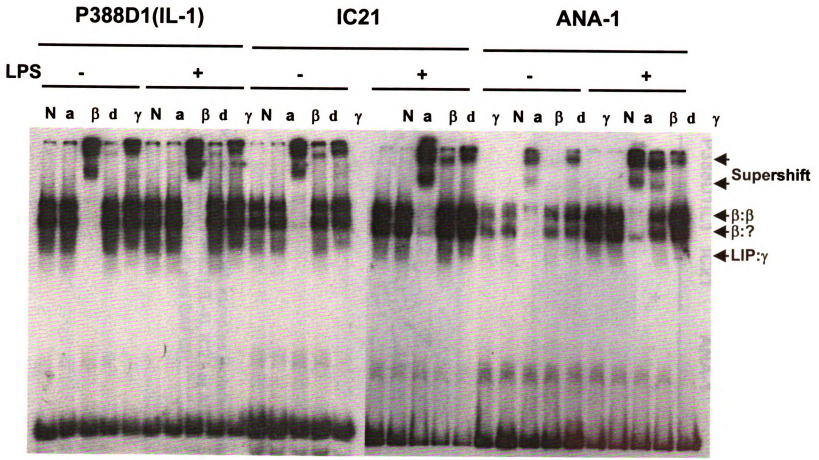


Figure 17. Both the IL-6 and DEI C/EBP binding motifs bound C/EBPγ-containing species. EMSA was performed using nuclear extracts from P388-Cβ cells and a labeled binding site oligonucleotides corresponding to the C/EBP consensus binding site, the IL-6 promoter C/EBP bindin site, and the DEI albumign C/EBP binding site. Binding reactions included normal rabbit IgG (N), C-terminus-specific anti-C/EBPβ (β), or C-terminus-specific anti-C/EBPγ (γ). Arrows labeled β:β, γ:γ, and β:γ indicate the positions of C/EBP:DNA complexes.

activity does not appear dependent upon differential binding of C/EBPγ to differing C/EBP binding sites. Both the IL-6 and DEI C/EBP binding motifs bound C/EBPγ-containing species in EMSA performed upon nuclear extracts from P388 cells overexpressing C/EBPβ (Fig. 17), 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 (Cooper et al 1995) led us to ask if its stimulatory activity was unique to that cell type or could be observed in other cell lineages that display LPS inducible IL-6 expression. In order to test this, we utilized several macrophages including P388D1(IL-1), IC21, and ANA-1. In these cell lines, only a relatively low proportion of C/EBP:DNA complexes are supershifted by anti-C/EBPγ in an EMSA (Fig. 18). LPS is a potent inducer of IL-6 expression in these cell lines (Fig. 19). Then, we utilized P388D1(IL-1) to test if C/EBPγ has stimulatory activity in this macrophage cell line. This macrophage cell line is actually a derivative of the original P388 B lymphoblast tumor (Bauer et al 1986). Transient transfections were performed where increasing amounts of C/EBPγ

Figure 18. EMSA was performed using nuclear extracts of P388D1(IL1), IC21, and ANA-1 cells that were untreated or LPS-treated for 4 hours. Binding reactions included normal rabbit IgG (N), anti-C/EBPα (α), carboxyl-terminus–specific anti-C/EBPβ (β), anti-C/EBPδ (δ), or carboxyl-terminus–specific anti-C/EBPγ (γ). Arrows labeled β:β, β:?, and LIP:γ indicate the positions of C/EBP:DNA complexes. "?" denotes an unidentified C/EBP dimerization partner. Arrows on the right also indicate supershifts.


P388D1(IL-1)
IC21
ANA-1
LPS
-
+
-
+
-
+
N a β d γ N a β d γ N a β d γ N a β d γ N a β d γ N a β d γ
Supershift
β:β
β:?
LIP:γ

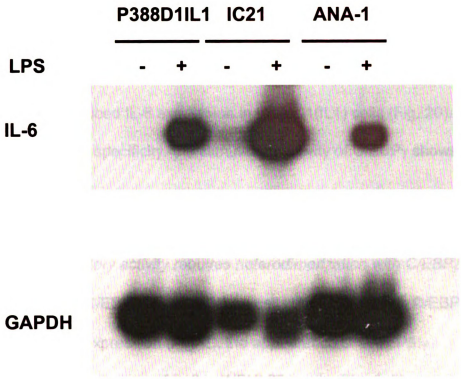


Figure 19. A northern blot of RNA samples isolated from untreated and LPS-treated P388D1(IL-1), IC21 or ANA-1 cells was successively hybridized for IL-6 and GAPDH.

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 (Fig. 20). 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-The heterodimerization of C/EBPβ with C/EBPγ in cells dependent upon C/EBPβ for LPS induction of IL-6 expression (Fig. 2), the appearance of C/EBPβ:γ heterodimers on LPS induction of IL-6 in WEHI 231 cells (Fig. 4, 5), and the predominance of C/EBPβ:γ heterodimers over a wide range of C/EBPβ expression in P388 cells (Fig. 6), led us to test whether C/EBPγ stimulatory activity in transfections with C/EBPβ requires heterodimer formation. To that end, we performed transient transfections with a chimeric C/EBPβ containing the leucine zipper of yeast GCN4. C/EBPβ-GCN4$_{LZ}$ (Fig. 1) can activate transcription from an albumin DEI site-driven reporter (Williams et al 1995), as well as the IL-6 promoter-reporter in conjunction with LPS treatment (Fig. 21C; see controls), and is unable to heterodimerize with C/EBPγ in vitro or in vivo (Parkin et al 2002). The heterologous leucine zipper prevents heterodimerization, but allows the chimeric protein to homodimerize. To verify expression, DNA binding, and the

heterodimeriztion properties of C/EBPβ-GCN4$_{LZ}$, western blot analysis and EMSA were performed using nuclear extracts of transiently transfected cells (Figs. 21A, 21B). Western analysis of nuclear extracts from P388 cells transfected with increasing quantities of C/EBPβ-GCN4$_{LZ}$ expression vector detected increasing quantities of a C/EBP-related protein at the expected molecular weight of approximately 38 kD (Fig. 21A). As can be seen in an EMSA of the same nuclear extracts, the overexpression of C/EBPβ-GCN4$_{LZ}$ fails to drive C/EBPγ into heterodimers (Fig. 21B), in contrast to C/EBPβ (Fig. 6). The major EMSA species associated with transfection of the C/EBPβ-GCN4$_{LZ}$ 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. 21B). 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-GCN4$_{LZ}$ 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. 21C). 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-GCN4$_{LZ}$ by itself supports LPS induction of the IL-6 promoter indicates that while C/EBPγ can augment

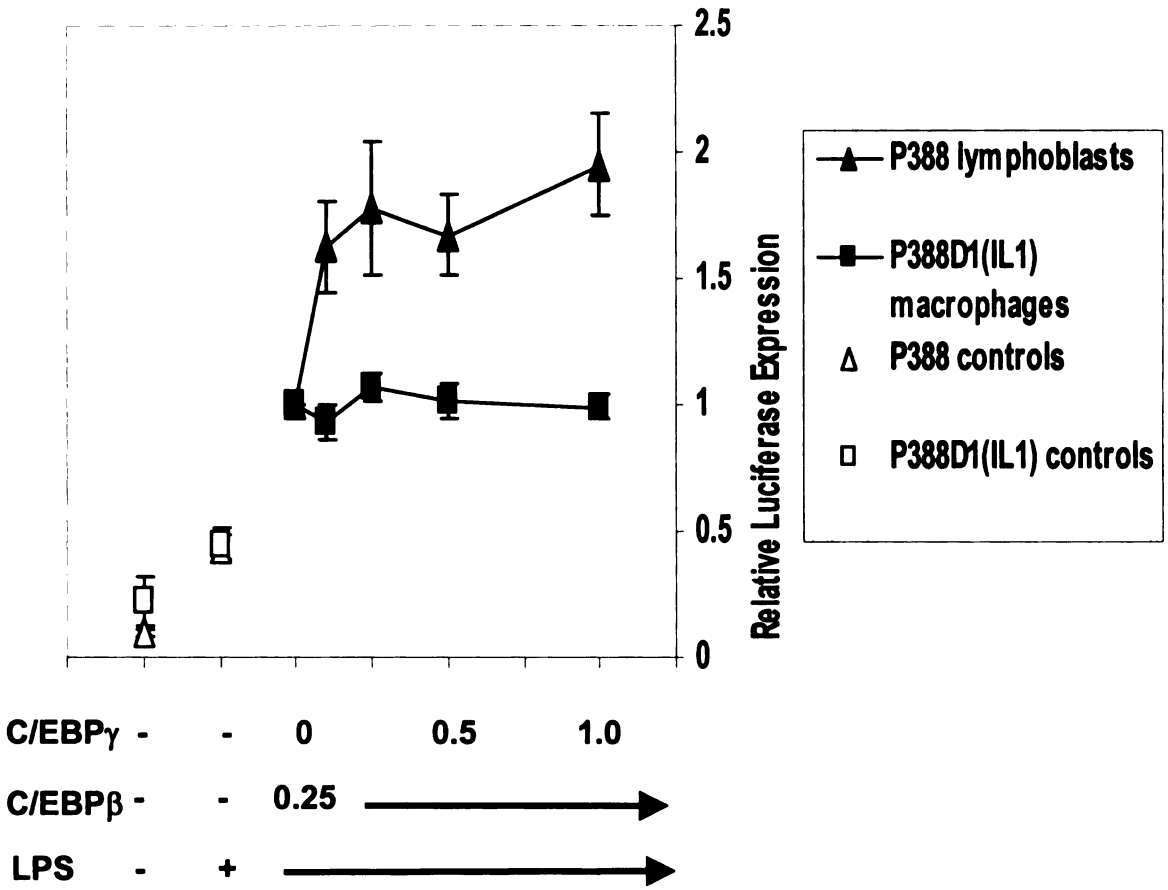


Figure 20. C/EBPγ lacks stimulatory activity in P388D1(IL1) macrophages. Transient transfections of P388D1(IL-1) cells were carried out in duplicate with the μg quantities of expression vectors and LPS treatment as indicated. Luminometer values were normalized as in Figure 12. The data presented are the mean of 3 experiments with standard error. The data for P388 lymphoblasts from Figure12 are presented for comparison.

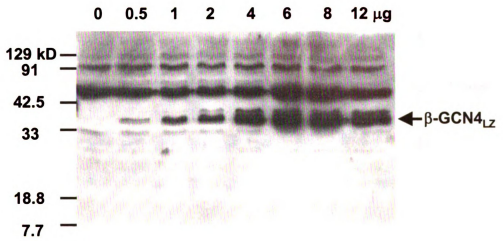


Figure 21. C/EBPγ stimulatory activity is dependent upon the formation of C/EBPβ:γ heterodimers. The replacement of the C/EBP leucine zipper in C/EBPβ with that of GCN4 blocked C/EBPγ activity. A, a western blot was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX-C/EBPβ-GCN4$_{LZ}$. The primary antibody used in the detection of C/EBPβ-GCN4$_{LZ}$ was amino-terminus–specific anti-C/EBPβ. An arrow marks the position of C/EBPβ-GCN4$_{LZ}$. The positions of protein standards are noted.

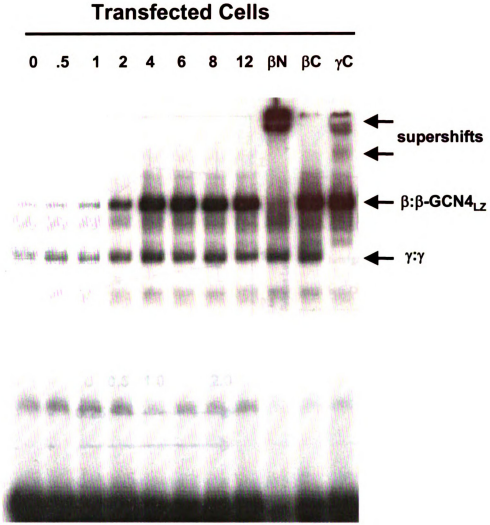
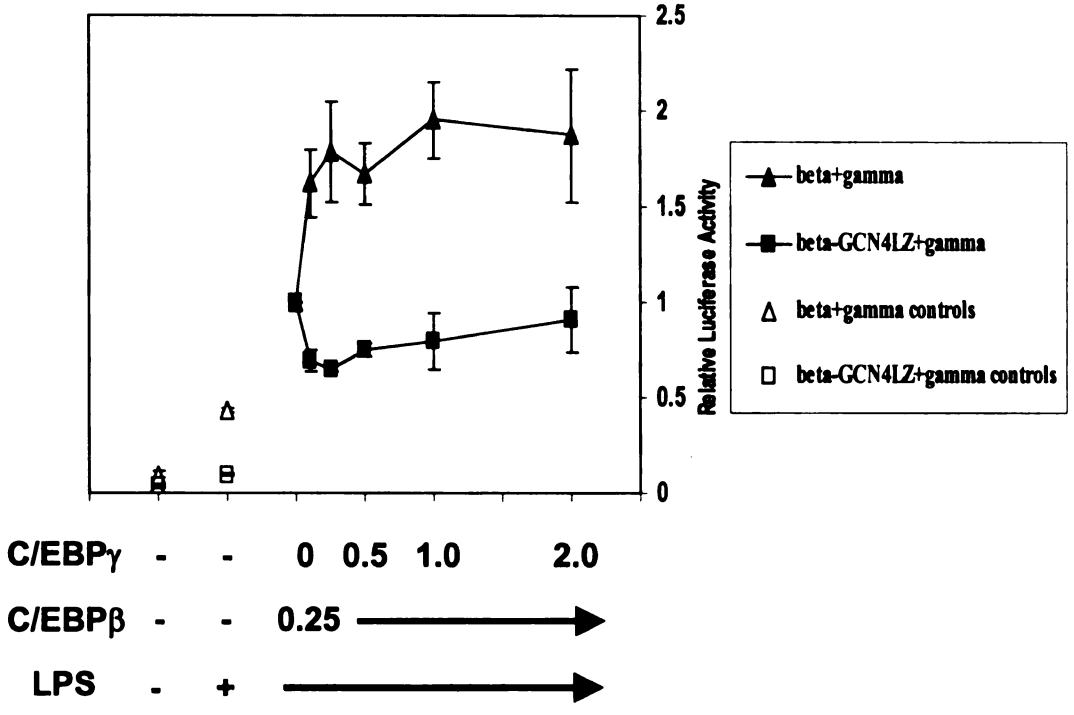


Figure 21. C/EBPγ stimulatory activity is dependent upon the formation of C/EBPβ:γ heterodimers. The replacement of the C/EBP leucine zipper in C/EBPβ with that of GCN4 blocked C/EBPγ activity. B, EMSA was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX- C/EBPβ-GCN4$_{LZ}$. The EMSA of 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), or carboxyl-terminus–specific anti-C/EBPγ (Cγ). Arrows labeled β-GCN4$_{LZ}$ and γ:γ indicate the positions of C/EBP:DNA complexes and supershifts. The C/EBPβ-GCN4$_{LZ}$ complex is only supershifted by amino-terminus-specific anti-C/EBPβ.



Figue 21. C/EBPγ stimulatory activity is dependent upon the formation of C/EBPβ:γ heterodimers. The replacement of the C/EBP leucine zipper in C/EBPβ with that of GCN4 blocked C/EBPγ activity. **C,** transient transfections of P388 cells were carried out in duplicate with the μg quantities of expression vectors and LPS treatment as indicated. Luminometer values were normalized as in figure 12. The data for C/EBPβ-$GCN4_{LZ}$+C/EBP (beta-$GCN4_{LZ}$+gamma) are the mean of 4 experiments with standard error. The data for C/EBPβ+C/EBPγ (beta+gamma) from figure 12 are presented for comparison.

C/EBPβ activity, formation of heterodimers containing C/EBPγ is not necessary for C/EBP activity on the IL-6 promoter.

C/EBPγ stimulatory activity resides with 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/EBPγ-ΔNco) was compared to intact C/EBPγ in the same experimental regime as described for Figure 6, 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-ΔNco, although lacking the 57-residue amino-terminus, had as much stimulatory activity as wild type C/EBPγ (Fig. 22A). An EMSA species that increased in abundance with increasing quantities of the C/EBPγ-ΔNco vector further indicated successful expression of C/EBPγ-ΔNco (Fig. 22B). Thus the amino terminus of C/EBPγ is unnecessary for its stimulatory activity.

Since C/EBPγ homodimers by themselves have no stimulatory activity (Fig. 15) and the ability of C/EBPγ to heterodimerize with C/EBPβ appears to be critical for its stimulatory activity (Fig. 21), 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/EBPγ-β_{LZ}). As a control for

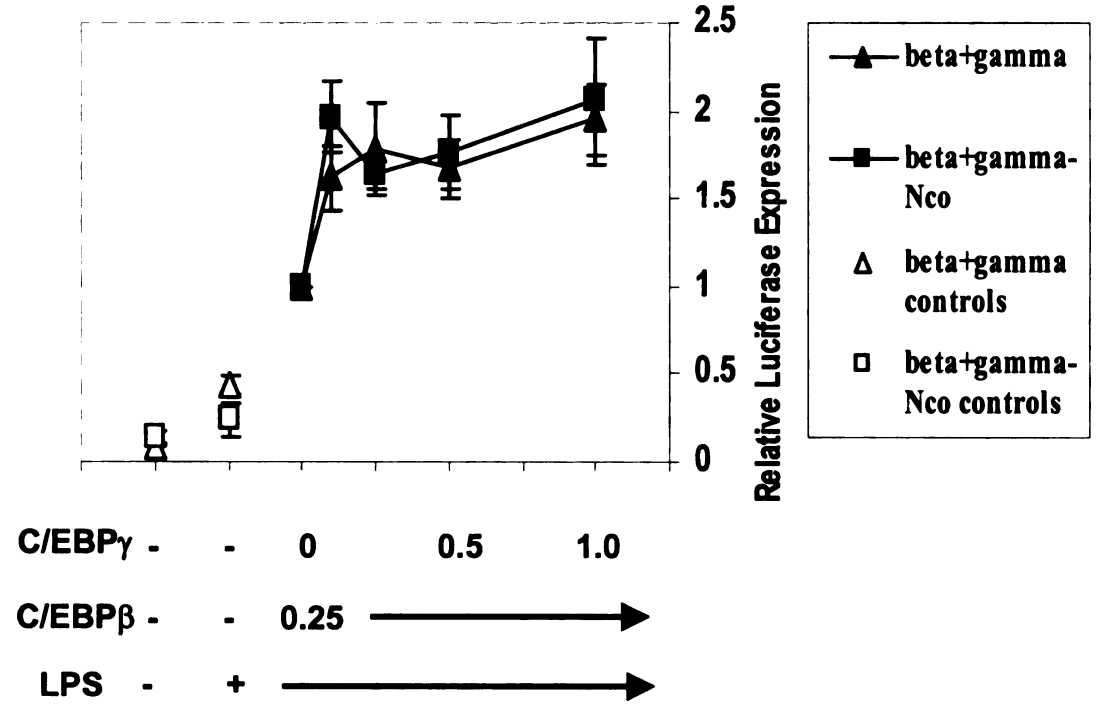


Figure 22. The amino-terminal region of C/EBPγ is not required for stimulatory activity. A, transient transfections of P388 cells were carried out in duplicate with μg quantities of expression vectors and LPS treatment as indicated. Luminometer values were normalized as described in figure 12. The data for C/EBPβ+C/EBPγ-ΔNco (beta+gamma-Nco) are the mean of 3 experiments with standard error.

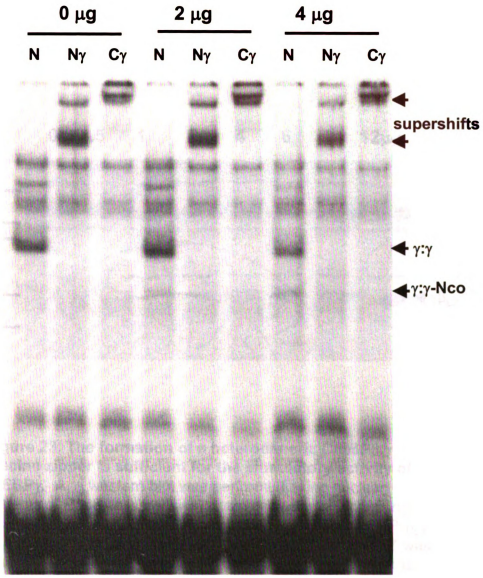


Figure 22. The amino-terminal region of C/EBPγ is not required for stimulatory activity. B, EMSA was performed using nuclear extracts of P388 cells transiently transfected with 0, 2, and 4 μg of pMEX-C/EBPγ-ΔNco. Binding reactions included normal rabbit IgG (N), amino-terminus-specific anti-C/EBPγ (Nγ), or carboxyl-terminus–specific anti-C/EBPγ (Cγ). Arrows labeled γ:γ -Nco and γ:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right indicate supershifts. The C/EBPγ-ΔNco complex is supershifted by both amino and carboxyl terminus-specific anti-C/EBPγ because it is heterodimeric with wild type C/EBPγ.

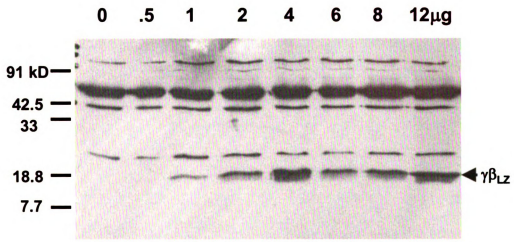
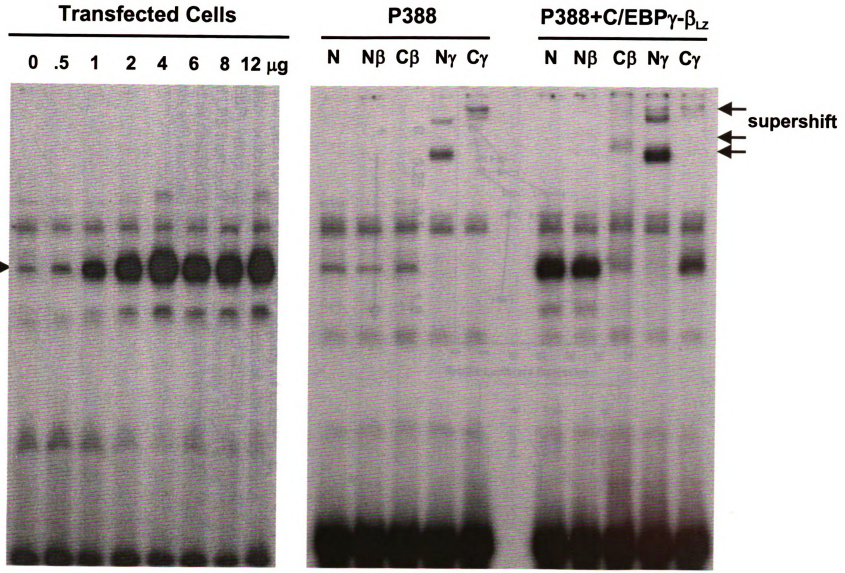


Figure 23. The formation of a heterodimeric C/EBPβ:γ leucine zipper is sufficient for the stimulatory activity of C/EBPγ. A, a western blot was performed using nuclear extracts of P388 cells transiently transfected with increasing quantities (0, 0.5, 1, 2, 4, 6, 8, and 12 μg) of pMEX-C/EBPγβ_{LZ}. The primary antibody used in the detection of C/EBPγ-βLZ was carboxyl-terminus–specific anti-C/EBPβ. An arrow marks the position of C/EBPγβ_{LZ}. The positions of protein standards are noted.

Figure 23. The formation of a heterodimeric C/EBPβ:γ leucine zipper is sufficient for the stimulatory activity of C/EBPγ. 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-β_{LZ}. 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 γ:γ and γ:γ-β_{LZ} indicate the positions of C/EBP:DNA complexes. Arrows on the right indicate supershifts. The C/EBPγ-β_{LZ} 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.


Transfected Cells
0 .5 1 2 4 6 8 12 µg
P388
N Nβ Cβ Nγ Cγ
P388+C/EBPγ-βLz
N Nβ Cβ Nγ Cγ
supershift
γ:γ,
γ:γ-βLz

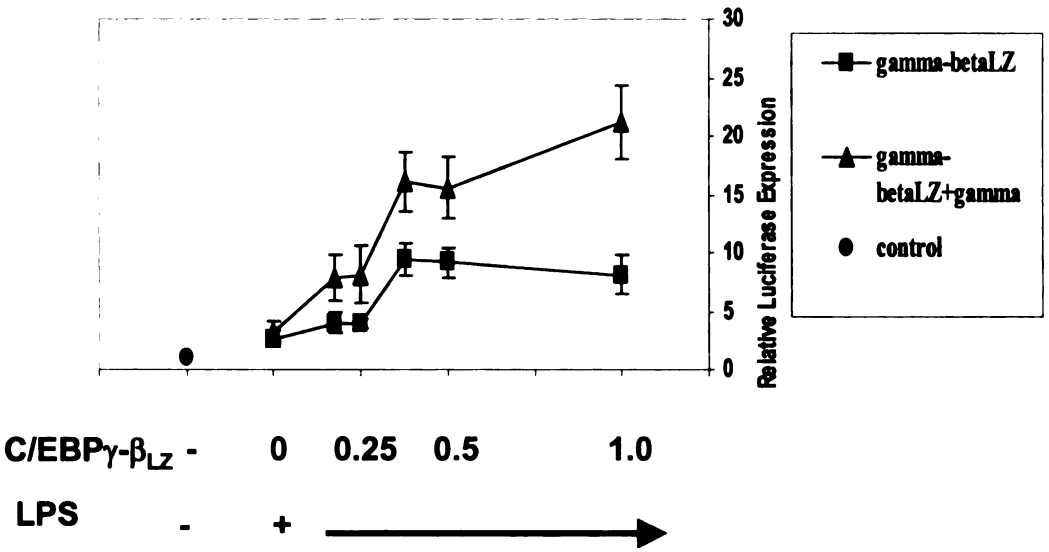


Figure 23. The formation of a heterodimeric C/EBPβ:γ leucine zipper is sufficient for the stimulatory activity of C/EBPγ. C, transient transfections were carried out in duplicate with and without 0.5 μg C/EBPγ vector, with the μg quantities of C/EBPγ-β_{LZ} expression vector and LPS treatment as indicated. Luminometer values were normalized as described in figure 9. The data are the mean of 3 experiments with standard error.

C/EBPγ-β_{LZ} 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/EBPγ-β_{LZ} expression vector (Figs. 23A, 23B, 23C). 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 weight of approximately 19 kD (Fig. 23A). A major EMSA species was detected in proportion to the amount of C/EBPγ-β_{LZ} expression vector (Fig. 23B). That species was supershifted with antibodies specific to the carboxyl terminus of C/EBPβ, the amino terminus of C/EBPγ and the carboxyl terminus of C/EBPγ, but not with antibody specific to the amino terminus of C/EBPβ (Fig. 23C). This is consistent with a heterodimerization between C/EBPγ-β_{LZ} and endogenous C/EBPγ. We tested the ability of C/EBPγ-β_{LZ} to support LPS induction of IL-6 with and without transfection of a vector expressing intact C/EBPγ (Fig. 23A). Surprisingly, in LPS-treated cells, the C/EBPγ-β_{LZ} expression vector by itself could support as much as 10-fold induction of the IL-6 promoter and the addition of 0.5 μg of C/EBPγ expression vector enhanced that stimulatory activity to 20-fold induction. While the stimulatory activity of C/EBPγ-β_{LZ} is less than that of intact C/EBPβ (40-fold for 1 μg of vector without C/EBPγ and 100-fold with C/EBPγ; see Fig. 9), the degree to which C/EBPγ augmented C/EBPγ-β_{LZ} activity was similar to its enhancement of C/EBPβ activity (about 2.5-fold). This suggests that C/EBPγ stimulatory activity resides in formation of a heterodimeric C/EBPβ:γ leucine zipper.

To further show that C/EBPβ leucine-zipper is sufficient to mediate LPS induction of the IL-6 expression in vivo, we performed stable transduction of P388 cells with a murine retroviral vector expressing C/EBPγ-β_{LZ}. These cells were compared to P388-Cβ cells as well as a control cells transfected with the same vector lacking an expressed insert (P388-Neo). The cells transfected for C/EBPγ-β_{LZ} expression were designated P388-Cγ-β_{LZ}. EMSA of nuclear extracts from two transfected cell pools verified proper expression of the stably transduced C/EBPγ-β_{LZ} genes. In comparison to nuclear extracts from P388-Neo, nuclear extracts from P388-Cγ-β_{LZ} yielded supershifted DNA-protein complexes upon incubation with antibody specific for the carboxyl terminus of C/EBPβ and the carboxyl terminus of C/EBPγ (Fig. 24). Next, P388-Neo, P388-Cβ and P388-Cγ-β_{LZ} 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 IL-6 mRNA. Confirming our transient transfection results, C/EBPγ-β_{LZ} itself is sufficient to confer on P388 cells the ability to induce IL-6 transcription in response to LPS (Fig. 25), although the IL-6 mRNAs were not induced to as high a level as with intact C/EBPβ. Furthermore, previous work in our lab showed that C/EBPβG_{LZ} had no activity in stable transfectants (Hu et al 2000). Taken together, these results suggest that the C/EBPβ:γ heterodimeric zipper in the absence of any conventional activation domains is the key determinant to support LPS induction of the IL-6 promoter.

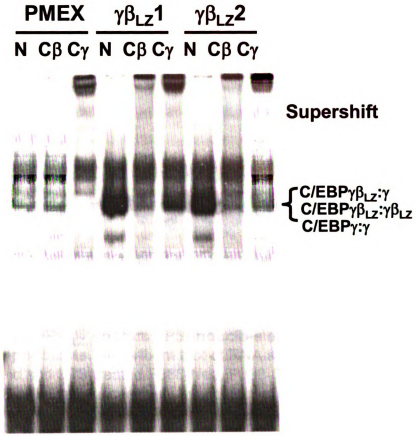


Figure 24. C/EBPγβ$_{LZ}$ is overexpressed in P388 cells. EMSA was performed using nuclear extracts of P388-Cγβ$_{LZ}$ cells. Binding reactions included normal rabbit IgG (N), C-terminus-specific anti-C/EBPβ (β) or anti-C/EBPγ (γ). Arrows on the right indicate the positions of C/EBP:DNA complex and supershifts. Themajor C/EBPγβ$_{LZ}$ complex are shifted by C/EBPβ-specific antibody. C/EBPγ-specific antibody only shifted part of this complex.

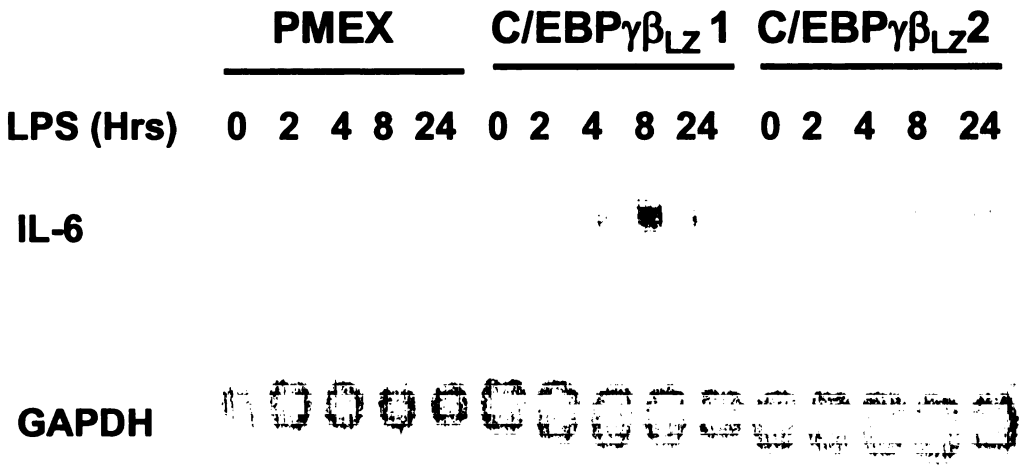


Figure 25. C/EBP$\gamma\beta_{LZ}$ confers LPS-inducible expression of IL-6 to P388 lymphoblasts. A northern blot of RNA samples isolated fromuntreated and LPS-treated P388-Neo, P388-C$\gamma\beta_{LZ}$ 1 and P388C$\gamma\beta_{LZ}$ 2 cells was successively hybridized for IL-6 and GAPDH.

DISCUSSION

The data presented in this paper demonstrate an activating role for C/EBPγ in transcription from the IL-6 and IL-8 promoters in B lymphoid cells. C/EBPγ, which in other contexts can inhibit activation by C/EBP family members (Roman et al 1990; Parkin et al 2002), was found to augment the C/EBPβ-dependent LPS stimulation of IL-6 and IL-8 promoter-reporters in P388 B lymphoblasts. This stimulatory activity of C/EBPγ is 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 derivatives.

The stimulatory activity of C/EBPγ was surprising, since it is generally accepted as being an inhibitor of C/EBP transcriptional activators (Cooper et al 1995; Cooper et al 1994). 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 (Cooper et

al 1992). Similarly, C/EBPγ synergizes with Stat6 and NF-κB p50/p65 to induce the germline gamma 3-immunoglobulin promoter in a B cell line (Pan et al 2000). C/EBP has also been found to enhance β-globin gene expression in collaboration with CP-1 (Wall et al 1996). 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 (Omori et al 1998). Thus, the role of C/EBPγ as a transcriptional activator does not seem unusual. It seems neither inherently an activator nor an inhibitor. Rather, the identity of its promoter context and dimerization partner may be the overriding features that govern the specific role of C/EBPγ in transcription. Heterodimerization with C/EBPγ has two effects on the ability of C/EBPβ to activate the IL-6 promoter: it inhibits C/EBPβ activity in the absence of LPS and enhances C/EBPβ transactivation in LPS stimulated cells. Therefore, we predict that in B cells the net effect of C/EBPγ is to greatly increase the index of LPS inducibility of the IL-6 promoter. This prediction could be tested in B lineage cells derived from C/EBPγ-deficient mice (Kaisho et al 1999).

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 (Matsuaka et al 1993; Stein et al 1993; Kunsch et al 1994). 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 of such a mechanism, the findings that C/EBPγ inhibits C/EBPβ

activation of the IL-6 promoter in the absence of LPS (Fig. 13A) and that this inhibitory effect is converted to a stimulatory effect by NF-κB p65 expression (Fig. 13B) are consistent with this. Furthermore, our previous studies found that the activity of C/EBPβ on the IL-6 promoter was dependent on an intact NF-κB site (Hu et al 2000). It is, however, unlikely that the stimulatory role of C/EBPγ is limited to promoters that exhibit synergy between C/EBPβ and NF-κB. Other promoters for which C/EBPγ stimulatory activity has been suggested, including immunoglobulin heavy chain (Cooper et al 1992; Pan et al 2000Wall et al 1996), β-globin (Wall et al 1996), and pp52 (Omori et al 1998), do not display synergistic regulation by C/EBPβ and NF-κB.

C/EBPγ stimulatory activity also displays cell-type specificity. This is also the case for the inhibitory activity of C/EBPγ (Parkin et al 2002). Stimulatory activity was seen in P388 B cells, but not in their macrophage derivatives, P388D1(IL-1) (Fig. 20). C/EBPγ is normally a minor component of the C/EBP family members expressed in these macrophages (Fig. 18), where C/EBPβ forms heterodimers with another as yet unidentified protein (Parkin et al in 2002). 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 heterodimeriztion. Our studies have certainly demonstrated that heterodimerization is critical for activity (Fig. 21C).

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 for autocrine IL-6 production to drive the maturation of B cells, while 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 (Kaisho et al 1999) 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 (Huang et al 1992; Wuyts et al 1996). In humans, however, both IL-6 and IL-8 are autocrine factors in myeloma tumor progression (Treon et al 1998; Shapiro et al 2001). 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 (Roman et al 1990), we have found C/EBPβ:γ and C/EBPδ:γ heterodimers to be the predominant form of C/EBP in LPS-stimulated WEHI 231 cells (Fig. 5), 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 (Parkin et al 2002). It will be worthwhile to evaluate whether C/EBPγ can stimulate target

genes that are already 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. 6). This may indicate a preference for heterodimeriztion between these C/EBP family members. This result cannot be explained by large pools of either monomeric C/EBPγ or unbound C/EBPγ 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. It is also 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. 23). Since C/EBPγ by itself is unable to support LPS induction of the IL-6 promoter (Fig. 15), this result demonstrates that the formation of a C/EBPβ:γ heterodimeric zipper in the absence of any conventional activation domains is 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 (Hu et al 2000). 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/EBP and NF-κB, and that C/EBPγ expression actually becomes inhibitory in the absence of NF-κB expression (Fig. 13A). 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-1β, serum amyloid A1, A2, A3, and $_1$-acid glycoprotein (Poli 1998). 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 (Nerlov et al 1994) 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 (Wegner et al 1992). Recently, the leucine zipper of transcription factor v-Myb has been found to regulate the commitment of hematopoietic progenitors (Karafiat et al 2001). 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 manner dependent upon leucine zipper dimerization partners.

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CHAPTER 3

C/EBPζ (CHOP/GADD153) IS A NEGATIVE REGULATOR OF LPS-INDUCED IL-6 EXPRESSION IN B CELLS

ABSTRACT

C/EBPζ was originally identified as a gene induced upon DNA damage and growth arrest. It has been shown to be involved in the cellular response to endoplasmic reticulum stress. Because of sequence divergence from other C/EBP family members in its DNA binding domain and its consequent inability to bind the C/EBP consensus-binding motif, C/EBPζ can act as a dominant negative inhibitor of other C/EBPs. C/EBP transactivators are essential to the expression of many proinflammatory cytokines and acute phase proteins, but a role for C/EBPζ in regulating their expression has not been described. We have found that expression of C/EBPζ is induced in response to LPS treatment of B cells at both the mRNA and protein levels. Correlating with the highest levels of C/EBPζ expression at 48 hours after LPS treatment, both the abundance of C/EBP DNA binding species and IL-6 expression are downregulated. Furthermore, ectopic expression of C/EBPζ inhibited C/EBPβ-dependent IL-6 expression from both the endogenous IL-6 gene and an IL-6 promoter-reporter. These results suggest that C/EBPζ functions as negative regulator of IL-6 expression in B cells and that it contributes to the transitory expression of IL-6 that is observed after LPS treatment.

INTRODUCTION

C/EBPs comprise a family of bZIP regulatory proteins containing two distinct domains: a basic region that binds to DNA, and an adjacent leucine-zipper region that enables homo- and heterodimerization of C/EBP proteins (Landschulz et al 1988). In mammalian species, the family consists of six unique members: C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, and C/EBPζ (reviewed by Lekstrom-Himes and Xanthopoulos 1998). *In vitro* studies have implicated both C/EBPβ and C/EBPδ as participating in the transcriptional activation of proinflammatory cytokines such as IL-6, as well as many acute phase proteins (reviewed by Akira 1997). Indeed, the promoter region of IL-6 contains C/EBP binding sites (Tanabe et al 1988). In addition, both C/EBPβ and C/EBPδ can activate an IL-6 promoter-reporter in transient expression assays (Kinoshita et al 1992; Matsuaka et al 1993).

C/EBPζ is a small nuclear protein that readily dimerizes with other members of the C/EBP family. However, the basic region of C/EBPζ deviates significantly from the consensus DNA binding domain defined by other members of the C/EBP family. It contains proline substitutions in two conserved residues, which are believed to be essential to the interaction of these proteins with consensus C/EBP DNA binding sites (O'Neil et al 1990; Schuman et al 1990). Indeed, C/EBPζ-C/EBP heterodimers fail to bind several known C/EBP sites *in vitro*. And when expressed in cells, C/EBPζ attenuates the ability of other C/EBP proteins to activate promoters containing such sites (Ron et al 1992). Based on

these findings, C/EBPζ was proposed to act as a dominant negative inhibitor of other C/EBPs (Ron et al 1992). However, recent studies have suggested that C/EBPζ-C/EBP heterodimers can activate several downstream target genes (Wang et al 1998) and that C/EBPζ-C/EBPβ heterodimers can specifically activate transcription of the murine carbonic anhydrase VI gene through a non-consensus binding site (Sok et al 1999).

C/EBPζ is transcriptionally activated and highly expressed following treatment of cells with a variety of growth arrest and DNA damaging conditions (Fornace et al 1989; Luethy et al 1990; Choi et al 1992; Carlson 1993). More generally, C/EBPζ has been shown to be inducible by agents that either directly (Bartlett et al 1992; Chen et al 1992; Price et al 1992; Halleck et al 1997) or indirectly (Carlson et al 1993; Marten et al 1994; Bruhat et al 1997) lead to impairment of the endoplasmic reticulum folding environment (i.e. ER stress). The C/EBPζ promoter contains a putative C/EBP-binding site suggesting that C/EBPζ itself is regulated by C/EBPs (Park et al 1992). Indeed, overexpression of C/EBPβ was found to transactivate expression of a C/EBPζ promoter-reporter in HepG2 hepatoma cells (Sylvester et al 1994).

In our previous studies, we found that the stable expression of C/EBPβ in a murine B lymphoblast cell line is sufficient to confer lipopolysaccharide (LPS)-inducible IL-6 expression, thus establishing an essential role for C/EBP transcription factors in IL-6 expression (Bretz et al 1994). We also found that C/EBPα, β, δ, and ε are largely redundant to each other in the LPS-induced expression of IL-6 (Hu et al 1998;Williams et al 1998). Since C/EBPζ, along with

various acute phase proteins, is induced in the livers of rats treated with LPS (Sylvester et al 1994), we wondered whether it might also be induced by LPS in B cells and thus have a role in the regulation of CEBP-dependent IL-6 expression. Here, we report that C/EBPζ is induced in response to LPS treatment of B cells and apparently contributes to the downregulation of IL-6 expression that occurs after the initial induction of IL-6 by LPS treatment. The highest levels of C/EBPζ expression at 48 hours after LPS treatment correlated with a dramatic decrease in the abundance of C/EBP DNA binding species, as well as the attenuation of IL-6 expression. The levels of nuclear C/EBPβ, the predominant activating C/EBP family member, are maintained and even modestly increase during the attenuation phase. However, C/EBPζ potently inhibits C/EBPβ-dependent IL-6 expression from both the endogenous IL-6 gene and an IL-6 promoter-reporter. Thus, C/EBPζ functions as negative regulator of IL-6 expression in B cells and contributes to the transitory expression of IL-6 that is observed after LPS treatment.

MATERIALS AND METHODS

Cells and cell culture- WEHI-231 cells are murine B cells (Gutman et al 1981) (American Type Culture Collection; CRL 1702). P388 cells are murine B lymphoblasts (Bauer et al 1986) (ATCC; CCL46). P388-Cβ cells and P388-Neo cells have been described previously (Hu et al 1998). WEHI-231 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 μM β-mercaptoethanol. P388 cells and their derivatives were cultured in RPMI 1640 medium supplemented with 5% FBS and 50 μM β-mercaptoethanol. Certain cultures were treated with LPS derived from *Escherichia coli* serotype 055:B5 (Sigma) added to 10 μg/ml. All cells were grown at 37 °C in 5% CO_2.

Transfections-Transductions of P388-Cβ with a G418-resistant vector encoding C/EBPζ were carried out by retroviral infection. Retrovirus stocks were prepared by transient expression of 293T cells (Pear et al 1993) that were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. 3 μg of the C/EBPζ retroviral expression vector were cotransfected with 3 μg of a packaging construct, pMOV-ψ (Mann et al 1983). Transfections were performed using DMRIE-C (Life Technologies) upon 80% confluent 293 T cells on 60 mm plates. Virus stocks were harvested 60 hours post-transfection by centrifuging the supernatants at 1500 rpm for 5 min followed by filtration through 0.45-μM-pore-size filters. Retroviral infections were performed by the addition of 3ml virus

stock to $2x10^6$ cells in the presence of 8 μg/ml polybrene (Sigma). The cells were then incubated at 37°C for 3 hours during which time the cells were resuspended every 30 min. The infected cells were then collected by centrifugation and resuspended in growth medium. After 24 hours, the cells were split to four 60 mm plates, G418 (Life Technologies) was added to a final concentration of 670 μg/ml, and selection of resistant cells proceeded for 7 to 10 days.

Transient transfections were conducted with $2x10^6$ cells, 6 μg of DNA, and 8 μl of DMRIE-C reagent (Life Technologies) in 1.2 ml of Opti-MEM medium (Life Technologies). The DNA was comprised of 1 μg of an IL-6 promoter-reporter, C/EBP expression vector, and pMEX plasmid to total 6 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 FBS. For the luciferase assays, the medium of certain transfections was supplemented with 10 μg/ml LPS after 24 hours. After 4 hours 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) and for β-galactosidase activity by using the Luminescent β-Galactosidase Genetic Reporter System II (Clontech). Otherwise, cells were directly harvested for RNA and nuclear extract after 24 hours transfection.

Expression vectors and promoter-reporters-For transient transfections, C/EBPβ was expressed from pMEX (Williams et al 1991), which utilizes the

Moloney murine sarcoma virus promoter. For transient transfections, C/EBPζ was expressed from pcDNA1 (Invitrogen), which uses the cytomegalovirus promoter (from Dr. David Ron, NYU School of Medicine). For stable transductions, a 9E10-myc-tagged C/EBPζ was expressed from pSRaMSVtkneo (Muller et al 1992), a retroviral vector derived from Moloney sarcoma virus (from Dr. David Ron, NYU School of Medicine).

The IL-6 promoter-reporter consists of the murine IL-6 promoter (Tanabe et al 1988) (-250 to +1) inserted into the luciferase vector, pXP2 (Nordeen 1988). The SV40 early promoter-reporter is a commercial product, pβgal-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 (Life Technologies) according to the manufacturer's directions. RNA's were electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to high stringency in 40 mM sodium phosphate/1% SDS/1mM EDTA at 65°C. Hybridization probes were prepared with a random priming kit (Life Technologies) with the incorporation of 5'-[-^{32}P] dATP (3000 Ci/mmol; DuPont-New England Nuclear). The C/EBPζ probe consisted of 0.6 kb partial murine cDNA (from David Ron, NYU School of Medicine). The IL-6 probe was a 0.65 kb murine cDNA (from Drs. N. Jenkins and N. Copeland, National Cancer Institute-Frederick). The glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) probe was a 1.3 kb rat cDNA (Fort et al 1985).

Western analysis-Nuclear extracts were prepared as described below. The extracts (60 μg) were adjusted to 1x Laemmli sample buffer (Laemmli 1970) 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).

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 $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% [vol/vol] NP-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 13,000 rpm for 60 sec at 4°C. Proteins were extracted from nuclei by 20 min incubation at 4°C with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 25% [vol/vol] 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 13,000 rpm for 30 min at 4°C and the supernatant extract was collected and stored at -80°C. The EMSA probes were double-stranded oligonucleotides containing a murine IL-6 C/EBP binding site (5'-

CTAAACGACGTCACATTGTGCAATCTTAATAAGGTT-3' annealed with 5'-TGGAAACCTTATTAAGATTGCACAATGTGACGTCGT-3'). The probe was labeled with the incorporation of 5'-[α-32P] dATP (3000 Ci/mmol; DuPont-New England Nuclear) and Klenow DNA polymerase. Underlined sequences correspond to the C/EBP binding motifs.

DNA binding reactions were performed at room temperature for 20 min in a 25 μl reaction mixture containing 6 μl of nuclear extract (1mg/ml in buffer C) and 5 μl of 5x binding buffer (20% [wt/vol] Ficoll, 50 mM HEPES [pH 7.9], 5mM EDTA, 5 mM dithiothreitol). The remainder of the reaction mixture contained 1 μg poly (dI-dC), 200 pg of probe (unless otherwise noted), bromophenol blue to a final concentration of 0.06% [wt/vol], and water to volume. For supershifts, nuclear extracts were preincubated with antibodies for 20 min at 4°C prior to the binding reaction. Samples were electrophoresed through 5.5% polyacrylamide gels in 1x TBE (90 mM Tris base, 90 mM boric acid, 0.5 mM EDTA) at 160 V.

Antibodies- Rabbit anti-C/EBPβ specific to the carboxyl terminus (C-19), rabbit anti-C/EBPζ specific to the amino terminus (F-168) and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Rabbit antibody specific to the carboxyl terminus of C/EBPγ was prepared against a synthetic peptide corresponding to this sequence (Parkin et al 2002).

RESULTS

C/EBPζ is induced by LPS treatment in B cells- We previously found that C/EBPα, β, δ, and ε are all capable of conferring LPS-inducible IL-6 transcription to P388 B lymphoblasts (Bretz et al 1994; Hu et al 1998; Williams et al 1998). P388 is a murine B lymphoblastic cell line (Bauer et al., 1986) that normally lacks C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε expression and has been a useful system for analyzing C/EBP protein function (Bretz et al., 1994; Hu et al., 1998; Williams et al 1998; Hu et al. 2000; Gao et al. in press). Although C/EBPs are expressed from constitutive vectors in this system, IL-6 expression is transitory with decreasing expression after 24 hours of LPS treatment (Fig. 1). In WEHI 231 B cells, a B cell line that has been used in several studies of IL-6 expression (Hobbs et al 1991; Macfarlane and Manzel 1998; Lee and Koretzky 1998; Venkataraman et al 1999), IL-6 induction by LPS is also transitory, decreasing after 24 hours treatment (Fig. 2).

Since C/EBPζ mRNA is markedly induced by LPS in the livers of rats during the acute phase response (Sylvester et al 1994), we examined whether LPS would also induce expression of this inhibitor to C/EBP activity in B cells. To that end, C/EBPζ expression was examined in RNA from P388-Cβ cells treated with LPS. P388-Cβ cells are P388 cells that have been transfected for C/EBPβ expression (Bretz et al., 1994; Hu et al., 1998). Northern analysis showed that C/EBPζ mRNA was highly induced in P388-Cβ cells following 24 hours treatment with LPS (Fig. 3). As expected, C/EBPβ mRNA was readily detected in

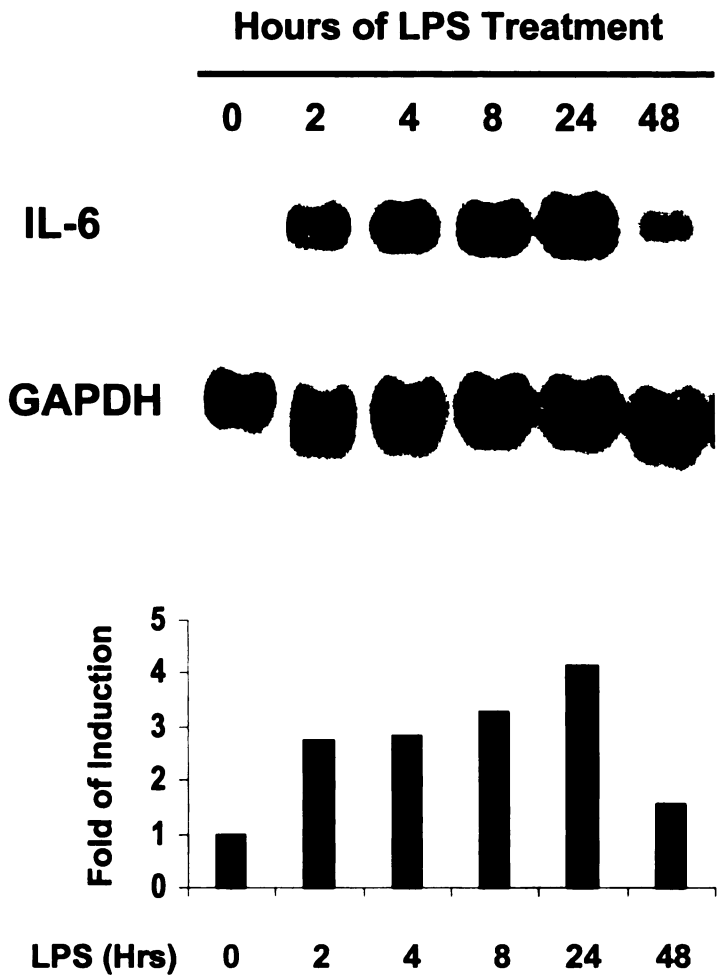


Figure 1. LPS induces IL-6 expression in P388-Cβ cells. Total RNA was isolated over time course of LPS treatment as indicated. Twenty microgram of RNA was analyzed on Northern blots that were successively hybridized to probes for IL-6 and GAPDH. Level of IL-6 mRNA (as determined by phosphoimager analysis) were expressed relative to GAPDH and graphed relative to control value (receiving no LPS) set at 1.

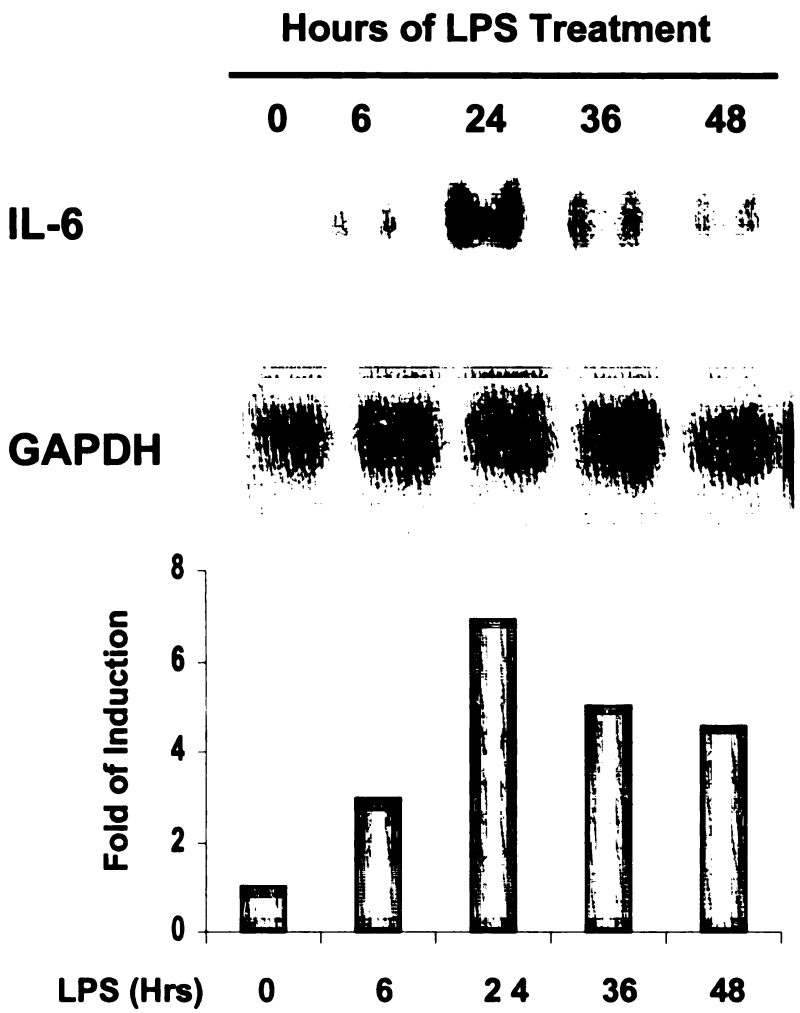


Figure 2. LPS induces IL-6 expression in WEHI231 cells. Total RNA was isolated over time course of LPS treatment as indicated. Twenty microgram of RNA was analyzed on Northern blots that were successively hybridized to probes for IL-6 and GAPDH. Level of IL-6 mRNA (as determined by phosphoimager analysis) were expressed relative to GAPDH and graphed relative to control value (receiving no LPS) set at 1.

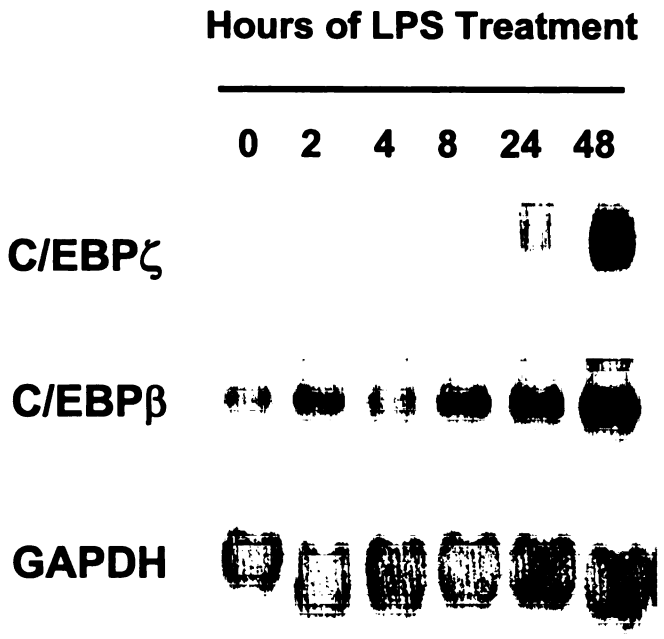


Figure 3. LPS induces C/EBPζ expression in P388-Cβ cells. Total RNA was isolated over time course of LPS treatment as indicated. Twenty microgram of RNA was analyzed on Northern blots that were successively hybridized to probes for C/EBPζ, C/EBPβ and GAPDH.

untreated cells, and its expression was further elevated in response to LPS treatment (Fig. 3), presumably due to activation of the retroviral promoter in the expression vector.

Having observed that C/EBPζ mRNA expression is induced by LPS in P388-Cβ cells, we sought to determine whether this induction resulted in increased expression of C/EBPζ protein. We also determined the expression levels of both C/EBPβ and C/EBPγ proteins, the other C/EBP proteins expressed by P388-Cβ cells. We had previously shown that C/EBPγ expression is stimulatory on the IL-6 promoter and that C/EBPβ exists largely as a heterodimer with C/EBPγ in these cells (Gao et al. in press). Therefore, the balance of expression of all three C/EBP proteins is relevant to the role of C/EBP as an activator of IL-6 expression. A western blot analysis of nuclear extracts isolated over a time course of LPS treatment of P388-Cβ cells revealed a time-dependent increase in both C/EBPζ and C/EBPβ proteins, while C/EBPγ expression was constant (Fig.4). The minor C/EBPβ-specific species may represent a modified form (e.g. phosphorylation), however both forms appear to be co-modulated. We also examined the expression of C/EBP proteins in nuclear extracts of WEHI 231 B cells. A western blot of a time course of LPS treatment showed both C/EBPζ and C/EBPβ proteins to be induced over time, while C/EBPδ exhibited a transitory induction and C/EBPγ exhibited a modest decrease in expression (Fig. 5). Two C/EBPβ-specific species are apparent at 36 and 48 hours. These likely represent 34 and 38 kD forms arising from alternative translational initiation. Both forms are reported to be transcriptional activators (reviewed in Johnson and

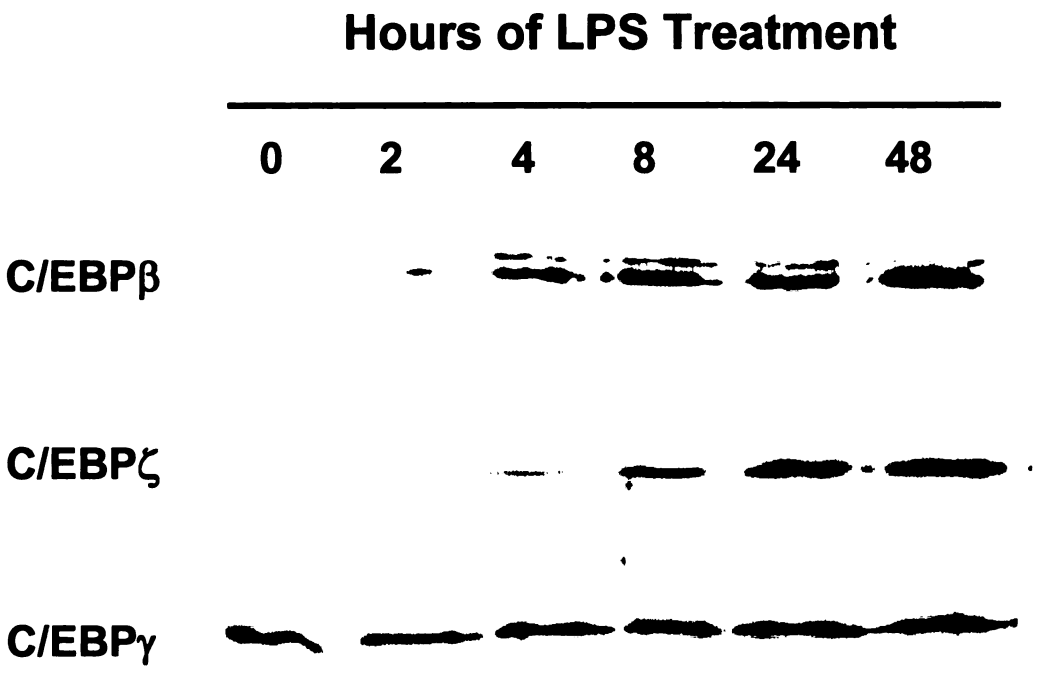


Figure 4. LPS induces C/EBPβ and C/EBPζ expression in P388-Cβ cells. Western analysis of nuclear extracts from a time course of LPS treatment as indicated. Proteins were detected with a carboxyl-terminal specific C/EBPβ, C/EBPζ or C/EBPγ antibody.

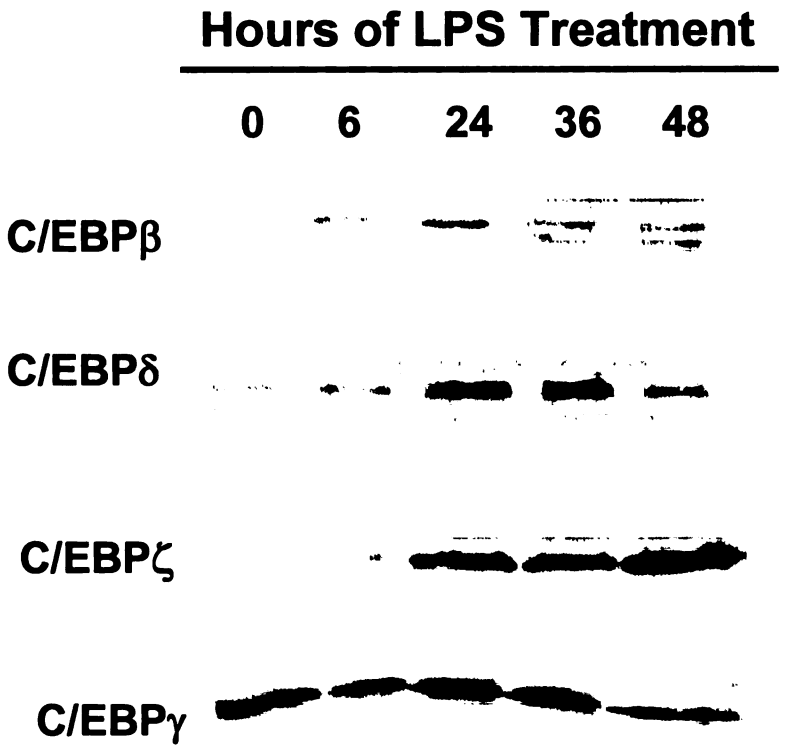


Figure 5. LPS induces C/EBPβ, C/EBPδ, and C/EBPζ expression in WEHI231 cells. Western analysis of nuclear extracts from a time course of LPS treatment as indicated. Proteins were detected with a carboxyl-terminal specific C/EBPβ, C/EBPδ, C/EBPζ or C/EBPγ antibody.

Williams 1994). The data demonstate that increased C/EBPζ expression is coincident with decreased IL-6 expression in both P388 lymphoblasts and WEHI 231 B cells.

C/EBPζ inhibits C/EBPβ DNA-binding in B cells -C/EBPζ possesses a leucine zipper dimerization domain and readily heterodimerizes with other C/EBPs (Ron et al 1992). However, the presence of two prolines in its DNA-binding domain disrupts its helical structure and prevents dimer binding to the consensus C/EBP DNA enhancer element (Ron et al 1992). Therefore, the upregulation of C/ EBPζ expression would be expected to cause the decreased binding of C/EBPβ to DNA. To determine whether this was the case, EMSA was performed using the same nuclear extracts derived from LPS treated P388-Cβ cells that were used for the western blot analysis of Figure 4. The binding of C/EBPβ:β and C/EBPβ:γ to the murine IL-6 C/EBP binding site decreased dramatically and steadily over time with LPS treatment (Fig. 6), even though the level of C/EBPβ protein in nuclear extracts increased over the same time course (Fig. 4). Similar results were obtained with nuclear extracts from WEHI231 cells. C/EBPβ:γ and C/EBPδ:γ binding, at first, increased with LPS treatment (Fig. 7), consistent with the induction of C/EBPβ and C/EBPδ expression observed in western analysis (Fig. 5). From 36 hours LPS treatment onward, the binding of C/EBPβ:γ and C/EBPδ:γ dramatically decreased, reaching levels below that of untreated cells (Fig. 7). To further address the ability of C/EBPζ to inhibit C/EBPβ DNA binding, P388 cells were stably transduced with a murine retroviral

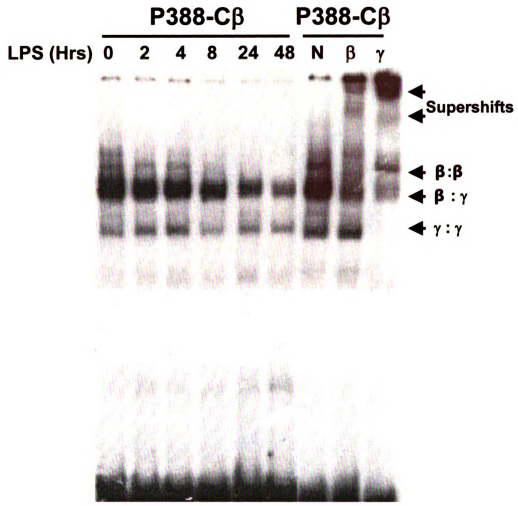


Figure 6. DNA-binding activity of nuclear extract containing C/EBPβ decreased following a time course of LPS treatment in P388-Cβ cells. EMSA was performed using nuclear extracts of P388-Cβ cells that were treated with LPS over a time course as shown and a labeled oligonucleotide corresponding to the IL-6 promoter C/EBP binding site. The EMSA of the no LPS treated P388-Cβ also was performed with binding reactions that included normal rabbit IgG (N), carboxyl-terminus specific anti-C/EBPβ (β), or carboxyl-terminus–specific anti-C/EBPγ (γ). Arrows labeled β:β, β:γ, and γ:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right also indicate supershifts. The C/EBPβ:β complex is supershifted by only C/EBPβ-specific antibodies, the C/EBPγ:γ complex by only C/EBPγ-specific antibodies, and the C/EBPβ:γ complex by both C/EBPβ-specific and C/EBPγ-specific antibodies.

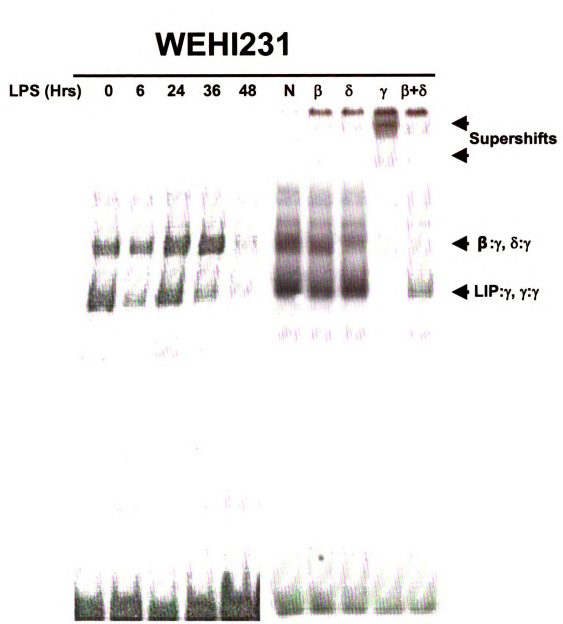


Figure 7. DNA-binding activity of nuclear extract containing C/EBPβ decreased following a time course of LPS treatment in WEHI231 cells. EMSA was performed using nuclear extracts of WEHI231 cells that were treated with LPS over a time course as shown and a labeled oligonucleotide corresponding to the IL-6 promoter C/EBP binding site. The EMSA of the 24 hours LPS treated WEHI231 also was performed with binding reactions that included normal rabbit IgG (N), carboxyl-terminus specific anti-C/EBPβ (β), anti-C/EBPδ (δ),carboxyl-terminus–specific anti-C/EBPγ (γ), or anti-C/EBPβ and anti-C/EBPδ (β+δ). Arrows labeled β:γ, δ:γ, LIP: γ and γ:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right indicate supershifts.

vector expressing C/EBPζ and the DNA binding ability of C/EBPβ in those transfectants compared to control transfectants. The population of cells transduced for C/EBPζ expression was designated P388-Cζ. Western blot analyses were used to verify proper expression of the stably transduced C/EBPζ gene. Anti-C/EBPζ detected two immunoreactive species in the transduced population (Fig. 8). The slower-migrating band that is absent in the control P388-Neo population corresponds to the 9E10-myc-tagged C/EBPζ protein, while the faster-migrating band corresponds to the 29 kDa endogenous C/EBPζ protein. To assess the impact of C/EBPζ on the DNA-binding ability of C/EBPβ, a C/EBPβ-expression vector was transfected into P388-Cζ and P388-Neo cells, and EMSA performed on nuclear extracts. C/EBPβ DNA binding was decreased significantly in P388-Cζ transfectants compared to P388-Neo control cells at both quantities of C/EBPβ vector used in the transient transfections (Fig. 9A), supporting the notion that C/EBPζ is the factor that diminishes C/EBPβ DNA-binding activity in B cells after lengthy LPS treatment. A western blot analysis was performed to verify that comparable levels of C/EBPβ were expressed in the transfected cells (Fig. 9B). It is worth noting that the C/EBPβ:γ heterodimers are more resistant to the C/EBPζ sequestration than are C/EBPβ:β homodimers.

C/EBPζ inhibits C/EBPβ-dependent LPS-induced expression of IL-6-

Having observed that C/EBPζ expression is induced by LPS treatment in B cells concomitant with attenuation of C/EBP DNA-binding activity and IL-6 expression

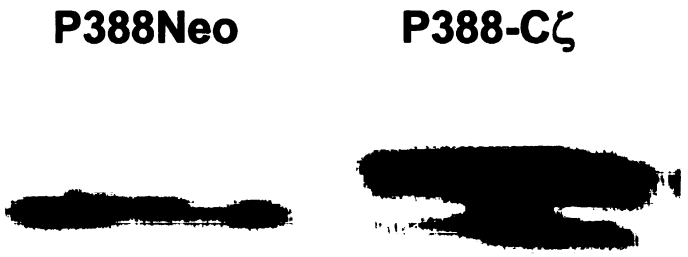
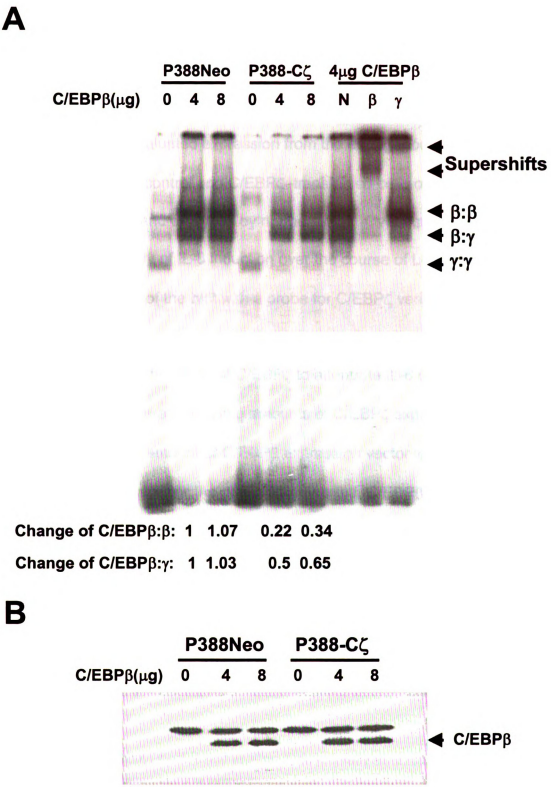


Figure 8. C/EBPζ is overexpressed in P388 cells. A western blot using C/EBPζ-specific antibody showed expression of myc-tagged C/EBPζ in P388 transductants of C/EBPζ (P388-Cζ) in comparison to control transductants (P388Neo).

Figure 9. Overexpression of C/EBPζ reduced DNA-binding activity of nuclear extract containing C/EBPβ. A. EMSA was performed using nuclear extracts of P388Neo and P388-Cζ cells that were transiently transfected with increasing amount of C/EBPβ as shown and a labeled oligonucleotide corresponding to the IL-6 promoter C/EBP binding site. The EMSA of 4μg C/EBPβ transfected P388Neo also was performed with binding reactions that included normal rabbit IgG (N), carboxyl-terminus specific anti-C/EBPβ (β), or carboxyl-terminus–specific anti-C/EBPγ (γ). Arrows labeled β:β, β:γ , andγ:γ indicate the positions of C/EBP:DNA complexes. Arrows on the right also indicate supershifts. The C/EBPβ:β complex is supershifted by only C/EBPβ-specific antibodies, the C/EBPγ:γ complex by only C/EBPγ-specific antibodies, and the C/EBPβ:γ complex by both C/EBPβ-specific and C/EBPγ-specific antibodies. **B.** western blot using C/EBPβ-specific antibody showed expression of C/EBPβ in P388-Cζ cells in comparison to P388Neo cells.


A
P388Neo
P388-Cζ
4μg C/EBPβ
C/EBPβ(μg) 0 4 8 0 4 8 N β γ
Supershifts
β:β
β:γ
γ:γ
Change of C/EBPβ:β: 1 1.07 0.22 0.34
Change of C/EBPβ:γ: 1 1.03 0.5 0.65
B
P388Neo
P388-Cζ
C/EBPβ(μg) 0 4 8 0 4 8
C/EBPβ

and that C/EBPζ inhibits C/EBPβ DNA-binding, we directly tested whether increased expression of C/EBPζ downregulates IL-6 expression. In order to address this question, we transiently transfected the C/EBPζ expression vector into P388-Cβ cells and evaluated expression from the endogenous IL-6 gene. RNAs were isolated from control and C/EBPζ–transfected cells over a time course of LPS treatment and analyzed by northern blot. Overexpression of C/EBPζ reduced the extent of IL-6 induction over the course of LPS treatment (Fig. 10). Rehybridization of the blot with a probe for C/EBPζ verified C/EBPζ expression (Fig. 10).

To further address the ability of C/EBPζ to attenuate IL-6 expression, transient transfections where increasing amounts of C/EBPζ expression vector were added to a constant amount of C/EBPβ expression vector were performed using the IL-6 promoter-reporter. As previously reported, C/EBPβ together with LPS stimulation elicits a robust induction of the IL-6 promoter (Fig. 11). However, increasing levels of C/EBPζ expression inhibited induction of the IL-6 promoter. The data demonstrate that the ability of C/EBPζ expression to attenuate C/EBPβ-dependent LPS induction of IL-6 expression from both the intact, endogenous IL-6 gene and a promoter-reporter construct.

Transitory NF-κB activation is not sufficient to explain attenuation of IL-6 expression-Because C/EBPβ and NF-κB act synergistically to induce IL-6

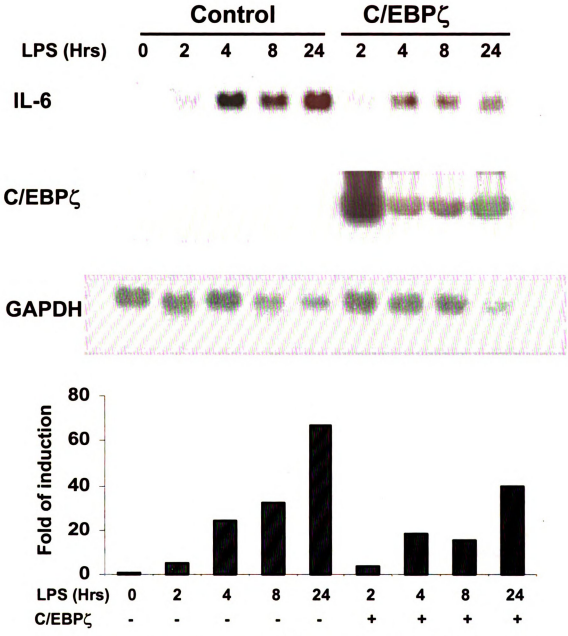


Figure. 10 Overexpression of C/EBPζ reduced the LPS-induced expression of IL-6 in P388-Cβ cells. Total RNA from P388-Cβ cells infected with a C/EBPζ expressing retrovirus or control vector were isolated over time course of LPS treatment as indicated. Twenty microgram of RNA was analyzed on Northern blots that were successively hybridized to probes for IL-6, C/EBPζ and GAPDH. Level of IL-6 mRNA (as determined by phosphoimager analysis) were expressed relative to GAPDH and graphed relative to control value (receiving no LPS) set at 1.

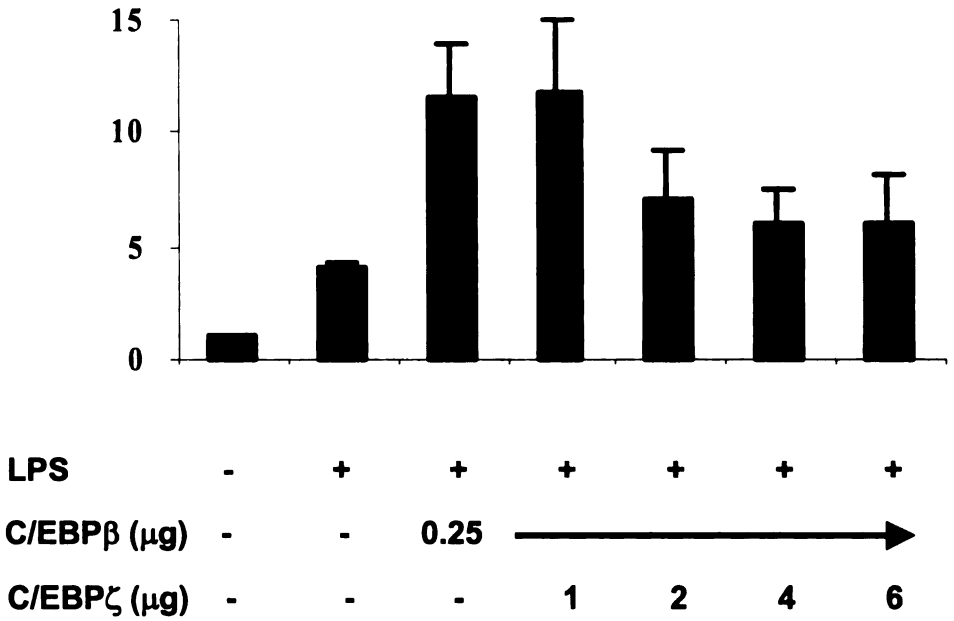


Figure 11. C/EBPζ inhibits the C/EBPβ-confered IL-6 induction upon LPS treatment. Transfections were carried out in duplicate without and with increasing amounts of C/EBPζ vector, with the μg quantities of C/EBPβ vector and LPS treatment as indicated. Luminometer values were normalized for expression from a co-transfected SV40 early promoter β-galactosidase-reporter. These values were then normalized to a relative value of 1 for cells receiving neither a C/EBP expression vector nor LPS.

transcription (Matsuaka et al 1993), it is plausible that the transitory nature of LPS-induced IL-6 expression is mediated by the transitory activation of NF-κB. To evaluate this possibility, the levels of NF-κB binding activity were assessed in WEHI231 cells treated over a time course with LPS. NF-κB binding activity was indeed transitory, but with more rapid kinetics than that observed for IL-6 expression. EMSA of nuclear extracts from the LPS-treated cells revealed increased NF-κB DNA-binding activity as early as 15 minutes LPS treatment (Fig. 12). NF-κB DNA-binding activity increased through 2 hours, and then declined to basal levels by 48 hours (Fig. 12). It should be noted that at 24 hours LPS treatment when IL-6 mRNA is at its peak level, NF-κB levels are only slightly above unstimualted levels. Thus the transitory nature of NF-κB activation is not likely the mechanism for attenuation of IL-6 induction.

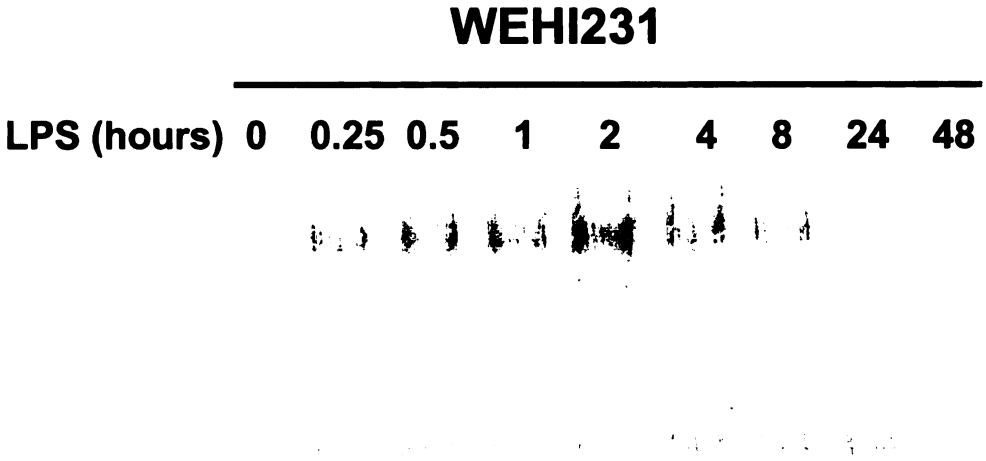


Figure 12. LPS induces NF-κB DNA binding of IL-6 promoter in WEHI231 cells. EMSA was performed using nuclear extracts of WEHI231 cells that were untreated or LPS-treated over a time course as shown and a labeled oligonucleotide corresponding to the IL-6 promoter NF-κB binding site.

DISCUSSION

The data presented in this paper suggest that C/EBPζ plays an inhibitory role in IL-6 expression, where its upregulation with lengthy LPS stimulation contributes to the transitory nature of LPS-induced IL-6 expression. C/EBPζ expression was found to be upregulated in two B cell lines after LPS stimulation. The highest levels of C/EBPζ expression correlated with the attenuation of IL-6 induction. The abundance of C/EBP DNA-binding species dramatically decreased with the induction of C/EBPζ expression and ectopic expression of C/EBPζ was found to suppress IL-6 expression from both the endogenous IL-6 gene, as well as an IL-6 promoter-reporter.

A large body of research supports a role for C/EBPβ and C/EBPδ in the regulation of IL-6 expression, as well as many other genes associated with the acute phase response (Akira et al 1990; Kinoshita et al 1992; Akira et al 1997; Poli 1998). Our own work has shown that the stable expression of C/EBPα, β, δ or ε in a murine B lymphoblast cell line is sufficient to confer LPS inducible IL-6 expression (Bretz et al 1994; Hu et al 1998; Williams et al 1998). More recently, we have found a stimulatory role for C/EBPγ in IL-6 expression (Gao et al in press). Although it has been demonstrated that expression of C/EBPζ is highly induced in liver during the acute phase response (Sylvester et al 1994), a role for C/EBPζ in IL-6 regulation or, more generally in the acute phase response, has not been described. C/EBPζ can act as a dominant negative inhibitor of other

C/EBPs (Ron et al 1992) and the effects upon IL-6 expression that we describe here are consistent with C/EBPζ playing an important role in the attenuation of the acute phase response by inhibiting the expression of C/EBP-regulated genes.

We have demonstrated that C/EBPζ expression is induced in response to LPS treatment in B cells. This is consistent with results obtained from LPS induction of the acute phase response in rat liver (Sylvester et al 1994). These earlier investigators found that the C/EBPζ promoter contains a putative C/EBP-binding site, suggesting that classical C/EBP transactivators could mediate LPS-induced C/EBPζ expression. Indeed, Sylvester et al (1994) showed that C/EBP-containing complexes bind to this site and that this binding activity increases in hepatic nuclear extracts from rats treated with LPS. They also showed that at least C/EBPα and C/EBPβ contribute to these complexes and that the relative contribution by C/EBPβ increases following LPS treatment. Furthermore, they showed that expression of a reporter plasmid containing the C/EBPζ promoter could be transactivated in a dose-dependent manner by a C/EBPβ expression vector in transient transfections into HepG2 cells. In support of the notion that C/EBP transactivators may induce expression of C/EBPζ, we found that C/EBPβ expression is induced in response to LPS treatment of WEHI231 B cells and that induction of C/EBPζ is delayed relative to that of C/EBPβ (Fig. 5). These data are consistent with the notion that the synthesis of C/EBPβ is a prerequisite for C/EBPζ induction. However, P388-Cβ cells still require LPS treatment for

induction of C/EBPζ expression suggesting more complex requirements for expression.

Regardless of the mechanism by which the C/EBPζ is upregulated by LPS, we have found that C/EBPζ inhibits the ability of C/EBPβ to bind DNA. The C/EBP DNA-binding activity of nuclear extracts decreases after an initial increase following LPS treatment in WEHI231 B cells (Fig. 7) and throughout LPS treatment of P388-Cβ cells (Fig. 6), even though western analysis of the same nuclear extracts shows increasing C/EBPβ expression (Figs. 4 and 5). This decrease correlates well with the increase in C/EBPζ expression observed in the same nuclear extracts. Furthermore, forced expression of C/EBPζ dramatically decreased the DNA-binding activity of C/EBPβ-containing complexes (Fig 9). It is interesting to note that the C/EBPβ:γ heterodimers appear more resistant to inhibition of binding than the C/EBPβ:β homodimers (Fig 9). We recently reported a stimulatory activity for C/EBPγ on the IL-6 promoter (Gao et al in press). C/EBPβ in P388-Cβ cells and both C/EBPβ and C/EBPδ in WEHI231 cells are largely present as heterodimers with C/EBPγ (Gao et al in press). At least some component of the stimulatory of C/EBPγ may reflect its ability to protect C/EBPβ from association with C/EBPζ.

The fact that forced expression of C/EBPζ inhibits the LPS induction of both an IL-6 promoter-reporter as well as the endogenous IL-6 gene strongly supports the model that upregulation of C/EBPζ with lengthy LPS stimulation attenuates IL-6 induction by directly antagonizing C/EBP binding to the IL-6

promoter. While our current study has not provided direct evidence by co-immune precipitation for the formation of heterodimers between C/EBPζ and C/EBPβ or C/EBPδ, our findings are totally consistent with this. Multiple mechanisms may certainly contribute to downregulation of IL-6 expression following its LPS induction in B cells. We have found that NF-κB activation is transitory (Fig. 12) and this may also attenuate IL-6 induction. However, NF-κB activation declines well before the attenuation in IL-6 expression that we observe suggesting that this is not a sufficient explanation. C/EBPδ itself shows transitory expression in WEHI231 cells and may also contribute to the attenuation of IL-6 induction. However, C/EBPδ is not expressed in P388-Cβ cells, which show similar kinetics of IL-6 expression. Therefore, transitory C/EBPδ expression is also not sufficient to explain attenuation of IL-6 expression. Glucocorticoids can downregulate the expression of IL-6 and other proinflammatory cytokines by either occlusion of transactivator binding, competition for limiting co-activators, or interference with interactions beween transcription factors and the basal transcription machinery (Ray et al 1990; De Bosscher et al 2000; Karin M 1998; Adcock et al 2001). Estrogen has also been reported to inhibit IL-6 expression by direct interaction with NF-κB and C/EBPβ (Stein and Yang 1995) and/or preventing NF-κB binding to the iL-6 promoter (Galien and Garcia 1997). Neither glucocorticoids nor estrogen are likely operating in the system described here which lacks the opportunity for endocrine effects.

The induction of C/EBPζ as an attenuator of the LPS induced, C/EBP-dependent expression of IL-6 suggests the possibility that C/EBPζ expression

may serve as a generalized attenuator of the acute phase response, many genes of which are C/EBP-regulated (Poli 1998). A test of this hypothesis will await a closer examination of the role of C/EBPζ in the regulation of genes such as hemopexin, haptoglobin, α_1-acid glycoprotein, serum amyloid A1, A2, and A3, complement C3, and C-reactive protein, which figure prominently in the acute phase response of hepatocytes.

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SUMMARY AND FUTURE DIRECTIONS

In Chapter 2, we have 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.

To further explore the role of C/EBPγ in IL-6 expression by B cells, an immediate question to be addressed in the future is: what is the mechanism of C/EBPγ stimulatory activity? To answer this, two models are worthy to be tested: 1) enhanced synergy with NF-κB. 2) enhanced recruitment of transcriptional coactivators.

Our studies show both the IL-6 and IL-8 promoters to be stimulated by C/EBPγ, but a simple promoter consisting of four tandemly arrayed C/EBP binding sites [(DE-I)4] was not stimulated by C/EBPγ. The TNF-α promoter

which does not display synergy between NF-κB and C/EBPβ was not stimulated by C/EBPγ either. Furthermore, in the absence of LPS treatment, C/EBPγ actually inhibited the limited activation of the IL-6 promoter that can be observed by transfection with C/EBPβ alone. Cotransfection with NF-κB p65 was sufficient to reverse the inhibition and allow C/EBPγ stimulatory activity to be observed in the absence of LPS stimulation. These data suggest that C/EBPγ plays a key role in the synergy between C/EBPβ and NF-κB. To test this, an IL-6 NF-κB consensus site will be inserted into the (DE-I)4 promoter reporter. Whether this promoter-reporter behaves similarly to the wild-type IL-6 promoter-reporter in transient transfections or not will be studied. Another approach is to test if there is a direct physical interaction between C/EBPγ and NF-κB by co-immunoprecipitation and GST pull-down.

While no co-activators have been reported in association with C/EBPγ, five coactivators have been reported in association with C/EBPβ: TIF1β, Rb, Nopp140, p300 and SWI/SNF chromatin-remodeling complex. Cotransfections will be performed to examine whether overexpression of any these coactivators augments LPS stimulation of the IL-6 promoter and whether they are more effective when coexpressed with additional C/EBPγ.

Another critical question to be addressed is: What is the physiological consequence of C/EBPγ-deficiency for IL-6 expression. For this point, LPS-induced IL-6 expression in B cells derived from C/EBPγ-deficient animals will be examined. These animals have normal levels of B lineage cells, although the function of C/EBPγ-deficient B cells has not been assessed (Kaisho et al 1999).

Primary splenic B cells, pre-B cells derived from long-term B cell culture of fetal liver cells, as well as immortalized B cell lines obtained from C/EBPγ-deficient and wild-type animals will be evaluated for their expression of C/EBP family members and their response to LPS stimulation. Transduction of ectopic C/EBPγ expression into C/EBPγ-deficient cell lines will allow a clearer assessment of the role of C/EBPγ expression without the complication of endogenous C/EBPγ that we have in P388 lymphoblasts.

In this report, we have also shown that C/EBPβ leucine-zipper is sufficient to mediate LPS induction of the IL-6 expression in vivo (Chapter 2, Fig 25). Furthermore, previous work in our lab showed that C/EBPβ:G_{LZ} has no activity in stable transfectant (Hu et al 2000). Taken together, these results suggest that the C/EBPβ:γ heterodimeric zipper in the absence of any conventional activation domains is the key determinant to support LPS induction of the IL-6 promoter. To further explore the intrinsic activity of the C/EBPβ and C/EBPγ heterodimer in our system, it is worthy to investigate the proteins with which the heterodimers interact. To that end, the proteomic approach can be utilized by using tagged C/EBPγβ_{LZ} expression vector in P388 B cells.

In Chapter 3, we have found that expression of C/EBPζ is induced in response to LPS treatment of B cells at both the mRNA and protein levels. Correlating with the highest levels of C/EBPζ expression at 48 hours after LPS treatment, both the abundance of C/EBP DNA binding species and IL-6 expression are downregulated. Furthermore, ectopic expression of C/EBPζ inhibited C/EBPβ-dependent IL-6 expression from both the endogenous IL-6

gene and an IL-6 promoter-reporter. These results suggest that C/EBPζ functions as negative regulator of IL-6 expression in B cells and that it contributes to the transitory expression of IL-6 that is observed after LPS treatment.

An immediate experiment in the future that will strengthen our results is to examine the formation of heterodimers between C/EBPζ and C/EBPβ or C/EBPδ by co-immune precipitation at different time after LPS treatment. An alternative approach is to test if C/EBP DNA-binding activity of nuclear extract at 24 hours of LPS treatment can be inhibited by mixing with a nuclear extract at 48 hours of LPS treatment.