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Hsien-Ming Hu

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Richard L. Schwartz
Major professor

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STUDIES OF C/EBP TRANSCRIPTION FACTORS IN MYELOMONOCYTIC CELL LINEAGES

By

Hsien-Ming Hu

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ABSTRACT

STUDIES OF C/EBP TRANSCRIPTION FACTORS IN MYELOMONOCYTIC CELL LINEAGES

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Hsien-Ming Hu

C/EBP-related proteins comprise a family of basic-region leucine zipper (bZIP) transcription factors. These proteins dimerize through a leucine zipper and bind to DNA through an adjacent basic region. Previous *in vitro* studies have implicated C/EBPβ in the regulation of many inflammation-associated genes, including proinflammatory cytokines. Recently, it was found that LPS stimulation of peritoneal macrophages from C/EBPβ-deficient mice led to a normal induction of a number of proinflammatory cytokines. Thus it is hypothesized that other C/EBP family members can support the expression of IL-6 and other proinflammatory cytokines.

In the first part of this study, experiments have been conducted to show that C/EBPα, C/EBPβ, and C/EBPδ are expressed in bone marrow-derived macrophages, and that all of them are available to support LPS-induced cytokine expression. When ectopically expressed in P388 B lymphoblasts, which normally lack the ability to express cytokines upon LPS stimulation, each of these C/EBP isoforms is capable of conferring LPS-inducible expression of IL-6 and MCP-1. These results demonstrate the redundancy of C/EBPα, C/EBPβ and C/EBPδ in supporting the LPS induction of IL-6 and MCP-1. In the second part of this study,

we have sought to identify the structural basis for this apparent redundancy. Surprisingly, we have found that P388 stably expressing truncated forms of C/EBPβ, that lack all regulatory domains and retain only the bZIP regions, are capable of inducing IL-6 and MCP-1 transcription in response to LPS. In contrast, transfectants expressing a C/EBP chimera, in which the leucine zipper of C/EBPβ is replaced with that of yeast transcription factor GCN4, have a reduced ability to induce IL-6 and MCP-1. Furthermore, a truncated form of C/EBPδ and, to a lesser extent, a truncated form of C/EBPα have both been shown to support LPS activation of the IL-6 promoter in transient transfection assays. Together, these results have implicated the leucine zipper domain in some function other than dimerization to known C/EBP isoforms, and have suggested that C/EBP redundancy in regulating cytokine expression may result from their highly related bZIP domains.

In chapter 4, the capabilities of C/EBP isoforms to induce myeloid-specific genes are investigated. Individual C/EBPs are ectopically-expressed in a pre-granulocytic cell line, 32Dcl3. The results demonstrate that a C/EBPα-C/EBPβ heterodimer is the most likely effector among other forms of C/EBP, in inducing the transcription of several primary granule product-encoding genes including myeloperoxidase, cathepsin G, and lysozyme.

Dedicated to my beloved family,

my wife, Yen-Hsueh Su

my lovely daughter, Alyssa

for their love and support

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I would like to express the deepest appreciation to my mentor, Dr. Richard Schwartz, for his guidance and encouragement. I am grateful to our collaborator Dr. Peter Johnson and his lab in National Cancer Institute for supplying reagents and for technical advice. I would also like to thank members of my guidance committee, Drs. Jerry Dodgson, Donald Jump, Water Esselman and Donna Koslowsky for their precious time and suggestions. And for the friendship and helpful discussions from my colleague Qiang Tian.

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LIST OF ABBREVIATIONS

C/EBP	CCAAT/enhancer binding protein
LTR	long terminal repeat
IL	interleukin
G-CSF	granulocyte-colony stimulating factor
M-CSF	macrophage-colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor
MCP-1	monocyte chemoattractant protein-1
LPS	lipopolysaccharide
LIP	liver inhibitory protein
bZIP	basic region-leucine zipper
FACS	fluorescence-activated cell sorting
TNF-α	tumor necrosis factor-α
IFN-γ	interferon-γ
iNOS	inducible nitric oxide synthase
EMSA	electrophoretic mobility shift assays

Chapter 1

Literature Review

1. C/EBP-related transcription factors:

C/EBP-related proteins comprise a family of basic-region leucine zipper (bZIP) transcription factors (Johnson et al. 1994). Members of the C/EBP family are highly homologous in their C-terminal dimerization and DNA binding domains, but are more divergent in the N-terminal transactivation domain. Homo- and heterodimers can be formed between any pair of the family members and bind to a similar DNA sequence (see Figure 1). Dimerization is a prerequisite for DNA-binding activity. The domain responsible for dimerization is a leucine zipper which is an alpha helix with a leucine residue every seven amino acids (Landschulz et al. 1988). The paired α-helices of the dimer associate in a parallel orientation through their hydrophobic surfaces to create a coiled-coil structure (O'shea et al. 1989). Not only can dimerization occur within the family, but heterodimerization with other activator protein families has also been demonstrated (Hsu et al. 1994). In addition, protein-protein interactions other than heterodimerization have been shown to occur between C/EBPs and many transcription factors. This phenomenon is postulated to be the basis of the synergistic effect between C/EBPs and these factors in many C/EBP-regulated promoters. A detailed review of this aspect of C/EBP function will be given in a later section. Located immediately to the N-terminal side of the zipper

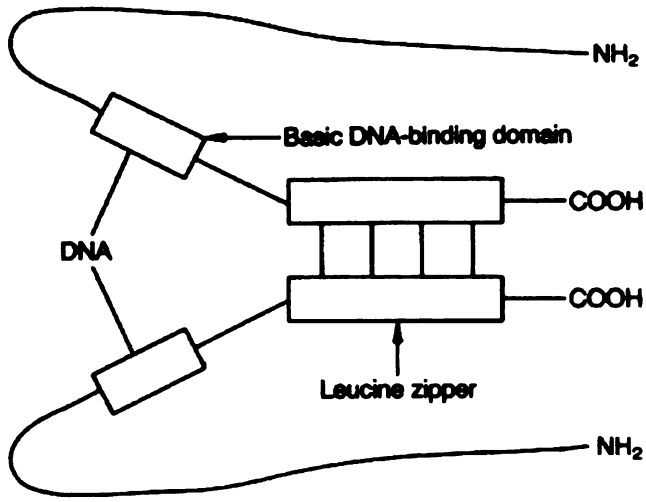

Figure 1. Structure of the C/EBP transcription factor.

region is a positively charged domain known as the basic region that functions as the DNA contact surface (Vinson et al. 1992). Dimerization juxtaposes the two basic regions which make specific contacts with DNA along the major grooves of the DNA duplex. The most conserved region between the family members is the basic-region. In fact, many residues which are critical in making contact with DNA are identical. Thus, it is not surprising to find that the DNA binding specificities of C/EBPs are very similar (Williams et al. 1991). The deduced consensus binding site for C/EBPs is : 5'-T(T/G)NNGNAA(T/G)-3' (Johnson et al. 1993). The C/EBP family is capable of activating *in vivo* transcription from promoters that contain such a consensus sequence. This sequence is found in the promoters of a number of genes that fall into five categories: inflammation-associated genes (including cytokines), liver-specific genes, adipocyte-specific genes, myeloid-specific genes and immunoglobulin genes (Akira et al. 1990, Brooks et al. 1992, Christy et al. 1989, Zhang et al. 1994).

The regulatory domain of C/EBPs is present at the N-terminal end of the proteins. Although this domain is not as conserved as the bZIP domain among family members, several clusters of sequence similarity have been identified in this region (Johnson et al. 1994). Mutations in these clusters abolish transactivation ability without affecting DNA-binding and dimerization (Friedman et al. 1990). In addition to these activation domains, a negatively-acting element has also been identified in the regulatory domain of C/EBPα, C/EBPβ and C/EBPε (Pei et al. 1991, Williams et al. 1995, Williamson et al. 1998). It is suggested that this element may function as an attenuator to keep the activation

domains in check. This block could be removed by extracellular signals which modify the attenuator by a post-translational mechanism, probably via phosphorylation.

Since the discovery of C/EBPα in 1988, five C/EBP-related proteins with physical and functional homology to C/EBPα have been identified. These C/EBPs are differentially expressed in many cell types. The variety of C/EBP family members and their potential for heterodimer formation could provide a large repertoire of transcription factors with complex *in vivo* regulatory features. In the following sections, I will review the identification, tissue distribution and regulatory functions of each member of the C/EBP family. The nomenclature used in this thesis for each C/EBP member is adopted from the one proposed by McKnight and colleagues (Cao et al. 1991). According to the order of discovery, they are designated as C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ and C/EBPζ.

1.1 C/EBPα

C/EBPα, the founding member of the family, was first identified in crude nuclear extract from adult rat liver (Johnson et al. 1987). It was discovered in a search for DNA-binding factors that recognize viral gene regulatory sequences that function ubiquitously, such as the CCAAT box and "enhancer core" elements of retroviral LTRs. Subsequently, it was found that these two elements have very weak homology to the C/EBP consensus, and that C/EBPα does not bind to these elements *in vivo* (Vinson et al. 1992). The fact that it's expression level is

high in liver cells, and that many liver-specific genes contain the C/EBP binding site in their cis-regulatory sequences, led to the realization that C/EBPα is an important transcription factor regulating liver-specific genes (Birkenmeier et al. 1989, Williams et al. 1991). C/EBPα is also very abundant in fat cells where it was found to be important for terminal differentiation (Christy et al. 1989, Kaestner et al. 1990). Several genes whose transcription is specific to differentiated adipocytes are regulated by C/EBPα. When the preadipocytic cell line 3T3-L1 is induced to differentiate by growth to confluency and exposure to hormones, the expression of C/EBPα is increased markedly (Birkenmeier et al. 1989, Christy et al. 1989). This finding implies that C/EBPα is an important regulator in the process of adipocyte differentiation. A proof that the induction of C/EBPα is essential for adipocyte differentiation comes from a study in which the expression of C/EBPα is suppressed by overexpression of antisense RNA for C/EBPα (Lin et al. 1992). 3T3-L1 cells expressing C/EBPα antisense RNA fail to undergo morphological differentiation and fat-specific genes are not induced.

C/EBPα is also shown to be expressed in early myeloid cells in the hematopoietic system. In addition, many myeloid-specific genes, such as granulocyte colony-stimulating factor (G-CSF) receptor (Smith et al. 1996), neutrophil elastase (Oelgeschlager et al. 1996) and myeloperoxidase (Ford et al. 1996), contain C/EBP binding sites in their promoters. Recent studies have shown that C/EBPα plays a critical role in granulocytic differentiation. A gene disruption experiment has revealed a lack of mature granulocytes in the blood of

C/EBPα knockout mice, while other blood cell types are not affected (Zhang et al. 1997).

1.2 C/EBPβ

C/EBPβ has also been reported as NF-IL6 (Akira et al. 1990), AGP/EBP (Chang et al. 1990), LAP (Descombes et al. 1990a), IL-6DBP (Poli et al. 1990) and CRP2 (Williams et al. 1991). It was discovered from a human cDNA library by its ability to bind the IL-1 responsive element in the IL-6 gene promoter (Isshiki et al. 1990). Other C/EBPβ homologs were later isolated from a variety of species, including mouse (Chang et al. 1990), rat (Descombes et al. 1990) and chicken (Katz et al. 1993). The rat C/EBPβ was cloned from a liver cDNA expression library screened with oligonucleotides containing IL-6 responsive *cis*-regulating elements from liver-specific acute phase response genes (Poli et al. 1990). Tissue distribution studies show that C/EBPβ is present at high level in the liver (Alam et al. 1992, Birkenmeier et al. 1989) and in the myelomonocytic lineages (including monocyte/macrophages and granulocytes) of the hematopoietic system (Scott et al. 1992, Katz et al. 1993). These results are consistent with a primary role for C/EBPβ in the regulation of acute phase proteins and inflammation-associated genes. C/EBPβ is also expressed in many other tissues, although at a lower level. The list includes ovarian granulosa cells (Sirois et al. 1993), differentiating adipocytes (Cao et al. 1991), pituitary cells (Wegner et al. 1992) and mammary epithelial cells (Robinson et al. 1998).

C/EBPβ has been closely linked to IL-6 expression and signaling. Its *trans*-activating potential is enhanced by IL-6 in transfected hepatoma cells, where it acts as an inducer of acute phase response genes (Akira et al. 1990). The induction of acute phase protein genes by IL-6 probably involves the activation of C/EBPβ, which binds to C/EBP recognition sites in the promoters of these genes. Although the primary mechanism of C/EBPβ regulation within the acute pnase response appears to be post-transcriptional (Ramji et al. 1993), C/EBPβ mRNA levels are also induced by IL-6 (Akira et al. 1990). C/EBPβ is also an important component in the regulation of genes specifically induced in activated macrophages by proinflammatory stimulants such as LPS, IL-6 and IL-1 (Natsuka et al. 1992). The regulation of inflammation-associated genes in activated macrophages will be discussed in detail in later sections.

A naturally existing truncated form of C/EBPβ, known as LIP, was reported by Descombes and Schibler (1990b). LIP lacks the first 131 amino acid residues in the N-terminal region of C/EBPβ, but retains the bZIP domain. LIP cannot activate transcription from C/EBP-dependent promoters because it lacks the transactivation domain. When cotransfected with C/EBPβ, LIP inhibits C/EBPβ-mediated transactivation of a target promoter, presumably, by competition for the DNA-binding site. Additionally, the LIP-C/EBPβ heterodimer is inactive or less active in comparison to the C/EBPβ homodimer. It was proposed that LIP is produced by a leaky ribosome scanning mechanism that occurs because initiation at the first AUG codon is inefficient. On the other hand LIP may be an

artifact of proteolysis in some systems (Baer et al. 1998). The biological significance and mechanisms regulating this C/EBPβ isoform remain elusive.

In this thesis, we have discovered that LIP is capable of activating IL-6 and MCP-1 transcription under conditions of LPS stimulation. This unexpected observation has led to the discovery that the leucine zipper region of C/EBPs may have a role other than simply serving as a dimerization domain. These studies will be described in detail in the third chapter.

1.3 C/EBPδ

The gene for C/EBPδ, alternatively known as NF-IL6β (Kinoshita et al. 1993) and CRP3 (Williams et al. 1991), was obtained by cross-hybridization using the C/EBP bZIP domain. Present at a very low level in many tissues, C/EBPδ mRNA can be increased dramatically by proinflammatory stimulants such as LPS and IL-6, suggesting a role in the regulation of the acute phase response and inflammation. Unlike C/EBPβ, the activation of C/EBPδ during the acute phase response occurs predominantly via increased transcription of the gene, rather than by post-translational modification of preexisting protein molecules (Ramji et al. 1993). In addition to the liver, C/EBPδ is expressed transiently in differentiating adipocytes (Cao et al. 1991), and is also present in the myelomonocytic lineages of the hematopoietic system (Scott et al. 1992).

1.4 C/EBPε

The gene for C/EBPε (originally named CRP1) was first cloned from a rat genomic DNA library by hybridization to C/EBPα. Because neither mRNA nor protein could initially be detected from a variety of tissues, the function and regulation of C/EBPε remained uncertain until recently. The gene for human (Chumakov et al. 1997) and murine (Antonson et al. 1996) C/EBPε were cloned and found to be expressed exclusively in cells of hematopoietic origin. It is proposed that C/EBPε may play a role in regulating myeloid differentiation because C/EBPε is found to be expressed only in early myeloid cells. Additionally, C/EBPε can transactivate reporter constructs containing myeloid-specific *c-mim* or human myeloperoxidase promoters (Chumakov et al. 1997). Most convincing in regard to its role in myeloid differentiation is the fact that C/EBPε-deficient mice fail to develop functional mature granulocytes (Yamanaka et al. 1997b). Consistent with this result, C/EBPε is up-regulated during granulocytic differentiation (Yamanaka et al. 1997a).

1.5 C/EBPγ

C/EBPγ (also known as Ig/EBP) was originally identified by its ability to bind to the Ig heavy chain promoter and Ig heavy chain enhancer (Roman et al. 1990). C/EBPγ is the only family member to be ubiquitously expressed. C/EBPγ can form heterodimers with other C/EBP family members and exhibits similar DNA-binding properties. However, because C/EBPγ lacks the N-terminal transactivation domain found in other C/EBP activators, it alone neither activates

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nor represses transcription. Instead, C/EBPγ is found to act as a dominant negative inhibitor of the C/EBP activators. Thus, the structure and the function of C/EBPγ is very similar to that of LIP. The ubiquitous expression of C/EBPγ appears to suggest that C/EBPγ simply acts as a buffer for C/EBP activators in many tissues, insuring that C/EBP activator activity is suppressed until its buffer capacity is exceeded by increasing C/EBP activator concentration.

1.6 C/EBPζ

C/EBPζ (also known as CHOP) was originally cloned based on its induction by DNA damaging agents and concomitant growth arrest (Fornace et al. 1989). Subsequently, it was found to exhibit homology to the other C/EBP proteins in the leucine zipper region. Considered a distantly related C/EBP protein, C/EBPζ not only lacks the activation domain found in other C/EBP activators, but is also devoid of the DNA binding domain. As a consequence, C/EBPζ by itself cannot form a homodimer and bind to the C/EBP consensus. But it can form heterodimers with other C/EBP family members and prevent them from binding to DNA, thus acting as a trans-dominant negative regulator of the C/EBP family (Ron et al. 1992). Although it is implicated in mediating the cell's response to environmental stress, the physiological function of C/EBPζ remains obscure.

In summary, C/EBP family members are versatile transcription factors expressed in a variety of tissues. They have been shown to regulate target genes that are critical for cell type-specific functions. In addition, accumulating evidence has also suggested their involvement in regulating growth/differentiation of many

different tissues. This thesis will focus on elucidating the role of various C/EBP proteins in the myelomonocytic lineages of the hematopoietic system. Thus, in the next two sections I will review the current literature on hematopoiesis and inflammation, the two processes in which C/EBPs have been implicated to play an important role.

2 Hematopoiesis:

2.1 Overview

Hematopoiesis is the process in which different types of blood cells arise from a common pluripotent stem cell. During this process, stem cells either renew themselves or differentiate along a number of pathways and generate mature blood cells with specialized functions. Two major groups of blood cells generated in the process are lymphoid cells, which include B and T lymphocytes, and myeloid cells, which include erythrocytes, megakaryocytes, mast cells, granulocytes, and macrophages. The structure of the hematopoietic system is shown in Figure 2. The stem cells are relatively few in number but can persist throughout life by undergoing proliferation to produce daughter stem cells. Early in hematopoiesis, a stem cell differentiates to either a lymphoid stem cell or a myeloid stem cell. Subsequent differentiation of lymphoid and myeloid stem cells generates committed progenitor cells for each cell type. Progenitor cells are restricted in their ability to produce a single type of mature blood cell. During terminal differentiation, changes in cell morphology and cell surface markers

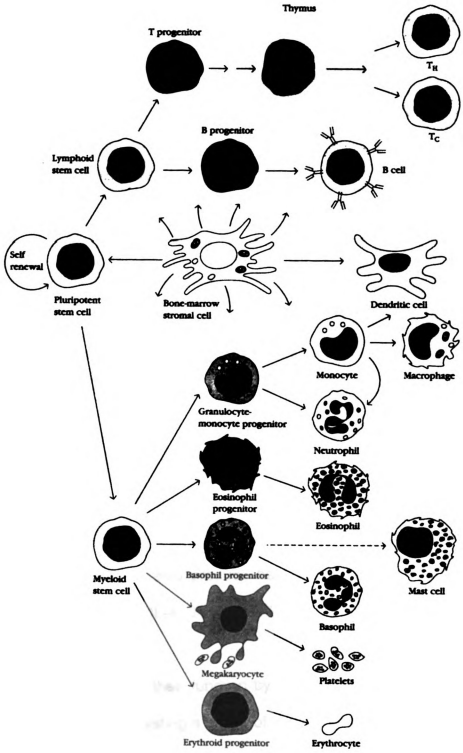

Figure 2. Origin of the hematopoietic cell lineages.

become evident and maturing cells also start to express genes critical to the functions of a specific lineage. These changes have allowed the development of such techniques as fluorescence-activated cell sorting (FACS) and histochemical staining to monitor and dissect the process of cell differentiation.

2.2 Growth factors

Bone marrow is the major hematopoietic organ in adults. In bone marrow, non-hematopoietic cells, known as stromal cells, support the growth and differentiation of the hematopoietic cells by providing a hematopoietic-inducing microenvironment consisting of a cellular matrix and either membrane-bound or secreted growth factors. Among the various growth factors, four colony stimulating factors (CSF), named for their ability to stimulate the formation of hematopoietic cell colonies in bone marrow culture, are the major regulators to promote survival, proliferation, differentiation, and maturation of myeloid cells. The list includes Multi-CSF (IL-3), GM-CSF, G-CSF, and M-CSF. In addition to bone-marrow stromal cells, other leukocytes such as activated T helper cells and activated macrophages, also produce hematopoietic growth factors known as cytokines. For example, IL-4, IL-5, IL-6, IL-7, IL-8, and IL-9 are also involved in hematopoiesis.

Growth factors exert their function by binding to their cognate receptors on the cell surface and activating a series of downstream signal transducing events. Eventually, the signals result in the activation or repression of transcription of a

selected group of genes necessary for lineage commitment and hematopoietic differentiation.

2.3 Myelomonocytic differentiation

Monocyte/macrophages and granulocytic neutrophils are two closely related cell lineages in the hematopoietic system. They share the same early developmental pathway from a pluripotent stem cell to a bipotential granulocyte-monocyte progenitor cell. Depending on the type and concentration of growth factors present in the microenvironment, the bipotential precursor cells can be induced to differentiate into either macrophages or neutrophils. M-CSF and GM-CSF are the major growth factors that act on the monocyte precursor, while G-CSF and GM-CSF are the inducers for the granulocyte progenitor. During granulocytic differentiation, there are easily identifiable morphological changes in the nucleus and cytoplasm, which can be assessed by Wright-Giemsa staining of cytospin slides. The morphology of a cell changes from a round and compact nucleus with relatively small cytoplasm to nucleus ratio (myeloblasts), to a bent or horseshoe-shaped nucleus (promyelocytes), and then to a segmented polymorphic nucleus with large cytoplasm to nucleus ratio (mature granulocytes). Other functional assays used to characterize granulocyte differentiation include histochemical staining for leukocyte alkaline phosphatase (LAP) and nitroblue tetrazolium (NBT). In addition, Northern blot analyses for the mRNA of primary granule myeloperoxidase is often used as an indication of the onset of

granulopoiesis, while the appearance of lactoferrin mRNA indicates a mature stage of the process.

The maturation of monocyte/macrophages can also be divided into several stages based on morphological and functional characteristics. In the bone marrow, early progenitor cells called monoblasts (compact and round nucleus with relatively small cytoplasm to nucleus ratio) divide and differentiate into promonocytes (relatively larger cytoplasm to nucleus ratio), which eventually become monocytes (round cytoplasm with irregular-shaped nucleus). Mature monocytes then enter and remain in the circulation for several hours before extravasating to various tissues to become differentiated macrophages (large irregular-shaped cytoplasm containing many vacuoles). Functional assays for lysozyme and naphthylacetate esterase, along with Northern analysis for the mRNA of *c-fms* (a proto-oncogene coding for M-CSF receptor), are frequently used for the evaluation of monocytic differentiation.

Due to difficulties in the isolation and purification of a large number of hematopoietic progenitor cells from bone marrow cells, the study of myeloid differentiation has mainly relied on the use of established cell lines which can be induced to differentiate by growth factors or chemical agents. For example, human cell lines HL60 (Collins et al. 1987) and U937 (Oberg et al. 1993), isolated from patients with acute myeloid leukemia, appear to be frozen at an immature stage of myeloid differentiation. They can be induced to differentiate either along the monocytic pathway with TPA, or along the granulocytic pathway with retinoic acid or DMSO. 32Dcl3 is another murine cell line commonly used to

study granulocytic differentiation (Valtieri et al. 1987). Isolated from mice infected by Friend virus, 32Dcl3 cells are immature pre-granulocytic cells. They require IL-3 for continuous proliferation without undergoing differentiation. Upon the replacement of IL-3 with G-CSF, a majority of the cell population can be induced to differentiate into mature granulocytes over a period of 10-14 days. Such inducible models have facilitated the study of mechanisms of myelomonocytic differentiation.

2.4 C/EBP proteins and myelomonocytic differentiation

Transcription factors play a major role in differentiation in a number of cell types, including the various hematopoietic lineages. To understand the process of myeloid differentiation, it is important to identify and characterize the transcription factors that activate target genes in the myeloid lineages. Several lines of evidence have suggested that members of the C/EBP family may play an important role in the regulation of myelomonocytic differentiation. First, in the hematopoietic system, C/EBPα, -β and –δ are highly expressed in myeloid, but not in erythroid and lymphoid cells (Scott et al. 1992). Recently, the human C/EBPε gene was cloned and found to be expressed exclusively in immature myeloid cells (Chumakov et al. 1997). Second, a unique temporal expression pattern of C/EBP isoforms has been observed in differentiating myelomonocytic cells (Scott et al. 1992). When murine 32D cells were induced to differentiate along the granulocytic pathway, it was found that C/EBPα was highly expressed in the early stage and downregulated with maturation, whereas C/EBPβ and

C/EBPδ were upregulated. Meanwhile, C/EBPε mRNA was found to be greatly induced during *in vivo* granulocytic differentiation of human primary CD34+ cells (Yamanaka et al. 1997a). Third, binding sites for C/EBPs have been shown to be critical for the activity of a number of myeloid-specific promoters, including myeloperoxidase (Ford et al. 1996), neutrophil elastase (Oelgeschlager et al. 1996), M-CSF receptor (Zhang et al. 1994), GM-CSF receptor (Hohaus et al. 1995) and G-CSF receptor (Smith et al. 1996). Although C/EBPα has been proposed to be the major regulator for these promoters, in many cases other C/EBP family members (C/EBPβ, -δ and –ε) have also been shown to be active.

Gene disruption experiments have provided more insights into the functions of C/EBP proteins in myeloid differentiation. In C/EBPα knock-out mice, analysis of the hematopoietic system has demonstrated a specific defect in production of granulocytic cells (Zhang et al. 1997) . C/EBPα null mice (-/-) do not produce any mature neutrophils, while other cell lineages including monocyte/macrophages, red blood cells and lymphoid cells are not affected. FACS analysis in embryonic and newborn animals confirmed that myeloid markers (Mac-1 and Gr-1) are greatly reduced, with normal B- and T-cell subsets. Expression of G-CSF receptor mRNA was also profoundly and selectively reduced. These data strongly suggest that C/EBPα has a critical role in granulocyte differentiation. This conclusion was further confirmed in a study in which C/EBPα was conditionally expressed in bipotential progenitor cells, HL-60 and U937 (Radomska et al. 1998). It was shown that conditional expression of C/EBPα in these cell lines was sufficient to induce granulocytic differentiation but not

monocytic differentiation. Moreover, induced expression of C/EBPα in bipotential precursors blocked their monocytic differentiation program. These results indicate that C/EBPα serves as a myeloid differentiation switch acting on bipotential precursors and directing them to mature to granulocytes.

Another C/EBP family member found to be involved in regulating myeloid differentiation is C/EBPε. C/EBPε-deficient mice have an increased number of granulocytic precursors in the blood and bone marrow. However, the number of Gr-1-positive mature granulocytes is markedly decreased (Yamanaka et al. 1997b). Thus, C/EBPε-deficient mice have a similar phenotype to their C/EBPα-deficient counterparts. During myeloid differentiation, C/EBPε is expressed slightly after C/EBPα, suggesting a sequential manner of regulation between these two activators. Indeed, ectopic expression of C/EBPα in myeloid progenitors induces the expression of C/EBPε mRNA and granulocytic differentiation (Radomska et al. 1998).

In contrast to C/EBPα and –ε, targeted disruption of C/EBPβ or –δ does not seem to cause adverse effects on myelopoiesis, consistent with a role of C/EBPβ and –δ in the inflammatory response rather than lineage commitment.

3. Inflammation

3.1 Introduction

Inflammation, which is initiated by tissue injury (usually caused by trauma or infection), comprises a series of adaptive responses which ultimately facilitate the clearance of infectious agents and the removal of injured tissues. Thus,

inflammatory responses serve as an important mechanism for the host to defend against the invasion of pathogenic microorganisms. The responses involved can be both localized and systemic. Three major events accompanying a local inflammatory response are (1) vasodilation, (2) increased capillary permeability, and (3) influx of phagocytic cells. On the other hand, a systemic response known as the acute phase response, includes the induction of fever, increased synthesis of hormones such as ACTH and hydrocortisone, increased production of a large number of hepatocyte-derived acute phase proteins, and increased production of white blood cells. The systemic response involves many different organs throughout the body. The key mediators that orchestrate these activities and link all these different cell types and organs together in a inflammatory response are cytokines. These cytokines are also termed proinflammatory cytokines due to their ability to promote an inflammatory response. Among the important proinflammatory cytokines are IL-1, TNF-α, IFN-γ, IL-6, and a group of polypeptides collectively known as chemokines.

3.2 Macrophage activation and acute phase response

The acute inflammatory response is initiated following activation of tissue macrophages. Macrophages become activated when stimulated by bacterial products such as lipopolysaccharides (LPS), or by cytokines produced by activated T cells such as IFN-γ and IL-1. Activated macrophages have increased phagocytic activity, increased microbicidal activity, and most importantly, increased secretion of proinflammatory cytokines. A list of these cytokines is

Table 1. Proinflammatory cytokines produced by activated macrophages.

Interleukin-1α (IL-1α)
Interleukin-1β (IL-1β)
Interleukin-6 (IL-6)
Interleukin-8 (IL-8)
Interleukin-11 (IL-11)
Interleukin-12 (IL-12)
Interferon-α (IFN-α)
Tumor necrosis factor-α (TNF-α)
Monocyte chemoattractant protein-1 (MCP-1)
Macrophage inflammatory protein-1α (MIP-1α)

given in Table 1. Proinflammatory cytokines produced by activated macrophages serve as the mediators of the inflammatory response by acting on many different tissues and organs. In a local inflammatory response, both IL-1 and TNF-α induce increased expression of cell-adhesion molecules (CAMs) on endothelial cells, resulting in increased adhesion of circulating white blood cells to vascular endothelial cells and their extravasation into the site of inflammation. IL-8 is noted by its ability to act as a potent chemotactic factor to attract neutrophils to vascular endothelial cells. IFN-α has also been shown to chemotactically attract macrophages to a site where antigen is localized. Several proinflammatory cytokines are also responsible for many systemic responses that occur in an acute inflammatory response. For example, IL-1, TNF-α and IL-6 can each act on the hypothalamus to induce a fever. And IL-6 along with IL-1 and TNF-α can induce production of acute phase proteins by the liver.

Acute phase proteins (APPs) are a family of approximately 30 plasma proteins produced in increased amounts by the liver in inflammation. Their concentrations in the serum can be raised by varying magnitudes, ranging from several to 1000-fold. The APPs serve multiple functions in a immune response. They have a role as immune mediators and inhibitors, and as scavengers of cell-derived products released from damaged tissues and macrophages. In addition, they are also involved in the healing process of the injured tissues. Studies of APP production by IL-6 stimulation in hepatocytes have revealed the important role of C/EBP transcription factors. It is well-established that the IL-6 signaling pathway greatly enhances the transactivation potential of C/EBPβ mainly by a

post-translational mechanism (Ramji et al. 1993). In the meantime, the expression of C/EBPδ mRNA is increased dramatically in hepatocytes stimulated by IL-6 (Akira et al. 1990). It has been shown that the promoters of APPs contain binding sites for C/EBP proteins, and that C/EBPs can transactivate the promoters from these genes (Ramji et al. 1993). Thus, it is believed that both C/EBPβ and C/EBPδ are the major regulators of the acute phase proteins produced by the liver in response to IL-6, and other cytokines as well.

3.3 Proinflammatory cytokines

Proinflammatory cytokines produced by activated macrophages, endothelial cells and fibroblasts are responsible for both local and systemic responses that occur during an acute immune response. Here I will summarize the general properties of several important proinflammatory cytokines.

3.3.1 Interleukin-6 (IL-6)

IL-6 is a polypeptide mediator with important roles in a wide variety of systems including regulation of the immune response, the acute phase response and hematopoiesis. Major cellular sources for IL-6 include monocyte/macrophages, endothelial cells and fibroblasts (Akira et al. 1992). They can be induced to produce IL-6 in response to a variety of stimuli such as LPS, virus infection, IL-1 and TNF-α. Detailed studies of the promoter region of the IL-6 gene have revealed several important *cis*-acting regulatory elements (Dendorfer et al. 1994). As shown in Figure 3, a c-fos/serum response element (SRE) contains two overlapping transcription control elements, NF-IL6 (C/EBPβ)

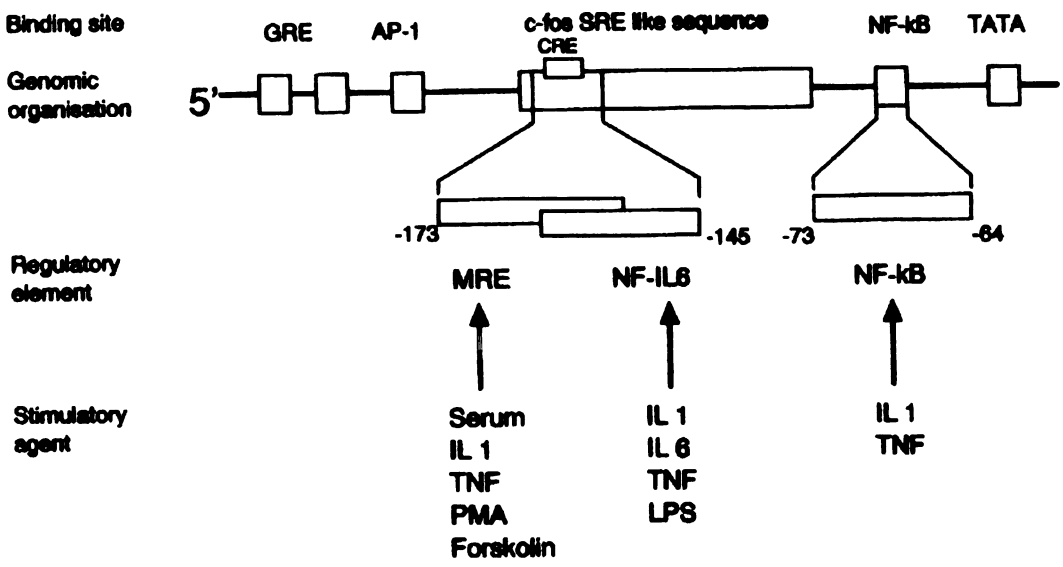

Figure 3. Cis-acting elements of the IL-6 promoter.

And the multiple response element (MRE). One NF-κB binding site is located on the 3' side of C/EBPβ binding element. Together, these elements account for the inducibility of IL-6 production by multiple stimuli. Other regulatory sequences located further upstream are two glucocorticoid response elements and one AP-1 binding site.

The major functions of IL-6 that are relevant to an acute immune response include acting as a main regulator for acute phase protein synthesis, and as a mediator of fever.

3.3.2 Interleukin-1 (IL-1)

IL-1 activity is actually composed of two polypeptide mediators of similar molecular size (17 kD) (Scales 1992). The IL-1 receptor binds both IL-1α and IL-β with a similar affinity. Many cell types have been shown to produce IL-1, the most studied being peripheral monocyte/macrophages. Other cell types capable of producing IL-1 include T and B lymphocytes, smooth muscle, endothelial and various brain cells. A variety of stimuli can induce IL-1 synthesis such as microbial products and cytokines.

Major cis-regulatory elements identified in the IL-1β promoter region are the binding sites for C/EBP, NF-κB, and AP-1, as well as the serum response element (SRE) (Zhang et al. 1993). Like IL-6, IL-1 can act on the hypothalamus to induce fever, and induce synthesis of acute phase proteins in hepatocytes. It also has a function in attracting phagocytic cells to the site of inflammation by inducing the expression of adhesion molecules in vascular endothelial cells.

3.3.3 Tumor necrosis factor-α (TNF-α)

TNF-α was originally identified as an endotoxin-induced serum factor that causes necrosis of tumors *in vivo* and tumor cell cytotoxicity *in vitro*. It was later shown to be a pleiotropic mediator involved in many immune responses. Its diverse actions in inflammation, in addition to cytotoxicity, include chemotactic activity for influx of phagocytic cells to inflammation sites, activation of endothelial cells and fibroblasts to produce various cytokines, and induction of fever (Tsuji et al. 1992).

The major source for TNF-α is macrophages. Like other proinflammatory cytokines, TNF-α can be induced by microbial products and cytokines. A number of transcription factors contribute to the regulation of the TNF-α gene. C/EBPβ has been shown to be important in TNF-α gene activation in myelomonocytic cells (Pope et al. 1994). A C/EBP binding site is located between 74 and 100 bp upstream of the transcription start site. The TNF-α promoter also contains several potential binding sites for additional transcription factors that may work in concert with C/EBPβ. They include binding sites for NF-κB, NF-AT, AP-1, AP-2, Ets, and Sp-1, as well as a cyclic AMP response element (CRE) (Zagariya et al. 1998).

3.3.4 Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 was first discovered in murine fibroblasts as a platelet-derived growth factor (PDGF)-induced immediate early response gene and designated as JE (Cocharin et al. 1983).It was later found by homology to be related to a number of cytokines and identical to the human monocyte chemoattractant protein-1

(MCP-1) (Rollins et al. 1989). MCP-1 belongs to a large family of cytokines with chemotactic activity, known as chemokines. These chemotactic cytokines are produced by macrophages, endothelial cells, and lymphocytes upon LPS, IL-1 or TNF stimulation. They are responsible for the influx of phagocytic cells from blood to tissue during inflammation. MCP-1 is chemotactic for monocyte/macrophages and T cells, with no activity on neutrophils.

Studies of the MCP-1 promoter have identified a binding site for NF-κB, which becomes occupied upon stimulation with IL-1β, TNF-α or TPA (Ueda et al. 1994). Induction by IL-1β also requires an AP-1 site for maximal activity (Martin et al. 1997). Although no LPS-responsive element has been identified in the MCP-1 promoter, our study has added C/EBPs to the list of transcription factors that are critical for MCP-1 expression (Bretz et al. 1994, Hu et al. 1998).

3.4 C/EBP proteins and the expression of proinflammatory cytokines

A C/EBP activity has been implicated in the regulation of many proinflammatory cytokine genes. The promoters for IL-6, IL-1α, IL-1β, IL-8, TNF-α, and G-CSF contain known or predicted C/EBP binding sites (Akira et al. 1990, Furutani et al. 1986, Shirakawa et al. 1993, Zhang et al. 1993). The LPS-induced expression of IL-1β and G-CSF requires one or more elements that bind a C/EBP-like activity (Shirakawa et al. 1993, Nishizawa et al. 1990). Furthermore, both C/EBPβ and C/EBPδ can transactivate a reporter gene regulated by the IL-6 promoter in transient expression assays. Indeed, we have previously shown that

overexpression of C/EBPβ confers upon a B-lymphoblastic cell line the ability to induce IL-6 and MCP-1 in response to LPS (Bretz et al. 1994).

In order to ascertain the specific role of C/EBPβ, knock-out mice were generated by gene targeting in two independent studies (Tanaka et al. 1995, Screpanti et al. 1995). C/EBPβ null mice appear to develop normally. An inspection of the ability of peritoneal macrophages derived from these animals to produce proinflammatory cytokines has shown that LPS stimulation leads to a normal induction of a number of proinflammatory cytokines, except for G-CSF. In fact, the expression level of IL-6 is increased. These results suggest that another C/EBP family member(s) can substitute for C/EBPβ. We have shown that this, indeed, is the case. C/EBPα and –δ are also expressed by macrophages, and either can activate the expression of IL-6 and MCP-1 in LPS-treated P388 lymphoblasts. These results will be presented in Chapter 2.

3.5 Other cooperating transcription factors

Many studies have demonstrated that C/EBP proteins can interact with a number of transcription factors bound to nearby binding sites on target promoters. A synergistic effect is often seen as a result of such interactions. Although the molecular mechanism underlying this synergism is not well understood, it is believed that protein-protein interactions between these transcription factors play a major role. C/EBPα has been shown to interact with a similar set of transcription factors in many myeloid-specific promoters. For example, two transcription factors, PU-1 and AML-1, can interact with C/EBPα

and synergistically activate the M-CSF (Zhang et al. 1994), G-CSF (Smith et al. 1996) and GM-CSF (Hohaus et al. 1995) receptor promoters. A similar regulatory scheme is observed in the promoters of neutrophil elastase (Oelgeschlager et al. 1996) and myeloperoxidase (Ford et al. 1996). A direct physical interaction between C/EBPα and AML-1 has also been demonstrated (Zhang et al. 1996).

Several transcription factors have been reported to have functional and/or physical interactions with C/EBPβ. The chicken homolog of C/EBPβ, NF-M collaborates with c-Myb in the combinatorial activation of two myeloid-specific genes, *mim-1* and lysozyme (Ness et al. 1993). C/EBPβ also works in conjunction with Sp-1 to activate the rat CYP2D5 gene that encodes a cytochrome P450 (Lee et al. 1994). Interestingly, C/EBPα cannot cooperatively activate the promoter with Sp-1. This is one of very few examples showing promoter specificity between C/EBP family members. It is shown that the specificity is determined by the leucine zipper and activation domains of C/EBPβ.

The most well-documented examples of interactions involving C/EBPβ come from studies of the Rel/NF-κB transcription factor family. NF-κB transcription factors are active as homo- and heterodimers composed of a variety of family members including NF-κB1(p50), NF-κB2(p52), c-rel, RelA(p65), and RelB (Baeuerle et al. 1994). The p50/p65 heterodimer is the most common form of NF-κB. NF-κB activity is regulated by nuclear and cytoplasmic partitioning. Several extracellular signals, including LPS and IL-1 and TNFα, induce NF-κB activity by causing the dissociation of NF-κB from an inhibitory subunit, IκB, whose function is to retain NF-κB protein in the cytoplasm. The release of NF-κB from IκB allows

the transcriptionally active NF-κB to translocate to the nucleus. Binding sites for NF-κB have been identified in the promoters of many inflammation-associated and acute phase genes. Thus, NF-κB and C/EBPs are often co-induced and regulate many of the same target genes including IL-1β (Shirakawa et al. 1993), TNF-α (Natsuka et al. 1992), IL-6 (Isshiki et al. 1990), inducible nitric oxide synthase (iNOS) (Lowenstein et al. 1993) , and IL-8 (Mukaida et al. 1990). In an effort to identify nuclear factors that interact with NF-κB, LeClair et al. (1992) screened a λ cDNA expression library with a radiolabeled NF-κB polypeptide. One of the genes identified was found to be C/EBPβ. A direct interaction between NF-κB and C/EBPβ was proposed to involve, respectively, the Rel homology and the leucine zipper domains. This result was confirmed by studies of the regulation of IL-8 (Stein et al. 1993b, Kunsch et al. 1994) and IL-6 genes (Matsusaka et al. 1993). In the IL-8 promoter, which contains adjacent binding sites for NF-κB and C/EBP, cooperative binding and synergy by NF-κB and C/EBP family members (including C/EBPα, -β and –δ) were demonstrated (Stein et al. 1993a). These effects are likely based on direct protein-protein interactions. Using transient transfection with a reporter construct driven by the IL-6 promoter containing binding sites for NF-κB and C/EBP, it was shown that NF-κB and C/EBPβ cooperatively transactivate the IL-6 promoter and that this synergy requires intact binding sites for both activators, as mutation in either site abolished the synergistic effect (Matsusaka et al. 1993). Together, these results indicate that NF-κB is an important partner of C/EBP proteins in regulating many inflammation-associated and acute phase genes.

We have shown that in our model system, the murine lymphoblast P388, NF-κB activity is induced by LPS stimulation (Hu et al. 1998), and have confirmed that NF-κB p65 cooperates with C/EBP family members in transactivating the IL-6 promoter (Tian and Schwartz, unpublished). Surprisingly, we have found that truncated forms of C/EBPβ, -δ and, to a lesser extent, -α which lack the activation domains but retain the basic region and leucine zipper are capable of transactivating the IL-6 promoter under conditions of LPS stimulation. The activation domains of C/EBP isoforms are apparently unnecessary for activation of the IL-6 promoter in response to LPS. These and other results that are presented in Chapter 3 show that at least some structural determinants for C/EBP activity reside in the leucine zipper domain. The leucine zipper region is thus implicated in some function other than dimerization to other known C/EBP family members. A model is proposed that the leucine zipper region, conserved among C/EBP family members, not only serves as a site for dimerization but also a site for physical interaction with NF-κB and/or additional transcription factors to form a higher-order ternary structure necessary for robust activation of the IL-6 promoter. Such a model may also explain the redundancy of C/EBPα, -β and -δ observed in the LPS-induction of IL-6 and MCP-1 that is described in Chapter 2.

4. Questions addressed in this thesis

C/EBPβ has long been considered a key regulator of genes associated with the inflammation and acute phase response, such as proinflammatory cytokines.

However, targeted gene disruption has shown that the macrophages from C/EBPβ knock-out mice are still capable of producing many proinflammatory cytokines, except for G-CSF, in response to LPS stimulation. This observation has raised the hypothesis that other C/EBP member(s) are functionally redundant to C/EBPβ and can replace the activity of C/EBPβ when it's not available. This question has been addressed and detailed in Chapter 2.

During the course of study of various truncated forms of C/EBPβ, we discovered unexpectedly that truncated forms of C/EBPβ which lack any conventional activation domain but retain the bZIP domain are capable of supporting LPS induction of IL-6 and MCP-1. This observation has led us to hypothesize that the redundancy of C/EBPs in regulating proinflammatory cytokine expression may result from their highly related bZIP domains. This part of the study has been described in Chapter 3.

Functional redundancy of C/EBP family members has also been observed in the regulation of other myeloid-specific genes such as those encoding the primary granule proteins expressed by the granulocytes. Multiple C/EBP family members are expressed by the granulocytes and their precursors in an overlapping manner. How the different forms of C/EBP dimers regulate these myeloid-specific genes is not well understood. We have tried to address this question by over-expressing individual C/EBP proteins in the pre-granulocytic cell line, 32D cl3. The expression of several granule proteins in these transfectants is investigated. A correlation between the induction of transcription and the major form of the C/EBP dimer is drawn and described in Chapter 4.

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Chapter 2

Redundancy of C/EBPα, -β, and –δ in Supporting the Lipopolysaccharide-Induced Transcription of IL-6 and Monocyte Chemoattractant Protein-1

Hsien-Ming Hu, Mark Baer, Simon C. Williams, Peter F. Johnson,

and Richard C. Schwartz

ABSTRACT

C/EBPα, -β and –δ are members of the CCAAT/enhancer binding protein family of transcriptional regulators. All three of these factors are expressed by bone marrow-derived macrophages, with the DNA binding activity of C/EBPβ and –δ increased by treatment with LPS while that of C/EBPα is decreased. We have ectopically expressed each C/EBP protein in P388 lymphoblasts. The expression of any of these transcription factor is sufficient to confer the LPS-inducible expression of IL-6 and monocyte chemoattractant protein-1 to lymphoblasts, which normally lack C/EBP factors and do not display LPS induction of proinflammatory cytokines. Thus, the activities of C/EBPα, -β and –δ are redundant in regard to the expression of IL-6 and monocyte chemoattractant protein-1. Since C/EBPβ-deficient mice have been reported to be largely normal in their expression of proinflammatory cytokines, it is likely that the lack of C/EBPβ is compensated for by the induction of C/EBPδ upon LPS treatment.

INTRODUCTION

C/EBP-related proteins comprise a family of basic region-leucine zipper transcription factors (reviewed in Johnson et. al. 1994). These proteins dimerize through a leucine zipper and bind to a consensus DNA motif through an adjacent basic region. C/EBP-related transcription factors have been implicated in the regulation of a number of proinflammatory cytokines as well as other gene products associated with the activation of macrophages by microbial products and cytokines. For example, the promoter regions of the genes for IL-6, IL-1α, IL-1β, IL-8, TNF-α, G-CSF, nitric oxide synthase, and lysozyme (Akira et al. 1990, Furutani et al. 1986, Lowenstein et al. 1993, Natsuka et al. 1992, Shirakawa et al. 1993, Zhang et al. 1993) contain C/EBP binding motifs. Furthermore, both C/EBPβ and –δ can *trans*-activate a reporter gene regulated by the IL-6 promoter in transient expression assays (Akira et al. 1990, Kinoshita et al. 1992). We have previously shown that the stable expression of C/EBPβ in a murine B lymphoblast cell line can confer the ability to induce IL-6 and MCP-1 expression with LPS (Bretz et al. 1994).

Two groups of investigators have recently generated mice deficient for C/EBPβ expression (Screpanti et al. 1995, Tanaka et al. 1995). Tanaka et al. (1995) found that LPS stimulation of peritoneal macrophages from such animals led to a normal induction of a number of proinflammatory cytokines, including IL-6. Basal levels of IL-6 mRNA were, in fact, elevated. These animals' macrophages, however, failed to express G-CSF mRNA in response to LPS stimulation.

Screpanti et al. (1995) found C/EBPβ deficient mice to have elevated levels of IL-6 expression, but did not otherwise report the ability of macrophages from those mice to produce proinflammatory cytokines. Consistent with the findings of Tanaka et al. (1995), ablation of C/EBPβ expression in human fibroblasts with either antisense- or ribozyme-mediated elimination of C/EBPβ mRNA blocked TNF-α induction of G-CSF, but not IL-6 expression (Kiehntopf et al. 1995).

The above results indicate that C/EBPβ is not necessary for the induction of IL-6 in the inflammatory response. However, the requirement of a C/EBP activity for LPS induction of IL-6 is very likely, since we have previously demonstrated a critical role for C/EBPβ in this process (Bretz et al. 1994). Several monocyte and macrophage cell lines have been reported to express both C/EBPβ and C/EBPδ (Bretz et al. 1994, Kinoshita et al. 1992), and immature myelomonocytic cell lines have also been reported to express C/EBPα (Scott et al. 1992). It is thus reasonable to propose that the expression of IL-6 and other proinflammatory cytokines by the macrophages of C/EBPβ-deficient mice is supported by C/EBPδ or, perhaps, C/EBPα. C/EBPα, C/EBPβ and C/EBPδ have all been reported to be functional in a heterologous transgenic rescue assay for a *Drosophila* C/EBP mutant, slow border cells (Rorth et al. 1994), but the functional redundance of C/EBPs in cytokine expression in mammalian cells has not been demonstrated. In this report we have directly compared the capacities of C/EBPα, C/EBPβ, and C/EBPδ to confer LPS-induced cytokine expression to a lymphoblastic cell line normally lacking this capability. Using stable transfection and endogenous cytokine genes containing a full complement of regulatory sequences, we show

that any of these C/EBPs can confer LPS-inducible expression of the genes encoding IL-6 and MCP-1. These results demonstrate the redundance of C/EBPα, C/EBPβ and C/EBPδ in supporting the LPS induction of IL-6 and MCP-1.

MATERIALS AND METHODS

Cells and cell culture: Bone marrow-derived macrophages were obtained from C57 Black/6 mice. Bone marrow was explanted from femurs into DMEM supplemented with 10% FCS, 10% heat-inactivated horse serum, and 20% L cell-conditioned medium at a density of 10^7 cells/ml in 25 ml on 150-mm tissue culture plates. After 48 h, the nonadherent cells were removed and replated at a density of 3 x 10^5 cells/ml in 10 ml on 100-mm tissue culture plates. Culture continued for 7 days, with a change of medium every 3 days.

P388 cells are murine B lymphoblasts (Bauer et al. 1986) (American Type Culture Collection, Rockville, MD; CCL46). P388-Cβ cells are P388-C2 cells previously described by Bretz et al. (1994). Cells were cultured in RPMI 1640 medium supplemented with 5% FCS and 50 μM 2-ME. Inductions were conducted with LPS derived from *Escherichia coli* serotype 055:B5 (Sigma Chemical Co., St. Louis, MO) added to 10 μg/ml.

Transfections: Transfections of G418-resistant vectors were conducted with 10^6 cells, 5 μg of DNA, and 40 μg of lipofectin (Life Technologies, Grand Island, NY) in 3 ml of Opti-MEM I medium (Life Technologies). Cells were incubated in the transfection mixture for 16 hr followed by the addition of RPMI 1640 supplemented with 20% FCS. After 72 h, the medium was replaced with the standard growth medium supplemented with G418 (Life Technologies) at 0.67 mg/ml. Transfections of puromycin-resistant vectors were conducted similarly with a selective concentration of puromycin (Boerhinger Mannheim, Indianapolis, IN) at 7 μg/ml.

Expression vectors: pSV(x)Neo is pZIP-NEO SV(x)1 (Cepko et al. 1984) and uses the promoter of Moloney murine leukemia virus. pSV(x)C/EBPα was constructed by insertion of the BamHI/KpnI fragment encoding rat C/EBPα from pMEXC/EBP (Williams et al. 1991) into the BamHI site of pSV(x)Neo with BamHI linkers. pSV(x)C/EBPβ was constructed by insertion of the BamHI fragment encoding rat C/EBPβ from pMEXCRP2 (Williams et al. 1991) into the BamHI site of pSV(x)Neo. To construct an expression for C/EBPδ, the sequences encoding murine C/EBPδ (Williams et al. 1991) were first inserted into the SphI and HindIII sites of pMEX (Williams et al. 1991) by a three-part ligation: one inserted fragment extended from a PCR-introduced SphI site 40 bp upstream of the C/EBPδ initiation codon to an ApaI site approximately 100 bp into the coding sequence, and the other fragment extended from the ApaI site to a PCR-introduced HindIII site just downstream of termination codon. The SphI/HindIII

fragment was then inserted with BamHI linkers into the BamHI site of pSV(x)Neo to produce pSV(x)C/EBPδ. The same BamHI fragment was inserted into the BamHI site of pBABE-Puro (Morgenstern et al. 1990) to construct pBABE-C/EBPδ.

Nucleic acid isolation and analysis: Total RNA isolated using TRIzol reagent (Life Technologies) according to the manufacturer's directions. RNAs were electrophoresed through 1% agarose/formaldehyde gels. Transfer to membranes were hybridized and washed to a stringency of 0.1% SSPE at 65°C. Hybridization probes were prepared with random priming kit (Life Technologies) with the incorporation of 5'-[α-32P]dATP (3000 Ci/mmol; DuPont-New England Nuclear, Newton, CT). The IL-6 probe was a 0.65-kb murine cDNA (from N. Jenkins and N. Copeland, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). The MCP-1 probe was a 0.58-kb murine cDNA (Rollins et al. 1988). The GAPDH probe was a 1.3-kb rat cDNA (Fort et al. 1985).

Western analysis: Nuclear extracts were prepared as described below. The extracts (20 μg) were adjusted to 1x Laemmli sample buffer (Laemmli 1970) and processed on a 12% PAGE gel. The gel was transferred to a Protran membrane (Schleicher and Schuell, Keene, NH), and Ag-Ab complexes were visualized with the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

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Electrophoretic mobility shift assay (EMSA): Nuclear extracts were prepared as described by Lee et al. (Lee et al. 1988), except that the samples were not dialyzed into buffer D. Protein was incubated with a double-stranded oligonucleotide probe containing an optimal C/EBP binding site (5'-GATCCTAGATATCCCTGATTGCGCAATAGGCTCAAAGCTG-3' annealed with 5'-AATTCAGCTTTGAGCCTATTGCGCAATCAGGGATATCTAG-3') or to a probe homologous to the NF-κB binding site of the Ig κ light chain enhancer (5'-TCGACTCCCTGGGGACTTTCCAGGCTCC-3' annealed with 5'-TCGAGGAGCCTGGAAAGTCCCCAGGGAG-3'). A probe containing a CTF/NF-1 consensus binding site (Landschulz et al. 1988) was used as a nonspecific competitor in some assays (5'-GATCCTTTGGCATGCTGCCAATATG-3' annealed with 5'-AATTCATATTGGCAGCATGCCAAAG-3'). Underlined sequences correspond to the binding motifs of the specified transcription factors. All binding reactions were performed at 23°C in a 25-μl mixture containing 6 μg of nuclear extract (1 mg/ml in buffer C), 6% (v/v) glycerol, 4% (w/v) Ficoll, 10mM HEPES (pH 7.9), 10 mM DTT, 0.25 μg of BSA, 0.06% (w/v) bromophenol blue, 1 μg of poly(dI-dC), and 1.25 ng of probe. Samples were electrophoresed through 5.5% polyacrylamide gels in 1x Tris-Borate (pH 8.3) and 0.5 mM EDTA at 150 V. For supershifts, nuclear extracts were preincubated with antisera for 30 min at 4°C before the binding reaction.

Antisera: Rabbit anti-C/EBPα was generated by immunization with a peptide corresponding to amino acids 253 to 268 of rat C/EBPα (Landschulz et al. 1988).

Rabbit anti-C/EBPβ was generated by immunization with a peptide corresponding to amino acids 1 to 12 of C/EBPβ (Williams et al. 1991) or was purchased from Santa Cruz Biotechnology (Santa Cruz, CA; C/EBPβ;C-19). Rabbit anti-C/EBPδ was obtained from M. Hannink (University of Missouri-Columbia) or was purchased from Santa Cruz Biotechnology (C/EBPδ; C-22). Rabbit anti-C/EBPε was purchased from Santa Cruz Biotechnology (CRP-1; C-22). Rabbit panCRP antiserum was generated by immunization with a peptide corresponding to a conserved motif within the basic region of C/EBP family members (Williams et al. 1995). Rabbit anti-p50 and anti-p65 were obtained from N. Rice (National Cancer Institute-Frederick Cancer Research and Development Center).

RESULTS

C/EBPα C/EBPβ and C/EBPδ are all expressed in primary bone marrow-derived macrophages. To determine which C/EBPs are expressed in primary macrophages, EMSAs were performed on the nuclear extracts of bone marrow-derived macrophages. Supershifts with specific antisera revealed both C/EBPα and C/EBPβ DNA binding activities before LPS stimulation (Fig. 1A). C/EBPβ became the predominant binding species after treatment with LPS for 4h; however, C/EBPα binding species were still present at a low level, and C/EBPδ binding species were induced (Fig. 1B). Thus, C/EBPα C/EBPβ and C/EBPδ are all potentially available to support the expression of inflammatory cytokines in

Figure 1. EMSA of C/EBPα, C/EBPβ, and C/EBPδ DNA binding activity in bone marrow-derived macrophages. *A*, No LPS treatment. *B*, Four-hour LPS treatment. Binding reactions included normal rabbit serum (N), C/EBPα antiserum (α), C/EBPβ antiserum (β), or C/EBPδ antiserum (δ). Some binding reactions, in addition to specific antisera, included 10-fold (10), 30-fold (30), and 100-fold (100) excess quantities of unlabeled C/EBP or CTF/NF1 binding oligonucleotides. The bar to the right indicates the positions of supershifted EMSA species.

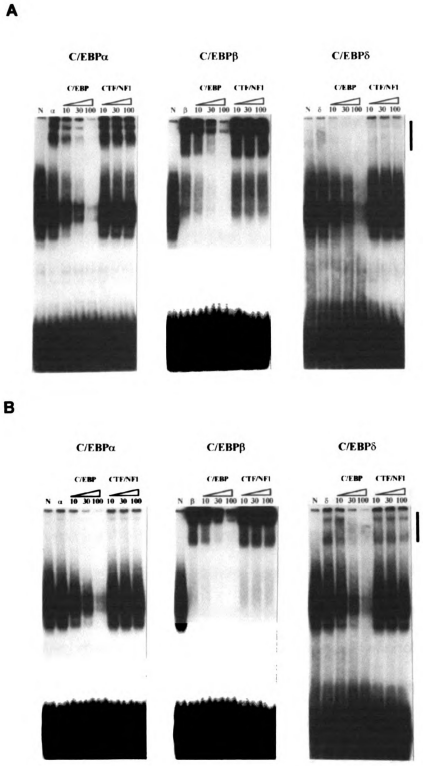

Figure 1. EMSA of C/EBPα, C/EBPβ, and C/EBPδ DNA binding activity in bone marrow-derived macrophages.

macrophages. To further ensure the specificity of our assay, competitions were performed with the unlabeled C/EBP binding site and an unlabeled CTF/NF-1 binding site. Both with (Fig. 1B) and without (Fig. 1A) LPS treatment, a 100-fold excess of the C/EBP binding site almost completely eliminated detectable C/EBP binding species, while a 100-fold excess of the CTF/NF-1 binding site barely reduced the abundance of such species. Since CRP-1 (C/EBPε) (Williams et al. 1991) has recently been reported to be a myeloid-specific transcription factor (Chumakov et al. 1997), we also examined whether this C/EBP family member was present in macrophages. EMSAs did not reveal CRP-1 (C/EBPε) binding activity in bone marrow-derived macrophages either before or after LPS stimulation (data not shown).

Ectopic expression of C/EBPα, C/EBPβ and C/EBPδ in P388 B lymphoblasts. We previously produced two transfectant populations of P388 cells that express C/EBPβ through a murine retroviral vector (P388-C2 and P388-C2-2) as well as a control population transfected with the same vector lacking an expressed insert (P388-Neo) (Bretz et al. 1994). P388 is a murine B lymphoblastic cell line (Bauer et al. 1986) that lacks C/EBPα, C/EBPβ and C/EBPδ expression (Bretz et al. 1994). To study the capacities of C/EBPα and C/EBPδ to support the expression of proinflammatory cytokines in comparison to C/EBPβ populations of P388 cells were transfected with pSV(X)C/EBPα or pSV(X)C/EBPδ. Pools of stably transfected cells were obtained after selection with G418. Cells transfected with

pSV(X)C/EBPα were designated P388-Cα, and cells transfected with pSV(X)C/EBPδ were designated P388Cδ. For consistency, the previous P388-C2 cells were designated P388-Cβ.

C/EBP expression in the transfected populations was initially characterized by EMSA (Fig. 2A). In comparison to nuclear extracts from P388-Neo, nuclear extracts from P388-Cβ, P388-Cδ, and P388-Cα yielded supershifted protein-DNA complexes upon incubation with antisera specific to C/EBPβ, C/EBPδ, and C/EBPα, respectively. The EMSA species that gave rise to the supershifts were also evident in the samples incubated with normal rabbit serum. This analysis did not reveal DNA binding activity for any C/EBP family members that had not been transfected into these populations in either the absence or the presence of LPS treatment. Supershift species for C/EBPα, -β, and –δ were only observed in cells transfected for their expression. Additionally, supershift species for CRP-1 (C/EBPε) were not observed in any of the transfectants.

As in the assays using extracts from bone marrow-derived macrophages, competitions were performed with the unlabeled C/EBP binding site and an unlabeled CTF/NF-1 binding site (Fig. 2B). All the supershifted protein-DNA complexes observed upon incubation with antisera specific to C/EBPβ, C/EBPδ, and C/EBPα were effectively competed by a 100-fold excess of the C/EBP binding site, while a 100-fold excess of the CTF/NF-1 binding site had little effect. The competition revealed a prominent protein-DNA complex that was not supershifted by specific antisera. but was effectively competed by the unlabeled C/EBP binding site. This species probably represents Ig/EBP (C/EBPγ), which is

Figure 2. Analyses of P388 cells stably transfected with C/EBPα, C/EBPβ or C/EBPδ expression vectors. Cell line nomenclature is described in *Results*. *A*, EMSA of C/EBP DNA binding activities in P388 transfectants with and without 4-h LPS treatment. Reactions included normal rabbit serum (N), C/EBPα antiserum (α), C/EBPβ antiserum (β), C/EBPδ antiserum (δ), or CRP-1 (C/EBPε) antiserum (ε). The positions of C/EBP-specific Ab supershifted species are indicated by arrowheads on the *right*. *B*, EMSA of C/EBP DNA binding activities in P388 transfectants in the presence of unlabeled competing oligonucleotide binding sites. Binding reactions included normal rabbit serum (N), C/EBPα antiserum (α), C/EBPβ antiserum (β), or C/EBPδ antiserum (δ). Some binding reactions, in addition to specific antisera, included 10-fold (10), 30-fold (30), and 100-fold (100) excess quantities of unlabeled C/EBP or CTF/NF1 binding oligonucleotides. The bar to the *right* indicates the positions of supershifted EMSA species. The asterisk marks the position of the likely Ig/EBP (C/EBPγ) EMSA species. *C*. Western blot analysis of C/EBP proteins derived from nuclear extracts of the transfectants. The positions of C/EBPα (α), C/EBPβ (β), C/EBPδ (δ), and cross-reactive material (CRM) are indicated by arrows on the *right*. The positions of m.w. markers are indicated on the *left*.

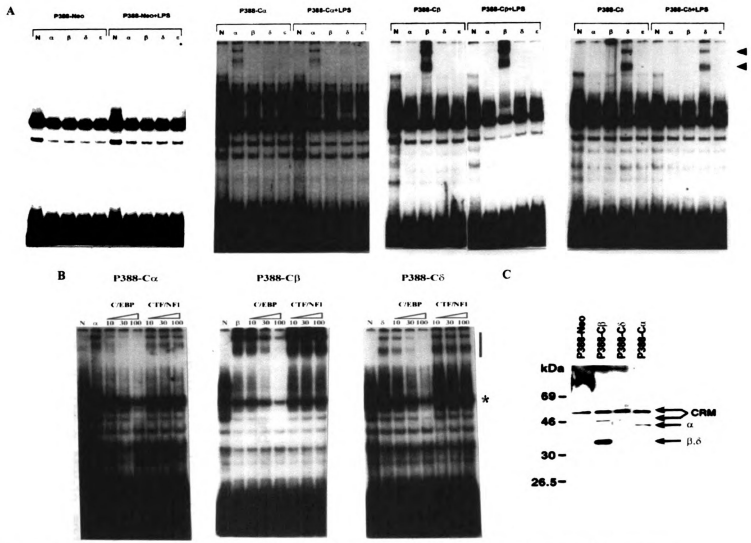

Figure 2. Analyses of P388 cells stably transfected with C/EBPα, C/EBPβ or C/EBPδ expression vectors.

highly expressed in P388 cells (data not shown) and other immature B cells (Roman et al. 1990).

Western blot analysis of nuclear extracts from the same transfected populations using panCRP antiserum confirmed expression of the C/EBPs from the transfected vectors (Fig. 2C). The immunogenic peptide used in generating panCRP antiserum is completely conserved among C/EBP family members (24); thus, this antiserum can be used for quantitative comparisons of protein levels between different C/EBP family members. C/EBPβ protein levels were much higher than those of C/EBPδ and C/EBPα (Fig. 2C) even though the abundance of EMSA species among the transfectants, particularly C/EBPβ and C/EBPδ was similar (Fig. 2A). This suggests a higher specific DNA binding activity for C/EBPδ. Successful transfection of the P388 populations was also confirmed by Southern blot and Northern blot analyses (data not shown).

LPS-induced cytokine expression is supported by C/EBPα and C/EBPδ as well as C/EBPβ. Cultures of P388-Cβ, P388-Cδ, and P388-Cα cells were treated with LPS over a time course of 0, 2, 4, 8, and 24 h, and RNA was isolated. A control population of P388 lymphoblasts transfected with pSV(X)Neo was also examined. Northern analyses and RNase protection assays were performed to detect transcripts encoding IL-6, MCP-1, IL- I α, IL- 10, TNF-α, MIP-1α, and G-CSF. Transcripts encoding GAPDH were also examined as a normalization control. LPS was found to induce transcripts for IL-6 and MCP-1 in P388-Cβ, P388-Cδ, and P388-Cα cells (Fig. 3). All three C/EBPs were quite effective in

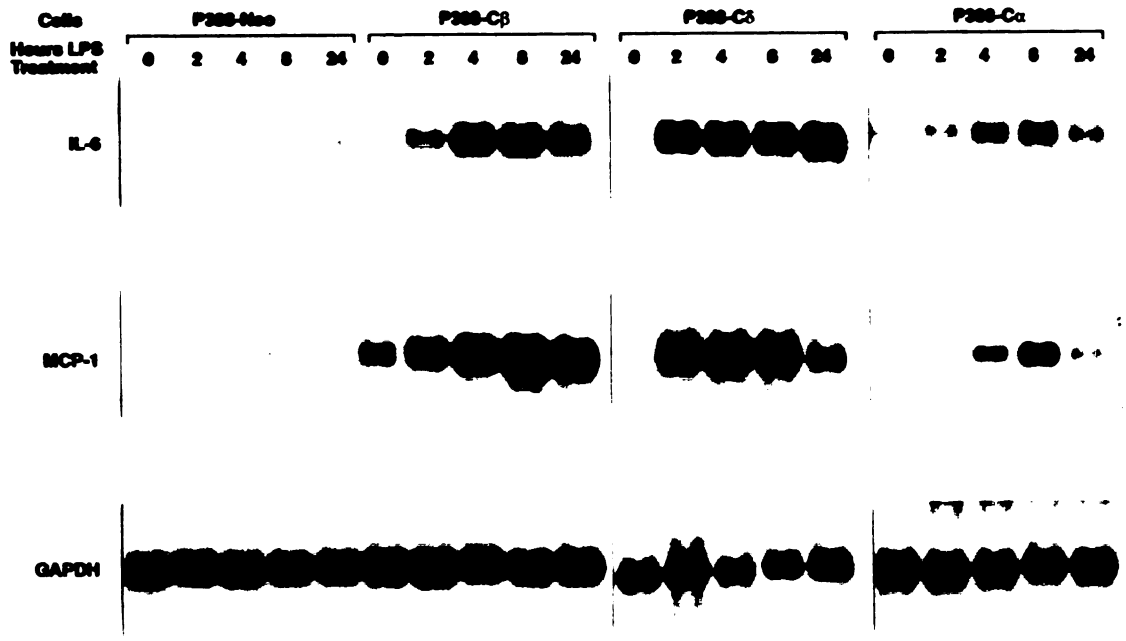

Figure 3. Northern analyses of IL-6 and MCP-1 expression in P388 transfectants. Total RNA was isolated over time course of LPS treatment as indicated. Ten micrograms of RNA was analyzed on Northern blots that were successively hybridized to probes for IL-6 and MCP-1, and GAPDH.

inducing IL-6 and MCP-1 RNAs. Induction was evident by 2 h of LPS treatment. with a decline by 24 h. The family members differed in the time required to reach peak levels of RNA: C/EBPδ transfectants required 2 h, and C/EBPβ and C/EBPα transfectants required as much as 8 h to reach peak levels. C/EBPδ may also be the most effective family member considering its relatively low abundance in P388-Cδ (Fig. 2B). C/EBPα may be the least effective, as P388-Cα cells show lower peak levels of IL-6 and MCP-1 RNAs. Also note that C/EBPβ expression is associated with significantly higher basal levels of MCP-1 transcripts than those seen with either C/EBPα or C/EBPδ expression. Transcripts encoding IL-1α, IL-1β, and G-CSF were not induced by LPS (data not shown), and weak LPS inductions of TNF-α and MIP-1α were not augmented in any of the C/EBP transfectants compared with those in P388-Neo cells (data not shown). These results were reproducible in similar independently transfected populations (data not shown). The various C/EBP family members thus differ subtly in their ability to support cytokine expression.

Coexpression of C/EBPδ with C/EBPβ augments the expression of IL-6 and MCP-1, but does not support the expression of additional proinflammatory cytokines. Since authentic macrophages were demonstrated to express multiple C/EBPs (Fig. 1), we sought to produce transfectants expressing multiple C/EBPs to test whether combinatorial expression confers augmented capacities to transcribe proinflammatory cytokine genes. In particular, we sought to produce cells coexpressing C/EBPβ and C/EBPδ because these DNA binding activities

were enhanced upon LPS treatment of bone marrow-derived macrophages (Fig. 1). To produce cells expressing both C/EBPβ and C/EBPδ, C/EBPδ was introduced into P388-Cβ cells with the murine retroviral vector pBABE-C/EBPδ. P388-Cβ cells were transfected with either pBABE-C/EBPδ or the parental vector lacking an expressed insert, pBABE-Puro. Pools of stably transfected cells were obtained after selection with puromycin. Cells doubly transfected with pSV(X)C/EBPβ and pBABE-C/EBPδ were designated P388-Cβ/δ and cells doubly transfected with pSV(X)C/EBPβ and pBABE-Puro were designated P388-Cβ/Puro.

Supershifting of EMSA species with specific antisera verified the expression of C/EBPβ and C/EBPδ in the doubly transfected population, while the control transfection population expressed only C/EBPβ (Fig. 4A). Successful transfection was also confirmed by Southern and Northern blot analyses (data not shown). When the LPS induction of IL-6 and MCP-1 RNAs was examined in these transfected populations. the level of expression was augmented in cells expressing both C/EBPβ and C/EBPδ compared with that in cells expressing only C/EBPβ (Fig. 4B). Densitometry revealed peak inductions of 2.3-fold for IL-6 and MCP-1 in cells expressing C/EBPβ, and peak inductions of 3.5-fold for IL-6 and 3.8-fold for MCP-1 were observed in cells coexpressing C/EBPδ and C/EBPβ (Table 1). Whether the coexpression of C/EBPδ and C/EBPβ augmented the LPS induction of IL-6 and MCP-1 in an additive or a synergistic manner is unclear. Since the previous data (Figs. 2C and 3) suggest that C/EBPδ may be more effective than C/EBPβ in supporting transcription of IL-6 and MCP-1 RNAs, the

Figure 4. Analyses of P388 cells stably transfected for dual expression of C/EBPβ and C/EBPδ. Cell line nomenclature is described in *Results*. *A*, EMSA of C/EBP DNA binding activities in P388 transfectants with and without 4-h LPS treatment. Reactions included normal rabbit serum (N), C/EBPα antiserum (α), C/EBPβ antiserum (β), C/EBPδ antiserum (δ), or CRP-1 (C/EBPε) antiserum (ε). The positions of C/EBP supershift species are indicated by arrowheads on the *right*. *B*, Northern analyses of IL-6 and MCP-1 expression. Total RNA was isolated over time course of LPS treatment and analyzed as described in Figure 3.

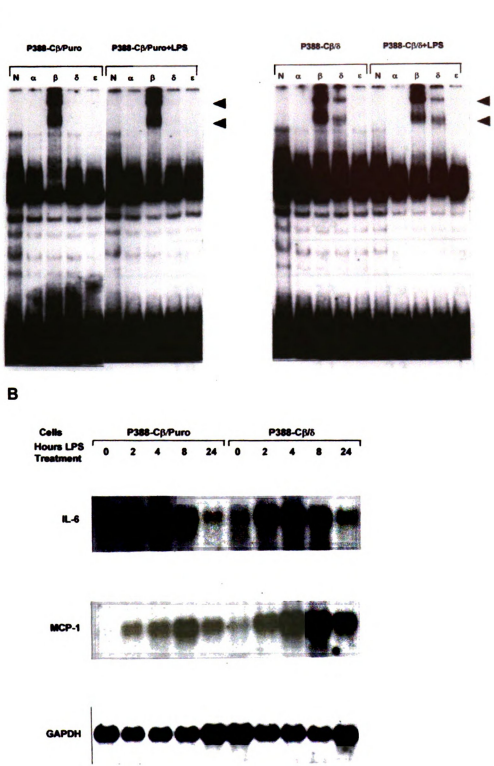

Figure 4. Analyses of P388 cells stably transfected for dual expression of C/EBPβ and C/EBPδ.

Table I. *Comparison of LPS induction of IL-6 and MCP-1 mRNA between P388-Cβ/Puro and P388-Cβ/δ*[a]

	LPS				
	0	2	4	8	24
P388-Cβ/Puro IL-6 RNA	1.0	1.6	2.3	1.9	0.8
P388-Cβ/δ IL-6 RNA	1.0	2.8	3.5	3.3	1.5
P388-Cβ/Puro MCP-1 RNA	1.0	1.7	2.1	2.3	1.1
P388-Cβ/δ MCP-1 RNA	1.0	2.4	3.7	3.8	2.2

[a] The autoradiograms presented in Figure 4*B* were analyzed by densitometer, and raw values for IL-6 and MCP-1 were normalized to values for GAPDH. The expression levels for each cell line were set at 1.0 at 0 h. LPS treatment and the tabulated values represent fold increases in expression.

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augmented expression of these mRNAs upon LPS induction may be solely dependent upon the added expression of C/EBPδ. Examination of IL-1α, IL-1β, TNF-α. MIP-1α. and G-CSF expression showed no effect of coexpression of C/EBPβ and C/EBPδ on the induction of RNAs encoding these cytokines (data not shown). These results were reproducible in a similar population of P388 cells independently transfected for coexpression of C/EBPβ and C/EBPδ (data not shown). Unexpectedly, in repeated attempts we were unable to obtain transfectants coexpressing C/EBPα and C/EBPβ or C/EBPα and C/EBPδ.

NF-κB (p50/p65) DNA binding activity is induced by LPS in the P388 transfectants. NF-κB has been implicated in the regulation of numerous cytokines that are expressed by macrophages in response to LPS (reviewed in Baeuerle et al. 1994, Grilli et al. 1993). In particular, mutation of an NF-κB binding site in the human IL-6 promoter completely abolished responsiveness to LPS (Dendorfer et al. 1994). Additionally, the IL-6 promoter (Matsusaka et al. 1993) and the IL-8 promoter (Matsusaka et al. 1993, Stein et al. 1993) are activated synergistically by C/EBPβ and NF-κB. The Importance of NF-κB in the expression of proinflammatory cytokines led us to determine whether NF-κB was indeed activated upon LPS treatment of P388 cells. The lack of cytokine induction in P388-Neo cells could be caused by an absence of NF-κB expression or activation. The inability of C/EBP transfectants of P388 cells to induce cytokines other than IL-6 and MCP-1 could be similarly explained. On the other hand, the ability of C/EBPβ to mediate a higher basal level of MCP-1 expression

than other C/EBPs could be caused by constitutive NF-κB activity in P388-Cβ cells. To address these issues, EMSAs were performed using a probe for NF-κB binding. As shown in Figure 5A, an LPS-induced EMSA species was observed in all transfectants, including the P388-Neo control. Formation of this LPS-induced species could be quantitatively blocked by either p50 or p65-specific antisera (Fig. 5B), showing that the major species induced is a p50/p65 heterodimer. Thus, NF-κB (p50p65) is translocated to the nucleus of P388 cells and is probably available to support the LPS-induced expression of proinflammatory cytokines. The inability of P388 cells to induce IL-6 and MCP-1 can be specifically attributed to the absence of C/EBP family members.

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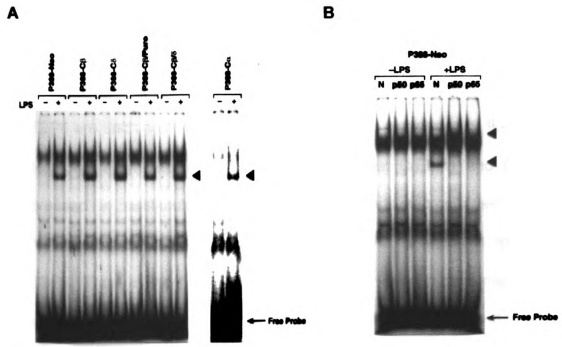

Figure 5. EMSA of NF-κB DNA binding activity in P388 transfectants. *A*. Cells were grown in the absence of LPS (-) or 4 h in the presence of LPS (+). The position of NF-κB EMSA species is indicated by arrowheads on the *right* of each panel. *B*. Nuclear extract of P388-Neo cells was treated with normal rabbit serum (N), p50 antiserum (p50), or p65 antiserum (p65). The arrowhead on the *right* indicates the position of NF-κB EMSA species.

DISCUSSION

The data presented in this paper demonstrate that C/EBPα. C/EBPβ, and C/EBPδ are each sufficient to confer LPS-inducible expression of IL-6 and MCP-1 to P388 B lymphoblasts. We have shown that C/EBPα and C/EBPβ are expressed in unstimulated bone marrow-derived macrophages, while LPS stimulation downregulates C/EBPα expression and up-regulates expression of C/EBPδ. Thus, all three of these C/EBPs are expressed in bone marrow-derived macrophages and could participate in the LPS induction of IL-6 and MCP-1. The observation of a largely normal cytokine response to LPS treatment in the macrophages of C/EBPβ-deficient mice (Tanaka et al. 1995) can be explained by the availability of C/EBPα and/or C/EBPδ. The induction of C/EBPδ by LPS in bone marrow-derived macrophages makes it a particularly attractive candidate for replacing C/EBPβ activity. In fact, C/EBPδ may be more effective than C/EBPβ in supporting the transcription of IL-6 and MCP-1 genes, since a relatively low level of its expression in P388-Cδ transfectants allows a quite vigorous induction of IL-6 and MCP-1. This induction is at least equal to that observed in P388-Cβ cells, which express a much higher level of C/EBPβ, and is more rapid. P388-Cδ cells also display a level of DNA binding similar to that of P388-Cβ cells, suggesting a higher sp. act. for DNA binding. C/EBPδ has previously been reported to be a stronger *trans*-activator than C/EBPβ using the human IL-6 promoter in a reporter construct (Kinoshita et al. 1992). The presence of a regulatory domain (RD2) in C/EBPβ that represses DNA binding

activity may explain its lower activity (Williams et al. 1995). On the other hand, C/EBPα appears less effective than C/EBPδ in inducing IL-6 and MCP-1 while being expressed at a similar level to C/EBPδ in transfectants. Additionally, C/EBPα DNA binding activity is reduced upon LPS treatment of bone marrow-derived macrophages, making it a less likely candidate to replace C/EBPβ activity in C/EBPβ-deficient mice. Collectively, the data suggest a prominent role for C/EBPδ in the LPS induction of inflammatory cytokines and implicate C/EBPδ as the most plausible activity to compensate for the lack of C/EBPβ in C/EBPβ-deficient animals.

The kinetics of LPS induction of IL-6 and MCP-1 mRNAs are generally similar among transfectants for the various C/EBP family members. Induction is evident by 2 h and declines by 24 h. There may be differences, however, in the time required to attain peak RNA levels among C/EBP family members. The C/EBPδ transfectants reached peak levels at 2 h compared with 4 or 8 h for C/EBPβ and C/EBPα transfectants. and the C/EBPβ/δ transfectants showed a dramatic induction by 2 h. Our previous studies (Bretz et al. 1994) found that the kinetics of proinflammatory cytokine mRNA production in a macrophage cell line, P388D1 (IL1), also reached peak RNA levels by 2 h. This may suggest the importance of C/EBPδ expression *in vivo*. Indeed, we have shown in this study that C/EBPδ is induced in LPS stimulation of bone marrow-derived macrophages. The delay in reaching peak RNA levels for C/EBPβ and C/EBPα transfectants may indicate a requirement for the induction of other factors for optimal expression with these C/EBPs. The delay may reflect the time required to induce and synthesize these

factors, or, on the other hand, the delay may simply indicate a lower rate of transcription requiring longer times to attain peak levels.

It is clear that LPS induction of IL-6 and MCP-1 mRNAs in our system operates through either the post-transcriptional activation of C/EBPs or the induction of a necessary cooperating transcription factor. EMSA analysis demonstrated C/EBP binding activity for the transfected genes before LPS treatment, and LPS treatment neither induced C/EBP family members other than those transfected nor increased the binding activity of the transfected C/EBPs. If LPS treatment is modulating the activity of C/EBPs in our system, it must be in a manner not evident in EMSA analysis. Other investigators have found in transient transfection studies of the IL-6 promoter that coexpression of C/EBPβ and NF-κB synergistically activates the IL-6 promoter (Matsusaka et al. 1993), and mutation of an NF-κB binding site in the human IL-6 promoter completely abolished responsiveness to LPS (Dendorfer et al. 1994). We have found that LPS induces NF-κB (p50/p65) in the P388 transfectants, and it is likely that this is the primary role of LPS in our system.

A synergism between the activities of C/EBPβ and C/EBPδ has been reported for the transient *trans*-activation of the human IL-6 promoter (Kinoshita et al. 1992). and we did observe that coexpression of C/EBPδ with C/EBPβ augments the LPS induction of IL-6 and MCP-1 mRNAs over that observed for C/EBPβ alone. It is unclear from our data whether that augmentation is synergistic or additive, since C/EBPδ by itself appears more active than C/EBPβ in supporting LPS induction of IL-6 and MCP-1. Interestingly, despite repeated attempts we

were unable to obtain transfectants doubly expressing C/EBPα and C/EBPβ or C/EBPα and C/EBPδ. Although bone marrow-derived macrophages coexpress these C/EBPs, we have not detected C/EBPα expression in any mature macrophage cell lines (our unpublished observation) (Bretz et al. 1994, Scott et al. 1992). These observations may indicate that C/EBPα expression is incompatible with the immortalization of mature macrophage cell lines. Consistent with this idea, C/EBPα has previously been shown to inhibit proliferation in adipocytes (Umek et al. 1991), hepatocytes (Hendricks-Taylor et al. 1995), and other cell types (Hendricks-Taylor et al. 1995).

Among the several cytokine mRNAs examined, only IL-6 and MCP-1 displayed robust LPS inductions. The lack of induction of other cytokines may reflect the requirement of transcription factors in addition to the C/EBP family for a full cytokine response. We have found that NF-κB (p50p65) is induced by LPS in the P388 transfectants, so such a deficiency must be attributed to other transcription factors. For instance, the murine IL-10 gene requires a novel 6-bp sequence (-2280 to -2275) in addition to C/EBP and NF-κB binding sites (Godambe et al. 1995). Furthermore, previous investigators have noted differences in the regulation of MCP-1, IL-1α, and IL-1β (Ohmori et al. 1990, Tannenbaum et al. 1989). For example, agents that elevate intracellular levels of cAMP suppress the LPS induction of MCP-1, but do not affect the induction of IL-1α, and actually enhance IL-1β induction. Stimuli other than LPS. such as IFN-γ, IL-1, or TNF, might also provide a more complete cytokine response through their ability to activate other transcription factors.

An alternative explanation for the lack of induction of cytokines other than IL-6 and MCP-1 may be the expression of Ig/EBP (C/ EBPγ) in P388 lymphoblasts. Ig/EBP has been reported to be a *trans*-dominant inhibitor of C/EBP family members (Cooper et al. 1995). It may block C/EBP activity on the promoters of those cytokine genes for which we do not observe activation. It will be of interest to assess the ability of Ig/EBP to inhibit C/EBP activation of the promoters for IL-6 and other proinflammatory cytokines in a transient expression system lacking endogenous Ig/EBP expression.

The expression of IL-6 and MCP-1, while both showing strong inductions with LPS, differ in regard to the basal levels of their mRNAs among the various C/EBP transfectants. In particular, MCP-1 displays an appreciable level of RNA in P388-Cβ in the absence of LPS. Since we do not observe NF-κB activity in the absence of LPS, it appears that MCP-1 does not require NF-κB for significant basal expression of its RNA. It will be of interest to compare the structure of the MCP-1 promoter to that of IL-6.

The data presented here lead us to predict that C/EBPδ expression may be crucial to supporting proinflammatory cytokine expression *in vivo*. Tanaka et al. (1995) did not find severe impairment of proinflammatory cytokine expression in C/EBPβ-deficient animals. We have now shown that while both C/EBPα and C/EBPδ can support the LPS activation of endogenous IL-6 and MCP-1 genes, the LPS activation of bone marrow-derived macrophages down-regulates C/EBPα activity and up-regulates C/EBPδ activity. C/EBPδ is thus the best candidate for the factor allowing C/EBPβ-deficient mice to display a largely

normal cytokine expression in response to LPS stimulation. A lack of C/EBPδ expression would be expected to reduce and/or delay peak expression of IL-6 and MCP-1 mRNAs. The development of knockout mice deficient in C/EBPδ expression and mice deficient in both C/EBPβ and C/EBPδ expression should provide the ultimate test of this issue.

Finally. why are there multiple C/EBP family members with seemingly redundant function within the inflammatory response? First, one should recognize that our system has only allowed examination of IL-6 and MCP-1 expression; promoter-specific functions of C/EBP family members are certainly possible for other genes. More significantly, differential function of C/EBP family members may become apparent under the influence of inflammatory stimuli other than LPS. It is clearly a high priority in future investigations to examine the C/EBP transfectants reported here under conditions of IL-1, IL-6, and TNF stimulation, since differences in function among C/EBP family members may derive from their linkages to different signal transduction pathways.

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Chapter 3

Conventional Activation Domains are Dispensable for the Role of C/EBPs in LPS Induction of IL-6 and MCP-1 Expression

Hsien-Ming Hu, Qiang Tian, Mark Baer,
Simon C. Williams, Peter F. Johnson, and
Richard C. Schwartz

ABSTRACT

C/EBP α, β, and δ are all expressed by bone marrow-derived macrophages. Ectopic expression of any of these transcription factors is sufficient to confer LPS-inducible expression of IL-6 and monocyte chemoattractant protein 1 (MCP-1) to lymphoblasts, which normally lack C/EBP factors and do not display LPS induction of proinflammatory cytokines. Thus, the activities of C/EBPα, β, and δ are redundant in regard to expression of IL-6 and MCP-1. Surprisingly, we have found that the bZIP regions of C/EBPβ and C/EBPδ are of themselves also capable of supporting LPS induction of IL-6 and MCP-1. The bZIP region of C/EBPα also shows modest activity. Furthermore, the naturally occurring transdominant negative inhibitor LIP is capable of supporting the LPS induction of IL-6 and MCP-1. Replacement of the leucine zipper of C/EBPβ with that of yeast GCN4 yields a chimeric protein that can dimerize and specifically bind to a C/EBP consensus sequence, but shows a markedly reduced ability to activate IL-6 and MCP-1. These results implicate the leucine zipper region in some function other than dimerization with known C/EBP family members in the activation of IL-6 and MCP-1 transcription, and suggest that C/EBP redundancy in regulating cytokine expression may result from their highly related bZIP domains.

INTRODUCTION

C/EBPα, β, and δ are members of the CCAAT/enhancer binding protein family of transcription factors (reviewed in Johnson and Williams, 1994). These proteins are basic region-leucine zipper transcriptional regulators that dimerize through their leucine zippers and bind to a consensus DNA motif through their adjacent basic regions. 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. For example the promoter regions of the genes for IL-6, IL-1α, IL-1β, IL-8, TNFα, G-CSF, nitric oxide synthase, and lysozyme (Akira et al., 1990; Furutani et al., 1986; Lowenstein et al., 1993; Natsuka et al., 1992; Shirakawa et al., 1993; Zhang and Rom, 1993) contain C/EBP binding sites. 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/EBPβ in a murine B lymphoblast cell line was sufficient to confer LPS-inducibility of IL-6 and monocyte chemoattractant protein 1 (MCP-1) expression (Bretz et al., 1994).

There have been a limited number of reports demonstrating specificity of a particular C/EBP family member for a given promoter. One example is the rat *CYP2D5* gene that encodes a cytochrome P450. It is transactivated cooperatively by C/EBPβ and SP1, but not C/EBPα (Lee et al, 1994). The specificity of this cooperativity is determined by the leucine zipper and activation

domains of C/EBPβ (Lee et al, 1997) Another case is promoter P1 of the proprotein processing enzyme furin (Ayoubi et al., 1994). It could be transactivated by C/EBPβ but not C/EBPα or C/EBPδ. Recently, it has been reported that C/EBPδ but not C/EBPβ, can transactivate the promoter for nerve growth factor (Colangelo et al., 1998). On the other hand, C/EBPα, β and δ are all functional in a heterologous transgenic rescue assay for a *Drosophila* C/EBP mutant, *slow border cells* (Rorth, 1994). Our own studies have demonstrated redundancy in the abilities of C/EBPα, β and δ to support LPS-induction of IL-6 and MCP-1 transcription (Hu et al., 1998).

A simple hypothesis for the redundancy of C/EBPs that we have observed is that their highly homologous bZIP domains (Williams et al., 1991) are all that is truly required of C/EBPs for activation of the genes for IL-6 and MCP-1. Against this hypothesis stand results obtained with a truncated form of C/EBPβ that initiates at Met 132 and lacks activation domains. This protein, referred to as LIP, could not activate transcription and, in fact, inhibited C/EBPβ-mediated transcriptional activation of a promoter derived from the DE-I site of albumin (Descombes and Schibler, 1991). In this report, we have surprisingly found that truncated forms of C/EBPβ that lack known activation domains, including the naturally occurring transdominant negative inhibitor LIP, are also capable of supporting LPS induction of IL-6 and MCP-1. Furthermore, a truncated form of C/EBPδ and, to a lesser extent, a truncated form of C/EBPα that similarly lack conventional activation domains have transcriptional activity on the IL-6 promoter. Replacement of the leucine zipper of C/EBPb with that of yeast GCN4

yields a chimeric protein that can dimerize and specifically bind to a C/EBP consensus sequence, but has reduced ability to activate IL-6 and MCP-1. These results implicate the leucine zipper domain in some function other than dimerization to known C/EBP family members in the activation of IL-6 and MCP-1 transcription, and suggest that C/EBP redundancy in regulating cytokine expression may result from their highly related bZIP domains.

MATERIAL AND METHODS

Cells and cell culture. P388 are murine B lymphoblasts (Bauer et al., 1986) (American Type Culture Collection; CCL46). P388-Cβ cells have been described previously by Hu et al. (1998). Cells were cultured in RPMI 1640 medium supplemented with 5% FCS and 50 μM 2-ME. Inductions were conducted with LPS derived from *Escherichia coli* serotype 055:B5 (Sigma) added to 10 μg/ml. Transfections. Stable transfections were conducted with $2x10^6$ cells, 5 μg of DNA, and 10 μl of DMRIE-C reagent (Life Technologies) in 1.2 ml of Opti-MEM I medium (Life Technologies). Cells were incubated in the transfection mixture for 16 h followed by the addition of 2.8 ml of the standard growth medium. After 24 hours, the medium was replaced with the standard growth medium supplemented with G418 (Life Technologies) at 0.67 mg/ml.

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 either 1 μg of the IL-6 promoter-reporter or the albumin DEI promoter-reporter, 1 μg of the SV40 early promoter-reporter, 0.1 μg of C/EBP expression vector, and pMEX plasmid to total 4 μg. Cells were incubated in the transfection mixture for 5 h followed by the addition of RPMI 1640 medium supplemented to 15% with FCS. 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 (Boehringer Mannheim) and for β-galactosidase activity by using the Luminescent β-Galactosidase Genetic Reporter System II (Clontech).

Expression vectors and promoter-reporters. For stable transfections, C/EBPs were expressed from pZIP-NEO SV(X)1 constructs (Cepko et al., 1984). C/EBP sequences were inserted into the BamHI site of the vector. Inserted sequences were transcribed from the Moloney murine leukemia virus promoter and the gene conferring G418-resistance was expressed from a subgenomic splicing product from the same promoter. For transient transfections, C/EBPs were expressed from pMEX (Williams et al., 1991), which also utilizes the Moloney murine leukemia virus promoter. The construction of C/EBP deletions and C/EBPβ:G_{LZ} have been described previously (Williams et al., 1991).

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 albumin DE-I promoter-reporter is $(DEI)_4$(-35alb)LUC (Williams et al. 1995) which is derived from pXP2 (Nordeen , 1988) and contains four copies of the DEI element upstream of the albumin minimal promoter. 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. RNAs were electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to a stringency of 0.1x SSPE at 65°C. Hybridization probes were prepared with a random priming kit (Life Technologies) with the incorporation of 5'-[α-32P]dATP (3000 Ci/mmol; DuPont-New England Nuclear). The IL-6 probe was a 0.65 kb murine cDNA (from N. Jenkins and N. Copeland, National Cancer Institute-Frederick Cancer Research and Development Center). The MCP-1 probe was a 0.58 kb murine cDNA (Rollins et al., 1988). The GAPDH probe was a 1.3 kb rat cDNA (Fort et al., 1985).

Western analysis. Nuclear extracts were prepared as described below. The extracts (50 μg) were adjusted to 1x Laemmli sample buffer (Laemmli, 1970) and

processed on a 12% PAGE gel. The gel was transferred to Protran membrane (Schleicher and Schuell), and Ag-Ab 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 14,000 x *g* for 20 sec at 4°C. Proteins were extracted from nuclei by incubation at 4°C with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 25% [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 14,000 x *g* for 15 min at 4°C and the supernatant extract was collected and stored at -70°C.

The EMSA probe was a double-stranded oligonucleotide containing an optimal C/EBP binding site (5'-GATCCTAGATATCCCTGATTGCGCAATAGGC-TCAAAGCTG-3' annealed with 5'-AATTCAGCTTTGAGCCTATTGCGCAATC-AGGGATATCTAG-3') labeled with the incorporation of 5'-[α-32P]dATP (3000 Ci/mmol; DuPont-New England Nuclear) and Klenow DNA polymerase. A probe containing a CTF/NF-1 biding site (Landschulz et al., 1988) (5'-GATCCTTTG-GCATGCTGCCAATATG-3' annealed with 5'-AATTCATATTGGCAGCATGCC-

AAAG-3') was used as a nonspecific competitor in some assays. Underlined sequences correspond to the binding motifs of the specified transcription factors.

DNA binding reactions were performed at room temperature in a 25 μl reaction mixture containing 6 μl of nuclear extract (1mg/ml in buffer C)] and 5 μl of 5 x 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), 1.25 ng of probe, bromophenol blue to a final concentration of 0.06% [wt/vol], and water to volume. For supershifts, nuclear extracts were preincubated with antibodies for 30 min at 4°C prior to the binding reaction. Samples were electrophoresed through 5.5% polyacrylamide gels in 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 (product C-19) and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Rabbit anti-C/EBPβ specific to the amino terminus was generated by immunization with a peptide corresponding to amino acids 1 to 12 of C/EBPβ (Williams et al., 1991).

RESULTS

Truncated forms of C/EBPβ that lack activation domains retain the ability to support LPS induction of IL-6 and MCP-1. We previously found that C/EBPα, β and δ were all effective in supporting the LPS-induced transcription of IL-6 and MCP-1 (Hu et al., 1998). In order to test whether this redundancy was based on the bZIP domain which is highly conserved among these C/EBP family members (Williams et al., 1991), we examined the expression of a truncated form of C/EBPβ (amino acids 192-276; C/EBP$\beta_{192\text{-}276}$) that lacks all conventional activation and regulatory domains and is simply the bZIP domain of C/EBPβ (Williams et al., 1995) (Figure 1). It was expected that the activity of C/EBP$\beta_{192\text{-}276}$ would be similar to a truncated form of C/EBPβ that initiates at Met 132 and lacks activation domains (Descombes and Schibler, 1991) (Figure 1). This protein, referred to as LIP, reportedly cannot activate transcription and, in fact, inhibits C/EBPβ-mediated transcriptional activation.

We performed stable transfections of P388 cells with murine retroviral vectors expressing either C/EBP$\beta_{192\text{-}276}$ or LIP, and compared those transfectants to P388-Cβ cells that had been transfected with a vector expressing C/EBPβ as well as a control population transfected with the same vector lacking a expressed insert (P388-Neo) (Bretz et al., 1994; Hu et al., 1998). P388 is a murine B lymphoblastic cell line (Bauer et al. 1986) that lacks C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε expression (Bretz et al., 1994; Hu et al., 1998). Populations of cells transfected for C/EBP$\beta_{192\text{-}276}$ expression were designated P388-C$\beta_{192\text{-}276}$, and

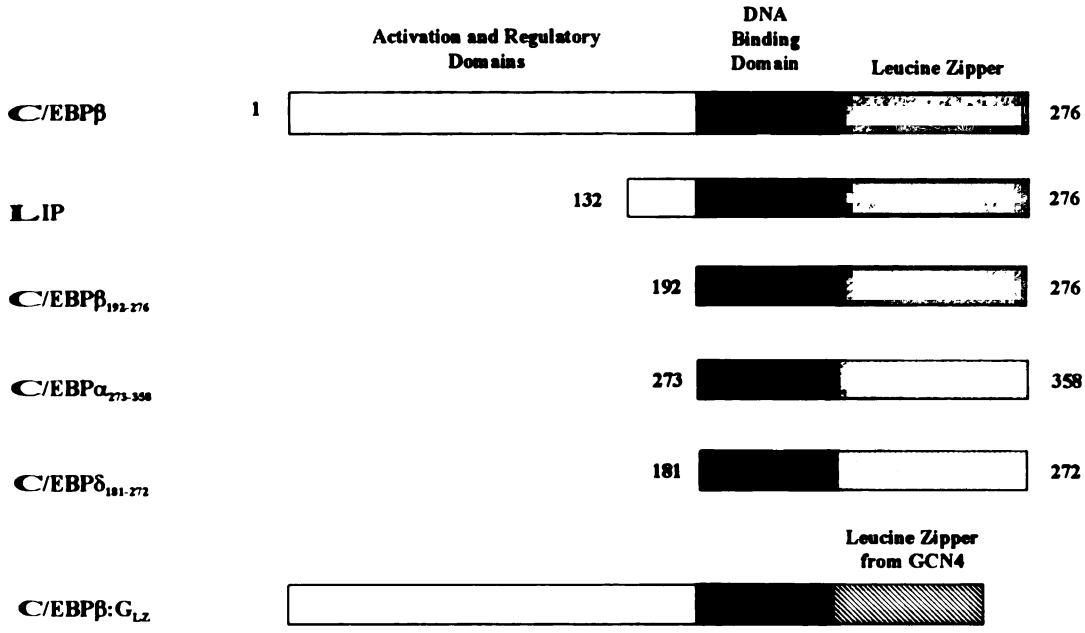

Figure 1. Structures of the various altered C/EBP isoforms used in the studies described in this paper.

populations transfected for LIP expression were designated P388-LIP. Surprisingly, P388-C$\beta_{192-276}$ cells behaved similarly to P388-Cβ cells in their ability to induce IL-6 and MCP-1 transcription in response to LPS (Figure 2). P388-LIP cells also showed activity in this assay. Their more modest activity can likely be attributed to the negative regulatory domains that are retained in LIP (Williams et al., 1995).

Electrophoretic mobility shift assays (EMSA) of nuclear extracts from the transfected populations, as well as western blot analyses were performed in order to verify proper expression of the stably transfected C/EBPβ genes. In comparison to nuclear extracts from P388-Neo, nuclear extracts from P388-Cβ, P388-C$\beta_{192-276}$, and P388-LIP yielded supershifted protein-DNA complexes upon incubation with an antibody specific for C/EBPβ (Figure 3). The EMSA species that gave rise to the supershifts were also evident in the samples incubated with normal IgG. As expected the C/EBPβ-specific EMSA species from P388-C$\beta_{192-276}$ and P388-LIP cells were of higher mobility than those of P388-Cβ cells reflecting the truncated structure of their C/EBPβ proteins. Additionally, the C/EBP$\beta_{192-276}$ and LIP EMSA species could only be supershifted with antibody specific to the carboxyl-terminus of C/EBPβ, while EMSA species of intact C/EBPβ could be supershifted by both amino- and carboxyl-terminus specific antibodies. To further ensure the specificity of the EMSA, competitions were performed with the unlabeled C/EBP binding site and an unlabeled CTF/NF-1 binding site (Figure 4). All of the supershifted protein-DNA complexes observed upon incubation with C/EBPβ-specific antibody were effectively competed by a 30-fold excess of the

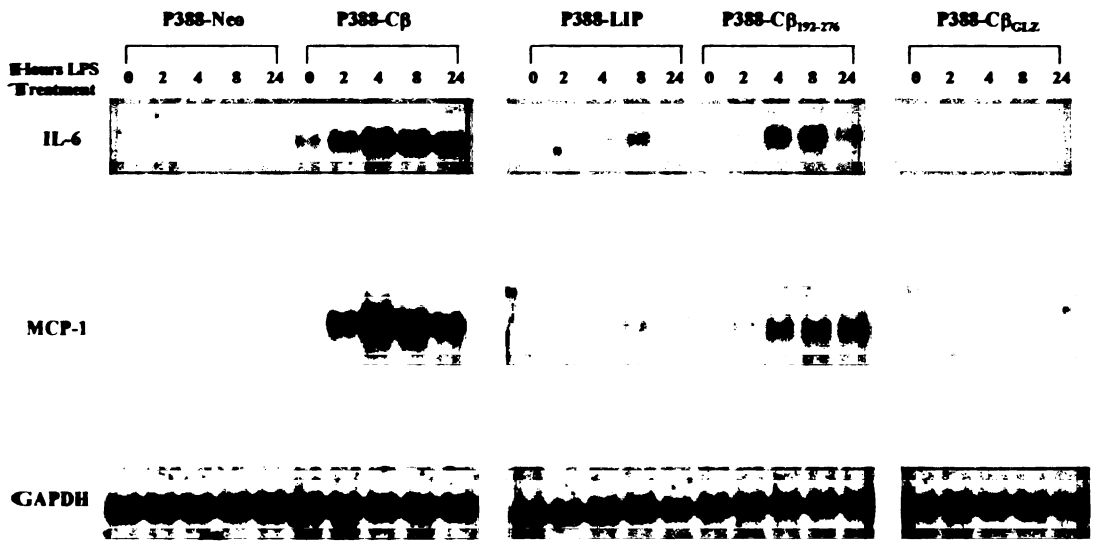

Figure 2. Transfectants of P388 that stably express C/EBPβ (P388-Cβ), LIP (P388-LIP), and C/EBPβ$_{192-276}$ (P388-Cβ $_{192-276}$) are stimulated by LPS to produce IL-6 and MCP-1 mRNAs, while cells that express C/EBPβ:G_{LZ} (P388-C β_{GLZ}) are not induced to produce these mRNAs. Northern analyses of IL-6 and MCP-1 expression in P388 transfectants. RNA was isolated over time courses of LPS treatment as indicated. Twenty micrograms of RNA analyzed on northern blots that were hybridized in parallel to probes for IL-6, MCP-1, and GAPDH.

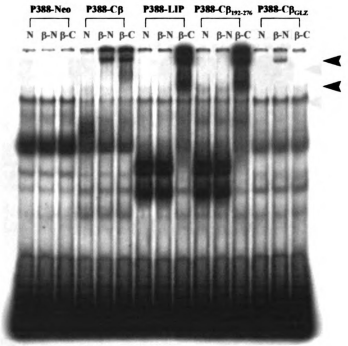

Figure 3. EMSA of C/EBP DNA binding activities in P388 cells stably transfected with C/EBPβ, LIP, C/EBPβ$_{192-276}$, and C/EBPβ:G$_{LZ}$ expression vectors. Cell line nomenclature is explained in *Results*. Binding reactions included normal rabbit IgG (N), amino-terminal-specific C/EBPβ antibody (β-N), or carboxyl-terminal-specific C/EBPβ antibody (β-C). The positions of C/EBP-specific antibody supershift species are indicated by arrowheads on the right.

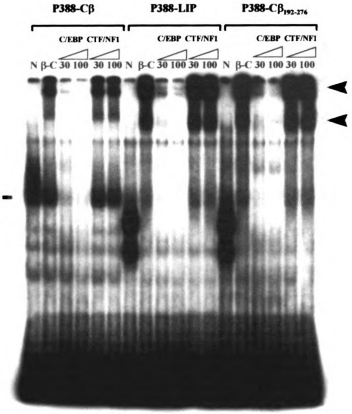

Figure 4. EMSA of C/EBP binding activities in P388 transfectants in the presence of unlabeled competing oligonucleotide binding sites. Binding reactions included normal rabbit IgG (N), amino-terminal-specific C/EBPβ antibody (β-N), or carboxyl-terminal-specific C/EBPβ antibody (β-C). Some binding reactions, in addition to specific antibody, included 30-fold and 100-fold excess quantities of unlabeled C/EBP or CTF/NF1 binding oligonucleotides. The positions of C/EBP-specific antibody supershift species are indicated by arrowheads on the right. The bar to the left indicates the position of the likely Ig/EBP (C/EBPγ) EMSA species.

C/EBP binding site, while a 100-fold excess of the CTF/NF-1 binding site had little effect. The prominent protein-DNA complex that was not supershifted by specific antibody, but was effectively competed by unlabeled C/EBP binding site is most likely Ig/EBP (C/EBPγ) (Hu et al., 1998), which is highly expressed in P388 cells and other immature B cells (Roman et al., 1990).

A western analysis of nuclear extracts from the transfected populations was performed in order to examine the actual levels of protein expression of the various forms of C/EBPβ (Figure 5). The levels of expression of C/EBP$\beta_{192\text{-}276}$ and LIP in transfected cells were much lower than that of C/EBPβ making their ability to support the LPS-induction of IL-6 and MCP-1 all the more remarkable. Interestingly, although the levels of protein expression for the truncated forms of C/EBPβ are much lower than that of intact C/EBPβ, their binding activity in the EMSA is quite high. C/EBP$\beta_{192\text{-}276}$ has been reported to have enhanced affinity for its binding site (Williams et al., 1995).

A chimeric form of C/EBPβ with a heterologous leucine zipper has no activity in stable transfectants. Since C/EBP$\beta_{192\text{-}276}$ retains only the DNA binding and leucine zipper domains of C/EBPb yet is still active on the IL-6 promoter, we decided to examine whether the leucine zipper domain contains determinants for activation other than those necessary for dimerization with other C/EBP family members. To that end, a chimeric C/EBPβ where the C/EBPβ leucine zipper was replaced with that of yeast GCN4 (C/EBPβ:G_{LZ}) was stably expressed in P388 cells (P388-Cβ_{GLZ}) (Figure 1). C/EBPβ:G_{LZ} retains wild type amino terminal activation and regulatory domains as well as the DNA binding domain , and has

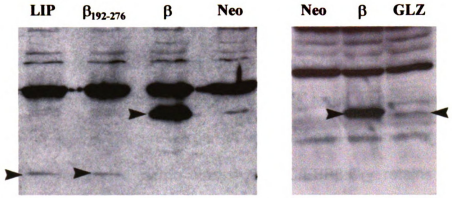

Figure 5. Western analysis of LIP, C/EBP$\beta_{192-276}$ ($\beta_{192-276}$) and C/EBPβ:G_{LZ} (GLZ) **expression** compared to that of C/EBPβ (β) in the P388 transfectants. Control **P388**-Neo (Neo) cells are included as a control. Arrowheads mark the position of **the C/EBP** proteins. The proteins on the left panel were detected with a carboxyl-**terminal**-specific C/EBPβ antibody, while the proteins on the right panel were **detected** with an amino-terminal-specific C/EBPβ antibody.

previously been shown to activate transcription from an albumin DE-I site-driven reporter (Williams et al., 1995). P388-Cβ_{GLZ} cells did not induce IL-6 or MCP-1 mRNA in response to LPS (Figure 2).

In order to verify that C/EBPβ:G_{LZ} was properly expressed in P388-Cβ_{GLZ} cells, both EMSA and western blot analyses were performed. EMSA revealed a protein-DNA complex that could be supershifted with amino-terminal specific but not carboxyl-terminus specific antibodies (Figure 3). This result was consistent with the replacement of the leucine zipper at the carboxyl-terminus of the C/EBPβ protein. This data also demonstrates that C/EBPβ:G_{LZ} retains the capacity to dimerize and is capable of binding to the C/EBP optimal binding site. As in the EMSA for the other stable transfectants, competitions were performed with the unlabeled C/EBP binding site and an unlabeled CTF/NF-1 binding site (Figure 6). Again, the supershifted protein-DNA complexes observed upon incubation with amino terminal C/EBPβ-specific antibody were effectively competed by a 30-fold excess of the C/EBP binding site, while a 100-fold excess of the CTF/NF-1 binding site had little effect. Western analyses of nuclear extracts from P388-Cβ_{GLZ} cells and other transfectant populations show that, while not expressed at as high a level as C/EBPβ, the C/EBPβ:G_{LZ} protein was expressed at a level comparable to C/EBP$\beta_{192\text{-}276}$ and LIP (Figure 5). All of the variant proteins have similar low levels of expression in comparison to C/EBPβ. The data suggest that some determinant for C/EBPβ activity on the IL-6 promoter apart from dimerization is located in the leucine zipper.

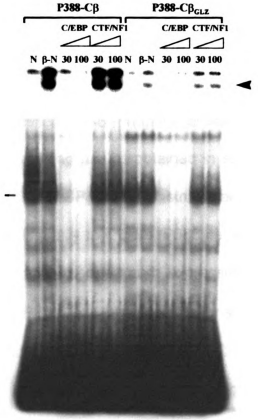

Figure 6. EMSA of C/EBP binding activities in P388 transfectants in the presence of unlabeled competing oligonucleotide binding sites. Binding reactions included normal rabbit IgG (N) or amino-terminal-specific C/EBPβ antibody (β-N). Some binding reactions, in addition to specific antibody, included 30-fold and 100-fold excess quantities of unlabeled C/EBP or CTF/NF1 binding oligonucleotides. The positions of C/EBP-specific antibody supershift species are indicated by the arrowhead on the right. The bar to the left indicates the position of the likely Ig/EBP (C/EBPγ) EMSA species.

C/EBPβ$_{192-276}$ and LIP are capable of supporting the LPS-induced activation of the IL-6 promoter in transient transfections. We decided to further examine the abilities of C/EBPβ$_{192-276}$, LIP, and C/EBPβ:G$_{LZ}$ to activate the IL-6 promoter in transient transfections with an IL-6 promoter-driven luciferase reporter. We wanted to confirm the surprising results obtained in stable transfections and to facilitate comparisons of C/EBPβ with its structural variants and analogous truncations of other C/EBP family members. P388 cells were cotransfected with an IL-6 promoter-luciferase reporter and expression vectors for C/EBPβ, C/EBPβ$_{192-276}$, LIP, or C/EBPβ:G$_{LZ}$. These transfections were carried out with and without LPS treatment. In addition to C/EBPβ, both C/EBPβ$_{192-276}$ and LIP were capable of supporting LPS-induction of the IL-6 promoter (Figure 7). While C/EBPβ transfectants with LPS treatment induced luciferase expression by a mean value of 12-fold over an untreated, "reporter-only" control , C/EBPβ$_{192-276}$ and LIP transfectants treated with LPS had levels of luciferase expression 8.3-fold and 6.2-fold of the control value, respectively. On the other hand, replacement of the leucine zipper in the chimeric C/EBPβ:G$_{LZ}$ showed a more dramatic reduction in the level of luciferase expression than loss of the activation domains, 4.8-fold of the control value. LPS stimulation by itself without transfection of a C/EBP expression vector produced a mean value of luciferase expression only 2.6-fold of the control value. The greater activity of C/EBPβ:G$_{LZ}$ observed in transient transfections in comparison to stable transfectants may be due to a higher level of expression in individual cells or differences in the extent of the promoter in the two assays. EMSA and western blot analysis did not

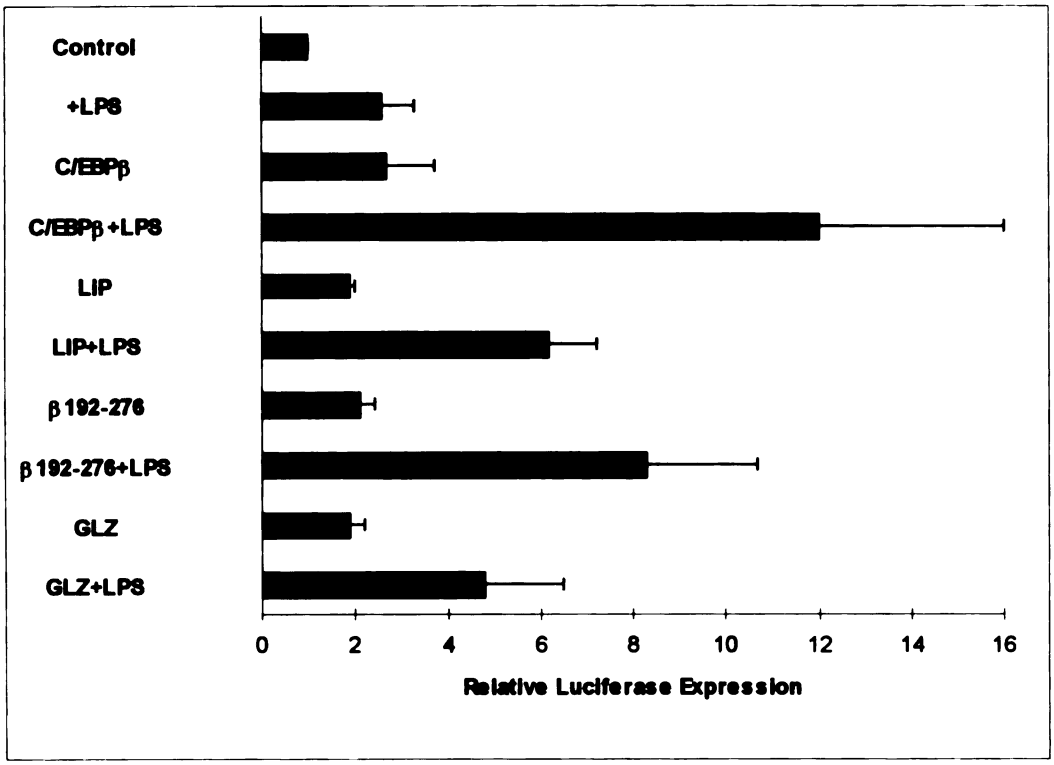

Figure 7. C/EBP$\beta_{192\text{-}276}$ (β192-276) and LIP, although lacking activation domains, can support the LPS induced activation of the IL-6 promoter in transient transfections of P388 cells. C/EBPβ:G_{LZ} (GLZ), possessing a heterologous leucine zipper, has reduced activity in comparison to C/EBPβ. Transient transfections were carried in out duplicate. 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 not receiving a C/EBP expression vector and untreated with LPS. The data presented are the mean of 3 experiments with their standard deviation.

detect C/EBPβ:G_{LZ} species in the transient assays, so its level of expression relative to the other forms of C/EBPβ could not be assessed as it was for the stable transfections (data not shown). Nonetheless, in either assay system, alteration of the leucine zipper domain has a greater impact on activity than complete loss of the conventional activation and regulatory domains(i.e. C/EBP$\beta_{192\text{-}276}$).

Other investigators may not have observed significant activity of C/EBP$\beta_{192\text{-}276}$ and LIP because they were tested on promoter-reporter constructs that are based on the DE-I site of the albumin promoter and were thus solely C/EBP-dependent (Descombes and Schibler, 1991; Williams et al., 1995). In order to test if C/EBP$\beta_{192\text{-}276}$ and LIP are, in fact, inactive on the albumin DE-I promoter in P388 cells, we performed transient transfections of C/EBPβ and its truncated forms with an albumin DE-I promoter-reporter (Figure 8). These transient assays show that LIP and C/EBP$\beta_{192\text{-}276}$ are, indeed, inactive on the simpler DE-I promoter, both in the presence and absence of LPS stimulation; presumably their activity on the IL-6 promoter is dependent on the interactions with other transcription factors that are available on the more complex promoter.

A truncated form of C/EBPδ analogous to C/EBP$\beta_{192\text{-}276}$ is capable of supporting the LPS-induced activation of the IL-6 promoter. We have previously shown that C/EBPα, β, and δ have virtually redundant activities in regards to the IL-6 promoter (Hu et al., 1998). These earlier results suggested that whatever structural feature that allows activity of C/EBP$\beta_{192\text{-}276}$ might be a general feature of C/EBPs. In order to test this hypothesis, we performed transient transfections

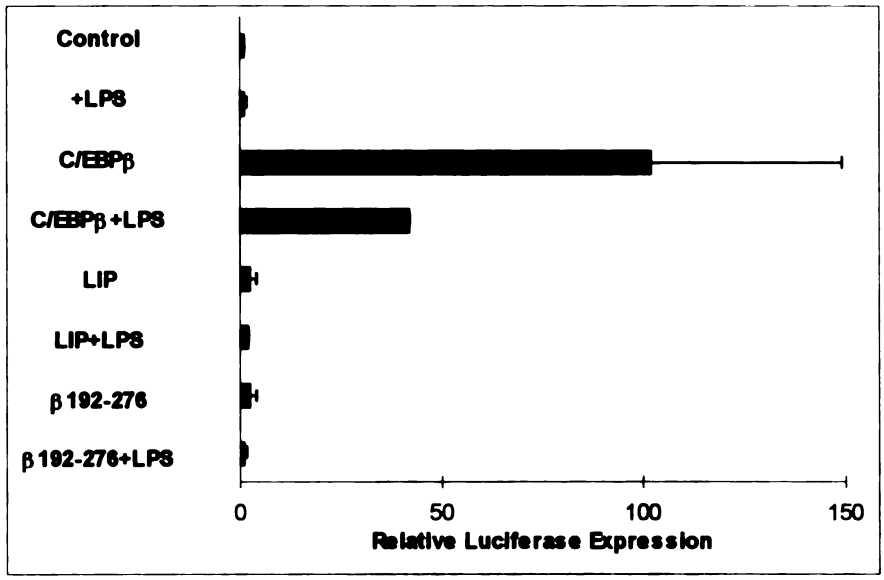

Figure 8. C/EBP$\beta_{192\text{-}276}$ (β192-276) and LIP fail to activate an albumin DEI site-reporter in transient transfections of P388 cells with and without LPS stimulation. Transient transfections were carried in out duplicate. Luminometer values were normalized for expression from a cotransfected SV40 early promoter-β–galactosidase reporter. These values were then normalized to a relative value of 1 for the cells not receiving a C/EBP expression vector and untreated with LPS. The data presented for transfections not treated with LPS are the mean of 3 experiments with their standard deviation. The data for transfections treated with LPS are the mean of one experiment with the standard deviation of the duplicate values.

with truncated forms of C/EBPα and C/EBPδ (C/EBP$\alpha_{273-358}$ and C/EBP$\delta_{181-272}$) in comparison to intact C/EBPβ in P388 cells (Figure 9). Again, the transfections were performed with and without LPS stimulation, and the expression vectors were cotransfected with an IL-6 promoter-luciferase reporter. C/EBP$\delta_{181-272}$ was almost as active as intact C/EBPβ. In this series of transfections, C/EBPβ transfectants with LPS treatment expressed luciferase at a mean value of 24-fold over an untreated, "reporter-only" control, while C/EBP$\delta_{181-272}$ transfectants treated with LPS had levels of luciferase expression of 20-fold over the control value. C/EBP$\alpha_{273-358}$ transfectants treated with LPS had levels of luciferase expression 7-fold over the control value and LPS treatment by itself without transfection of a C/EBP expression vector produced a mean value of luciferase expression only 3.8-fold of the control value. EMSA and western blot analysis did not detect either C/EBP$\delta_{181-272}$ or C/EBP$\alpha_{273-358}$ species, so their level of expression relative to C/EBPβ could not be assessed (data not shown). The modest level of activation with C/EBP$\alpha_{273-358}$ is consistent with the more modest activity of intact C/EBPα observed in LPS inductions of the endogenous IL-6 promoter in stable transfections (Hu et al., 1998).

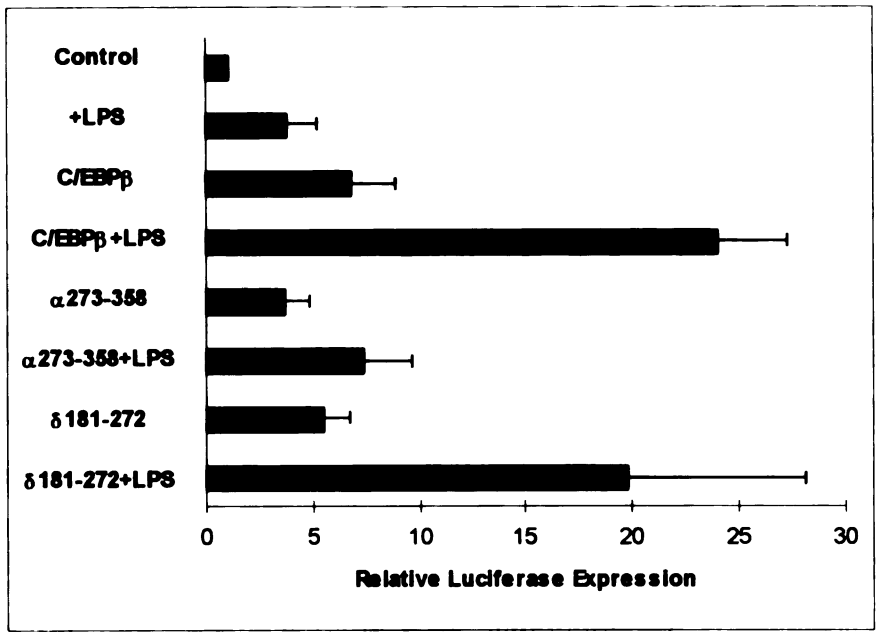

Figure 9. C/EBP$\delta_{181\text{-}272}$ (δ181-272), although lacking an activation domain, can support the LPS induced activation of the IL-6 promoter in transient transfections of P388 cells. C/EBP$\alpha_{273\text{-}358}$ (α273-358) has modest activity in comparison to C/EBPβ. Transient transfections were carried out in duplicate. 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 not receiving a C/EBP expression vector and untreated with LPS. The data presented are the mean of 3 experiments with their standard deviation.

DISCUSSION

The data presented in this paper demonstrate that the conventional activation domains of C/EBPβ (Williams et al., 1995) and C/EBPδ (P. F. Johnson, unpublished results) are dispensable for their roles in the LPS-induced activation of IL-6 and MCP-1 expression. Both C/EBP$\beta_{192\text{-}276}$ and LIP, truncated forms of C/EBPβ lacking their first 192 and 132 amino acids respectively, are capable of supporting the LPS-induced activation of IL-6 and MCP-1 transcription both in stable and transient transfections of P388 lymphoblasts. Transient transfections showed that C/EBP$\delta_{181\text{-}272}$, a similarly truncated form of C/EBPδ was also effective in activating the IL-6 promoter with LPS stimulation. A truncated form of C/EBPα, C/EBP$\alpha_{273\text{-}358}$, also showed modest activity.

The activity of the bZIP domains of C/EBP isoforms and of LIP is quite surprising. LIP, particularly, has been found to have very little or no transcriptional activity (Descombes and Schibler, 1991; Cooper et al., 1994; Cooper et al., 1995; Williams et al., !995). Previous investigators may not have observed this activity because they used different forms of C/EBPβ, different reporters and/or different cell types in their transfection systems. Matsuaka et al. (1993), using an embryonic carcinoma cell line, failed to observe activity of an internally deleted form of C/EBPβ on the IL-6 promoter. This mutant, however, would have retained one of three activation domains, as well as sequences that inhibit transactivation potential and mediate cell specificity (Williams et al., !995). Others have assayed LIP and C/EBP$\beta_{192\text{-}276}$ on promoter-reporter constructs

based on the DE-I site of the albumin promoter (Descombes and Schibler, 1991; Williams et al., 1995) or other tandem arrangements of C/EBP binding sites (Cooper et al., 1994; Cooper et al., 1995), all of which are solely C/EBP-dependent. We also find that these forms of C/EBPβ are inactive on a DE-I albumin-based promoter in P388 lymphoblasts (Figure 8). Presumably, the activity that we have observed is dependent on the interactions with other transcription factors that are available on the more complex IL-6 and MCP-1 promoters.

There is good evidence that C/EBPβ and NF-κB synergistically activate the IL-6 promoter (Matsuaka et al., 1993). C/EBPα, β, and δ have been shown to synergize with NF-κB in activating the IL-8 promoter (Stein and Baldwin, 1993; Kunsch et al. 1994). This synergy may not only involve binding of the factors to their cognate binding sites, but direct physical association through their respective basic region-leucine zipper (bZIP) and Rel homology domains (Stein et al, 1993; Stein and Baldwin, 1993). The basis for the activity of truncated C/EBPs on the IL-6 promoter may rest in the capacity of the bZIP domain by itself to effect synergy with NF-κB in the absence of C/EBP activation domains. We have only observed robust activation of the IL-6 promoter by C/EBPs under conditions of LPS stimulation (Bretz et al., 1994; Hu et al., 1998; this paper) and LPS stimulation does indeed induce NF-κB binding activity in P388 cells (Hu et al. 1998). Future experiments will need to examine the ability of truncated forms of C/EBPβ, as well as point mutants within the bZIP domain, to synergize with NF-κB in activating the IL-6 promoter.

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Another possible mechanism by which a C/EBP bZIP domain could of itself support the LPS induction of IL-6 and MCP-1 expression is as a structural component in "enhanceosome" assembly (Thanos and Maniatis, 1995; Merika et al., 1998). Perhaps even in the absence of any inherent activation potential, the bZIP domain through its occupation of the C/EBP binding site on the IL-6 promoter allows enhanceosome assembly. Arguing against this is the fact that untransfected P388 cells, which cannot effectively express IL-6 upon LPS induction, exhibit C/EBPγ (Ig/EBP) DNA binding activity (Hu et al., 1998). C/EBPγ is a transdominant negative regulator of transcription that binds to the C/EBP consensus binding site (Roman et al., 1990; Cooper et al. 1995). Presumably C/EBPγ could perform the role of simply occupying the C/EBP binding site with a bZIP domain.

Whatever the mechanism of bZIP activity in the LPS induction of IL-6 and MCP-1 expression, our findings suggest that the basis for C/EBP redundancy in the activation of these genes (Hu et al., 1998) resides in this well-conserved region that is shared by all C/EBP isoforms. Our experiments replacing the leucine zipper of C/EBPβ with that of GCN4 suggest that the critical structural feature for activity of bZIP domains may be further localized to the leucine zipper itself. The replacement of the leucine zipper that produces the chimeric C/EBPβ:G_{LZ} protein shows a greater decrement in activity in comparison to intact C/EBPβ than removal of the activation domains. There is some uncertainty in the interpretation of the result because the levels of C/EBPβ:G_{LZ} expression are below that for intact C/EBPβ. On the other hand, the level of C/EBPβ:G_{LZ}

expression is similar to that of C/EBP$\beta_{192\text{-}276}$ expression (Figure 5), which is a far more potent transcriptional activator (Figures 2 and 7). It is likely that the leucine zipper possesses critical determinants for the activity of C/EBPs on the IL-6 promoter other than mediating dimerization to known C/EBP family members. For example, the leucine zipper might mediate dimerization to an as yet unknown dimerization partner with inherent activation activity or, as proposed above, it might mediate the synergistic activation of NF-κB activity. The leucine zippers of C/EBP proteins have previously been implicated in functions beyond dimerization. The leucine zipper of C/EBPα has been shown to mediate cell type specificity of albumin promoter activation (Nerlov and Ziff, 1994). This effect can be mediated by a mutant in the nonhydrophobic face of the zipper. Another instance of a non-dimerization function residing in the leucine zipper is that of serine 276 of human C/EBPβ (Wegner et al., 1992). Phosphorylation of this serine confers calcium-regulated transcriptional stimulation to a promoter that contains binding sites for C/EBPβ. In future experiments, it will be useful to examine single amino acid substitutions in the leucine zipper not only because they may more sharply delineate critical structural features, but because these altered forms of C/EBPβ may provide levels of expression more comparable to that of intact C/EBPβ and thus allow more direct comparisons of activity.

Finally, our results surprisingly show a significant capacity for LIP to support the LPS induction of IL-6 and MCP-1 expression. LIP has previously been proposed to be a transdominant negative inhibitor of transcription (Descombes and Schibler, 1991). It is proposed that high levels of LIP observed in fetal liver

constitute a mechanism for inhibiting the activity of other C/EBP isoforms in hepatocytes that are not yet terminally differentiated. The regulation of LIP expression has also been proposed to play a role in the regulation of lactation-associated genes such as β-casein (Raught et al., 1995). More recently, LIP expression has been correlated neoplastic transformation of mammary tissue and has been proposed as a prognostic indicator for breast cancer because of its overexpression in breast tumors that were negative for the estrogen and progesterone receptors (Raught et al., 1996; Zhanow et al., 1997). The central theme of these models is that the expression of LIP in immature proliferating cells suppresses the activity of C/EBPβ and other isoforms in activating the expression of gene products associated with terminal differentiation. It is clear from the findings reported here that conditions favoring LIP expression would not universally down-regulate C/EBPβ-regulated genes, but would be permissive for the expression of IL-6, MCP-1, and genes with a similar promoter structure. IL-6 and MCP-1 are certainly genes whose expression has been associated with the function of mature terminally differentiated cell types. The findings reported here of LIP activity in the expression of these genes, as well as a recent report that LIP can be the product of proteolysis associated with certain isolation procedures (Baer et al. 1998), call for a reexamination of the role of LIP. It will be a high priority in future investigations to examine the activity of LIP on the promoters of other genes encoding proinflammatory cytokines, as well as several milk protein genes that are apparently regulated by C/EBPβ (Robinson et al., 1998). The

correlation of LIP activity to promoter structure may provide clues to the mechanism of bZIP activity in the absence of conventional activation domains.

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Chapter 4

Regulation of the Expression of Primary Granule Proteins by the C/EBP Transcription Factor Family

Hsien-Ming Hu and Richard C. Schwartz

ABSTRACT

C/EBP transcription factors have been implicated in the tissue-specific and temporal regulation of a number of genes encoding primary granule proteins in granulopoiesis. In order to evaluate the abilities of various C/EBP isoforms to regulate the transcription of primary granule genes, we have ectopically expressed C/EBPα, -β, -δ, and -ε individually in the granulocytic progenitor cell line, 32D cl3. 32D cl3 cells transfected for C/EBPβ expression (32D-Cβ) have the greatest enhancement of mRNA levels for the genes encoding myeloperoxidase (MPO), cathepsin G (Cat G), and lysozyme (LZ). They display levels of expression similar to the G-CSF-induced 32D cl3 parental cells. Transfectants for expression of other C/EBP isoforms also show a modest increase in the mRNA levels of the aforementioned genes. EMSA and Western blot analyses suggest that a C/EBPα-C/EBPβ heterodimer is the predominant form of C/EBP present in the nucleus of 32D-Cβ cells. The predominance of the C/EBPα-C/EBPβ heterodimer and increased binding of this form of C/EBP in 32D-Cβ cells suggest that the C/EBPα–C/EBPβ heteodimer is an important regulator of the primary granule-associated genes.

INTRODUCTION

Neutrophilic granulocytes (also known as polymorphonuclear cells, or PMNs) are specialized phagocytic cells characterized by their distinct mutilobed nuclei and granulated cytoplasms. Neutrophils carry two types of granules (reviewed in Borregaard et al. 1993). Primary or azurophilic granules which contain peroxidase, lysozyme and various hydrolytic enzymes are formed first at the promyelocyte stage of granulopoiesis. Secondary granules which contain collagenase, lactoferrin and lysozyme develop at the later myelocyte stage. The expression of granule-associated genes appears to be regulated primarily at the level of transcription (Grisolano et al. 1996). Most of the primary granule genes are transcriptionally activated at the beginning of the promyelocyte stage and are transcriptionally repressed at the transition to the myelocyte stage. The mechanism that controls this special temporal expression is not well understood.

The identification of common *cis*-acting DNA elements and transcription factors in the regulation of many primary granule genes has shed some light on the mechanism of their stage- and tissue-specific expression (Friedman, 1996). Among the important transcription factors that have been found to be involved in the regulation of many primary granule-associated genes is the C/EBP family. C/EBP proteins belong to a family of basic region-leucine zipper transcription factors with highly homologous DNA binding and dimerization domains (reviewed in Johnson et. al. 1994). Homo- and heterodimers are readily formed within the family and bind to a similar DNA target site. Within the hematopoietic system,

high level expression of C/EBPs is restricted to the myelomonocytic lineages. Indeed, multiple C/EBP family members are expressed in differentiating myelomonocytic cells and show a distinct temporal expression pattern (Scott et al. 1992). Several studies have shown that the promoters of the genes encoding myeloperoxidase (MPO) (Zhu et al. 1994), neutrophil elastase (NE) (Oelgeschlager et al. 1996), azurocidin (Friedman 1996), and myeloblastin (Zimmer et al. 1992), all components of the primary granule, contain known or predicted binding sites for C/EBP. C/EBPα is shown to bind to the C/EBP site in the NE promoter and transactivate the NE promoter alone or cooperatively with other transcription factors including c-Myb, PU.1 and AML-1 (Oelgeschlager et al. 1996). C/EBPα is proposed to be the major form of C/EBP that regulates the NE gene because it is the most active among C/EBP family members in transient cotransfection experiments. C/EBPβ and –δ are also active, although to a lesser extent. A distal enhancer, which contains multiple C/EBP binding sites, has been shown to be responsible in part for the tissue- and stage-specific expression of the MPO gene (Zhu et al. 1994). It is suggested that C/EBPβ and C/EBPδ are the two major C/EBP isoforms that bind to these C/EBP binding sites and activate the transcription of the MPO gene; C/EBPα is mainly present in earlier precursor cells that do not express MPO (Ford et al. 1996). Thus, it remains uncertain as to which C/EBP family member is the major regulator of these primary granule-associated genes. The answer may be more complicated than is suggested by prior studies, as multiple C/EBPs are expressed by the differentiating granulocytic cells in an overlapping manner. So far, four C/EBP family members,

including C/EBPα, -β, -δ, and –ε have been found to be expressed during granulopoiesis. The variety of possible homo- and heterodimers may present a very complex regulatory scheme for the roles C/EBPs in the expression of primary granule-associated genes.

We have tried to address this issue by individually over-expressing C/EBPα, -β, –δ, and –ε in the murine 32D cl3 cell line (Valtieri et al. 1987), which is an immature myeloid precursor that has not yet expressed granule proteins. Stable transfection of a C/EBPβ expression vector appears to induce the greatest enhancement in the levels of primary granule protein mRNAs. Furthermore, we have found that the C/EBPα–C/EBPβ heterodimer is the predominant form of C/EBP in these transfectants. Increased levels of C/EBPα-C/EBPβ binding detected in nuclear extracts of cells transfected for C/EBPβ expression suggest that the C/EBPα-C/EBPβ heterodimer is an important regulator of the genes encoding primary granule proteins.

MATERIALS AND METHODS

Cells and transfection. The murine 32D cl3 cells (Valtieri et al. 1987) were maintained at 37°C in a 5% CO2 environment in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS and 10% WEHI3-conditioned medium as a source of interleukin-3 (IL-3). For induction of granulocytic

differentiation, cells were washed twice with phosphate-buffered saline (PBS) and plated in IMDM supplemented with 15% heat-inactivated FCS and 15% WEHI274.1-conditioned medium as a source of G-CSF. Stable transfection of 32D cl3 cells was accomplished by using a liposome-mediated transfection protocol. Briefly, 10^7 cells were incubated with 4 μg of DNA and 15 μl of DMRIE-C reagent in 1.2 ml of Opti-MEM I medium for 12 hr followed by the addition of 2 ml of IMDM supplemented with 15% FCS and 15% WEHI3-conditioned medium. After 36 hr, the medium was replaced by the standard growth medium supplemented with G418 at 1.0 mg/ml. Stably transfected cells were maintained in the presence of G418 at 0.25 mg/ml.

Expression Vectors. pSV(x)Neo is pZIP-NEO SV(x)1 (Cepko et al. 1984) (Fig. 1) and expresses inserted sequences from the promoter of Moloney murine leukemia virus. This vector expresses the gene for neomycin (neo) resistance through alternative splicing of a transcript from the same promoter. pSV(x)C/EBPα was constructed by insertion of the BamHI/KpnI fragment encoding rat C/EBPα from pMEXC/EBP (Williams et al. 1991) into the BamHI site of pSV(x)Neo with BamHI linkers. pSV(x)C/EBPβ was constructed by insertion of the BamHI fragment encoding rat C/EBPβ from pMEXCRP2 (Williams et al. 1991) into the BamHI site of pSV(x)Neo. To construct an expression vector for C/EBPδ, the sequences encoding murine C/EBPδ (Williams et al. 1991) were first inserted into the SphI and HindIII sites of pMEX (Williams et al. 1991) by a three-part ligation: one inserted fragment extended from a PCR-

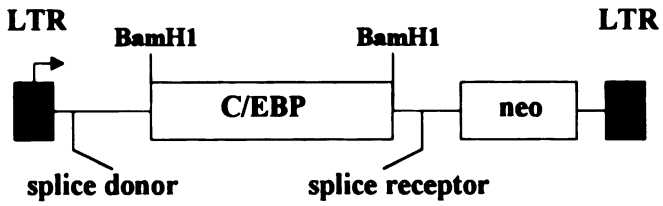

Figure 1. Structure of the retroviral vector pSV(X)Neo containing the C/EBP cDNA inserted at a BamH1 site. LTR, LTRs are derived from the murine Moloney virus. The transcription start site is indicated by the arrowhead. The donor and receptor sites for alternative splicing are also indicated.

introduced SphI site 40 bp upstream of the C/EBPδ initiation codon to an ApaI site approximately 100 bp into the coding sequence, and the other fragment extended from the ApaI site to a PCR-introduced HindIII site just downstream of termination codon. The SphI/HindIII fragment was then inserted with BamHI linkers into the BamHI site of pSV(x)Neo to produce pSV(x)C/EBPδ. To construct an expression vector for C/EBPε, the sequences encoding rat C/EBPε were excised from pMEXC/EBPε (Williams et al. 1998) with HindIII, and the 5' overhangs were made blunt using Klenow polymerase, and a BamHI linker was added. The resulting fragment was inserted into the BamHI site of pSV(x)Neo to generate pSV(x)C/EBPε.

Nucleic acid isolation and analysis. Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's directions. RNAs were electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to a stringency of 0.1% SSPE at 65°C. Hybridization probes were prepared with a random priming kit (Life Technologies) with the incorporation of 5'-[α-32P]dATP (3000 Ci/mmol; DuPont-New England Nuclear, Newton, CT). The MPO probe was a 0.8-kb murine cDNA (Friedman et al. 1991). The lysozyme probe is a 1.0-kb human cDNA. The cathepsin G probe was a 0.5-kb murine cDNA cloned by differential display PCR of control and C/EBPβ transfectants of 32D cl3 (Tian and Schwartz, unpublished). The GAPDH probe was a 1.3-kb rat cDNA (Fort et al. 1985). The C/EBPα probe was a 1.0-kb murine genomic DNA fragment.

Western analysis. Nuclear extracts were prepared as described below. The extracts (50 μg) were adjusted to 1x Laemmli sample buffer (Laemmli 1970) and processed on a 12% PAGE gel. The gel was transferred to a Protran membrane (Schleicher and Schuell, Keene, NH), and Ag-Ab complexes were visualized with the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

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 14,000 x *g* for 20 sec at 4°C. Proteins were extracted from nuclei by incubation at 4°C with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 25% [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 14,000 x *g* for 15 min at 4°C and the supernatant extract was collected and stored at -70°C.

The EMSA probe was a double-stranded oligonucleotide containing an optimal C/EBP binding site (5'-GATCCTAGATATCCCTGATTGCGCAATAGGC-TCAAAGCTG-3' annealed with 5'-AATTCAGCTTTGAGCCTATTGCGCAATC-AGGGATATCTAG-3') 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 binding motifs of the specified transcription factors.

DNA binding reactions were performed at room temperature in a 25 μl reaction mixture containing 6 μl of nuclear extract (1mg/ml in buffer C)] and 5 μl of 5 x 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), 1.25 ng of probe, bromophenol blue to a final concentration of 0.06% [wt/vol], and water to volume. For supershifts, nuclear extracts were preincubated with antibodies for 30 min at 4°C prior to the binding reaction. Samples were electrophoresed through 5.5% polyacrylamide gels in 1x TBE (90 mM Tris base, 90 mM boric acid, 0.5 mM EDTA) at 160 V.

Antibodies: Rabbit antibodies against C/EBPα (14AA), C/EBPβ (C-19), C/EBPδ (C-22) and C/EBPε (C-22) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Ectopic expression of C/EBP family members in 32D cl3 cells. 32D cl3 is an interleukin 3 (IL-3)–dependent hematopoietic precursor cell which can be induced to differentiate into neutrophilic granulocyte by G-CSF (Valtieri et al.

1987). This cell line has provided a useful system to study the process of neutrophil differentiation (Laneuville et al. 1991, Kreider et al. 1992, Patel et al. 1993). In order to compare the abilities of the C/EBP family members to induce granule-associated genes, a murine retroviral expression vector pSV(x)Neo was used to ectopically express C/EBP isoforms in 32D cl3 cells. Pools of G418-resistant cells were isolated. Cells transfected with pSV(X)C/EBPα, pSV(X)C/EBPβ, pSV(X)C/EBPδ or pSV(X)C/EBPε were designated 32D-Cα, 32D-Cβ, 32D-Cδ and 32D-Cε, respectively. Control populations transfected with empty vectors were also generated and designated 32D-Neo. For each construct, two independent G418-resistant populations were examined in order to ensure reproducibility of our findings. To verify that the introduced vectors were properly transcribed, total RNA was isolated from these transfectants and analyzed by Northern blotting. As shown in Fig. 2, the C/EBP transfectants produced two transcripts specific to the gene for neo resistance. As predicted, one transcript is the full length of the retroviral genome from the 5' LTR to the 3' LTR, and the other is a shorter transcript resulting from alternative splicing.

<u>Elevated expression of primary granule protein mRNAs in 32D cl3 transfectants</u>. Total RNA was isolated from cultures of 32D-Cα, -Cβ, -Cδ, and –Cε as well as the 32D-Neo control cells. As a positive control, total RNA was also isolated from 32D cl3 cells induced by G-CSF for 3 and 7 days. Northern analyses were performed to detect the transcripts encoding several granule-associated genes including myeloperoxidase (MPO), cathepsin G (Cat G), lysozyme (LZ), and

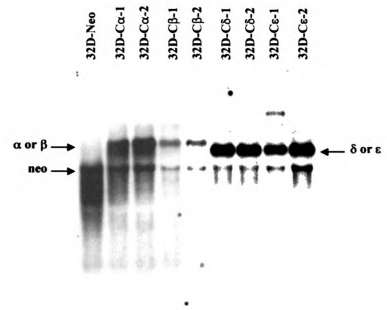

Figure 2. Northern blot analysis of 32D transfectants. Twenty microgram of total RNA was analyzed on a northern blot and was hybridized to a probe for neomycin (neo). The positions of the transcripts containing various C/EBPs as well as neo are indicated by the arrowheads.

lactoferrin (LF). Transcripts encoding GAPDH were also examined as a normalization control. As shown in Fig. 3, a strong induction of MPO transcription is observed in 32D cl3 cells transfected for C/EBPβ expression. Transfectants for C/EBPα, C/EBPδ and C/EBPε expression showed a more modest elevation in the level of MPO expression in comparison to the 32D-Neo control cells. The level of MPO expression in 32D-Cβ cells is similar to that of 32D cl3 cells treated with G-CSF for 3 or 7 days. To quantify the level of expression, Northern blots were analyzed using an Ambis radioanalytic scanner. All values for MPO expression were normalized to those of GAPDH as a control for loading (Table 1). A 5.4 fold induction of MPO mRNA expression is observed for 32D-Cβ cells compared to control cells. The fold of induction for 32D-Cα, -Cδ, and -Cε is 2.5, 2.7, and 3.3, respectively (Table 1).

Cathepsin G, a serine protease expressed in the primary granule, has been found to be coexpressed with MPO during granulopoiesis (Hanson et al. 1990). A Northern analysis in Fig. 3 shows that Cat G transcription is also induced in the 32D cl3 transfectants in a manner very similar to that of MPO. One difference, however, is that 32D-Neo control cells express very little, if any, Cat G mRNA, making the enhanced expression in the transfectants even more robust. Quantitation shows that the induction of Cat G mRNA in 32D-Cβ is 10.2 fold, while 2.6, 3.1 and 3.2 fold inductions are seen for 32D-Cα, -Cδ and –Cε, respectively (Table 1).

Lysozyme is a component of both primary and secondary granules (Borregaard et al. 1993). The presence of a C/EBP binding site in the promoter of the LZ

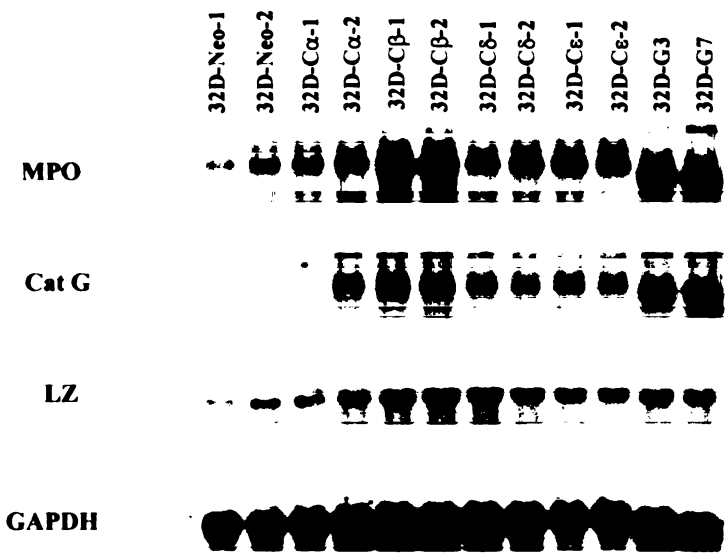

Figure 3. Induction of granule proteins myeloperoxidase (MPO), cathepsin G (Cat G), and lysozyme (LZ) in 32D cl3 transfectants. Total RNA was isolated from transfectants of 32D cl3 that express C/EBPα (32D-Cα), C/EBPβ (32D-Cβ), C/EBPδ (32D-Cδ) and C/EBPε (32D-Cε). RNA was also isolated from the vector controls (32D-Neo) and 32D cl3 parental cells induced by G-CSF for 3 days (32D-G3) and 7 days (32D-G7). Twenty micrograms of RNA was analyzed on northern blots and hybridized in parallel to probes for MPO, Cat G, LZ, and GAPDH. For each construct, two independent transfectants were analyzed.

Table 1. Induction of MPO, Cat G and LZ transcriptions in 32D cl3 transfectants*

Transfectants or treatment	Fold of activation		
	MPO	Cat G	LZ
32D-Neo	1.0	1.0	1.0
32D-Cα	2.5	2.6	1.5
32D-Cβ	5.4	10.2	3.2
32D-Cδ	2.7	3.1	2.0
32D-Cε	3.3	3.2	1.5
32D-G3	6.2	12.2	2.9
32D-G7	6.9	13.2	1.9

*The blots presented in Figure 3 were analyzed by Ambis scanner, and raw data for MPO, Cat G and LZ were normalized to value for GAPDH. The expression levels for 32D-Neo control were set as 1.0. The data shown are average of two experiments.

gene has been demonstrated (Goethe et al. 1994). It has also been shown that the avian C/EBPβ homolog, NF-M can transactivate the LZ promoter in concert with c-Myb (Ness et al. 1993). Fig. 3 shows that 32D cl3 cells transfected for C/EBPα, β, δ and ε expression have a rather modest elevation in their expression of lysozyme mRNA compared to the control populations. Quantitatively, an increase of 3.2 fold is seen in 32D-Cβ cells, and 2.0 fold in 32D-Cδ cells, while 1.5 fold increase in expression are observed for 32D–Cα and –Cε transfectants. The expression of lactoferrin (LF), a protein of the secondary granule (Rado et al. 1987), was also examined in 32D transfectants. LF RNA was not detectable in any of the transfectants (data not shown), a result consistent with the findings that LF is induced only at the mature stage of granulopoiesis and that LF is probably not regulated by C/EBPs (Friedman et al. 1991).

Our results clearly show that 32D cl3 populations transfected for C/EBPβ expression have elevated levels of transcripts encoding MPO, Cat G and LZ. 32D cl3 transfectants for C/EBPα, δ or ε expression show more modest elevations in the levels of these transcripts.

EMSA of 32D cl3 transfectants. The expression of multiple C/EBP isoforms in 32D cl3 cells (Scott et al. 1992) coupled with the ectopic expression of individual isoforms from introduced expression vectors could yield a diverse repertoire of homo- and heterodimeric C/EBP transcription factors. To determine whether there is any correlation between the levels of primary granule mRNA and the

pattern of C/EBP-binding activities, EMSAs were performed on nuclear extracts isolated from the transfectants. Specific antibodies against individual C/EBP family members were used to identify the corresponding C/EBP protein-DNA complexes. Endogenous expression of C/EBPα and C/EBPβ, as well as a minor amount of C/EBPδ is observed in 32D-Neo control cells with specific antibodies that yield supershifted protein-DNA complexes. The total amount of C/EBPβ–DNA complex appears to be somewhat more abundant than that for C/EBPα.

In 32D-Cα cells, C/EBPα binding activity is increased compared to control cells, presumably, due to expression from the transfected vector, while the level of C/EBPβ binding activity remains similar to that of control cells. An inspection of the C/EBPβ binding activity in 32D-Cβ cells surprisingly does not reveal any increase in C/EBPβ-DNA complexes. On the other hand, the C/EBPα binding activity in 32D-Cβ cells is strongly increased, probably to a level even higher than that of 32D-Cα. This suggests that the ectopic expression of C/EBPβ may induce expression of the endogenous C/EBPα gene. EMSA for 32D-Cδ cells clearly demonstrates the expected increase in C/EBPδ binding activity. Although the 32D-Cδ EMSA may be somewhat underloaded, there is a relatively low abundance of C/EBPα binding activity compared to other transfectants. Lastly, an increase of C/EBPε binding activity is observed in 32D-Cε cells, while the levels of C/EBPα and C/EBPβ binding activities are similar to those in 32D-Neo cells. A comparison of supershifts with antibodies to C/EBPα and C/EBPβ

reveals protein-DNA species (marked by bar number 1 in Fig. 4) that are reactive with either antibody as opposed to species reactive only to anti-C/EBPβ (marked bar number 2 in Fig. 4) This suggests that the majority of C/EBPα is present as a heterodimer with C/EBPβ.

While our EMSAs clearly demonstrate the expected increase in C/EBPα, C/EBPδ and C/EBPε binding activities in their corresponding transfectants, a more accurate assessment of C/EBPβ expression is required. Although 32D-Cβ cells express RNA from the transfected C/EBPβ expression vector, no increase in C/EBPβ binding activity is detected. Western blot analysis was performed to more accurately examine the abundance of C/EBPβ and other C/EBP family members in these transfectants.

Western analysis of 32D cl3 transfectants. Western analyses of nuclear extracts from the transfected populations were performed in order to assess the actual levels of C/EBP protein (Fig.5). Antibody to C/EBPα detect two immunoreactive species. The slower-migrating band corresponds to the 42 kDa full-length C/EBPα protein, while the faster-migrating band corresponds to the 30 kDa polypeptide resulting from alternative translation of the C/EBP mRNA. As predicted, 32D-Cα shows an increase in C/EBPα protein expression. Unexpectedly, the expression levels of C/EBPα are also strongly enhanced in 32D-Cβ and –Cδ transfectants. The C/EBPα protein level of 32D-Cβ is even higher than that of 32D-Cα. This result suggests that ectopic expression of

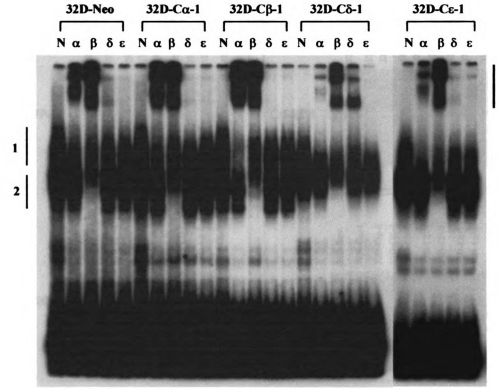

Figure 4. EMSA of C/EBP DNA binding activities in 32D cl3 cells stably transfected with C/EBPα, C/EBPβ, C/EBPδ and C/EBPε expression vectors. Reactions included normal rabbit serum (N), C/EBPα antibody (α), C/EBPβ antibody (β), C/EBPδ antibody (δ), or C/EBPε antibody (ε). The bar to the *right* indicates the positions of supershifted EMSA species. The bars to the *left* indicate the positions of protein-DNA complexes reactive with both C/EBPα and C/EBPβ antobodies (1), and protein-DNA complexes only reactive with C/EBPβ antibody (2).

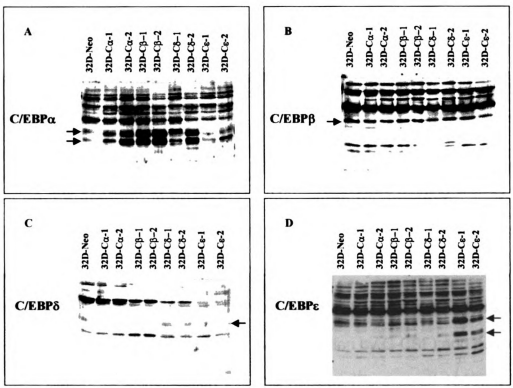

Figure 5. Western blot analyses of C/EBP proteins derived from nuclear extracts of the transfectants. The nomenclature for each cell line is explained in *Results*. Specific antibodies were used to detect *A*. C/EBPα, *B*. C/EBPβ, *C*. C/EBPδ, and *D*. C/EBPε. The positions of C/EBP proteins are indicated by the arrowheads.

Another surprising result is obtained in western blot analyses for C/EBPβ. The expression level of C/EBPβ protein is not increased in either of the C/EBPβ transfectants. In fact, all of the transfectants have a similar level of C/EBPβ expression. Since the Northern blot demonstrates expression of the C/EBPβ expression vector at the RNA level (Fig. 2), one possible reason for our inability to detect any increase in either C/EBPβ protein or DNA binding activity in the nuclear extract of 32D-Cβ is that the protein is retained in the cytoplasm. In support of this view, it has been shown in immature myeloid cells that C/EBPβ is largely retained in the cytoplasm in an unphosphorylated form. It is possible that it requires an extracellular signal (i. e. the stimulation by G-CSF) and subsequent phosphorylation for translocation into the nucleus (Ford et al. 1996). Western blots using C/EBPδ and C/EBPε antibodies show increased protein expression in the corresponding 32D transfectants, as expected.

Induction of endogenous C/EBPα expression in C/EBPβ and C/EBPδ transfectants. It was unclear from EMSA and western analyses as to what mechanism mediates the strong induction of C/EBPα protein in 32D-Cβ and –Cδ transfectants. In order to determine whether level of endogenous C/EBPα mRNA is increased, a northern analysis was performed. As shown in Fig. 6, a radiolabeled probe for C/EBPα detects two transcripts of different sizes. The longer species represents the transcript from the transfected vector, while the shorter one represents the endogenous C/EBPα mRNA. Clearly, the endogenous C/EBPα mRNA shows increased expression in both 32D-Cβ and

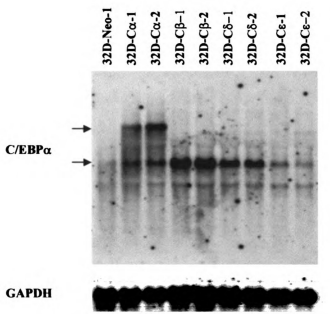

Figure 6. Northern blot analysis of C/EBPα expression in 32D transfectants. Twenty micrograms of RNA was analyzed on a northern blot and hybridized to a probe for C/EBPα. The position of C/EBPα transcripts are indicated by the arrowheads. The upper one indicates the transcript derived from the transfected expression vector while the lower one indicates the endogenous C/EBPα.

–Cδ cells relative to 32D-Neo control cells. This result shows that C/EBPα mRNA levels are upregulated by either C/EBPβ or C/EBPδ expression and suggests a transcriptional mechanism for this increase.

DISCUSSION

The tissue and stage-specific expression of the granule-associated genes in granulopoiesis has been shown to be regulated mainly at the level of transcription (Friedman et al. 1991, Yoshimura et al. 1992, Sturrock et al. 1996). The C/EBP transcription factor family has been implicated in the regulation of primary granule-associated genes (Ford et al. 1996, Oelgeschlager et al. 1996, Sturrock et al. 1996). We have directly compared the abilities of C/EBPα, -β, -δ and –ε to induce the transcription of the genes encoding primary granule-associated proteins including MPO, Cat G, and LZ in the murine myeloid precursor cells, 32D cl3. The data presented in this report show that ectopic expression of C/EBPβ in 32D cl3 results in a dramatic increase in the levels of MPO and Cat G mRNA, levels similar to those of G-CSF-induced 32D cl3 cells. A more modest increase in LZ mRNA is also observed. In addition, transfectants for C/EBPα, -δ and –ε expression all show a small but significant increase in the transcripts encoding MPO, Cat G and LZ compared with the control cells. The elevated transcription is most likely a direct effect of the elevated expression of C/EBP proteins resulting from their ectopic expression, as binding sites for C/EBP have been identified in the promoter of the LZ gene, as well as the distal enhancer of the MPO gene. Interestingly, no C/EBP binding site has been found in the immediate 5'-region of the Cat G promoter. Our results strongly suggest the involvement of C/EBPs in the regulation of Cat G transcription. It is very likely that a functional C/EBP binding site is present further upstream or downstream of

the previously characterized Cat G promoter. On the other hand, the possibility cannot be ruled out that the ectopically expressed C/EBPs may have induced other transcription factors which work in concert with C/EBP proteins in the promoters of the primary granule-associated genes. For example, it is suggested that the transcription of the murine NE gene requires the cooperation of PU.1, c-Myb and C/EBPs for maximal expression.

Our data have shown that 32D cl3 cells transfected for C/EBPβ expression have the highest level of transcription for the MPO, Cat G and LZ genes among other transfectants. EMSA and Western analyses, however, did not reveal an increase in the level of C/EBPβ protein in nuclear extracts of 32D-Cβ cells. Instead, a large increase in the level of C/EBPα is observed. It is clear that the total level of C/EBP proteins and, particularly, the level of C/EBPα-C/EBPβ heterodimer DNA binding activity in the nucleus are increased compared with the 32D cl3 control cells. In fact, in control cells, 32D-Cβ cells and all transfectants with the exception of 32D-Cδ, C/EBPα-C/EBPβ heterodimers appear to be the predominant form of C/EBP detected by EMSA. This suggests that the increase in C/EBPα-C/EBPβ heterodimers caused the up-regulation of the transcripts encoding MPO, Cat G and LZ. The observation that 32D-Neo cells have a considerable level of endogenous C/EBPα and C/EBPβ but do not express high levels of transcripts for MPO, Cat G and LZ may suggest a threshold for the concentration of C/EBPα-C/EBPβ in the nucleus to induce a high level of transcription of these genes. The fact that the levels of these transcripts are not as high in 32D-Cα as they are in 32D-Cβ may be explained by C/EBPα-C/EBPβ

being the most active form of C/EBP in regulating these genes. The increased amount of C/EBPα by itself would not be expected to increase the abundance of C/EBPα-C/EBPβ. Rather, both isoforms would need to be elevated as in the case of 32D-Cβ. The more modest induction of transcription for the MPO, Cat G and LZ in 32D-Cδ and –Cε may reflect the weaker activities of C/EBPδ- or C/EBPε-containing C/EBP dimers. The relative lack of C/EBPα DNA binding activity in 32D-Cδ may be a reflection of C/EBPα-C/EBPδ heterodimers having weaker DNA binding activity than C/EBPα-C/EBPβ heterodimers. This could be tested by in vitro binding assays using recombinant C/EBPα, β and δ and heterodimers thereof.

It is intriguing that although Western analyses did not detect an increase of C/EBPβ protein in nuclear extracts of 32D-Cβ cells, the cells have the strongest elevation in the mRNA levels of several primary granule genes. This may reflect post-transcriptional regulatory mechanisms of C/EBPβ activity. Ford et al. (1996) have shown that C/EBPβ is unphosphorylated and localized in the cytoplasm of early progenitor cells, but exists as a phosphorylated form capable of binding to its cognate DNA site in the nuclei of granulocyte-committed cells. Furthermore, G-CSF-induced granulocytic differentiation of multipotential progenitor cells results in the functional recruitment of C/EBPβ to the nucleus. It is thus possible that C/EBPβ is indeed elevated in the 32D-Cβ cells but is mainly confined to the cytoplasms. The only change in C/EBPβ binding that we can observe is an increase in the amount of C/EBPα-C/EBPβ detected. Any change in the nuclear

concentration of C/EBPβ and/or DNA-binding activity of the C/EBPβ homodimer may have been too subtle to be detected by our EMSA and Western blot analyses. If C/EBPβ is grossly overexpressed in the cytoplasm of 32D cl3 cells, then cytoplasmic contamination of the nuclear compartment might obscure changes in C/EBPβ that would otherwise be detectable. Future analyses should determine C/EBPβ levels in the cytoplasm and the procedure for preparation of nuclear extracts should be modified to eliminate cytoplasmic contamination. Since two independent 32D-Cβ transfectant populations behaved very similarly, it is very unlikely that this is an artifact of clonal variation.

We have shown that the level of C/EBPα protein is highly elevated in the nucleus of 32D-Cβ cells and, to a lesser extent, is also elevated in the 32D-Cδ cells. Northern blot analyses suggest that this induction occurs at the level of transcription. This suggests that C/EBPβ and C/EBPδ, either as homodimers or as heterodimers with C/EBPα itself, are capable of inducing the transcription of the endogenous C/EBPα gene. In support of this view, a functional C/EBP binding site has been identified in the promoter of the murine C/EBPα gene. This C/EBP site was found to be responsible for the autoregulation of C/EBPα in the liver cells. In addition to C/EBPα, C/EBPβ and C/EBPδ can also bind to this site. All three C/EBP isoforms can transactivate a reporter construct driven by the C/EBPα promoter. It is very likely that the C/EBP binding activities expressed during myelopoiesis are not only expressed in a sequential manner (Scott et al. 1992), but regulate their own expression as well. .

Finally, the observation that the C/EBPα-β heterodimer is the only form of

C/EBP that is detectably increased in P388-Cβ cells suggests that this form is responsible for the increased expression of MPO, Cat G, and LZ observed in these cells. This conclusion is also consistent with the notion that the upregulation of C/EBPα and –β coincide with the activation of primary-granule associated genes, when 32D cl3 is induced to differentiate along the granulocytic pathway by G-CSF. It would be informative to compare the EMSA pattern of G-CSF induced cells to that of 32D-Cβ. Additionally, transient transfections of cells with various C/EBP expression vectors, either individually or in combination, and a MPO or Cat-G promoter-reporter would be informative as to the relative efficacy of the various forms of C/EBP in supporting transcription of these genes.

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CONCLUSIONS

The results presented in Chapter 2 show that C/EBPα and –δ, as well as C/EBPβ are expressed by bone marrow-derived macrophages and are all potentially available to support the expression of proinflammatory cytokines in macrophages. It is demonstrated that C/EBPα, -β and –δ are all capable of supporting the LPS-inducible transcription of IL-6 and MCP-1 in P388 lymphoblasts, which normally lack C/EBP factors and do not display LPS induction of proinflammatory cytokines. These results suggest that a largely normal cytokine response to LPS in the macrophages of C/EBPβ-deficient mice can be explained by the availability of C/EBPα and/or C/EBPδ. The induction of C/EBPδ by LPS in bone marrow-derived macrophages makes it a particularly attractive candidate for replacing C/EBPβ activity.

Although C/EBPα, -β and –δ are largely redundant in the LPS-inducible expression of proinflammatory cytokines, specific roles for these C/EBP isoforms in the regulation of other inflammation-associated genes are certainly possible. Especially, inflammatory stimuli other than LPS, such as IFN-γ, IL-1, IL-6 and TNFα might provide a more complete cytokine response by altering the level of activity and/or specificity of the same C/EBP isoforms that are available during LPS stimulation through post-translational modification (e.g. phosphorylation). Alternatively, these stimuli may activate other transcription factors that are essential for inducing a broad spectrum of proinflammatory cytokines. It will be of interest in future experiments to examine the abilities of these inflammatory

stimuli to induce other proinflammatory cytokines in the P388 transfectants generated in this study.

The data presented in Chapter 3 show that the bZIP regions of C/EBPβ and C/EBPδ are of themselves capable of supporting LPS induction of IL-6 and MCP-1. The bZIP region of C/EBPα also shows modest activity. Furthermore, the naturally occurring transdominant negative inhibitor LIP is capable of supporting the LPS induction of IL-6 and MCP-1. Replacement of the leucine zipper of C/EBPβ with that of yeast GCN4 yields a chimeric protein that can dimerize and specifically bind to a C/EBP consensus sequence, but shows a markedly reduced ability to activate IL-6 and MCP-1. These results implicate the leucine zipper region in some function other than dimerization with known C/EBP family members in the activation of IL-6 and MCP-1 transcription, and suggest that C/EBP redundancy in regulating cytokine expression may result from their highly related bZIP domains.

NF-κB has been implicated as an important partner of C/EBP proteins in regulating the genes of proinflammatory cytokines. There is good evidence that C/EBPα, -β and -δ and NF-κB synergistically activate the IL-6 and IL-8 promoters. This synergism may not only involve binding of the transcription factors to their cognate binding sites in the promoters, but direct physical association of the factors through their respective bZIP and Rel homology domains. We have shown that the activation domains of C/EBPs are not necessary for their activities in LPS induction of IL-6 and MCP-1. It is very likely that the bZIP domain that is highly conserved in the C/EBP isoforms may serve

to enhance the activity of NF-κB on these promoters. It is also possible that the physical interaction of the bZIP domain with nuclear factors in addition to or other than NF-κB may be critical to its activity.. It will be informative in future experiments to examine what structural components of the truncated forms of C/EBPs are required for their physical interactions with NF-κB p50 and p65. In addition, the abilities of truncated forms of C/EBPs to cooperate with other nuclear proteins may also be worthy of investigation. The techniques of co-immunoprecipitation and immunoaffinity chromatography could be applied to these questions.

The results in Chapter 4 show that 32D cl3 cells transfected for C/EBPβ expression (32D-Cβ) have the greatest enhancement of mRNA levels for the genes encoding myeloperoxidase (MPO), cathepsin G (Cat G), and lysozyme (LZ) compared to transfectants for other C/EBP isoforms. A level of expression similar to the G-CSF-induced 32D cl3 parental cells is observed in these primary granule protein encoding genes. Other transfectants also show a modest increase in the level of these mRNA. EMSA suggests that the C/EBPα-C/EBPβ heterodimer is the predominant form of C/EBP present in the nucleus of 32D-Cβ cells and that this form of C/EBP DNA binding activity is increased. The C/EBP/α–C/EBPβ heteodimer is likely an important regulator of the primary granule-associated genes.

An experiment for the immediate future that will strengthen our results is to examine the C/EBPβ expression level in the cytoplasmic compartment of 32D-Cβ cells compared with that of control cells. Also necessary is an improvement of the

current protocol for the isolation of nuclear proteins to avoid cytoplasmic contamination. A more accurate assessment of the concentration of C/EBPβ and C/EBPβ DNA binding activity can then be achieved by Western analyses and EMSA. The elucidation of the mechanism by which the C/EBPβ and –δ containing C/EBP isoforms induce the expression of endogenous C/EBPα mRNA may also contribute to the understanding of the special temporal expression pattern of C/EBP proteins observed during granulopoiesis.