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Two Main Control Elements are Responsible for the Activation of the Murine Interleukin 12 p40 Promoter: Studies Reveal C/EBP β and C/EBP δ To Have Trans-Activation Functions

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Abstract

The heterodimeric cytokine Interleukin 12 (IL-12) is critical for T-helper 1 responses. The gene encoding the IL-12 p40 subunit is induced in macrophages following exposure to bacterial products. This study provides a functional analysis of the p40 promoter control elements in the murine macrophage cell lines RAW 264.7 and J774. Results show that two immune response associated transcription factors, C/EBP β and C/EBP δ , are induced by lipopolysaccharide in these cell lines. The most critical cis-acting element is located between positions -96 and -88 relative to the transcription start site. Electrophoretic mobility shift assays reveal LPS-inducible functional binding of both in vitro and cell line produced C/EBP β and C/EBP δ to the critical element. In vitro transcription/translation of the putative control elements suggest the heterodimer C/EBP β (LAP)/C/EBP δ (LIP) to function in vivo. In addition, transient transfection assays show the C/EBP β isoform LAP to activate transcription of the IL-12 p40 promoter, while C/EBP δ (LIP) shows transcriptional inhibition. The results suggest that C/EBP proteins play critical roles in the bacterial induction of the IL-12 p40 promoter.

Introduction

The cytokine Interleukin 12 (IL-12) is synthesized by macrophages upon exposure to bacterial products and intracellular pathogens. A main function of IL-12 is to mediate the signalling for differentiation of effector T-helper 1 (Th1) cells and to secrete the Th1 cytokines, IL-2 and IFN- γ (29). Th1 cells take part in cell-mediated immunity associated with intracellular microbes (1, 17). IL-12 has been shown to generate a Th1 response against human pathogens such as *Mycobacterium tuberculosis* (32), *Mycobacterium leprae* (25), and *Leishmania species* (19), and during the pathogenesis of the human immunodeficiency virus (5, 6, 7). Because of the role of IL-12 in the pathogen-induced immune response, its expression may be important for the development of vaccines against several intracellular pathogens (2). While the induction of IL-12 by intracellular pathogens instigates the protective host Th1 response, overexpression of Th1 cytokines and IL-12 may take part in the formation of chronic autoimmune and inflammatory diseases (24). Thus, an elucidation of macrophage expression of IL-12 may supply information concerning the pathogenesis of infectious and inflammatory diseases and may aid in the development of novel approaches for manipulation of the immune response.

Two covalently linked glycosylated chains, p40 and p35, constitute the IL-12 heterodimer. The subunits are encoded by distinct genes and together make up the biologically active p70 heterodimer (13, 21, 31). The p35 gene is induced in several tissues (13). p40 is strongly induced by intracellular bacteria and bacterial products (8) and its mRNA is found in macrophages and other cells that synthesize IL-12 (13).

This paper presents the results of a functional analysis of the murine IL-12 p40 promoter in the RAW 264.7 and J774 cell lines. The study implicates two main control elements involved in LPS-induced promoter activity. Both C/EBP β and C/EBP δ show significant nuclear induction by lipopolysaccharide. The two transcription factors also show selective binding of the IL-12 p40 promoter in vitro. In vitro expression and electrophoretic mobility shift assays provide evidence that the precise heterodimeric element responsible for activation of p40 may be composed of C/EBP β (LAP) and C/EBP δ (LIP) subunits. In addition, overexpression of C/EBP β (LAP) causes for increased transcription of the IL-12 p40 promoter in transient transfection assays, while overexpression of C/EBP δ (LIP) results in decreased transcription. These findings aid in the exposition of the principle mechanisms involved in the regulation of this important cytokine.

Materials and Methods

Nuclear extracts and DNA binding assays

The method of Dignam et al. (10) as previously described (14) was used to prepare RAW 264.7 nuclear extracts with the following modifications: 1 μ g of aprotinin (Sigma) per ml, 0.5 μ g of leupeptin (Sigma) per ml, 1 μ M pepstatin (Sigma), and 1mM phenylmethylsulfonyl fluoride (Sigma). Annealed single-stranded oligonucleotides with 5' GATC overhangs (Operon) that were gel purified were used to make electrophoretic mobility shift assay (EMSA) probes. The Klenow enzyme was used to fill in two hundred nanograms of probe with [α -³²P]dGTP and [α -³²P]dCTP. A NucTrap purification column (Stratagene) was used to purify the labeled probes. Wild-type IL-12 p40 EMSA probe sequence is displayed below (see Fig. 4A). Binding for C/EBP EMSAs was optimized at room temperature for 45 min, and EMSA products were separated on 5% acrylamide-1 \times Tris-glycine-EDTA gel run at 4 $^{\circ}$ C for 3 h at 150 V. 2 μ l of antibody was added to extracts for supershift experiments, in addition to poly (dI-dC) and binding buffer 30 minutes prior to addition of labeled probe at room temperature. Santa Cruz Biotechnology, Inc. supplied polyclonal antibodies. Kodak X-AR 5 film was used to expose dried gels at -80 $^{\circ}$ C with an intensifying screen.

Cell lines and reagents

Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco) (assayed for low endotoxin activity) was utilized to maintain the RAW 264.7 and J774 murine macrophage lines (American Type Culture Collection). LPS was obtained from Sigma Chemicals.

Plasmids

pBluescript KS+ (Stratagene) was used to isolate a 10kb murine IL-12 p40 genomic clone. DNA sequencing and restriction mapping was employed to determine the location of the promoter region and intron-exon structure. The sequence of one kilobase of the promoter region was determined (Sequenase, version 2) and was found to match that previously

published (18). Chloramphenicol acetyltransferase (CAT) assay p40 promoters were cloned from pGL2B into pCAT basic (Promega) modified to contain a *KpnI* site and a *BglII* site in the polylinker. C/EBP β isoforms LIP (pSCT-LAP with a deleted *NcoI* fragment) and LAP (pSCT-LAP) expression plasmids containing a cytomegalovirus (CMV) promoter/enhancer were originally constructed by Patrick Descombes and Uelie Schibler (9) and were obtained from Andrew Henderson and Kathryn Calame. An endotoxin-free plasmid purification system (Qiagen) was used to purify all plasmids used in transient transfections.

Transfections

A previously described protocol (26) was employed with modifications to transiently transfect RAW 264.7 cells by electroporation. For CAT transfections, 7.5×10^6 cells were suspended in 200 μ l of DMEM with 10% FBS plus 20 μ g of CAT plasmid and 1 μ g of HSP- β -galactosidase reporter (provided by Bradley Cobb, UCLA). Cells were electroporated in a 0.4-mm-long cuvette (Bio-Rad) in a Bio-Rad Gene Pulser at 250 V and 960 μ F. The cells were incubated for 10 min at room temperature and were washed with 5 ml of PBS and resuspended in 1 ml of DMEM. Each transfection product was divided into two 500- μ l aliquots, and each was diluted to 2.5 ml with medium in a six-well plate. 50 μ g of total protein from cell extracts were used to perform CAT assays, as per the Promega protocol. A PhosphorImager (Molecular Dynamics) was employed to quantitate the conversion of [14 C]chloramphenicol to its acetylated forms.

Results

C/EBP β and C/EBP δ are induced by lipopolysaccharide in the murine macrophage cell line RAW 264.7

An immunoblot assay utilizing anti-C/EBP β antibody reveals no protein in either the unactivated or LPS-activated cytoplasm (Fig. 1A, lanes 1 and 2). Basal levels of C/EBP β are seen in the unactivated nucleus (lane 3), and the protein is upregulated in the LPS-activated nucleus (lane 4). Previous findings have described C/EBP β as capable of activating the IL-12 p40 promoter upon LPS exposure (9). An upregulation of C/EBP β induced by LPS may be necessary for C/EBP β to exert its effects on the p40 promoter. C/EBP δ is not present in the cytoplasm or in the unactivated nucleus (Fig. 1B, lanes 1-3), but protein levels are significantly induced in the LPS-activated nucleus (lane 4). The precise role of C/EBP δ in the immune regulation cascade has yet to be elucidated. However, upregulation of the protein in response to lipopolysaccharide suggests C/EBP δ to play some role in the LPS-induced regulation process. C/EBP α is not present in the unactivated or LPS-activated cytoplasm or nucleus of the RAW 264.7 macrophage cell line (Fig. 1C). Lack of upregulation of C/EBP α does not necessarily exclude the possibility that it is a member of the immune regulation system, (since a post-translational modification of endogenous C/EBP α may be sufficient for activation).

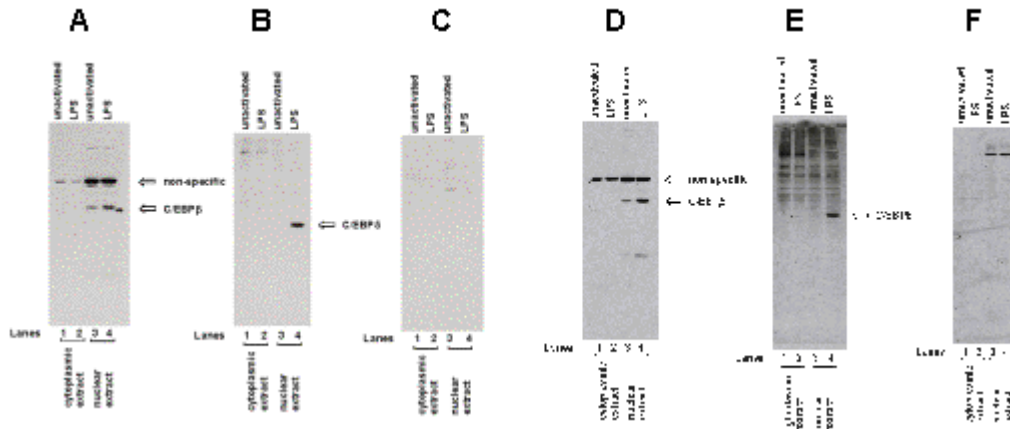


Figure 1. C/EBP β and C/EBP δ are induced by lipopolysaccharide in the RAW 264.7 and J774 murine macrophage cell lines. (A) An immunoblot assay was performed with cytoplasmic and nuclear extracts from unactivated RAW 264.7 cells and from cells activated with LPS (5 μ g/ml) for 4 h. 6 μ g of extract was hybridized at room temperature with anti-C/EBP β primary antibody (Santa Cruz Biotechnology) for 1 h, and then with secondary antibody for 1 h. The hybridization complexes were treated with ECL reaction for 1 min, and were exposed to film. (B) Similar immunoblot assay hybridizing with anti-C/EBP δ primary antibody. (C) Similar immunoblot assay using anti-C/EBP α antibody. (D-F) Immunoblot assays using the same respective methods as described in A-C, with J774 cells as the source of cytoplasmic and nuclear extract.

C/EBP β and C/EBP δ are induced by lipopolysaccharide in the J774 murine macrophage cell line

Consistent with the results obtained in the RAW 264.7 cell line, C/EBP β is produced at a basal level in the nucleus and is upregulated upon exposure of the cells to lipopolysaccharide (Fig. 1D, lanes 3 and 4). Similar results in the J774 cell line confirm that the findings in the RAW 264.7 cells are not specific for this cell line, and may implicate C/EBP β as a universal response element in the immune regulation cascade. Also consistent with findings in the RAW 264.7 cell line is the upregulation of C/EBP δ in J774 cells (Fig. 1E). Again, the protein is completely absent from cytoplasmic and unactivated nuclear extracts (lanes 1-3) and significant production of protein is observed in the activated nucleus (lane 4). Immunoblot results for C/EBP α are analogous to those in RAW 264.7 cells, and reveal no protein in the unactivated or activated cytoplasmic or nuclear extracts (Figure 1F, lanes 1-4).

The C/EBP β (LAP) isoform homodimer is larger than the C/EBP δ homodimer

Previous studies have described the presence of two distinctly translated forms of C/EBP β , LAP, which is larger, and LIP, smaller (9). Functionally, LIP has been shown to bind to the LAP binding site on DNA and serve as an inhibitor due to its lack of a transcriptional activation domain (9). EMSA experiments have revealed 3 complexes associated with C/EBP β , the fastest-migrating complex being composed of LIP homodimers, the intermediate complex containing LIP-LAP heterodimers, and the slowest-migrating complex composed of LAP homodimers. The LAP and LIP proteins are further able to heterodimerize with other proteins (e.g. LAP is able to heterodimerize with C/EBP α and C/EBP δ). To determine the precise heterodimeric partners responsible for activation of the IL-12 p40 promoter, in vitro expressed C/EBP β (LAP), C/EBP β (LIP), and C/EBP δ were combinatorially incubated, and the resulting dimerization products were run in EMSA experiments utilizing a radioactive probe consisting of

the p40 promoter (see below). Previous experiments have shown both C/EBP β (LAP) and C/EBP δ to be similar in size. An immunoblot assay using these proteins expressed in vitro was performed to distinguish between C/EBP β (LAP) and C/EBP δ based on electrophoretic mobility. Results reveal that the C/EBP β (LAP) homodimer has slower mobility on the poly-acrylamide gel (Fig. 2A, lane 5). C/EBP δ shows slightly higher mobility (lane 1). The C/EBP β (LIP) homodimer is significantly smaller than either of the above mentioned proteins, and runs to a lower position on the gel (lane 6).

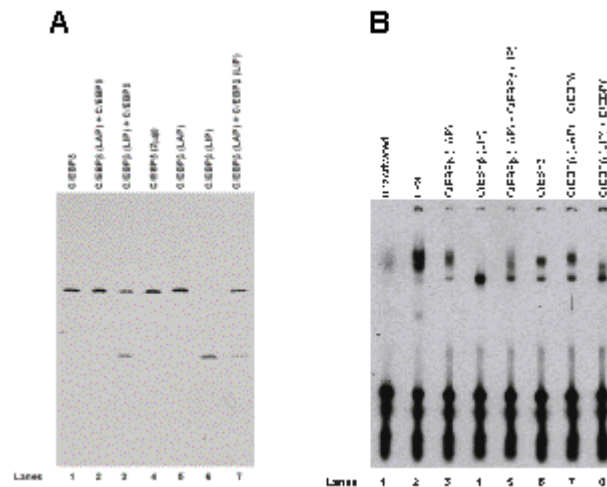


Figure 2. The transcription activator of the IL-12 p40 promoter may be a C/EBP β (LAP)/C/EBP β (LIP) heterodimer. (A) An immunoblot assay was performed with in vitro transcribed/translated presumptive IL-12 p40 activators. 6 μ g of protein was incubated with C/EBP β and C/EBP δ primary antibody (Santa Cruz Biotechnology) for 1 h, and then with secondary antibody for 1 h. An ECL reaction was performed and the complexes were exposed to film. (B) An EMSA was performed with nuclear extracts from unactivated RAW 264.7 cells (lane 1) and from cells activated with LPS (5 μ g/ml) for 4 h (lane 2), and with in vitro transcribed/translated presumptive IL-12 p40 transcription activators (lanes 3-8). Labeled probe was incubated with 6 μ g of protein and 2 μ g of poly (dI-dC) at room temperature for 45 min, and electrophoresis followed.

The transcription activator of the IL-12 p40 promoter may be a C/EBP β (LAP)/C/EBP β (LIP) heterodimer

An EMSA experiment was employed using lanes consisting of J774 cell line nuclear extracts and in vitro expressed presumptive IL-12 p40 promoter transcription factors. Protein mixtures were incubated with a radioactively labeled DNA oligonucleotide probe consisting of the IL-12 p40 promoter. The in vitro expressed C/EBP β (LAP)/C/EBP β (LIP) heterodimer (Fig. 2B, lane 5) runs to a similar position on the acrylamide gel as the principle bands of the J774 unactivated (lane 1) and LPS-activated (lane 2) nuclear extracts. The correspondence in size of the C/EBP β (LAP)/C/EBP β (LIP) heterodimer with the active proteins in the nuclear extract may suggest the presence of this heterodimer as an active transcription factor in vivo. The C/EBP β (LIP)/C/EBP δ heterodimer (lane 8) shows similar correspondence in size and may be a secondary contributor to p40 activation. Possible minor contribution may come from C/EBP β (LIP) homodimers (lane 4).

C/EBP β (LAP) is a transcriptional activator of the IL-12 p40 promoter, while *C/EBP β* (LIP) shows inhibition of transcription

To determine the effects of *C/EBP β* (LAP) and *C/EBP β* (LIP) on the IL-12 p40 promoter in RAW 264.7 cells, promoter activity was studied by a transient transfection assay. The IL-12 p40 promoter (containing the -355-to-+55 region) was cloned from pGL2B into a CAT reporter plasmid (pCAT basic; Promega), and transiently transfected into RAW 264.7 cells along with varying amounts of *C/EBP β* isoform expression plasmids^{3/4}LAP (PSCT-LAP) or LIP (PSCT-LAP with a deleted *Nco*I fragment). To promote overexpression in vivo, each expression plasmid contained a cytomegalovirus (CMV) promoter/enhancer, a synthetic promoter containing multiple SP1 binding sites upstream of consensus TATA and initiator elements.

Overexpression of 0.1 μ g of LIP decreased both unactivated and LPS-activated promoter activity by twofold, and 1.0 μ g of LIP caused for slightly greater inhibition (Fig. 3).

Overexpression of 0.1 μ g of LAP increased transcription of the unactivated p40 promoter by nearly fourfold and had a less dramatic effect on LPS-induced transcription. Increased amounts of LAP in LPS-activated cells caused for a decrease in promoter activity possibly due to nonspecific squelching. These results suggest *C/EBP β* (LIP) to inhibit transcription of the IL-12 p40 promoter and *C/EBP β* (LAP) to promote transcription.

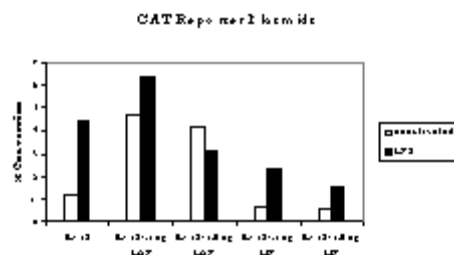


Figure 3. Effects of overexpressed *C/EBP β* isoforms LIP and LAP on p40 promoter activity. 15 μ g of the -355-to-+55 IL-12 p40 promoter-CAT reporter was cotransfected in transient assays into RAW 264.7 cells with expression plasmids for LAP and LIP (9) containing a CMV promoter/enhancer. The amounts of the LIP and LAP plasmids cotransfected are indicated below the graph. Addition of a CMV promoter/enhancer-luciferase plasmid normalized plasmid amounts to 20 μ g per transfection. Twenty-four hours after transfection, cells were either not activated or were activated with 5 μ g of LPS per ml for 24 h.

C/EBP β (LAP) is the primary contributor to binding of IL-12 p40 promoter, with *C/EBP δ* contributing to a smaller degree

A radiolabeled double-stranded oligonucleotide probe spanning the sequence from position -110 to -69 (Fig. 4A) was prepared to elucidate the proteins that bind to the putative *C/EBP* site between nucleotides -96 and -88 of the murine p40 promoter. An EMSA supershift experiment was performed incubating this probe with unactivated and LPS-activated J774 cell line nuclear extracts and in vitro transcribed/translated *C/EBP β* (LAP) and *C/EBP δ* . Unactivated nuclear extract incubated with *C/EBP β* antibody (Fig. 4B, lane 5) shows the presence of a slow-mobility complex and a greatly decreased intensity of the principle active protein double-band. This result implicates *C/EBP β* present to a substantial degree in the unactivated nuclear extract, and shows that endogenous *C/EBP β* shows considerable binding to the p40 promoter

probe. These results are consistent with previous information suggesting C/EBP β as the principle trans-acting factor inducing expression of the IL-12 p40 promoter (9). The two complexes were strongly induced by LPS (lane 4), further suggesting the role of C/EBP β as a functional component of the lipopolysaccharide-induced immune response. LPS-activated nuclear extract incubated with C/EBP δ antibody shows a smaller, yet evident decrease in intensity of the principle active band in both unactivated (lane 3) and LPS-activated (lane 6) nuclear extracts, with LPS-activated extracts again showing inducibility. These results suggest C/EBP δ to have a smaller, although still significant functional trans-acting relation to the p40 promoter, and also ascribes to C/EBP δ a possible role in LPS-induced p40 regulation. It should be noted that there is a seeming inconsistency in these results in that although the C/EBP δ antibody is decreasing the intensity of the principle active band (lane 6) there is no residual band representing C/EBP δ in the lane consisting of nuclear extract incubated with C/EBP β antibody (lane 5). This inconsistency can be explained by the fact that the Santa Cruz Biotechnology anti-C/EBP β antibody may bind to both C/EBP β and C/EBP δ . To test this hypothesis, in vitro transcribed/translated C/EBP δ was incubated with anti-C/EBP β antibody and run on an EMSA gel. The results (lane 11) show that the anti-C/EBP β antibody does bind to C/EBP δ by the appearance of a slow-mobility complex and a disappearance of the band representing C/EBP δ . The specificity of the anti-C/EBP β antibody for both C/EBP β and C/EBP δ misrepresents the results of this EMSA experiment by giving the impression that the principle active double band in the J774 nuclear extract is composed solely of C/EBP β . To obtain a more accurate estimation of the relative amounts of C/EBP β and C/EBP δ actually present in these extracts, it was important to resolve the double specific activity of the anti-C/EBP β antibody.

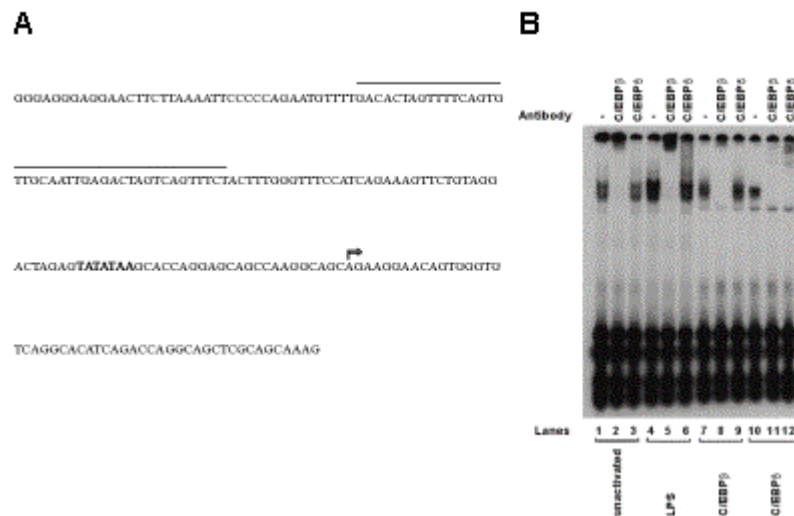


Figure 4. EMSA shows LPS-inducible binding of C/EBP β , and to a smaller degree C/EBP δ , to the p40 promoter. (A) Oligonucleotide probe location within the p40 promoter. The thick line represents the probe spanning the putative C/EBP site (-110/-69). The TATA box is indicated by boldfaced letters and the transcription start site is indicated by the arrow. (B) An EMSA was performed with 5 mg of nuclear extract from unactivated (lanes 1-3), LPS-activated (lanes 4-6) cells, and in vitro expressed C/EBP β (lanes 7-9) and C/EBP δ (lanes 10-12). 2 mg of poly(dI-dC) and extract were incubated for 30 min at room temperature without (lanes 1, 3, 6, 9) or with 2 ml of the following polyclonal antibodies (Santa Cruz Biotechnology): anti-C/EBP β (lanes 2, 5, 8, and 11), and anti-C/EBP δ (lanes 3, 6, 9, and 12). Subsequent to this incubation, labeled probe -110/-69 was added to the mixture and incubated for 45 min at room temperature prior to electrophoresis.

The specificity of the Santa Cruz Biotechnology anti-C/EBP β antibody for both C/EBP β and C/EBP δ is concentration-independent

An EMSA supershift experiment using in vitro expressed C/EBP δ incubated with increasing concentrations of anti-C/EBP β antibody (Fig. 5A, lanes 11-15) shows complete binding of the anti-C/EBP β antibody to C/EBP δ at antibody levels as low as .25 μ l. These results show that the specificity of the anti-C/EBP β antibody for C/EBP δ is not due to excessive concentrations of the antibody in the assay. The dual specificity of the anti-C/EBP β antibody may be due to flawed commercial preparation of either the primary antigen or the antibody itself. To continue with these experiments it was essential to use an antibody which binds solely to C/EBP β . For this reason, a C/EBP β antigen was prepared and purified in the laboratory and antibody was made against this particular antigen. This specially prepared anti-C/EBP β antibody was used in the following experiment to determine if binding to C/EBP δ would be precluded.

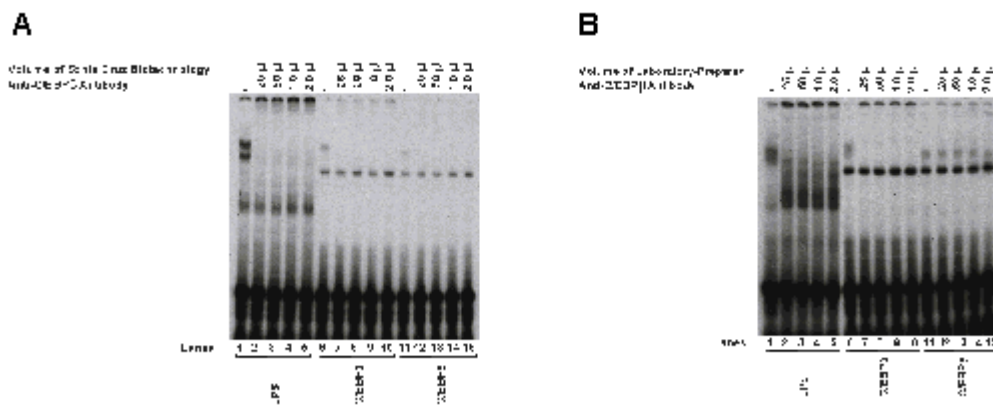


Figure 5. EMSAs demonstrate the double specific activity of the Santa Cruz Biotechnology anti-C/EBP β antibody, and the single specificity of the laboratory-prepared anti-C/EBP β antibody. (A) An EMSA was performed with 5 μ g of nuclear extract from LPS-activated cells (lanes 1-5) and in vitro expressed C/EBP β (lanes 6-10) and C/EBP δ (lanes 11-15). Extract and 2 μ g of poly(dI-dC) were incubated for 30 min at room temperature without (lanes 1, 6, and 11) or with (lanes 2-5, 7-10, and 12-15) increasing concentrations of anti-C/EBP β antibody (Santa Cruz Biotechnology). Following incubation, labeled probe -110/-69 was added, and incubation proceeded for 45 min at room temperature prior to electrophoresis. (B) A similar EMSA was performed using laboratory-prepared anti-C/EBP β antibody in place of the Santa Cruz Biotechnology stock.

Laboratory-prepared anti-C/EBP β antibody shows specificity only for C/EBP β

An EMSA supershift experiment was performed using LPS-activated J774 cell line nuclear extracts and in vitro expressed C/EBP β and C/EBP δ . The lanes consisting of nuclear extract and in vitro expressed C/EBP β (Fig. 5B, lanes 1-10) serve as controls and show expected binding of the putative antibody to proteins present in the incubation. The lanes consisting of in vitro expressed C/EBP δ (lanes 11-15) serve as the functional assay, the results of which show quite conclusively the absence of antibody binding at levels as high as 2.0 μ l. Its specificity only for C/EBP β confirmed, the laboratory- prepared anti-C/EBP β antibody was used

in subsequent experiments in place of the non-specific commercial version.

C/EBP δ binds to the IL-12 p40 promoter

An EMSA supershift experiment using J774 nuclear extracts was performed to determine the relative amount of C/EBP δ which binds to the p40 probe. The lane consisting of LPS-activated nuclear extract incubated with the specially prepared anti-C/EBP β antibody shows the presence of a light-intensity double band at the same vertical position on the gel as the active bands in the LPS-activated nuclear extract (Fig. 6, compare lanes 8 and 6). This band represents residual levels of C/EBP δ bound to the radiolabeled probe. Thus, the specially prepared anti-C/EBP β antibody is able to bind to and shift only the C/EBP β protein and any C/EBP δ present in the extract remains at its original position. These results show quite clearly the relative amounts of bound C/EBP β and C/EBP δ , with C/EBP β present to a great degree, and C/EBP δ present at a smaller, yet recognizable level. The lanes consisting of LPS-activated cell line nuclear extracts with antibodies for either C/EBP α (lane 7) or C/EBP ϵ (lane 10) show no decrease in active band intensity relative to extracts incubated without antibody (lane 6). This data suggests that C/EBP α and C/EBP ϵ may not take part in the trans-activation of the p40 promoter.

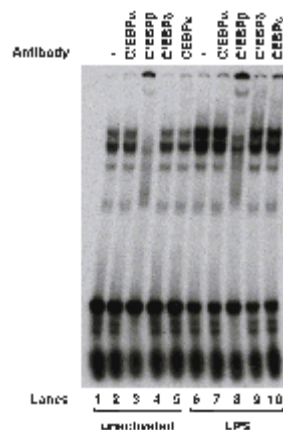


Figure 6. EMSA reveals more precisely the extent of binding of both C/EBP β and C/EBP δ to the IL-12 p40 promoter. C/EBP α and C/EBP ϵ show no binding to the p40 promoter. An EMSA was performed with 5 μ g of nuclear extract from unactivated (lanes 1-5) and LPS-activated (lanes 6-10) cells. Extract and 2 μ g of poly(dI-dC) were incubated for 30 min at room temperature without (lanes 1 and 6) or with 2 μ l of the following polyclonal antibodies: anti-C/EBP α (lanes 2 and 7), laboratory-prepared anti-C/EBP β (lanes 3 and 8), anti-C/EBP δ (lanes 4 and 9), and anti-C/EBP ϵ (lanes 5 and 10). After incubation, labeled probe -110/-69 was added and incubation continued for 45 min at room temperature prior to electrophoresis.

In the murine macrophage cell line J774, active C/EBP β is induced between 2 and 4 hours after LPS activation.

An immunoblot assay was performed using J774 cell line nuclear extracts probed with anti-C/EBP β antibody for increasing periods of time following LPS activation (Fig. 7A). Results show basal levels of C/EBP β in unactivated nuclear extracts. These levels remain constant until the period between 2 and 4 hours after LPS activation, at which point the initial induction of

C/EBP β occurs. By 8 hours after LPS activation, protein concentration reaches maximum levels and is maintained into 24 hours. Previous studies have shown that C/EBP β is able to activate the IL-12 p40 promoter within 1 hour of LPS activation (9). The requirement of at least 2 hours for the upregulation of C/EBP β excludes the possibility that an increase in protein concentration is necessary for initial p40 activation. These results suggest some other mechanism of initial C/EBP β activation, such as a post-translational modification of proteins present in the cell. An upregulation of C/EBP β may, however, be necessary to maintain activation of the IL-12 p40 promoter beyond 2 hours of LPS activation.

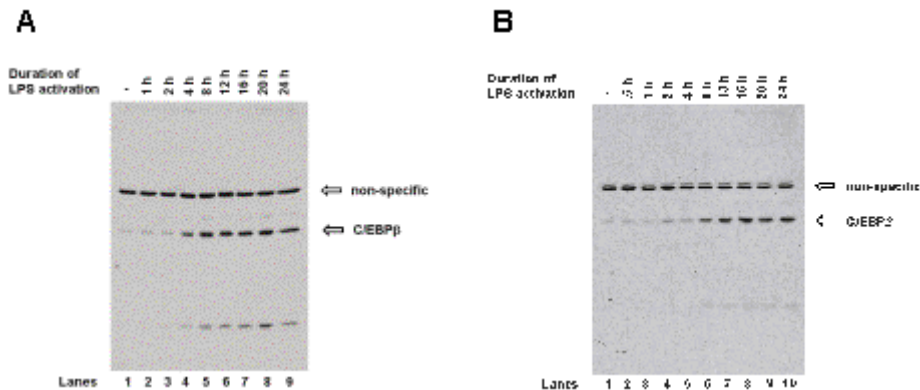


Figure 7. Immunoblot assays demonstrate the LPS-induced upregulation of C/EBP β in both the J774 murine macrophage cell line and in primary cells. (A) An immunoblot assay was performed with nuclear extracts from J774 cells activated for increasing periods of time with lipopolysaccharide (5 μ g/ml). 5 μ g of extract was hybridized at room temperature with anti-C/EBP β antibody for 1 h, and then with secondary antibody for 1 h. The hybridization complexes were treated with ECL reaction for 1 min, and were exposed to film. (B) A similar immunoblot assay using primary cells as the source of nuclear extract.

In primary cells, induction of active C/EBP β occurs between 4 and 8 hours after LPS activation

Consistent with results obtained in the J774 cell line, primary cell C/EBP β is present at basal levels in the unactivated nucleus and is induced to a constant maximum level following LPS activation (Fig. 7B). In primary cells, the initial induction occurs between 4 and 8 hours and continues into 16 hours, at which point net protein production ceases. Protein levels are maintained into 24 hours.

Discussion

A functional analysis of the murine IL-12 p40 promoter has been carried out, providing information contributing to our understanding of the molecular proceedings that generate a Th1 immune response. In nuclear extracts, DNA-binding activities involving C/EBP β were induced by LPS. C/EBP δ DNA-binding activities were induced somewhat less. A heterodimer of the alternatively transcribed forms of C/EBP β , LAP and LIP, has been implicated to provide most of the DNA binding activity in vivo. Finally, C/EBP β (LAP) has been shown to be a transcription activator, while C/EBP β (LIP) has inhibitory effects.

C/EBP β has been shown in previous studies to be induced in monocytes and macrophages

(22, 30) and has been suggested to take part in the activation of the genes for cytokines expressed in myeloid cells, including IL-1, IL-6, IL-8, tumor necrosis factor alpha, and monocyte inflammatory protein 1 α (MIP-1 α) (4, 11, 12, 16, 20, 27). Potential mechanisms for the regulation of C/EBP β activity have been suggested, although conclusive determination is yet to be established (3, 9). In one study, mice with a disrupted C/EBP β gene demonstrated decreased IL-12 production and increased susceptibility to infection with intracellular organisms, implicating an IL-12 regulation function to C/EBP β (24). In another study, mice with a disrupted C/EBP β gene were also susceptible to infection but did not show a decrease in IL-12 production, suggesting that other control elements, perhaps C/EBP δ , may compensate for the non-functional C/EBP β (28).

In summary, this study provides characterization of IL-12 p40 gene expression with respect to trans-acting control elements. IL-12 p40 expression is a crucial event in the development of a protective immune response to intracellular pathogens, and the dysfunctional regulation of this expression may cause for the Th1 response in chronic inflammatory disorders such as rheumatoid arthritis and inflammatory bowel disease. The elucidation of IL-12 regulatory mechanisms will provide information about the pathogenesis of infectious and autoimmune diseases and may suggest novel methods for manipulating the immune response.

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