Differential Expression of Three C/EBP Isoforms in Multiple Tissues during the Acute Phase Response*

(Received for publication, December 12, 1991)

Tawfiq Alam, Mi Ra An, and John Papaconstantinou†

From the Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

Eucaryotic organisms possess natural defense processes triggered by stress factors such as injury, infection, and inflammation. The acute phase response is an early defense mechanism during which striking changes in protein synthesis occur in the liver and other tissues. The altered expression of many acute phase protein genes is at the transcriptional level. Some of these genes have DNA binding sites for the CCAAT/enhancer binding protein (C/EBP) family of transcription factors. We report here that in vivo expression of three isoforms of C/EBP is dramatically changed during the acute phase response. The steady-state mRNA levels of C/EBPα decreased significantly in the liver, lung, and fat tissues of lipopolysaccharide (LPS)-treated mice; moreover, nuclear run-off transcription assays indicated a decrease in the rate of C/EBPα gene transcription in isolated liver nuclei. The steady-state levels of C/EBPβ and a new isoform, C/EBPγ, were dramatically increased in many tissues within 4 h following LPS treatment. The rates of transcription of these two genes were only minimally altered in liver but significantly increased in kidney nuclei isolated from stimulated animals. Thus, the C/EBP isoforms exhibit differential mechanisms in their responses to LPS in various tissues and are likely to play an important role in mediating the transcriptional activation of genes involved in the acute phase response.

The acute phase response represents the body’s well orchestrated defense against bacterial and viral infection, inflammation, trauma due to surgery, thermal injury, and neoplastic disease. The systemic response includes endocrine, neurological, and metabolic changes involving an increased secretion of steroid and peptide hormones (cytokines), which in turn mediate a dramatic change in the synthesis of serum proteins, named the acute phase reactants (1–4). Although the primary site of synthesis of most acute phase reactants is the liver, other tissues, such as choroid plexus of the brain, kidney, yolk sac, and placenta, exhibit a lesser degree of involvement (5, 6).

The in vivo stimulation of the liver during the acute phase response is mediated by several cytokines which are released by activated monocytes and macrophages. It is now well established that the major regulator of the acute phase response is interleukin-6 (IL-6), a 26-kDa protein produced by monocytes, macrophages, T-cells, fibroblasts, mast cells, keratinocytes, and a variety of tumor cells (2, 3, 7). IL-6 can induce the synthesis of fibrinogen, haptoglobin, α1-acid glycoprotein, and other proteins. In addition to cytokines, glucocorticoids are also important in mediating the acute phase response. In vivo experiments with adrenalectomized animals suggest that glucocorticoids are not absolutely essential but are required for the maximal stimulation of acute phase reactants (8–10). Thus, glucocorticoids can act synergistically with cytokines.

Recent studies have indicated that the role of cytokines and glucocorticoids in the regulation of acute phase reactant genes is based on their ability to regulate the activity of specific trans-acting factors. C/EBP, a well characterized trans-acting factor involved in cell differentiation and energy metabolism (11–13), is known to bind to the cis-elements of a wide spectrum of genes; moreover, the genes of several acute phase reactants, such as α1-acid glycoprotein, albumin, α1-antitrypsin, and angiotensinogen are known to have C/EBP binding sites (14–18). These studies indicate that the activity of these genes is regulated by the interaction of C/EBP with corresponding cis-acting promoter sequences. At least four isoforms of C/EBP, i.e. C/EBPα, C/EBPβ, C/EBPγ, and C/EBPδ, have been identified. Furthermore, these isoforms have been shown to cross-dimerize and bind to DNA with similar specificity (19–22). In the mouse, C/EBPα, C/EBPβ, and C/EBPδ, are encoded by separate genes located on different chromosomes (22).

As an approach to understand the molecular mechanisms of regulation of the acute phase response in vivo, we conducted experiments to determine whether the expression of C/EBPα, C/EBPβ, and C/EBPδ isoforms are regulated by LPS in various tissues. We demonstrate that C/EBPβ and especially C/EBPδ, a novel member of the family, are highly inducible in multiple tissues during the acute phase response; in contrast, C/EBPα is down-regulated in some tissues. We also present evidence that both transcriptional and post-transcriptional mechanisms are involved in the altered expression of the C/EBP isoforms.

MATERIALS AND METHODS

Animals—Two-month-old BALB/c mice (19–20 g) were obtained from Charles River Laboratories. Animals were injected intraperitoneally with bacterial LPS in pyrogen-free saline. Animals were sacrificed by cervical dislocation at indicated time points, and various organs were quickly excised, frozen on dry ice, and stored at −70 °C. Isolation and Northern Analysis of RNA—Total RNAs were isolated according to the procedures of Chomczynski and Sacchi (23). Equal amounts of RNA were resolved by electrophoresis through a formaldehyde/agarose denaturing gel (1.4%) buffered in 0.02 M MOPS, 1 mM EDTA, pH 7.4. The integrity of the RNA and equal

* This investigation was supported by a grant from the Shriners Burns Institute, Galveston Unit (to J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom reprint requests should be addressed: Dept. of Human Biological Chemistry and Genetics, 613 Basic Science Bldg., Rt. F-43, University of Texas Medical Branch, Galveston, TX 77550. Tel.: 409-772-5761; Fax: 409-772-6193.

1 The abbreviations used are: IL, interleukin; C/EBP, CCAAT/enhancer binding protein; LPS, lipopolysaccharide; MOPS, 4-morpholino-propanesulfonic acid; NF-IL-6, nuclear factor IL-6.
loading was tested by comparison of rRNA intensities in ethidium bromide-stained gels. The RNAs were transferred overnight from the gels to nitrocellulose membranes and baked for 2 h at 80 °C under vacuum (24). The filters were probed with radiolabeled cDNAs, washed, air-dried, and exposed to Kodak XAR-5 film in the presence of intensifying screen at -70 °C (24). The filters were reprobed with radiolabeled DNA that hybridizes with 18 S rRNA as a control for loading.

**Quantitation of mRNA Levels**—Relative amounts of mRNAs for the C/EBP isoforms were determined by densitometric analyses of Northern blot autoradiographs, using the transmitting scanning densitometer (BVI 400 biological analysis system). The steady-state levels of C/EBP mRNAs were normalized to the 18 S RNA signals.

**Nuclear Run-off Transcription Analysis**—Nuclear run-off assays were performed using nuclei isolated from livers and kidneys as described previously (25). Briefly, the animals were sacrificed, and nuclei were isolated from fresh tissue homogenates by a sucrose gradient ultracentrifugation at 4 °C (26). The nuclear pellets were washed in cold storage buffer (50 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 5 mM MgCl₂, and 40% glycerol). In vitro run-off transcription was carried out immediately on equal numbers of isolated nuclei in the presence of [α-³²P]UTP. Transcriptions were stopped by treatment with DNase I and proteinase K. Following purification, a total of 1 × 10⁶ cpm of elongated nascent RNA, per assay, was hybridized to a variety of denatured single-stranded cDNAs (5 μg) immobilized on nitrocellulose filters. The filters were washed with 2× SSC at 65 °C for 1 h, 2× SSC with 10 μg/ml boiled Rnase A at 37 °C for 15 min, and 2× SSC at 37 °C for 1 h. The washed filters were exposed to x-ray films. The relative transcription rates were determined by densitometric analyses of the autoradiographs.

**RESULTS**

Following a systemic injury the liver responds with a striking increase or decrease in the synthesis of a subset of serum proteins, named the acute phase reactants (1, 2, 4). The genes for several acute phase reactants are transcriptionally regulated during the acute phase response (27-30). Recently, the liver-enriched transcription factor C/EBP, and related proteins have been shown to bind to the cis-elements of certain acute phase reactant genes, including those for α₁-acid glycoprotein, hemopexin, angiotensinogen, and albumin (14-18, 20). In view of the demonstrated regulation of acute phase reactant genes by C/EBP, it was of interest to determine whether the expression of C/EBP isoforms in the liver are altered during acute inflammation. We analyzed the steady-state mRNA levels of the three isoforms using cDNAs for rat C/EBPα and mouse C/EBPβ and C/EBPδ. Northern blot analysis revealed that the sizes of mouse C/EBPα, C/EBPβ, and C/EBPδ mRNAs are approximately 2.7, 1.5, and 1 kilobases, respectively; moreover, no cross-hybridization was detected (Fig. 1). As reported earlier, the constitutive level of C/EBPα mRNA in the liver was relatively high (22, 31); however, within 4 h of induction of acute phase response by LPS, the mRNA level of C/EBPα decreased significantly (Fig. 1). Repeated experiments with several animals indicated that the steady-state level of C/EBPα mRNA at the 4-h time point was between 20 and 50% of control. In contrast, a 4-fold induction in the expression of C/EBPδ was observed at the 4-h time point, followed by a gradual decline. Interestingly, the most dramatic acute phase inducibility was seen with C/EBPδ. The expression of C/EBPβ in the control liver was very low, but within 4 h after LPS treatment a 70-fold increase in the mRNA level was detected. C/EBPδ mRNA levels declined sharply following a peak at 8 h.

To investigate if the expression profile of the C/EBP family of transcription factors is altered in other tissues during the acute phase response, we isolated RNAs from various tissues. Representative Northern blot analyses are shown in Fig. 2. Also, time course analyses of C/EBP mRNA levels after stimulation with LPS are presented in Fig. 3. Similar to earlier reports, C/EBPα mRNA in control animals was found at abundant levels in fat tissue and at relatively lower levels in intestine, kidney, and lung (22, 31). In the untreated animals, a significant level of C/EBPδ mRNA was observed in the lung and intestine. During the acute phase response, the levels of C/EBPα mRNA decreased in the lung and fat. In all other tissues tested, C/EBPα mRNA levels were elevated slightly or remained unchanged. In contrast, C/EBPβ expression was highly inducible by LPS treatment in non-hepatic tissues, most notably in the spleen (30-fold), fat (22-fold), heart (10-fold), and kidney (9-fold). The dramatic induction of C/EBPδ was not limited to the liver; in tissues where the constitutive expression of C/EBPδ was relatively low, such as kidney, spleen, and heart, changes ranging from 151- to 18-fold were observed (Fig. 3). The induction of the C/EBPδ mRNA level was relatively modest in the lung and intestine where the constitutive expression was relatively high. A 42-fold increase in C/EBPδ mRNA in the brain suggests that the extracellular signals for acute phase response can traverse the blood brain barrier.

To determine whether the observed changes in the mRNA levels of the C/EBP isoforms were based on transcriptional or post-transcriptional mechanisms, nuclear run-off assays were performed. Nuclei isolated from the liver and kidney were incubated with [α-³²P]UTP. The elongated nascent RNA was hybridized to the various denatured cDNA probes for C/EBP immobilized to nitrocellulose filters. Probes for the ribosomal RNA gene were also included in the filters as internal controls. The transcript levels for the C/EBP isoforms were normalized to the ribosomal RNA gene. In the uninduced liver nuclei, the rates of transcription of C/EBPα

![Fig. 1. Expression of C/EBPα, C/EBPβ, and C/EBPδ mRNAs in the liver during the acute phase response. The time (h) after LPS treatment (100 μg, intraperitoneally) is indicated. Equivalent amounts of RNA from each sample were used for Northern blot analysis. The filters were probed with radiolabeled cDNA clones for C/EBPα (A), C/EBPβ (B), and C/EBPδ (C). A 3-day exposure is shown. As control, each filter was reprobed with a cDNA that hybridizes with 18 S rRNA (D). The positions of the 28 and 18 S rRNA are indicated by arrows.](image1)

![Fig. 2. Northern blot analysis of RNA from various tissues demonstrating the differential expression of C/EBPα, C/EBPβ, and C/EBPδ during the acute phase response.](image2)
and C/EBPβ were relatively higher than of C/EBPδ (Fig. 4A). However, transcription of C/EBPα decreased significantly (57% of control) in the liver nuclei from LPS-treated (4-h postinjection) animals. This result indicates that the decreased expression of C/EBPα observed during the acute phase response is partly due to a reduction in the rate of transcription of this gene in hepatocytes. In contrast, although the mRNA levels of C/EBPβ and in particular C/EBPδ were very high in LPS-treated liver, the rates of transcription of these two genes were increased only slightly; thus, post-transcriptional events, such as mRNA processing and stabilization, may play a role in these mRNA pool changes. In the kidney nuclei the transcription rate of C/EBPα was unaltered (Fig. 4B). The steady-state level of C/EBPδ mRNA was decreased by approximately 9-fold in the kidney following LPS stimulation; a similar increase (6-fold) in the rate of transcription of C/EBPδ gene occurred in the induced kidney nuclei. The rate of transcription of C/EBPδ gene increased by approximately 4-fold in the kidney nuclei from LPS-treated animals; therefore, the 151-fold rise in C/EBPδ mRNA in this tissue is likely due to both transcriptional and post-transcriptional events. Furthermore, the changes in the transcription rates suggest that the three C/EBP genes contain cytokine- and/or glucocorticoid-responsive elements.

**DISCUSSION**

In this study we present evidence that during the acute phase response the C/EBP family of transcription factors is differentially expressed. Mediators of the acute phase response, in particular IL-1, IL-6, and glucoorticoids, through their receptors evoke a signaling cascade that ultimately activates or represses gene expression. The dramatic changes in the expression of C/EBP isoforms suggest that the genes for these trans-acting factors are also influenced by mediators of acute inflammation. These trans-acting factors in turn regulate the expression of other genes, i.e. they may play a fundamental role in the regulation of genes required to maintain physiological homeostasis in various tissues during a pathological insult. This may represent another important role played by C/EBPs in addition to their well-characterized function in cell growth and differentiation (12, 22, 32).

The C/EBPs are members of a class of transcription factors, termed bZIP proteins, characterized by a basic domain linked to heptad leucine repeats required for dimerization (33, 34). C/EBPα, the first leucine zipper protein to be identified, can trans-activate the genes for serum albumin (14, 35), stearoyl acyl-CoA desaturase-1 and 422/a P2 protein (12, 36), and the insulin-responsive glucose transporter (37). C/EBPα is enriched in the liver and may play a role in the transcription of several liver-specific genes. For instance, it binds avidly to several sites of the albumin promoter (21, 35). Previous studies have indicated a striking transient decrease in albumin gene transcription during the acute phase response (7). The decreased expression in the liver of C/EBPα described here may contribute to this down-regulation of the albumin gene.

Our results indicate that the expression of C/EBPβ increases rapidly after LPS treatment in a wide variety of tissues. Recently, NF-IL-6, a human homolog of C/EBPβ, was also shown to be highly inducible by interleukins and LPS (19). NF-IL-6 binds to the IL-1-responsive element of
the IL-6 gene; consequently, the expression of IL-6, which participates in host defense reaction, rises rapidly during viral or bacterial infection. In addition, IL-6-dependent DNA binding protein, a rat homolog of C/EBPβ, has been shown to bind to the cis-elements of the hepatic acute phase reactant genes, hemopexin and haptoglobin (20). It has been shown that C/EBPβ displaces C/EBPα from the acute phase responsive element of the mouse α1-acid glycoprotein gene and that the binding activity of C/EBPβ increases dramatically in LPS-treated mice (16). The increased expression of C/EBPβ mRNA demonstrated in the present study strongly suggests that de novo synthesis of C/EBPβ is required for the induction of acute phase reactant genes, including the α1-acid glycoprotein gene.

Although C/EBPβ mRNA levels are dramatically increased by LPS, we find that the induction of C/EBPβ mRNA, a novel member of the family, is much more striking. In control animals, C/EBPβ mRNA was barely detectable in the kidney, liver, spleen, brain, and adipose tissues; however, within 4 h of LPS treatment, inductions ranging from 20- to 150-fold are seen in these tissues. Recently, increased expression of C/EBPβ was observed during very early adipocyte differentiation (22). At present the function of this newly identified transacting factor is unknown, although the dramatic inducibility of C/EBPβ suggests that this protein will play an important role in gene regulation in various tissues during acute inflammation.

The acute phase inducibility of hepatic genes has been well studied (3, 4); however, the effects of mediators of inflammation on other tissues are at present poorly understood. It was interesting to establish that non-hepatic tissues are also involved in the inflammatory process. We anticipate that the induction of the C/EBP isoforms is required for the activation of many different genes in various tissues. For instance, a recent report indicates that C/EBPβ (NF-IL-6) induces transcription of the cellular proto-oncogene c-fos (38). Thus, it is very likely that the differential expression of C/EBP isoforms are required to maintain the basic integrity of cells during a pathological event.

Acknowledgments—We are grateful to M. A. Hillesheim for her assistance in the preparation of this manuscript. We also thank Drs. S. L. McKnight and Zhaodan Cao for the C/EBP cDNA clones.

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