ELSEVIER

CYTOKINE

www.elsevier.com/locate/issn/10434666

Cytokine 37 (2007) 119–127

C/EBPβ serine 64, a phosphoacceptor site, has a critical role in LPS-induced IL-6 and MCP-1 transcription

Chauncey J. Spooner [a,1], Thomas Sebastian [b], Jon D. Shuman [b,2], Srinivasan Durairaj [c], Xiangrong Guo [a], Peter F. Johnson [b], Richard C. Schwartz [a,*]

[a] *Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA*
[b] *Laboratory of Protein Dynamics and Signaling, NCI-Frederick, Frederick, MD 21702-1201, USA*
[c] *Department of Physiology, Michigan State University, East Lansing, MI 48824, USA*

Received 9 June 2006; received in revised form 14 January 2007; accepted 5 March 2007

Abstract

C/EBPβ is a member of the CCAAT/enhancer binding protein family of transcription factors and has been shown to be a critical transcriptional regulator of various proinflammatory genes, including IL-6 and MCP-1. Serine 64 in the transactivation domain of C/EBPβ has recently been identified as a *Ras*-induced phosphoacceptor site. The integrity of serine 64 along with threonine 189 is important for the Ha-ras^{V12}-induced transformation of NIH3T3 cells, however no target genes dependent upon serine 64 for their expression have been reported. In order to evaluate a potential role of serine 64 in C/EBPβ-regulated cytokine expression, we expressed a form of C/EBPβ with an alanine substitution at serine 64 (C/EBPβ$_{S64A}$) in P388 murine B lymphoblasts, which lack endogenous C/EBPβ expression and are normally unresponsive to LPS for expression of IL-6 and MCP-1. In comparison to wild type C/EBPβ, which robustly supports the LPS-induced expression of IL-6 and MCP-1, C/EBPβ$_{S64A}$ was severely impaired in its ability to support the LPS-induced transcription of IL-6 and MCP-1. Furthermore, LPS stimulation increased the level of phosphorylation detected at serine 64. Thus, serine 64, probably through its phosphorylation, is a critical determinant of C/EBPβ activity in the transcription of IL-6 and MCP-1.

Keywords: Gene regulation; C/EBPβ; IL-6; MCP-1; Lipopolysaccharide

1. Introduction

C/EBPs comprise a family of basic region-leucine zipper (bZIP) transcription factors that, as a prerequisite for DNA-binding through their basic regions, dimerize through their adjacent C-terminal leucine zippers to form either homodimers or heterodimers with other C/EBP family members [1]. While the C/EBPs are well conserved through their bZIP regions, they exhibit more limited conservation in their N-terminal activation and regulatory domains [1]. C/EBPα, β, δ, and ε commonly function as strong transcriptional activators, while C/EBPγ and ζ generally act to inhibit C/EBP transcriptional activators [2]. C/EBPζ:C/EBP heterodimers can also activate transcription through a non-consensus binding site [3,4], and recent work has shown that C/EBPγ as a heterodimer with C/EBPβ can augment transcription in a promoter and cell-specific manner [5]. C/EBP binding sites have been identified in the promoter regions of numerous cytokine and other proinflammatory genes, including IL-6 [6], IL-12 [7], TNFα [8], IL-1-β [9], IL-8 [10]), MCP-1 [11], G-CSF [12], myeloperoxidase [13], inducible NO synthase [14], and lysozyme [15]. Moreover, the activities of C/EBPβ and C/EBPδ are influenced by various inflammatory stimuli, including LPS [16,17], IL-6 [18], IL-1 [6], and TNFα

* Corresponding author. Fax: +1 517 353 8957.
E-mail address: schwart9@msu.edu (R.C. Schwartz).
[1] Present address: Department of Molecular Genetics & Cell Biology, The University of Chicago, Chicago, IL 60637, USA.
[2] Present address: Brewton-Parker College, Mount Vernon, GA 30445, USA.

1043-4666/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2007.03.001

[19], suggesting an important role for C/EBPs in mediating the inflammatory response. We previously demonstrated that the stable expression of C/EBPα, β, δ, or ε was capable of conferring LPS-induced IL-6 and MCP-1 expression to a murine B lymphoblast cell line [20–22].

While C/EBPs are quite divergent in their amino-termini, three separate activation domain modules (ADM1, 2, and 3) are shared by C/EBPα, β, δ, and ε [23,24]. The functional importance of amino-terminal determinants in C/EBP-mediated transcriptional regulation is supported by the fact that C/EBP$\beta_{153–297}$ (LIP), a truncated form of C/EBPβ that initiates at methionine 153 and lacks amino-terminal activation domains, can function as a repressor of C/EBP transcriptional activity by either directly competing with activating forms for C/EBP binding sites or as a dominant negative inhibitor by heterodimerization with activating C/EBP family members [25]. Moreover, C/EBPγ, which also lacks amino-terminal activation motifs, was reported to inhibit C/EBP transcriptional activity by either forming inactive heterodimers with C/EBPα and β, or by competing with activating C/EBP isoforms [26,27].

The amino-terminal domains modulate C/EBPβ activity by providing substrates for protein–protein interactions and post-translational modifications. Amino-terminal activation domains in C/EBPβ are reported to associate with the coactivator p300 [28–30] and the chromatin remodeling complex SWI/SNF [31], influencing the regulation of C/EBP-dependent promoters. In addition, various signal transduction pathways impact C/EBPβ activity at amino-terminal substrates. TPA-induced activation of the protein kinase C pathway results in phosphorylation of rat C/EBPβ at serine 105 and enhanced C/EBPβ transactivation activity [32]. This site has also been shown to function as a substrate for TGF-α-induced phosphorylation by p90 ribosomal S kinase, which increases C/EBPβ activity and hepatocyte proliferation [33]. *Ras*-mediated phosphorylation of rat C/EBPβ at a MAPK site located at threonine 189 enhances transactivation of an IL-6 promoter–reporter target promoter, and mutations of the residues that comprise this site reduce *Ras*-mediated C/EBPβ activation [34]. This MAPK substrate is also responsive to growth hormone-induced phosphorylation and an alanine substitution at threonine 189 inhibits c-fos promoter activation in response to growth hormone [35].

Recently, serine 64 of rat C/EBPβ was identified as a *Ras*-induced, proline-directed phosphoacceptor site [36]. The same study found that both serine 64 and threonine 189 are substrates for phosphorylation by Cdk2 and Cdc2, and possibly other cyclin-dependent kinases [36]. While inferferon-γ stimulation has recently been shown to induce target genes through the activation of C/EBPβ by dephosphorylation of serine 64 [37], no target genes have been identified that are dependent upon serine 64 phosphorylation. LPS stimulation has been implicated in *Ras* activation by several studies using dominant negative inhibitors of *Ras* [38–40], as well as inhibitors of farnesylation [41]. Recently, LPS activation of *Ras* has been directly demonstrated [42]. Viewed in this context, the *Ras*-induced phosphorylation of C/EBPβ serine 64 [36] implicates this residue as a potentially important motif in the activation of proinflammatory target genes. In this study, we examined the role of C/EBPβ serine 64 in the transcription of two proinflammatory cytokines, IL-6 and MCP-1. We have identified serine 64 in the amino-terminal ADM2 of C/EBPβ, a residue that is highly conserved among C/EBP family members, as a critical functional determinant for C/EBPβ-mediated activation of both IL-6 and MCP-1 expression.

2. Materials and methods

2.1. Cell culture

P388 cells are murine B lymphoblasts [43] (American Type Culture Collection; CCL 46). P388-Neo and P388-Cβ cells have been described previously [44]. P388 cells were cultured in RPMI 1640 medium supplemented to 5% FCS and 50 μM 2-ME. WEHI 274.1 cells (American Type Culture Collection; CRL 1679) are a murine monocytic cell line. WEHI 274.1 cells were cultured in DMEM supplemented to 10% FCS and 50 μM 2-ME. All IL-6 and MCP-1 inductions were conducted with LPS derived from *Escherichia coli* serotype 055:B5 (Sigma) added to 10 μg/ml.

2.2. Expression vectors and promoter–reporters

For transient transfections, C/EBPβ (LAP) was expressed from pMEX [45], which utilizes the Moloney murine sarcoma virus promoter. C/EBPβ_{S64A} was expressed from pcDNA3.1 [36], which utilizes the CMV immediate-early promoter. For stable retroviral transductions, C/EBPβ (LAP) and C/EBPβ_{S64A} were expressed from pZIP-NEO SV(X)1, a retroviral vector derived from Moloney murine leukemia virus [46]. In order to prepare the C/EBPβ_{S64A} form of this vector, C/EBPβ_{S64A} [36] was excised from pcDNA3.1 as an *Eco*RI/*Hin*dIII fragment and was transferred into the *Bam*HI site of pZIP-NEO SV(X)1 [46] with *Bam*HI linkers. 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.

The IL-6 promoter–reporter consists of the murine IL-6 promoter (−250 to + 1) inserted into the luciferase vector pXP2 [44]. The MCP-1 promoter–reporter consists of the murine MCP-1 promoter [47] (−322 to +59) inserted into the luciferase vector pGV-β2 (Toyo Ink Mfg. Co., Ltd.). The SV40 early promoter–reporter is a commercial product, p-βgal control (Clontech), where the SV40 early promoter and enhancer sequences are cloned upstream and downstream, respectively, of the *lacZ* gene.