CCAAT/Enhancer-binding Protein Activates the CD14 Promoter and Mediates Transforming Growth Factor β Signaling in Monocyte Development

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Transcription factors from the CCAAT/enhancer-binding protein (C/EBP) family play important roles in myeloid cell differentiation. CD14 is a monocyte/macrophage differentiation marker and is strongly up-regulated during monocytic cell differentiation. Here, we report the direct binding of C/EBP to the monocyte-specific promoter of CD14. Transactivation analyses demonstrate that C/EBP family members significantly activate the CD14 promoter. These data indicate that C/EBP is directly involved in the regulation of CD14 gene expression. When myelomonoblastic U937 cells are treated with vitamin D₃ and TGF-β, they differentiate toward monocytic cells. Using specific antibodies against different C/EBP family members in electrophoretic mobility shift assays and Western blot assays, we have identified a specific increase in the DNA binding and the expression of C/EBPa and C/EBPβ during U937 monocyto/macrophage cell differentiation, and we found C/EBPa and C/EBPβ bind to the promoter in heterodimer. Furthermore, with stably transduced cell lines, we demonstrate that the C/EBP binding site in the CD14 promoter plays a critical role for mediating TGF-β signaling in the synergistic activation of CD14 expression by vitamin D₃ and TGF-β during U937 differentiation. This may indicate that C/EBPs have important functions in the process of TGF-β signal transduction during monocytic differentiation.

CD14 is highly expressed on the surface of monocytes/macrophages and is strongly up-regulated during the differentiation of mononuclear precursors into monocytes (14–16). Therefore, the regulation of CD14 expression has been investigated as a model to provide critical insight into the mechanisms of monocytic differentiation. During monocye differentiation, the expression of CD14 is specifically and strongly up-regulated at the transcription level (17). The upstream sequence of the CD14 gene shows a strong tissue-specific promoter activity (10). Functionally, CD14 is a receptor for the complex of lipopolysaccharide and its binding protein (18); the recognition between CD14 and the complex is a crucial step in triggering macrophage function during bacterial infection (19). Recently, CD14 has also been reported to play an important role in apoptosis (20, 21).

C/EBPs are a family of transcription factors containing an activation domain, a DNA-binding basic region, and a leucinerich dimerization domain (22). C/EBPa was initially identified in the liver (23), where it was found to be important for cell-specific gene expression and differentiation (24, 25). There are currently six known members of the C/EBP transcription factor family. C/EBP regulates the expression of three critical growth factor receptors for myeloid cell development, namely, macrophage-CSF, granulocyte-CSF, and granulocyte/macrophage-CSF (8, 26, 27). Knockout experiments in mice provide direct evidence to demonstrate that C/EBPs play critical roles in hematopoietic cell development (28–33).

In the present study, we have identified a C/EBP site in the critical region of the CD14 promoter and investigated the expression of the CD14 gene under the regulation of C/EBP. Furthermore, we have analyzed the regulation of CD14 expression during vitamin D₃- and TGF-β-induced monocyte differentiation with U937 cells. Our results demonstrate that C/EBP is an important transcription factor for CD14 promoter activity and that the C/EBP binding site is crucial for the synergistic signaling from vitamin D₃ and TGF-β to induce monocyte differentiation, and we are the first to demonstrate that C/EBPs mediate TGF-β signaling in a model of monocyte development.

MATERIALS AND METHODS

Cell Culture—Human promonocytic THP-1 cells (American Type Culture Collection (ATCC) TIP202, Manassas, VA) were grown in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Sigma), 2 mM l-glutamine (Life Technologies, Inc.), and 2 × 10⁻³ M 2-mercaptoethanol (Sigma). Human myelomonoblastic U937 cells (ATCC, CRL 1593) were maintained in RPMI 1640 medium with 10% fetal bovine serum and 2 mM l-glutamine. Human cervical carci-

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‡ The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; TGF, transforming growth factor; CSF, colony-stimulating factor; bp, base pair(s); PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; RSV-hGH, Rous sarcoma virus promoter directing human growth hormone gene expression.
The cells were subsequently harvested for nuclear protein preparation. pCMV-hC/EBP THP-1 and U937 cells were cultured to a concentration of bovine serum albumin, and 1 μg of poly(dI-dC) at room temperature for 20 min. For competition analysis, a 50-fold molar excess (except as described previously) of poly(dI-dC) was purchased. Total RNA was isolated using the Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). Northern blot analysis was performed as described previously. CD14 cDNA was labeled by the random priming method. The blot was washed twice with 0.2× SSC, 0.1% SDS for 20 min at 65 °C. Autoradiography was performed using Kodak BioMax MR film at ~80 °C with Kodak BioMax MS intensifying screens. Quantitation of related mRNA levels was performed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**The Putative C/EBP Site Is Important for CD14 Promoter Activity**—In a previous study (10), we defined the proximal promoter of the CD14 gene, in which four regions of the CD14 proximal promoter were protected by nuclear proteins from monocyctic cells in DNase I footprinting analysis. Three Sp1 sites were located in this region. The Sp1 binding site at bp −110 is critical for the tissue-specific activity of the CD14 promoter. However, the function of a protected site further upstream (bp −121 to −154) and the identity of the protein interacting with this site remained unknown. In order to elucidate this protein/DNA interaction, the sequence of this region was analyzed. We found that the sequence between bp 128 and −136 (TATTGCAAT) of the noncoding strand was almost identical to the C/EBP consensus sequence TTNGNNAAT (43). To verify the functional importance of the putative C/EBP site for CD14 promoter activity, six base pairs (bp −129 to −135) in the core C/EBP binding site were mutagenized by PCR-mediated site-specific mutagenesis from TGCAAT in the CD14 promoter luciferase reporter gene construct, p-227CD14-luc, to GGTACC and 5′-GGGGATTCAATGTCAGG-3′. To verify the functional importance of the putative C/EBP site for CD14 promoter activity, six base pairs (bp −129 to −135) in the core C/EBP binding site were mutagenized by PCR-mediated site-specific mutagenesis from TGCAAT in the CD14 promoter luciferase reporter gene construct, p-227CD14-luc, to GGTACC and 5′-GGGGATTCAATGTCAGG-3′. To verify the functional importance of the putative C/EBP site for CD14 promoter activity, six base pairs (bp −129 to −135) in the core C/EBP binding site were mutagenized by PCR-mediated site-specific mutagenesis from TGCAAT in the CD14 promoter luciferase reporter gene construct, p-227CD14-luc, to GGTACC and 5′-GGGGATTCAATGTCAGG-3′. To verify the functional importance of the putative C/EBP site for CD14 promoter activity, six base pairs (bp −129 to −135) in the core C/EBP binding site were mutagenized by PCR-mediated site-specific mutagenesis from TGCAAT in the CD14 promoter luciferase reporter gene construct, p-227CD14-luc, to GGTACC and 5′-GGGGATTCAATGTCAGG-3′. To verify the functional importance of the putative C/EBP site for CD14 promoter activity, six base pairs (bp −129 to −135) in the core C/EBP binding site were mutagenized by PCR-mediated site-specific mutagenesis from TGCAAT in the CD14 promoter luciferase reporter gene construct, p-227CD14-luc, to GGTACC and 5′-GGGGATTCAATGTCAGG-3′.
nuclear protein(s) showed a strong interaction with oligo A (Fig. 2A, lane 2). The major shifted complex (labeled with C/EBP) can be efficiently competed with unlabeled self oligonucleotide and a C/EBP binding oligonucleotide from the M-CSF receptor promoter (Fig. 2A, lanes 3 and 4) (8). In contrast, non-C/EBP binding oligonucleotides with the consensus sequences of Sp1 or PU.1 failed to compete with labeled oligo A (Fig. 2A, lanes 5 and 6). In addition, nuclear extracts from nonmonocytic HeLa cells do not form the same complex with oligo A (Fig. 2A, lane 7). C/EBPs are not normally expressed in HeLa cells (7, 8). These results demonstrate that the protein(s) binding to bp −115 to −144 of the CD14 promoter belongs to the C/EBP family.

C/EBPs are a family of transcription factors that have high homologies at their C-terminal dimerization domains and DNA binding domains. In order to verify which C/EBP member interacts with the CD14 promoter, specific antibodies against different members of the C/EBP family were used in an EMSA. As shown in Fig. 2B, a strong supershifted band was observed with the C/EBPα antibody, and the original shifted band was significantly reduced in the presence of the C/EBPα antibody (Fig. 2B, lanes 2 and 3). The antibodies against C/EBPβ and C/EBPδ also reacted with the DNA-C/EBP complex (Fig. 2B, lanes 4 and 5). However, in comparison to the C/EBPα antibody, the reduction of the complex by C/EBPβ and C/EBPδ antibodies was less significant. As negative controls, the antisera against Sp1 and normal rabbit serum were used and do not have any effect on the DNA-C/EBP complex (Fig. 2B, lanes 6 and 7). The results indicate that C/EBPα, -β, and -δ are able to bind to the CD14 promoter. Furthermore, C/EBPα is the major C/EBP family member interacting with the CD14 promoter in promonocytic THP-1 cells.

**C/EBPs Transactivate the CD14 Promoter in C/EBP negative CV-1 Cells**—To further demonstrate the importance of C/EBPs for CD14 promoter activity, C/EBP transactivation experiments were conducted in the C/EBP negative cell line CV-1 (Fig. 3). Compared with CD14 promoter activity in the absence of any C/EBP expression, C/EBPs activated the CD14 promoter 11-fold, whereas C/EBPβ and C/EBPδ activated the promoter 4.8- and 8.5-fold, respectively. When the construct with the mutated C/EBP site was used in parallel experiments, C/EBPα, -β, and -δ significantly lost their ability to transactivate the CD14 promoter. These data indicate that C/EBPα, -β, and -δ can activate the CD14 promoter and that this transactivation by C/EBPs is through the identified C/EBP site at bp −129 to −136 of the CD14 promoter.

**C/EBP Binding Is Enhanced during Monocyte Differentiation**—The above results demonstrate that C/EBPs are important transcription factors for CD14 expression. To further study the role of C/EBP in CD14 expression during monocyte differentiation, U937 cells were treated with vitamin D3, a C/EBP binding oligonucleotide for Sp1 (lane 5), and an oligo for PU.1 (lane 6) were used in the assays. B, C/EBPα is the major C/EBP that interacts with the CD14 promoter. The radiolabeled double-stranded oligo A was incubated in the absence (lane 1) or the presence of 5 μg of nuclear extracts from THP-1 cells (lanes 2–7). One μg of antisera against various transcription factors (C/EBPα lane 3, C/EBPβ lane 4, C/EBPδ (lane 5), Sp1 lane 6, or normal rabbit serum (lane 7)) was added to the binding reaction to identify the transcription factor in the DNA-protein complex. The asterisk marks a relatively nonspecific complex.
expression was elevated upon such induction. CD14 mRNA was detected by Northern blot analysis when cells were treated with vitamin D₃ (Fig. 4, lanes 2 and 3). CD14 expression was highly up-regulated when cells were treated with both vitamin D₃ and TGF-β (Fig. 4, lanes 5 and 6) but not by TGF-β (lane 4). Densitometry analysis revealed a 30-fold increase relative to treatment by vitamin D₃ alone. To investigate whether C/EBP is involved in the induction of CD14 expression, an EMSA was performed with nuclear extracts from untreated and variously treated U937 cells, as shown in Fig. 5. Under both treatments, an increase of C/EBP binding to the CD14 promoter was detected. Treatment with a combination of vitamin D₃ and TGF-β showed a much higher level of the increase than is seen in treatment with vitamin D₃ alone (Fig. 5, lanes 2, 7, and 12). To identify which C/EBP family members were responsible for the increase of C/EBP binding during monocyte differentiation, antibodies against C/EBPα, -β, -δ, -ε were independently added to the reaction mixtures. The results showed that the majority of the binding before the inductions was C/EBPβ (Fig. 5, lanes 2–6). C/EBPα and C/EBPβ were the major contributors in the increase of the DNA-C/EBP complex after the cells were treated with vitamin D₃ or vitamin D₃ plus TGF-β (Fig. 5, lanes 7–16). Because the addition of either C/EBPα or C/EBPβ antibody removed almost the entire complex (Fig. 5, lanes 8, 9, 13, and 14), it indicates not only that C/EBPα and C/EBPβ are the major binding factors but also that they form heterodimers.

C/EBP Expression Is Increased during U937 Differentiation toward Monocyte—To investigate the reason for the elevation of C/EBP binding during monocyte differentiation, Western blot analyses were performed with nuclear proteins from U937 cells after various inductions. The expression of C/EBPα and C/EBPβ was increased when U937 cells were treated with either vitamin D₃ or TGF-β (Fig. 6). However, the strongest increases of both C/EBP factors were seen upon treatment with both vitamin D₃ and TGF-β. Densitometry analysis revealed a 13-fold increase for both C/EBPα and C/EBPβ. It has been reported that phosphorylation regulates the ability of C/EBPs to interact with DNA (47–50). Therefore, we also treated these nuclear extracts with a phosphatase. The dephosphorylation did not alter the increasing interaction between C/EBP and the CD14 promoter during monocyte differentiation (data not shown). These data indicate that the increased production or accumulation in the nuclei of C/EBPs and C/EBPβ is the major contributor for the increase of C/EBP binding to the CD14 promoter.

C/EBPs Stimulate Transcription from the CD14 Promoter and Mediate TGF-β Signaling in Vivo—We have demonstrated that C/EBPs can transactivate the CD14 promoter in CV-1 cells and the amounts of C/EBPα and C/EBPβ in the nuclei are increased during induced U937 cell differentiation. In order to investigate the in vivo function of C/EBPs in the regulation of the CD14 promoter, we stably transfected U937 cells with pXPA (vector construct), p-227CD14-luc, and p-227CD14(m135)-luc, which contains the CD14 promoter with a mutated C/EBP binding site. Four of the positive transfectants from each DNA construct were treated...
with $1 \times 10^{-7}$ M vitamin D$_3$ and 1 ng/ml TGF-β for 24 h to induce monocyte differentiation. The results revealed that the activity of the CD14 promoter in the induced transfectants by vitamin D$_3$ and TGF-β was 8.6-fold higher than in the uninduced transfectants (Fig. 7). However, if the C/EBP binding site in the CD14 promoter was mutated, the elevation of CD14 promoter activity by vitamin D$_3$ and TGF-β was significantly lower to 2.5-fold. The transfectants with the vector construct pXP2 did not show any induction. This result indicates that C/EBP transcription factors contribute to the regulation of CD14 expression during vitamin D$_3$ and TGF-β-induced monocyte differentiation. To further distinguish the role of C/EBP in such signal transduction, the same p-227CD14-luc and p-227CD14(m135)-luc stably transfected cell lines were also treated with TGF-β or vitamin D$_3$ independently. As shown in Fig. 7, TGF-β treatment alone did not have any effect on the promoter activity, whereas vitamin D$_3$ treatment enhances the promoter activity 2–3-fold. The increase in promoter activity in vitamin D$_3$ treated cells is about the same as that seen in the response of the mutant promoter to vitamin D$_3$ plus TGF-β treatment. The data from these in vivo stably transfected cell line analyses demonstrate that C/EBP is involved in the synergy of TGF-β and vitamin D$_3$ signaling and mediated TGF-β signaling but not in vitamin D$_3$-induced CD14 up-regulation.

**Fig. 6.** Western blot analysis of C/EBP expression in treated and untreated U937 cells. Eighty μg of nuclear protein from wild type U937 cells or U937 cells treated with $1 \times 10^{-7}$ M vitamin D$_3$, 1 ng/ml TGF-β, or both as labeled on the top of the panels were electrophoresed and transferred to a membrane. The blot was incubated with antiserum against C/EBPα and then stripped and incubated with antiserum against C/EBPβ.

**DISCUSSION**

In normal hematopoiesis, pluripotent stem cells proliferate and differentiate to become cells of different lineages. This process is tightly regulated by gene expression under the control of signaling from various growth factors and cytokines. Our previous work has demonstrated that monocyte-specific CD14 expression is regulated at the transcriptional level and the CD14 proximal promoter is involved in the tissue-specific regulation of CD14 gene expression. Here, we report the analysis of the protein-DNA interaction in a critical region of the CD14 proximal promoter and demonstrate that C/EBP is the transcription factor involved in this interaction. Furthermore, using transient transfection analyses in a nonmonocytic cell line, C/EBP shows a strong transactivation of the CD14 promoter. Most interestingly, data from stably transfected U937 cell lines indicate that C/EBP plays an important role during the synergistic activation of monocyte differentiation induced by vitamin D$_3$ and TGF-β and mediates TGF-β signaling.

C/EBPs have been shown to play important roles in different cellular events (32). Their expressions during hematopoiesis are highly regulated (31, 32, 43, 51). In relatively undifferentiated U937 cells or THP-1 cells, C/EBPα is the major C/EBP in the interaction with CD14 promoter (Figs. 2B and 5). However, in the vitamin D$_3$ and TGF-β-treated, relatively differentiated U937 cells, the results indicate that instead of C/EBPα alone, C/EBPα and C/EBPβ, which form heterodimer (Fig. 5), are the two major C/EBP members involved in the interaction with the CD14 promoter in monocytic cells. These observations suggested that during monocyte maturation, the role of C/EBPβ is enhanced and critical; moreover, the expression of C/EBPβ is elevated. These results are supported by recent reports that targeted inactivation of C/EBPβ in mice causing macrophage dysfunction, impaired tumor killing ability and lymphoproliferative disorder (29, 30). Furthermore, induced expression of C/EBPβ can result in the expression of several monocytic genes, including CD14 (52). C/EBPα knockout mice died within 8 h after birth due to hypoglycemia (25). We have analyzed C/EBPα knockout mice and demonstrated the importance of C/EBPα for granulocytic cell maturation during fetal liver hematopoiesis (28). Mature monocytes were present in the blood of fetal and newborn mice. The data from knockout mice indicate that the C/EBPα is a critical factor for granulocytic but not monocytic cell maturation. However, a recent report regarding fet al liver hematopoiesis demonstrated that liver-derived macrophages arise from a precursor that is also common to lymphocytes (53). Therefore, the role of C/EBPα during normal bone marrow-derived myeloid cell differentiation remains to be investigated.

All members of the C/EBP family contain a highly homologous basic region for DNA binding and a leucine zipper region for dimerization. Their N-terminal activation domains are relatively diverse and can interact with different proteins (54, 55). We observed the change of the forms of C/EBP binding to CD14 promoter during U937 differentiation from homodimer to heterodimer. It is possible that the interaction of the same DNA sequence with different homo- or heterodimers of C/EBP precisely regulates their target gene expression at different stages of proliferation, differentiation, and function. To elucidate this possibility, we have performed transient transfection analyses in CV-1 cells with the combination of various amounts of C/EBPα and C/EBPβ expression constructs. No differences have been detected in such an assay (data not shown). However, it is possible that in primary hematopoietic cells, the effect of the combination of these two factors may facilitate normal monocyte differentiation. As shown in Fig. 6, C/EBPα, -β, and -δ can independently activate the CD14 promoter. Therefore, it is also possible that in the absence of one factor
TGFB, which is produced by cells present in the bone marrow and the hematopoietic microenvironment of the liver, inhibits cell proliferation and promotes cell differentiation (56). By itself, TGFB cannot promote U937 differentiation in our assay (data not shown), but it can dramatically enhance the efficiency of vitamin D3 in induction of monocyte differentiation. TGFB signaling has been shown to be mediated by Sp1 in the p15 promoter (57), the a2U collagen promoter (58), and the p21 promoter (59, 60). However, Sp1 is not directly responsible for TGFB signaling in the CD14 promoter, although Sp1 is very important for the tissue specificity of CD14 promoter and vitamin D3-induced up-regulation of CD14 promoter activity (10, 17). As shown in Fig. 7, we observed that p-227 (m135)CD14-luc mutation construct lost the response to TGFB stimulation although it contains three intact Sp1 binding sites. We could also not detect the interaction between Sp1 and C/EBP with EMSA (data not shown). These data indicate that C/EBP binding site at bp -135 of the CD14 promoter is critical for TGFB signaling during monocyte development and may mediate TGFB signaling during monocyte differentiation.

As shown in Figs. 5 and 6, in U937 cells upon treated with a combination of vitamin D3 and TGFB to induce monocyte development, the level of C/EBPa and C/EBPb and their binding to CD14 promoter are highly increased. It has been reported that the DNA binding abilities of C/EBPa and C/EBPb are attenuated by TGFB signaling in a rat intestinal epithelial cell line (61) and that C/EBPb binding activity is increased by TGFB signaling in mouse hepatocytes (62). This indicates that TGFB signaling also has tissue specificity. Further studies will be required to analyze how C/EBPa and C/EBPb expression is regulated during TGFB- and vitamin D3-induced monocyte differentiation.

The results presented here and in previous studies demonstrate the important functions of C/EBP and Sp1 in the regulation of CD14 promoter activity (10, 17). In the stable cell lines, the synergistic enhancement of CD14 promoter activity by vitamin D3 and TGFB is 8.5-fold over the control (Fig. 7). The increase in endogenous CD14 expression is significantly higher than 8.5-fold during monocyte differentiation. This indicates that the CD14 promoter is responsible for the induction of CD14 expression during monocyte differentiation, other regulatory element(s) beyond the promoter are also involved in the up-regulation. Such interpretation is also suggested by our analysis of CD14 gene expression in transgenic mice (63). One of the candidate factors is PU.1. PU.1 knockout mice have an absolute block in monocyte development (5, 6, 64). Moreover, PU.1 has been demonstrated to play a critical role in the expression of monocyte-specific genes, such as macrophage-CSF receptor (9, 65, 66), CD11b (67), CD18 (68), and macrophage scavenger receptor (69). Analysis of the contribution of other regulatory elements will provide important insights into the response of monocyte differentiation signals.

In conclusion, we have identified a functional critical C/EBP site in CD14 promoter, demonstrated that this C/EBP site can mediate TGFB signaling during monocyte differentiation, and related vitamin D3 and TGFB signal pathways to the changes in C/EBP expression. Further studies will be necessary to analyze whether these results from in vitro cell lines reflect the in vivo events. Moreover, because TGFB and C/EBP both are involved in the regulation of cell cycle (70–73), the analysis of CD14 gene expression in response to vitamin D3 and TGFB signaling will provide a valuable opportunity to study the cross-talk between cell cycle regulation and differentiation and the mechanism of vitamin D3 and TGFB signaling during monocyte differentiation.

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