Representational difference analysis using myeloid cells from C/EBPε deletional mice

Tetsuya Kubota, Seiji Kawano, Doris Y. Chih, Yasuko Hisatake, Alexey M. Chumakov, Hirokuni Taguchi, and H. Phillip Koeffler

C/EBPε is a recently cloned member of the C/EBP family of transcriptional factors. Previous studies demonstrated that the expression of this gene is tightly regulated in a tissue specific manner; it is expressed exclusively in myeloid cells. C/EBPε-deficient mice developed normally but failed to generate functional neutrophils and eosinophils, and these mice died of opportunistic infections sug- gesting that C/EBPε may play a central role in myeloid differentiation. To identify myelomonocytic genes regulated by the C/EBPε gene, we performed representational difference analysis (RDA), a polymerase chain reaction (PCR)-based subtractive hybridization using neutrophils and macrophages from wild-type and C/EBPε knockout mice. We identified a set of differentially expressed genes, in-cluding chemokines specific to myelomonocytic cells. Several novel genes were identified that were differentially expressed in normal myelomonocytic cells. Taken together, we have found several genes whose expression might be enhanced by C/EBPε. (Blood. 2000;96: 3953-3957)

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Introduction

The human CCAAT/enhancer binding protein (C/EBP) family of transcription factors includes C/EBPα,[1] C/EBPβ,[2-7] C/EBPγ,[8] C/EBPδ,[3,9,10] C/EBPε,[11,12] and C/EBPζ.[13] These proteins have highly conserved C-terminal basic amino acid-rich regions with 3′ flanking leucine zipper domains that are essential for DNA binding and dimerization, but these proteins differ in their N-terminal regions.[14,15] The members of this family are believed to bind the same recognition sites on DNA, having the consensus sequences 5′-ATTGCGCAAT-3′.[16] Previous studies have suggested that the C/EBP proteins interact with some transcriptional factors, including c-Myb,[17] cAMP response element-binding protein (CREB),[18] Fos-Jun family,[19] and NF-κB.[20]

The human C/EBPε gene was recently cloned and mapped to chromosome 14q11.2.[11,12] Differential splicing and using alternative promoters can generate a total of 4 proteins with calculated molecular masses of 32, 30, 27, and 14 kd.[12,21] The functional significance of these variants remains unclear.[22] Previous studies demonstrated that the expression of this family was tightly regulated in a tissue-specific manner.[23] C/EBPα, C/EBPβ, and C/EBPδ are all expressed during hematopoiesis, suggesting that these proteins may regulate a variety of hematopoietic-related genes.[24] C/EBPε is exclusively expressed in the myeloid and T-lymphoid lineages, especially during granulocytic differentiation.[11,12,21,25] A previous study using C/EBPε-deletional mice demonstrated that these mice developed normally but failed to generate functional neutrophils and eosinophils, and died of opportunistic infections between 3 and 5 months after birth, suggesting that C/EBPε may play a central role in myeloid differentiation.[26] C/EBPε-deficient neutrophils possess distinctive defects, including a lack of secondary granule proteins, which may contribute to the loss of normal responses to inflammatory stimuli.[27,28] In addition, studies have shown that retinoic acid (RA) can dramatically enhance the expression of C/EBPε in acute promyelocytic leukemia (APL) cells as they are induced to differentiate, suggesting that C/EBPε is a retinoic acid response gene that helps mediate terminal differentiation of APL cells.[21,29,30] Meanwhile, a previous study showed that genes coding for macrophage inhibitory protein 1 α (MIP-1α), MIP-1β, and the macrophage colony-stimulating factor (M-CSF) receptor have been implicated as transcriptional targets of C/EBPε in mice.[31]

To identify other myelomonocytic target genes regulated by the C/EBPε gene, we performed a representational difference analysis (RDA) using peritoneal lavage cells from wild-type and C/EBPε knockout mice. Several differentially expressed genes, including chemokines, were identified, suggesting that C/EBPε could enhance chemokine gene expression in mice.

Materials and methods

Mice and thioglycollate challenge

C/EBPε −/− mice were generously provided by Dr K. G. Xanthopoulos and Julie Lekstrom Himes (NIH, the former now at Aurora Bioscience, La Jolla, CA)[26] and wild-type mice (129SV) were obtained from Harlan Sprague Dawley, Inc (Indianapolis, IN) and maintained in a pathogen-free condition. Their genotypes were evaluated, as previously reported.[26] Twenty C/EBPε −/− mice at 1 month of age and 20 age-matched wild-type mice received 2 mL of 4% thioglycollate broth (Sigma Chemical Co, St Louis, MO) by intraperitoneal injection, as previously reported.[26] Twenty-four hours later, all mice were killed by neck dislocation, and peritoneal exudate cells were harvested by lavage with 8 mL of Hanks' balanced salt solution (Life Technologies, Inc, Gaithersburg, MD) on ice. Total cell

numbers were counted and cytospins of lavage fluid cells were stained with Wright-Giemsa and the percentage of neutrophils and macrophages was determined by light microscopy.

RNA preparation and complementary DNA synthesis

Total RNA was isolated from the peritoneal lavage cells of 20 wild-type and 20 knockout mice using TRIzol (Life Technologies, Inc). To minimize individual variation, the RNAs from either all the wild-type mice or all the knockout mice were mixed together and used for RDA analysis. Complementary DNA (cDNA) samples of tester (containing differentially expressed transcripts in wild-type mice) and driver (containing transcripts also expressed in C/EBPϵ-knockout mice) were synthesized and amplified in parallel using SMART PCR cDNA Synthesis Kit (CLONTECH, Palo Alto, CA), as per the manufacturer's instructions.

Representational difference analysis

RDA was performed using PCR-Select cDNA Subtraction Kit (CLONTECH), as described previously.[32-34] Subtracted nested-polymerase chain reaction (PCR) products were cloned into a plasmid using an Original TA cloning kit (Invitrogen, Carlsbad, CA) and electroporated into competent *Escherichia coli*. Finally, subtracted cDNA libraries were constructed. To make 2 sets of membrane filters, *E coli* colonies were blotted onto nylon membranes and duplicated. Probes from either tester or driver cDNA were generated from the same subtracted nested-PCR product by separating adaptors with restriction enzyme *Rsa*I. After probes were purified using the GENECLEAN II kit (BIO 101 Inc, La Jolla, CA), membranes containing cDNA libraries were hybridized with either tester or driver probe in a standard condition. By comparing signals between 2 blots, differentially expressed clones were picked and cultured. Plasmids were isolated with a standard method and sequenced with Thermo Sequenase II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech, Inc, Cleveland, OH), as per manufacturer's protocol. Sequence data were identified via the GenBank.

Virtual Northern blot and Northern blot analyses

Five hundred nanograms of PCR-amplified (nonsubtracted) cDNAs were electrophoresed on agarose gels (1.2%), and Southern blotted onto nylon membranes. These filters were hybridized at 65°C for 3 hours using a Rapid-hyb buffer (Amersham Life Science) with [α-^{32}P] dCTP-labeled DNA probes, which were obtained by RDA. Filters were rinsed to a final stringency of 0.25 × SSC and exposed to a Kodak X-Omat or a Biomax film (Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen. To obtain a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe for control hybridization, murine cDNAs were amplified with specific primers for GAPDH included in the PCR-Select cDNA subtraction kit (CLONTECH), cloned into plasmid using the TA cloning method, and sequenced for integrity. Clones that showed no significant difference, as determined by virtual Northern blot analysis, were excluded for further consideration.

Ten micrograms of each total RNA sample were electrophoresed on agarose formaldehyde gels and transferred onto nylon membranes. The blots were hybridized at 65°C overnight with [α-^{32}P] dCTP-labeled DNA probes generated by RDA using a standard method.[32-34] Filters were rinsed to a final stringency of 0.25 × SSC and exposed to a Kodak X-Omat or a Biomax film (Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen.

Results

Thioglycollate challenge

Twenty C/EBPϵ-knockout mice at the age of 1 month and 20 age-matched wild-type mice received thioglycollate intraperitoneally, and peritoneal lavage fluids were collected after 24-hour stimulation. Total cell numbers in the fluid were not different

between control and knockout (control, $[2.1 \pm 0.9] \times 10^7$; knockout, $[2.7 \pm 2.0] \times 10^7$, respectively). In addition, the ratios of the granulocyte (G) to macrophage (M) populations showed no difference between these 2 groups (control, G:M = 65.6 ± 9.5: 34.4 ± 9.5; knockout, G:M = 61.0 ± 9.2 :39.1 ± 9.2, respectively).

Identification of differentially expressed genes between C/EBPϵ +/+ and −/− peritoneal cells determined by representational difference analysis

After RDA was used to isolate cDNA fragments that were expressed specifically in normal neutrophils/macrophages, a total of 174 clones were isolated, grown up, and sequenced. These fragments, which were theoretically differentially expressed, were sequenced and compared with known GenBank sequences via a BLAST search to determine their identity. Homology analysis revealed that these clones consisted of 33 clones with different nucleotide sequences. The abundant clones were: Early T-Lymphocyte Activation-1 protein (ETa-1) (28.2%), MIP-1γ (10.2%), cathepsin L (6.3%), C10 chemokine (6.3%), and galactose/ N-acetyl galactosamine (Gal/GalNAc)-specific lectin (1.7%). The other clones were not so frequent as these (data not shown). Interestingly, C/EBPϵ was not identified. Fifteen clones disclosed no major homology to previously known sequences, suggesting that these genes might be unknown. The unknown clones 1, 2, and 3 of the 15 unknown clones showed partial homology to asialoglycoprotein receptor gene (87 of 407 bases), proapoptotic protein (siva) gene (50 of 340 bases), and pig alveolar macrophage chemotactic factor II gene (26 of 420 bases), respectively.
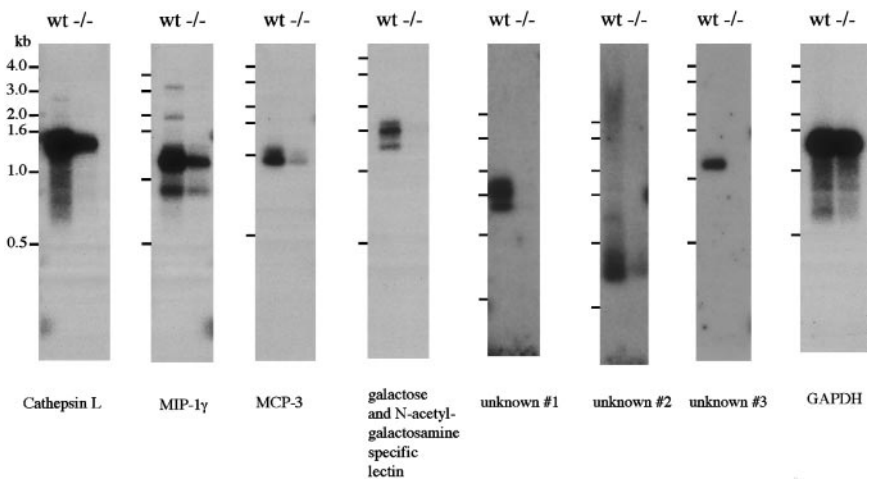
Expression of genes in C/EBPϵ −/− mice

To confirm that these fragments were differentially expressed in normal cells, virtual Northern blot analyses using PCR-amplified cDNAs were initially carried out; this approach was used because the amount of RNA from peritoneal lavage cells was limited. Figure 1 shows that cathepsin L, MIP-1γ, monocyte chemotactic protein-3 (MCP-3), and Gal/GalNAc-specific lectin were differentially expressed in normal cells, as measured by virtual Northern blot. Cathepsin L was strongly expressed in the wild-type; however, the difference between wild-type and knockout mice was modest. MIP-1γ was also strongly expressed in the wild-type and weakly expressed in the knockout mice. MCP-3 was modestly expressed in the wild-type and very weakly expressed in the knockout mice. Gal/GalNAc-specific lectin was not expressed in the knockout mice, as determined by virtual Northern blot. Interestingly, unknown clones 1, 2, and 3 were almost exclusively expressed in the wild-type mice. Unexpectedly, another 26 clones, including ETa-1, C10 chemokine, and some unknown genes, did not show a significant difference between wild-type and knockout mice, as examined by virtual Northern blot. Thus, these clones were excluded for further consideration. Northern blot analysis using total RNA from peritoneal cells of wild-type and knockout mice and using cathepsin L, MIP-1γ, and MCP-3 cDNAs as probes confirmed that these genes were differentially expressed in wild-type mice, as measured by the "real" Northern blot (Figure 2). These results are summarized in Table 1.

Discussion

The expression of the C/EBPϵ gene is regulated in a strictly lineage-specific manner. It is exclusively expressed in myeloid cells.[11,12,25] Expression is highest around the promyelocyte stage of

Figure 1. Virtual Northern blot analysis. Total RNAs were extracted from cells in the peritoneal lavage fluid. cDNAs were synthesized and polyclonally amplified. Five hundred nanograms of PCR-amplified (nonsubtracted) cDNAs were electrophoresed on agarose gels (1.2%), and Southern blotted onto nylon membranes. These filters were hybridized at 65°C for 3 hours using Rapid-hyb buffer (Amersham Life Science) with [α-³²P] dCTP-labeled cDNA probes. Filters were rinsed to a final stringency of 0.25 × SSC and exposed to a Kodak X-Omat or a Biomax film at −70°C with an intensifying screen. Probes used in this study were generated from the cDNA library made by RDA. GAPDH was used as a control of expression. Size markers on the left expressed in kilobase. GenBank accession nos. BE846999, BE8470001, and BE847000 for unknown #1, #2, and #3, respectively.

Cathepsin L   MIP-1γ   MCP-3   galactose and N-acetyl-galactosamine specific lectin   unknown #1   unknown #2   unknown #3   GAPDH

development but C/EBPϵ is detectable in monocytes/macrophages and related cell lines, as well as granulocytes in mice.[31] C/EBPϵ-deficient mice develop functionally defective neutrophils.[27,28] Previous studies revealed that C/EBPϵ could transactivate the expression of neutrophil-mediated genes, including lactoferrin and gelatinase.[27,28] Another study showed that C/EBPϵ could enhance the expression of the M-CSF receptor as well as chemokines, suggesting that macrophage-related genes may also be targets for transcriptional regulation by C/EBPϵ in mice.[31] To identify other potential targets of C/EBPϵ, we performed PCR-based RDA analysis using peritoneal cells from C/EBPϵ knockout mice compared with those from wild-type animals. One hundred and seventy-four clones were isolated; sequencing showed that 33 independent genes were identified. However, virtual Northern blot analysis revealed that only 7 of the 33 genes had a differential expression pattern, indicating that the number of false-positive genes using PCR-based RDA analysis is relatively high. The extremely abundant genes contained in the tester cDNA could remain after subtractive hybridization, and these clones would be included in the cDNA library.

We have found that 4 known genes (cathepsin L, MIP-1γ, MCP-3, Gal/GalNAc-specific lectin) plus 3 unidentified genes were differentially expressed in wild-type mice using the virtual Northern blot, suggesting that C/EBPϵ may enhance their expression. The results of the Northern blot analysis paralleled the virtual Northern blot data. The 4 known genes are all expressed in macrophages and are important to their catabolism and/or inflammatory activities. Interestingly, C/EBPϵ was not detected by RDA, suggesting that the expression level of C/EBPϵ was much lower than that of chemokines in activated macrophages. Because C/EBPϵ is preferentially expressed in promyelocytes and myelo-

cytes, these macrophage-related transcripts detected by subtraction may reflect secondary events due to C/EBPϵ deficiency.

Cathepsin L (also called macrophage cysteine proteinase [MCP], major excreted protein [MEP]) is a member of the papain superfamily of lysosomal cysteine proteinase with a major role in intracellular protein catabolism.[35] Cathepsin L has the greatest collagenolytic and elastinolytic activity in vitro of any of the cathepsins. It is expressed in various cell types, including macrophages, fibroblasts, and also malignant cells.[35,36]

MIP-1γ and MCP-3 are 2 members of the C-C-chemokine family. Chemokines have been subdivided, based on the position of their conserved cysteine residues, into the C-X-C and the C-C groups, the latter includes MIP-1α, MIP-1β, MIP-1γ, RANTES, MCP-1, MCP-2, MCP-3, and C10.[37,38] These chemokines are homologous to one another. Not only do they mediate chemotaxis, they also modulate a variety of functional properties of leukocytes, including the activation of the contractile cytoskeleton, the transient rise in intracellular Ca^{++} concentration, exocytosis, and the expression of adhesion molecules. The MIP-1γ can activate both neutrophils and monocytes/ macrophages.[39,40] The MCP-3 can also act on macrophages, activated T lymphocytes, eosinophils, and basophils.[41,42] These chemokines appear to regulate inflammation

Table 1. Genes differentially expressed in wild-type mice compared with C/EBPϵ-deletional mice

Description	Virtual Northern blot	GenBank accession no.
Cathepsin L	+	M20495
MIP-1γ	+	U49513
MCP-3	+	S71251
Galactose/N-acetylgalactosamines-pecific lectin	+	S36676
Unknown (no homology) no. 1	+	BE846999
Unknown (no homology) no. 2	+	BE847001
Unknown (no homology) no. 3	+	BE847000

MIP-1γ: macrophage inflammatory protein-1γ; MCP-3: monocyte chemotactic protein-3.

Cathepsin L   MIP-1γ   MCP-3   GAPDH
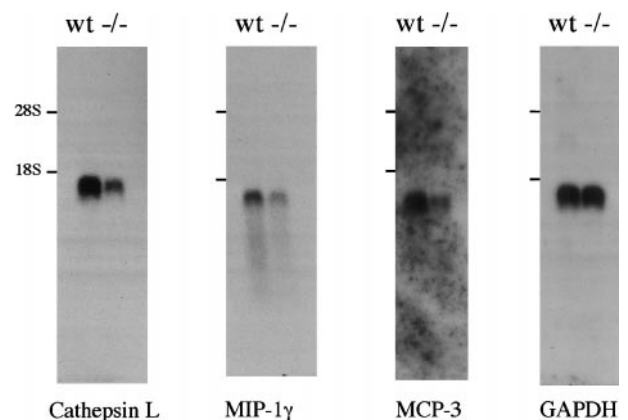
Figure 2. Northern blot analysis. Ten micrograms of total RNA sample were electrophoresed on agarose formaldehyde gels and transferred onto nylon membranes. The blots were hybridized with [α-³²P] dCTP-labeled cDNA probes at 65°C overnight using a standard method. Filters were rinsed to a final stringency of 0.25 × SSC and exposed to a Kodak X-Omat or a Biomax film at -70 °C with an intensifying screen. After sequential stripping of the probes by a standard method, the blot was rehybridized with a GAPDH probe as a control.

by the selective recruitment and activation of leukocyte populations. We have found that consistent with decreased expression of these chemokines, as also previously reported,[27] neutrophil migration was impaired in granulocytes lacking C/EBPε. When 2-month-old C/EBPε knockout mice were used for thioglycollate stimulation, much fewer (less than 1 of 10) leukocytes appeared in the peritoneal lavages, compared with numbers found in the wild-type mice at the same age (data not shown). Our results suggest that the reduced expression of these chemokines in C/EBPε knockout mice may partly contribute to diminished ability to migrate and impaired functions of macrophages and neutrophils.

Gal/GalNAc-specific lectin (also called asialoglycoprotein-binding protein [ASGP-BP]) is expressed on macrophages and is inducible with thioglycollate injection.[43,44] Nonstimulated macrophages showed negligible levels of expression.[44] This protein is located on the cell surface with its COOH terminus on the extracellular side. It is homologous to the hepatic asialoglycoprotein receptor (rat hepatic lectin).[43] Gal/GalNAc-specific lectin is responsible for carbohydrate-mediated endocytosis and plays an important role in cell-to-cell adhesion. Our results suggest that the absence of the Gal/GalNAc-specific lectin may contribute to the impaired ability of neutrophils and macrophages to migrate correctly in the C/EBPε knockout mice.

Experimentally induced overexpression of C/EBPε in a murine macrophage cell line enhanced the expression of MCP-1 as well as MIP-1α and MIP-1β, suggesting that these genes may be targets of regulation by C/EBPε.[31] However, our RDA analysis did not detect these genes. To evaluate the difference of expression between knockout and wild-type mice, virtual Northern blots were hybridized with ^{32}P-labeled MCP-1, MIP-1α, and MIP-1β. No significant differences were noted in the expression of these 3 genes in the macrophages and neutrophils of wild-type and C/EBPε-deletional mice (data not shown). The experimental approach between our studies and those that overexpressed C/EBPε in the macrophage cell line are very different. Taken together, expression of MCP-1, MIP-1α, and MIP-1β does not require C/EBPε, although overexpression of this transcription factor may enhance their levels.

This study has found 3 previously unidentified, unknown genes that have prominent differences in expression between C/EBPε knockout and wild-type mice. These genes have some homologies to known, macrophage-related genes. Characterization of these genes is ongoing.

Taken together, we have found several genes that were differentially expressed in macrophages and granulocytes of wild-type but not C/EBPε-deletional mice, suggesting that C/EBPε may enhance their expression. Although we do not know whether C/EBPε can directly or indirectly enhance the expression of these genes, our results expand the potential targets of C/EBPε. As C/EBPε is expressed mostly during granulocytic differentiation, C/EBPε may be enhancing these macrophage-related genes indirectly. Further studies. including reporter assays, will be needed to appreciate fully the role of C/EBPε in control of these genes.

Acknowledgments

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