Cooperation between PU.1 and CAAT/Enhancer-binding Protein β Is Necessary to Induce the Expression of the MD-2 Gene

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Myeloid differentiation factor 2 (MD-2) binds Gram-negative bacterial lipopolysaccharide with high affinity and is essential for Toll-like receptor 4-dependent signal transduction. MD-2 has recently been recognized as a type II acute phase protein. Plasma concentrations of the soluble form of MD-2 increase markedly during the course of severe infections. Its production has recently been recognized as a type II acute phase protein, MD-2, in nonmyeloid cells cooperatively with C/EBPα, a classical IL-6-inducible TF.

Efficient innate recognition of bacterial endotoxin lipopolysaccharide (LPS) requires its interaction with various humoral and cell-surface proteins, including LPS-binding protein, CD14, myeloid differentiation factor-2 (MD-2), and transmembrane Toll-like receptor 4 (TLR4). Recent data have shed light on the biochemical interactions between LPS and the host proteins forming its receptor. LPS binds MD-2 by inserting its acyl chains into a hydrophobic groove (1). MD-2 binds to TLR4, but there is no evidence showing direct LPS binding to TLR4 nor transfer of LPS to TLR4. Mice lacking the MD-2 gene are resistant to LPS but are more vulnerable to live Gram-negative bacteria (2). The recent crystallization of the LPS:MD-2:TLR4 complex clearly shows that MD-2 is the critical receptor protein for LPS, accommodating five acyl chains of LPS into its hydrophobic pocket, and is necessary for the ligand-dependent dimerization of MD-2:TLR4 complexes triggering cell activation (1, 3).

MD-2 is a 160-amino acid protein found at the cell surface of myeloid cells, bound to TLR4, and as a soluble protein in body fluids such as plasma, urine, and lung edema fluid (4). It has an N-terminal 16-residue secretion signal sequence and two canonical N-glycosylation sites. Human MD-2 gene (ENSG00000154589; National Center for Biotechnology Information (NCBI) gene ID 23643) is located on chromosome 8q21.11 and is composed of 5 exons. Three different MD-2 cDNA variants can be found in data bases, but only one, the reference sequence (RefSeq; NM015364) seems to be relevant. In mice, the existence of a Md-2 splice variant lacking exon 3 was reported (AY641431) (5). MD-2 is mainly found as a multimeric protein in solution. However, only the monomeric form of soluble MD-2 (sMD-2) complexed with LPS is able to stimulate cells in a TLR4-dependent manner (6).

Soluble MD-2 concentrations increase markedly in plasma from patients with severe sepsis or septic shock (4, 7, 8). We have recently shown that sMD-2 was a type II acute phase protein up-regulated by interleukin 6 (IL-6) and an opsonin for Gram-negative bacteria (9). Soluble MD-2 binds Gram-negative bacteria enhancing both phagocytosis and intracellular bacterial killing (9–11). MD-2 is produced by various cell types and found in virtually all organs (12–15). Because MD-2 is secreted by hepatocytes during an acute phase response (8, 9), we postulated that it could represent a prototypic example of an inducible opsonin participating in the defense against Gram-negative bacterial infections. This motivated us to study the
MD-2 Gene Regulation

TABLE 1
Primers sequences
Primer sequences are expressed as 5′ to 3′; f, forward primer; r, reverse primer.

| Analysis of MD-2 transcripts | P1 | P2 | P3 | P4 |
|------------------------------|----|----|----|----|
|                              | ATT TGG TCT GCA ACT CAT CC | TTC TGG TTC ACC CAT GCC | AAG ATC CAA AGG ATT ATT GCA CA | GGC TTC CCA GAA ATA GCT TC |

| Mutated hP-MD2 constructs | −911 mPU.1−1 f | −911 mPU.1−1 r | −911 mPU.1−2 f | −911 mPU.1−2 r | −911 mNF-IL-6 f | −911 mNF-IL-6 r |
|---------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                            | CTC TTT TTC TAC AGG GAG TGC TCA AAT G | CAT TTT AGC ACT CTC TCT TTT AAA GGA AGA G |CAA AAA AAG AGA GAA CAG TGG GAT AGG | CCA TAC CCA CTT TCT CTT CTT TTT TTT TTA TAT A |

| Chromatin immunoprecipitation | P1 f | P2 r | P3 = PU.1−1 + 2 f | P4 = PU.1−1 + 2 r | P5 f | P6 r | P7 = NF-IL-6 f | P8 = NF-IL-6 r | P9 f | P10 r | Luciferase f | Luciferase r |
|-------------------------------|-----|------|------------------|------------------|-----|-----|----------------|----------------|-----|------|-----------|-----------|
|                              | CAG ACC AGC AGC AGC ATT AG | AAT TTT TCC ACC GAG TGA G | AAG TGA TCC ACC TGC CTC AG | AAG CTT TAC AAA TGC AAA GAG GA | CCA CTT CTG CTC CCC AGA TA | GCA GGT GCA TCA CTT GAG GT |

| Sequencing primers | pGL3 f | pGL3 r |
|--------------------|--------|--------|
|                    | AGT GCA AGT GCA GGT GCC AGA AC | TAC CGG AAT GCC AAG ATT CC |

regulatory mechanisms responsible for MD-2 expression. The aim of our study was primarily to characterize the molecular determinants controlling basal and IL-6-induced MD-2 expression in human hepatocytic cells.

EXPERIMENTAL PROCEDURES

**Cells**—Human hepatocytic HepG2, promonocytic THP-1, human bladder carcinoma (ECV), HL-60, and SW620 cell lines were obtained from the ATCC (Manassas, VA). HepG2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine, 10 mM HEPES, 50 μg/ml gentamycin, and 50 units/ml penicillin. THP-1 cells were grown in the same medium except that Dulbecco’s modified Eagle’s medium was substituted for RPMI 1640 (Amimed, Bioconcept, Basel, Switzerland). In some experiments cells were cultured with 10 ng/ml recombinant human IL-6 (R&D, Minneapolis, MN) for 6 h when looking at mRNA levels or promoter activity and 24 h when looking at MD-2 protein secretion (9). In some promoter activity experiments, transfected HepG2 cells were stimulated with 10 ng/ml phorbol ester (PMA, Sigma) and 10⁻⁷ M 1α,25-dihydroxyvitamin D₃ (Calcitriol, Leo Pharma, Ballerup, Denmark).

**Analysis of MD-2 Transcripts**—The MD-2 reference sequence (Ly96 RefSeq; NM_015364) was obtained from the NCBI website (www.ncbi.nlm.nih.gov). Gene analysis and putative transcripts identification were performed using NCBI AceView at www.ncbi.nlm.nih.gov (16). Three putative MD-2 mRNA sequences were found in data bases. To determine which of these transcripts existed in vivo, cDNA were prepared from mRNA extracted from HepG2, THP-1, HL-60, human bladder carcinoma, and SW620 cells. Briefly, mRNA were extracted from cell lysates using TriZol® (Invitrogen) and treated with DNase (Ambion, Foster City, CA) according to the manufacturers’ protocols. Complementary DNA was obtained by reverse transcription using equivalent volumes of random primers and oligo-DT, RNasin treatment, and ImProm II reverse transcriptase (Promega, Madison, WI). PCR was performed on all cDNAs using primers discriminating the three possible transcripts (Table 1). The PCR protocol included a 45-cycle amplification using the IQ5 thermocycler and the SYBR Green supermix (Bio-Rad). Melting curves and agarose gel electrophoresis were used to ascertain the presence of only one amplicon after PCR.

**Human MD-2 Promoter**—The nucleotide sequence of the human MD-2 gene 5′-flanking region was obtained from ~1 kilobase of genomic DNA contained within a contig (NT_008183.17) encompassing part of Homo sapiens chromosome 8q13.3. The prediction of transcription factor (TF) binding sites was done by matching data obtained from three online TF analysis software programs: Alibaka (17), Signalscan (18), and TFSEARCH ((19). Interspecies conservation of sequences for transcription factor binding was assessed by aligning MD-2 promoter sequences from human, mouse, rabbit, and torus (eEnsembl) using CustalW2 at EMBL-EBI.

**Quantitative PCR**—Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase (Ambion). Reverse transcription was performed as previously described. Quantitative PCR was performed using the IQ5 thermocycler (Bio-Rad) and the following TagMan probes (MD-2, Hs00209771_m1; PLI, Hs00231368_m1; glyceraldehyde-3-phosphate dehydrogenase, Hs99999905_m1) with the universal PCR Mastermix (Applied Biosystems, Foster City, CA).

**RLM-5′-RACE**—MD-2 transcription starting site (TSS) was determined by RLM 5′-end cDNA amplification with First-Choice RLM-RACE (Ambion). RLM-5′-RACE is designed to amplify cDNA only from full-length, capped mRNA (20). Briefly, to generate 5′-end partial cDNA, 5 μg of total RNA
from HepG2 cells stimulated or not with IL-6 was treated with calf intestine alkaline phosphatase and tobacco acid pyrophosphatase to generate decapped mRNA. After the 5′-RACE adapter, 5′-GCU GAU GCC GAU GAU UGA ACA CUG CGU UUG CUG CUC AUG AAA-3′, was attached to decapped mRNA and treatment with RNase inhibitor was performed. A, processed mRNA was reverse-transcribed with random decamers and Moloney murine leukemia virus reverse transcriptase. Then outer nested PCR was performed using specific outer adapter primers, 5′-GCT GAT GCC GAT GGA TGA ACA CTG-3′ and a MD-2 gene-specific primer (GSP), GSP-1, 5′-GGA TGA GTT GCA GAC CCA ATA-3′. GSP-1 primer was specific to the MD-2 exon 1 (NCBI blast: NT_008183.18 Hs8_8340). An inner-nested PCR was then performed with inner adapter primer 5′-CGC GGA TCC GAA CAC TGG GTT TGC TGG CT TGA TG-3′ and inner MD-2 gene-specific primer, GSP-2, 5′-TGGA AAG AAA ACA GGG TGG AA-3′. The nested PCR product was then ligated with T4 DNA ligase in pGEM-T easy vector (Promega) and cloned into “library efficient” DH5α Escherichia coli (Invitrogen). Inserts from 20 bacterial colonies from two independent experiments (10 from unstimulated and 10 from IL-6-stimulated HepG2 cells) were sequenced using SP6 primers. Sequencing was done using the ABI 3130xl sequencer and BigDye Terminator v1.1/3.1 (Applied Biosystems).

Cap Analysis of Gene Expression (CAGE)—CAGE data bases (fantom3 and Roslin) regroup data from genome-wide human and mouse transcriptomes (21). MD-2 (LY96) transcriptome analysis was performed using the following data files: T01F0101386D and T08F04796B5A.

Construction of Human MD-2 Synthetic Promoters—A ~1-kilobase genomic PCR fragment of the human MD-2 proximal promoter (hPMD-2) was subcloned using the NcoI site of the multiple cloning site of the pGL3-Basic luciferase vector (9). The pGL3-B vector containing hPMD-2 was cloned into DH5α E. coli, amplified, and extracted using the EndoFree Plasmid Maxi kit (Qiagen). Sequential deletions of the ~911 human MD-2 promoter were generated using the following restriction enzymes: Pvull, Bst11071, Bsu361, BlnI, Bst Ap-1 (activated protein 1), MscI, HindIII (New England Biolabs, Ipswich, MA). This led to the generation of the following synthetic human MD-2 promoters: −783 hPMD-2, −644 hPMD-2, −542 hPMD-2, −398 hPMD-2, −186 hPMD-2, and +69 hPMD-2 (Fig. LA). Point mutations in the −911 hPMD-2 promoter were performed using site-directed mutagenesis (Stratagene, La Jolla, CA, Fig. LA). The primers used to generate mutated NF-IL-6, PU.1-1, PU.1-2, and PU.1-1+2 response elements are shown in Table 1. The correct sequences of all deleted and mutated hPMD-2 plasmids were confirmed by DNA sequencing using pGL3-specific primers (Promega, Table 1).

Reporter Gene Assay—HepG2 (10⁡5 cells/ml) cells were seeded into 24-well culture plates the day before transfection. A total of 500 ng of pGL3 plasmid containing various hPMD-2 constructs driving the Firefly luciferase were transiently transfected in HepG2 cells using FuGENE 6 (Roche Diagnostics) together with 100 ng of the pRL-TK plasmid expressing constitutively Renilla luciferase. Transfected cells were cultured for an additional 24 h and stimulated with IL-6 for another 6 h.

THP-1 cells (ATCC) were transfected using the JetPei reagent (Polyplus, Illkirch, France) according to the manufacturer’s protocol. Cell lysates were assayed for Firefly and Renilla luciferase activity using the dual luciferase reporter assay (GloMax 20/20 luminometer, Promega). Firefly luciferase activity was normalized with Renilla luciferase activity and expressed as relative luciferase activity (arbitrary units). All experiments were performed at least in triplicate and repeated at least three times in independent experiments.

Chromatin Immunoprecipitation (ChIP)—ChIP experiments were performed according to the manufacturer’s protocol (Upstate, Temecula, CA). Briefly, 5 × 10⁵ HepG2 cells were seeded into each well of a 6-well plate, grown to 80% confluence, and treated or not with IL-6 for another 4 h. Cells were then fixed with 1% formaldehyde for 10 min at 37 °C, washed with ice-cold phosphate-buffered saline containing a protease inhibitor mixture (Complete Mini; Roche Diagnostics), lysed, and sonicated to obtain genomic DNA fragments of about 200–400 bp of length (controlled by agarose gel electrophoresis, data not shown). Immunoprecipitation was performed overnight at 4 °C using 0.5 µg/ml goat anti-human PU.1 N-19 polyclonal antibody and 1 µg/ml rabbit anti-human C/EBPβ Δ198 polyclonal antibody or their respective isotype controls (Endogen, Woburn, MA and Santa Cruz, Santa Cruz, CA) followed by precipitation using protein A coupled to agarose beads. After washing, treatment with high salt and proteinase K (Fermentas, Burlington, ON), immunoprecipitated DNA fragments were recovered by classical phenol/chloroform extraction and ethanol precipitation. Quantitative PCR was performed on immunoprecipitated DNA fragments using specific sets of primers to amplify PU.1 and NF-IL-6 sequences (Table 1). Values were expressed as percent of amplified chromatin obtained after immunoprecipitation with the TF-specific antibody (respectively, anti-PU.1 and anti-C/EBPβ) to input chromatin, normalized to amplified chromatin immunoprecipitated with IgG. ChIP experiments were also performed in HepG2 cells transfected with synthetic and mutated hPMD-2 (−911 hPMD-2; mutated NF-IL-6 hPMD-2; mutated PU.1-1+2 hPMD-2). The normalization of transfection efficiency between different conditions was done by quantitative PCR on the luciferase gene (Table 1).

PL.1 RNA Interference—HepG2 cells were seeded in 6-well plates and grown to 50% confluence. Then PU.1 gene silencing was performed with 2.2 pmol/well of PU.1 or scramble small interfering RNA (respectively, sc-36330 and sc-37007; Santa Cruz). Silencing or scramble RNA was transfected using the interferin reactant following the manufacturer’s protocol (Polyplus). After 24 h of transfection, cells were stimulated or not with IL-6 for an additional 24 h, then whole cell lysates and cell supernatants were immunoblotted.

Western Blots—Five × 10⁵ HepG2 cells were seeded into each well of 6-well plates and grown to 60% confluence in RPMI containing 10% fetal calf serum. Cells were treated with IL-6 for 24 h. Conditioned supernatants were then collected, and cells were immediately lysed in loading buffer containing SDS (Invitrogen). Proteins from supernatants and cell lysates were separated using SDS-PAGE in reducing condition and electroblotted onto nitrocellulose membranes (Bio-Rad). MD-2 immunoblot were performed according to previously published method (sc-20668
MD-2 Gene Regulation

A

| Genebank Number of clones | 5' | 3' |
|----------------------------|----|----|
| 22                        | P1 | P4 |
| 2                         | P2 | P3 |
| 1                         | E1 | E2 |
|                            | E3 | E4 |
|                            | E5 |

NM_015364
ENSG00000154589
A1424897
AW978895
BM918324

B

IL-6
- + - + - + - +

P1P4

P2P4

P3P4

FIGURE 1. Putative MD-2 transcript variants. A, a total of 25 MD-2 clones have been sequenced and published in GenBank™, including a major isoform (22 clones) and 2 minor isoforms (2 and 1 clones, respectively). B, the PCR sequencing of five different human cell line cDNA obtained in cells treated or not with IL-6 yielded a single MD-2 transcript, corresponding to the major isoform (RefSeq NM_015364, ENSG00000154589), i.e. the minor isoforms were not found in these cell lines. Primers used for discriminative PCR are labeled P1, P2, P3, and P4. This experiment is representative of three independent experiments. ECV, human bladder carcinoma.

Antibody, Santa Cruz) (9). PU.1 immunoblot were performed as followed: membranes were blocked for 1 h in pH 7.6 Tris-buffered saline (TBS) containing 0.1% Tween20 (TBST) and 5% nonfat dry milk. Membranes were then incubated overnight at 4 °C in blocking buffer containing 0.5 μg/ml goat anti-human PU.1 N-19 polyclonal antibody (Santa Cruz) followed by an incubation with 1/25,000 dilution of a horseradish peroxidase-conjugated donkey anti-goat antibody in TBST, 5% nonfat dry milk. Immunoblotted proteins were revealed using the SuperSignal West Femto® maximum sensitivity substrate (Pierce) on film. Membranes were then stripped using 0.2% NaOH at room temperature for 4 min and re-blocked for 1 h in TBST, 5% nonfat dry milk to be re-blotted with anti-α-actin antibody (“housekeeping” protein for normalization the MD-2 signal in cell lysate). Stripped membranes were incubated with 0.2 ng/ml horseradish peroxidase-conjugated anti-human α-actin mAb (Millipore, Billerica, MA; MAB1501) in TBST, 5% nonfat dry milk and revealed with a 1/3000 dilution of a horseradish peroxidase-conjugated anti-mouse antibody (Pierce).

RESULTS

Only One MD-2 Transcript Is Found in Human Epithelial and Monocytic Cell Lines—We first aimed at determining whether different MD-2 transcription variants existed in human cells. We used the NCBI AceView mRNA model as a non-redundant and comprehensive representation of the cDNA sequence data in public repositories (GenBank™ and dbEST). Bioinformatics analysis using AceView predicted the “classical” MD-2 transcript (reference sequence, RefSeq) plus two additional putative transcript variants (Fig. 1A). A first putative variant was identified in two clones (A1424897 and AW978895), lacking exon 1 with a larger exon 2 as compared with the RefSeq. A second variant was also found (clone BM918324) in which the only difference with RefSeq was the absence of exon 2. To investigate whether these variants existed in human cells, we performed a PCR screening of cDNAs from five different human cell lines: promonocytic THP-1 and HL-60 cells, epithelial hepatic HepG2, bladder human bladder carcinoma, and colonic SW620 cells stimulated or not with IL-6 for 6 h. Despite a large number of PCR amplification cycles (45 cycles), only the RefSeq cDNA (NM_015364) transcript was found in all cells stimulated or not with IL-6 (Fig. 1B).

MD-2 Gene Transcription Starting Site and Putative Alternate Promoters—We next studied the MD-2 TSS in cells stimulated or not with IL-6. 5'-End cDNA amplification using RLM-5’-RACE was performed on HepG2 cells stimulated or not with IL-6. Ten PCR products per condition were sequenced. Of those 20 products, 18 MD-2 amplicons had a high chromatography profile and were used for further analyses. TSS were dispersed within a 100-bp window surrounding the RefSeq TSS (+1). After IL-6 stimulation, TSS were centered within a 30-bp window located 50 bp downstream of the RefSeq TSS, which did not corresponded to TSS in non-stimulated condition (Fig. 2A). This suggested that transcription started at a different starting site in cells stimulated with IL-6. Our data suggested that at least three alternate promoters existed: 1) one including a TATA box (+1-TATA+5) and a TSS located 26 bp downstream of the RefSeq TSS (+1); 2) a TSS located 60 bp upstream of the RefSeq +1; 3) a fluctuating non-TATA TSS located within a 30-bp region downstream of RefSeq +1. These results refine data obtained with classical 5’-RACE analysis that had already identified two of the three alternate promoters (data not shown). These data are in accordance with the analysis of the CAGE data base (Riken’s Institute and Centers) (21), where mouse Md-2 CAGE tags identified (in IL-6 non-stimulated condition) two promoters located within a 50-bp window, one being TATA-dependent. Interestingly,
A

\[-911\] ttataaaaga gggagcccac tcctccgggg gccgtggacc agtaccagtc catggcctgt taggaatctg gttggtttct
\[-831\] agcacagca ttaatgcagag ggaaaaggag tgcctgtctg tttgttatttt caatggagctgat gctctgttgc ccaggctgga
\[-751\] taatgcagag gggagcccac tcctccgggg gccgtggacc agtaccagtc catggcctgt taggaatctg gttggtttct
\[-671\] agacagtggt gccatcttgg ctcactgcaa cctctgcctc ccagataaag tgaagctcct gcctcagcct cctgaatagc
\[-591\] tgggattaca ggcatgcact accacacctg gctaattttt gtatttttag tagagatgtg tttcatcatg tcagccaggc
\[-511\] taatgcagag gggagcccac tcctccgggg gccgtggacc agtaccagtc catggcctgt taggaatctg gttggtttct

B

Mouse
Chr1: 16857117-16857217 (+)

Mouse
Chr8: 75066092-75066209 (+)

FIGURE 2. Analysis of the proximal human MD-2 promoter, TSS, and CAGE analysis. A, initiation of transcription of the human MD-2 gene. Eighteen positive clones were obtained from a total of 20 clones in two independent RLM-5’-RACE experiments and were used to determine the MD-2 TSS in HepG2 cells. Most TSSs obtained by RLM-5’-RACE were located within a 100-bp window. Specific TSS were found whether cells were treated with (#) or without (*) IL-6. The analysis of putative transcription binding sites identified three proximal PU.1 binding sites as well as a unique NF-IL-6 site. Restriction enzyme cleavage sites to produce promoter deletion mutants (see Fig. 3) are shown in this panel.

B, mouse Md-2 TSS tags obtained from CAGE-Riken data base indicated that Md-2 seems to have different TSS in myeloid (black boxes) versus non-myeloid (gray boxes) cells. Mouse Md-2 had TSS found within the same 50-bp windows in non-myeloid cells similar to those found in human hepatocytic HepG2 cells (panel A). Mouse Md-2 had TSS found within 28-bp upstream of a putative TATA box in myeloid cells. Existing CAGE data on human MD-2 TSS tags is limited and do not allow comparison with data obtained with murine cells.
Md-2 TSS seemed to be different in mouse myeloid and non-myeloid tissues. In murine macrophages Md-2 is preferentially transcribed using a TATA-dependent TSS, whereas in lung and liver TSS were spread out within a 30-bp window upstream of the RefSeq with no TATA box identified (Fig. 2B). Information on human MD-2 CAGE tags is unfortunately limited in the Riken database and does not allow a comparison of our results with this data bank. Altogether, these data, however, strongly suggest that the transcription initiation of MD-2 is dependent on three tissue-specific alternate promoters, with one fluctuating promoter preferentially used after IL-6 stimulation.

PU.1 and C/EBPβ Regulate MD-2 Expression in HepG2 Cells—We have recently shown that MD-2 was a type II acute phase protein (9). In this paper we observed a concomitant activity of the synthetic −911 proximal MD-2 promoter (−911 hPMD-2) and the transcription of endogenous MD-2 mRNA followed by the production and the secretion of the MD-2 protein by HepG2 cells after stimulation by IL-6 (9). Herein, we extend those findings, showing a dose-dependent effect of IL-6 on the MD-2 promoter activity. In addition, MD-2 promoter activity was also induced by PMA but not by 1,25-dihydroxyvitamin D3 (Fig. 3A).

Our initial approach to identify putative transcriptional regulatory elements was to transfect HepG2 cell with a −911 hPMD-2 driving a luciferase reporter gene. It was previously shown that the activation profile of the −911 hPMD-2 was similar to that of a −2 kilobase promoter activity after IFN-γ or PMA stimulation (12, 22). To identify possible critical regions in MD-2 promoter controlling MD-2 basal and IL-6-stimulated expression (9), we constructed hPMD-2 of various lengths. Using restriction enzyme digestions, we obtained six sequential deletions from the −911 proximal hPMD-2 that were subcloned into the pGL-3 plasmid (driving the Firefly luciferase gene, Fig. 3B).
MD-2 Gene Regulation

could be located in close proximity to the transcription initiation region (Fig. 3B). After stimulation with IL-6, the induction of the promoter was lost after the deletion of the proximal −542 promoter region (Fig. 3B). Bioinformatics analysis of the human MD-2 promoter region predicted three putative PU.1 binding sites within the −186/+69 region. Two of these putative PU.1 binding sites (positive strand, −89/−84 and −49/−44) were highly conserved among MD-2 sequences from different mammals, which was not the case for the most distal, negative strand PU.1 binding site (Fig. 3C). PU.1 is known to be a critical regulator of the expression of numerous genes involved in innate immunity, including IL-1β, TLR4, and CD14 (23–25). This together with the proximal location of these PU.1 binding sites in the MD-2 promoter and the results obtained with serial deletions made this transcription factor a likely regulator of MD-2 expression. The IL-6 inducibility of luciferase was conserved in the −542 but lost in the −389 promoter construct. Importantly, a putative NF-IL-6 site (C/EBPβ binding site) was identified in the promoter sequence between −542 and −389 (−515/−509, Fig. 3B). Noteworthy, this specific NF-IL-6 binding site was not found in the promoter regions of mouse, rabbit, and torus MD-2 promoters (Fig. 3C). To further test the role of these transcription factor binding sites in the regulation of basal and IL-6-induced MD-2, we per-

These vectors were transiently transfected into HepG2 cells stimulated or not with IL-6 for 6 h, and luciferase activity was measured. A basal transcriptional activity could be measured with promoter constructs larger than −186, suggesting that regulatory elements controlling basal MD-2 transcription

![Image](https://example.com/image.png)
formed site-directed mutagenesis of the full-length MD-2 promoter to modify these sites in such a way that they did not bind their respective transcription factors anymore (26, 27). Full-length −911 hPMD-2 driving the luciferase gene with point mutations in NF-IL-6 and PU.1 responding elements were transiently transfected in HepG2 cells; luciferase activity was measured in cell lysates from cells treated or not with IL-6 (Fig. 3D). Basal (unstimulated) promoter activity was strongly reduced after mutation of one or the other or both of the putative PU.1 binding sites (−911 mPUDU.1-1, −911 mPUDU.1-2, −911 mPUDU.1-1+2 hPMD-2), whereas IL-6 induction seemed to be maintained. IL-6 induction was lost after the mutation of the NF-IL-6 putative binding site (−911 mNF-IL-6 hPMD-2, Fig. 3D). Binding of PU.1 and C/EBP to their putative binding sites was assessed by ChIP assay (Fig. 3E). PU.1 was shown to bind to the putative DNA region containing the two PU.1 binding sites both in unstimulated and IL-6-stimulated cells. Interestingly, IL-6 treatment increased PU.1 binding. In contrast, C/EBP was shown to bind to the region containing NF-IL-6 only after IL-6 stimulation. We next determined whether PU.1 was necessary for sMD-2 production. PU.1 RNA interference was performed in HepG2 cultured with or without IL-6. PU.1 silencing was shown to interfere with sMD-2 production after IL-6 stimulation (Fig. 3F).

C/EBPβ Enhances PU.1 Binding after IL-6 Stimulation—We next investigated why PU.1 ligation to its binding site was enhanced by IL-6 treatment. Using quantitative PCR, we found that PU.1 mRNA was not increased by IL-6 treatment. This ruled out the possibility that the observed increased PU.1 binding after IL-6 treatment was because of an increase in PU.1 expression (Fig. 4A). It remained a possibility that PU.1 binding was increased by IL-6 treatment through cooperation with C/EBPβ. This was further investigated by ChIP experiments (Fig. 4B). Immunoprecipitation of C/EBPβ allowed us to amplify a region containing the proximal PU.1 binding sites and, this, only after IL-6 treatment. Conversely, immunoprecipitation of PU.1 brought down a DNA sequence amplifiable by primers of the NF-IL-6 binding site (data not shown). Altogether, these data strongly suggested that C/EBPβ bound to and cooperated with PU.1 at the level of the proximal MD-2 promoter. To validate these important results and exclude nonspecific PCR amplifications (because of large stretches of immunoprecipitated chromatin), we quantitatively amplified five continuous regions covering the whole −911 hPMD-2. Regions containing NF-IL-6 and the PU.1 binding sites were significantly more amplified than neighboring regions (Fig. 4C). No significant amplification was observed when we attempted to amplify a region at the junction between exon 1 and intron 1 (negative control).

C/EBPβ Cooperates with PU.1 to Induce MD-2 Expression—We next determined whether C/EBPβ binding to PU.1 required the distal NF-IL-6 site or if C/EBPβ bound PU.1 independently of the NF-IL-6 site (Fig. 5A). To answer this, we performed a ChIP experiment on HepG2 cells transiently transfected with the −911 hPMD-2 or the same synthetic promoter with a point mutation in the distal NF-IL-6 site (−911 mNF-IL-6 hPMD-2). Quantitative PCR was performed using both primer sets designed for NF-IL-6 and PU.1 sequences (Fig. 5B). C/EBPβ did not bind to the promoter containing the mutated NF-IL-6 site after IL-6 treatment, confirming the specificity of the binding of C/EBPβ to this consensus sequence. C/EBPβ bound to the PU.1 sites in cells transfected with the −911 hPMD-2 plasmid, in accordance with prior observations in untransfected HepG2 cells. When cells were transfected with the hPMD-2 promoter containing a point mutation in the NF-IL-6 site, C/EBPβ did not bind to the PU.1 site anymore. This indicated that NF-IL-6 site was necessary for C/EBPβ binding to PU.1, suggesting cooperation between these two distant binding sites.

PLU.1 and C/EBPβ Also Regulate MD-2 Expression in Human Myeloid THP-1 Cells—Because MD-2 is expressed in myeloid cell where it plays a critical role in LPS binding and TLR4 activation, we also investigated the role of PU.1 and C/EBPβ in human promonocytic THP-1 cells. Human MD-2 promoters of various lengths transfected into these cells also identified the −186 proximal region to be critical for basal promoter activity (data not shown). Similarly to HepG2 cells, site-directed...
PCR amplification of the luciferase gene. This is one experiment representa-
cells showed that both PU.1 and C/EBP chromatin immunoprecipitated with IgG (model 1); chromatin sequence (Fig. 4).

**DISCUSSION**

Herein, we describe a unique transcriptional regulatory mechanism of the acute phase MD-2 protein production. We show that IL-6-dependent up-regulation of MD-2 depends on C/EBPβ binding to a distal NF-IL-6 binding element, cooperating with PU.1 binding to its proximal sites. To our knowledge it is the first report implicating PU.1 as a critical regulator of gene expression in nonmyeloid cells and showing that PU.1 is at the same time a regulator of innate immune receptors such as CD14 and TLR4 in myeloid cells and of MD-2, an acute phase protein made by the liver. In addition, we also show 1) that a single MD-2 transcript is found in human epithelial and myeloid cell lines, 2) that a tissue-specific alternate promoter regulates MD-2 expression in human and mouse, 3) the existence of a specific fluctuating transcription start site of MD-2 when cells are treated with IL-6, and 4) that MD-2 expression is dependent on the PU.1 transcription factor in both myeloid and nonmyeloid cell lines.

MD-2 is a type II acute phase protein and an opsonin for Gram-negative bacteria enhancing bacterial phagocytosis and killing by neutrophils (9). However, molecular mechanisms regulating MD-2 expression are basically unknown. Interferon γ, a prototypic cytokine skewing the immune response toward a pro-inflammatory Th1 response, was shown to induce surface expression of TLR4 and MD-2 in human monocytes and macrophages (28). Interferon γ seemed to up-regulate MD-2 expression via the JAK-STAT (Janus kinases and signal transducers and activators of transcription) pathway in intestinal epithelial and endothelial cells (12). IL-10 was recently shown to be a potent inducer of MD-2 in human monocytes, but the precise mechanism is still unknown (29). Recently, Li et al. characterized PMA-induced MD-2 expression in promono-
cytic HL-60 cells (22). They showed that both extracellular signal-regulated kinase and Jun kinase (JNK) pathways were required for PMA-induced MD-2 expression via the binding of Elk-1, a member of the E26 transformation-specific (ETS) family, to a specific motif located in a proximal region of the human MD-2 promoter (−185/−171). In our work another member of the ETS family, PU.1, was shown to bind the MD-2 promoter at the close proximity of an Elk-1 binding site. The proximity of these two critical transcription factors activated by two different agonists (PMA and IL-6) strongly suggests that this region contains critical regulatory elements. We confirmed the activating effect of PMA on human MD-2 promoter in HepG2 cells (Fig. 3A). Interestingly, Li et al. (22) observed a reduced activity of the −935 and −2371 promoter of human MD-2 after stimulation of cells by the PMA, as compared with a full activation when the promoter was shorter (−451). These results suggested that a repressor may be located upstream of −451 bp in the MD-2 promoter. Although the agonist was different, we found a similar reduced activity of the MD-2 promoter when it was longer than −542 bp compared with its shorter versions. Altogether, these data are compatible with the existence of a putative transcription factor repressing MD-2 expression after PMA and IL-6 treatment induced within the region between −644 and −542. Further studies are needed to identify this repressor.

Although TNF-α, and IL-1β are potent inducer of inflammatory genes and genes of the innate immunity such as IL-8 and CD14, they do not induce directly MD-2 expression (9, 12). Endotoxin injection was shown to induce an increase in plasma soluble MD-2 (sMD-2) in humans. It remains to be investigated whether this is because of a direct effect of LPS or an effect secondary to the induction by LPS of IL-6 and/or interferon γ (8). The presence of low levels of sMD-2 protein in normal plasma and its increased levels in acute phase plasma suggest an important role in innate immune response against microorga-
MD-2 Gene Regulation

A

FIGURE 6. PU.1 and C/EBPβ are critical regulators of MD-2 expression in promonocytic THP-1 cells. A, site-directed mutagenesis of PU.1 and C/EBPβ binding sites of synthetic -991 human MD-2 promoter transfected into THP-1 cells treated or not with IL-6. These data showed that both PU.1 sites as well as NF-IL-6 site are required for basal MD-2 promoter activity in myeloid THP-1 cells. PU.1 binding sites and NF-IL-6 mutations reduced basal MD-2 promoter activity and impaired IL-6 inducibility. Data are expressed as Firefly fluorescence relative to Renilla fluorescence ± S.D. This experiment was performed in triplicate and is representative of three independent experiments. a.u., arbitrary units. B, ChIP experiments; C/EBPβ immunoprecipitation (IP) in myeloid THP-1 cells. Similarly to what was observed in HepG2 cells, these data showed that PU.1 and C/EBPβ bound to their respective responding elements essentially after IL-6 treatment. Additionally, C/EBPβ bound to the PU.1 sequence after IL-6 treatment (C/EBPβ, immunoprecipitation with anti-C/EBPβ; PU.1, immunoprecipitation with anti-PU.1; IgG, IgG immunoprecipitation with an isotype control). Values were expressed as percent of amplified chromatin obtained after immunoprecipitation with the TF-specific antibody (anti-PU.1, and anti-C/EBPβ; dark column) to input chromatin, normalized to amplified chromatin immunoprecipitated with IgG (white column) ± 1 S.D. One experiment is representative of three.

nisms. Understanding the mechanisms that regulate MD-2 expression may, therefore, help to identifying a defective pathway in host response to Gram-negative pathogen. Significant genetic variations involving MD-2 expression regulation have, however, not been reported in humans yet.

PU.1 (or Sp1) belongs to the transcription factor family containing an ETS domain (30). PU.1 has been extensively described as a key transcription factor in hematopoiesis and macrophage differentiation. Although principally expressed in myeloid cells, PU.1 has also been shown to regulate gene expression in nonmyeloid cells, such as in HepG2 cells, murine mesangial cells, and fibroblasts (31–33). PU.1 was identified as a key regulatory factor of TLR4, CD14, and IL-1β expression in myeloid cells (23, 25, 34, 35). The identification of the critical role of PU.1 in MD-2 expression was, therefore, not a complete surprise. In our study PU.1 was found to be essential for MD-2 expression in both human hepatocyte-like HepG2 and myeloid THP-1 cells. In contrast to TLR4, where PU.1 regulates both human and murine gene, MD-2 expression in mouse alveolar macrophages does not seem to be controlled by PU.1 (23). This is supported by CAGE data shown in the present work, suggesting that mouse MD-2 gene regulation is tissue-specific. CAGE diagrams strongly suggest that MD-2 TSS is TATA-dependent in murine myeloid cells, whereas in mouse lung and liver the initiation of MD-2 expression is controlled by a non-TATA TSS. However, the absence of CAGE data in humans did not allow us to conclude on TSS for MD-2 dependent on the tissue origin (Fig. 2B).

Alternate promoter activity is now recognized as an important mechanism governing tissue-specific expression profiles (36). The role of transcription activation windows regulating tissue specificity in innate immune genes was recently reported by Lichtinger et al. (37). These investigators showed that the initiation of human and murine TLR4 transcription arouse from two alternate promoters responsible for both species and tissue specificities (37). Our data are in line with those findings, suggesting that the same promoter windows of MD-2 expression exist in human and in mice nonmyeloid cells. This transcription initiation mechanism is dependent on a unique cooperative effect of PU.1 and C/EBPβ that serves as a basis of the acute phase induction of MD-2 by human hepatocytes.

PU.1 was shown to interact with a variety of transcription factors, such as Tax, high mobility group box 1 (HMG1), or members of the CAAT/enhancer-binding protein (C/EBP) family such as C/EBPα, C/EBPβ, and C/EBPδ (32, 38–41). Members of the C/EBP family possess a characteristic leucine zipper and a basic domain in their C-terminal regions, the bZIP domain. bZIP is essential for DNA binding and homo- or heterodimeric interactions with various transcription factors such CREB (cAMP response element binding), Fos, Jun/AP-1 (activated protein 1), and ATF (activating transcription factor). bZIP can also bind other transcription factors such as NF-κB, pRB, Sp1, Stat3, Stat5, and PU.1. Interaction of PU.1 with co-regulatory partner proteins is a unifying feature of transcription factors containing an ETS domain (30). The winged helix-turn-helix domain of PU.1 was shown to interact with the C/EBPβ bZIP region, inducing synergistic transcriptional activation in 3T3 cells (39). However, the combined role of C/EBPδ and PU.1 in the context of natural promoter in vivo was not investigated. C/EBPα has been shown to have an opposite effect of that of CEBPδ, inhibiting PU.1 activity by interfering with the binding of c-Jun with the activation site of PU.1 (40, 42). Yang et al. (43) showed that C/EBPβ activation of IL-1β expression depended on PU.1 in HeLa cells. They showed in vitro an interaction between the winged helix-turn-helix domain of PU.1 and the bZIP region of C/EBPβ. In their study, a dominant-negative NF-IL-6 binding
site was shown to repress PU.1-dependent activation of the IL-1β core promoter.

Our study brings additional data and a novel understanding as to how PU.1 may cooperate with another member of the C/EBP family, namely C/EBPβ. Using different approaches including chromatin immunoprecipitation and gene silencing techniques, we show that IL-6 induces a positive cooperation between PU.1 and NF-IL-6 binding sites and their respective TF leading to an increase in the transcription of the acute phase MD-2 protein. C/EBPβ protein is up-regulated by various stimulants, such as LPS, IL-6, IL-1β, dexamethasone, glucagon, and interferon γ (44). This transcription factor is, therefore, likely to play an important role in the gene regulation of proteins such as MD-2, implicated in first line of innate immune responses. Interestingly, C/EBPβ knockout mice have a defect in macrophage-driven tumoricidal and bactericidal activities and are highly susceptible to Salmonella typhi and Listeria monocytogenes infections (45, 46). One may speculate that bacterial susceptibility to Salmonella of C/EBPβ knockout mice could be related to a defective MD-2-dependent opsonophagocytosis (9).

A transcriptional effect directly dependent on a cooperative mechanism between C/EBPβ and PU.1 had not been previously reported. It is our hypothesis that the recruitment and stabilization of the transcription machinery may require chromatin structure modification triggering cooperation between C/EBPβ and PU.1 while binding to their respective binding elements. Recently, Grondin et al. (47) showed in murine monocytic cell lines that c-Jun homodimers (a transcription factor containing a bZIP region) could bind PU.1 and C/EBPβ to function as a transcription enhancer by facilitating the assembly of the RNA polymerase II preinitiation complex. This supports our observation that the initiation of MD-2 transcription induced by IL-6 requires a specific alternate TSS region as well as other reports showing that PU.1 may participate in the transcription initiation of genes with GC-poor and/or TATA-less 5′-regulatory regions, as illustrated by the ability of PU.1 to bind in vitro to the transcription factor TFIID (48, 49). PU.1 could, therefore, carry a dual role in the same cell lineage. In myeloid cells it drives precursor cell differentiation and, as shown in our work, also cooperates with other transcription factors to up-regulate proteins of the innate immunity such as MD-2 in response to appropriate agonists (IL-6).

In conclusion, we describe herein a unique cooperation mechanism between PU.1 and C/EBPβ in regulating MD-2 expression in response to the acute phase inducer IL-6. Although MD-2 expression is ubiquitous among human organs and tissues (15), the critical role of PU.1 and C/EBPβ in controlling MD-2 expression may be central to the specific tissue response to inflammatory stimuli and bacteria. It is also the first description of a transcriptional regulatory role of PU.1 in a non-myeloid tissue such the liver for a key function; that is, the up-regulation of an acute phase protein. Understanding the intimate mechanisms of the transcriptional regulation of proteins of the innate immunity (opsonins and acute phase reactants) is potentially important for the development of modulators of innate immune responses to bacterial infections.

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MD-2 Gene Regulation

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