Cooperative interaction of NF-κB and C/EBP binding sites is necessary for manganese superoxide dismutase gene transcription mediated by lipopolysaccharide and interferon-γ

Kayoko Maehara[a,b], Tadao Hasegawa[a], Hengyi Xiao[a], Akihide Takeuchi[a,c], Ryoichi Abe[a,1], Ken-ichi Isobe[a,*]

[a] *Department of Basic Gerontology, National Institute for Longevity Sciences, 36-3 Gengo, Morioka-cho, Obu, Aichi 474-8522, Japan*
[b] *Domestic Research Fellow from Japan Science and Technology Corporation, Tokyo, Japan*
[c] *Department of Immunology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8520, Japan*

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Abstract Expression of the manganese superoxide dismutase (Mn-SOD) is induced by pro-inflammatory cytokines. We investigated the *cis*-acting elements within a tumor necrosis factor-responsive element (TNFRE) which was identified in the second intron of the murine Mn-SOD gene. Site-directed mutagenesis, reporter plasmid transfection studies and electrophoretic mobility shift assays demonstrated that inducible transcription factors enhanced the transcriptional activity of the Mn-SOD gene through the TNFRE. The cooperation between proteins binding to the newly identified NF-κB and C/EBP sites led to synergistic gene transcription. This report provides the first evidence that cooperation between two distinct *cis*-acting elements may be required for induction of Mn-SOD gene expression mediated by lipopolysaccharide and interferon-γ.

Key words: Manganese superoxide dismutase; Nuclear factor-κB; CCAAT/enhancer binding protein; Lipopolysaccharide

1. Introduction

The superoxide dismutases (SODs) are important metalloenzymes which scavenge superoxide radicals via disproportionation [1]. Three types of SODs with distinctive distributions are known to exist. The copper-zinc SOD (CuZn-SOD) is found mainly in the cytosol of eukaryotes, the iron SOD (Fe-SOD) is found in prokaryotes, eukaryotic algae and higher plants, and the manganese SOD (Mn-SOD) is found in both prokaryotes and the mitochondria of eukaryotes. Expression of CuZn-SOD and Fe-SOD is constitutive, whereas Mn-SOD is inducible by various stimuli, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), lipopolysaccharide (LPS), interferon-γ (IFN-γ), or X-irradiation [2–4].

Recently, a TNF-responsive element (TNFRE) has been identified within the second intron of the Mn-SOD gene [5,6]. DNA-binding assays using TNFRE showed that C/EBP β binds to the 5′ region of the TNFRE and both C/EBP β and NF-κB bind to the 3′ region. Transfection assays demonstrated that the 5′ C/EBP-related region was responsive to TNF, whereas the 3′ NF-κB-related site was not [6].

To identify the critical *cis*-acting elements interacting with NF-κB, we focused on a 30-bp region within the 5′ C/EBP-related region which responded to LPS and IFN-γ. Site-directed mutagenesis, transfection studies, and electrophoretic mobility shift assays were performed. We identified NF-κB as a component of the inducible complex that binds to the NF-κB binding site within the 5′ C/EBP-related region. In addition, the NF-κB site was shown to work in concert with the C/EBP site to enhance gene transcription induced by either LPS alone, or LPS in combination with IFN-γ.

2. Materials and methods

2.1. Materials

Cell culture reagents and calf serum (CS) were obtained from Gibco BRL (Life Technologies Inc., New York, USA). Recombinant murine IFN-γ was purchased from R&D Systems (Minneapolis, MN, USA), LPS was from Sigma (St. Louis, MO, USA). Anti-C/EBP α, β and δ, anti-CRP-1, anti-p65 and p50 NF-κB antibodies and a NF-κB consensus oligonucleotide were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A rat Mn-SOD cDNA probe was a gift from Dr. Ho (Wayne State University, Detroit, MI) [7]. A λ genomic library prepared from *Bam*HI-, *Mbo*I-, *Bgl*II-, or *Sau*3A-digested DNA isolated from the spleen cells of B6/CBA F1J mice was purchased from Stratagene (La Jolla, CA, USA).

2.2. Cell culture

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% CS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Plasmid construction

The genomic library was screened according to the standard procedure with murine Mn-SOD probes recognizing distinct regions of the gene. The plasmid constructions were generated by polymerase chain reaction (PCR) amplification with the genomic Mn-SOD clone as a template. The 5′ primer (the positions of the 5′ base are indicated relative to the Mn-SOD transcription initiation site in parentheses) (+2119) 5′-CGCGGATCCGGGGGCATCTAGTGGAGAAG-3′ was used with the 3′ primer (+2420) 5′-TTTTGACGTCGACGCGAGCTCTGGCTCCACA-3′ in a standard amplification reaction with cycling temperatures of 94, 58, and 74°C. The PCR product was digested with *Bam*HI and *Sal*I, gel-purified, and ligated into a *Bam*HI-*Sal*I-digested pGL3 promoter vector (Promega, Madison, WI, USA). Plasmids containing mutations within the A site (pGL3pro-mA) and the B site (pGL3pro-mB) were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) (Fig. 1B).

*Corresponding author. Fax: (81) (562) 44-6591.
E-mail: kenisobe@nils.go.jp

[1] Present address: Department of Biochemistry, School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-0072, Japan.

Abbreviations: Mn-SOD, manganese superoxide dismutase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; TNFRE, TNF-responsive element; IL, interleukin; NF-κB, nuclear factor-κB; C/EBP, CCAAT/enhancer binding protein; EMSA, electrophoretic mobility shift assay; WT, wild type

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The following pairs of oligonucleotides were used to create the mutant sites (mutated residues are underlined): mA: 5′-CCAAGAGAGGGAAATGACTAGTCATTCTGGAAATTTTAC-3′, 5′-GTAAAATTTCCAGAATGACTAGTCATTTCCCTCTCTTGG-3′, mB: 5′-CCACATGACTAGTCTTTTACTTGC-3′, 5′-GCAAGTAAAAGACTAGTCATGTGG-3′. All plasmids were sequenced to confirm their fidelity.

2.4. Transfection assays

Transient transfection of NIH3T3 cells was carried out using SuperFect reagent (Qiagen, Hilden, Germany). In general, the day before transfection, 4×10^5 cells were plated in 12-well tissue culture plates, which were supplemented with fresh medium before transfection. A total of 2.5 μg of DNA per plate was mixed with 10 μl of SuperFect reagent and allowed to form complexes for 15 min in serum- or antibiotics-free medium before being added to the cells. After transfection, the cells were placed in serum-free medium for 10 h, and then 0.1 or 1 μg/ml LPS, 100 U/ml IFN-γ, both 1 μg/ml LPS and 100 U/ml IFN-γ, or control buffer was added for an additional 14 h. Cells were harvested, and assayed by the Dual-Luciferase Reporter Assay System (Promega) using a luminometer (E.G. and G. Berthold, Germany). Promoter activities were expressed as relative light units (RLU), normalized against the activity of the pRL-thymidine kinase (TK) control vector (Promega).

2.5. Nuclear extract preparation

Nuclear extracts were prepared by the method described by Dignam et al. [8]. In brief, cells with or without 4 h treatment with either or both 0.1 μg/ml LPS and 100 U/ml IFN-γ were washed twice in ice-cold PBS, then suspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and incubated on ice for 10 min. After centrifugation at 2000 rpm, the cell pellet was resuspended in buffer A and lysed by five strokes with a glass Dounce homogenizer. The nuclei were collected by centrifugation at 27 000 rpm for 20 min at 4°C in an Optima TLX Ultracentrifuge rotor (Beckman). The pellet was resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 550 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin). The nuclear pellet was incubated on ice for 30 min, followed by centrifugation at 27 000 rpm for 20 min. The supernatant was dialyzed against buffer C (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) for 3 h at 4°C. The dialysate was collected by centrifugation at 27 000 rpm for 20 min at 4°C. The protein concentrations of the nuclear fractions were determined by the Bradford assay with the Bio-Rad protein assay dye reagent, and all extracts were stored at −70°C.

2.6. Electrophoretic mobility shift assays (EMSA)

The DNA binding reaction was performed for 20 min at 4°C in a volume of 10 μl, containing 1–2 μg of nuclear extract, 2 μl of 5× binding buffer (1×: 10 mM Tris, pH 7.5, 4% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.05 μg/μl poly(dI-dC)) with or without a 200-fold molar excess of an unlabeled competitor DNA. Synthetic complementary oligonucleotides with a G overhang were annealed and labeled with [α-^{32}P]dCTP using the Klenow fragment. The radiolabeled probe was added for an additional incubation period of 20 min. For supershifts, nuclear extracts were preincubated with antibodies for 20 min at 4°C. DNA binding reactions were separated on 5% native polyacrylamide gels. Gels were dried, and subjected to autoradiography. The following pairs of oligonucleotides were used (mutated residues are underlined) (Fig. 1C): SOD-C/EBP-κB: 5′-GGAAATATTACCACATTCTGGAAATTTTAC-3′, 5′-GGGTAAAATTTCCAGAATGTGGTAATATTT-3′; SOD-κB, 5′-CACATTCTGGAAATTTTACTTGCAATAAG-3′, 5′-GCTTATTGCAAGTAAAATTTCCAGAATGTG-3′; Mut-κB, 5′-CACATGACTAGTCTTTTACTTGCAATAAG-3′, 5′-GCTTATTGCAAGTAAAAGACTAGTCATGTG-3′.

3. Results

3.1. Determination of the regulatory DNA elements required for transcriptional activity of the Mn-SOD enhancer

To test the effects of LPS and IFN-γ on the enhancer elements of Mn-SOD, a 302-bp region (TNFRE) of intron 2 (+2119 to +2420) was subcloned into the pGL3 promoter vector (pGL3pro), which contains the basic, enhancerless simian virus 40 promoter instead of the Mn-SOD promoter. Lysates from the cells transfected with the pGL3pro produced only low luciferase activity both in untreated cells and treated cells. Lysates from the cells transfected with pGL3pro-WT showed a 10-fold or 8-fold induction following treatment with 0.1 or 1 μg/ml LPS respectively, a 2-fold induction with 100 U/ml IFN-γ alone, and a 15-fold induction with 1 μg/ml LPS and 100 U/ml IFN-γ relative to the basal activity

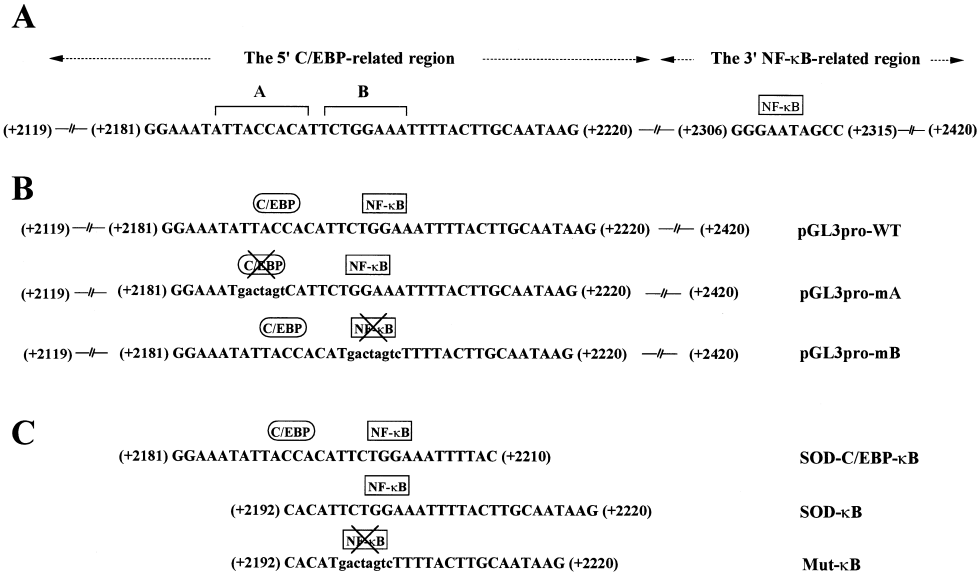

Fig. 1. A: A schematic representation of the Mn-SOD enhancer. The locations of sites A and B are indicated by brackets. The positions of the bases are indicated relative to the Mn-SOD transcription initiation site (+1). B: Sequences of wild-type and mutant Mn-SOD reporter vectors transfected into the NIH3T3 cells, as well as the locations of the C/EBP- and NF-κB-binding sites. Wild-type enhancer sequences are indicated by capital letters. A crossed out site indicates that the site has been mutated to a non-binding sequence, with the mutated sequence indicated by lower-case letters. C: Sequences of the oligonucleotides for EMSA.

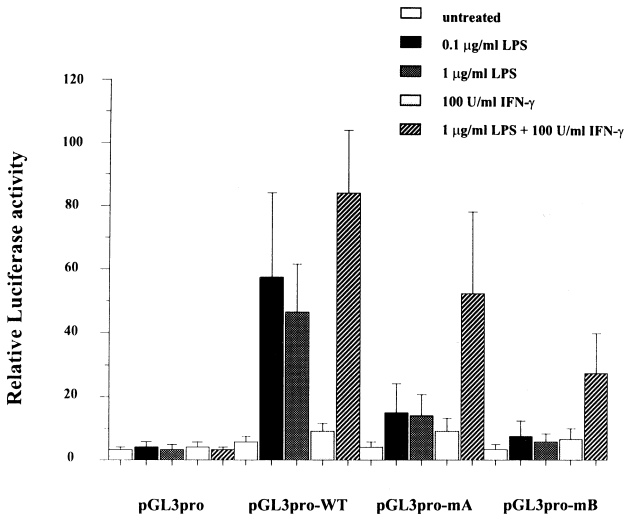

Fig. 2. Functional analysis of the Mn-SOD enhancer. NIH3T3 cells were transfected with pGL3pro, pGL3pro-WT, pGL3pro-mA, or pGL3pro-mB plasmids. The average promoter activities were generated from three separate experiments. The S.E.M.s are indicated by the error bars. Luciferase activities were normalized for the transfection efficiency with the cotransfected pGL3-TK control vector.

of this construct in untreated cells (Fig. 2). These results were consistent with those of the Northern blot analysis (data not shown).

To determine which *cis*-elements show significant enhancer activity, we focused on a 30-bp region containing two adjacent, putative C/EBP-binding motifs which were termed C/EBP-1 and C/EBPX by Jones et al. [6]. In this study, C/EBP-1 and C/EBPX were designated the A site and the B site, respectively (Fig. 1A). Lysates from the cells transfected with pGL3pro-mA showed a 4-fold induction following treatment with 0.1 or 1 μg/ml LPS alone, a 2-fold induction with 100 U/ml IFN-γ alone, and a 13-fold induction with 1 μg/ml LPS and 100 U/ml IFN-γ (Fig. 2). Although LPS-induced transcriptional activity was significantly decreased, co-stimulation with LPS and IFN-γ maintained the high transcriptional activity, which was about 60% that of pGL3pro-WT. LPS-induced enhancer activity was completely abolished in the cells transfected by pGL3pro-mB. The induction by co-stimulation with 1 μg/ml LPS and 100 U/ml IFN-γ was also decreased significantly in cells transfected with pGL3pro-mB. These data indicate that LPS- and IFN-γ-induced transcriptional activation of Mn-SOD is mediated primarily by the B site, but depends on a potent synergistic effect by factors interacting with both the A site and B sites within the TNFRE.

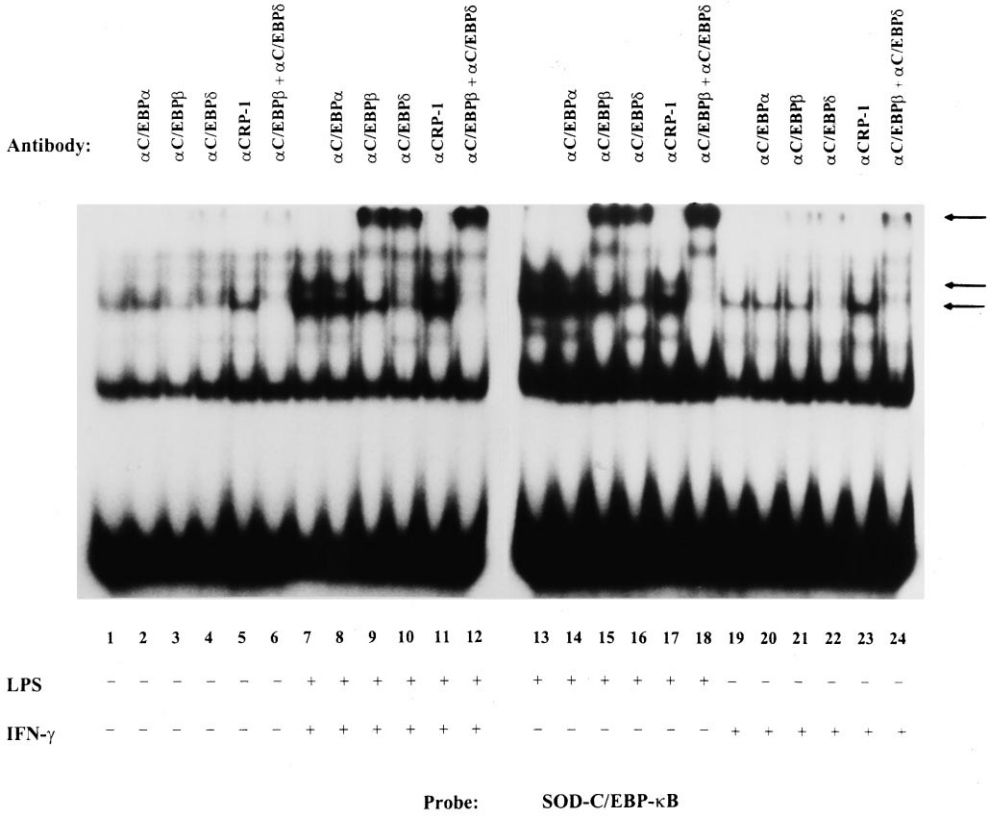

Fig. 3. Anti-C/EBP antibodies supershift the SOD-C/EBP-κB-protein complexes. EMSA using a ^{32}P-labeled SOD-C/EBP-κB probe incubated with the nuclear extracts from NIH3T3 cells which were left untreated (lanes 1–6), treated with 0.1 μg/ml LPS and 100 U/ml IFN-γ for 4 h (lanes 7–12), treated with 0.1 μg/ml LPS for 4 h (lanes 13–18), or treated with 100 U/ml IFN-γ for 4 h (lanes 19–24). Antibodies to C/EBP α (lanes 2, 8, 14, and 20), C/EBP β (lanes 3, 6, 9, 12, 15, 18, 21, and 24), C/EBP δ (lanes 4, 6, 10, 12, 16, 18, 22, and 24), or CRP-1 (lanes 5, 11, 17, and 23) were added to the extracts prior to the addition of the probe. The arrows indicate the SOD-C/EBP-κB-protein complexes and the supershifted complex.

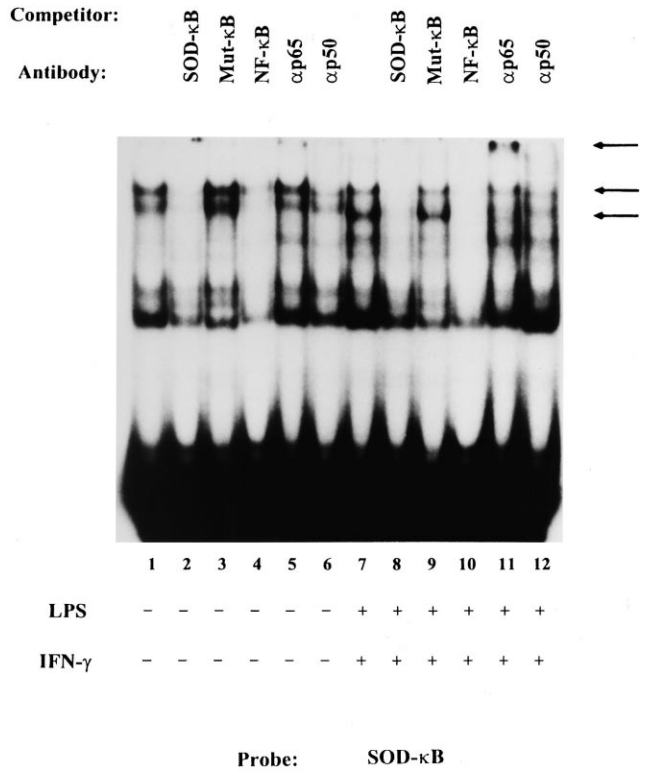

Fig. 4. Identification of NF-κB proteins binding to the B site. EMSA using a ^{32}P-labeled SOD-κB probe incubated with the nuclear extracts from NIH3T3 cells either untreated (lanes 1–6), or LPS- and IFN-γ-cotreated for 4 h (lanes 7–12). A 200-fold molar excess of a SOD-κB oligonucleotide (lanes 2 and 8), a Mut-κB oligonucleotide (lanes 3 and 9), or a NF-κB consensus oligonucleotide (lanes 4 and 10) was used as an unlabeled competitor. Antibodies to the p65 subunit (lanes 5 and 11) or the p50 subunit (lanes 6 and 12) were added to the extracts prior to the addition of the probe. The SOD-κB-protein complexes and the supershifted complex are indicated.

3.2. C/EBP proteins bind to the A site in vitro

To identify factors that interact with the 30-bp region encompassing the A and B sites, EMSAs were carried out with nuclear extracts prepared from the NIH3T3 cells which were untreated and treated with either or both 0.1 μg/ml LPS and 100 U/ml IFN-γ. Nuclear extracts from control and treated cells interacted with ^{32}P-labeled SOD-C/EBP-κB oligonucleotides, forming several bands (Fig. 3). Anti-C/EBP β and δ antibodies were able to supershift the medium-sized bands of complexes, especially complexes from cells treated with LPS alone (lanes 15, 16, and 18) or with LPS in combination with IFN-γ (lanes 9, 10, and 12). However anti-C/EBP α and anti-CRP-1 antibodies had no effect. We performed supershift analysis using ^{32}P-labeled SOD-κB oligonucleotides containing only the B site instead of the SOD-C/EBP-κB probe. When the nuclear extracts prepared from untreated and treated cells were incubated with the SOD-κB probe, none of the antibodies of C/EBP bound to the complexes (data not shown). These results indicate that both β/β and β/δ isoforms of C/EBP binds to the A site.

3.3. p65 and p50 NF-κB bind to the B site in vitro

To characterize the nuclear factors which bind to the B site, we performed competition and supershift EMSA using the SOD-κB probe. Binding of the complexes to the SOD-κB probe was sequence-specific since it was blocked by an excess of an unlabeled SOD-κB oligonucleotide but not by an excess of an unlabeled oligonucleotide containing a mutation within the B site (Mut-κB) (Fig. 4, lanes 7–9). We also used a NF-κB consensus oligonucleotide as an unlabeled competitor. This oligonucleotide prevented formation of the SOD-κB-protein complexes (lane 10), suggesting a possible involvement of NF-κB in the assembly of the complexes. To confirm the possibility, supershift analysis was carried out with the SOD-κB probe. Antibody to p65 supershifted the complexes (lane 11) and antibody to p50 partially disrupted the DNA binding of the complex (lane 12). These results suggested NF-κB as the inducible binding factor interacting with the B site despite the lack of identification of canonical NF-κB sites within this region.

4. Discussion

In this study, we identified the critical *cis*-acting element interacting with NF-κB, distinct from the previously described NF-κB transcription factor-binding site.

We showed here that the 5′ C/EBP-related region of the TNFRE is necessary for LPS and IFN-γ induction using a transfection assay. The importance of the C/EBP-related binding sites, the A site and the B site, which regulate Mn-SOD gene expression, was demonstrated by the ability of mutations at these sites to substantially reduce transcriptional activity induced by LPS alone or LPS in combination with IFN-γ. Expression of C/EBP β and C/EBP δ is elevated in several tissues as a part of the acute phase response [9,10]. Transfection studies and EMSAs demonstrated that LPS-mediated activation of C/EBP δ in addition to C/EBP β is important for LPS-mediated transcriptional activation of Mn-SOD through the A site, the C/EBP protein-binding site.

Mutation of the B site reduced LPS-mediated transcriptional activity to nearly the control level, suggesting that the B site is indispensable for LPS-mediated transcriptional activation through the TNFRE. Despite the lack of identification of a classic NF-κB-binding site around the B site, we found evidence that NF-κB proteins could bind to the B site, using competition and supershift EMSAs. The B site has a strong homology with regulatory regions, such as IFNc-C3 and IFN-γ κB of the human IFN-γ gene [11], FP-b of the macrophage inflammatory protein 1α promoter [12], and IL-6κB of the interleukin-6 (IL-6) gene promoter [13], which bind to NF-κB proteins in vitro. Interestingly, several promoters of immune or acute phase-responsive genes, such as the IL-6 gene [14], the interleukin-8 (IL-8) gene [15], and the angiotensinogen gene [16], have adjacent or overlapping binding sites for NF-κB and C/EBP. Such interactions may be implied by the close proximity of the NF-κB- and C/EBP-family binding sites in the Mn-SOD enhancer.

IFN-γ alone had little effect on the NF-κB and C/EBP binding to the A and B sites, and did not induce Mn-SOD gene expression through transcriptional activation. However, IFN-γ augments the ability of LPS to induce the gene expression and the transcriptional activation of Mn-SOD, as well as interleukin-12 (IL-12) p40 [17] and inducible-type NO synthase [18,19]. A significant supershift was seen with extracts from cells treated with both LPS and IFN-γ for 20 min using antibody to p65 (data not shown), suggesting that co-stimulation with LPS and IFN-γ could enhance IκB degradation or NF-

κB translocation to increase effective NF-κB activity and Mn-SOD gene expression. Furthermore, the induction by LPS and IFN-γ was retained in both mutants, although it was reduced in pGL3pro-mB, indicating that there is a potential for additional interactions between the NF-κB site and other factors binding sites. These theories remain to be studied.

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