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Research Article

Sp1-associated activation of macrophage inflammatory protein-2 promoter by CpG-oligodeoxynucleotide and lipopolysaccharide

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Abstract. Macrophage inflammatory protein-2 (MIP-2) is a C-X-C chemokine that is important in recruiting neutrophils to inflammatory sites. Our previous reports demonstrated that lipopolysaccharide (LPS) or CpG-oligodeoxynucleotide (CpG-ODN) rapidly induce MIP-2 gene expression in the macrophage cell line, RAW 264.7. Here, we show that the DNA sequence of the MIP-2 promoter between –114 and +14 is sufficient for strong promoter activity in LPS- or CpG-ODN-stimulated RAW 264.7 cells. Importantly, comprehensive mutant analysis reveals that an Sp1 element in the promoter region between –114 and –94 is essential for synergistic MIP-2 promoter activation by NF-κB and c-Jun regardless of the presence of an AP-1 site. By combining deletion or site-specific mutant analysis with immunocomplex assays, we also confirmed that Sp1 mediates the recruitment of transcription factors NF-κB and c-Jun in LPS- or CpG-ODN-treated RAW 264.7 cells. Several lines of experimental evidence imply that the Sp1-binding element is an important determinant of MIP-2 promoter activity, and that NF-κB, c-Jun and Sp1 can functionally cooperate to elicit maximal activation of the promoter.

Key words. MIP-2; LPS; CpG-ODN; Sp1; NF-κB; c-Jun.

Macrophage inflammatory protein-2 (MIP-2) is a C-X-C chemokine functionally analogous to human interleukin (IL)-8 [1–3]. MIP-2 was first described as a ~6-kDa heparin-binding protein secreted from the mouse macrophage cell line RAW 264.7 after stimulation with Gram-negative bacterial lipopolysaccharide (LPS) [1, 2]. The following cell types have been suggested for the production of MIP-2: macrophages [3, 4], epithelial cells [5, 6], bone marrow endothelial cells [7], astrocytes [8] and mast cells [9]. The production of MIP-2 in response to LPS is of particular clinical importance because MIP-2 helps to recruit neutrophils during LPS-induced lung inflammation [10, 11]. The upregulated production of MIP-2 has been associated with inflammatory diseases such as arthritis, glomerulonephritis and sepsis [12–14].

The immediate recognition of bacteria and their products early in host defense is mediated by an ancient immune response that uses conserved pattern recognition receptors to distinguish the pathogen-associated molecular pattern signatures of bacterial components [15, 16]. A wide variety of bacterial components including LPS, bacterial DNA, peptidoglycan and lipoteichoic acid can stimulate the innate immune responses [16]. LPS is the major component of the outer surface of Gram-negative bacteria,

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and is a typically potent immune activator. Toll-like receptor 4 (TLR4) mediates the LPS-induced activation of the innate immune system [17, 18]. Bacterial DNA and synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) are also recognized by the innate immune cells [19, 20]. Immune activation by CpG-ODN depends on TLR9 which shares a high degree of homology with TLR4 [21, 22]. The TLR/IL-1 receptor (IL-1R) signaling triggered by LPS or CpG-ODN results in the recruitment of the common adaptor protein MyD88 [23, 24]. Activation of the MyD88/IRAK pathway stimulates the transcription factors NF-κB and AP-1, which in turn enhance the transcriptional upregulation of genes downstream from the κB motif [25, 26].

In our previous studies [27, 28], LPS and CpG-ODN have been identified as powerful mediators of MIP-2 gene expression in RAW 264.7 cells. Computer-assisted and experimental analysis demonstrate the existence of potential LPS- or CpG-ODN-responsive transcriptional elements in the 531-bp 5′-untranslated region of the mouse MIP-2 gene. These elements include two copies of the AP-1 response element and one NF-κB binding site. The involvement and synergistic action of the transcription factors c-Jun and NF-κB have been observed in LPS- or CpG-ODN-induced activation of MIP-2 gene expression in RAW 264.7 cells.

Here, we performed further experiments to understand the detailed mechanisms involved in these phenomena. Experimental evidence shows that Sp1 is another key transcription factor in the expression of the MIP-2 gene, and that the synergistic action of NF-κB and c-Jun requires the Sp1-binding element in the MIP-2 promoter. By combining immunocomplex assays with deletions and site-specific mutant analysis of the MIP-2 promoter, we also confirmed that Sp1 is essential for recruiting the transcription factor NF-κB and c-Jun in LPS- or CpG-ODN-stimulated RAW 264.7 cells.

Materials and methods

Cell culture and reagents

The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC, Manassas, Va.), and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Viability was assayed using trypan blue dye exclusion and was typically greater than 95%. Cells were maintained until passage 20, and then discarded.

To obtain the desired concentration, *Escherischia coli* LPS (Sigma, St. Louis, Mo.) was suspended in sterile water and added to the cells. Phosphorothioate backbone-modified oligodeoxynucleotide was purchased from GenoTech (Daejon, Korea). The CpG-ODN 1826(S) consisted of 20 bases containing two CpG motifs (underlined): TCCATGACGTTCCTGACGTT. The LPS content of ODN was less than 1 ng LPS/mg ODN as measured by a *Limulus* amebocyte assay (Whittaker Bioproducts, Walkersville, Md.). The rabbit anti-NF-κB p65 polyclonal antibody was purchased from Delta Biolabs (Campbell, Calif.), and anti-NF-κB p65 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-Sp1 and anti-c-Jun monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, Mass.). Dr. H. Nakshatri (Indiana University, Indianapolis, Ind.) kindly provided the expression vectors encoding NF-κB p65 and c-Jun.

Construction of luciferase reporter plasmids

The MIP-2 promoter fragments –187 to +14, –124 to +14, –114 to +14, –94 to +14, and –71 to +14 were amplified by polymerase chain reaction using mouse genomic DNA (Clontech, Palo Alto, Calif.) as a template with the following primer sets: 5′ primer, MIP-2(–187) 5′-GGGTACCATAGTGGAAGGGCAG-3′, MIP-2(–124) 5′-CGGTACCCACTCAGCTTAGGGG-3′, MIP-2(–114) 5′-CGGTACCTTAGGGGCGGGGCTCTG-3′, MIP-2(–94) 5′-CGGTACCTTCCTGATGAGGGGACCC-3′, MIP-2(–71) 5′-CGGTACCTGAGCTCAGGGAATTTCC-3′; 3′ primer, MIP-2(+14) 5′-GCTCGAGGGGCCATGGCGCT-3′. These fragments were ligated into *Kpn*I and *Xho*I sites of the luciferase reporter plasmid pGL3-Basic vector (Promega, Madison, Wis.) yielding the reporter constructs pMIP-2(–187)-*Luc*, pMIP-2(–124)-*Luc*, pMIP-2(–114)-*Luc*, pMIP-2(–94)-*Luc*, and pMIP-2(–71)-*Luc*. To introduce site-specific mutations in NF-κB- and Sp1-binding sites, the transcription factor recognition sites were abrogated and changed to the *Cla*I site for NF-κB binding site or to the *BamH*I site for the Sp1-binding site by a two-step PCR mutagenesis method [27]. The method uses 5′-primer MIP-2(–187) or MIP-2(–124), and 3′-primer MIP-2(+14), along with primers that encode the following sequences in sense or antisense orientation: mNF-κB, $^{-74}$GAGCTCAatcgATTTCCCTGGT^{-53}; mSp1, $^{-117}$CAGCTTAGGatCcGGGCTCTGT^{-96}. Mutated sites are indicated with lowercase letters. The promoter sequences including the mutations were ligated into *Kpn*I and *Xho*I sites of the pGL3-Basic vector, thereby yielding the reporter constructs pMIP-2(–187)mNF-κB-*Luc*, pMIP-2(–187)mSp1-*Luc*, pMIP-2(–124)mNF-κB-*Luc*, and pMIP-2(–124)-mSp1-*Luc*. DNA sequencing helped to verify the nucleotide sequences of these constructs.

Transfection and luciferase assay

A day before the transfection, RAW 264.7 cells were passed into 12-well plates at a concentration of 2×10^5 cells/well. The cells were transfected with FuGene 6 Transfection Reagent (Roche, Indianapolis, Ind.) in DMEM with 10% FBS in accordance with the manufacturer's

instructions. For each comparison between constructs, we confirmed the equivalent transfection efficiency by cotransfecting the promoterless *Renilla* luciferase vector pRL-null (Promega) as an internal control [29]. After transfection, cells were placed in complete medium for 24 h prior to LPS (200 ng/ml) or CpG-ODN (3 µM) treatment for 6 h. Cells were harvested, washed and lysed by freeze-thawing three times, and luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) with a TD-20/20 luminometer (Tuner Designs, Sunnyvale, Calif.) according to the manufacturer's specifications. Individual assays were normalized for *Renilla* luciferase activity and the data are presented as a fold increase in activity relative to the empty-vector control. Data are from two or three independent experiments performed in duplicate or triplicate with similar results. Standard errors are indicated.

Immunoprecipitation and Western blotting

RAW 264.7 cells were treated with 200 ng/ml LPS or 3 µM CpG-ODN 1826(S). After 30 min, the cells were harvested and lysed at 4 °C in an immunoprecipitation (IP) lysis buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and Complete Protease Inhibitor Cocktail (Roche)]. Cell debris was removed by centrifugation, and cell lysates were incubated with the anti-Sp1 antibody for 2 h at 4 °C. Protein A-Sepharose CL-4B [10% (v/v) slurry; Amersham Pharmacia Biotech, Piscataway, N. J.)] was added to antibody-containing cell lysates, and the reaction mixtures were subjected to an additional 2 h of incubation at 4 °C. Immunocomplexes collected by centrifugation were washed twice in an IP wash buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 and Complete Protease Inhibitor).

The complexes were resuspended in an SDS-PAGE sample buffer, denatured, and subjected to SDS-PAGE in 10% reducing polyacrylamide gels. The separated proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, Calif.) for Western blot analysis. The membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 and 2% bovine serum albumin for 1 h, and incubated with an anti-NF-κB p65, anti-Sp1 or anti-c-Jun antibody for 2 h at room temperature. Immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa.) and an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay

Nuclear extracts from untreated and LPS- or CpG-ODN 1826(S)-treated cells were prepared as described elsewhere [30]. The MIP-2 promoter fragment encompassing –124 to –28 [MIP-2(–124/–28) probe] was amplified by PCR using the full-length MIP-2 promoter as a template with the following primer sets: 5′ primer, MIP-2(–124) 5′-CGGTACCCACTCAGCTTAGGGG-3′ and 3′ primer, MIP-2(–28) 5′-GCACGATGTCTGGAAAAGCCC-3′. For the electrophoretic mobility shift assay (EMSA), 20,000 cpm of ^{32}P-labeled probe was incubated with 20 µg of nuclear extract proteins in 20 µl of binding buffer containing 10 mM HEPES, pH 7.9, 65 mM NaCl, 1 mM dithiothreitol, 0.2 mM EDTA, 0.02% NP-40, 50 µg/ml poly(dI·dC):poly(dI·dC) and 8% glycerol at room temperature for 30 min, and then resolved on 4% polyacrylamide gels containing 0.5 × TBE (1 × TBE is 89 mM Tris borate and 1 mM EDTA, pH 8.0) and 2.5% glycerol. Competitor DNAs for consensus AP-1, Sp1, and NF-κB sites were 5′-CGCTTGATGACTCAGCCGGAA-3′, 5′-ATTCGATCGGGGCGGGGCGAGC-3′, and 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Santa Cruz Biotechnology), respectively. Oligonucleotide competition was performed by preincubating nuclear extracts with the cold probe (50-fold excess) and poly(dI·dC):poly(dI·dC) for 30 min at room temperature before addition of the labeled probe. c-Jun, Sp1 or NF-κB antibody supershift assays were carried out by adding 1 µg antibody to the reaction mixture for 30 min at 4 °C prior to the addition of the labeled probe.

Results

AP-1 binding element-independent and synergistic activation of the mouse proximal MIP-2 promoter by NF-κB and c-Jun

In our previous studies [27, 28], we demonstrated that the transcription factors NF-κB p65 and c-Jun are required for MIP-2 gene expression in RAW 264.7 cells. To determine the contributions of the NF-κB p65- and AP-1-binding elements in the promoter region, we employed several reporter constructs shown in figure 1A and performed the promoter analysis after transfection with the expression vectors for c-Jun or NF-κB p65 (fig. 2B) [and ref. 27]. The results suggested that the transcription factor NF-κB and its recognition site are closely involved when NF-κB and c-Jun synergistically induce the expression of the MIP-2 gene (fig. 1B). Interestingly, even in the absence of the AP-1-binding element, c-Jun enhanced the promoter activity in synergy with NF-κB if the NF-κB-binding site was present in the MIP-2 promoter. These observations indicate that NF-κB cooperates with c-Jun somehow independently of the AP-1-binding site, though the mode of cooperation remains to be determined. In this study, we investigated the molecular mechanism involved in this phenomenon.

Identification of an Sp1-like element in the MIP-2 promoter by deletion analysis

To identify other DNA elements necessary for synergistic MIP-2 promoter activation, a series of MIP-2 promoter

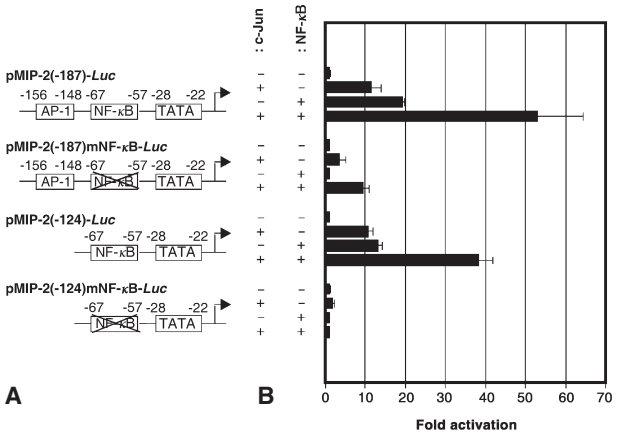

Figure 1. Effects of the transcription factors NF-κB p65 and c-Jun on MIP-2 promoter activation. (*A*), The positions of the NF-κB and AP-1 sites. Each mutated promoter fragment was ligated into the luciferase reporter plasmid pGL3 basic vector. (*B*), Activation of the mutant promoters in response to ectopical expression of NF-κB p65 and c-Jun. RAW 264.7 cells were transiently cotransfected with each reporter construct and plasmid that expresses NF-κB p65, c-Jun or both for 24 h. Cultures were harvested and assayed for luciferase activity. The results are presented as fold activation compared with control vector alone.

mutants that contained successive deletions from the 5′ end were cloned into luciferase reporter constructs (fig. 2A). Following cotransfection of the RAW 264.7 cells with the deletion reporter construct and the c-Jun expression vector, the luciferase activity was reduced to a background level when the MIP-2 promoter was deleted at a sequence between –114 and –94 (Fig. 2B). Luciferase activity of pMIP-2(–94)-*Luc* induced by NF-κB was decreased by 76% compared to that of pMIP-2(–187)-*Luc*, and the synergistic response of NF-κB and c-Jun was also reduced dramatically (fig. 2B).

LPS and CpG-ODN have been identified as powerful mediators of MIP-2 gene expression in RAW 264.7 cells [27, 28]. Therefore, to confirm the regulatory potential in the sequence between –114 and –94 of the MIP-2 promoter, we examined the promoter activity in response to LPS and CpG-ODN 1826(S). As the promoter activities of pMIP-2(–124)-*Luc* and pMIP-2(–114)-*Luc* were much lower than that of pMIP-2(–187)-*Luc*, the AP-1 site is presumed to be required for the maximal promoter activation induced by LPS or CpG-ODN. Nonetheless, LPS- or CpG-ODN-induced promoter activation in the absence of the

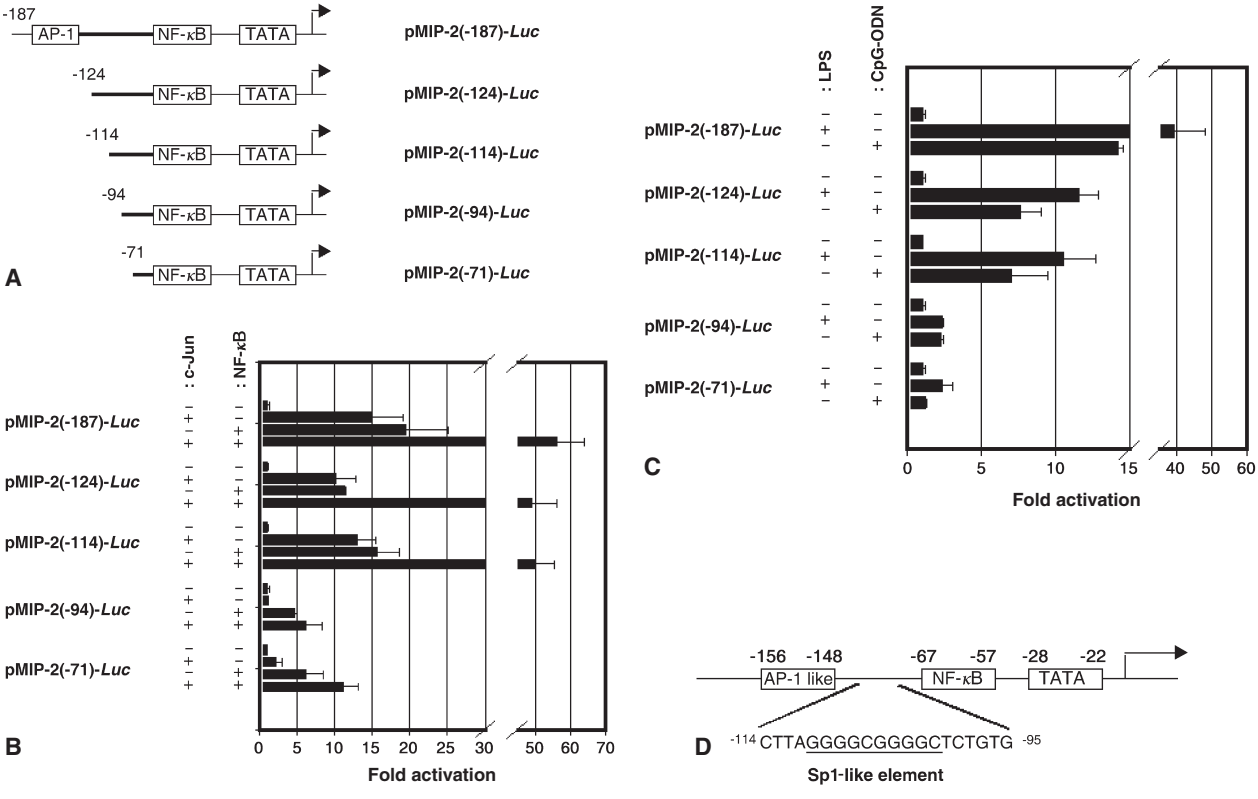

Figure 2. Effects of the 5′ serial deletion mutations on the activity of the MIP-2 promoter. (*A*) The structure of a series of 5′ deletion mutants of the MIP-2 promoter. (*B*) RAW 264.7 cells were transfected with each reporter construct and the indicated expression vectors (0.1 µg each) that encode NF-κB p65 or c-Jun. The results are presented as fold activation compared with the control expression vector. (*C*) LPS or CpG-ODN 1826(S) induction of the promoter constructs. RAW 264.7 cells were transfected with each reporter construct and cultured in the presence of 200 ng/ml LPS or 3 µM CPG-ODN 1826(S) for 6 h. The fold activation represents the ratio of the luciferase activity in stimulated and unstimulated cells. (*D*), Position and sequence of the Sp1-like element.

AP-1 site was still more than sevenfold compared to the unstimulated control (fig. 2C). Further deletion of the sequence between –114 and –94 substantially decreased the LPS- and CpG-ODN-induced luciferase activity (Fig. 2C). These results indicate that the DNA sequence between –114 and +14 is sufficient for the strong promoter activation by LPS and CpG-ODN, and at least one critical DNA element may be present in this region. We postulated that this element plays a critical role in synergistically activating the MIP-2 promoter by NF-κB and c-Jun in the absence of the AP-1 element. A computer-assisted analysis of the –114 to –94 of the MIP-2 gene reveals an element similar to a recognition site for the Sp1 protein (fig. 2D). Although Sp1 is expressed ubiquitously, it helps induce several genes, including monocyte chemoattractant protein 1 (MCP-1), TLR2 and IL-10 [31–34].

The contribution of the Sp1-binding site to MIP-2 promoter activity

To evaluate the possibility that the Sp1-like element contributes to the activity of the MIP-2 promoter, we performed mutational analysis starting from the two promoter constructs pMIP-2(–187)-*Luc* and pMIP-2(–124)-*Luc*. As shown in figure 3A, the two constructs have similar basal promoter activities and presence of the AP-1 site seems unnecessary for the basal promoter activity. When a mutation of the NF-κB-binding site was introduced in the presence of the AP-1 site [pMIP-2(–187)-mNF-κB-*Luc*], the mutation had minimal effect on the promoter activity (Fig. 3A). However, in the absence of the AP-1 site [pMIP-2(–124)mNF-κB-*Luc*], the same mutation reduced the promoter activity by 75%. In contrast, a mutation in the Sp1-like element markedly decreased the promoter activity regardless of the AP-1 site: compared to each wild-type construct, the promoter activity was 32% for pMIP-2(–187)mSp1-*Luc* and 9% for pMIP-2(–124)mSP1-*Luc* (fig. 3A). Therefore, we believe that the Sp1-like element is critical for basal activation of the promoter, and NF-κB is also required, especially in the absence of the AP-1 site.

To determine whether the Sp1-like element is required for LPS or CpG-ODN-induced MIP-2 promoter activation, mutant constructs were transiently transfected into RAW 264.7 cells and treated with LPS-or CpG-ODN 1826(S). Here again, we observed much higher promoter activity of pMIP-2(–187)-*Luc* compared to the AP-1 site-deleted

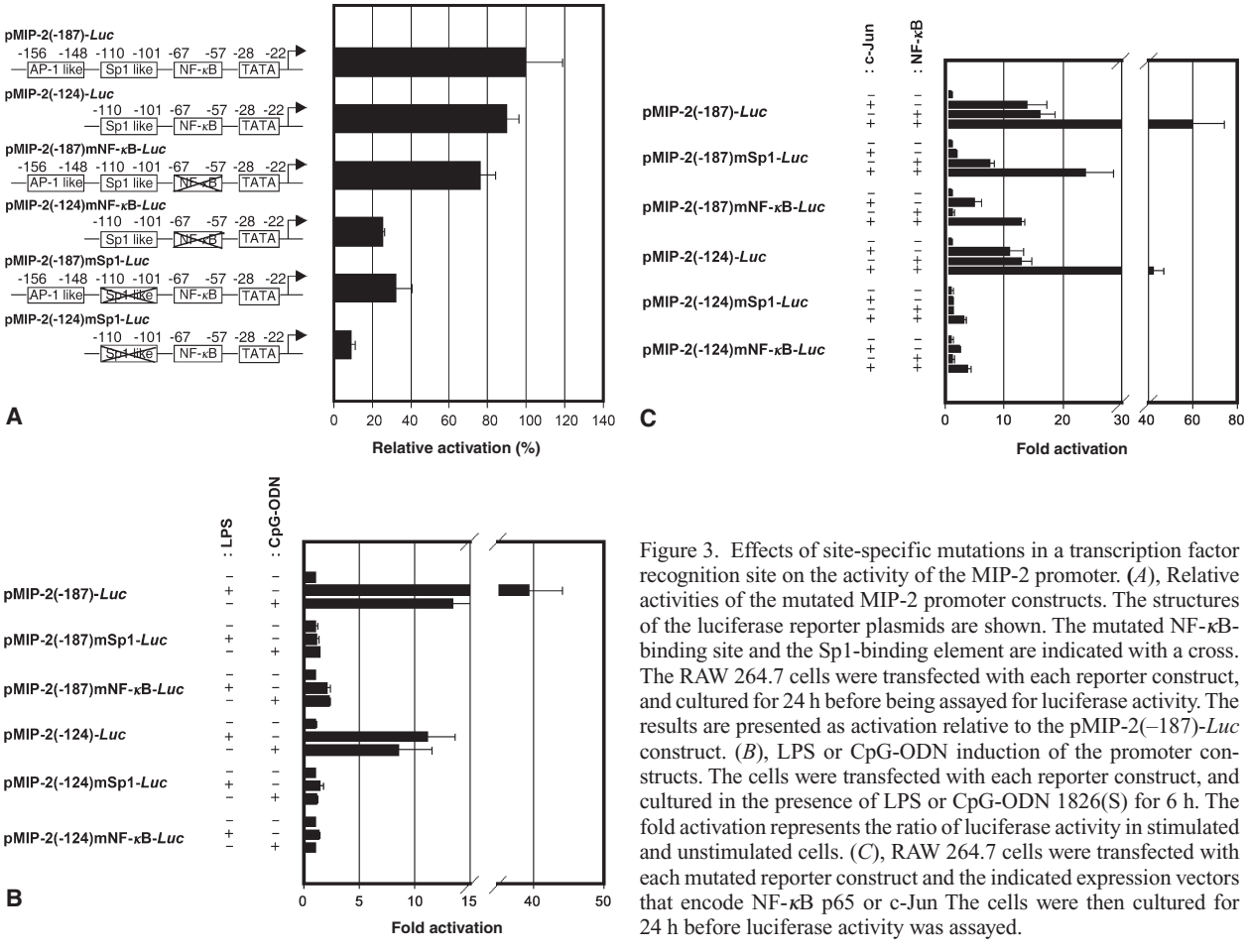

Figure 3. Effects of site-specific mutations in a transcription factor recognition site on the activity of the MIP-2 promoter. (*A*), Relative activities of the mutated MIP-2 promoter constructs. The structures of the luciferase reporter plasmids are shown. The mutated NF-κB-binding site and the Sp1-binding element are indicated with a cross. The RAW 264.7 cells were transfected with each reporter construct, and cultured for 24 h before being assayed for luciferase activity. The results are presented as activation relative to the pMIP-2(–187)-*Luc* construct. (*B*), LPS or CpG-ODN induction of the promoter constructs. The cells were transfected with each reporter construct, and cultured in the presence of LPS or CpG-ODN 1826(S) for 6 h. The fold activation represents the ratio of luciferase activity in stimulated and unstimulated cells. (*C*), RAW 264.7 cells were transfected with each mutated reporter construct and the indicated expression vectors that encode NF-κB p65 or c-Jun The cells were then cultured for 24 h before luciferase activity was assayed.

pMIP-2(–124)-*Luc*. Mutations of the Sp1 or NF-κB sites in pMIP-2(–187)-*Luc* and pMIP-2(–124)-*Luc* markedly reduced the promoter activities (fig. 3B). We therefore conclude that LPS- or CpG-ODN-induced MIP-2 promoter activation is mediated by the concerted action of NF-κB- and Sp1-binding sites, and that the AP-1 site is required for the maximal activation.

To further confirm our observations, we examined the involvement of Sp1-associated with NF-κB and c-Jun in transcriptional upregulation of the MIP-2 promoter by analyzing the site-specific mutant constructs after ectopic expression of NF-κB p65 and c-Jun (fig. 3C). The wild-type promoter pMIP-2(–187)-*Luc* showed a strong luciferase activity after cotransfection with NF-κB p65 or c-Jun expression vectors. However, a mutation in the Sp1-binding site of pMIP-2(–187)-*Luc* reduced the activity of the promoter by 88% in response to c-Jun and by 53% in response to NF-κB p65, even though the AP-1- and NF-κB-binding elements were intact (fig. 3C). Moreover, the mutation reduced the synergistic activation of the promoter by c-Jun and NF-κB p65 to 40% compared to that of the wild-type construct.

We then investigated the role of the Sp1 element in the absence of the AP-1 site (pMIP-2(–124)-*Luc*). The ectopic expression of NF-κB p65 or c-Jun or both caused promoter activation of pMIP-2(–124)-*Luc* with an efficiency comparable to pMIP-2(–187)-*Luc* (fig. 3C). In other words, c-Jun can stimulate the MIP-2 promoter that has no AP-1-binding site. When a mutation of the Sp1 site was introduced into the construct, the ectopic expression of c-Jun and NF-κB each failed to activate the MIP-2 promoter (fig. 3C). These results indicate that Sp1 is a potential regulator in MIP-2 promoter activation caused by the stimulation with c-Jun and NF-κB.

The Sp1 element is required for NF-κB- and c-Jun-dependent MIP-2 promoter activation following stimulation with LPS or CpG-ODN

Previously, we found that the MIP-2 promoter is maximally activated when stimulated by LPS or CpG-ODN in the presence of ectopically expressed NF-κB and c-Jun [27, 28]. Therefore, we endeavored to verify whether the Sp1 element is required for the NF-κB- and c-Jun-dependent response of the MIP-2 promoter to LPS and CpG-ODN. For the verification, activities of the Sp1 site-mutated MIP-2 promoter constructs were monitored in the presence of ectopically expressed NF-κB p65 or c-Jun (figs. 4, 5). The treatment of RAW 264.7 cells with LPS increased the promoter activity by approximately 39-fold for MIP-2(–187)-*Luc* and 9-fold for MIP-2(–124)-*Luc* (fig. 4). The LPS-induced transactivation of the MIP-2 promoter was dramatically increased by ectopic expression of c-Jun (fig. 4A), NF-κB p65 (fig. 4B), or both (fig. 4C). The mutations in the NF-κB- and Sp1-binding sites markedly reduced the promoter activation stimulated by LPS in the

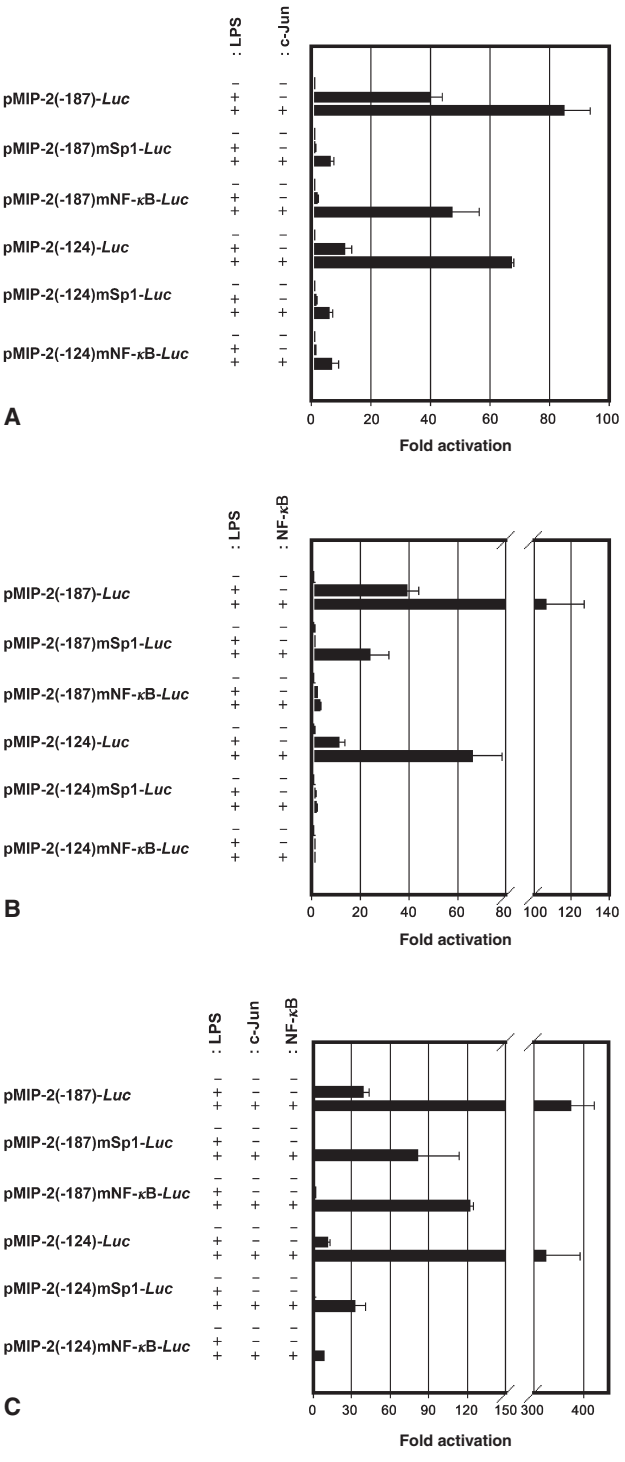

Figure 4. Effects of mutation in the NF-κB and Sp1 sites on the transcriptional activation of the MIP-2 gene in response to LPS after ectopic expression of NF-κB and c-Jun. RAW 264.7 cells were transfected with each reporter construct and plasmid that expresses c-Jun (*A*), or NF-κB p65 (*B*) or both (*C*). After 24 h, the cells were stimulated with 200 ng/ml of LPS for 6 h. Cultures were harvested and assayed for luciferase activity.

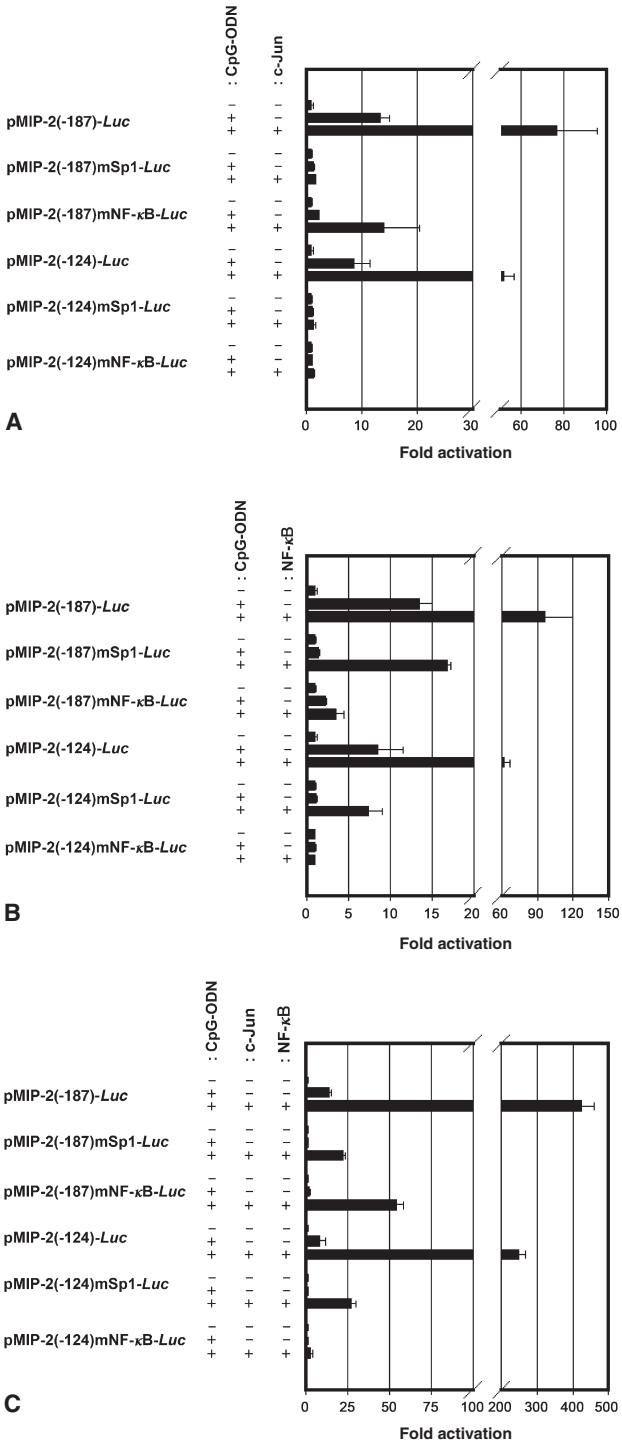

Figure 5. Effects of mutation in the NF-κB and Sp1 sites on the transcriptional activation of the MIP-2 gene in response to CpG-ODN after ectopic expression of NF-κB and c-Jun. RAW 264.7 cells were transfected with each reporter construct and plasmid that expresses c-Jun (*A*), NF-κB p65 (*B*) or both (*C*). After 24 h, the cells were stimulated with 3 µM of CpG-ODN 1826(S) for 6 h. Cultures were harvested and assayed for luciferase activity.

presence of the ectopic expression of NF-κB p65, c-Jun or both (fig. 4). Figure 5 shows that the CpG-ODN-induced activation of the MIP-2 promoter was markedly increased by cotransfection of NF-κB, or c-Jun, or both. It also shows that the CpG-ODN responsiveness of the MIP-2 promoter is dependent on the NF-κB-binding site and the Sp1 element. Mutation in the Sp1-binding site abolished the promoter activities caused by CpG-ODN in the c-Jun-transfected cells (fig. 5A), and also reduced promoter activation in the NF-κB p65-transfected cells by more than 85% compared to the activation of the wild-type promoter (fig. 5B). The mutation in the Sp1 site also substantially reduced the synergistic responses to the ectopic expressions of c-Jun and NF-κB p65 in CpG-ODN-stimulated cells (fig. 5C). These results indicate that the Sp1-binding element is needed for activating the MIP-2 promoter cooperatively with NF-κB and c-Jun in LPS- or CpG-ODN-stimulated RAW 264.7 cells.

Sp1-mediated recruitment of the transcription factors NF-κB and c-Jun in mouse macrophages stimulated with LPS or CpG-ODN

We identified the Sp1-binding site as an essential element for both NF-κB- and c-Jun-dependent activation of the MIP-2 promoter. To determine whether c-Jun and NF-κB interact directly with Sp1 in LPS- or CpG-ODN-treated RAW 264.7 cells, immunocomplex assays was performed. Antiserum to Sp1 protein was used to generate immunocomplexes from the lysates of RAW 264.7 cells treated with LPS or CpG-ODN 1826(S) for 30 min. Immunoprecipitated proteins were separated by SDS-PAGE, Western blots were prepared, and then probed with antiserum to NF-κB p65 or to c-Jun. Figure 6 shows the appearance of NF-κB p65 and c-Jun in the immunocomplexes generated with Sp1 antisera in the LPS- or CpG-ODN 1826(S)-treated cells. These results indicate that Sp1 is physically

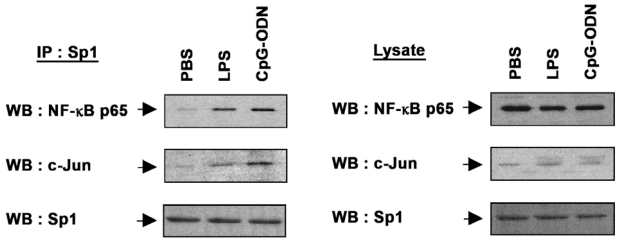

Figure 6. Complex formation of Sp1 with NF-κB p65 and c-Jun in RAW 264.7 cells stimulated with LPS- or CpG-ODN. Proteins were extracted from LPS or CpG-ODN 1826(S)-stimulated RAW 264.7 cells, and immunoprecipitated with anti-Sp1 antibody. Immunocomplexes were separated on SDS-PAGE, transferred onto nitrocellulose membrane, and Western blot analysis was performed with the indicated antibodies (left panels). The cell lysates were analyzed by Western blotting using the same antibodies to confirm that the initial protein amount used for immunoprecipitation was equal in each sample (right panels).

associated with NF-κB and c-Jun in the LPS- or CpG-ODN-stimulated RAW 264.7 cells.

To determine whether NF-κB p65, c-Jun and Sp1 actually bind to the MIP-2 promoter, and to further clarify the nuclear interaction between them for the MIP-2 promoter activation, we performed EMSA. Since we had demonstrated the cooperative action of NF-κB p65, c-Jun and Sp1 in the minimal promoter pMIP-2(–124)-*Luc*, we used the promoter sequence –124 to –28 containing Sp1- and NF-κB-binding sites [MIP-2(–124/–28)] as a probe. EMSA results indicated protein-DNA complexes induced by LPS or CpG-ODN (fig. 7A). To identify the protein-DNA complexes, competition assays were carried out using cold consensus sequences of AP-1, Sp1 or NF-κB sites. As shown in figure 7B, complex 1 and complex 2 disappeared in the presence of NF-κB competitor and Sp1 competitor, respectively. Further analysis was performed with supershift assays using antibodies specific to NF-κB p65, c-Jun or Sp1. A new supershift band appeared with concurrently decreased complex 1 in the presence of NF-κB p65 antibody. Similarly the complex 2 disappeared by reacting with Sp1 antibody. These results provide clear evidence that Sp1 and NF-κB directly bind to their corresponding sites in the MIP-2 promoter region. Since an AP-1 site competitor and c-Jun antibody did not affect the complex formation, c-Jun is likely involved in the promoter activation via protein-protein interaction after the Sp1-binding.

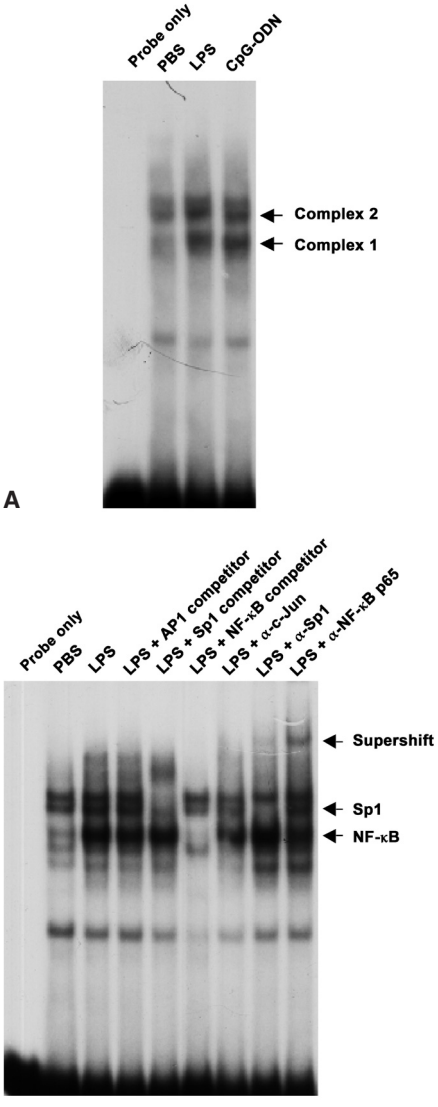

Figure 7. Direct binding of Sp1 and NF-κB p65 to the MIP-2 promoter region. (*A*) Nuclear extracts from untreated and LPS- or CpG-ODN 1826(S)-treated cells were analyzed by EMSA. DNA-protein complexes are denoted by arrows. (*B*) Nuclear extracts from LPS-treated cells were analyzed by EMSA in the presence or absence of cold consensus oligonucleotides for Sp1-, NF-κB- or AP-1-binding sites as competitors. For supershift assays, antibodies to Sp1, NF-κB p65 or c-Jun were included in the reaction mixture.

Discussion

The chemokine MIP-2 is produced by several cell types such as macrophages, epithelial cells, bone marrow endothelial cells, astrocytes and mast cells [3–9]. The MIP-2 gene is expressed in response to microbial infection and by stimulation of cells with LPS, CpG-ODN and proinflammatory cytokines such as IL-1 and tumor necrosis factor-α [35–37]. Previously, we showed that LPS and CpG-ODN upregulate mouse MIP-2 gene expression in the mouse macrophage cell line RAW 264.7 [27, 28]. The AP-1 element and NF-κB-binding site were identified in the proximal promoter of the mouse MIP-2 gene, and the promoter region between –187 to +14 from the transcription start site has been implicated for the basal activity of the MIP-2 promoter. Here, we present the first evidence that the Sp1-binding element of the MIP-2 gene is necessary for mediating the synergistic activation of the minimal MIP-2 promoter by NF-κB and c-Jun in RAW 264.7 cells.

Characterized as a constitutive transcription factor, Sp1 is considered a regulator of basal promoter activity [38, 39]. It stimulates transcriptional initiation by supporting the formation of a functional preinitiation complex that comprises RNA polymerase II, transcription factors, and the target DNA element [39–41]. It functionally interacts with other sequence-specific transcription factors. Examples include such ubiquitous factors as Oct-1, NF-κB, c-Jun and E2F-1, but also tissue-specific regulators such as MEF-2 [42–48]. When combined with Sp1, these transcription factors can synergistically activate the transcription of various target genes. Recent data suggest that Sp1 binding and transactivation can be modulated by a variety of stimuli, and that Sp1 mediates the effects of various cytokines and LPS [31, 34, 48, 49, 50].

Our previous results proved that NF-κB and c-Jun contribute to LPS- or CpG-ODN-induced MIP-2 gene ex-

pression in RAW 264.7 cells. In this study, the MIP-2 promoter was activated by ectopic expression of c-Jun even in the absence of the AP-1-binding site. Furthermore, the synergistic response was observed by stimulation with both c-Jun and NF-κB p65 in the same context. These results suggest that NF-κB is functionally associated with c-Jun independently of the AP-1-binding site, and this possibility was demonstrated by the deletion analysis and immunocomplex assay. For MIP-2 promoter activation by NF-κB, or c-Jun, or both, we used deletion analysis to identify one critical DNA element in the region between –114 and –94 bp 5′ of the transcription start site. When the sequence of the promoter region was analyzed by a computer-associated search, an Sp1-binding element showed up. Furthermore, mutation of the Sp1 site in the MIP-2 promoter reduced the promoter activity to less than 37% of the baseline, suggesting that the MIP-2 promoter does not function properly without Sp1. Experimental evidence suggests that the Sp1 binding element of the MIP-2 promoter is necessary for LPS- and CpG-ODN-induced transcriptional activity. We also found that, even in the absence of the AP-1 site, the NF-κB and Sp1 sites are sufficient to mediate the synergistic activation by NF-κB and c-Jun.

Treatment of RAW 264.7 cells with extracellular stimuli such as LPS, CpG-ODNs, and proinflammatory cytokines activates the cytoplasmic signal transduction pathways that lead to nuclear translocation and activation of NF-κB and AP-1 [23, 27, 51, 52]. Our data show that NF-κB and c-Jun can augment the MIP-2 promoter activation induced by LPS and CpG-ODN through the Sp1-binding site. In this context, we have demonstrated three things. First, mutation of the Sp1-binding element that affects its interaction abrogates the synergistic activation by NF-κB and c-Jun and, second, the nuclear-targeted NF-κB and c-Jun can make a complex with Sp1 protein in the nucleus to induce MIP-2 gene expression in LPS- or CpG-ODN-stimulated RAW 264.7 cells. Third, we observed direct binding of NF-κB or Sp1 to its corresponding binding element. As c-Jun does not bind directly to the promoter region that lacks the AP-1-binding site, one can postulate that c-Jun is involved in the promoter activation via protein-protein interaction. One possible explanation of the interplay of these transcription factors is the recruitment of the transcriptional coactivator p300/CBP to Sp1, c-Jun and NF-κB, as suggested previously by Wang and Chang, [48]. This interplay may increase DNA recognition and transcriptional activation, which result in the synergistic activation of the MIP-2 promoter by NF-κB and c-Jun in an Sp1-dependent manner in the absence of an AP-1-binding site.

Induction of MIP-2 in response to LPS or CpG-ODN is likely to be an important mediator of the chemotactic activity of neutrophils and to contribute to inflammatory diseases. This work demonstrated the involvement and synergistic action of the transcription factors c-Jun and NF-κB in LPS- or CpG-ODN-induced activation of the MIP-2 gene promoter even in the absence of the AP-1 site. Interestingly, the Sp1 mediates the recruitment of the activated c-Jun and NF-κB to transactivate the MIP-2 gene in LPS- or CpG-ODN-stimulated RAW 264.7 cells. The cooperation and synergism between these transcription factors may have an advantage in amplifying and ensuring the generation of inflammatory signals. In summary, the Sp1-binding element appears to be an important determinant of MIP-2 promoter activity, and NF-κB, c-Jun and Sp1 can cooperate functionally to elicit activation of the promoter. Future studies should gain further insight into this aspect of promoter activation.

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