Myeloid differentiation factor 88-dependent transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: role of NF-κB and p38

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**Running title:** MyD88-dependent regulation of COX-2 transcription by CpG DNA
SUMMARY

CpG DNA induces macrophage Cyclooxygenase-2 (COX-2) production. In the present study, we have investigated a biochemical signaling pathway and transcription factors responsible for transcriptional regulation of the COX-2 gene expression induced by CpG DNA. CpG DNA-induced COX-2 promoter activity was completely inhibited by an endosomal acidification inhibitor (chloroquine), a TLR9 antagonist inhibitory CpG DNA (iCpG DNA), or overexpression of a dominant negative (DN) form of MyD88. In contrast, overexpression of DN-IRAK1 or DN-TRAF6 only partially inhibited CpG DNA-induced COX-2 promoter activity and NF-κB activation, indicating the presence of additional signaling modulators downstream of MyD88. CpG DNA-induced COX-2 promoter activity was substantially suppressed in cells overexpressing super-suppressive IκB (IκB-AA), DN-p38, or DN-CREB. In addition, COX-2 promoter-luciferase reporters with alterations in predicted cis-acting transcriptional regulatory elements revealed that C/EBP, Ets-1, NF-κB, and CREB binding sites are essential for optimal COX-2 expression in response to CpG DNA. Conclusively, these results demonstrate that endosomal DNA processing and TLR9/MyD88-dependent activation of NF-κB and p38 are required for transcriptional regulation of COX-2 expression induced by CpG DNA, and suggest that IRAK and/or TRAF6 may be a diverging point for NF-κB activation in response to CpG DNA in RAW264.7 cells.
INTRODUCTION

Arthritis is one of the most common diseases in which inflammation and tissue destruction are driven by an autoimmune process. It has been speculated that bacterial infection may be involved in the pathogenesis of arthritis (1, 2). Indeed, bacterial products such as bacterial DNA are present in the synovial fluid of patients with some types of arthritis (3, 4). Like other bacterial products, unmethylated CpG motifs (GACGTT for murine, GTCGTT for human) in bacterial DNA are capable of activating innate immune cells, such as monocytes/macrophages and dendritic cells (DCs), that play a critical role in directing the host immune response to infection (reviewed in ref. 5). The ability of bacterial DNA to activate innate immunity can be mimicked by synthetic oligodeoxynucleotides containing the unmethylated CpG motif (CpG DNA). CpG DNA directly activates DCs and macrophages/monocytes to secrete cytokines and chemokines including TNF-α, IFN-α/β, IL-6, IL-10, IL-12, MIP1α, MIP1β, IP-10 and RANTES, to express increased levels of costimulatory molecules, and to increase antibody-dependent cellular cytotoxicity (ADCC) activity, antigen presentation and cross priming (6-13). Some of these cytokines, such as IFN-γ, TNF-α, IL-1, IL-6, and IL-12, have been shown to be implicated in arthritis. In addition, recent studies have demonstrated that CpG DNA induces the release of arachidonic acid (AA), which is converted to prostaglandin E2 (PGE2) by cyclooxygenase (COX), the production of PGE2, and expression of COX-2 (14, 15). Furthermore, CpG DNA-mediated PGE2 production is dependent on COX-2 (14, 15). Considering the critical role of PGE2 and COX-2 in the pathogenesis of arthritis, CpG DNA-mediated induction of COX-2 expression may be a key factor in infection-mediated arthritis.
The molecular mechanisms by which CpG DNA mediates immune cell activation are not completely understood at the present time. CpG DNA is endocytosed by leukocytes, acidified, and then recognized by Toll-like receptor 9 (TLR9), a pattern recognition receptor (PRR), in an endosomal compartment (6, 16, 17). Upon recognition of CpG DNA, TLR9 recruits the adaptor molecule myeloid differentiation factor 88 (MyD88), through an interaction between C-terminal Toll/IL-1 receptor (TIR) domains found in both TLR9 and MyD88. This recruitment of MyD88 to the TIR domain of TLR9 initiates a signaling pathway that sequentially involves IL-1R-associated kinases (IRAKs) and tumor necrosis factor-α receptor-associated factor 6 (TRAF6) (16-20). Studies using gene-deficient mice and RAW264.7 cells transiently transfected with the dominant negative (DN) forms of these molecules have indicated that this MyD88-mediated signaling pathway is essential for CpG DNA-induced activation of NF-κB and mitogen-activated protein kinases (MAPKs) including c-Jun NH2-terminal kinase (JNK) and p38 (16, 18, 21). In addition to this MyD88-mediated signaling pathway, DNA activated protein kinase (DNA-PK) and intracellular reactive oxygen species (ROS) have also been demonstrated to be required for CpG DNA-mediated activation of NF-κB and JNK, which in turn lead to the expression of various oncogenes and proinflammatory cytokines (6, 22).

COX, which catalyzes the conversion of AA into PGs, is a rate-limiting step in the synthesis of PGs, a lipid mediator that contributes to the development of inflammatory responses and is considered to be an important mediator in the pathophysiologic processes of arthritis (23-27). Two different isoforms of COX have been identified: COX-1 and COX-2 (28). COX-1 is expressed constitutively in most tissues and may be responsible for housekeeping functions. In contrast, COX-2 is not detectable in most normal tissues or resting immune cells, but its expression can be induced by cytokines, growth factors, and bacterial products including
endotoxin and CpG DNA (14, 15, 29). The regulation of COX-2 expression occurs at both transcriptional and post-transcriptional levels (30-47). Several consensus sequences, including binding sites for NF-κB, CCAAT enhancer-binding protein β (C/EBPβ), cAMP-responsive element binding protein (CREB), and a TATA-box, are found in the 5’ region of the COX-2 gene and regulate COX-2 expression in response to a variety of stimuli in different species and cell types (38, 44, 48, 49). NF-κB has been reported to play a key role in LPS-mediated induction of COX-2 expression (50). In addition, mitogen activated protein kinases (MAPKs) and the small GTP-binding proteins Ras and Rac have also been demonstrated to be involved in the regulation of COX-2 expression (51). CpG DNA induces activation of the transcription factors NF-κB and CREB as well as all three MAPKs [extracellular signal-regulated kinase (ERK), JNK, and p38] in macrophages (52-54). However, the relative contribution of these different signaling modulators and transcription factors to CpG DNA-induced COX-2 transcription in macrophages has not been studied. In the present study, we have investigated whether transcriptional regulation of COX-2 expression by CpG DNA is dependent on the endosomal processing of CpG DNA and the TLR9/MyD88-dependent signaling pathway, and we have identified cis-acting transcriptional regulatory elements critical for CpG DNA-induced COX-2 expression.
EXPERIMENTAL PROCEDURES

**Oligodeoxynucleotides.** Nuclease resistant phosphorothioate oligodeoxynucleotides (S-ODN) were purchased from Operon (Alameda, CA) and further purified by ethanol precipitation. S-ODN had no detectable endotoxins by Limulus assay. The sequences of S-ODN used are 5’TCCATGACGTTCTGACGTT3’ (CpG DNA: ODN1826), 5’TCCAGGACTTCTCTCAGGTT3’ (non-CpG DNA: ODN1982), and 5’T CCTGGCGGGGAAGTG3’ (inhibitory CpG DNA: iCpG DNA: ODN2088).

**Cell lines, culture conditions, and reagents.** RAW264.7 cells (ATCC, Rockville, MD) were maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and cultured at 37°C in a 5% CO2 humidified incubator. All culture reagents were purchased from Life Technologies (Gaithersburg, MD). LPS (Salmonella minnesota Re 595) was purchased from Sigma Chemical Co. (St. Louis, MO). LPS purity was confirmed by its inability to induce IL-6 production in macrophages isolated from the LPS-unresponsive C3H/HeJ mouse strain. SB202190, U0126, and SP600125 were purchased from Calbiochem (La Jolla, CA).

**Plasmids.** The COX-2 promoter region containing 963 base pairs (-963/+1) was amplified by polymerase chain reaction (PCR) using genomic DNA as a template. The resulting COX-2 promoter region was cloned into the pGL3 basic luciferase expression vector (Promega, Madison, WI) to generate a wild type COX-2 promoter-luciferase reporter (COX2-promoter-luc). Deletion mutants of the COX-2 promoter were generated by PCR using the wild type COX-2 promoter-luciferase reporter construct as a template. Each of the resulting COX-2 promoter deletion fragments, Δ-700 (-700/+1), Δ-320 (-320/+1), or Δ-52 (-52/+1), were cloned into the pGL3 basic luciferase expression vector to generate deletion mutant COX-2 promoter-
luciferase reporters. *Cis*-acting element response sites in COX-2 promoter region were identified by sequence analysis using the TFSEARCH {Searching Transcription Factor Binding Sites (ver 1.3)} software program. Putative *cis*-acting element response sites in the COX-2 promoter are C/EBP-(3) (-900/-895), Ets-1 (-800/-794), NF-κB (-402/-392), C/EBP-(2) (-135/-130), C/EBP-(1) (-90/-86), AP-1 (-65/-60), and CRE (-60/-56). The presence of NF-κB (-402/-392), C/EBP-(2) (-135/-130), C/EBP-(1) (-90/-86), AP-1 (-65/-60), and CRE (-60/-56) sites in the COX-2 promoter region has also been previously reported (35, 38, 48). Site-directed mutagenesis was performed to modify each *cis*-acting element response site in the COX-2 promoter region of the wild type COX-2 promoter-luciferase reporter using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Wild type and modified sequences of each *cis*-acting element response site in COX-2 promoter region are listed in Table 1.

cDNA fragments encoding p38, dominant negative (DN) IRAK-1 (amino acids 1-215), or DN-TRAF6 (amino acids 305-531) were amplified by PCR using murine cDNA as a template. DN-IRAK-1 or p38 encoding cDNA fragments were cloned into pEF6/V5-His-TOPO (Invitrogen, Carlsbad, CA) for N-terminal histidine tagging, and then subcloned into the bicistronic expression vector pIRES2-EGFP (Clontech, Palo Alto, CA), which expresses green florescent protein as a selection marker. DN-TRAF6 encoding cDNA fragments were cloned into pIRES2-EGFP. The DN-CREB expression construct pCMV-CREB-S133A was purchased from Clontech (Palo Alto, CA). The MEK1 expression construct pUSE-MEK1 and control vector pUSE were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The DN-MyD88 expression construct pIRES2-EGFP-DN-MyD88 was provided by Dr. S.-C. Hong (Indiana Univ., Indianapolis, IN). An AP-1-β-galactosidase construct and pCDNA3-JNK1
expression construct were provided by Dr. G. Koretzky (University of Pennsylvania, Philadelphia, PA). Expression constructs encoding DN-p38, DN-JNK1, and DN-MEK1 were generated from wild type p38, JNK1, and MEK1 expression constructs by site-directed mutagenesis using Stratagene’s QuickChange kit, according to the manufacturer’s protocol. DNA sequences of all cloned and mutated genes were confirmed by DNA sequencing analysis and were identical with the previously reported sequences. All PCR primers used for cloning and mutagenesis were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) and listed in Table 2.

**Transient Transfection and reporter gene assays.** RAW 264.7 cells (1.5x10^6 cells/plate) were plated into 60-mm cell culture plates and then incubated for 48 hr to reach approximately 80% confluence. Cells were co-transfected with pRL-TK-luciferase (1 µg) and wild type or mutant COX-2 promoter-luciferase reporter constructs (1 µg) using FuGene6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s protocol. In some experiments, cells were co-transfected with an equal amount (2 µg) of control empty vector (pIRES-EGFP, pCDNA3, or pUSE), DN-MyD88, DN-IRAK1, DN-TRAF6, DN-MEK1, DN-p38, DN-JNK1, IκB-AA, or DN-CREB and reporter gene COX-2 promoter-luciferase (1 µg) plus pRL-TK-luciferase (1 µg), NF-κB-luciferase (1 µg) plus pRL-TK-luciferase (1 µg), CREB-luciferase (1 µg) plus pRL-TK-luciferase (1 µg), or AP-1-β-galactosidase (2 µg) and then incubated for 6 hr. Transfected cells were pooled and washed 3 times with culture media. Cells (1x10^6 cells/1.5-mm cell culture plate) were stimulated with medium, CpG DNA (6 µg/ml), non-CpG DNA (6 µg/ml), or LPS (50 ng/ml) for 12 hr. In some experiments, transfected cells were pretreated with medium, iCpG DNA (6-12 µg/ml), Chloroquine (2.5 µg/ml), DMSO, U0126 (1.25 µM), SB202190 (2.5 µM), or SP600125 (5 µM) for 15 min before stimulation. β-galactosidase and
luciferase activities in cell extracts were analyzed according to manufacturers’ protocols using Galacto-Light Plus Reporter gene assay (Tropix, Bedford, MA) and Dual-Luciferase Reporter Assay System (Promega, Madison, WI), respectively. Luciferase activity was normalized using pRL-TK-luciferase activity (Renilla luciferase activity) in each sample. For AP1-β-galactosidase assay, equal concentrations of cell lysates were used.

**Preparation of whole cell lysates and Western blot analysis.** RAW264.7 cells (2×10⁶ cells/ml) were pretreated with DMSO or SP600125 (5 µM) for 30 min and then stimulated with medium or CpG DNA (6 µg/ml) for 45 min. Whole cell lysates were prepared as previously described (55). To detect the presence of a specific protein or the phosphorylation status of a specific protein, equal amounts (15 µg/lane) of whole cell lysates were subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS and then western blots were performed as previously described (55). As a loading control, blots were probed for actin using an actin-specific antibody purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies specific for the phosphorylated forms of c-Jun, ERK, JNK, or p38 were purchased from Cell Signaling (Beverly, MA).
RESULTS

CpG DNA up regulates COX-2 promoter activity. Expression of COX-2 is regulated by both transcriptional and post-transcriptional mechanisms (30-47). A recent study has demonstrated that CpG DNA induces COX-2 expression (14, 15). However, the mechanism by which CpG DNA induces COX-2 expression remains unexplored. To investigate the mechanism by which CpG DNA induces COX-2 expression at the transcriptional level, the proximal 963 base pairs of the mouse COX-2 promoter were cloned into a luciferase reporter vector and this COX2-promoter reporter construct (COX2-promoter-luc) was transiently transfected into RAW264.7 cells. The transfected cells were stimulated with various concentrations of CpG DNA or control non-CpG DNA for 12 hr and then luciferase activity was measured. As shown in Figure 1A, CpG DNA induced COX-2 promoter-luciferase activity in a dose dependent manner. In contrast, control non-CpG DNA failed to induce COX2-promoter-luciferase activity. This result indicates that CpG DNA induces COX-2 expression at the transcriptional level by up regulating COX-2 promoter activity.

Many stimulatory effects of CpG DNA have been reported to be specifically blocked by a TLR9 antagonist, inhibitory CpG DNA (iCpG DNA) (56-58). To investigate whether iCpG DNA can also inhibit CpG DNA-induced COX-2 transcription in macrophages, RAW264.7 cells were transfected with the COX2-promoter-luc reporter and then stimulated with medium, CpG DNA, control non-CpG DNA, or LPS in the presence or absence of iCpG DNA. As shown in Figure 1B, both CpG DNA and LPS induced COX2-promoter-luciferase activity. CpG DNA-induced COX2-promoter-luciferase activity was completely abolished in the presence of an equal concentration of iCpG DNA. In contrast, LPS-induced COX2-promoter-luciferase activity was not suppressed by iCpG DNA. This result demonstrates that CpG DNA used for this study...
is free from LPS contamination and that CpG DNA induces transcriptional activity of COX-2 promoter in a sequence-dependent manner.

*Endosomal acidification is required for the CpG DNA-mediated induction of COX-2 promoter activity.* It has been previously demonstrated that CpG DNA is endocytosed by leukocytes and endosomal acidification/processing/maturation of CpG DNA is required for its action (6, 17, 52, 55). To investigate whether this endosomal processing of DNA is also required for the CpG DNA-mediated transcriptional regulation of COX-2 expression, RAW264.7 cells were transfected with the COX-2-promoter-luc reporter. Cells were stimulated with CpG DNA or LPS in the presence or absence of chloroquine, an inhibitor of endosomal acidification. As demonstrated in Figure 2, CpG DNA-induced COX-2 promoter-luciferase activity was completely inhibited by chloroquine. In contrast, chloroquine failed to suppress LPS-induced COX-2-promoter-luciferase activity. These results indicate that CpG DNA induces COX-2 promoter activity through an endosomal acidification-dependent pathway.

*CpG DNA induces COX-2 promoter activity through a TLR9/MyD88-dependent signaling pathway.* It has recently been demonstrated that after endocytosed, CpG DNA is co-localized in an endosomal compartment with TLR9 (17). Although there is no direct evidence that CpG DNA directly binds to TLR9, TLR9 is believed to be a receptor for CpG DNA. After recognizing CpG DNA, TLR9 binds to MyD88 and initiates a cascade of signal transduction that is mediated through MyD88 downstream effectors including IRAK1 and TRAF6 (21, 18). We have investigated the regulatory role of signaling modulators of the TLR9/MyD88 signaling pathway in CpG DNA-induced COX-2 transcription. To investigate whether CpG DNA induces COX-2 promoter activity through a MyD88-dependent pathway, RAW 264.7 cells were transiently co-transfected with a DN-MyD88 overexpression vector and COX-2 promoter-luc
reporter vector. As demonstrated in Figure 3A, overexpression of DN-MyD88 completely inhibited CpG DNA-induced COX-2 promoter-luc activity. In addition, overexpression of DN-MyD88 also completely inhibited CpG DNA induction of both AP-1 and NF-κB transcriptional reporters (Fig. 3B). These results demonstrate that MyD88 is required for CpG DNA-mediated induction of COX-2 transcription.

Binding of MyD88 to TLR9 through their TIR domain interaction leads to the recruitment and activation of IRAK-1 (21). To determine whether IRAK-1 is required for CpG DNA-induced COX-2 promoter activity, RAW 264.7 cells were transiently co-transfected with a DN-IRAK-1 overexpression vector and a COX-2 promoter-luc reporter vector. Overexpression of DN-IRAK-1 resulted in substantial, but not complete, inhibition of CpG DNA-induced COX-2 promoter-luc activity (Fig. 4A). In addition, CpG DNA-mediated NF-κB activation was also partially inhibited by overexpression of DN-IRAK-1 (Fig. 4B). However, overexpression of DN-IRAK-1 completely abolished activation of AP-1 induced by CpG DNA, indicating that overexpression of DN-IRAK-1 was sufficient to inhibit the function of CpG DNA and that IRAK-1 is required for CpG DNA-mediated AP-1 activation (Fig. 4B). Taken together, these results indicate that IRAK-1 is necessary for the optimal induction of COX-2 promoter activity by CpG DNA and that IRAK-1 may be a diverging point for NF-κB activation in the CpG DNA-signaling pathway.

In the TLR9/MyD88-signaling pathway, activated IRAK-1 interacts with TRAF6, which in turn leads to the activation of NF-κB and MAPK (16, 18, 21). To investigate whether TRAF6 is necessary for CpG DNA-induced COX-2 promoter activity, RAW 264.7 cells were transiently co-transfected with a DN-TRAF6 overexpression vector and COX-2 promoter-luc reporter vector. As demonstrated in Figure 5A, CpG DNA-induced COX-2 promoter-luciferase activity
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was only partially inhibited in cells overexpressing DN-TRAF6. In addition, CpG DNA-mediated NF-κB activation was also only partially inhibited by overexpression of DN-TRAF6 (Fig. 5B). Transfecting increased concentrations of the DN-TRAF6 expression vector did not further inhibit CpG DNA-induced COX-2 promoter-luc activity or NF-κB-luciferase activity (data not shown). In contrast, overexpression of DN-TRAF6 completely abolished CpG DNA-induced AP-1 activation, demonstrating that the incomplete inhibition of NF-κB and COX-2 promoter activities by DN-TRAF6 was not due to an ineffective dominant negative function (Fig. 5). These results demonstrate that TRAF6 is essential for CpG DNA-mediated AP-1 activation and is necessary for the optimal induction of COX-2 promoter activity and NF-κB activation by CpG DNA.

MAP kinase p38, but not ERK and JNK, plays a critical role in the CpG DNA-mediated transcriptional regulation of COX-2 expression. Our results show that overexpression of DN-TRAF6 only partially inhibits CpG DNA-induced COX-2 promoter activity while it completely abolishes AP-1 activation induced by CpG DNA (Fig. 5). Previously we have demonstrated that CpG DNA-mediated AP-1 activation is dependent on activated MAPKs including ERK, JNK, and p38 (53, 54). These findings suggest that TRAF6 may contribute to optimal COX-2 promoter induction by leading to the activation of MAPKs, which in turn activate one or more transcription factors involved in the regulation of COX-2 expression. Therefore, we have investigated whether MAPKs play a functional role in CpG DNA-mediated transcriptional regulation of COX-2 expression. To determine whether activated MAPKs are required for induction of COX-2 promoter activity by CpG DNA, RAW264.7 cells transfected with the COX-2 promoter-luc reporter were pretreated with SB202190 (a p38 inhibitor), U0126 (a MEK inhibitor), or SP600125 (a JNK inhibitor) before CpG DNA stimulation. As shown in Figure
6A, CpG DNA-mediated COX-2 promoter luciferase activity was substantially inhibited in the presence of the p38 inhibitor SB202190, indicating the essential role of p38 in CpG DNA-induced COX-2 expression. In contrast, CpG DNA-induced COX-2 promoter luciferase activity was not suppressed by U0126 or SP600125. Of note, SP600125 specifically inhibited phosphorylation of JNK and c-Jun without effecting phosphorylation of ERK or p38 in RAW264.7 cells (Fig.6B). The specificity of U0126 and SB202190 in our experimental system has previously been reported (54).

To confirm the finding that p38, but not ERK and JNK, is required for CpG DNA-induced COX-2 promoter activity, RAW264.7 cells were cotransfected with a COX-2 promoter-luc reporter and expression vectors encoding DN-p38, DN-MEK1, or DN-JNK. CpG DNA-induced COX-2 promoter-luciferase activity was substantially reduced by overexpression of DN-p38 (Fig. 6C). In contrast, overexpression of neither DN-MEK1 nor DN-JNK inhibited CpG DNA-induced COX-2 promoter-luciferase activity (Figs. 6D and 6E). AP-1 reporter activity induced by CpG DNA was completely inhibited in RAW264.7 cells overexpressing either DN-p38, DN-MEK1, or DN-JNK (Figs. 6C, 6D, and 6E). This indicates that the expressed levels of DN-p38, DN-MEK1, and DN-JNK were sufficient to inhibit the function of CpG DNA. These results demonstrate that p38 activated by CpG DNA contributes to COX-2 transcription.

**NF-κB and CREB are required for CpG DNA-induced COX-2 promoter activity.** It has previously been demonstrated that p38 activated by CpG DNA leads to the activation of transcription factor CREB (54). In addition to an NF-κB consensus site, a CREB-responsive CRE site is also present in the COX-2 promoter region. To investigate whether NF-κB and/or CREB are required for CpG DNA-induced COX-2 promoter activity, RAW264.7 cells were co-transfected with a COX-2 promoter-luc reporter and a super-repressive IκB (IκB-AA) expression
vector and/or a DN-CREB expression vector. As shown in Figures 7A and 7B, overexpression of either IκB-AA or DN-CREB substantially inhibited CpG DNA-induced COX-2 promoter-luciferase activity. Moreover, co-expression of both IκB-AA and DN-CREB completely inhibited CpG DNA-induced COX-2 promoter-luciferase activity (Fig. 7C). As expected, overexpression of IκB-AA and DN-CREB completely suppressed the CpG DNA-induced NF-κB-luciferase activity and CREB-luciferase activity, respectively (Figs. 7A and 7B). To further confirm the requirement of NF-κB and CREB for CpG DNA-induced COX-2 promoter activity, we modified NF-κB (-402/-392) and CRE (-59/-52) consensus sites in the COX-2 promoter region using site-directed mutagenesis. As demonstrated in Figure 7D, mutations at either NF-κB or CRE consensus sites substantially diminished the COX-2 promoter-luciferase activity in response to CpG DNA stimulation. Furthermore, double mutations at NF-κB and CRE binding sites completely abolished COX-2 promoter-luciferase induction by CpG DNA (Fig. 7D). These results demonstrate that both NF-κB and CREB are required for CpG DNA-induced transcriptional activity of COX-2 promoter.

*The C/EBPβ and Ets-1 sites are essential for optimal COX-2 promoter activity induced by CpG DNA.* About 1,000 bp of the 5’-flanking region of the mouse COX-2 gene contain various putative transcriptional response elements as described previously (35, 38, 48). Based on sequence analysis, this region contains predicted consensus binding sites for transcription factors C/EBPβ, Ets-1, NF-κB, AP-1, and CREB. To determine whether cis-acting elements in the COX-2 promoter other than NF-κB and CREB binding sites are necessary for CpG DNA-induced COX-2 transcription, we generated a series of deletion mutants and site-directed mutants of COX-2 promoter-luciferase reporter constructs. Three truncated COX-2 promoter-luciferase reporter constructs generated from wild type full-length (-963 to +1 bp) COX-2 promoter-
Luciferase reporter were ∆-700 (containing bp -700 to +1), ∆-320 (containing bp -320 to +1), and ∆-52 (containing bp -52 to +1) (Fig. 8). The ∆-700 COX-2 promoter-luciferase reporter contains NF-κB (-402/-392), two proximal C/EBP (-135/-130 and -90/-86), AP-1 (-65/-60), and CREB (-60/-56) consensus binding sites, but an Ets-1 (-800/-974) site and a distal C/EBP (-900/-895) site were deleted from the full-length COX-2 promoter-luciferase reporter. The ∆-320 COX-2 promoter-luciferase reporter contains AP-1, CREB, and two proximal C/EBP consensus binding sites but lacks an Ets-1 site, NF-κB site, and a third distal C/EBP site. The ∆-52 COX-2 promoter-luciferase reporter contains only a TATA box. To identify promoter regions involved in CpG DNA-induced COX-2 transcription, RAW264.7 cells were transfected with full-length, ∆-700, ∆-320, or ∆-52 COX-2 promoter-luciferase reporters. As shown in Figure 8, CpG DNA induced an approximately 30-fold increase in the activity of the full-length COX-2 promoter-luciferase reporter as compared to the basal unstimulated level. Deletion of a 263 bp region from the 5’ end of the COX-2 promoter region (Δ-700), a region containing a distal C/EBP and Ets-1 sites, reduced the CpG DNA-induced COX-2 promoter-luciferase activity by approximately 45%. This indicates that the region between –963 and –700 bp is required for the optimal expression of COX-2 in response to CpG DNA and that an Ets-1 site and/or the distal C/EBP site may be essential cis-acting elements regulating CpG DNA-induced COX-2 expression. As expected, further deletion of 380 bp from the 5’ end of the Δ-700 COX-2 promoter (Δ-320), removing an NF-κB site, led to a substantial reduction (approximately 70% reduction of the full-length COX-2 promoter-luciferase activity) in CpG DNA-induced COX-2 promoter-luciferase activity, indicating the critical role of NF-κB (Fig. 8). Deletion of an additional 268 bp from the 5’ end of the Δ-320 COX-2 promoter (Δ-52), thus removing an AP-1 site, a CRE site, and two proximal C/EBP sites, completely abolished COX-2 promoter-luciferase activity in response to
CpG DNA (Fig. 8). This indicates that the −320 to −52 bp region in the COX-2 promoter contains important cis-acting elements, which may be C/EBP, AP-1, and/or CREB binding sites.

Since our study with COX-2 promoter deletion mutant reporters indicated the possible involvement of cis-acting elements other than NF-κB and CREB (Fig. 8), we introduced site-directed mutations in each of the predicted cis-acting element response sites in the full length (-963 to +1 bp) COX-2 promoter. A mutation in putative C/EBPβ-(3) (-900/-895) and C/EBP-(2) (-135/-130) sites reduced CpG DNA-induced COX-2 promoter-luciferase activity to 78% and 49%, respectively, as compared with the luciferase activity of the wild-type COX-2 promoter (compare b and c to a in Fig. 9). In contrast, a mutation at the proximal predicted C/EBP-(1) site (-90/-86) had no effect on CpG DNA-induced COX-2 promoter-luciferase activity (compare a and d in Fig. 9). These results indicate that C/EBPs, presumably C/EBPβ, may play a functional role in CpG DNA-mediated COX-2 expression through C/EBP-(3) and C/EBP-(2) sites but not through the C/EBP-(1) site in the COX-2 promoter region. Site-directed modification of the Ets-1 (-800/-794) site diminished CpG DNA-induced COX-2 promoter-luciferase activity to 64% of the luciferase activity of the wild-type COX-2 promoter (compare a and e in Fig. 9). Double mutation of both C/EBP-(3) and Ets-1 sites led to only a modest further decrease in promoter activity as compared with either single site mutation alone (compare f to b and e in Fig. 9). The CpG DNA-induced luciferase activity of the C/EBP-(3)/Ets-1 doubly mutated COX-2 promoter reporter was comparable with that of the Δ-700 COX-2 promoter reporter (Figs. 8 and 9f).

Mutation of the predicted AP-1 (-65/-60) site reduced the CpG DNA-induced COX-2 promoter-luciferase activity to 34% of the value for the wild-type reporter (compare g to a in Fig. 9). This result indicates that AP-1 may be an essential CpG DNA responsive cis-acting element in the COX-2 promoter region. Taken together, these results demonstrate that in addition to NF-κB...
and CREB, C/EBPβ, Ets-1, and AP-1 are necessary for the optimal induction of COX-2 promoter activity in response to CpG DNA.
DISCUSSION

Elevated levels of COX-2 and PGE2 have been observed in osteoarthritic and rheumatoid arthritic synovia and are associated with the disease process (59). Bacterial infection has been speculated to be involved in the pathogenesis of arthritis (1, 2). It has previously been demonstrated that CpG DNA, a pathogen associated molecular pattern (PAMP), induces expression of COX-2 that is responsible for CpG DNA-induced PGE2 release in the macrophage-like cell line RAW264.7 cells (14, 15). In the present study, we have investigated the molecular mechanisms by which CpG DNA induces COX-2 expression at the transcriptional level in RAW264.7 cells.

CpG DNA induces increased activity of the COX-2 promoter in a dose-dependent manner, indicating that CpG DNA-induced COX-2 expression is due, at least in part, to increased transcription of the COX-2 gene (Fig. 1A). This CpG DNA-mediated COX-2 transcription appears to utilize the TLR9/MyD88-signaling pathway. TLR9 involvement was shown indirectly by using a TLR9 antagonist, iCpG DNA (Fig. 1B). The recently identified iCpG DNA specifically blocks the effects of CpG DNA, including all proximal signaling events in B cells and macrophages such as activation of NF-κB and the MAPKs (56-58 and Yi, unpublished data). Although there is no formal demonstration that either CpG DNA or iCpG DNA directly binds to TLR9, and the precise molecular mechanism by which iCpG DNA blocks activity of CpG DNA has not been revealed, these previous studies suggest that iCpG DNA may compete with CpG DNA for binding to TLR9. Utilization of TLR9 by CpG DNA for induction of COX-2 gene transcription was also supported by demonstrating that chloroquine, an endosomal acidification inhibitor, completely blocked CpG DNA-induced COX-2 promoter activity (Fig. 1C). It has previously been demonstrated that endosomal acidification or
processing of CpG DNA precedes the CpG DNA-mediated generation of reactive oxygen species (ROS), MAPK activation, and NF-κB activation that lead to the expression of various oncogenes, costimulating factors, proinflammatory cytokines and chemokines (6, 14, 52, 55). Moreover, a recent study (17) has shown that CpG DNA and TLR9 are co-localized in an endosomal compartment and that TLR9 recruits MyD88 to an endosomal compartment upon CpG DNA stimulation. This MyD88 endosomal recruitment by TLR9 can be blocked by inhibitors of endosomal acidification or maturation, such as chloroquine and bafilomycin A. These studies suggest that an endosomal acidification inhibitor such as chloroquine abolishes the biologic effects of CpG DNA by inhibiting binding of CpG DNA to TLR9 and subsequent recruitment of MyD88 to TLR9.

TLRs recruit one or more adapter molecules that contain a TIR domain, such as MyD88, TIR-containing adapter protein (TIRAP), and TIR-containing adaptor molecule (TICAM)-1 (16, 60-62). TLR2 and TLR4 recruit MyD88 and TRIAP while TLR3 recruits MyD88 and TICAM-1 (60-62). To date, however, MyD88 is the only known adapter molecule that is recruited by TLR5, TLR7, and TLR9 (16, 60-62). Recent studies have demonstrated the absolute requirement of MyD88 for CpG DNA-mediated activation of NF-κB, JNK, and p38 and subsequent biologic effects including proinflammatory cytokine production and B cell proliferation (16, 18). Our results showed that transient overexpression of DN-MyD88 led to complete inhibition of CpG DNA-induced COX-2 promoter activity as well as CpG DNA-induced transcriptional activities of AP-1 and NF-κB (Fig. 3). These results indicate that like expression of proinflammatory cytokine genes, the induction of COX-2 expression by CpG DNA is also dependent on the recruitment of MyD88 to TLR9.
MyD88 bound to TLRs recruits IRAK-1 via a death domain-death domain interaction. IRAK-1 is activated at the receptor complex, becomes rapidly phosphorylated, and leaves the receptor complex to interact with the adapter molecule TRAF6 (63-66). CpG DNA has also been demonstrated to employ IRAK-1 and TRAF6 via a TLR9/MyD88-dependent mechanism (18, 21). Overexpression of DN-IRAK-1 inhibits CpG DNA-induced NF-κB activation in HEK293 cells when transiently co-transfected with mouse TLR9 (21). In contrast to this previously reported finding in HEK293 cells, transient overexpression of DN-IRAK-1 led to only partial inhibition of CpG DNA-induced COX-2 promoter activity and NF-κB transcriptional activity, while it completely inhibited CpG DNA-induced AP-1 transcriptional activity in RAW264.7 cells (Fig. 4). Our results indicate that IRAK-1 is required for the activation of AP-1 but additional signaling modulators other than IRAK-1 may function downstream of MyD88 to regulate NF-κB activation and COX-2 expression in RAW264.7 cells stimulated with CpG DNA. Fibroblast cells from IRAK-1 deficient mice show dramatically diminished, but not completely abolished, activation of JNK, p38, and NF-κB, and production of IL-6 and IFN-γ in respond to IL-1 or IL-18 (67, 68). In addition, deletion of IRAK-1 does not affect the ability of mice to develop delayed-type hypersensitivity or clear infection with the intracellular parasite, *Listeria monocytogenes* (68). This might be explained by redundancy in the IRAK family. Three additional IRAK family members, IRAK-2, IRAK-4 and IRAK-M, have been reported (19, 20, 63, 69). Indeed, IRAK-4-deficient mice are severely impaired in their response to IL-1, LPS, peptidoglycan, poly(I:C), and CpG DNA, indicating the indispensable role of IRAK-4 in IL-1 receptor and TLR signal transduction (20). Of note, the role of IRAK-2 in TLR9 signal transduction is currently unknown, and IRAK-M has been shown to negatively regulate TLR9 signaling (19). Together with our results, these findings indicate the presence of
an additional IRAK-1-independent pathway for IL-1, IL-18, and TLR signal transduction. Therefore, it is possible that multiple redundancies in the IRAKs and/or other interacting signaling modulators may participate in CpG DNA-induced NF-κB activation and COX-2 gene transcription.

In IL-1R and TLR pathways, TRAF6 forms a signaling complex with activated IRAKs, transforming growth factor-β-activated kinase (TAK)1, TAK-1 binding protein (TAB)1, TAB2, and evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) (70, 71). TRAF6 has been demonstrated to be required for IL-1- and LPS-induced JNK and NF-κB activation (72, 73). Overexpression of DN-TRAF6 has also been shown to inhibit CpG DNA-induced NF-κB activation in HEK293 cells when transiently co-transfected with mouse TLR9 (21). In addition, transient overexpression of DN-TRAF6 substantially inhibits CpG DNA-induced IκB phosphorylation and JNK activation and completely abolishes IL-12p40-promoter activity in RAW264.7 cells (18). Moreover, our results showed that CpG DNA failed to induce transcriptional activity of AP-1 when DN-TRAF6 was overexpressed in RAW264.7 cells (Fig. 5B). However, transient overexpression of DN-TRAF6 led to only partial inhibition of CpG DNA-induced COX-2 promoter activity and NF-κB transcriptional activity in RAW264.7 cells (Fig. 5). Our results indicate that TRAF6 is indispensable for the activation of AP-1 but additional signaling modulators may be necessary for NF-κB activation and COX-2 expression in RAW264.7 cells in response to CpG DNA stimulation. Thus, the TLR9/MyD88 signaling pathway may bifurcate at, or before, the level of TRAF6, leading to a divergent pathway regulating NF-κB and the COX-2 promoter.

Previous studies have demonstrated that depending on the type of cells, stimuli, and species, each MAPK has a different regulatory role in COX-2 gene transcription. JNK plays a
critical regulatory role in human COX-2 gene transcription in macrophages activated by Human herpesvirus 6 (HHV-6) (36) or LPS (35), and in murine MC3T3-E1 osteogenic cells stimulated with PGE2 or TNF-α plus IL-1β (42). In contrast, LPS-induced murine COX-2 promoter activity in RAW264.7 cells is dependent on ERK (43). Both ERK and JNK are required for COX-2 promoter activity induced by IgE receptor aggregation in mast cells (74). ERK1/2 and p38 play a role in regulating HSP60-induced expression of COX-2 in macrophages (74). Our results showed that of the three MAPKs, only p38, but not ERK and JNK, played a critical role in the CpG DNA-induced COX-2 promoter activity (Fig. 6). We previously have demonstrated that p38 contributes to CpG DNA-induced AP-1 and CREB activation (53, 54). In addition, our results demonstrated that overexpression of DN-CREB or mutation of the CRE site in the COX-2 promoter substantially inhibits the COX-2 promoter activity in response to CpG DNA (Figs. 7B and 7D). Taken together with the previous findings, our results indicate that CpG DNA-activated p38 may contribute to COX-2 transcription by leading to the activation of CREB that plays an important role in the COX-2 promoter activity.

In addition to the CREB binding site, the COX-2 gene contains binding sites for a number of important transcription factors, including C/EBP (-900/-895, -135/-130, and -90/-86), Ets-1 (-800/-794), NF-κB (-402/-392), and AP-1 (-65/-60). One or more of these cis-acting elements have been shown to be crucial for COX-2 transcription in response to various stimuli including LPS, phorbol ester, TNF-α, forskolin, follicle-stimulating hormone, hypoxia, and shear stress (30-44). The regulatory roles of these cis-acting elements in the COX-2 promoter depend on the type of cells, stimuli, and species. Although the role of NF-κB in LPS-mediated COX-2 promoter regulation in murine macrophages is controversial (35, 38, 39, 50), NF-κB plays a critical role in COX-2 expression induced by TNF-α, hypoxia, and lipoteichoic acid (32, 34, 45).
Likewise, our results demonstrated that NF-κB plays an indispensable role in the CpG DNA-induced COX-2 promoter activity. Overexpression of a super-suppressive IκB mutant (IκB-AA) or mutation of the NF-κB binding site in the COX-2 promoter resulted in substantial inhibition of COX-2 promoter activity in response to CpG DNA (Figs. 7A and 7Da). In addition, overexpression of both IκB-AA and DN-CREB completely suppressed CpG DNA-induced COX-2 promoter activity (Fig. 7C). Moreover, double mutation of both NF-κB and CRE cis-acting element consensus sites showed complete abrogation of COX-2 promoter activity in response to CpG DNA (Fig. 7Dc). These results demonstrate that the cooperation of these two transcription factors is required for the transcription of COX-2 induced by CpG DNA.

CpG DNA-inducible COX-2 promoter activity was impaired by deletion of a promoter region encompassing C/EBP site 3 (-900/-895) and the Ets-1 site (-800/-974) or by site directed mutation of the Ets-1 site, C/EBP site 2 (-135/-130), or C/EBP site 3 (-900/-895) (Figs. 8 and 9). These results indicate that in addition to CREB and NF-κB, C/EBPs (presumably C/EBPβ) and Ets-1 are also necessary for optimal induction of COX-2 promoter activity by CpG DNA. However, intact C/EBP and Ets-1 sites were insufficient to induce the COX-2 promoter in the absence of CREB and NFκB mediated effects (Figs. 7C and 7Dc). Thus, C/EBP and Ets-1 may serve more in an auxiliary capacity to augment promoter induction initiated by CREB and NFκB. Although there has been no previous demonstration that p38 activated by CpG DNA leads to the activation of C/EBPβ or Ets-1, it has been demonstrated that activation of p38 by various other stimuli leads to the activation of C/EBPs and Ets-1 (38, 75, 76). Taken together with previous findings, our results indicate that in addition to CREB activation, CpG DNA-activated p38 may contribute to COX-2 expression by leading to the activation of C/EBPs and Ets-1 transcription factors that play an important role in COX-2 promoter activity.
It has been demonstrated that AP-1 plays a crucial role in fluid shear stress-induced COX-2 promoter activity in osteoblastic MC3T3-E1 cells (44). Our results also showed that mutation in the AP-1 binding site in COX-2 promoter region resulted in substantial decreases in the COX-2 promoter activity in response to CpG DNA (Fig. 9g). On the other hand, our results also showed that inhibition of ERK or JNK activation using pharmacological inhibitors or overexpression of DN-MEK1 or DN-JNK1 completely abolished CpG DNA-mediated AP-1 activation yet failed to inhibit CpG DNA-induced COX-2 promoter activity (Fig. 6). These results suggest that perhaps factors other than the canonical AP-1 proteins may bind to and regulate the predicted AP-1 site in the COX-2 promoter, or that the AP-1 site mutation may interfere with regulatory elements adjacent to the AP-1 site, such as CRE, in the COX-2 promoter.

In summary, the present study demonstrates that CpG DNA induces COX-2 expression by transcriptional mechanisms. CpG DNA-mediated transcriptional regulation of COX-2 expression is dependent on endosomal acidification/processing of CpG DNA and a TLR9/MyD88-signaling pathway, which is completely inhibited by a TLR9 antagonist iCpG DNA or by overexpression of DN-MyD88. The MyD88 downstream effectors IRAK1 and TRAF6 only partially contribute to the transcriptional regulation of COX-2 expression induced by CpG DNA. Among the three MAPKs, only p38 (which in turn leads to CREB activation) positively regulates CpG DNA-induced COX-2 expression at the transcriptional level. Transcription factors NF-κB and CREB play an indispensable regulatory role in CpG DNA-induced COX-2 promoter activity. In addition, COX-2 promoter-luciferase reporters with alterations in predicted cis-acting transcriptional regulatory elements revealed that an Ets-1 and
two C/EBP binding sites are also necessary for optimal COX-2 expression in response to CpG DNA.
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FOOTNOTE

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Table 1. Sequences changed by Site-directed mutagenesis processing

| Element          | WT       | Mut        |
|------------------|----------|------------|
| C/EBP(3) (-900/-895) | TTTTATCA  | TTTTATCA   |
|                  | AA       | GG         |
| Ets-1 (-800/-794) | TACATTC   | GAACAGGG  |
|                  | CCGT     | CGT        |
| NF-κB (-402/-392) | GGGGATTC  | GGGGTGTAT  |
|                  | TCCC     | TCA        |
| C/EBP(2) (-135/-130) | TCTTGCG   | TGTCGTCG   |
|                  | AA       | GA         |
| C/EBP(1) (-90/-86) | TGGGGAAG  | CGGGTGAAG  |
|                  | AG       | AG         |
| AP-1 (-65/-60)    | ACAGAGT   | ACAGAGT    |
|                  | TCA      | TGC        |
| CREB(-60/-56)     | CACTACGT  | CACTGATTCA |
|                  | TCA      | TCA        |
### Table 2 The primers used for cloning and mutagenesis

| Forward / Reverse |  
|------------------|---
| **DN-IRAK-1**    | 5'-TACGTAATGGCCGGGGGCGG-3'  
|                  | 5'-TTCTTCAGAGAAGTTGCAAGCTTGGCGAACAT-3'  
| **P38**          | 5'-GAAGATGTCAGAGGAGGAAG-3'  
|                  | 5'-ACAGAAACCAGGTGCACA-3'  
| **DN-JNK1**      | 5'-GGAACGAGTTTTATGATGCGCCTTTCTGCTGAGATCGCTACTAC-3'  
|                  | 5'-GTAGTAGGCGAGTCACCAAGGCGCCATCATACGATCGTTCC-3'  
| **DN-p38**       | 5'-CACACTGATGATGAGATGCGAGCTTGGCTGGCTACAGGT-3'  
|                  | 5'-ACCTGGTAGCCACAGGCTGCTCCCTACTCATGTCGCTG-3'  
| **DN-MEK**       | 5'-GCCAGCTATTGACACGCCATGGCCACGCCCTGCTGGGGAAC-3'  
|                  | 5'-GTTCACGCGGCTGTCGCTGACAGCTGCCACCC-3'  


Figure legends

Figure 1. CpG DNA-dependent induction of COX-2 promoter activity. RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and COX-2 promoter-luciferase reporter. Transfected cells were pooled and washed 3 times with culture media. Cells were stimulated with various concentrations (0-12 µg/ml) of CpG DNA or non-CpG DNA for 12 hr (Panel A) or stimulated with medium, CpG DNA (6 µg/ml), or LPS (50 ng/ml) in the presence or absence of iCpG DNA (6 or 12 µg/ml) for 12 hr (Panel B). Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity (Renilla luciferase activity) in each sample. Data present the mean ± SD of triplicates. The experiment was done three times with similar results.

Figure 2. Endosomal acidification is required for the CpG DNA-mediated induction of COX-2 promoter activity. RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and COX-2 promoter-luciferase reporter. Transfected cells were pooled and washed 3 times with culture media. Cells were stimulated with medium, CpG DNA (6 µg/ml), or LPS (50 ng/ml) in the presence or absence of an inhibitor of endosomal acidification chloroquine (2.5 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity (Renilla luciferase activity) in each sample. Data present the mean ± SD of triplicates. The experiment was done three times with similar results.

Figure 3. CpG DNA induces COX-2 promoter activity through a MyD88-dependent signaling pathway. RAW264.7 cells were transiently co-transfected with empty vector or plasmids encoding DN-MyD88 and COX-2 promoter-luciferase reporter + pRL-TK-luciferase (for Panel A), AP-1-β galactosidase (for Panel B) or NF-κB-luciferase + pRL-TK-luciferase (for
Panel B). Transfected cells were washed and then incubated in complete media for 6 hr to allow expression of DN-MyD88. Cells were stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. AP1-β-galactosidase activities in cell extracts were analyzed using Galacto-Light Plus Reporter gene assay for β-galactosidase and normalized by equal concentrations of cell lysates used in each sample. Data present the mean ± SD of triplicates. The experiment was done three times with similar results.

**Figure 4. Overexpression of DN-IRAK1 substantially inhibits CpG DNA-mediated induction of COX-2 promoter activity.** RAW264.7 cells were transiently co-transfected with empty vector or plasmids encoding DN-IRAK1 and COX-2 promoter-luciferase reporter + pRL-TK-luciferase (for Panel A), AP-1-β galactosidase (for Panel B) or NF-κB-luciferase + pRL-TK-luciferase (for Panel B). Transfected cells were washed and incubated in complete media for 6 hr to allow expression of DN-IRAK1. Cells were stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. AP1-β-galactosidase activities in cell extracts were analyzed using Galacto-Light Plus Reporter gene assay for β-galactosidase and normalized by equal concentrations of cell lysates used in each sample. Data present the mean ± SD of triplicates. The experiment was done three times with similar results.

**Figure 5. Overexpression of DN-TRAF6 partially inhibits CpG DNA-mediated induction of COX-2 promoter activity.** RAW264.7 cells were transiently co-transfected with empty vector or plasmids encoding DN-TRAF6 and COX-2 promoter-luciferase reporter + pRL-TK-luciferase (for Panel A), AP-1-β galactosidase (for Panel B) or NF-κB-luciferase + pRL-TK-luciferase (for
Panel B). Transfected cells were washed and incubated in complete media for 6 hr to allow expression of DN-TRAF6. Cells were stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. AP1-β-galactosidase activities in cell extracts were analyzed using Galacto-Light Plus Reporter gene assay for β-galactosidase and normalized by equal concentrations of cell lysates used in each sample. Data present the mean ± SD of triplicates. The experiment was done three times with similar results.

Figure 6. p38 MAP kinase plays a critical role in the CpG DNA-mediated induction of COX-2 promoter activity. Panel A. RAW264.7 cells were co-transfected with COX-2 promoter-luciferase vector (1 µg) and pRL-TK-luciferase vector (0.5 µg). Transfected cells were pooled and washed 3 times with culture media. Cells were treated with DMSO, SB202190 (2.5 µM), U0126 (1.25 µM), or SP600125 (5 µM) for 15 min and then stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean ± SD of triplicates. The experiment was done three times with similar results. Panel B. RAW264.7 cells (2x10^6 cells/ml) were treated with DMSO or SP600125 (5 µM) for 15 min and then stimulated with medium or CpG DNA (6 µg/ml) for 45 min. Equal amounts of whole cell lysates (15 µg/lane) were subjected to SDS-PAGE and then western blots were performed using a specific antibody against the phosphorylated form of c-Jun (pc-Jun), JNK (pJNKp52 and pJNKp46), ERK (pERK1 and pERK2) or p38 (pp38). Actin was used as a loading control. The experiment was done three times with similar results. Panel C, D, and E. RAW264.7 cells were transiently co-transfected with empty vector or plasmids encoding DN-p38 (for Panel C), DN-MEK (for Panel D), or DN-JNK1 (for Panel E) and COX-2
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promoter-luciferase reporter + pRL-TK-luciferase or AP-1-β galactosidase. Transfected cells were washed and incubated in complete media for 6 hr to allow expression of DN-p38, DN-MEK, or DN-JNK1. Cells were stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. AP1-β-galactosidase activities in cell extracts were analyzed using Galacto-Light Plus Reporter gene assay for β-galactosidase and normalized by equal concentrations of cell lysates used in each sample. Data present the mean ± SD of triplicates. The experiment was done three times with similar results.

**Figure 7. NF-κB and CREB are required for the CpG DNA-induced COX-2 promoter activity. Panel A.** RAW264.7 cells were transiently co-transfected with empty vector or plasmids encoding super-suppressive IκB (IκB-AA) and COX-2 promoter-luciferase reporter + pRL-TK-luciferase or NF-κB-luciferase reporter + pRL-TK-luciferase. **Panel B.** RAW264.7 cells were transiently co-transfected with empty vector or plasmids encoding DN-CREB and COX-2 promoter-luciferase reporter + pRL-TK-luciferase or CREB-luciferase reporter + pRL-TK-luciferase. **Panel C.** RAW264.7 cells were transiently co-transfected with empty vector or plasmids encoding IκB-AA+DN-CREB and COX-2 promoter-luciferase reporter + pRL-TK-luciferase. Transfected cells (for Panel A, B, and C) were washed and incubated in complete media for 6 hr to allow expression of IκB-AA and/or DN-CREB. Cells were stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean ± SD of triplicates. The experiment was done three times with similar results. **Panel D.** RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and wild type COX-2 promoter-luciferase reporter, or COX-2 promoter-luciferase
reporter without NF-κB binding site (a), CREB binding site (b), or both NF-κB and CREB binding sites (c). NF-κB binding site and/or CREB binding sites were removed from the COX-2 promoter-luciferase reporter by mutating these sites using site-directed mutagenesis as described in the Experimental Procedures Section. Transfected cells were washed and stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (% of luciferase activity of wild type COX-2 promoter-luciferase reporter in the cells stimulated with CpG DNA) ± SD of triplicates. The experiment was done three times with similar results.

Figure 8. Identification of CpG DNA responsive regions in COX-2 promoter. RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and full length or 5’-deletion mutants (Δ-700, Δ-320, or Δ-52) COX-2 promoter-luciferase reporters. Transfected cells were washed and stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (% of luciferase activity of wild type COX-2 promoter-luciferase reporter in the cells stimulated with CpG DNA) ± SD of triplicates. The experiment was done three times with similar results.

Figure 9. The C/EBP, Ets-1, and AP-1 sites contribute to the optimal induction of COX-2 promoter activity by CpG DNA. RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and wild type COX-2 promoter-luciferase reporter (a), or COX-2 promoter-luciferase reporter without C/EBP-(3) (-900/-895; b), C/EBP-(2) (-135/-130; c), C/EBP-(1) (-90/-86; d), Ets-1 (-800/-794; e), C/EBP-(3) and Ets-1 (f), or AP-1 (-65/-60; g). Transfected cells were washed and stimulated with medium or CpG DNA (6 µg/ml) for 12 hr. Luciferase
activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (% of luciferase activity of wild type COX-2 promoter-luciferase reporter in the cells stimulated with CpG DNA) ± SD of triplicates. The experiment was done three times with similar results.
FIGURES

Fig. 1

A.

B.

Fig. 2
Fig. 3

A.

\[ \text{COX2 promoter-Luc (Fold)} \]

\[ \text{medium} \quad \text{CpG} \]

\[ \text{Empty vector} \quad \text{DN-MyD88} \]

B.

\[ \text{RLU (Fold)} \]

\[ \text{medium} \quad \text{CpG} \]

\[ \text{AP-1-b-galactosidase} \quad \text{NF-kB-luciferase} \]

\[ \text{Empty vector} \quad \text{DN-MyD88} \]

Fig. 4

A.

\[ \text{COX2 promoter-Luc (Fold)} \]

\[ \text{medium} \quad \text{CpG} \]

\[ \text{Empty vector} \quad \text{DN-IRAK-1} \]

B.

\[ \text{RLU (Fold)} \]

\[ \text{medium} \quad \text{CpG} \]

\[ \text{AP-1-b-galactosidase} \quad \text{NF-kB-luciferase} \]

\[ \text{Empty vector} \quad \text{DN-IRAK-1} \]
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Fig. 5

A.

B.

Fig. 6

A.

B.

C.

D.

E.
Fig. 7

A. Full length

B. Full length

C. Full length

D. Full length
Fig. 8

[Diagram showing transcription factors binding sites and COX2-promoter-Luc activity

Fig. 9

[Diagram showing various constructs and COX2-promoter-Luc activity]
Myeloid differentiation factor 88-dependent transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: Role of NF-κB and p38
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