Transcriptional Induction of Matrix Metalloproteinase-9 in the Chondrocyte and Synoviocyte Cells Is Regulated via a Novel Mechanism: Evidence for Functional Cooperation between Serum Amyloid A-Activating Factor-1 and AP-1

Alpana Ray, B. Sonny Bal and Bimal K. Ray

*J Immunol* 2005; 175:4039-4048; doi: 10.4049/jimmunol.175.6.4039

http://www.jimmunol.org/content/175/6/4039

References

This article cites 35 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/175/6/4039.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Transcriptional Induction of Matrix Metalloproteinase-9 in the Chondrocyte and Synoviocyte Cells Is Regulated via a Novel Mechanism: Evidence for Functional Cooperation between Serum Amyloid A-Activating Factor-1 and AP-1

Alpana Ray, B. Sonny Bal, and Bimal K. Ray

Increased expression of matrix metalloproteinase-9 (MMP-9) by IL-1β and TNF-α is regarded as a key factor in the degradation of cartilage during arthritis. However, the underlying molecular mechanism of this induction process especially in the cells of the joint capsule remains elusive. Chondrocytes and synoviocytes, the resident cells of joint capsule, markedly increase transcription of MMP-9 in response to IL-1β- and TNF-α-mediated stimulation. Using progressively deleted and mutant promoter constructs of MMP-9, we show that serum amyloid A-activating factor (SAF)-1, a novel transcription factor, and the AP-1 family of proteins cooperatively regulate cytokine-mediated induction of MMP-9 in the resident cells of the joint capsule. In the MMP-9 promoter, SAF-1 and AP-1 DNA-binding elements are present in close proximity with only 14 nucleotides apart. SAF-1 DNA-binding activity is increased in both cytokine-stimulated cells as well as in osteoarthritic cartilage tissues. Although overexpression of SAF-1 could increase expression of the MMP-9 promoter and endogenous MMP-9 gelatinolytic activity, for maximal induction of MMP-9 gene concurrent participation of SAF-1 and AP-1 is required. Mutation of either one of these two elements resulted in a severe reduction in cytokine responsiveness of MMP-9 promoter and compromised the transactivation potential of both SAF-1 and AP-1. Simultaneous requirement for two distinct DNA-binding elements suggests that SAF-1 and AP-1 function in a mutually beneficial manner acting as essential coactivators to drive cytokine-mediated transcriptional activation of MMP-9. The Journal of Immunology, 2005, 175: 4039–4048.

Matrix metalloproteinase (MMP)−1−9 is a member of the degrading MMP family. Overexpression of these proteins is linked with various pathological diseases that involve connective tissue destruction and breakdown of the extracellular matrix (ECM) (1, 2). MMP also plays a critical role in a number of physiological processes such as embryonic development, wound repair, endometriosis, and bone growth. There are over 20 members of this family, which are further grouped into five subfamilies according to their properties and substrate specificities. Because of powerful proteolytic ability, the activity of the MMP is regulated by several mechanisms among which control of gene expression is the primary mode of regulation. This action is followed by activation of proenzymes by proteolytic processing of the N-terminal inhibitory domain and inactivation of enzyme activity by naturally occurring tissue inhibitor of metalloproteinases. Under pathogenic condition dysregulation in the gene expression is shown to be the primary cause of excessive synthesis of this family of proteins.

MMP-9 has the ability to cleave a variety of ECM components including denatured collagen (gelatin) generated by thermal denaturation at body temperature after specific cleavage of triple helix region of fibrillar collagen molecules by collagenases, types I, IV, and XI collagen, aggrecan, elastin, and fibronectin (3, 4). This protein is shown to be present in excessive amounts in a number of pathologic conditions including metastasis of cancer, arthritis, atherosclerosis, and ulcerating corneas (5–9). In all of these conditions the cellular level of MMP-9 is significantly increased primarily via increased gene expression. Thus considerable focus is given to shed light on the mechanism of MMP-9 synthesis in the diseased state.

MMP-9 is synthesized by a variety of cell types, but due to its extensive proteolytic ability, normal expression of MMP-9 is very low and tightly regulated. The expression of pro-MMP-9 in most cells can be induced by cytokines like IL-1β, TNF-α, tumor promoting agent, TGF-β growth factor, LPS, oncogenes, and the agents that stimulate cell migration (10–14). After secretion into the ECM, the activity of MMP-9 is further regulated by its conversion to the processed active form and inactivation by specific tissue inhibitor of metalloproteinase. Most of the mechanistic studies available on understanding induction of MMP-9 are concentrated on its activation mechanism under oncogenic condition and oncprotein mediated malignant transformation (15–17). The proximal promoter of MMP-9 is highly conserved and shown to contain multiple functional elements including a proximal AP-1 element, which is a hallmark of many MMP promoters, and NF-κB, PEA3/ets, Sp1, and a GT box element (15, 16). Three motifs, AP-1, NF-κB, and Sp1 are shown to contribute positively to the induction of MMP-9 expression in response to tumor promoting
agent and TNF-α cytokine in osteosarcoma and HT1080 fibrosarcoma cells (15). In contrast, AP-1 and the GT box element were shown to be necessary for v-Src mediated activation of MMP-9 in HT1080 fibrosarcoma cells (16). The stimulation of MMP-9 by ras oncogene in OVCAR-3 malignant ovarian cells is shown to be mediated through a MEK1-independent signaling pathway and require multiple transcription factor binding elements (17). Transcriptional up-regulation of MMP-9 during spontaneous epithelial (S) to neuroblast (N) conversion by SK-N-SH neuroblastoma cells involved in enhanced invasivity is shown to be dependent on GT mediated through a MEK1-independent signaling pathway and regulated in HT1080 fibrosarcoma cells (18). The stimulation of MMP-9 by ras has been shown to be necessary for v-Src mediated activation of MMP-9 in HT1080 fibrosarcoma cells (15). In contrast, AP-1 and the GT box element were shown to be necessary for v-Src mediated activation of MMP-9 in HT1080 fibrosarcoma cells (15). In contrast, AP-1 and the GT box element were demonstrated (15). These plasmids were a gift of Dr. M. Seiki (Institute of Medical Science, University of Tokyo, Tokyo, Japan) and obtained from Dr. D. Boyd (MD Anderson Cancer Center, Houston, TX). Progressive deletion constructs of 670wtMMP-9 promoter were synthesized by PCR amplification using different upstream primers starting at positions 570-, 400-, 241-, 190-, and 20- and a single downstream primer ending at position +54 of MMP-9. Amplified DNA molecules were ligated to pBLCAT3 vector DNA. The pcDSAF-1 (27), pcD-Fos, pcD-Cjun, pcDNF-κB, and pcDSP1 expression plasmids were prepared by inserting a full length cDNAs of each transcription factor under the control of CMV promoter in pcDNA3 vector (In vitrogen Life Technologies). The mutant SAF-1 expression plasmid pcDSAF-1(mt) was constructed by inserting an out-of-frame SAF-1 cDNA that does not produce any SAF-1 protein.

**Materials and Methods**

**Cartilage specimens**

Articular cartilage specimens were collected from the hip and joints of five dogs (one each of Australian shepherd, Labrador retriever, hound mix, Labrador, and a mixed breed), whose age and body weight ranged from 2 to 7 years and 23 to 31 kg, respectively. All of these animals developed spontaneous OA, and were undergoing orthopaedic surgery at the University of Missouri Veterinary Medical Teaching Hospital (Columbia, MO). Normal cartilage specimens were obtained from three cadavers. All procedures were approved by the Animal Care and Use Committee. Severity of the osteoarthritic changes in all cartilage specimens was graded using the Outerbridge System (28) and verified by histologic examination.

**Cell lines**

Chondrocyte cell HTB-94 (SW1353) was derived from a primary grade II chondrosarcoma of the right humerus of a patient. Rabbit synovial fibroblast cell, HIG-82 (CRL 1832) was derived from rabbit joint tissue. These cells were obtained from American Type Culture Collection and grown in DMEM containing a high concentration of glucose (4.5 g/L) and supplemented with 7% FBS. For the collection of conditioned medium, cells were changed to serum-free medium and 48 h later the conditioned medium was collected.

**Transfection analysis**

HTB-94 and HIG-82 cells were transfected by a calcium phosphate method as described (29). Briefly, cells were seeded at 50% confluency and 24 h later, the cells were transfected by adding chloroform-acetonitrile (CAT) reporter plasmid DNA together with pSVβ-galactosidase (Promega) plasmid DNA. The pSVβ-galactosidase DNA was used to monitor the efficiency of transfection and to normalize the cell extracts used for CAT assay. In some cotransfection reactions, expression plasmid DNA for SAF-1, c-Fos, c-Jun, NF-κB, Sp1, or revSAF-1 was included. After 16 h incubation with DNA, cells were trypsinized and fresh growth medium was added. In some reactions, the cells were stimulated with human IL-1β (1000 U/ml), which is equivalent to 5 ng/ml or TNF-α (40 pg/ml). The cells were harvested 24 h later and CAT activity was measured as described (29). β-Galactosidase activity was assayed with the substrate ONPG (o-nitrophenyl-β-D-galactopyranoside) as described (29). Cell extracts containing equivalent amount of β-galactosidase activity were used for CAT assay. Before the CAT assay, each cell extract was heated at 60°C for 10 min to inactivate nonspecific acetylase activity. Different amounts of cDNA used in the transfection assay had no effect on β-galactosidase expression. All transfections were performed in duplicate and the experiments were repeated at least three times. The data for CAT activity assays represent the mean and SE of the mean derived from three independent experiments.

**Plasmid constructs**

The 670wtMMP-9CAT, 670Sp1mutMMP-9CAT, 670NF-κBmutMMP-9CAT, 670AP1mutMMP-9CAT, and 670GTmutMMP-9CAT constructs have been previously described (15). These plasmids were a gift of Dr. M. Seiki (Institute of Medical Science, University of Tokyo, Tokyo, Japan) and obtained from Dr. D. Boyd (MD Anderson Cancer Center, Houston, TX). Progressive deletion constructs of 670wtMMP-9 promoter were synthesized by PCR amplification using different upstream primers starting at positions 570-, 400-, 241-, 190-, and 20- and a single downstream primer ending at position +54 of MMP-9. Amplified DNA molecules were ligated to pBLCAT3 vector DNA. The pcDSAF-1 (27), pcD-Fos, pcD-Cjun, pcDNF-κB, and pcDSP1 expression plasmids were prepared by inserting a full length cDNAs of each transcription factor under the control of CMV promoter in pcDNA3 vector (In vitrogen Life Technologies). The mutant SAF-1 expression plasmid pcDSAF-1(mt) was constructed by inserting an out-of-frame SAF-1 cDNA that does not produce any SAF-1 protein.

**Nuclear extracts and EMSA**

Nuclear extract from normal and osteoarthritic cartilage tissues, HTB-94, and HIG-82 cells were obtained as described earlier (27). For EMSA, MMP-9 probe was prepared by radiolabeling a double-stranded MMP-9 oligonucleotide containing nucleotides from position −71 to −39, which has the following sequence: 5′-ACCTTCGCCTGTCAGGAGGGGGCGTTACAGG-3′. Labeling of DNA was performed by filling in the overhangs at the termini with Klenow fragment of DNA polymerase, incorporating [α-32P]dCTP as a probe. EMSAs were performed following a standard protocol described earlier (27). In some binding assays, competitor oligonucleotides were included in the reaction mixtures. Competitor oligonucleotides used were: SAF-1 oligonucleotide 5′-CCCTCCTCCCTCCAGCACGAGCTCCATGG-3′, Sp1 oligonucleotide 5′-GGGGATTCTGGAAA-3′, GT box oligonucleotide 5′-GGAGGAGGTGGGGTGC-3′, and nonspecific oligonucleotide 5′-TCGAACTCGAATTCAGACGAGCTATGTT-3′. For Ab interaction studies, anti-SAF-1 or anti-Sp1 Ab (1 μl of a 10-fold diluted Ab) was added to the binding reaction mixture during a preincubation for 30 min on ice. Anti-Sp1 Ab was obtained from Santa Cruz Biotechnology, and a polyclonal anti-SAF-1 Ab was developed in rabbit using, purified mouse SAF-1, which was further purified by ammonium sulfate fractionation and affinity chromatography using protein G-agarose. This anti-SAF-1 Ab recognizes multiple epitopes of full-length SAF-1 and does not cross-react with other known proteins.

**Quantitation of MMP-9 mRNA**

Total RNA prepared from normal and osteoarthritic canine cartilage tissues was amplified by RT-PCR using a kit from Invitrogen Life Technologies. MMP-9 primers used for PCR amplification were 5′-GGCTGCAAA GAGACTTTTCTTC-3′ (forward) and 5′-CAAGGACGAGCTAGAC CACTTCG-3′ (reverse). MMP-9 forward primer is present in exon 10 whereas MMP-9 reverse primer is present in exon 13. Primer sequences for β-actin were 5′-GGGATCGGCGGCAAGAAGACTCC-3′ (forward) and 5′-AGGACGACGTCGATGACCAC-3′ (reverse). The amplified products using MMP-9 and β-actin primers were 380 and 285 nucleotides, respectively. Reaction mixtures were first denatured at 94°C for 1 min. The PCR condition was 94°C for 30 s, 55°C 30 s, 72°C 45 s for 20 cycles. To ensure that amplification was in the linear range, PCR was terminated at 20 cycles. PCR products were resolved by 1.4% agarose gel electrophoresis.
Equal protein (20 µg) of synovial fluid collected from normal and osteoarthritic dogs or conditioned medium from cultured cells were fractionated in a 15% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, SDS was removed by dialysis with 2.5% Triton X-100 buffer and incubated in 50 mM Tris-HCl, pH 7.5, 10 mM CaCl2, 1 mM ZnCl2, 1.25% Triton X-100 buffer for 16 h at 37°C. The gels were stained with Coomassie blue.

**Immunohistochemical analysis**

Immunohistochemical staining was performed using anti-MMP-9 Ab (Sigma-Aldrich) and anti-SAF-1 rabbit IgG, prepared as described above, as primary Ab. HRP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) was used as the secondary Ab. As control preimmune rabbit IgG was used. Tissue sections were cut 5 µm thick, deparaffinized in xylene, and rehydrated in graded ethanol solutions followed by washing with a buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl). Endogenous peroxidase activity was quenched by immersion in 3% H2O2 in methanol for 20 min followed by rinses in buffer. The slides were then incubated in 0.1% trypsin using a chemiluminescence detection kit (Amersham Biosciences). Bands were detected by blotting was performed using 1/1000 dilution of anti-MMP-9 (Sigma-Aldrich) and anti-SAF-1 rabbit IgG, prepared as described above. Slides were incubated overnight at 4°C with either anti-SAF-1 IgG or preimmune IgG as a control at a concentration of 1.0 µg/ml. The slides were rinsed twice in washing buffer (50 mM Tris-HCl, (pH 7.5), 0.15 M NaCl, 0.05% Tween 20), and then incubated with secondary Ab. Bound primary Ab was detected using a HRP method with substrate-chromogen solution. Sections were counterstained with Mayer’s hematoxylin solution.

**Western immunoblot analysis**

Cell extracts (50 µg protein) or conditioned media were fractionated in a 5/11% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. For evaluation of the relative amount of proteins in each lane, proteins were stained with Ponceau S solution (Sigma-Aldrich). Immunoblotting was performed using 1/1000 dilution of anti-MMP-9 (Sigma-Aldrich), or anti-c-Fos or anti-c-Jun (both from Santa Cruz Biotechnology) and anti-SAF-1 Ab (prepared as described above). Bands were detected by using a chemiluminescence detection kit (Amersham Biosciences).

**Results**

**MMP-9 level in cartilage and synovial fluids of osteoarthritic joints**

Immunohistochemical analysis of articular cartilage tissue from knee joint of dogs with and without OA indicated that MMP-9 protein level is almost undetectable in the normal cartilage tissue but is highly increased in osteoarthritic cartilage (Fig. 1A). To determine the level of MMP-9 proteolytic activity we used gelatin zymography. In this assay we compared the gelatin breakdown ability of MMP-9 in the synovial fluid of normal and osteoarthritic dogs. Compared with normal joint synovial fluid (Fig. 1B, lane 1), synovial fluid of dogs with OA (Fig. 1B, lane 2) contains much higher levels of 92- and 88-kDa MMP-9 gelatinases. In contrast, there was no change in the MMP-2 gelatinase activity migrating at 72 kDa position. These findings indicated that MMP-9 is differentially expressed during the disease. To examine whether increased gelatinolytic activity of MMP-9 is due to a corresponding increase in the MMP-9 protein level, Western blot analysis was performed. Results shown in Fig. 1C revealed that although normal canine synovial fluid contains almost undetectable levels of MMP-9 protein, synovial fluid of dogs with OA contains this protein at an appreciable level. Densitometric analysis of the Western blot data indicated that MMP-9 protein level is significantly higher in dogs with OA compared with levels of normal animals (Fig. 1D). Next, limited-cycle RT-PCR using RNA isolated from canine normal and osteoarthritic cartilage tissues was performed to determine whether increase of MMP-9 protein is a consequence of increased MMP-9 mRNA level (Fig. 1E). Absence of any amplified product (Fig. 1E, lane 1) indicated that MMP-9 mRNA level is very low in normal cartilage. However, its level is substantially increased in the osteoarthritic cartilage (Fig. 1E, lane 2).

**IL-1β and TNF-α responsive elements of MMP-9 in chondrocyte and synoviocyte cells**

TNF-α and IL-1β, which are present at high levels in arthritic joints, are regarded as major cytokines that mediate increased gene expression in arthritis. At present little is known on the induction mechanism of TNF-α- and IL-1β-mediated MMP-9 expression in the resident cells of joint capsule. We used a series of CAT reporter constructs containing progressively deleted MMP-9 promoter DNA (Fig. 2A) to understand the regulatory mechanisms governing TNF-α- and IL-1β-mediated induction of MMP-9 in HTB-94 chondrocyte and HIG-82 synovial cells. The longest reporter, containing MMP-9 promoter sequence from nucleotide position −670 to +54, was strongly stimulated by both IL-1β and TNF-α (Fig. 2B). The promoter containing sequences from −570 to +54 of MMP-9 exhibited moderate decrease in both basal level of transcription as well as in the level of cytokine stimulation. Deletion of sequences up to nucleotide position −400 showed small but noticeable further reduction of cytokine responsiveness compared with that of −570/+54 reporter. With additional deletion of sequences up to nucleotide position −90, 2- to 3-fold stimulatory response to both cytokines was consistently observed. However, when the promoter sequences were deleted up to nucleotide position −20, there was a precipitous drop in the cytokine responsiveness, suggesting the presence of critical cytokine-responsive elements within nucleotide position −90 and −20. A
similar response of the cytokines was also observed when HIG-82 synoviocyte cells were used, indicating that both cell types use the same promoter elements for cytokine induction of MMP-9 (Fig. 2C). It was, however, noticeable that the basal transcriptional activity of HIG-82 cells is substantially higher than that of HTB-94 cells. This effect could be due to a potential difference in the endogenous pool of transcriptional machinery among these two cell types. Together, these results indicated the presence of multiple regulatory elements, mapping to positions -670 to -570, -570 to -400, and -90 to -20 of the MMP-9 promoter, are involved in regulating IL-1β- and TNF-α-mediated induction in chondrocyte and synoviocyte cells. Comparison of sequences at these three regions indicated the presence of one consensus NF-κB binding site between -600 and -590 (16), one Sp1 binding site between -562 and -555 (16), one AP-1 binding site between -79 and -73 (16), and a GT box (15) homologous to the so-called retinoblastoma control element (30) between -57 and -45.

Mutational analysis revealed that AP-1, GT box, and NF-κB elements mediate IL-1β and TNF-α response

To determine the contribution of NF-κB, Sp1, AP-1, and GT box elements in the context of surrounding sequences, we constructed
single site mutant reporters containing specific mutation in the designated region. The response of these mutant reporters to TNF-α and IL-1β stimulation was monitored by transfection in HTB-94 and HIG-82 cells (Fig. 3). Mutation of the NF-κB element considerably reduced both the basal transcription and the inductive effect of IL-1β and TNF-α. However, the stimulatory response was not totally abrogated suggesting that downstream elements could also be involved in promoter activation. In contrast, mutation of either AP-1 or GT box element virtually eliminated response to both cytokines and to the basal transcriptional activity. The response of Sp1 site mutation showed considerably less effect on MMP-9 transcription. These results suggested that IL-1β- or TNF-α-mediated stimulation of MMP-9 is primarily regulated by AP-1, GT box, and NF-κB elements.

Identification of a GT box binding protein in cytokine-stimulated cells and osteoarthritic cartilage extracts

The GT box sequence in the MMP-9 promoter is highly conserved, except that GGGTGGGG sequence in the mouse and rat is replaced by GGGGAGGGG in the human. As our studies indicated a regulatory role of the GT box sequence in mediating cytokine stimulation, we examined whether any nuclear protein interacts with this region of MMP-9. To determine this effect, DNA-binding assay was performed using MMP-9 promoter region spanning nucleotide position −71 to −39 that encompasses the GT box element (−57/−45) as a probe. Nuclear extracts prepared from untreated, TNF-α- and IL-1β-stimulated HIG-82 cells were used. As seen in Fig. 4A, lane 1, proteins of untreated cell nuclear extract formed two faint DNA-protein complexes, termed as complex “b” and complex “c”. There was marked increase in the intensity of these two complexes when equal protein amounts of nuclear extract of IL-1β-stimulated (Fig. 4A, lane 2) or TNF-α-stimulated (Fig. 4A, lane 3) cells were used. In addition another faint DNA-protein complex termed as “a” was seen to be formed by cytokine-treated cells. Formation of these complexes was inhibited by the GT box binding oligonucleotide (Fig. 4A, lanes 6 and 7) but not by the nonspecific oligonucleotide (Fig. 4A, lanes 4 and 5). These data indicated that cytokine-stimulated synoviocyte cells contain GT box binding proteins. Nuclear extracts prepared from cytokine-stimulated HTB-94 chondrocyte cells also exhibited the presence of similar proteins (data not shown).

We next investigated whether osteoarthritic cartilage contains GT box binding nuclear protein. One faint DNA-protein complex, complex 1 as seen in Fig. 4B, lane 1, was formed with normal cartilage nuclear extract but osteoarthritic cartilage nuclear extract formed two highly prominent DNA-protein complexes, complex 1 and complex 2 (Fig. 4B, lane 2). Both complexes 1 and 2 were inhibited by the addition of GT box oligonucleotide (Fig. 4B, lane 3) but not by nonspecific oligonucleotide (Fig. 4B, lane 4). Comparison of the migration pattern of DNA-protein complexes formed by HIG-82 cells and cartilage tissue extract indicated that complex 1 comigrates with the complexes b and c, whereas complex 1 comigrates with complex a. Occasionally, complex 2 is seen to be migrated as two very closely spaced complexes (data not shown). These results clearly indicated that stimulation of a DNA-binding activity interacting with GT box regulatory element of MMP-9 occurs during cytokine stimulation and under osteoarthritic condition in the cartilage.

Characterization of the GT box binding protein

Because the GGGTGGGG sequence could be potential binding element of at least two transcription factors, SAF-1 (27) and Sp1 (31), we examined which of these proteins might interact with the GT box. Competitor oligonucleotides containing SAF-1 and Sp1 binding sequences and specific Abs to SAF-1 and Sp1 were used in the DNA-binding assay using TNF-α-stimulated HIG-82 (Fig. 5, lanes 1–4) and osteoarthritic cartilage (Fig. 5, lanes 5–8) nuclear extracts. No change in the complex formation was seen with Sp1 oligonucleotide (Fig. 5, lanes 1 and 5) or Sp1 Ab (Fig. 5, lanes 2 and 6), which indicated that Sp1 is not involved in the formation of any of these complexes. SAF-1 oligonucleotide inhibited complex b, complex c (Fig. 5, lane 3), and complex 2 (Fig. 5, lane 7). Further, complexes b and c (Fig. 5, lane 4) and complex 2 (Fig. 5, lane 8) were supershifted by SAF-1 Ab. Complex a (Fig. 5, lanes 1–4) and complex 1 (Fig. 5, lanes 5–8) remained unaffected by all of these agents suggesting that their formation involves neither Sp1 nor SAF-1 binding. Using Abs to a number of different transcription factors in the DNA-binding assay, we were unable to detect any ablation/supershift of this DNA-protein complex (data not shown). Thus the identity of the protein involved in the formation of complex a and complex 1 still remains unknown. Similar results were obtained when IL-1β-stimulated nuclear extract was used in the DNA-binding assay (data not shown). Together these results suggested that induction of SAF-1 activity in cytokine-treated cells and in osteoarthritic cartilages results in its enhanced interaction with the GT box element of MMP-9. This finding raises the question whether increased binding of SAF-1 is responsible for the cytokine-mediated induction of MMP-9 expression because GT box has been found to be an essential promoter element for MMP-9 expression in response to cytokine action (Fig. 3).

SAF-1 induces MMP-9 expression in chondrocyte and synoviocyte cells

To assess the role of SAF-1 in MMP-9 expression, this transcription factor was ectopically expressed in both HTB-94 and HIG-82
SAF-1 is responsible for cytokine-mediated activation of MMP-9

Activation of MMP-9 promoter by SAF-1 suggested that SAF-1 might also activate endogenous MMP-9 expression. To test, conditioned medium of SAF-1 and mutant SAF-1-overexpressing cells were examined for MMP-9-specific gelatinolytic activity (Fig. 7). Untreated HTB-94 cells showed virtually no MMP-9 but high level of MMP-2-specific gelatinolytic activity (Fig. 7A, lane 1). However, in the SAF-1-transfected cells, marked increase of 92-kDa MMP-9 gelatinase activity was observed (Fig. 7A, lane 2). Similar increase in the MMP-9 activity was also evident in the SAF-1-transfected HIG-82 cells (Fig. 7A, compare lanes 8 and 9). In HIG-82 cells, the active MMP-9 band migrating at 88 kDa position was also quite apparent (Fig. 7A, lane 9). In contrast to this finding, overexpression of mutant SAF-1 did not stimulate MMP-9 expression (Fig. 7A, lanes 3 and 10), indicating the specificity of SAF-1 action. As a positive control, equivalent amount of conditioned medium of TNF-α (Fig. 7A, lanes 4 and 11) and IL-1β (Fig. 7A, lanes 5 and 12) cells were tested. Considering that only ~30% cells are usually transfected, it was assuring to note that SAF-1-transfected cells produced almost same level of induction as that obtained with these two proinflammatory cytokines. When the cells were transfected with pCMV-revSAF-1 plasmid there was considerable loss of the stimulatory effects of the cytokines (Fig. 7A, compare lanes 6, 7, 13, and 14 with lanes 4, 5, 11, and 12, respectively). Western immunoblot analysis (Fig. 7B) further indicated that the protein level of MMP-9 in both chondrocyte (HTB-94) and synoviocyte (HIG-82) cells is increased by SAF-1 whereas MMP-2 protein level remains the same. Together, these results suggested that SAF-1 stimulates MMP-9 expression in both chondrocyte and synoviocyte cells.

Synergistic transactivation of MMP-9 promoter by SAF-1 and AP-1

Results of Fig. 3 have shown that mutation of either the SAF-1 binding GT box element (~57/~45) or the adjacently located AP-1 (~79/~73) element severely inhibits cytokine-mediated stimulation of MMP-9 expression. This finding suggested that these two transcription factors may function in a mutually beneficial manner. Increasing evidence suggests that control of transcription of mammalian gene is complex and involves participation of a number of transcription factors. Under diseased condition, multiple inflammation responsive transcription factors are activated and exert their combined effect in modulating expression of the candidate gene. NFκB (~599/~590) and Sp1 (~562/~557) elements, which are present distally from AP-1 and SAF-1.

FIGURE 5. SAF-1 transcription factor interacts with GT box element of MMP-9 promoter. Nuclear extracts (NE), 10 μg, from TNF-α-treated HIG-82 cells (lanes 1–4) and osteoarthritic canine cartilage tissues (lanes 5–8) were incubated with a 32P-labeled MMP-9 (~70/–10) DNA. In addition, 100-fold molar excess of Sp1 oligonucleotide (lanes 1 and 5), 100-fold molar excess of SAF-1 oligonucleotide (lanes 3 and 7), anti-Sp1 Ab (lanes 2 and 6), and anti-SAF-1 Ab (lanes 4 and 8) were included as indicated. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel. Arrow indicates supershifted DNA-protein complex.

FIGURE 6. Dose-dependent stimulation of MMP-9 promoter by ectopic expression of SAF-1. A, HTB-94 chondrocyte cells were transfected with 1.0 μg of 670MMP-9CAT or 670GmutMMP-9CAT reporter plasmid either alone or with increasing concentrations (0.1, 0.2, and 0.4 μg) of pcDSAF-1 or pcDSAF-1(mt) expression plasmid DNA. B, HIG-82 synoviocyte cells were cotransfected with 1.0 μg of either 670MMP-9CAT or 670GmutMMP-9CAT reporter plasmid alone or with increasing concentrations (0.1, 0.2, and 0.4 μg) of pcDSAF-1 or pcDSAF-1(mt) expression plasmid DNA. Fold induction of the CAT activity in the cotransfected cells relative to that of reporter plasmid alone was determined and plotted as relative CAT activity. The result represents mean and SE of the mean derived from three independent experiments. Presence and absence of a specific plasmid are denoted by “+” and “−”, respectively.
showed moderate effect on MMP-9 stimulation. Although MMP-9 promoter contains two additional distally located AP-1 elements, only the proximal AP-1 site (−79/−73) was shown to be regulatory (16). This earlier finding suggests potential cross-talk between this AP-1 site and the neighboring GT box element, which is now found to be the binding site of SAF-1. To test whether simultaneous presence of these transcription factors modulates MMP-9 transcription, we examined the effect of coexpression of these proteins in the cells (Fig. 8). Interestingly, coexpression of SAF-1 and AP-1 family members demonstrated a synergistic level of induction that was much higher than the additive value when these two groups of proteins were expressed separately. In contrast, coexpression of SAF-1 and NF-κB or AP-1 and NF-κB showed a less than additive level of activation, and coexpression of SAF-1 and Sp1 showed slight but consistent antagonistic effect (Fig. 8B).

**Mutation of either AP-1 or SAF-1 element in the MMP-9 promoter severely abrogates mutual responsiveness**

The synergy between SAF-1 and AP-1 family members on MMP-9 transcription was further studied by two independent sets of experiments. In the first set, HTB-94 cells were cotransfected with the CAT reporter and increasing amounts of either SAF-1 or c-Jun expression plasmids or a combination of both SAF-1 and c-Fos plus c-Jun expression plasmids at different concentrations (Fig. 9A). Interestingly, simultaneous presence of SAF-1 and AP-1 proteins stimulated MMP-9 promoter in a synergistic dose-dependent manner. Western blot analysis indicated dose-dependent expression of SAF-1, c-Jun, and c-Fos proteins in transfected cells (Fig. 9B). In the second set of experiments, we used three different CAT reporter DNA (wild-type MMP-9CAT, AP-1 mutant MMP-9CAT, and GT box mutMMP-9CAT as described in Fig. 3) to transfect HTB-94 cells alone or in combination of each amount of SAF-1 and c-Fos plus c-Jun expression plasmids (Fig. 9C). Although SAF-1 and c-Fos plus c-Jun plasmids were individually able to activate wild-type MMP-9 promoter and together they acted in a synergistic fashion, mutation of any one of these two elements not only inhibited transactivation potential of the respective transcription factor but also inhibited the stimulatory effect of the other protein whose binding site was not mutated. Western blot analysis was performed, which verified that none of these results were due to any variation of the expression of SAF-1 and c-Jun plus c-Fos proteins in transfected cells (data not shown).

Together, these results suggested that participation of both SAF-1 and AP-1 and their respective binding elements are necessary for induction of MMP-9. Transcriptional induction of MMP-9 from ectopic expression of only SAF-1 or c-Jun plus c-Fos proteins is most likely due to the use of the limited endogenous pool of protein that acts in synergy with the ectopically expressed partner. In the event of mutation of either the SAF-1 or AP-1 element, the
inability of endogenous protein to interact with the MMP-9 promoter results in the loss of synergy.

MMP-9, SAF-1, c-Jun, and c-Fos are present at high levels in osteoarthritic cartilage

In light of the involvement of SAF-1 and AP-1 transcription factors in the induction of MMP-9 in osteoarthritic cartilage, one begs the question about the level of these proteins in the diseased tissue. Therefore to assess these levels, immunohistochemical analysis of cartilage tissue was performed (Fig. 10). This experiment indicated that expression pattern of SAF-1, c-Jun, and c-Fos is similar to that of MMP-9. The levels of these proteins are much higher in osteoarthritic cartilage compared with normal cartilage. Together these results indicate that increased activity of SAF-1 and AP-1 and their cooperative action gives rise to the induction of MMP-9 in the osteoarthritic cartilage.

**FIGURE 9.** SAF-1- or AP-1-mediated transactivation of MMP-9 requires both elements. A. HTB-94 chondrocyte cells were transfected with (+) 1.0 μg of 670MMP-9CAT reporter DNA and increasing concentrations (0.1, 0.2, and 0.4 μg) of pcDSAF-1 or pcDe-Fos plus pcDe-Jun (c-Fos+c-Jun) expression plasmids, as indicated. In addition, some transfection assays contained constant amounts (0.5 μg) of either pcDSAF-1 or pcDe-Fos plus pcDe-Jun expression plasmids, as indicated. CAT activity was measured as described in Materials and Methods and fold induction of the CAT activity in the cotransfected cells relative to that of reporter plasmid alone was determined and plotted as relative CAT activity. The result represents mean and SE of the mean derived from three independent experiments. B. Fifty micrograms of protein per lane from the cells transfected with increasing concentrations (0.1, 0.2, and 0.4 μg) of pcDSAF-1 or pcDe-Fos plus pcDe-Jun expression plasmids were subjected to Western blot analysis using anti-SAF-1, anti-c-Jun, and anti-c-Fos Abs, as indicated. C. Three reporter plasmids carrying either 670 bp of wild-type MMP-9 promoter sequences (wt MMP-9CAT), or specific mutation at AP-1 site (AP-1 mutMMP-9CAT) or specific mutation at SAF-1 site (SAF-1 mutMMP-9CAT) were cotransfected with 0.5 μg of either pcDSAF-1 or pcDe-Fos plus pcDe-Jun (c-Fos+c-Jun) expression plasmids, alone, or in combination, as indicated. Relative CAT activity was determined and plotted as described in A. The result represents mean and SE of the mean derived from three independent experiments. Presence and absence of a specific plasmid are denoted by “+” and “−,” respectively.

**FIGURE 10.** Increased levels of MMP-9, SAF-1, and AP-1 in osteoarthritic cartilage tissue. Immunohistochemical analysis of age-matched canine normal and osteoarthritic cartilage tissue sections was performed as described in Materials and Methods. Serial sections were immunostained with anti-MMP-9 Ab (a and a’) at magnification ×100; anti-SAF-1 Ab (b and b’) at magnification ×100; anti-c-Fos Ab (c and c’) at magnification ×100; and anti-c-Jun Ab (d and d’) at magnification ×100.

**Discussion**

We have identified a novel mechanism that delineates induction of MMP-9 by cytokines in the resident cells of joint capsule and show that MMP-9 is a target of and regulated by transcription factor SAF-1. This is evident by 1) demonstration of SAF-1 binding element at the conserved proximal promoter region of MMP-9 and specific interaction of SAF-1 to the site, 2) increased levels of SAF-1 DNA-binding activity in osteoarthritic nuclear extract, 3) SAF-1-dependent activation of MMP-9 promoter and endogenous MMP-9 gene, and 4) increase of SAF-1 in osteoarthritic cartilage. Further, we provide evidence of transcriptional cooperativity between AP-1 and SAF-1 in maximal stimulation of MMP-9 expression. Mutation of either element alone in the MMP-9 promoter severely diminished cytokine responsiveness, suggesting that the two elements have a mutually
dependent and a cooperative relationship. Interdependency of these regulatory transcription factors in the expression of MMP-9 could have pathophysiological significance in the manifestation of arthritis.

Although increased MMP-9 proteolytic activity due to induction of this protein in chondrocyte cells and synovial lining cells by specific cytokines is implicated in the degradation and damage of cartilage, specific mechanisms controlling induction of MMP-9 are still sketchy. The systematic analysis of serially truncated and specifically mutated reporter constructs identified the requirement of multiple regulatory elements for IL-1β- and TNF-α-mediated induction of MMP-9 (Figs. 2–5), in which the role of SAF-1 was earlier unknown. The SAF-1 DNA binding site positioned between −57 and −46 in human MMP-9 promoter is highly conserved and located within a region that was previously identified as the GT box element (16). The GT box element, in several previous reports, was shown to be critical in mediating MMP-9 induction during oncogenic and oncoprotein-mediated malignant transformation (16–18), but the protein interacting at this region was never identified. This report is the first revealing a nuclear protein in cytokine-induced chondrocyte and synoviocyte cells as well as in osteoarthritic cartilage, which interacts with the GT box element of the human MMP-9. The GGGGA/TGGGG sequence of the GT box, which could potentially act as a Sp1 binding element, does not interact with Sp1 that is present in the chondrocyte, synoviocyte, or osteoarthritic cartilage nuclear extracts. This was evident by the failure of Sp1 oligonucleotide or anti-Sp1 Ab to inhibit the formation of specific complexes (Fig. 5).

In addition to the identification of MMP-9 as a target of the inflammation-responsive novel transcription factor, SAF-1, this report provides the first evidence of functional cooperation between the SAF-1 and AP-1 family of proteins in achieving maximal MMP-9 induction. AP-1 transcription factors belong to a family whose members contain a basic leucine zipper DNA-binding domain. Members of this family include c-Fos, Fra-1, FosB, c-Jun, JunB, and JunD. AP-1 family members form homo- or heterodimeric complexes to bind to specific DNA elements in the gene promoter. Protein kinases such as JNK and p38 MAPK are known to phosphorylate and activate AP-1 family members (32). In this study we show that transcriptional induction of MMP-9 involves mutually dependent and functionally synergistic actions of AP-1 and SAF-1. We have demonstrated this effect by cotransfection of wild-type MMP-9 promoter with expression plasmids and with mutant MMP-9 promoter constructs (Figs. 8 and 9) and also by loss of cytokine responsiveness (Fig. 3). Mutation of either transcription factor binding site in the context of surrounding sequences diminishes the ability of SAF-1 or AP-1 to drive MMP-9 promoter function. These results clearly show that both factors require cooperation from the other in regulating MMP-9 expression. Transcriptional induction of MMP-9 by only SAF-1 or AP-1 family of proteins is most likely due to the use of the limited endogenous pool of protein that acts in synergy with the ectopically expressed partner. In the event of mutation of any of these elements, endogenous protein being unable to interact with the MMP-9 promoter cannot facilitate other transcription factor action. Such a symbiotic relationship was not seen between NF-κB and AP-1 or NF-κB and SAF-1 or SAF-1 and Sp1 (Fig. 8). In fact, SAF-1 and Sp1 modestly antagonized the function of the other. Incidentally, SAF-1 and Sp1 exhibited cooperative relationship in augmenting serum amyloid A gene expression (33). These apparently opposing effects suggest that functional relationship between SAF-1 and Sp1 is gene-specific and depends on the surrounding sequences as well. Overall, our findings introduce an important step into the developing model of MMP-9 regulation during cytokine-mediated induction in chondrocyte and synoviocyte cells.

The synergistic relationship between SAF-1 and AP-1 suggests that even under mild inflammatory conditions, the outcome of a relatively low level of induction of these two transcription factors would result in a much higher level of MMP-9 protein. In contrast, inhibition of any single transcription factor activity would significantly lower the level of MMP-9 expression. There are several possible mechanisms by which synergy between SAF-1 and AP-1 may be achieved, and elucidating the mechanism for such symbiotic relationship is an important area for future investigations. First possible mechanism is that close proximity of the AP-1 and SAF-1 element is responsible for mediating the functional cooperation between these two groups of proteins. For optimal interaction with the MMP-9 promoter, it is possible that interaction of each transcription factor with DNA in vivo may be enhanced by cooperative recruitment and stabilization or by an alteration in local DNA structure, which is fostered by the simultaneous interaction of both transcription factors. A second possible mechanism is that a physical, protein-protein interaction between SAF-1 and AP-1 family members is responsible for mediating functional cooperation. The accounts of protein-protein interaction between transcription factors in the regulation of gene expression are continuously growing. The AP-1 transcription factor has been reported to interact with a wide variety of transcription factors, including NF-κB (34), Smads (35), and NFAT (36), but no report on interaction between SAF-1 and AP-1 is available.

In summary, identification of SAF-1 as the transcription factor interacting with the GT box element, which was earlier known to play a crucial role in MMP-9 expression, provides a new insight into the induction mechanism of this gene in the pathogenesis of OA. Furthermore, the functional cooperation between SAF-1 and AP-1 not only solidifies the role of SAF-1 as a regulator of MMP-9 but also supports a more complex model of SAF-1-mediated regulation, one which involves cooperation with neighboring response elements.

**Acknowledgments**

We are grateful to Motokazu Seiki and Douglas Boyd for generous gifts of MMP-9 promoter constructs. We also thank James A. Goodrich for generous gifts of pET-c-Jun and pET-c-Fos plasmid DNA and James L. Cook for providing canine tissue samples. We thank Arvind Shakhya for assistance in several experiments.

**Disclosures**

The authors have no financial conflict of interest.

**References**


