Transcriptional Synergy Mediated by SAF-1 and AP-1

CRITICAL ROLE OF N-TERMINAL POLYALANINE AND TWO ZINC FINGER DOMAINS OF SAF-1

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Previously we determined that inflammation responsive transcription factors AP-1 and SAF-1 synergistically regulate transcriptional induction of the MMP-1 gene. The present study investigated the underlying molecular mechanism of cooperativity between these two different groups of transcription factors. We present evidence that knockdown of SAF-1 by small interfering RNAs inhibits AP-1-mediated increase of human MMP-1 expression. The two key members of the AP-1 family of proteins, c-Fos and c-Jun, and SAF-1 form a ternary protein complex, which has markedly higher DNA binding activity than either a SAF-1 homodimer or a c-Fos/c-Jun heterodimer. The increased DNA binding activity of the ternary complex is translated into a striking enhancement of their transcriptional activity by which synergistic transcriptional induction of MMP-1 expression is achieved. The SAF-1-c-Fos-c-Jun ternary complex efficiently promotes transcription from both SAF-1 and AP-1 sites of human MMP-1 promoter. The physical interaction between SAF-1 and AP-1 was demonstrated both in vitro by Far-Western and antibody pulldown assays with recombinant proteins and in vivo by chromatin immunoprecipitation (ChIP), re-ChIP, and co-immunoprecipitation analyses. Two distinct but adjacent domains in SAF-1 are involved in protein-protein contact with c-Fos and c-Jun; one domain resides within two N-terminal polyalanine tracts, and the other is present within the first two zinc finger motifs. Together these findings delineate the mechanism of synergy and the essential role of SAF-1 and AP-1 in up-regulating human MMP-1 expression under various inflammatory conditions.

A crucial role of matrix metalloproteinase-1 (MMP-1)3 in the pathogenesis of various diseases associated with extracellular matrix (ECM) degradation is well established (1–3). MMP-1 is a prominent member of more than 26 structurally related matrix MMP proteins that in concert are capable of degrading all components of the ECM at physiological pH (4, 5). Although all MMPs possess some ECM-degrading capacity, the initial cleavage of triple helical collagen can be done only by a small subset of this family of which MMP-1 is one. Normal expression of MMP-1 is thus at a very low level and is highly regulated. Under many pathogenic conditions, MMP-1 protein concentration is markedly increased, primarily by transcriptional induction of the MMP-1 gene. Increase of MMP-1 mRNA by many inflammatory stimuli such as cytokines, growth factors, and tumor promoting agents is still a major challenge in successful inhibition of this molecule for the treatment of all MMP-1-related pathogenicities (6).

Several transcription factors have been implicated thus far for transcriptional induction from the MMP-1 gene; these include activator protein 1 (AP-1) (7) and serum amyloid A-activating factor 1 (SAF-1) (8). AP-1 comprises a group of proteins containing a basic leucine zipper DNA-binding domain. AP-1 family members consist of the Fos (c-Fos, Fra-1, Fra-2, FosB) and Jun (c-Jun, JunB, and JunD) proteins (9, 10). Typically, AP-1 protein dimers bind to AP-1-binding elements, and two subunits are formed either by heterodimerization or homodimerization. In cells, the Fos/Jun heterodimer is the most predominant form of AP-1, which has a very high affinity for an AP-1 DNA-binding element, whereas the Jun/Jun homodimer exhibits a very low affinity for the same DNA. AP-1 proteins are transcriptionally induced by the action of various serum and growth factor regulated factors. For example, the c-fos gene is transcriptionally induced by the mitogen-activated protein (MAP) kinase-activated Elk transcription factor, a member of the ternary complex factor (TCF) family, (11, 12) and by protein kinase C during oncogenic Ha-Ras-mediated induction (13). SAF-1 transcription factor is a member of the multiple Cys2-His2-type zinc finger proteins, which also are activated in response to various inflammatory stimuli, including cytokines, phorbol 12-myristate 13-acetate, lipopolysaccharide, and oxidized low density lipoproteins (14–17). Phosphorylation of SAF-1 via MAP kinase, protein kinase C, or protein kinase A markedly increases its DNA binding and transactivation potential (18–20).

The activation of AP-1 and SAF-1 by similar signal transduction cascades, the MAP kinase and protein kinase C pathways, suggests that these transcription factors may function in a cooperative manner and/or may be dependent on each other to exert their full transactivation potential. A recent study from our laboratory indicates that concurrent participation of AP-1 and SAF-1 is necessary for full transcriptional induction of
canine MMP-1 (21). The SAF-1 and AP-1 DNA-binding elements are well conserved in the MMP-1 promoter among many species, which further underscore their importance. Functional cooperation between candidate proteins, in general, is mediated by protein-protein interaction, which is most effective when the DNA-binding elements of the involved transcription factors are proximally located. In correlation with this hypothesis, several studies are available that demonstrate the ability of nuclear proteins to bind each other and to mediate cooperative DNA binding and promoter activation when their respective binding elements are juxtaposed (22, 23). However, in human or canine MMP-1, SAF-1 and AP-1 DNA-binding elements are not in close proximity but are more than 250 nucleotides apart. To understand the mechanism of cooperation between SAF-1 and AP-1, we have extended the study of human MMP-1, and in this report we present evidence for a physical interaction between these proteins, the formation of a ternary protein complex with markedly high DNA binding ability and a synergistic level of transactivation potential. Further, this study provides, for the first time, an identification of novel structural motifs in the SAF-1 transcription factor for protein-protein contact with AP-1 family members. These findings extend the paradigm that cellular machinery utilizes concurrent activation of a network of transcription factors and their cooperative interaction for efficient and optimal activation of genes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HTB-94 human chondrocyte cells derived from a primary grade II chondrosarcoma were cultured in Dulbecco’s modified Eagle’s medium containing high glucose, 100 units/ml penicillin, and 100 units/ml streptomycin supplemented with 7% fetal calf serum. Transfection assays were performed as described previously (8). The pSV-β galactosidase (Promega) plasmid DNA was used as an internal control and was assayed as described (24). Cells were harvested 24 h post-transfection, and CAT activity was measured as described previously (24). All transfection experiments were performed at least three times.

**Plasmids**—MMP-1(–518/+63)-CAT reporter was constructed by ligating the human MMP-1 genomic DNA sequence from nucleotide position –518 to +63 into pBLCAT3 vector (25). Mutant MMP-1-CAT reporters were constructed by ligating the respective mutant DNA fragments generated by megaprimer PCR into pBLCAT3 vector. Mutant SAF-1 and AP-1 oligonucleotide sequences used were 5′-CCTTGTGCTGACAATGCGGAATGTAAC-3′ and 5′-GGATGTTATAAAGCAGATCCTTAGACAGGC-3′, respectively. The underlined bases represent altered sequences. The SAF-1 CAT (26) or AP-1 CAT reporter plasmids contained three tandem copies of SAF-1 or AP-1 DNA-binding elements into pBLCAT2 vector. Various SAF-1 fragments were constructed by PCR, and wherever it became necessary, initiator ATG and TGA stop codons were added to the coding sequences. The PCR-amplified DNAs were ligated to the appropriate expression vectors, including pcDNA3 (Invitrogen), pGEX-5X-1 (GE Healthcare), PRSET (Invitrogen), and pAR (27). Primer sets used for synthesizing various SAF-1 derivatives, indicated by beginning and ending amino acids in parentheses, were as follows: SAF-1(1–477), 5′-ATGTTCCCGTGTTCCTTGCACGTG-3′ and 5′-AGCTCACAAGGGTGGGGAGAGCTTGTG-3′; SAF-1(1–192), 5′-ATGTTCCCGTGTTCCTTGCACGTG-3′ and 5′-TCAGAGAACGGAGGAATGTTG-3′; SAF-1(1–90), 5′-ATGTTCCCGTGTTCCTTGCACGTG-3′ and 5′-TCAGAGAACGGAGGAATGTTG-3′; SAF-1(90–192), 5′-ATGTTCCCGTGTTCCTTGCACGTG-3′ and 5′-TCAGAGAACGGAGGAATGTTG-3′; SAF-1(192–301), 5′-ATGTTCCCGTGTTCCTTGCACGTG-3′ and 5′-TCAGAGAACGGAGGAATGTTG-3′; SAF-1(301–413), 5′-ATGTTCCCGTGTTCCTTGCACGTG-3′ and 5′-TCAGAGAACGGAGGAATGTTG-3′; SAF-1(413–477), 5′-ATGTTCCCGTGTTCCTTGCACGTG-3′ and 5′-TCAGAGAACGGAGGAATGTTG-3′.

**Protein Preparation**—Bacterially expressed proteins were purified by affinity chromatography using nickel-agarose (Invitrogen) or glutathione-Sepharose column chromatography following the manufacturer’s protocol. pET-cJun and pET-cFos plasmid DNAs were obtained from James A. Goodrich, and the c-Jun/c-Fos heterodimer was prepared as described (28).

**GST Pulldown Assay**—GST-tagged full-length SAF-1 or truncated SAF-1 proteins and control GST proteins (50 pmol each) were mixed individually with glutathione-Sepharose 4B beads (Amersham Biosciences), incubated for 1 h at 4 °C in binding buffer (12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 100 mM NaCl, 1 mM EDTA), and washed extensively (five times) with binding buffer. Next, GST-SAF-1, truncated GST-SAF-1, or control GST protein immobilized to Sepharose beads was mixed with bacterially expressed c-Jun/c-Fos heterodimer, incubated for 1 h at 4 °C, and washed extensively with binding buffer plus 0.05% Nonidet P-40. The proteins were eluted using elution buffer (50 mM Tris–HCl, pH 8.0, 100 mM reduced glutathione) or boiled in protein sample loading buffer containing 1% SDS plus β-mercaptoethanol, analyzed by SDS-PAGE using 11% polyacrylamide gel, and visualized by Coomassie Blue staining.

**Far-Western Assay**—Equal amounts of protein were electrophoresed on 11% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated in a modified HEPES binding buffer (25 mM HEPES, pH 7.9, 25 mM NaCl, 5 mM MgCl2, 5 mM β-mercaptoethanol) containing 6 mM guanidine hydrochloride for 10 min at room temperature. Membrane-bound denatured proteins were renatured by sequential incubation of the membrane for five cycles (10 min each) in buffers that were diluted each time (1:1) with HEPES binding buffer without any guanidine hydrochloride. The membrane received a final rinse twice in HEPES binding buffer and then were incubated in a blocking solution (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5% bovine serum albumin) at 4 °C overnight, further incubated in overlay buffer (0.5% bovine serum albumin, 0.25% gelatin, 1% Nonidet P-40, 10 mM NaCl, 1 mM EDTA, 5 mM

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**Interaction between SAF-1 and AP-1**

Further, this study provides, for the first time, an identification of novel structural motifs in the SAF-1 transcription factor for protein-protein contact with AP-1 family members. These findings extend the paradigm that cellular machinery utilizes concurrent activation of a network of transcription factors and their cooperative interaction for efficient and optimal activation of genes.
β-mercaptoethanol, 20 mM HEPES, pH 7.5) containing 32P-labeled pAR-SAF-1 at room temperature for 4 h, washed four times for 10 min each in a washing buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% sarkosyl), dried, and autoradiographed. The pAR-SAF-1 protein was prepared by cloning a full-length SAF-1 cDNA in pAR vector (27) that contains a FLAG tag and a heart muscle kinase phosphorylation site, and purified protein was radioactively labeled with [γ-32P]ATP and heart muscle kinase (Sigma).

siRNA Experiments—HTB-94 cells were transfected in duplicate with predesigned human MAZ/SAF-1-specific siRNAs (Dharmacon Research, Lafayette, CO) using Oligofectamine (Invitrogen) according to the manufacturer’s protocol. As negative controls, mock transfection (no siRNA) and nonspecific scrambled siRNAs were used. Forty-eight hours after transfection, cells in each treatment groups were harvested and divided into two parts. Also conditioned media were collected for measuring secreted MMP-1 and MMP-2 proteins. Total RNA was isolated from one portion of the cells using the guanidinium thiocyanate method (29), and RT-PCR was performed using an RT-PCR kit from Invitrogen. The PCR primer sequences specific for the genes examined and the predicted using an RT-PCR kit from Invitrogen. The PCR primer sequences specific for the genes examined and the predicted using an RT-PCR kit from Invitrogen.

Electrophoretic Mobility Shift Assay—Equal amounts (0.1 μg) of bacterially expressed proteins were used for an electrophoretic mobility shift assay, which was performed as described previously (17). Protein concentrations were measured by the Bradford method (30). Radiolabeled probes were prepared by labeling double-stranded oligonucleotides with [α-32P]dCTP. In some assays, anti-SAF-1 or anti-c-Fos and anti-c-Jun (Santa Cruz Biotechnology) antibodies were added during a 30-min preincubation on ice prior to the addition of the labeled probe. Anti-SAF-1 antibody was prepared as described (8). In some assays, 25 pmol of the following oligonucleotides were added in the preincubation mixtures as competitor oligonucleotides: SAF-1 oligo, 5’-CCCTCTCTCTCACCACACAGCCCGCATGG-3’; AP-1 oligo, 5’-CGCCTGGATGACTGACCGAAGA-3’; and nonspecific oligo, 5’-TGTCGAAATCAGAAATCAGAA-3’.

Chromatin Immunoprecipitation (ChIP) and Re-ChIP Assays—ChIP was performed with a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY) following the manufacturer’s protocol. Briefly, HTB-94 cells (2 × 106 each) were stimulated with IL-1β (500 units/ml). Twenty-four hours later, formaldehyde (1% final concentration) was added directly to the culture medium and incubated at 37 °C for 10 min for cross-linking. Cells were lysed in 200 μl of cell lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) with a protease inhibitor mixture and sonicated to generate ~500-bp long DNA fragments, and the supernatants were diluted with a dilution buffer (20 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.01% SDS, and protease inhibitors). The solutions were precleared with a salmon sperm DNA/protein G-agarose slurry and then incubated with anti-SAF-1 antibody or a mixture of anti-c-Fos and anti-c-Jun antibodies (Santa Cruz Biotechnology) at 4 °C for 16 h with rotation. The immune complexes were precipitated with salmon sperm DNA/protein G-agarose. The agarose beads were washed sequentially in a low salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% SDS), a high salt wash buffer (same as low salt wash buffer except containing 500 mM NaCl), LiCl wash buffer (20 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% deoxycholate, and protease inhibitors), and 20 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer. Immune complexes were extracted from the beads with elution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, and 50 mM NaHCO3) at 65 °C for 4 h.

The Re-ChIP assays were performed as described (31). Briefly, the eluant of the primary immunocomplex obtained with the first antibody was diluted 10-fold with dilution buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, and protease inhibitors) and then further subjected to immunoprecipitation with the second antibody. Cross-linking was reversed, and nucleic acids were isolated from the eluates by proteinase K and RNase A treatments followed by phenol extraction and ethanol precipitation. Purified DNA was subjected to PCR using primers covering the AP-1 or SAF-1 DNA-binding regions in the human MMP-1 promoter. PCR products were resolved in a 2% agarose gel and visualized by ethidium bromide staining. The primers used for amplification of the SAF-1 element in human MMP-1 (162 bp) were 5’-GAACTTCCGACGCTGTGGTTCGACGAGCC-3’ (sense) and 5’-GAACTTCCGACGCTGTGGTTCGACGAGCC-3’ (antisense), and those for amplification of the AP-1 element in human MMP-1 (132 bp) were 5’-CTGAAATTCCCATCCATCTTATGACGTCGACGACG-3’ (sense) and 5’-CTGAAATTCCCATCCATCTTATGACGTCGACGACG-3’ (antisense).

Immunoprecipitation and Western Blotting—HTB94 cells, untreated or treated with IL-1β, were lysed using a lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). The proteins were immunoprecipitated with anti-SAF-1 antibody, anti-c-Jun plus anti-c-Fos antibody, or control nonimmune IgG as indicated. Samples were fractionated in SDS-11% PAGE and electrophoresed onto a nitrocellulose membrane. Immunoblotting was performed using anti-c-Fos, anti-c-Jun, or anti-SAF-1 antibody as the primary antibody and horseradish peroxidase-
Interaction between SAF-1 and AP-1

conjugated goat anti-rabbit IgG as the secondary antibody. Bands were detected using a chemiluminescence detection system (Amersham Biosciences).

RESULTS

SAF-1 Knockdown Affects AP-1-mediated Transcriptional Increase from MMP-1 Gene—To determine the extent of functional cooperation between SAF-1 and AP-1, we used siRNAs directed against SAF-1 and examined the consequence of endogenous SAF-1 inhibition on AP-1-mediated up-regulation of the human MMP-1 promoter. Treatment of HTB-94 cells for 48 h with SAF-1 siRNAs successfully decreased the level of endogenous SAF-1 mRNA as well as protein, as compared with untreated or scrambled siRNA-treated cells (Fig. 1, A and B). Having determined the specificity of SAF-1 siRNAs, we investigated whether knockdown of SAF-1 would show any effect on IL-1β-induced increase of MMP-1 expression. Both RT-PCR analysis (Fig. 1C) and Western blot analysis (Fig. 1D) indicated that reduction of endogenous SAF-1 in response to SAF-1 siRNA treatment of cells substantially reduces the IL-1β-mediated increase of MMP-1 mRNA and protein levels. The specificity of SAF-1 siRNAs on MMP-1 expression was verified by examining MMP-2 protein levels in both untreated and IL-1β-treated cells. There was no detectable change in MMP-2 protein level between scrambled siRNA and SAF-1 siRNA-treated cells during IL-1β treatment or with no treatment (Fig. 1D).

In the next experiment, we examined the effect of AP-1 overexpression on the MMP-1-CAT reporter gene in SAF-1-silenced cells. HTB-94 cells were cotransfected with MMP-1(-518/+63)-CAT reporter and an equimolar mixture of c-Fos and c-Jun expression plasmids. Intriguingly, the cells treated with SAF-1 siRNAs failed markedly, by more than 50%, to support c-Fos/c-Jun-mediated induction of MMP-1-CAT reporter expression, whereas cells treated with scrambled siRNAs showed no such inhibitory effect (Fig. 1E). These results suggested a requirement for SAF-1 protein to facilitate AP-1 action and optimal AP-1-mediated transcriptional induction of the MMP-1-CAT reporter.

SAF-1 and AP-1 Synergistically Induce MMP-1 Gene Expression—For further assessment of a possible combined regulatory role of SAF-1 and AP-1 in promoting MMP-1 expression, HTB-94 cells were transfected with MMP-1-CAT reporter and expression plasmids of SAF-1 and AP-1. Clearly, simultaneous overexpression of both SAF-1 and AP-1 (c-Fos/c-Jun) expression plasmids synergistically increased MMP-1-CAT reporter transcription (Fig. 2A). The synergy between SAF-1 and AP-1 was not due to any aberrant level of expression from transfected plasmids, as Western blot analysis revealed a dose-dependent increase of SAF-1, c-Fos, and c-Jun proteins in transfected cells (Fig. 2B). These results suggested that SAF-1 and AP-1 act in synergy to induce transcription of the MMP-1 promoter. To better understand how SAF-1 and AP-1 participate in the context of two respective promoter elements of the MMP-1 gene, single and double site mutant promoters were used (Fig. 2C). IL-1β-mediated induction of MMP-1-CAT reporter was partially inhibited when the individual SAF-1 or AP-1 site was mutated. Mutation of both sites resulted in a near complete loss of responsiveness to IL-1β. These results, showing that the participation of IL-1β activated transcription factors at both the SAF-1 and AP-1 sites to promote induction of the MMP-1 gene, were in accord with the previous observation indicating a requirement for both of these proteins.

Physical Interaction between SAF-1 and AP-1 in Vitro—We contemplated that the cooperativity between SAF-1 and AP-1 might result from a physical interaction between these proteins. To verify this idea, two sets of in vitro experiments, a Far-Western assay and a GST pulldown assay, were performed. The Far-Western assay showed a clear interaction between
Interaction between SAF-1 and AP-1

with GST-SAF-1 protein immobilized to a glutathione-Sepharose column and were eluted by a molar excess of glutathione (Fig. 3B, lane 5). However when used alone, His-c-Fos or His-c-Jun protein showed no interaction with GST-SAF-1 (Fig. 3B, lanes 6 and 7). The AP-1 proteins also did not interact with control GST protein (Fig. 3B, lane 8). Taken together, these results indicated a physical interaction between SAF-1 and AP-1 proteins and further suggested that, in solution, only heterodimers of c-Fos/c-Jun interact efficiently with SAF-1.

In Vivo Interaction between SAF-1 and AP-1—To detect the physical interaction between SAF-1 and AP-1 in vivo, coimmunoprecipitation experiments were performed. The SAF-1 antibody coimmunoprecipitated c-Fos and c-Jun proteins along with SAF-1 in both untreated and IL-1β-treated cells, albeit at different levels (Fig. 4A). In a reciprocal experiment, SAF-1 protein was immunoprecipitated by a mixture of c-Fos and c-Jun antibodies from both untreated and IL-1β-treated cells, indicating in vivo interaction between SAF-1 and members of the AP-1 family. The specificity of this assay was verified by using control IgG, which showed no coimmunoprecipitation of c-Fos, c-Jun, or SAF-1. For further verification, we performed ChIP analysis using untreated and IL-1β-treated cells (Fig. 4B). PCR amplification of the AP-1-binding element (Fig. 4B, lane 6) and the SAF-1-binding element (Fig. 4B, lane 12) in a cross-linked chromatin assay of IL-1β-stimulated cells indicated active interaction of a SAF-1/c-Fos-c-Jun complex with the MMP-1 promoter at both the SAF-1 and AP-1 sites during IL-1β stimulation of cells. In unstimulated cells, the interaction between SAF-1 and AP-1 proteins seemed to be much less, as the intensity of PCR-amplified products was low compared with the products obtained from IL-1β-stimulated cell chromatin DNA (Fig. 4B, compare lanes 3 with 6 and 9 with 12). The specificity of the ChIP assay was verified using control IgG-precipitated chromatin, where no PCR-amplified product was visible (Fig. 4B, lanes 2, 5, 8, and 9).
The interaction of transcription factors to a gene promoter is of a dynamic nature. Therefore, to examine whether SAF-1 and AP-1 proteins are simultaneously localized in the MMP-1 promoter, we performed a re-ChIP assay. The cross-linked chromatin from untreated and IL-1β-treated cells was immunoprecipitated with the first antibody, and the resulting immunocomplex was then eluted and further subjected to immunoprecipitation with the second antibody or control antibody. The re-ChIP results indicate that the proximal promoter region of MMP-1 is occupied by SAF-1 and AP-1 proteins only under conditions of IL-1β stimulation (Fig. 4C, lanes 1 and 7). Very little to almost no interaction between SAF-1 and AP-1 proteins was detected in unstimulated cells (Fig. 4C, lanes 1 and 7). Also, no PCR-amplified product was seen when re-Chip analysis was performed using control IgG (Fig. 4C, lanes 2, 5, 8, and 11). Taken together, these results indicated the simultaneous recruitment of SAF-1 and AP-1 to the MMP-1 proximal promoter region and suggested that they may participate in cytokine induction of the MMP-1 gene in vivo.

**Ternary Complex of SAF-1-c-Fos-c-Jun Shows Markedly Higher DNA Binding Ability**—Protein-protein interaction between SAF-1 and c-Fos/c-Jun proteins prompted us to examine the DNA binding activity of the SAF-1-c-Fos-c-Jun ternary complex. In the first experiment, the efficacy of the DNA binding activity of bacterially expressed proteins was tested. Alone, bacterially expressed His-c-Fos (Fig. 5B, lane 1) and His-c-Jun (Fig. 5B, lane 2) interacted very poorly with the radioactive AP-1 element of the MMP-1 promoter, but an equimolar mixture of c-Fos/c-Jun proteins showed a considerable level of DNA-protein interaction with the same probe (Fig. 5B, lane 3). These results are in correlation with other previous observations that reported optimal DNA binding of the AP-1 element with only the c-Fos/c-Jun dimer. The c-Fos/c-Jun DNA-protein complexes were not competed by a nonspecific oligonucleotide but efficiently competed with a molar excess of nonradioactive AP-1-binding oligonucleotide (Fig. 5B, lanes 4 and 5). Having determined the DNA binding efficiency of bacterially expressed proteins, we included an equal amount of bacterially expressed purified GST-SAF-1 protein in the DNA binding assays. GST-SAF-1 protein did not interact with the radioactive AP-1 element (Fig. 5B, lane 6) but, together with His-c-Fos and His-c-Jun proteins, formed a very prominent SAF-1-c-Fos-c-Jun DNA-protein complex (Fig. 5B, lane 8). The level of SAF-1-c-Fos-c-Jun complex was much higher than the c-Fos/c-Jun complex formed with the same AP-1 probe (Fig. 5B, compare lanes 7 and 8). The specificity of the ternary complex was verified by using anti-SAF-1 antibody, which caused a reduction in the level of the SAF-1-c-Fos-c-Jun ternary complex (Fig. 5B, lane 9), and by a mixture of c-Fos and c-Jun antibodies, which caused supershifting of the complex (Fig. 5B, lane 10).

In a reciprocal experiment, the extent of SAF-1-c-Fos-c-Jun ternary complex formation at the SAF-1 DNA-binding element was examined (Fig. 5E). The interaction of GST-SAF-1 protein with the radiolabeled SAF-1 element was found to be modest, and there was no interaction of c-Fos and c-Jun proteins (Fig. 5E, lanes 1 and 2). However, when the same SAF-1 protein was mixed with c-Fos and c-Jun proteins, it interacted with the probe with much higher affinity and formed two DNA-protein complexes.
Interaction between SAF-1 and AP-1

FIGURE 5. Superior DNA binding ability of the ternary complex of c-Fos, c-Jun, and SAF-1. A, the sequence of the AP-1 DNA-binding element in the human MMP-1 promoter used as probe in DNA binding assays. B, purified bacterially expressed His-c-Fos, His-c-Jun, and GST-SAF-1 proteins were incubated singly or in combination with 32P-labeled AP-1 oligonucleotide as indicated. In some reactions, a molar excess of unlabeled nonspecific oligonucleotide (lane 4) or of unlabeled AP-1 oligonucleotide (lane 5), anti-SAF-1 antibody (Ab; lane 9), or a mixture of anti-c-Fos and anti-c-Jun antibodies (lane 10) was included. The SAF-1-c-Fos-c-Jun ternary complex, c-Fos-c-Jun, and supershifted complexes are indicated. C, Western blot analysis to determine the input of proteins in the DNA binding assay. Purified bacterially expressed His-c-Fos, His-c-Jun, and GST-SAF-1 proteins, as used in the DNA binding assays, were subjected to Western blot analysis using anti-c-Fos, anti-c-Jun, and anti-SAF-1 antibodies as indicated. D, the sequence of the SAF-1 DNA-binding element in the human MMP-1 promoter used as a probe in the DNA binding assays. E, purified bacterially expressed GST-SAF-1, His-c-Fos, and His-c-Jun proteins, singly or in combination, were incubated with 32P-labeled SAF-1 oligonucleotide as indicated. In some reactions, a molar excess of unlabeled nonspecific oligonucleotide (lane 4), a mixture of anti-c-Fos and anti-c-Jun antibodies (lane 5), a molar excess of unlabeled SAF-1 oligonucleotide (lane 6), or anti-SAF-1 antibody (lane 7) were included. The SAF-1-c-Fos-c-Jun ternary complex, SAF-1-SAF-1 complex, and the supershifted complex are indicated.

Complexes, one of which was composed of SAF-1-c-Fos-c-Jun and the other of SAF-1-SAF-1 proteins (Fig. 5E, lane 3). The SAF-1-c-Fos-c-Jun DNA ternary complex was supershifted by c-Fos/c-Jun antibodies, whereas the SAF-1-SAF-1 complex remained unaffected (Fig. 5E, lane 5). The addition of a molar excess of SAF-1 oligonucleotide (Fig. 5E, lane 6) or anti-SAF-1 antibody (Fig. 5E, lane 7) substantially inhibited the formation of both DNA-protein complexes. These results showed that the SAF-1-c-Fos-c-Jun ternary complex can bind to either a SAF-1 or an AP-1 DNA element with a markedly higher efficiency.

Functional Interaction of SAF-1 with c-Fos/c-Jun Promotes Synergistic Transcriptional Activation from SAF-1 or AP-1 Element—To correlate whether increased DNA binding of the SAF-1-c-Fos-c-Jun ternary complex leads to increased transcriptional function, a series of transfection assays was performed. Simultaneous expression from SAF-1, c-Fos, and c-Jun plasmids synergistically increased MMP-1(-518/+63)-CAT, AP-1-CAT, or SAF-1-CAT reporter activity (Fig. 6A). Exogenous expression of SAF-1, c-Fos, and c-Jun proteins in transfected cells was verified by Western blot analysis (Fig. 6B), which showed no discrepancy in the expression pattern of transfected plasmids. There was a modest increase in the MMP-1 protein level in pcDSAF-1/pcDc-Fos/pcDc-Jun plasmid-transfected cells (Fig. 6B). These reporter assays clearly indicated that the synergism between SAF-1 and c-Fos/c-Jun proteins can be achieved by the engagement of the ternary complex at either the AP-1 or SAF-1 element and further suggested that synergism between SAF-1 and AP-1 proteins may be applicable to other genes that contain only one DNA-binding element, SAF-1 or AP-1, in their promoters.

Mapping of the AP-1 Protein-interacting Domains in SAF-1—To define the domains of SAF-1 involved in interaction with c-Fos/c-Jun proteins, a series of GST fusion proteins containing different regions of SAF-1 was prepared (Fig. 7A). Among different SAF-1 proteins, fragments 1–477, 1–192, 192–413, 192–360, and 90–301 showed a good interaction with AP-1 proteins (Fig. 7B, lanes 1–5), whereas the two terminal 1–90 and 413–477 fragments and negative control GST protein did not show any interaction with the c-Fos/c-Jun protein mixture (Fig. 7B, lanes 6–8). Further delineation of the domains indicated the presence of two independent AP-1-interacting modules in SAF-1 (Fig. 7B, lanes 9 and 10). One domain is centered within the two N-terminal polyalanine tracts (amino acids 90–192), and the other domain resides within the first two zinc fingers (amino acids 192–301). Coomassie Blue staining was performed to verify the input and integrity of different GST-SAF-1 proteins (Fig. 7C). It was interesting to note that the SAF-1(90–301) fragment containing both interacting domains displayed a somewhat lower level of interaction with AP-1 proteins than the SAF-1(192–360) fragment. Nonetheless, these results clearly mapped the AP-1 protein-interacting domains of SAF-1.
DISCUSSION

Our results show that inhibition of endogenous SAF-1 activity achieved by small interfering RNA molecules leads to the inhibition of AP-1-mediated transcriptional induction of human MMP-1 gene. These results clearly indicated a functional cooperation between SAF-1 and AP-1 proteins. Protein-protein interaction and cooperativity between transcription factors leading to synergistic activation or repression of gene expression is an important mode of gene regulation. We have demonstrated that a physical interaction, both in vitro and in vivo, between SAF-1 and AP-1 transcription factors results in the formation of a SAF-1-c-Fos/c-Jun ternary protein complex. The ternary complex has a markedly higher DNA binding ability, which leads to synergistic activation of transcription from the natural MMP-1 promoter as well as from synthetic promoters containing either the SAF-1 or AP-1 response element. These novel findings imply that, upon activation by various inflammatory stimuli, SAF-1 and AP-1 together can promote transcription of a gene even in the absence of their own interacting element in the promoter; the target gene needs to contain only one, SAF-1 or AP-1, DNA-binding site to receive transcriptional stimulation from the synergistic interaction between these two proteins.

The accumulating evidence indicates that expression of a gene is regulated by the combined actions of a number of transcription factors and that interaction between them is a critical regulatory part of gene expression. Cooperativity and transcriptional synergy between transcription factors are believed to be useful in the execution of an exponentially large number of regulatory decisions. From DNA binding assays, it became evident that the SAF-1-c-Fos/c-Jun ternary complex has a many-fold higher affinity for the AP-1 element than the dimers of c-Fos/c-Jun, as almost no c-Fos/c-Jun DNA complex was seen (Fig. 5B). Reciprocally, the SAF-1-c-Fos/c-Jun ternary complex preferentially interacted with a SAF-1 element, although in this case some SAF-1 DNA complex was also seen (Fig. 5E). We reason that the affinity of the ternary complex for the AP-1 element is much higher than for a SAF-1 element. Nonetheless, preferential formation of the SAF-1-c-Fos/c-Jun ternary complex further attests that cooperative interaction between these two groups of proteins is functionally superior.
The AP-1 family of proteins is shown to interact with a variety of other transcription factors, including NF-κB (32), C/EBP (33), Ets (34), Cbfa1 (35), and STAT-3 (36). In comparison, reports on SAF-1 interacting with other proteins are not so abundant. In yeast two-hybrid assays, SAF-1 was shown to interact with the catalytic subunit of protein kinase A (37). Another report (15) indicates a functional cooperation between SAF-1 and Sp1 leading to synergistic activation of the serum amyloid A gene. However, in this study we found no evidence of a physical interaction between SAF-1 and Sp1.

The nature of cooperative interaction was further defined from various transient transfection assays (Figs. 2 and 6). Partial inhibition of cytokine-induced transcription of mutant MMP-9 promoters containing altered AP-1 or SAF-1 element or activation of synthetic promoters driven from multimeric AP-1 or SAF-1 elements clearly showed that a SAF-1:AP-1 complex can exert a biological function through either of these response elements. However, in an earlier study we observed a somewhat different form of cooperativity between SAF-1 and AP-1 proteins in regulating human MMP-9 gene expression (38). In the MMP-9 promoter SAF-1 and AP-1 elements are present in very close proximity, placed within 14 nucleotides of each other. Mutation of either SAF-1 or AP-1 element markedly affects cytokine-mediated induction of the MMP-9 promoter and also compromises the ability of SAF-1 and AP-1 to activate transcription from mutant MMP-9 promoters (38). Such different results, perhaps a gene-specific effect, could be explained by taking into consideration (i) the close spacing between SAF-1 and AP-1 that may have created a certain helical arrangement and steric hindrance and/or (ii) the participation of other proteins interacting at nearby regions in the human MMP-9 promoter. For example, a specific helical phasing is shown to facilitate the physical interaction between AP-1 with Cbfa1 transcription factors in regulating collagenase-3 promoter expression (39) and between STAT3 and AP-1 in regulating transcription factors in regulating collagenase-3 promoter and also in activation of gene transcription (44).

In summary, we have provided a molecular basis for a better understanding of synergistic gene regulation by SAF-1 and AP-1 transcription factors, which in addition to the MMP-1 promoter may be applicable to other genes that contain these transcription factor DNA-binding elements. It remains to be seen whether other factors capable of interacting with SAF-1 or AP-1 also participate in forming multimeric transcription complexes, thereby allowing sequential enhancement and maximal target gene expression in response to different stimuli.

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Interaction between SAF-1 and AP-1

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