**Induction of Serum Amyloid A (SAA) Gene by SAA-activating Sequence-binding Factor (SAF) in Monocyte/Macrophage Cells**

EVIDENCE FOR A FUNCTIONAL SYNERGY BETWEEN SAF AND Sp1

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Serum amyloid A (SAA), a member of the acute-phase group of proteins, is synthesized in the body in response to inflammatory signals. Normally, SAA is expressed at an undetectable level, but infection, tissue injury, or even a prolonged high fat diet can highly induce the synthesis of this protein (1–3). SAA is a well conserved protein among various species. In mammals, SAA comprises multiple isoforms and is coded by multiple genes. Increase of SAA biosynthesis is due primarily to its increased transcription (9). In addition to transcriptional induction, several reports have indicated that mRNA stability also contributes to the enhanced expression of SAA in mouse (10) and human (11, 12). Studies have shown that C/EBP and NF-κB are involved in regulating SAA gene expression in human (13), mouse (14), rat (15), and rabbit (16–18). Recently, a new promoter called SAA-activating sequence (SAS) is identified to control expression of a rabbit SAA gene in some nonhepatic cells (19). By promoter function analysis, we previously showed that SAS promoter element can regulate LPS-mediated SAA gene induction in monocyte/macrophage cells (20). This study, however, did not reveal whether the SAS-binding factor (SAF) is directly involved in potentiating SAA transcription. In the present study, we have used a cloned cDNA of SAF transcription factor2 and have shown that transient overexpression of SAF can activate a rabbit SAA2 gene promoter in monocyte/macrophage cells. We also present evidence for heteromeric complex formation between SAF and Sp1. Furthermore, we demonstrate that cooperative interaction between SAF and Sp1 synergistically activates SAA gene expression through the SAS element.

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**MATERIALS AND METHODS**

- **Plasmid Constructs**—Plasmid construct pSAS-CAT was prepared by ligating SAA genomic DNA sequences from −280 to −226 into plasmid vector pHLCAT2 (21). Plasmid pmtSAS-CAT, a mutant derivative of pSAS-CAT, contained the mutated DNA sequence (5′-CAAGACGTGCATGAGCTCCCAATGAGTCGACGGCTGAATCG-3′) ligated into pHLCAT2 vector. The underlined bases indicate substitution. Plasmid pOXAS-CAT was constructed by ligating three tandem copies of the SAA promoter sequences from −254 to −226. The selected clones were analyzed by DNA sequencing. Bacterial extracts containing SAF were carried out as described (25). The selected clone was lysogenized in host E. coli (26). The selected clone was lysozymized in host E. coli and was grown in LB medium, induced by isopropyl-β-D-thiogalactopyranoside, and harvested by centrifugation. The cells were resuspended in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA-D-thiogalactopyranoside, and harvested by centrifugation. The cells were resuspended in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA.

- **Cell Culture and Transfection**—Monocyte cell THP-1 was obtained from the American Type Culture Collection and maintained in suspension in RPMI 1640 containing 10% fetal calf serum. For induction, cells were stimulated with 10 μg of LPS/ml and were grown for different lengths of time (as indicated). For transient transfection of THP-1 cells, 2 × 10^7 cells were transfected with 10 μg of plasmid DNA by the DEAE-dextran method (23). Rabbit synoviocyte cells HIG-82, obtained from ATCC, were transfected by calcium-phosphate method (24). Chloroform-acetone-ethanol-extracted (CAE) DNA was isolated from bacterial extracts containing SAF. The selected clone was ligated into pBluescript II vector. Plasmid DNA was isolated from bacterial extracts containing SAF and was grown in LB medium, induced by isopropyl-β-D-thiogalactopyranoside, and harvested by centrifugation. The cells were resuspended in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA and were used for transient transfection.

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EDTA, 0.5 mM PMSF, 0.5 mg/ml benzamidine, 2.5 M urea and lysed by repeated freeze-thaw cycles. The cell extracts were partially purified by passing through a DE-52 column. Flowthrough fractions that contain SAF DNA binding activity were dialyzed against 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml benzamidine.

**Nuclear Extracts and Electromobility Shift Assays**—Nuclear extracts were prepared from uninduced and LPS-induced THP-1 cells essentially following a method described previously (20). Protein concentrations were measured as described (27). DNA binding assays were performed following a standard protocol described earlier (17) with 32P-labeled double-stranded DNA probe. In some binding assays, competitor oligonucleotides were included in the reaction mixture. For antibody interaction studies, anti-Sp1 (Santa Cruz Biotechnology) and anti-SAF antiserum (20) were added to the reaction mixture during a preincubation period of 30 min on ice. Purified Sp1 protein was obtained from Promega Corporation.

**RESULTS**

**Transactivation of SAA Promoter by SAF in Monocyte/Macrophage Cells**—SAA gene transcription is induced in monocyte/macrophage cells by LPS (20, 28). We previously showed that nonhepatic transcriptional induction of SAA is primarily regulated by SAS promoter located between −280 and −226 nucleotide sequences (19). In keeping with the promoter activity, using THP-1 monocyte cell nuclear extract, specific DNA-protein complex binding to this promoter was detected. The DNA-binding factor was characterized as SAF (20). These studies, although demonstrating involvement of SAF in DNA binding to SAA promoter, did not determine the transactivation potential of this factor in SAA gene expression.

To assess the in vivo role of SAF in SAA gene transcription, we used a transient expression system in which a cloned cDNA of SAF transcription factor2 was overexpressed in THP-1 monocyte/macrophage cells. A reporter gene, pSAS-CAT, containing one copy of the promoter element of the SAA gene from −280 to −226, was transfected alone or with an expression plasmid containing SAF cDNA under the control of CMV promoter. As shown in Fig. 1, overexpression of SAF increased the reporter gene expression in a dose-dependent manner. The level of reporter gene activation was much higher on a reporter gene containing multiple copies of the SAS sequence. The transactivating effect of pCMV-SAF was negligible on pmtSAS-CAT reporter gene containing mutated sequence of SAA element. These results indicate that SAF is involved in the expression of SAA gene in monocyte/macrophage cells.

**Multiple DNA Binding Activities of SAF Are Induced in THP-1 Monocyte/Macrophage Cells by LPS**—To test how LPS treatment of monocyte cells affects DNA binding activity of SAF, gel mobility shift assays were performed with untreated and LPS-treated THP-1 monocyte/macrophage cell nuclear extracts. As the DNA probe, we used a minimal binding region, sequences from −254 to −226 base pairs, containing SAF-binding elements that produced a better resolution of the DNA-protein complexes than that obtained with the larger probe (−280/−226) used earlier (20). Interestingly, the shorter DNA-binding element (−254/−226) gave rise to multiple DNA-protein complexes designated as a, c, d, and e by the control untreated THP-1 nuclear extract (Fig. 2, lane 1). It is possible that nuclear proteins interacted less efficiently with the larger probe (−280/−226) due to the steric hindrances posed by extra 5′-flanking sequences. Occasionally, we have seen a slightly higher level of complex d in control untreated THP-1 cell extract than that seen in lane 1. This pattern was changed when the probe was incubated with same protein amount of LPS-treated (48 h) THP-1 cell nuclear extract (lane 2). LPS treatment increased the intensities of complexes a and d severalfold and caused the appearance of a new complex designated as b that was not formed by untreated cell nuclear extract (lane 1).

The intensity of minor complex e, seen in lane 1, was reduced in LPS-treated nuclear extract. This complex often showed variability in its intensity; its level decreased variably during LPS treatment of the cells. Intensity of complex c remained virtually unchanged during LPS treatment. Competition with a molar excess of a nonradioactive homologous probe inhibited...
The patterns of DNA-protein complexes formed by LPS-24 h post LPS treatment and reached the maximum level at 24 h. Intensity of the complexes a, b, and d began increasing at 4 h respectively, lane 8 arrow (mNuclear factors that form DNA-protein complexes.

anti-Sp1 antibody (lanes 4–9 complexes a, b, and d but not complex c (Fig. 2, lanes 4–9). At a very high level of the homologous probe, some reduction of the intensity of complex c was seen (data not shown). This suggested that complex c was formed by a very low affinity DNA-protein interaction between a nuclear factor and the SAA promoter. In some experiments, we have noticed the appearance of some faster migrating DNA-protein complexes that are sequence-specific, as judged from efficient competition by excess unlabeled homologous probe.

Kinetics of LPS-mediated Increase in the DNA Binding Activity of SAF.—Because the above experiment indicated LPS-mediated increase in the levels of multiple DNA-protein complexes, namely a, b, and d, we sought to determine the kinetics of activation of these DNA-binding proteins. Nuclear extracts were prepared from THP-1 cells that were incubated in the presence of LPS for various lengths of time (Fig. 3, lanes 1–4). Intensity of the complexes a, b, and d began increasing at 4 h post LPS treatment and reached the maximum level at 24 h. The patterns of DNA-protein complexes formed by LPS-24 h and LPS-48 h nuclear extracts were not very different (compare between lanes 3 and 4). The level of complex c remained virtually unchanged during LPS exposure of the cells. Some additional minor bands composed of faster migrating DNA-protein complexes that are sequence-specific, as judged from efficient competition by excess unlabeled homologous probe (described earlier in Fig. 2), were also detected. Preincubation of LPS-24 h nuclear extract with anti-SAF antibody completely inhibited complexes a, b and d and partially blocked complex c formation (Fig. 3, lanes 6 and 7). In the SAF DNA-binding element, a Sp1 transcription factor DNA-binding sequence, a 5’-CCACCC-3’ sequence element, is embedded (see legend of Fig. 2). To determine if any of these complexes are formed by Sp1, we used anti-Sp1 antibody (lane 8). Sp1 antibody partially supershifted the complex a and had no effect on complexes b and d. The addition of nonspecific preimmune serum also had no effect on any of these complexes (lane 9). In a separate experiment when both anti-SAF and anti-Sp1 antibodies were included in a single reaction, complete inhibition of complex a as well as complexes b and d were noted (data not shown). These results taken together indicated that complex a is formed by the combined interaction of Sp1 and SAF, whereas complexes b and d are formed by SAF only. It is noteworthy to mention that complex a has a higher affinity for the SAA element because it was most efficiently competed with the homologous probe (Fig. 2, lanes 3–9).

Interaction of SAF and Sp1 Transcription Factors with SAS Promoter Element.—In light of the facts that Sp1 can interact with the SAS promoter and Sp1 is present ubiquitously in almost all cell types, we addressed whether Sp1 and SAF can simultaneously interact with SAS element and, if so, what is the functional consequence of such an interaction. In vitro interaction of these transcription factors was studied using a fractionated system where both of these factors could be provided in different combinations. DNA binding assays were performed with a SAF protein preparation obtained from a λgt11 recombinant phage containing SAF cDNA and affinity column purified Sp1 available from a commercial source (Promega). As the probe we used the SAS element, sequences from −254 to −226. The addition of bacterially produced SAF fusion protein formed a single DNA-protein complex (Fig. 4, lane 1). The anti-SAF antibody considerably inhibited this complex formation (lane 4), which indicated the specific nature of this complex. It should be noted that the cloned SAF-gal fusion protein produced only one complex with SAS element as opposed to several DNA-protein complexes (a, b, d, and e) formed by the crude nuclear extract of THP-1 cells (Figs. 2 and 3). This difference could arise due to the presence of either several different isoforms of SAF family in THP-1 cells or due to other factors present in the THP-1 cells that may have some influence in SAF binding or both. In comparison to SAF, purified Sp1 protein interacted very weakly, almost at an undetectable level with SAS DNA (lane 2). This SAS-Sp1 complex was detectable only after prolonged exposure of the autoradiogram (data not shown). When we added both SAF and Sp1 proteins
in the DNA binding assay (lane 3), two DNA-protein complexes, one migrating with SAF-specific complex and the other migrating quite slowly, were detected. Although SAF antibody considerably reduced the levels of both of these two complexes (lane 6), the Sp1 antibody supershifted the slower migrating complex (lane 7). These results indicated that the top complex in lane 3 is a heteromer of SAF and Sp1 and the bottom complex is composed of only SAF. It was interesting that although Sp1 alone could not form a very visible complex with SAS promoter DNA, in the presence of SAF it formed a heteromorphic SAF-Sp1 complex that was readily detectable.

Because protein-protein interactions between transcription factors often lead to both high level and cell-specific gene activation, we evaluated the rate of heteromorphic SAF-Sp1 complex formation with SAS DNA. The rate of SAF-Sp1 heteromeric complex formation was favored (Fig. 5, lanes 1–4) when a constant amount of SAF was used in conjunction with increasing amounts of Sp1. Addition of increasing levels of Sp1 recruited more SAF to form the SAF-Sp1 heteromer, and because a constant amount of SAF was provided, this somewhat lowered the level of SAF-specific complex (lanes 2–4). In a reciprocal experiment, an increasing dose of SAF in the presence of a constant amount of Sp1 again favored the formation of SAF-Sp1 heteromer rather than the SAF-specific complex (lanes 5–8).

Synergistic Transactivation of SAS Promoter Element by SAF and Sp1—To evaluate the in vivo effect of SAF and Sp1 interaction in the transcriptional activation of SAA gene, we performed cotransfection experiments using pSAS-CAT reporter plasmid and expression vectors encoding SAF and Sp1. Transfection assays were conducted in HIG-82 cells because these cells have lower levels of endogenous Sp1 and SAF activity than that present in THP-1 cells (data not shown). Lower levels of endogenous SAF and Sp1 made it easier to observe transactivation in these transfection experiments. Transfection of pSAS-CAT reporter gene with SAF expression plasmid yielded about 6-fold activation, whereas the same amount of Sp1 expression plasmid transactivated the reporter gene about 2.5-fold over the control (Fig. 6). However, combination of equal amounts of both SAF and Sp1 activated the pSAS-CAT reporter gene at a level much higher than that expected from their additive value (Fig. 6). The expression plasmid, pSVSp1-FX (containing a frameshift mutation in the Sp1 coding region), had no positive transactivating effect.

The transactivation potential of the SAF-Sp1 heteromer was further analyzed by cotransfecting cells with SAS-promoter containing reporter plasmid plus a combination of a constant amount of SAF and increasing amounts of Sp1 expression plasmids. The results, described in Fig. 7A, revealed a synergistic dose-dependent transactivation of the reporter gene. Western blot analysis (data not shown) was performed, which verified that the increase of reporter gene expression was proportional to increasing Sp1 protein expression in the transfected cells, whereas the expression level of SAF protein remained same. In a reciprocal experiment, similar synergistic transactivation was seen with a fixed amount of Sp1 and an increasing dose of SAF expression plasmids (Figs. 7B). Western blot analysis verified that the increase in reporter gene expression is proportional to increased SAF production with similar levels of Sp1 production (data not shown). In both experiments, the observed induction level of the reporter gene was always higher than that achieved by a simple additive effect of the two transcription factors. These results suggested that Sp1 may cooperate with SAF and synergize its transactivating ability.

**DISCUSSION**

SAA, a member of the acute phase protein group is gaining more attention in the pathogenesis of atherosclerosis because recent studies have suggested that SAA, by displacing apoA1 from HDL, is capable of affecting lipoprotein metabolism (4). Also, SAA is found to be a constituent of the atherosclerotic lesions (29), which is composed of macrophage-derived foam cells. In an effort to control the SAA production as a therapeutic measure against pathophysiological inflammatory conditions, we undertook this investigation. The mechanism of SAA gene induction was studied, particularly in monocyte/macrophage cells, which play a key role in atherogenesis. In this report we have shown that SAF, a zinc finger transcription factor, interacts with the SAA promoter, and cooperates with Sp1 to synergize the transactivating ability of the latter.
factor is involved in the regulation of SAA induction in THP-1 monocyte/macrophage cells. Also, we provide evidence for physical and functional interactions between the transcription factors SAF and Sp1 that have a synergistic transactivating ability on SAS promoter.

SAS promoter containing −280 to −226 base pairs sequences of SAA gene is induced in response to LPS treatment of THP-1 cells. SAF has been found to interact with this element, and this protein was detected in both hepatic and several nonhepatic cells (19, 20). In an effort to understand whether such interaction of SAF with SAS element results in transcriptional induction, we have studied the transactivation potential of a cloned SAF gene on a reporter gene carrying a SAS element. Increasing reporter CAT activity as more of the SAF cDNA is cloned SAF gene on a reporter gene carrying a SAS element.

In the competition assay, preincubation of nuclear extract with increasing amounts of pSVSp1-F plasmid DNA (2 μg) plus increasing amounts of pSVSp1-F (2 μg) plus increasing amounts of pCMV-SAF plasmid DNA.

![Image](505x750) **Fig. 7.** Transcriptional synergy between SAF and Sp1. HIG-82 cells were cotransfected with pSAS-CAT reporter plasmid and plasmids expressing either Sp1 or SAF or in combination as indicated in the figure. A, pSAS-CAT reporter plasmid (10 μg of DNA) was transfected alone or cotransfected with constant amount of pCMV-SAF plasmid DNA (2 μg) plus increasing amounts of pSVSp1-F plasmid DNA. B, pSAS-CAT reporter plasmid (10 μg of DNA) was cotransfected with pSVSp1-F (2 μg) plus increasing amounts of pCMV-SAF plasmid DNA.

is a superior transactivator of SAA than SAF or Sp1 alone. Accumulative evidence indicates that the specific high level expression of a gene is achieved through the combined actions of a group of transcription factors. In fact, previous studies showed that under some inflammatory conditions NF-κB and C/EBP synergistically transactivate SAA expression (18). Sp1 was recently shown to potentiate the transactivation role of GATA-1, a major erythroid transcription factor (30). The mechanism by which Sp1 potentiates SAF activity is not yet known. It is tempting to speculate that the SAF-Sp1 heteromeric complex establishes a stable contact with basal transcription initiation complex with the help of Sp1 when interacting with the SAS element via SAF. Indeed, the glutamine-rich activation domains of Sp1 were found to bind to a glutamine-rich domain of TAFII 110, which is a major protein component of basal transcription initiation complex (31, 32), and such interaction helps to mediate transcription activation possibly via conformational or covalent modification of the basal transcription complex (reviewed in Ref. 33). SAF-Sp1 interaction may play a critical role in this process where SAF via interacting with the SAS promoter of SAA gene recruits Sp1 to the vicinity of basal transcription complex formed at the TATA box and facilitates Sp1 interaction to TAFII 110.

In summary, our data show that SAF activates SAA gene induction in THP-1 monocyte/macrophage cells. The in vitro biological consequence of the fact that SAF can functionally cooperate with Sp1, a ubiquitously present transcription factor, and synergistically transactivate SAS promoter remains to be determined.

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