Involvement of an SAF-like Transcription Factor in the Activation of Serum Amyloid A Gene in Monocyte/Macrophage Cells by Lipopolysaccharide†

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Received September 29, 1996; Revised Manuscript Received January 21, 1997⊗

ABSTRACT: Serum amyloid A (SAA) has been linked to atherosclerosis because of its ability to remodel high-density lipoprotein by the depletion of apolipoprotein A1, its ability to bind cholesterol, and its presence in the atherosclerotic plaques of coronary and carotid arteries. In the present study, we investigated the induction mechanism of SAA gene in THP-1 monocyte/macrophage cells which play a critical role in the development of atherosclerotic fatty streak and plaque formation. We and others have shown that SAA gene is induced in monocyte/macrophage cells by lipopolysaccharide (LPS). By promoter function analysis, we show that the SAA promoter sequence between −280 and −226 can confer LPS responsiveness. Gel electrophoretic mobility shift assay detected an induced DNA-binding activity in these cells in response to LPS. Characterization of the DNA-binding protein by UV cross-linking, Southwestern blot, and antibody ablation/supershift assays revealed that it is similar to a recently reported nuclear factor designated SAF. These results demonstrated that LPS-mediated SAA gene induction in monocyte/macrophage cells is primarily due to the induction of SAF activity.

Serum amyloid A (SAA)[1] proteins, a member of the acute phase protein family are found in the circulation as major apolipoprotein components of high-density lipoprotein (HDL) during inflammation (Malle et al., 1993; Sipe, 1992). Normally SAA proteins are present in small amounts, but their concentration can increase up to 1000-fold within 24 h as part of a response to various inflammatory conditions (Kushner, 1982). Such an increase of SAA concentration is a consequence of transcriptional induction mediated by several proinflammatory cytokines including IL-1, IL-6, and TNF-α released during inflammation (Mackiewicz et al., 1988; Sipe, 1992). Normally, the serum level of SAA returns back to the low basal level rapidly within 48 h of the initial inflammatory signal. However, a persistent higher level of SAA is seen under chronic inflammatory conditions and it leads to several diseases including rheumatoid arthritis, osteoarthritis, and secondary amyloidosis (Sipe, 1992). High-level induction of SAA and its association with HDL during inflammation suggests that SAA also has a role in lipid metabolism or transport during host−response to injury (Kisilevsky & Subrahmanyan, 1992). Recent evidence suggests that a higher than normal level of SAA may contribute to the risk of atherosclerosis by altering the HDL metabolism and cholesterol transport (Kisilevsky & Subrahmanyan, 1992; Cabana et al., 1989; Steinmetz et al., 1989; Rosenson, 1993).

SAA biosynthesis primarily takes place in the liver. The protein is then released into the bloodstream as a constituent of HDL. Besides the liver, SAA is expressed at various levels by several other cell types including spleen, kidney, lung, adipocytes, and macrophage. However, the extrahepatic synthesis of SAA, specially by monocyte/macrophage cells, is quite significant as SAA has been found to be one of the components of aortic lesions. The arterial fatty streak, an early and reversible precursor to advanced atherosclerotic lesions, is thought to be made up of mostly monocyte-derived macrophages that have taken up modified lipoproteins to become lipid-enriched foam cells (Ross, 1986). The atherosclerotic lesions contain monocyte/macrophage, lipid, apolipoproteins, SAA, and immunoglobulin M (Lusis & Navab, 1993). In response to inflammatory conditions or under the influence of cytokines or LPS, SAA synthesis is induced in peritoneal macrophage and in various monocyte/macrophage cell lines. As SAA has the ability to remodel the HDL and alter cholesterol transport and has been detected in the atherosclerotic lesions of coronary and carotid arteries (Meek et al., 1994), understanding the regulation of SAA biosynthesis in these cell types becomes important.

Studies on the mechanism of the transcriptional induction of SAA gene have shown the involvement of several regulatory transcription factors whose binding elements are present in the 5′-flanking promoter region (Betts et al., 1993; Edbrooke et al., 1989; Huang & Liao, 1994; Li & Liao, 1992; Ray et al., 1995; Ray & Ray, 1993, 1994). Recent studies have identified a novel SAA-activating sequence, SAS, which is essential for the transcriptional induction of SAA gene in several nonhepatic cells (Ray & Ray, 1996). Following IL-6 activation, the induction of a transcription factor called SAS-associated factor (SAF) binding to this element was detected by gel mobility shift assay (Ray & Ray, 1996). In the present study, we examined how SAA expression is regulated in the monocyte/macrophage cells. As a model, human monocytic THP-1 cells were chosen because these cells retained many of the properties of monocyte/macrophage cells and, like native monocyte-derived macrophage, differentiate to mac-

† This work was supported by USPHS grant DK49205 and funds from the College of Veterinary Medicine, University of Missouri.

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: SAA, serum amyloid A; LPS, lipopolysaccharide; SAS, SAA activating sequence; SAF, SAS associated factor; CAT, chloramphenicol acetyl transferase; IL, interleukin; TNF, tumor necrosis factor; HDL, high-density lipoprotein.

S0006-2960(96)02459-2 CCC: $14.00

rophage cells in response to LPS. By promoter function analysis and DNA-binding electromobility shift assay, we show that LPS-mediated induction of SAA gene in THP-1 cells is primarily regulated by SAF transcription factor.

MATERIALS AND METHODS

Plasmid Constructs. Plasmid construct pSAS-CAT2 was prepared by ligating SAA genomic DNA sequences from −280 to −226 into plasmid vector pBLCAT2 (Luckow & Schutz, 1987). A mutant derivative of pSAS-CAT2, pSASmt-CAT2 contained the mutated DNA sequence (5′-CAAGACGGTCACTAGACTCCCAATGAGTCGAGACCGTCGACATCC-3′) ligated into pBLCAT2 vector. Underlined bases indicate substitution. pSAS × 3 CAT2 was constructed by ligating three tandem copies of the SAA promoter sequences from −280 to −226. The selected clones were analyzed by DNA sequencing to verify their authenticity and orientation.

Cell Culture and Transfection. THP-1 cells were obtained from the American Type Culture Collection and maintained in suspension in RPMI-1640 containing 10% fetal calf serum (FCS). No antibiotic was added in the growth medium. For RNA preparation, cells were centrifuged, resuspended in RPMI-1640 containing 10% FCS and dexamethasone (1 μM), and grown in two 75 cm^2 flasks for 24 h. For induction, cells were stimulated with 10 μg of LPS per mL and were grown in 100 mm dishes for additional 24 h. For transient transfection of THP-1 cells, 2×10^7 cells were transfected with 10 μg of plasmid DNA by the DEAE−dextran method (Sambrooke et al., 1989), cultured for 48 h, and then exposed to LPS for additional 12 h. Cell extracts were prepared by the freeze−thaw procedure and protein concentration was measured as described (Bradford, 1976). Following heating to 60 °C for 10 min using equal protein amount of each cell extracts, chloramphenicol acetyl transferase (CAT) activity was determined as described previously (Ray & Ray, 1994). All transfection experiments were performed at least three times.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from uninduced and lipopolysaccharide (LPS)-induced THP-1 cells by using the guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). Fifty microgram of each sample of RNA was fractionated in a 1% agarose gel containing 2.2 M formaldehyde and transferred onto nylon membrane, and the blot was hybridized to a ^{32}P-labeled SAA cDNA probe (Ray & Ray, 1991). The same membrane was subsequently hybridized to an actin cDNA probe to evaluate the quality and quantity of each RNA sample on the membrane.

Oligonucleotides. The sequences of the oligonucleotides synthesized for use as competitors were as follows:

NF-κB: 5′-GATCCATGGGGAATTCCCCATG-3′

Sp1: 5′-TCGACTGGGCGGAGTCTGGA-3′

For synthesizing double-stranded oligonucleotides, complementary strands of the oligonucleotides were heated to 95 °C for 2 min in 50 mM Tris, pH 7.4, 60 mM NaCl, 1 mM EDTA and allowed to cool to room temperature slowly in about 2−3 h. Complementary oligonucleotides were designed to generate 5′ overhangs which were filled in with Klenow fragment of DNA polymerase, incorporating [α-^{32}P]-dCTP as the probe.

Nuclear Extracts and Electromobility Shift Assays (EMSA). A total of 2×10^8 cells were stimulated with 10 μg of LPS per mL for 24 h. Nuclear extracts were prepared from uninduced and LPS-induced THP-1 cells essentially following a method described previously (Dignam et al., 1983) with modifications. The cells were harvested by centrifugation at 500*g* and washed three times in phosphate-buffered saline (PBS). The cells were resuspended in 300 μL of buffer A (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.5 μg/mL each of leupeptin, antipain, pepstatin, 0.1 μg/mL chymostatin, 0.3 TIU/mL aprotinin, 0.5 mg/mL benzamidine). The suspension was kept on ice for 15 min, vortexed vigorously for 10 s and centrifuged in a microfuge at 3000 rpm for 1 min. The supernatant was saved as postnuclear supernatant and the pellet was resuspended in 300 μL of buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5 μg/mL each of leupeptin, antipain, pepstatin, 0.1 μg/mL chymostatin, 0.3 TIU/mL aprotinin, 0.5 mg/mL benzamidine) and incubated on ice for 30 min with occasional stirring. The suspension was then centrifuged in a microfuge at 7500 rpm for 3 min and the supernatant was saved as the nuclear extract. Protein concentrations were measured using the method previously described (Bradford, 1976). DNA-binding assays were performed following a standard protocol described earlier (Ray & Ray, 1994) with ^{32}P-labeled double-stranded DNA probe as described in the text and figure legends. The labeling of DNA was performed by filling in the overhangs at the termini with Klenow fragment of DNA polymerase, incorporating [α-^{32}P]dCTP as the probe. In some binding assays, competitor oligonucleotides were included in the reaction mixture. For antibody interaction studies, anti-Sp1 and anti-NF-κB antisera (both from Santa Cruz Biotechnology) and anti-SAF antiserum (prepared as described below) were added to the reaction mixture during a preincubation period of 30 min on ice. Purified NFκB (p50) and Sp1 proteins were obtained from Promega Corp.

Preparation of SAF Antibody. SAF antibody was developed in mice by using a purified preparation of this protein as an antigen. The protein was isolated from rabbit liver. Both nuclear and postnuclear supernatant fractions were subjected to ammonium sulfate precipitation by the addition of 0.35 g of solid $(NH_4)_2SO_4$/mL. Precipitated proteins were collected by centrifugation, resuspended in buffer A (20 mM Hepes, pH 7.9, 25 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.5 mM PMSF), dialyzed against the same buffer at 4 °C for 6−8 h and assayed for SAF DNA-binding activity. We observed that SAF activity is present in both the nuclear fraction and postnuclear supernatant. The active fraction was subjected to heparin−Sepharose CL-6B (Pharmacia) chromatography for further purification. Briefly, about 20 mg of protein was loaded onto a 10 mL heparin−Sepharose CL-6B column. The bound protein was eluted in a stepwise manner using buffer A containing 0.1, 0.25, 0.5, and 1.0 M KCl. SAF DNA-binding activity was detected primarily in the 0.25 M KCl fraction. Partially purified SAF (the 0.25 M KCl fraction of heparin−Sepharose column) was further purified by oligonucleotide affinity chromatography. The sequence of the SAS oligonucleotide was 5′-CCCCTTCCTCTCCAC-

CCACAGCC-3′. The complementary strand's sequence was 5′GGGGGCTGGGTGGGTGGAGAGGAAG-3′. This double-stranded oligonucleotide corresponds to positions −255 to −230 of SAA promoter. A 250 μg portion of double-stranded oligonucleotide was phosphorylated and self-ligated. The 5′ overhangs of multimerized SAS oligonucleotides were then filled with Klenow and biotinylated dCTP (Sigma) and coupled to 2 mL of streptavidin−agarose (Sigma). After adjustment to 100 mM KCl with column buffer and addition of 2 μg of poly(dI-dC) per mL, the pooled active fractions from the heparin−Sepharose chromatography were loaded and the bound protein was eluted with a 0.2−1.0 M linear KCl gradient. This process resulted in at least 300-fold purification. To generate antiserum against SAF, the pooled purified fraction from DNA-affinity chromatography was injected into mice with Fraund's incomplete adjuvant. Before immunization, the same mice were bled and the serum served as preimmune serum control.

UV Cross-Linking and Southwestern Assay. A synthetic template oligonucleotide containing SAA DNA sequence from −280 to −226 was annealed to a 15-mer oligonucleotide complementary from one end of the template and extended with the Klenow fragment of DNA polymerase in the presence of 5′-bromodeoxyuridine and [α-^{32}P]dCTP. The labeled DNA was purified from a 6% polyacrylamide gel and then used as the probe in binding assays. Following the binding reaction that was carried out using a standard protocol (Ray & Ray, 1996), the reaction mixtures were exposed to ultraviolet light (300 nm) for 15 min and then incubated with 0.5 units of DNase I (Promega Corporation) for 15 min at 37 °C. The reaction was stopped by adding an equal volume of a buffer containing 0.125M Tris HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) β mercaptoethanol, 6 M urea, and 10% (v/v) glycerol and heated at 95 °C for 12 min, and the samples were then fractionated in an 11% SDS−polyacrylamide gel.

Southwestern assay was performed essentially by following the method as described (Miskimins et al., 1985). Nuclear extract (100 μg of protein) was fractionated in an 11% SDS−polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated in 10 mM Tris-HCl, pH 7.0, 50 mM NaCl, 20 mM EDTA, 0.1 mM DTT, 4 M urea buffer for 2 h. Next, it was preincubated for 1 h in binding buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.1 mM $ZnCl_2$, 1 mM DTT) containing 5% nonfat dry milk. After preincubation, the membrane was incubated with radiolabeled concatenated double-stranded SAS oligonucleotide in the binding buffer for 15 h at 4 °C. The membrane was washed three times 15 min each in binding buffer, dried, and autoradiographed.

Western Blot (Immunoblot) Assay. Nuclear extracts (50 μg of protein) were fractionated in an SDS−11% polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane. Immunoblotting with anti-Sp1 and anti-NF-κB antisera was performed as described earlier (Ray & Ray, 1994).

RESULTS

SAA mRNA Is Induced by LPS in THP-1 Monocyte/Macrophage Cells. To study the regulation of SAA induction in THP-1 monocyte/macrophage cells, we first determined the induction level of SAA mRNA in these cells

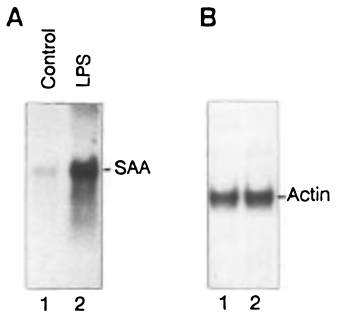

FIGURE 1: SAA mRNA is induced in THP-1 cells in response to bacterial LPS. THP-1 cells were incubated in the presence of LPS (10 μg/mL) for 24 h. Total RNA was prepared, subjected to Northern blot analysis (50 μg of RNA was loaded in each lane), and hybridized using SAA cDNA as a probe. As control, the same blot was hybridized with an actin cDNA probe. Lane 1 contains RNA prepared from uninduced THP-1 cells, and lane 2 contains RNA prepared from LPS-treated THP-1 cells.

(Figure 1). Bacterial lipopolysaccharide (LPS), which causes release of cytokines including IL-1, IL-6, and TNF-α, is a known inducer of the SAA gene both *in vivo* and *in vitro* (Mackiewicz et al., 1988; Meek et al., 1992; Ray et al., 1995; Urieli-Shoval et al., 1994). THP-1 cells cultured in the presence of LPS (10 μg/mL) synthesized a much higher level of SAA mRNA (lane 2) than the control untreated cells (lane 1). Addition of LPS caused differentiation of THP-1 cells to macrophage-like characteristics and the cells adhered to the surface. Constitutive SAA mRNA synthesized by THP-1 cells under the present culturing condition was minimal and the induction of SAA by LPS was manyfold. This result is consistent with some earlier observations (Meek et al., 1992; Urieli-Shoval et al., 1994) and established that SAA gene is indeed induced by LPS in the present culturing conditions in THP-1 monocyte/macrophage cells.

Analysis of the Rabbit SAA Promoter. Previously we have shown that SAA gene induction in at least two nonhepatic cells, e.g., lung and synoviocyte cells, is primarily regulated by SAS promoter (Ray & Ray 1996). To know whether SAS regulates SAA gene induction in THP-1 cells, we first examined the promoter function activity of this element. The reporter gene pSAS-CAT (−280/−226) which contains the SAS DNA sequences was used in transient transfection assays (Figure 2). In the presence of LPS (10 μg/mL), chloramphenicol acetyl transferase activity of pSAS-CAT was induced about 5-fold. The inducive effect was more pronounced when the reporter gene contained multimerized units of SAS. Under the same condition, a reporter gene containing mutated sequence of SAS was not induced by LPS. This result indicated the active role of SAS in regulating LPS-mediated SAA gene induction in monocyte/macrophage cells.

Identification of a LPS-Inducible Nuclear Protein in THP-1 Cell Nuclear Extract. Since transcription of the reporter gene driven by SAS promoter was induced, it seemed logical to seek, in LPS-induced THP-1 cells, for the transcription factor(s) that can interact with this promoter. THP-1 cells were incubated in the presence of LPS (10 μg/mL) for various lengths of time, and nuclear extracts were prepared from uninduced and LPS-induced cells. Using uninduced THP-1 cell nuclear extract and SAS promoter sequence from −280 to −226 as the probe, a weak DNA−protein complex was seen (Figure 3A, lane 1). However, when we used the same protein amount of various LPS-

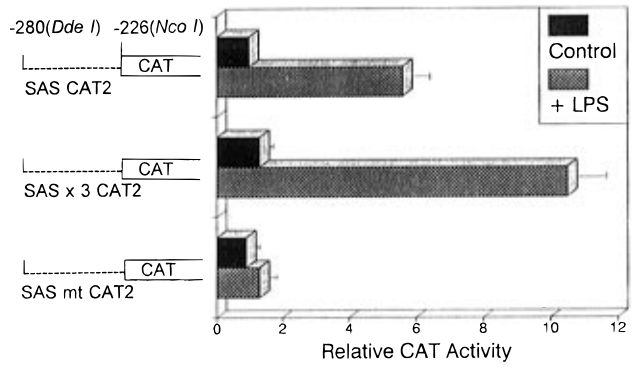

FIGURE 2: Analysis of SAA promoter activity in THP-1 cells. A physical map of the SAA promoter region containing sequences from *Dde*I to *Nco*I site was ligated to the pBL CAT2 vector. SAS X 3 CAT2 contains three tandem copies of the SAA promoter sequence from −280 to −226. SAS mt CAT2 contains a single copy of the mutated sequence of SAA promoter (−280 to −226). The mutated sequence is described in Materials and Methods. THP-1 cells were transfected with 10 μg DNA of the indicated plasmids. For induction, transfected cells were incubated in the presence of bacterial LPS (10 μg/mL). The details of transfection are described in Materials and Methods. The results represent average of three separate experiments.

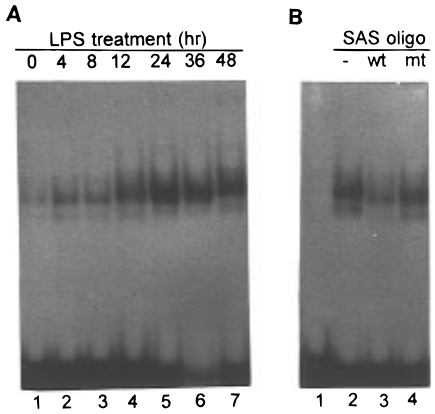

FIGURE 3: Detection of DNA−protein complexes using electrophoretic mobility shift assay. (A) Nuclear extracts (10 μg protein) prepared from THP-1 cells, both uninduced (lane 1) and LPS-induced for 4−48 h (lanes 2−7), were incubated with SAS probe, a ^{32}P-labeled SAA DNA fragment containing the SAA promoter sequence from −280 to −226. The resulting complexes were resolved in a 6% nondenaturing polyacrylamide gel. One major DNA−protein complex was seen. (B) Competition analysis of the DNA−protein complex formed by the LPS 24 h induced THP-1 cell nuclear extract (10 μg protein) and ^{32}P-labeled SAS probe. Wild-type (wt) SAS oligonucleotide (oligo) containing sequences from −280 to −226 and a mutant (mt) derivative whose sequence is given in Materials and Methods were used as competitors in some binding assays (lanes 3 and 4, respectively). Lane 2 contains no competitor oligo and lane 1 contains probe only.

induced THP-1 cell nuclear extracts, the intensity of this DNA−protein complex was increased severalfold (Figure 3A, lanes 2−7). This result indicated the induction of a DNA-binding protein activity in LPS-treated THP-1 cells. The level of induction of this protein appeared to be a maximum at 24 h, which remained at a similar level for up to 48 h. Inhibition of this DNA-binding activity by the wild-type SAS oligonucleotide (Figure 3B, lane 3) but not by a mutated oligonucleotide (Figure 3B, lane 4) established that this DNA-binding activity is highly specific in nature. Complete inhibition of the DNA-binding activity was achieved by the addition of increasing concentrations of the wild-type SAS oligonucleotide (data not shown).

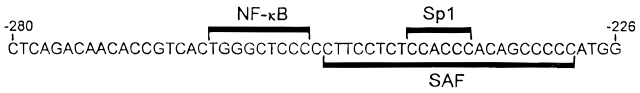

FIGURE 4: SAA promoter sequence. DNA sequence of the SAA promoter region spanning −280 to −226 is shown. Homologies to known transcription factor binding elements are indicated.

Characterization of the LPS-Inducible Nuclear Factor. Inspection of the DNA sequence in the SAA promoter region between −280 and −226 (Figure 4) revealed the presence of a 5′-CCACCC-3′ sequence, which in its opposite orientation (5′-GGGTGG-3′) appears as a potential Sp1 binding element. The optimal binding element for Sp1 has a 5′-GGGCGG-3′ consensus sequence (Letovsky & Dynan, 1989). Also, a TGGGCTCCC sequence where the NF-κB transcription factor can potentially interact is present in this region. We examined if indeed Sp1 and NF-κB transcription factors can interact with these potential Sp1 and NF-κB binding elements. Three different approaches were used to address this question. (i) Purified Sp1 (Figure 5A, lanes 1 and 3) or NF-κB (Figure 5B, lane 1) proteins formed DNA−protein complexes with the SAA promoter (−280/−226). (ii) Competition assays with oligonucleotides containing a consensus Sp1 (Figure 5A, lane 2) or a consensus NF-κB (Figure 5B, lane 2) binding sites effectively abolished the DNA−protein complex formation with the SAA probe. (iii) Addition of Sp1 antibody (Figure 5A, lane 4) or NF-κB antibody (Figure 5B, lane 3) supershifted the DNA−protein complexes. These results, taken together, strongly indicated that purified Sp1 and NF-κB transcription factors can interact with the SAA promoter (−280/−226).

To determine if Sp1 and NF-κB in the THP-1 cell nuclear extract are responsible for the formation of the DNA−protein complex seen in Figure 3, we conducted a competition DNA-binding assay using LPS-treated THP-1 nuclear extract and a molar excess of either Sp1 or NF-κB binding site oligonucleotides. Addition of Sp1 (Figure 5C, lane 2) or NF-κB (Figure 5C, lane 3) binding site oligonucleotide did not reduce the level of the major DNA−protein complex. For further verification we conducted antibody ablation/supershift experiment using antibodies directed against Sp1 and NF-κB. As can be seen, preincubation of LPS-treated THP-1 cell nuclear extract with anti-Sp1 antibody (Figure 5C, lane 4) or anti-NF-κB antibody (Figure 5C, lane 5) showed the presence of a minor but distinct supershifted band. This result indicated that Sp1 and NF-κB, present in the THP-1 nuclear extract, could bind weakly to the SAA promoter. Such a weak interaction might be due to the degenerated lower affinity binding elements for Sp1 and NF-κB in the SAA promoter or could be due to very low amounts of these transcription factors in our THP-1 cell nuclear extract preparation. First possibility was tested by comparing the DNA-binding activities of Sp1 and NF-κB using their consensus high-affinity binding elements as probes. As seen in Figure 5D lanes 1−8, purified Sp1 and NF-κB interacted at a much lower level with the SAA promoter than their respective consensus high-affity binding elements. In a reciprocal experiment, we used LPS-treated THP-1 nuclear extract in the DNA-binding assay with the consensus Sp1 and NF-κB probes and compared the DNA-binding activity with that of SAA probe. THP-1 nuclear extract produced a high level of specific DNA−protein complexes with both Sp1 and NF-κB probes, and such DNA−protein complexes

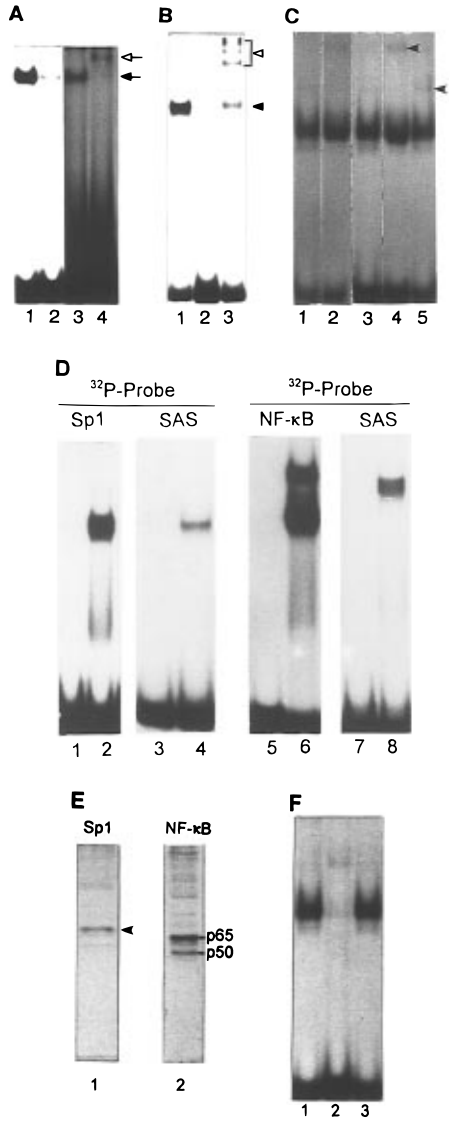

FIGURE 5: Characterization of the LPS-induced DNA−protein complex. (A) Purified Sp1 protein (0.1 unit) was incubated with a ^{32}P-labeled SAA DNA fragment (−280/−226) containing the SAS promoter (lanes 1−4). Lanes 1 and 3 contain no competitor oligonucleotide, lane 2 contains a 20-fold molar excess of unlabeled Sp1 binding oligonucleotide and lane 4 contains Sp1 antibody. The migration position of DNA−Sp1 complex is indicated by a solid arrow and the antibody-mediated supershifted complex is indicated by an open arrow. (B) Purified NF-κB protein (0.1 unit of p50) was incubated with the ^{32}P-labeled SAA DNA fragment (−280/−226) (lanes 1−3). Lane 1 contains no other addition, lane 2 contains 20-fold molar excess of unlabeled NF-κB binding oligonucleotide, lane 3 contains NF-κB antibody. Migration position of the DNA−NF-κB complex and the antibody-mediated supershifted complex are indicated by closed and open arrowheads respectively. (C) Nuclear extract (10 μg of protein) prepared from LPS-induced THP-1 cells was incubated with the ^{32}P-labeled SAA DNA fragment (−280/−226) (lanes 1−5). Lane 1 contains no other addition. Lanes 2 and 3 contain a 20-fold molar excess of unlabeled Sp1 and NF-κB binding oligonucleotides, respectively. Lanes 4 and 5 contain Sp1 and NF-κB antibodies, respectively. Arrow heads indicate the appearance of noticeable supershifted complexes in lanes 4 and 5. (D) Binding efficiencies of Sp1 and NF-κB proteins to SAA promoter. ^{32}P-labeled probes (equivalent molar amounts) containing either a high-affinity Sp1 binding element (lanes 1 and 2) or a SAA DNA fragment (−280/−226) called SAS (lanes 3 and 4) were compared for the binding of purified Sp1 protein (Promega). Lanes 1 and 3 contain probe only, and lanes 2 and 4 contain 0.1 unit of purified Sp1 protein. ^{32}P-labeled probes (equivalent molar amounts) containing either a high-affinity NF-κB binding element (lanes 5 and 6) or a ^{32}P-labeled SAA DNA fragment (−280/−226) containing the SAS element (lanes 7 and 8) were compared for the binding of purified NF-κB (p50) protein. Lanes 5 and 7 contain probe only, and lanes 6 and 8 contain 0.1 unit of purified NF-κB (p50) protein (Promega). (E) Western blot analysis of LPS-treated THP-1 cell nuclear extract for detection of transcription factors, Sp1 and NF-κB. Nuclear extract was fractionated in a denatured polyacrylamide gel, electrophoretically transferred to nitrocellulose membrane and probed separately with antisera to Sp1 and NF-κB-(p50 + p65) as described in Materials and Methods. (F) SAF interaction to SAS element. LPS-treated THP-1 nuclear extract was incubated with the ^{32}P-labeled SAA DNA fragment (−280/−226) containing SAS element (lanes 1−3). In some binding reactions either anti-SAF antibody (lane 2) or preimmune serum (lane 3) were included.

were inhibited only by the wild-type competitor oligonucleotide (data not shown), indicating the presence of active Sp1 and NF-κB transcription factors in the THP-1 nuclear extract preparation. To further verify their presence in LPS-treated THP-1 cell nuclear extract we employed Western blot analysis (Figure 5E). Detectable levels of these transcription factors were present in THP-1 nuclear extract. These results, taken together, indicated that Sp1 and NF-κB binding elements present in the SAA promoter between sequences −280 and −226 are less than optimal since only a highly purified, essentially homogeneous preparation of Sp1 and NF-κB could only moderately interacted with it. Thus the major DNA−protein complex formed by THP-1 nuclear extract, seen in Figures 3 and 5C, is not composed of these two transcription factors.

Previously we showed that a cytokine inducible nuclear protein SAF, can interact with SAA promoter (−280/−226) (Ray & Ray, 1996). We generated antibody against this protein. As seen in Figure 5F (lanes 1−3), the specific DNA−protein complex was partly inhibited and partly supershifted (lane 2) by an antibody directed against a purified preparation of SAF protein but not by the preimmune serum (lane 3). These results demonstrated that although Sp1 and NF-κB can weakly interact with the SAA promoter, the major DNA−protein complex formed by LPS-induced THP-1 cell nuclear extract was formed by a SAF-related protein.

UV Cross-Link and Southwestern Analysis. To determine the tentative molecular weight of the SAF-like factor in THP-1 cells, we performed a UV cross-linking experiment (Figure 6A). DNA-binding assay was conducted using LPS-induced THP-1 cell nuclear extract and a bromodeoxyuridine labeled SAS probe (−280/−226). The incubation product was irradiated with UV light and the sample was analyzed by SDS−PAGE. The proteins cross-linked to the labeled SAA probe were visualized by autoradiography. One protein migrating at about the 55 kDa position was seen.

In a different approach, we performed Southwestern analysis. Nuclear proteins of LPS-treated THP-1 cells were fractionated by SDS−polyacrylamide gel electrophoresis. The proteins were then transferred electrophoretically to a nitrocellulose membrane and hybridized using a radioactive concatenated SAS oligonucleotide probe (Figure 6B). The radioactive SAS probe hybridized with a protein of molecular weight of approximately 55 kDa.

DISCUSSION

Recent studies have linked SAA in the pathogenesis of atherosclerosis: (i) SAA is identified in the atherosclerotic lesions of coronary and carotid arteries as well as in the

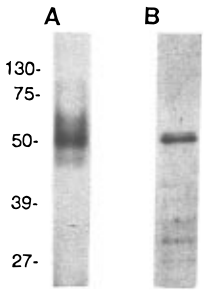

FIGURE 6: Characterization of SAS-binding factor in LPS-induced monocyte/macrophage cells. (A) UV cross-linking assay. Nuclear extract (10 μg of protein) prepared from LPS-treated THP-1 cells was incubated with a bromodeoxyuridine-containing ^{32}P-labeled SAA DNA fragment (−280/−226). After irradiation with UV light, the reaction products were fractionated in an 11% SDS−polyacrylamide gel. Molecular masses of standard protein markers are indicated in kilodaltons. (B) Southwestern blot assay. Nuclear extract (100 μg of protein) from LPS-treated THP-1 cells was fractionated in an 11% SDS−polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane and hybridized with ^{32}P-labeled concatenated double-stranded SAS oligonucleotide probe. Migration positions of molecular weight markers are the same as in panel A.

smooth muscle and endothelial cells (Meek et al., 1994); (ii) SAA has been shown to bind cholesterol (Liang & Sipe, 1995); (iii) SAA can influence and alter HDL-mediated cellular cholesterol efflux (Banka et al., 1995); (iv) a high-fat diet or injection of mildly oxidized LDL, which eventually leads to atherosclerotic plaque development, induce SAA synthesis (Liao et al., 1994). Although at a lower level, SAA is known to be expressed and induced at various extrahepatic locations. In humans, SAA mRNA is documented in atherosclerotic lesions, cultured vascular cells, and cultured monocyte/macrophage cell lines (Meek et al., 1992, 1994; Urieli-Shoval et al., 1994). Since macrophages play a key role in mediating inflammation and atherogenesis, understanding the regulation of SAA biosynthesis in these cell types becomes quite important. Prior studies have shown that hepatic and nonhepatic expressions of SAA gene are regulated via different promoter elements (Ray & Ray 1996). Induction of SAA expression in at least two nonhepatic cells, e.g., lung and synoviocyte, is regulated by a novel promoter element termed the SAA-activating sequence or SAS. Here, we assessed whether SAS promoter element controls SAA gene induction in monocyte/macrophage cells. A combination of DNA−protein interaction and promoter function analysis were used to investigate this aspect.

As a representative of monocyte/macrophage cells, the human monocytic leukemia cell line THP-1 was chosen, because these cells retained many properties of native monocyte/macrophage cells and have been widely used as an *in vitro* model for monocyte/macrophage cells. These cells contain the characteristic monocyte surface markers, produce lysozymes, are phagocytic, can be differentiated into macrophage like cells, and also convert into foam cells in response to coculture with lipoproteins (Banka et al., 1991). LPS showed a profound effect in inducing SAA gene expression in these cells (Figure 1). Our functional studies using the SAS promoter demonstrated that similar to lung and synoviocyte cells (Ray & Ray, 1996), this region of the SAA promoter can promote increased SAA gene transcription in monocyte/macrophage cells in response to LPS addition in the culture medium.

To identify potential transcription factor acting through SAS promoter, we employed electrophoretic mobility shift assay. This assay (Figure 3) has shown that a factor, present in the THP-1 cell nuclear extract, forms a DNA−protein complex with the SAS promoter (−280/−226). Additional studies on the characterization of this factor, described in Figures 5F and 6, have indicated that it is similar to SAF, a transcription factor reported recently (Ray & Ray, 1996). Increased SAA gene transcription together with the increased DNA-binding activity of SAF, suggests that SAF plays a positive regulatory role in SAA gene expression in monocyte/macrophage cells. SAF was previously shown to be important for the transcriptional induction of SAA gene in lung and synoviocyte cells (Ray & Ray, 1996) in response to proinflammatory cytokine, IL-6. LPS is known to activate cytokine expression including IL-6 production by monocyte/macrophage cells (May et al., 1988; Ray & Ray, 1995). It is likely, therefore, that SAF activation in THP-1 cells by LPS involves participation of a cytokine like IL-6. Further studies along this line are currently underway.

THP-1 cells seem to contain a low level of constitutive SAF-like activity (Figure 3A, lane 1). It will be interesting to know whether both a constitutive and an inducible isoform of this protein exists in the THP-1 cells. The gradual increase of SAF DNA-binding activity by LPS suggests that this protein may be *de novo* synthesized upon LPS stimulation, although we cannot rule out the possibility that such an increase in the DNA-binding activity could arise from the stabilizing effect of LPS. LPS is shown to regulate mRNA stability as well as translation of a number of cytokines and protooncogenes including TNF, IL-1, interferon, and GM-CSF (Thorens et al., 1987).

DNA-binding studies, described here, clearly demonstrated that one major complex is formed by the interaction of SAA probe (−280/−226) and nuclear extract from LPS-treated THP-1 cells. Also, we found that Sp1 and NF-κB can interact, albeit weakly, with SAA promoter at this region (Figure 5). Close proximity of NF-κB- and SAF-binding elements and overlapping SAF and Sp1 binding sites in the SAA promoter are quite significant because they can provide a considerable concentration of regulatory transcription factors within the local environment. As transcription factors often either synergize or antagonize each other's transactivating ability via protein−protein interaction, the close proximity of several transcription factor binding elements in a gene promoter provides a unique mode of regulation. Recent studies have shown that Sp1 and NF-κB, by physically interacting with each other, can activate HIV gene transcription (Perkins et al., 1994). Similar protein−protein interaction between the transcription factors SAF, Sp1, and NF-κB is yet to be seen.

In summary, our study has shown that LPS-mediated SAA gene induction in the monocyte/macrophage cells is regulated by the SAS element present within −280 to −226 bases of the SAA promoter. This study indicated the induction of the DNA-binding activity of SAF by LPS in the monocyte/macrophage cells. Further experiments using the cloned gene of SAF will determine how this transcription factor is activated by LPS. Experiments along this line are currently in progress.

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BI9624595