Activation of Sp1 and Its Functional Co-operation with Serum Amyloid A-activating Sequence Binding Factor in Synoviocyte Cells Trigger Synergistic Action of Interleukin-1 and Interleukin-6 in Serum Amyloid A Gene Expression*

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The serum amyloid A (SAA) protein has been implicated in the progression and pathogenesis of rheumatoid arthritis through induction of collagenase activity in synovial fibroblast cells that line the joint tissues. We demonstrate that SAA is synergistically induced in synovial cells by interleukin (IL)-1 and IL-6 that are present at significantly high level in the synovial fluid of arthritis patients. These cytokines induced phenotypic changes in synovial cells, promoting protrusion and increased cellular contact. Induction of SAA under this condition is mediated by promoter elements located between −254 and −226, which contains binding sites for transcription factors Sp1 and SAA activating sequence binding factor (SAF). Mutation of these sequences abolishes SAA promoter response to IL-1 and IL-6. The role of Sp1 in SAA induction was demonstrated by increased DNA binding activity, phosphorylation, and increased protein content of Sp1 during cytokine treatment. Sp1 interacts with the SAA promoter in association with SAF as an SAF-Sp1 heteromeric complex. Furthermore, using a phosphatase inhibitor, we demonstrated increased transactivation potential of both Sp1 and SAF as a consequence of a phosphorylation event. These results provide first evidence for cytokine-mediated activation of Sp1 in synovial fibroblast cells and its participation in regulating SAA expression by acting in conjunction with SAF.

Serum amyloid A (SAA), a member of the acute-phase protein group, is implicated in the pathophysiology of several chronic inflammatory diseases including rheumatoid arthritis, amyloidosis, and atherosclerosis. Its level increases up to 1,000-fold in response to both acute and chronic inflammatory conditions. Consistent with this observation, the plasma concentration of SAA in arthritis patients may range from 1 to 1,000 μg/ml or more (1, 2), and SAA has been found in abundance in the synovial fluid recovered from inflamed joints of rheumatoid arthritis patients (3, 4). Current hypothesis suggests that locally synthesized SAA by synovial fibroblast cells in the inflamed joints acts as an autocrine inducer of matrix metalloproteinase-1 (collagenase), the only enzyme that degrades interstitial collagen I, II, and III at neutral pH and causes extensive joint erosion (5, 6). Synovial fluid samples obtained from the knee joints of rheumatoid arthritis patients have been found to contain high levels of IL-1, IL-6, and a few other proinflammatory cytokines that may play a critical role in the manifestation of chronic inflammation and articular destruction (7). Presence of high levels of proinflammatory cytokines could possibly trigger local production of SAA in the synovial fibroblast cells. Indeed, it has been shown that in synovial fibroblast cells, IL-1 can induce SAA expression (6). However, the molecular mechanism by which IL-1-mediated SAA gene induction occurs is not well understood. Moreover, the combinatorial effect of IL-1 and IL-6, the two cytokines found in abundance in synovial fluid of rheumatoid arthritis patients, in SAA gene expression in synovial fibroblast cells is also unknown. The present study was undertaken to elucidate the mechanism by which IL-1 and IL-6 stimulate SAA in synovial cells.

Because of its link with rheumatoid arthritis, amyloidosis, and atherosclerosis, the mechanism of SAA gene induction for the past few years has been a subject of intense investigation by many laboratories, including ours. SAA biosynthesis during acute-phase response is highly induced in the liver, and its concentration drops rapidly to a low background level within a few days. The increase of SAA biosynthesis during inflammation is due largely to its increased transcription (8). Stability of mRNA also contributes to the enhanced expression of SAA in mouse (9) and human (10, 11). Studies on SAA gene transcription indicated involvement of C/EBP (12–17) and NF-κB (15, 18–20) in human, mouse, rat, and rabbit. Induction of common promoter elements in these species indicates a signaling and response pathway that presumably remained conserved during evolution. Although liver is a major site of SAA expression, extrahepatic tissues are also involved in expressing this gene. Such a local production of SAA is linked to the pathogenesis of several diseases. For example, in rheumatoid arthritis, SAA biosynthesis in the synovial cells of joint tissue is highly induced (3–6). Also SAA expression in macrophage cells (21–23) and in aortic smooth muscle cells (24) is likely to play a significant role in atherosclerosis. Recent studies indicate that SAA is also synthesized in the brain (25). Although it is expressed in both hepatic and extrahepatic cells, the expression level of SAA has been found to vary markedly depending on the cell types (26) and on the nature of inflammatory agents (27).

Part of the difference lies with the complexity of the SAA gene with the presence of multiple isoforms in many species (reviewed in Ref. 28). In humans, for example, multiple SAA...
isoforms, designated as SAA 1–4 have been reported among which SAA4 is constitutively expressed, SAA3 is not expressed, and SAA1 and SAA2 isoforms are expressed with varying degrees of induction in different tissues. In mouse, five SAA isoforms have been reported. Mouse SAA1 and SAA2 isoforms are expressed and induced predominantly in the liver, whereas SAA3 isoform is induced in multiple tissues during inflammation. Similar to human SAA4, mouse SAA5 is constitutively expressed, although mouse SAA4, which is analogous to human SAA3, is not expressed. In rabbit so far three isoforms of SAA have been reported. Nonhepatic induction of mouse SAA3 has been extensively studied, which revealed that C/EBP-δ and SAA enhancing factor are major regulators (13). In rabbit, besides C/EBP and NF-κB, a novel transcription factor termed as SAP is involved in the cytokine-induced expression of rabbit SAA2 gene (29). In this report, we provide the first evidence of the involvement of Sp1 in inducing rabbit SAA2 gene expression in response to IL-1 and IL-6 cytokines in synovial cells.

MATERIALS AND METHODS

Cell Culture and Transfection—Rabbit synoviocyte (HIG82) cells were obtained from American Type Culture Collection. HIG82 cells were cultured in Dulbecco’s modified Eagle’s medium containing high glucose (4.5 g/liter) supplemented with 7% fetal calf serum. HIG82 synoviocytes were derived from the interarticular soft tissue of the knee joint of a normal female New Zealand White rabbit. These cells have retained many of the features and, similar to the tissue of origin, are activated by phorbol myristic acid and express genes coding for enzymes such as collagenase, gelatinase, and cascinase. For induction, HIG82 cells were stimulated with IL-1β (200 units/ml), IL-6 (1000 units/ml), or both for 48 h.

Transient transfections of HIG82 cells were carried out by the calcium phosphate method (30). Transfections were carried out using a mixture of DNAs containing 10 μg of chloramphenicol acetyltransferase (CAT) reporter plasmid, 2 μg of plasmid pSV-β-gal (Promega) as a control for measuring transfection efficiency, and carrier DNA so that the total amount of DNA in each transfection remained constant at 16 μg. Cells were harvested 48 h post-transfection, and CAT activity was determined from cell extracts as described previously (30). For CAT assays, extracts were heated at 60 °C for 10 min to inactivate endogenous acetylase and assayed for β-galactosidase activity. Different effectors used in the transfection assay had no effect on β-galactosidase expression. An equivalent amount of each cell extract was used in the determination of CAT activity. All values reported have been corrected for background activity, which was determined from mock-transfected cells. All transfection experiments were performed at least three times.

Phalloidin staining—HIG82 cells were cultured as described above. Cells were washed in PBS, fixed in 1% glutaraldehyde in 0.08 M sodium cacodylate buffer (pH 7.4) at room temperature, and washed in PBS. The cells were then stained with rhodamine-conjugated phalloidin for 30 min, washed in PBS, and the coverslips were mounted on slides for viewing with fluorescence microscope.

Results

Synergistic Induction of SAA by IL-1 and IL-6—To determine the pattern by which SAA is expressed in synovium exposed to cytokines, rabbit synovial cells (HIG82) were incubated in the presence of IL-1, IL-6, and IL-1 plus IL-6, and the level of SAA mRNA was measured by Northern blot analysis. As shown in Fig. 1, IL-1 alone had a higher stimulatory effect than IL-6, whereas the combination of IL-1 and IL-6 synergistically activated SAA mRNA expression.

Cytokine Treatment Triggers Phenotypic Changes in Synovial Fibroblast Cells—Cytokine-treated cells exhibited striking morphological changes when visualized using a phase contrast microscope (data not shown). To further verify the cytokine-induced cytoskeletal structural modification, we have analyzed the cells by scanning electron microscopy (Fig. 2). This analysis revealed long protrusions in cytokine-treated cells that extend from one cell and interact with surrounding cells in culture. Such protrusions are characteristic of cells attempting to es-

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establish a connection with other cells to form an association that are seen in cells undergoing differentiation. This process is facilitated by the polymerization of actin that forms filamentosus or F-actin giving rise to polymorphic shapes of the cells with pseudopod-like protrusions (35). Formation of such structural configuration was evaluated by rhodamine-conjugated phallodin staining for F-actin and visualized by fluorescence microscopy as described (36). Results of this experiment, shown in Fig. 3, revealed significant formation of F-actin bundles, a thread-like extensions (Fig. 3, B-D). Normally slender and elongated protrusions of F-actin are seen in control HIG82 cells (Fig. 3A). Upon exposure to cytokines, these become bulky and readily fuse with those from the adjacent cells creating a connection between the cells. These results demonstrated a distinct change in the morphology of synovial cells in response to cytokines like IL-1 and IL-6.

The Promoter Region between −254 to −226 Is Essential for Optimal IL-1 Plus IL-6 Response—Cytokine-induced cellular changes significantly altered SAA transcription causing accumulation of this transcript. In an effort to identify the cytokine-response element of SAA promoter, a series of 5′-deletion constructs were prepared (Fig. 4A). These constructs were transiently transfected in rabbit synovial cells, followed by the addition of IL-1 plus IL-6. The results from the transfection assay indicated that deletion of sequences from −314 to −280 has no effect on cytokine-mediated CAT reporter gene induction. In contrast, significant reduction in CAT gene induction was noticed when the region between −280 and −193 was deleted. Further removal of upstream sequences abolished any measurable effect of cytokines. The results of functional promoter analysis suggested that sequences between −280 and −193 were most important for IL-1 plus IL-6 mediated inducible expression of the SAA gene in rabbit synovial cells. Further analysis of the cytokine-inducible region was done by preparing some additional deletion constructs. As shown in Fig. 4B, a reporter gene containing sequences between −254 and −226 retained full responsiveness to IL-1 plus IL-6 stimulation. Mutation in this region resulted in a loss of responsiveness to cytokine stimulation. These results supported the involvement of sequences between −254 and −226 in the cytokine activation of the SAA gene in synovial cells.

Characterization of Nuclear Factors Induced by IL-1 and IL-6—To investigate the nuclear factors in cytokine-activated synoviocyte that might interact with the above cytokine-responsive element, we performed DNA binding assays using SAA promoter (−254/−226) and various cytokine-treated synovial cell nuclear extracts. Control, untreated nuclear extract formed three major complexes, termed as a, b, and c (Fig. 5A, lane 1). The same protein amount of nuclear extracts from IL-1, IL-6, and IL-1 plus IL-6-induced cells formed five complexes, a, b, c, d, and e (Fig. 5A, lanes 2–4). These results showed that cytokines can induce some nuclear proteins that form inducible DNA-protein complexes like a and b with SAA promoter. Noticeably, the levels of a and b were highest in cells treated with IL-1 plus IL-6, whereas the levels of constitutively present complexes c, d, and e remained the same. The specificity of these DNA-protein complexes was determined by competition with a molar excess of unlabeled homologous probe (Fig. 5B, lanes 2, 8, 14, and 20) and mutated probe (Fig. 5B, lanes 3, 9, 15, and 21). Because complex c was not inhibited by the homologous probe, it indicated that this complex was formed by either a nonspecific or a very low affinity interaction of a protein with SAA promoter. Polyclonal antibodies were used to determine the identity of these nuclear proteins. This region of the SAA promoter contains an element where the transcription factor SAF binds. Also a potential Sp1 binding domain is located in this region of the SAA promoter. Antisera to these two transcription factors were used. A complete inhibition of complexes b, d, and e and partial inhibition of complex a were obtained using antibody directed against SAF protein (Fig. 5B, lanes 4, 10, 16, and 22). At a higher concentration of the anti-SAF antibody, complex a was also completely inhibited (Fig. 5C). Anti-Sp1 antibody partially supershifted only complex a (Fig. 5B, lanes 5, 11, 17, and 23) and at a higher

**Fig. 1. Northern blot analysis of SAA mRNA in synoviocyte cells.** HIG82 cells were grown in control medium (lane 1) or in the presence of IL-1 (lane 2), IL-6 (lane 3), or IL-1 plus IL-6 (lane 4) for 48 h. Total RNA (50 μg) prepared from these cells were fractionated in a formaldehyde-agarose gel, transferred onto nylon membrane, and hybridized to 32P-labeled SAA cDNA probe. The same membrane was reprobed with 32P-labeled β-actin cDNA for qualitative and quantitative evaluation of the RNA samples.

**Fig. 2.** Scanning electron microscopy of synoviocyte cells following cytokine treatment. HIG82 cells were grown in culture medium described under “Materials and Methods” in the absence (panel A), or presence of IL-1 (panel B), IL-6 (panel C), or IL-1 plus IL-6 (panel D) for 48 h. Cells were processed for scanning electron microscopy as described under “Materials and Methods.” The white bars in each panel represent 10 nm.
concentration, completely supershifted complex a (Fig. 5C). Complete inhibition or supershifting of complex a by anti-SAF or anti-Sp1 antibodies indicate that complex a is formed by a combined interaction of SAF and Sp1 with the SAA probe and not because of a comigration of two independent complexes formed by SAF or Sp1. Also, in a previous study using affinity purified Sp1 protein (23), we noticed that Sp1 itself does not bind to the SAA promoter. Other three complexes, b, d, and e, are formed by SAF-like proteins. Taken together, these results suggested that activation of SAA promoter after cytokine stimulation is closely related to the induction of both SAF and Sp1 factors, in which the combination of IL-1 and IL-6 had the most stimulatory effect. Noticeably, no DNA-protein complex was detected that was formed by Sp1 alone, indicating that Sp1 cannot directly bind to the SAA promoter.

**Increased Level of DNA Binding Activity in Cytokine-induced Synovial Cells**—A high level of complex a, which is formed by both SAF and Sp1, potentially could arise because of an increase of DNA binding activities of these two transcription factors or it could be because of only SAF activation. To determine the contribution of Sp1 in the formation of complex a, we assayed the level of Sp1 DNA binding activity in various cell nuclear extracts (Fig. 6). Because the SAA promoter (−254 to −226) is interacted by both SAF and Sp1 proteins, we chose a high-affinity and highly specific Sp1 DNA binding element as a probe for measuring Sp1 activity to avoid any functional interference from SAF proteins present in the nuclear extracts. Moreover, a previous study (23) indicated that Sp1 does not bind by itself to the SAA promoter. With control nuclear extract, three faint DNA-protein complexes, a, b, and c, in which complex a is major, were detected (lane 1). Cytokine treatment increased the level of complex a with the highest activity seen in IL-1 plus IL-6-treated cells (lanes 2–4). The levels of the other two minor complexes, b and c, were only slightly increased during cytokine treatment. The formation of these complexes was completely abolished by the presence of an excess of unlabeled homologous Sp1 oligonucleotide (Fig. 6B, lanes 2, 7, and 12) but not by the presence of unlabeled mutated Sp1 oligonucleotide (Fig. 6B, lanes 3, 8, and 13). Anti-Sp1 antibody supershifted only complex a (Fig. 6B, lanes 4, 9, and 14), whereas nonspecific antibody had no effect on these complexes (Fig. 6B, lanes 5, 10, and 15). These results showed that complex a is formed by Sp1, and complexes b and c may be formed by any of the other Sp1-like proteins such as Sp2, Sp3, and Sp4 (37). Thus, Sp1 is highly induced in synoviocyte cells particularly in response to IL-1, and a synergistic induction is achieved by the combined action of IL-1 and IL-6.

**Western Blot Analyses of SAF and Sp1**—The increase in SAF and Sp1 DNA binding activities led us to examine their protein levels. Western blot analysis of untreated and various cytokine-treated nuclear extracts with anti-SAF antibody revealed that the level of SAF protein is essentially the same in these four cell extracts (Fig. 7A). However, the protein levels of Sp1 was seen to be appreciably higher in cytokine-treated cells (Fig. 7B).
To verify that this is not because of any difference in total protein content loaded in the gel, we stained the gel containing fractionated nuclear proteins with Coomassie Blue. As seen in Fig. 7C, in all cases, the same amounts of nuclear proteins of comparable quality were used. Also, SAF content in Fig. 7A served as an internal normalization control. These results indicated that increased SAF activity in response to cytokine treatment of HIG82 cells most likely involve a post-transla-

Fig. 5. Electrophoretic mobility shift assay for the detection of DNA binding activity in cytokine-treated synoviocyte cells. A, 32P-labeled SAA element (−254/−226) was incubated with 10 μg of nuclear extracts prepared from uninduced (lane 1), IL-1 treated (lane 2), IL-6 treated (lane 3), and IL-1 plus IL-6 treated (lane 4) HIG82 cells. DNA-protein complexes were resolved in a 6% native polyacrylamide gel. Detectable complexes are designated as a through e. B, the abovementioned DNA probe and four different nuclear extract preparations, described in panel A, were incubated in the presence of 50-fold molar excess of either wild-type (wt) or mutant (mt) competitor oligonucleotides containing SAA sequences as described under “Materials and Methods.” In some reactions, nuclear extracts were preincubated with anti-SAF antibody (1 μl of a 10-fold diluted stock), anti-Sp1 antibody (1 μl of the stock), or nonspecific (NS) serum. The arrow indicates the migration position of supershifted complexes in lanes 11, 17, and 23. C, 32P-labeled SAA element (−254/−226) was incubated with 10 μg of nuclear extract prepared from IL-1 plus IL-6-treated HIG82 cells (lanes 1–3). In lane 2, nuclear extract was preincubated with anti-SAF antibody (3 μl of a 10-fold diluted stock), and in lane 3, nuclear extract was preincubated with anti-Sp1 antibody (3 μl of the stock) before the addition of the radioactive probe.

Fig. 6. Cytokine-mediated induction of Sp1 activity in synoviocyte cells. A, nuclear extracts (10 μg of protein) prepared from untreated control (lane 1), IL-1 treated (lane 2), IL-6 treated (lane 3), or IL-1 plus IL-6 treated (lane 4) cells were incubated with 32P-labeled high affinity Sp1-binding oligonucleotide. The resultant DNA-protein complexes were resolved in a 6% native polyacrylamide gel. Three detectable complexes are designated as a, b, and c. B, The DNA-protein complexes formed by nuclear extracts from IL-1 treated (lanes 1–5), IL-6 treated (lanes 6–10), and IL-1 plus IL-6 treated (lanes 11–15) HIG82 cells were further characterized by using competitor oligonucleotides and anti-Sp1 antibody. 50-fold molar excess of either wild-type (wt) or mutant (mt) Sp1-binding oligonucleotides were added as competitors. Anti-Sp1 antibody was added at a concentration of 1 μl of the stock. As a control in antibody inhibition/supershift assay, nonspecific serum was included in some assays. The arrows indicate the migration position of the supershifted complex in lanes 4, 9, and 14.
included that at least two types of SAF proteins are present in synovial cells. One type is sensitive to phosphatase treatment, whereas the second type can interact with SAA promoter even if dephosphorylated. Interestingly, the DNA binding ability of the inducible SAF proteins that form complexes a and b is affected by dephosphorylation. In contrast, dephosphorylation had no effect on the DNA binding activities of the constitutively present SAF proteins. Noticeably, some additional faster migrating bands appeared when interaction of complexes a and b was prevented (Fig. 9A, lanes 2 and 3).

To analyze the effect of phosphorylation on Sp1 DNA binding activity, the same dephosphorylated nuclear extracts were in-

To determine whether the observed changes in protein content of SAF and Sp1 correlated with the mRNA expression, we performed a Northern blot analysis (Fig. 8). Consistent with the Western immunoblot analysis, SAF transcript level remained essentially same in all four cell preparations. This indicated that a post-translational event is probably responsible for the enhanced SAF activity in response to cytokine treatment of synovial cells. With regard to Sp1, an increased expression in synovial cells is evident.

Phosphorylation Reduces the DNA Binding Activities of SAF and Sp1—Phosphorylation of transcription factors plays an important role in the cytokine response. We therefore assessed the role of phosphorylation in mediating increased DNA binding activity of SAF during IL-1 plus IL-6 induction of cells. Nuclear extracts were first dephosphorylated using increasing concentrations of calf intestinal alkaline phosphatase and then used for DNA-protein interaction studies. As seen in Fig. 9A, dephosphorylation severely inhibited formation of the two cytokine-inducible complexes, a and b. Formation of the two constitutive complexes, d and e, was not affected by dephosphorylation, but a change in their relative migration was noticed. There was no change in the level or migration pattern of complex c. To rule out the possibility of degradation of nuclear proteins by any contaminating proteases present in the phosphatase enzyme, we dephosphorylated IL-1 plus IL-6-treated nuclear extract in the presence of two concentrations of phosphatase inhibitors. Inclusion of phosphatase inhibitors restored the formation of complexes a and b (Fig. 9A, lanes 4 and 5), which indicated that indeed phosphorylation is required for their formation. Based on the DNA binding ability, we concluded that at least two types of SAF proteins are present in synovial cells.
washed and grown for 48 h in fresh medium in the presence or absence of 10 nM OA. Cell extracts were prepared and CAT activity was measured.

Finding, OA was found to enhance transactivation potential of SAF and Sp1 (Fig. 10, panel A and B). Nuclear extracts, prepared from HIG82 cells grown in the absence (lane 1) or in the presence of 10 nM of okadaic acid for 24 h (lane 2) were used in the binding assay performed as described under “Materials and Methods.” For transient transfection, HIG82 cells were co-transfected with pSAA – 254– 226CAT reporter plasmid (10 μg of DNA) and 2 μg of DNA of either pCMV SAF (panel C) or pCMV Sp1 (panel D). Following overnight incubation with the plasmids, the cells were washed and grown for 48 h in fresh medium in the presence or absence of 10 nM OA. Cell extracts were prepared and CAT activity was measured as described under “Materials and Methods.” Results represent an average of three separate experiments.

Binding Activities of SAF and Sp1—To further verify the role of phosphorylation, we treated HIG82 cells with okadaic acid (OA), a potent protein phosphatase inhibitor. Nuclear extracts prepared from untreated and OA-treated cells (10 nM final concentration, for 24 h) were used in DNA binding assays for SAF and Sp1 (Fig. 10, A and B). Augmentation of DNA binding of both transcription factors by OA indicated that a phosphorylation event potentiates these activities. Consistent with this finding, OA was found to enhance transactivation potential of both SAF (Fig. 10C) and Sp1 (Fig. 10D) in transient transfection assays.

**DISCUSSION**

The results presented here focus on understanding the molecular basis of SAA gene induction in synovial fibroblast cells in response to combined stimulation by IL-1 and IL-6 cytokines. A synergistic increase of SAA expression was noted when synovial cells were exposed to these two cytokines. The novel findings obtained during this study are: (i) involvement of Sp1 in mediating SAA gene induction; (ii) induction of Sp1 in synovial cells during cytokine exposure; and (iii) association of Sp1 with SAF in mediating synergistic increase of SAA expression during IL-1 plus IL-6 stimulation of the cells.

Expression of SAA in the synovial cells is implicated in the induction of collagenase enzyme that is associated with the joint tissue destruction in rheumatoid arthritis (6). SAA, being a member of the type 1 acute-phase protein group, is synergistically activated by IL-1 plus IL-6. However, synergistic activation of SAA by IL-1 and IL-6 is only reported in liver cells (38, 39). Incidentally, the synovial fluid of arthritic patients contains, among other molecules, high levels of both IL-1 and IL-6. In such a milieu, a relatively higher level of SAA expression can increase local collagenase production (6) and most likely increases the severity of the disease. We have demonstrated that, indeed, the combined presence of IL-1 and IL-6 has a much higher stimulatory effect on SAA gene expression in synovial fibroblast cells. We also noticed some morphological changes during cytokine exposure of these cells (Figs. 2 and 3), evidenced by the ability of cytokine-treated cells to form extensive elongated protrusions composed of filamentous actin. Associated with the changes in cellular morphology, we provided evidence of activation and induction of two transcription factors, SAF and Sp1, in synovial cells that are involved in altered gene expression such as that of SAA. Comparable phenomenon has been observed in other cell types. For example, onset of differentiation in preadipocytes induced by exogenous hormonal agents, triggers activation of C/EBP-α (40). C/EBP-α in turn up-regulates expression of 422 adipose P2 and glucose transporter 4 genes.

The mechanism of SAA induction was investigated by transfection of synovial cells with various SAA promoter-CAT constructs followed by induction with IL-1 and IL-6. The region between –254 and –226 was found to be most responsive to IL-1 plus IL-6 stimulation. Another region between –193 and –135 was seen to contain some cytokine responsiveness (Fig. 4). Two C/EBP binding sites are located in this region that earlier were found to be highly active in regulating SAA gene induction in cultured liver cells and also in liver tissues (12–18). No effect of NF-κB, a known regulator of SAA in liver cells (15, 18–20), was noticed. For further characterization of the promoter element between –254 and –226, DNA binding assays were conducted that detected the formation of specific inducible DNA-protein complexes. Antibody-mediated ablation/supershift of these complexes suggested the involvement of Sp1 and SAF transcription factors in the formation of inducible complexes, which appeared following cytokine stimulation of the cells (Figs. 5 and 6). It should be noted that some nuclear factors, which were constitutively present in the cells, belong to the SAF family of proteins. However, constitutively present SAF isoforms are distinctly different in terms of their DNA binding abilities from the cytokine-activated SAF isoforms.
Cytokine-activated SAF proteins are highly sensitive to dephosphorylation (Fig. 9A), which inhibits their interacting ability to the SAA promoter. These results demonstrated that cytokine-inducible SAF isoforms may require phosphorylation to interact with the SAA promoter.

An important result of this study is the identification of Sp1 as a mediator of cytokine response for SAA gene induction. Involvement of Sp1 is documented by DNA-protein interaction (Fig. 5). Following cytokine addition, the DNA binding activity of Sp1 was increased and IL-1 plus IL-6 had the most stimulatory effect (Figs. 5 and 6). Increased Sp1 DNA binding activity correlated very well with increased SAA mRNA expression and increased SAA promoter activity. Higher DNA binding activity of Sp1 following cytokine treatment of HIG82 cells is partly because of an increase in Sp1 protein level (Fig. 7B). Sp1 protein level is known to be increased during development (41), cellular differentiation (41), and SV40 infection (42).

The loss of DNA binding activity of Sp1 following dephosphorylation (Fig. 9B) and potentiation of this activity by okadaic acid (Fig. 10B) suggest that phosphorylation of Sp1 is helpful for its DNA binding function as well as for its transactivation potential. The significance of phosphorylation of Sp1 has recently been demonstrated in several observations where strong circumstantial evidences suggested that phosphorylated Sp1 is an active moiety in transcription. Phosphorylation of Sp1 at the serine 131 position is found to be important for supporting activity to the SAA promoter. These results demonstrated that phosphorylation of Sp1 is helpful as a mediator of cytokine response for SAA gene induction.

A constitutive transcription factor required only for the main-synovial fibroblast cells during inflammatory conditions.

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