Purification and Characterization of the Serum Amyloid A3 Enhancer Factor*

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Serum amyloid A (SAA) is a major acute-phase protein synthesized and secreted mainly by the liver. In response to acute inflammation, its expression may be induced up to 1000-fold, primarily as a result of a 200-fold increase in the rate of SAA gene transcription. We showed previously that cytokine-induced transcription of the SAA3 gene promoter requires a transcriptional enhancer that contains three functional elements: two CCAAT/enhancer-binding protein (C/EBP)-binding sites and a third site that interacts with a constitutively expressed transcription factor, SAA3 enhancer factor (SEF). Each of these binding sites as well as cooperation among their binding factors is necessary for maximum transcription activation by inflammatory cytokines. Deletion or site-specific mutations in the SEF-binding site drastically reduced SAA3 promoter activity, strongly suggesting that SEF is important in SAA3 promoter function. To further elucidate its role in the regulation of the SAA3 gene, we purified SEF from HeLa nuclear extracts to near homogeneity by using conventional liquid chromatography and DNA affinity chromatography. Ultraviolet cross-linking and Western experiments indicated that SEF consisted of a single polypeptide with an apparent molecular mass of 65 kDa. Protein sequencing and antibody supershift experiments identified SEF as transcription factor LBP-1c/CP2/LSF. Cotransfection of SEF expression vector with SAA3-luciferase reporter resulted in approximately a 5-fold increase in luciferase activity. Interestingly, interleukin-1 treatment of SEF-transfected cells caused dramatic synergistic activation (31-fold) of the SAA3 promoter. In addition to its role in regulating SAA3 gene expression, we provide evidence that SEF could also bind in a sequence-specific manner to the promoters of the α2-macroglobulin and Aα-fibrinogen genes and to an intrinsic enhancer of the human Wilm’s tumor 1 gene, suggesting a functional role in the regulation of these genes.

The defense processes initiated in most vertebrates after infection or tissue injury are termed the acute-phase response (1). One characteristic of this response is changes in the circulating plasma protein profile, reflecting the synthesis and secretion of proteins involved in immune function and wound repair (2). After tissue injury or infection, macrophages and monocytes near the damaged site detect the infectious agent or damaged cells and respond with a first wave of synthesis of cytokines, mainly of interleukin-1 (IL-1) and tumor necrosis factor. These first-wave cytokines trigger the surrounding cell types, such as fibroblasts and blood vessel endothelial cells, to respond with an amplified second wave of cytokine synthesis, which includes a large amount of IL-6. A significant amount of these cytokines is transported in the blood stream and triggers the acute-phase response in target tissues such as the liver. The liver is one of the major targets for these proinflammatory cytokines because it has the largest number of cells with cytokine receptors as well as a high density of receptors per cell (3–5). The liver responds to the cytokine stimulation by a burst of synthesis of acute-phase plasma proteins. The magnitude of the changes in the relative plasma concentrations of these proteins ranges from less than 2-fold to several hundredfold after acute inflammation.

Elevated expression of acute-phase genes is regulated primarily at the transcriptional level. Analyses of many acute-phase gene promoters have revealed two general types of regulatory cis-acting elements in the transcriptional induction by cytokines: the binding sites for constitutive factors such as C/EBPα, hepatocyte nuclear factor 1, and hepatocyte nuclear factor 3 and the binding sites for inducible transcription factors such as C/EBPβ, NFκB, and signal transducer and activator of transcription proteins (STATS). In most cases, full transcriptional activation of these acute-phase gene promoters requires the combined action of a constitutive factor and an inducible transcription factor or factors. For example, induction of β-fibrinogen by IL-6 requires the cooperative interaction of three transcription factors: the constitutively expressed transcription factor hepatocyte nuclear factor 1, the IL-6-inducible C/EBPβ protein, and an unidentified IL-6-responsive factor (6). In the promoter of the C-reactive protein gene, the binding site for members of the C/EBP family and hepatocyte nuclear factor 1 are required for full promoter activity after cytokine induction (7, 8).

The serum amyloid A (SAA) gene family belongs to one of the major acute-phase proteins. In mice, there are four SAA genes (SAA1, SAA2, SAA3, and SAA5) and a pseudogene (9–11). The

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1 The abbreviations used are: IL-1, interleukin-1; SAA, serum amyloid A; SEF, SAA3 enhancer factor; DRE, distal response element; C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility shift assay; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; STAT, signal transducer and activator of transcription protein; LC, liquid chromatography; MS, mass spectrometry; CID, collision-induced dissociation; WT1, Wilm’s tumor 1.
SAA plasma concentration rises from 0.5 μg/ml to more than 1000 μg/ml 24 h after injection of bacterial lipopolysaccharide (12). SAA circulates as an apolipoprotein of high density lipoprotein particles, and at the peak of inflammation, it constitutes up to 20% of the total protein in the high density lipoprotein particles (12). SAA has been suggested to play a role in reverse cholesterol transport of high density lipoprotein by affecting the activity of the enzyme lecithin-cholesterol acyltransferase, which converts cholesterol to cholesteryl esters (13). However, continuous overproduction of SAA associated with chronic inflammation often results in secondary amylodosis, an incurable and frequently fatal disorder (14).

The large increase in the hepatic synthesis of SAA is primarily a consequence of dramatically increased transcription of SAA genes (10, 15). Thus, transcriptional induction of SAA genes is an excellent model system for studying differential gene expression in response to a specific stimulus. To dissect the molecular mechanisms of SAA gene regulation, we have studied the promoters of the rat SAA1 (16, 17) and mouse SAA3 genes (18–20). Our studies of the rat SAA1 promoter have shown the functional importance and cooperative interaction between NFκB and C/EBP proteins in cytokine-induced expression. Our studies of the mouse SAA3 promoter demonstrated that a 350-bp promoter fragment was necessary and sufficient to confer cytokine responsiveness. Two elements were identified in this 350-bp promoter fragment: a proximal response element, which contains two adjacent C/EBP binding sequences that enhances SAA3 gene expression in liver-derived cells, and a distal response element (DRE), which confers responsiveness to cytokine induction and has properties of an inducible transcription enhancer (19). We demonstrated that DRE consists of three functionally distinct elements: the A element, a weak binding site for C/EBP family proteins; the B element, which also interacts with C/EBP family proteins but with a much higher affinity; and the C element that interacts with a constitutive nuclear factor, which was named SAA3 enhancer factor (SEF). Deletions and site-specific mutation studies revealed that all three elements are required for maximum promoter activity. Deletions and mutations of the C element drastically reduce both basal and inducible activities of SAA3 promoter. Furthermore, although the C element does not interact with C/EBP directly and mutation of this element does not alter C/EBP binding to elements A and B, mutation of the C element nevertheless dramatically reduces the transcriptional activation of the SAA3 promoter by C/EBP (20). Taken together, these functional studies clearly demonstrated that SEF is a critical component in the regulation of SAA3 promoter activity. To further our understanding of SAA3 gene regulation, we purified and characterized SEF from HeLa nuclear extracts and provide some evidence that SEF may play a broad role in regulating other gene promoters.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Nuclear Extracts—HeLa cells were grown in suspension in Spinner’s minimum essential medium supplemented with 5% (v/v) bovine calf serum (HyClone). The cells were maintained by daily dilution with fresh complete medium to 4.5 × 10⁶ cells/ml and were grown to a density of 9 × 10⁶ cells/ml before harvesting. Nuclear extracts were prepared as described previously (21). The cell pellet from 12 liters of cells was resuspended with 5 volumes of hypotonic buffer (10 mM HEPES, pH 7.6, 1.5 mM MgCl₂, 10 mM KCl, 1 mM benzamidine, and freshly added 0.2 mM phenylmethylsulfonyl fluoride and 14 mM β-mercaptoethanol) and dialyzed to a 160-mL DEAE-Sepharose column at a flow rate of 3 mL/min. After loading, the column was washed extensively with Buffer A, and SEF activity was subsequently eluted with 0.2 M NaCl in Buffer A. The DEAE eluates were loaded directly onto a 50-mL heparin-agarose column at a flow rate of 1 mL/min. After washing with 0.2 M NaCl in Buffer A, the bound SEF activity was eluted with 0.5 M NaCl in Buffer A. The eluate from the heparin-agarose column was then diluted to 0.25 mM NaCl with Buffer A before being applied to a phenyl-Sepharose column (2.5 × 10⁶ mol). The phenyl-Sepharose column was washed sequentially with Buffer A containing 0.25 M NaCl and Buffer A containing 0.25 M NaCl and 30% ethylene glycol before the SEF activity was eluted with Buffer A containing 65% ethylene glycol. The eluate from phenyl-Sepharose column was first diazylated in TEG buffer (20 mM Tris-HCl, pH 9.0, 0.1 mM EDTA, 10% glycerol, 0.05% Nonidet P-40, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 14 mM β-mercaptoethanol) for 2 h and then diazylated in TEG buffer containing 0.1 mM NaCl for an additional 2 h. The diazylated sample was mixed directly with the DNA affinity beads. The amount of beads and poly(dI-dC) used in the incubation depended on the amount of protein in the phenyl-Sepharose eluate. In general, approximately 100 μg of protein was incubated with 1.5 mg of DNA affinity beads and 50 μg of poly(dI-dC). This mixture was incubated in a roller at 4 °C for 30 min before being subjected to magnetic separation. After the magnetic separation, the SEF-bound magnetic beads were washed twice by resuspension in TEG buffer containing 0.1 mM NaCl. SEF binding activity was then eluted from the DNA affinity beads with 0.4 mM NaCl in TEG buffer. Unless otherwise stated, all electrophoretic procedures were performed at 4 °C. Protein concentrations were measured by the Bradford assay (23), and SEF activities were determined by electrophoretic mobility shift assays (EMSA).

EMSA—A [3P]-labeled C element DNA containing a SAA-binding site (4 × 10⁹ cmp) was incubated with protein samples from different stages of purification to assess SEF activity (20). Approximately 1–2 μg of protein was incubated with the radioactively labeled probe in TEG buffer containing 100 mM NaCl for 30 min at 4 °C. In assays with affinity-purified SEF samples, 5 μM acetylated bovine serum albumin was included in the reaction buffer to minimize nonspecific loss of SEF protein. After incubation, the reaction mixtures were loaded onto a 5% polyacrylamide gel (19.1 cross-linking ratio) in glycine buffer and subjected to electrophoresis at 200 V for 90 min at 4 °C. The gel was dried before autoradiography. The SEF activity was quantified with a PhosphorImager (Molecular Dynamics). One unit of SEF binding activity was defined as the amount of protein required to retard 10% of the labeled DNA under our standard assay conditions. Rabbit polyclonal antibody (anti-LCL) raised against an N-terminal peptide, LPLADEVIESGLVQD, corresponding to amino acid residues 7 to 21 (30) was used in antibody supershift experiments. DNA-affinity-purified SEF was incubated with [3P]-labeled DNA in the presence of rabbit anti-LCL antiserum (1:1200 dilution) or preimmune serum for 30 min at 4 °C. The reaction mixtures were then subjected to electrophoresis as above.

Electrophoresis and Silver Staining—SDS-PAGE was performed as described by Laemmli (24), and protein sizes were determined by comparison with prestained molecular weight markers (Bio-Rad). Electrophoresis and silver staining were performed to detect protein bands.
phosphorylation was performed at 165 V for 4.5 h. Silver staining was performed according to the instructions in the silver staining kit (Sigma). The gels for protein profiles were fixed in 30% ethanol and 10% glacial acetic acid. After exposure to silver nitrate, each gel was treated with developer to control the level of staining. When the desired staining intensity was reached, the gel was fixed and photographed.

**UV Cross-Linking of Purified SEF to 32P-Labeled C Element**—Affinity-purified SEF was incubated with 25 ng of poly(dI-dC) and 5 × 10^5 cpm of a 5-bromodeoxuryridine-substituted, uniformly labeled SEF-binding site in a 50-μl reaction mixture (25). The mixture was incubated at 4°C for 30 min with or without a 100-fold molar excess of wild-type or mutant SEF-binding oligonucleotides. The incubation mixture was then either first separated on a 5% nondenaturing polyacrylamide gel and then exposed to UV radiation or directly exposed to UV radiation for 7 min from a UV transilluminator (254 nm, 7000 milliwatts/cm^2) at a distance of 4 cm. After separation on a 7.5% SDS-polyacrylamide gel, the proteins directly involved in binding to the DNA were identified by autoradiography.

**Southwestern Assay**—The southwestern assay was performed by the method of Philippe (26). Briefly, the DNA affinity-purified proteins were separated on a 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, denatured, and then reanimated in sequential dilutions of guanidine-HCl (5, 1.5, 0.75, 0.38, and 0.175 M) and in binding buffer (25 mm HEPES, pH 7.9, 3 mm MgCl₂, 50 mm KCl, and 0.1% nonfat milk in binding buffer) for 1 h to block the nonspecific sites. After gentle mixing for 2 h at 4°C, the membranes were washed in binding buffer, and the protein that bound the probe was visualized by autoradiography.

**Mass Spectrometric Sequencing**—Protein sequencing using mass spectrometry was carried out as described (27). Briefly, DNA affinity-purified material accumulated from approximately 200 liters of HeLa cells was resolved by SDS-PAGE. The Coomassie Blue-stained 65-kDa protein band was in-gel-digested with trypsin, and the recovered peptides were analyzed using an electrospray ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) coupled on-line with a capillary liquid chromatograph (Magic 2002, Michrom BioResources, Auburn, CA). A 0.1 × 50-mm MAGICMS C18 column (5-μm particle diameter, 200-Å pore size) with mobile phases of A (methanol:water:acetic acid, 5:94:1) and B (methanol:water:acetic acid, 85:14:1) was used with a gradient of 2–98% mobile phase B over 2.5 min followed by 98% B for 2 min at a flow rate of 50 μl/min. The flow was split with a Magic precolumn capillary splitter assembly (Michrom BioResources), and 1 μl/min was directed to the 100-μm column. The LC/MS was programmed to run in a data-dependent fashion. That is, the mass spectrometer was switched to the MSMS mode to acquire collision-induced dissociation (CID) spectra once an ion signal was detected to exceed a preset value in the MS mode during the entire LC run. Data derived from the CID spectrum were used to search a compiled protein data base that was composed of the protein data base NR and a six-frame-translated Expressed Sequence Tag data base to identify the protein.

**Plasmids and Oligonucleotides**—A DNA fragment containing 306 bp of the 5’-flanking region and 45 bp of the untranslated exon 1 region of mouse SAA3 promoter was inserted into the Smal site of the pGL3-Basic vector (Promega) to generate the pSAA3(−306)/Luc construct. The SEF cDNA was obtained by reverse transcription-polymerase chain reaction (Roche Molecular Biochemicals) and was inserted into the XhoI site of pCS2+ MT vector (28), which contains six copies of the myc epitope fused in-frame at the N terminus of SEF. The integrity of this construct was confirmed by sequencing of the entire coding region.

**Transient Transfection Assay**—HepG2 cells were cultured in basal medium consisting of minimum essential medium and Waymouth MAB (3:1, v/v) plus 10% fetal calf serum (29) and were passaged at confluence by trypsinization once a week. pSAA3(−306)/Luc reporter was cotransfected with either SEF expression vector or empty vector into HepG2 cells using FuGENE method (Roche Molecular Biochemicals). Approximately 16 to 20 h after transfection, cells were stimulated with basal medium or 100 units of IL-1/ml. Cell extracts were assayed for protein content, and the luciferase activity was quantitated according to manufacturer’s procedures.

RESULTS

**Purification of SEF**—Originally identified in HepG2 and Hep3B cells, SEF activity was subsequently detected at high levels in several other cell types, including HeLa cells (20). As HeLa cells can be easily cultured and grown as cell suspensions to a high cell density, we chose to use HeLa nuclear extracts as our starting material for the purification of SEF. Steps in the purification were carried out as described under “Experimental Procedures.” Protein eluates from each purification step were assayed for SEF binding activities using end-labeled C element containing the SEF-binding site as probe. As shown in Fig. 1, nuclear extracts and eluates from DEAE, heparin, and phenyl-Sepharose columns all showed strong SEF binding activities. Moreover, the binding activity is sequence-specific because the SEF-DNA complex could be completely inhibited by an excess of wild-type C element but not by the mutated C element. Although the DEAE-Sephalac and heparin steps only modestly increased the specific activity of SEF (Table I), they nevertheless efficiently concentrated the SEF activity and also eliminated some of the major contaminants in the crude nuclear extracts.

**Phenyl-Sepharose Chromatography**—The steps that achieved the most significant purification were the phenyl-Sepharose and DNA affinity chromatography steps. More than 90% of the protein from the heparin-agarose column either did not bind to the phenyl-Sepharose column or was eluted in the 30% ethylene glycol, 0.25 M NaCl wash (Fig. 2A). Only about 6% of the protein loaded remained on the column and was eluted with 65% ethylene glycol. Some of the C element binding activity that apparently migrated at the same position as SEF was found in the flow-through fraction. There are two possible explanations for this observation, which are that the column capacity was insufficient for the amount of protein loaded, or the C element binding activity in the flow-through fraction may not be SEF but some interfering protein or proteins. To test the first possibility, we collected the flow-through and reloaded it onto a freshly prepared phenyl-Sepharose column. The binding activity was again recovered in the flow-through fraction; no binding activity was detected in the 30 and 65% ethylene glycol eluates (data not shown). The binding activity in the flow-through fraction was therefore not due to overloading of the column but rather may be due to another binding protein or proteins with properties different from those of SEF. To determine the sequence specificities of this binding activity, com-
Purification and Identification of SEF

SEF activity was measured by EMSA.

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* One unit of SEF activity is defined as the amount of protein required to retard 10% of labeled DNA under standard assay conditions.

**TABLE I**

Purification of SEF from HeLa cell nuclear extracts

FIG. 2. Phenyl-Sepharose chromatography. A, elution profile of C element binding activities from phenyl-Sepharose column as detected by EMSA. The reaction mixture contained 4 μl of input sample, 10 μl of flow-through fraction (FT), 10 μl of 30% ethylene glycol eluate (30% EG), and 4 μl of 65% ethylene glycol eluate (65% EG). The numbers denote the fraction numbers after each elution step. The SEF-DNA complex is indicated. NS, nonspecific binding. B, competition analysis of C element binding activities in different fractions from phenyl-Sepharose chromatography. Protein samples (flow-through fraction (FT), 30% ethylene glycol (30% EG), and 65% ethylene glycol (65% EG) eluates) were incubated with labeled C fragment with or without a 100-fold molar excess of wild-type (WT) or mutant (mt) SEF oligos as competitors. The numbers denote the fraction numbers after each elution step. The positions of SEF and nonspecific (NS) complexes are indicated.

DNA Affinity Chromatography—To facilitate DNA affinity purification, we sought to define some parameters that would minimize protein degradation, preserve the integrity of the DNA affinity beads, and at the same time maintain maximum SEF binding. We examined the effects of various concentrations of EDTA, NaCl, and poly(dI-dC) on the ability of SEF to bind DNA. Our results showed that SEF binding activities were at or near optimal levels under a wide range of concentrations (2 to 18 mM EDTA, 50 to 110 mM NaCl, and 50 to 100 μg of poly(dI-dC)) (data not shown). Therefore, buffers used in DNA affinity chromatography included 10 mM EDTA, 100 mM NaCl, and 50 μg of poly(dI-dC) to maximize specific SEF binding and at the same time limit binding of nonspecific proteins to DNA affinity beads.

Because ethylene glycol severely interfered with SEF binding in our EMSA, it may therefore also affect binding of SEF to the DNA affinity beads and greatly reduce the efficiency of the DNA affinity column. To circumvent this problem, the 65% ethylene glycol eluate was dialyzed at 4 °C sequentially in TEG buffer containing 0.1 M NaCl for an additional 2 h before incubation with the DNA affinity beads.

To confirm that the binding activities detected in the 0.4 M NaCl eluate were specific for SEF binding to the C element and to determine the efficiency of the DNA affinity column, we performed EMSA assays. As shown in Fig. 3, the wild-type C element oligonucleotides effectively competed for binding, but the mutant C element did not. Approximately 50% of the input SEF binding activity was recovered from this step (Table I).

To assess the purity of SEF at each purification step, protein eluates from each column were analyzed by silver staining. As shown in Fig. 4, a substantial amount of protein was removed by the phenyl-Sepharose column, although many proteins still remained. The bulk of the nonspecific proteins from phenyl-Sepharose column did not bind to the DNA affinity column. Two major protein bands and

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several minor bands were recovered in the DNA-affinity eluate when poly(dI-dC) was not included in the wash (Fig. 4, lane 5). After an additional wash with poly(dI-dC), only three major protein species, with apparent molecular weights of 140, 105, and 65 kDa, remained (Fig. 4, lane 6). Overall, approximately 20% of the SEF activity was recovered, resulting in a 4500-fold purification (Table I).

**The 65-kDa Protein Band Possesses SEF Binding Activity**—Because three major protein species remained in the DNA affinity eluate, we performed UV cross-linking and Southwestern experiments to identify the protein that possesses the SEF binding activity. Results from the UV cross-linking experiment revealed one major DNA-protein complex on polyacrylamide gels with the adjusted protein molecular mass of approximately 65 kDa (Fig. 5A). Formation of this protein-DNA complex could be specifically competed by oligonucleotide containing the wild-type SEF-binding site but not by the mutant oligonucleotide. Similar results were obtained with in-gel UV cross-linking (data not shown). To confirm these findings, we analyzed the SEF-binding activity in the DNA-affinity purified samples by Southwestern analysis. Consistent with our UV cross-linking results, the polypeptide that bound to the radiolabeled, oligomericized wild-type C element (Fig. 5B, lane 1) but not the mutant probe (Fig. 5B, lane 2) was estimated to be 65 kDa. Taken together, our results indicate that the 65-kDa protein purified by the DNA affinity chromatography indeed possesses SEF binding activity.

**Identification of SEF as LBP-1c/CP2/LSF**—To determine the identity of this 65-kDa SEF protein, two peptides, Peptide-1 and Peptide-2, from the trypsin digestion were sequenced by mass spectrometry. The amino acid sequences obtained from both peptides were found to match exactly with two regions from the transcription factor LBP-1c/CP2/LSF (30–32). Peptide-1, with the amino acid sequence KLGELPEINGK, corresponds to amino acids 103 to 115 in LBP-1c, and Peptide-2, with the amino acid sequence AETNDSYHILK, corresponds to residues 491 to 502 (30). In addition, the molecular mass of SEF and its ubiquitous tissue distribution characteristics are also consistent with it being LBP-1c/CP2/LSF. To further determine whether SEF and LBP-1c/CP2/LSF are indeed identical and are antigenically related, specific rabbit polyclonal antibodies against the N-terminal peptide of LBP-1c/CP2/LSF were used in supershift experiments with purified SEF protein. As shown in Fig. 6, purified SEF formed a strong SEF-DNA complex with 32P-labeled C element. The addition of anti-LCL antibodies, but not preimmune serum, completely supershifted the SEF-DNA complex. Taken together, our results demonstrated that SEF is identical to LBP-1c/CP2/LSF. The function of SEF in the regulation of SAA3 promoter, we cotransfected HepG2 cells with wild-type pSAA3(-306)/Luc reporter gene along with a SEF expression plasmid. As shown in Fig. 7, cotransfection of SEF increased the luciferase activity.
was 32P-labeled and incubated with purified SEF in EMSA assays. The activity of the control and noncotransfected cells, to which a value of 1.0
were cotransfected with 0.5 g of pSAA3(-306)/Luc and 1 g of SEF expression plasmids. Transfected cells were treated with medium alone (control) or with IL-1 (50 ng/ml). The results were normalized to the expression plasmids. Transfected cells were treated with medium alone

Purification and Identification of SEF

The DNA affinity chromatography column was by far the most efficient step. The basis of DNA affinity chromatography is the differential sensitivity of sequence-specific and nonspecific DNA-protein interactions to increases in the ionic strength of the buffer conditions (38, 39). Ideally, the protein samples would be loaded onto a DNA affinity column at an ionic strength optimal for specific protein binding and minimal for nonspecific interactions. Because the affinity of the SEF-binding site is such that the DNA affinity column must be loaded at

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by nearly 5-fold. Interestingly, although IL-1 alone induced the reporter gene activity by approximately 10-fold, stimulation of SEF-transfected cells with IL-1 resulted in dramatic synergistic activation of the SAA3 promoter with more than a 31-fold increase in luciferase activity. Consistent with its important functional role, mutations in the SEF binding site greatly reduced SAA3 promoter activity (20). Taken together, our data indicated that SEF is an important regulatory component at the SAA3 gene promoter and appears to cooperate with other IL-1-inducible factor(s) to confer the dramatic up-regulation in SAA3 gene expression.

Binding of SEF to the α2-Macroglobulin and Apo-fibrinogen Promoters and Wilms Tumor 1 Intronic Enhancer—Since SEF binding activities could be detected in nearly all cell lines and tissues examined (20), we sought to identify other potential target genes that may be regulated by SEF. A computer search for sequences homologous to SEF-binding sites identified several genes that contain SEF-like binding sequences. To determine whether SEF binds to these sequences, the oligonucleotides that correspond to the sequences from the α2-macroglobulin (33–35) and Apo-fibrinogen (36) promoters and the WT1 intronic enhancer (37) were end-labeled and used as probes in the EMSA assays. As shown in Fig. 8, when incubated with partially purified SEF, all three probes formed intense protein-DNA complexes that could be specifically competed by wild-type but not by mutated SEF binding oligonucleotides, suggesting that SEF may have a functional role in the regulation of these genes.

DISCUSSION

We previously demonstrated that a 350-bp promoter fragment from the mouse SAA3 gene was necessary and sufficient to confer cytokine-induced expression in hepatoma cells (19). Deletion studies identified a DRE that is responsible for the cytokine response and has the properties of an inducible transcriptional enhancer (20). We also demonstrated that the DRE consists of three functionally distinct elements: the A element, a weak binding site for C/EBP family proteins; the B element, which also interacted with C/EBP family proteins but with a much higher binding affinity; and the C element, which interacted with a novel constitutive nuclear factor, SEF (20). Each of these binding sites is required for maximum transcription activation by inflammatory cytokines. Deletion and site-specific mutations of the SEF binding site drastically reduced both the basal and inducible activities of the SAA3 promoter. Therefore, to understand the molecular mechanisms by which SEF regulates the SAA3 promoter, perhaps by cooperating with other transcription factors, we performed studies aimed at determining the identity of the SEF protein. Here, we have described the purification and initial characterization of SEF from HeLa nuclear extracts. By several chromatographic steps, including DNA affinity, we purified SEF to near homogeneity. The purified SEF had the same DNA-binding specificities as the HeLa and HepG2 nuclear extracts; they bound with identical DNA sequence specificity. Although all the purification steps contributed to SEF purification, the most important steps were the phenyl-Sepharose and DNA affinity chromatography. In the phenyl-Sepharose step, the amount of contaminating proteins was greatly reduced after sequential washes of the column. More importantly, two major nonspecific DNA binding activities that were still present after the DEAE-Sepharose and heparin-agarose columns were efficiently removed by the phenyl-Sepharose column.

The DNA affinity chromatography column was by far the most efficient step. The basis of DNA affinity chromatography is the differential sensitivity of sequence-specific and nonspecific DNA-protein interactions to increases in the ionic strength of the buffer conditions (38, 39). Ideally, the protein samples would be loaded onto a DNA affinity column at an ionic strength optimal for specific protein binding and minimal for nonspecific interactions. Because the affinity of the SEF-binding site is such that the DNA affinity column must be loaded at relatively low salt concentrations, a potential problem arose because of saturation of a limited number of binding sites by an
FIG. 8. Binding of SEF to the αa-macroglobulin and Ao-fibrinogen promoters and the WT1 intronic enhancer. One μg of 65% ethylene glycol eluate from phenyl-Sepharose column (65% EG) was used in the assays. The probes were labeled with [α-32P]dATP and were the α2M probe (5'-GAGCAAGAATTTCTGG-3') from the αa-macroglobulin promoter (33–35) (A), the AaF probe (5'-GAGCAGAAATTTCCTGGAATGCCTGTGGT-3') from the Aa-fibrinogen promoter (30) (B), and the WT1 probe (5'-CGGCCGGCCGGCTCGCGTATGGTTTGCCT-3') from the WT1 intronic enhancer (37) (C). The competitors used were either the probes themselves or wild-type (WT) or mutant (mt) SEF binding sequence from the SAA3 promoter.

excess of nonspecific DNA-binding proteins. We circumvented this problem by enriching the SEF activity using a series of conventional chromatographic separations before DNA affinity chromatography and by the addition of nonspecific competitor DNA to the pool of proteins. This strategy allowed us to achieve a more than 100-fold purification in a single purification step.

Several lines of evidence suggested that the 65-kDa polypeptide purified by DNA chromatography under native conditions is the SEF activity that specifically binds to the C element. First, the 65-kDa polypeptide was one of the major bands present in an amount sufficient to account for the observed SEF binding activity. Second, the photoactivated protein-DNA cross-linking experiments detected a 65-kDa polypeptide that could be specifically competed by the wild-type oligo containing the SEF-binding site but not by the mutant. Third, a Southwestern assay showed that the 65-kDa polypeptide bound only to the wild type and not to the mutant multimerized probes.

Protein sequencing and antibody supershift experiments identified SEF as the transcription factor LBP-1c/CP2/LSF (30–32). LBP-1, CP2, and LSF were initially identified as cellular factors that bind at multiple sites in the human immunodeficiency virus long terminal repeat (40–42), whereas it cooperates with YY1 to repress human immunodeficiency virus-1 long terminal repeat activity (45). Furthermore, it was shown recently that inducers of cell growth can up-regulate the DNA binding activity of LBP-1c/CP2/LSF in human peripheral T lymphocytes, suggesting this factor may participate in regulating growth-responsive genes (46). Our finding that SEF is involved in the regulation of SAA3 gene transcription adds yet another dimension to its diverse cellular functions. We showed previously that deletion or mutation of SEF-binding site drastically decreased basal SAA3 promoter activity as well as its responsiveness to cytokine induction. Moreover, mutation of the SEF-binding site rendered the promoter nonsensitive to transactivation by C/EBPα, even though such mutation did not alter C/EBP binding to the adjacent C/EBP binding sites (20). Recently, we found that transactivation of SAA3 promoter by NFκB p65 was dependent on a functional SEF-binding site, suggesting that NFκB p65 may be recruited to the SAA3 promoter complex by SEF through protein-protein interaction. Taken together, it is tempting to speculate that the dramatic induction of SAA3 expression by IL-1 and tumor necrosis factor may be the consequence of cooperative interactions between constitutively expressed transcription factor SEF and cytokine-inducible transcription factors C/EBP and NFκB. Consistent with this idea is our finding that stimulation of SEF-transfected cells with IL-1 resulted in a dramatic synergistic induction of the luciferase activity. It is interesting to note that in addition to SAA3, we also identified SEF-binding sites in the promoters of αa-macroglobulin and Ao-fibrinogen and in the intronic enhancer of the human WT1 gene. In the rat αa-macroglobulin promoter, SEF binds to a site near the STAT3-binding site. Similarly, the SEF-binding site in the WT1 intronic enhancer is adjacent to the binding site of the hematopoietic transcription factor GATA-1. Given the close proximities of the SEF-binding site to the binding sites of these transcription factors, SEF may also cooperate with these transcription factors to regulate expression of their target genes. Future studies will aim to understand the molecular mechanisms by which SEF regulates the transcription of these and other genes involved in various cellular, immunological, and developmental processes.

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