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Serum Amyloid A Gene Expression under Acute-Phase Conditions Involves Participation of Inducible C/EBP-β and C/EBP-δ and Their Activation by Phosphorylation

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Serum amyloid A (SAA) is a plasma protein whose synthesis is markedly increased in the liver during the inflammatory process. Previous analysis of SAA promoter function implicated the involvement of the CCAAT/enhancer-binding protein (C/EBP) in controlling this process. In this study, using antibodies against three C/EBP isoforms in DNA-binding and Western blot (immunoblot) assays, we found that in response to inflammatory signals, both C/EBP-δ and C/EBP-β are induced and that their interactions with the SAA promoter element are necessary for the increased SAA gene expression. Cotransfections of liver cells with an SAA promoter-linked reporter chloramphenicol acetyltransferase gene and murine sarcoma virus-expressed C/EBP-δ or C/EBP-β confirm such phenomena. The increased transactivating ability in the presence of the cellular phosphatase inhibitors okadaic acid and sodium orthovanadate, coupled with the observation that dephosphorylation severely inhibits the DNA-binding ability in vitro, implicates a role of phosphorylation in the regulation of the activities of the C/EBP-δ isoform. Consistent with these findings, we have detected higher levels of DNA-binding activity of C/EBP-δ prepared from cells treated with phosphatase inhibitors. We also present evidence that C/EBP-δ is a phosphoprotein. These results suggest that C/EBP-δ is regulated by phosphorylation and, in conjunction with C/EBP-β, is one of the major proteins responsible for the increased transcription of the SAA gene in response to inflammatory stimuli.

Serum amyloid A (SAA) is the precursor of amyloid A protein, which is one of the major components usually found in amyloid fibrils isolated from patients with secondary amyloidosis (16, 32). Its synthesis in liver increases 1,000-fold in response to inflammatory stimuli. Such a large increase in hepatic SAA synthesis is attributed primarily to the transcriptional induction of this gene. Different cytokines, including interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha, either alone or in combination, have been shown to be capable of inducing SAA gene transcription (12). In experimental animals, induction has also been achieved by the administration of turpentine oil or bacterial lipopolysaccharide (LPS) (27, 29, 30). Thus, expression of the SAA gene is regulated by many substances that trigger inflammation.

Studies on the mechanism of the transcriptional induction of the SAA gene have revealed the presence of several regulatory elements in the 5′-proximal promoter region. A functional NF-κB element has been found in human (9, 10), rat (21), and rabbit (30) SAA genes. In LPS-induced acute-phase rabbit liver, an active NF-κB transcription factor has also been detected (30). In addition to the NF-κB element, the rat SAA_1 gene has been found to contain several CCAAT/enhancer-binding protein (C/EBP)-like binding sites (21). Similar studies on the mouse SAA_3 gene have also revealed the presence of two adjacent C/EBP-like binding sites (20). Both of these studies on rat and mouse SAA genes have demonstrated binding of a constitutively expressed C/EBP-like factor to the promoter element of the SAA gene, and no change in the DNA-binding ability was detected when either unstimulated or stimulated liver cells (Hep3B cells) were used. This is puzzling because SAA gene expression is extremely low in the liver under normal unstimulated conditions and becomes high only upon stimulation. Thus, these studies remained inconclusive as to the role of C/EBP in the overall process of induction of SAA gene expression in acute inflammatory conditions.

The appearance of inflammation-responsive specific inducible C/EBP-like factors that interact with the SAA gene in experimentally induced acute-phase rabbit liver has recently been demonstrated (27). Multiple highly induced factors which interact with two neighboring C/EBP-like elements in the rabbit SAA gene were found to be different proteins since they exhibited variable heat tolerance. This observation suggested that multiple inducible factors probably represent different members of the C/EBP family of transcription factors. Two of these family members, C/EBP-β and C/EBP-δ, also known as NF-IL6 and NF-IL6β, have been shown to be induced in hepatic as well as nonhepatic tissues by LPS or inflammatory cytokines (1, 7, 18). The involvement of C/EBP-β in the regulation of α_1-acid glycoprotein (3, 19, 25, 26, 28) and C/EBP-δ in the regulation of the complement C3 gene (17) has been recently reported. These transcription factors have also been implicated in the regulation of genes involved in cell differentiation and in inflammatory and immune responses and act as transactivators involved in signal transduction.

In this study, we demonstrate that C/EBP-β and -δ are the two major C/EBP family members which are induced in the liver and elicit acute-phase conditions. These two factors, along with the constitutively present C/EBP-α, interact with the SAA promoter element. Involvement of these factors in SAA gene expression has been demonstrated by transactivation assays using a reporter gene containing the SAA promoter element and expression plasmids containing various C/EBP isoforms. To evaluate the role of phosphorylation-mediated activation, we have examined the effects of two protein phosphatase inhibitors, okadaic acid and sodium orthovanadate, on the transactivating role of C/EBP isoforms. Our study shows

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that C/EBP-δ is activated by phosphorylation and that such a modification is required for its DNA-binding ability.

MATERIALS AND METHODS

Plasmids. Plasmid pSAA-CAT, containing the SAA promoter region from −193 to −136, which had previously been characterized as the cytokine-responsive element of the SAA gene (27), was constructed by ligating one copy of this sequence in the pBLCAT2 vector (22). A mutant derivative, pmtSAA-CAT, was also constructed by ligating the modified sequences of this region (5′-^{193}GGCCTTCATAGACTAC ACAACTAGGCACGGGATCTGCGCATCACGCAACCCT GTATGT^{136}-3′) to the pBLCAT2 vector. Underlined nucleotides represent the mutated bases. These constructs were verified by DNA sequence analysis to determine their authenticity and orientation. MSV (murine sarcoma virus)-C/EBP-α, MSV-C/EBP-β, and MSV-C/EBP-δ plasmids were kindly provided by S. L. McKnight (Tularik, San Francisco, Calif.) (7). In transfection assays, we used the pJFCAT1 vector (11) as a control.

Cell cultures and transfection assays. Liver cells (BNL CL.2; obtained from the American Type Culture Collection) were grown in Dulbecco's modified Eagle medium (DMEM) containing high glucose (4.5 g/liter) supplemented with 10% fetal calf serum. Cells were seeded in 60-mm-diameter dishes at a density of 10^4 cells per plate; the following day, the medium was changed. Transfections were carried out by the calcium phosphate method (13) with a mixture of DNAs containing 5 to 10 μg of reporter chloramphenicol acetyltransferase (CAT) plasmid, 2 μg of plasmid pSV-β-gal (obtained from Promega Corporation), and carrier plasmid DNA to keep the total amount of DNA constant. In cotransfection experiments with expression vectors containing the genes for C/EBP-α, C/EBP-β, and C/EBP-δ, various amounts of these plasmids (1 to 10 μg) replaced with an equal amount of the carrier DNA to keep the total amount of DNA constant at 15 μg. Twenty-four hours later, the cells were washed once with phosphate-buffered saline (PBS), shocked with 15% glycerol in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline for 1 min, and refed with fresh medium or medium supplemented with 25% conditioned medium (CM) for cytokine stimulation. The cells were grown for an additional 24 h, and cell extracts were prepared. Extracts were assayed for β-galactosidase activity, and then appropriate amounts of extract normalized for β-galactosidase activity were used in the CAT assay as described earlier (27). CM was prepared by incubating either the primary culture of rabbit peripheral monocytes or human monocyte U937 cells in medium containing LPS (20 μg/ml) for 24 h. The media were aspirated and centrifuged, and the supernatant was stored at −70°C.

Preparation of nuclear extracts and DNA-binding assays. The acute-phase condition in New Zealand White male rabbits was developed by a single injection of concentrated turpentine oil (1 ml/kg of body weight) in the dorsal lumbar region. Nuclear extracts were prepared from normal and acute-phase-induced (collected at various time points after the injection) rabbit livers essentially by the method of Dignam et al. (8), with minor modifications as described previously (27). Also, nuclear extracts were prepared from BNL cells grown to confluency in regular growth medium as described above and then incubated, prior to cell harvest, either in the absence or in the presence of 100 nM okadaic acid and 100 μM sodium orthovanadate for 6 h. The protein concentrations of the nuclear extracts were measured as described previously (5), divided into small aliquots, and stored at −70°C. DNA-binding assays were performed as described previously (27) with a double-stranded DNA fragment from −193 to −136 of the SAA promoter region labeled by filling in the overhangs at the termini with the Klenow fragment of DNA polymerase, incorporating [α-^{32}P]dATP as the probe.

Nuclear extracts (2 to 10 μg of protein) were preincubated for 10 min at 25°C in a reaction mixture containing 10 mM HEPES (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM $ZnCl_2$, 0.05% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, and 50 μg of poly(dI-dC) per ml. The labeled DNA probe was added, and incubation in a total volume of 10 μl was continued for additional 30 min at 25°C. DNA-protein complexes were fractionated in a 6% nondenatured polyacrylamide gel with recirculation of running buffer (7 mM Tris-HCl [pH 7.5], 3 mM sodium acetate, 1 mM EDTA). For antibody interaction studies, antibodies against C/EBP-α, C/EBP-β, or C/EBP-δ (a gift of S. L. McKnight) were added to the reaction mixture during the preincubation period.

Western blot (immunoblot) assay. Nuclear extracts (30 μg of protein) were fractionated on a sodium dodecyl sulfate (SDS)–11% polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was then blocked in PBS–0.05% Tween 20 supplemented with 5% (wt/vol) casein (Sigma) at room temperature for 1 h. The primary antibodies to C/EBP isoforms were diluted 1:1,000 in PBS–0.05% Tween–1% bovine serum albumin and incubated for 1 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Gibco BRL) was used as the secondary antibody. Bands were detected by using a chemiluminescence detection system (Renaissance; NEN).

Dephosphorylation of nuclear extracts. Dephosphorylated nuclear extracts were prepared by incubating both uninduced and induced nuclear extracts with calf intestinal alkaline phosphatase (CIP; Boehringer Mannheim) at room temperature for 30 min. The amount of enzyme used ranged from 0.5 to 2.5 U/10 μg of protein of nuclear extract. In some experiments, in addition to phosphatase, nuclear extracts were incubated in the presence of a combination of phosphatase inhibitors (50 mM NaF, 1 mM sodium vanadate, and 5 μM okadaic acid). These treated nuclear extracts were subsequently used in the DNA-binding assays as described above.

In vivo phosphorylation of C/EBP-δ. Cultured BNL CL.2 liver cells were transfected with expression plasmid MSV-C/EBP-δ. The cells were metabolically labeled with $^{32}P_i$ (0.5 mCi/ml) in phosphate-free DMEM for 2 h either in the presence or in the absence of phosphatase inhibitors (100 nM okadaic acid and 100 μM sodium orthovanadate). Prior to the labeling procedure, the transfected cells were grown in DMEM supplemented with 5% fetal calf serum for 24 h. The ^{32}P-labeled cells were harvested, washed quickly in phosphate-free DMEM to remove excess radioactivity, and lysed by adding hot lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [vol/vol] Nonidet P-40, 1.0% [wt/vol] SDS, 3 mM sodium orthovanadate, 2.5 mM phenylmethylsulfonyl fluoride). After lysis, the lysates were heated at 95°C for additional 5 min, and the SDS concentration in the lysates was reduced to 0.05% (wt/vol) by adding lysis buffer without SDS. The ^{32}P-labeled C/EBP-δ was isolated by immunoprecipitation with an anti-C/EBP-δ antibody and protein A agarose. The immunoprecipitated C/EBP-δ was heated at 95°C in a buffer containing 1% SDS, fractionated in an SDS–12% polyacrylamide gel, and detected by autoradiography.

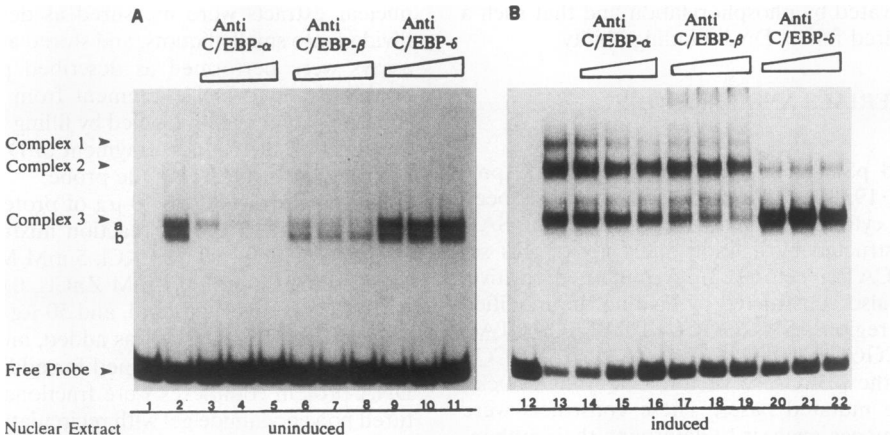

FIG. 1. Characterization of the C/EBP isoforms involved in formation of the DNA-protein complexes with the SAA promoter element. DNA-binding assays were performed with a ^{32}P-labeled SAA promoter DNA fragment (−193 to −136) in the presence of antisera to various C/EBP isoforms. Three concentrations (0.5, 1, and 2 μl of a 1:10 dilution) of antibody for each isoform were used. No cross-reactivity between the antisera was noticed. (A) Nuclear extracts (10 μg of protein) from untreated normal rabbit liver were incubated with increasing amounts of anti-C/EBP-α (lanes 3 to 5), anti-C/EBP-β (lanes 6 to 8), and anti-C/EBP-δ (lanes 9 to 11). Lane 1 contained probe only, and lane 2 contained no antiserum. (B) Nuclear extracts (10 μg) from 24-h acute-phase-induced (turpentine-injected) rabbit liver were incubated in the presence of increasing amounts of anti-C/EBP-α (lanes 14 to 16), anti-C/EBP-β (lanes 17 to 19), and anti-C/EBP-δ (lanes 20 to 22). Lane 12 contained the probe only, and lane 13 contained no antiserum.

RESULTS

SAA gene expression under acute-phase conditions involves participation of C/EBP-δ and C/EBP-β isoforms. Previous analysis of SAA gene expression (27) showed that inducible transcription factors with similarity to the C/EBP family interact with the cytokine-responsive promoter region (−191 to −140) and thereby induce the expression of this gene enormously under acute-phase conditions. In an effort to identify which isoforms of C/EBP are induced, we used antibodies specific to the three C/EBP family members, α, β, and δ, in DNA-binding assays. The uninduced liver nuclear extract formed two DNA-protein complexes, 3a and 3b, with the SAA promoter element (Fig. 1, lane 2). As seen in lanes 3 to 5, a C/EBP-α-specific antibody completely inhibited the formation of complex 3b and at higher concentrations blocked the formation of complex 3a also. An antibody specific to C/EBP-β had little to no effect on complex 3b but inhibited the formation of complex 3a (lanes 6 to 8). The C/EBP-δ-specific antibody did not alter the formation of either of these two complexes (lanes 9 to 11), implying that transcription factor C/EBP-δ either is absent or remains inactive in the uninduced liver nucleus. The result also indicates that complex 3b is formed solely by C/EBP-α and that complex 3a is a heteromer of C/EBP-α and C/EBP-β. Thus, the existence of both C/EBP-α and -β in the uninduced liver nucleus is evident.

The pattern of DNA-protein complexes changed dramatically when acute-phase-induced liver nuclear extract was used (Fig. 1, lane 13). Instead of the two closely migrating complexes 3a and 3b as seen with uninduced liver nuclear extract, only one complex migrating closely at the position of complex 3a, and two additional complexes, complex 1 and complex 2, were noticed. The C/EBP-δ-specific antibody almost completely neutralized the formation of both of these acute-phase-specific complexes 1 and 2 (lanes 20 to 22). At a higher concentration of the C/EBP-δ-specific antibody, the formation of complex 2 was completely inhibited (data not shown). These results indicate the appearance of C/EBP-δ in acute-phase liver and its participation in the formation of these two complexes. This antibody had no effect on the formation of complex 3a, whereas the C/EBP-β-specific antibody supershifted complex 3a (lanes 17 to 19), suggesting that C/EBP-β is responsible for the formation of this complex. Interestingly, the composition of complex 3a in the induced liver is distinct from that in the uninduced liver. While in the uninduced liver it is formed by C/EBP-α and -β, it is composed mainly of C/EBP-β in the induced liver. This observation may indicate a possible modification in the binding characteristics of C/EBP-β under induced conditions. Additional findings, described below, substantiate this possibility. An antibody specific to C/EBP-α inhibited the formation of complex 1 only at a very high concentration (lanes 14 to 16). Complex 1 formation was also affected by the anti-C/EBP-δ antibody (lanes 20 to 22), indicating that DNA-protein complex 1 is a possible heteromer composed of C/EBP-δ and C/EBP-α. The anti-C/EBP-β antibody also partly inhibited the formation of complex 1 (lanes 16 to 18) but not in a dose-dependent manner. Even at a very high concentration of the C/EBP-β antibody, formation of complex 1 could not be completely inhibited (data not shown). This finding suggests that CEBP-β may not be directly involved in complex 1 formation. These findings demonstrate that under turpentine-induced acute-phase conditions, one of the major changes in the nuclear factors that bind to the cytokine-responsive promoter elements of the rabbit SAA gene is the interaction of C/EBP-δ with this promoter region. In addition, modification in the binding characteristics of C/EBP-β is observed.

Time course of appearance of the induced C/EBP isoforms. The results described above prompted us to determine whether the induced C/EBP isoforms were synthesized de novo or translocated to the nucleus following a covalent modification in response to the acute-phase signal. Nuclear extracts were prepared from uninduced as well as 1-, 4-, and 24-h acute-phase-induced livers and used in DNA-binding assays (Fig. 2B). The C/EBP-δ-specific DNA-binding activity (complexes 1 and 2) began to appear at 4 h and increased considerably within 24 h (lanes 3 and 4) following the onset of acute-phase conditions. A nonspecific complex that migrates between complexes 2 and 3a is often detected in acute-phase

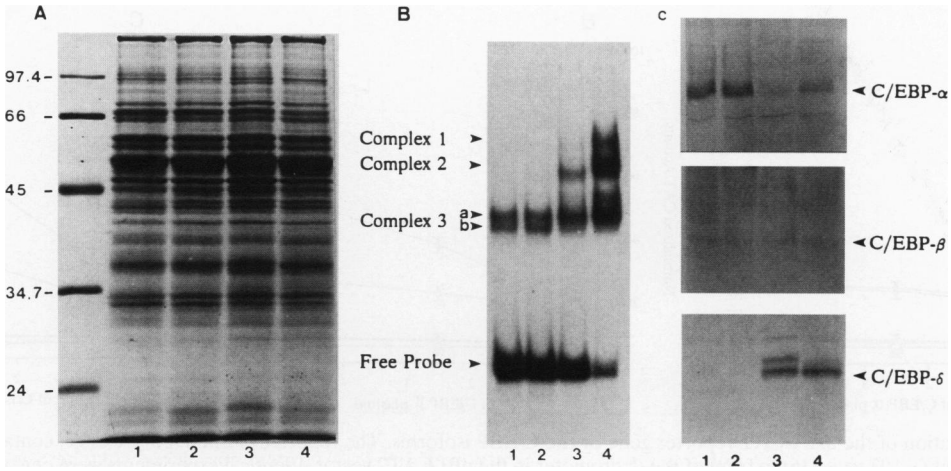

FIG. 2. Time course of appearance of the induced C/EBP isoforms. (A) SDS-PAGE analysis of the nuclear extracts prepared from various rabbit livers collected after 0, 1, 4, and 24 h (lanes 1 to 4, respectively) of turpentine injection. A 50-μg sample of protein from each extract was applied to a 12% polyacrylamide gel and stained with Coomassie blue for visualization. Molecular masses of standard protein markers are indicated in kilodaltons. Similar patterns of proteins in lanes 1 to 4 indicate that they are of equal quality and suitable for comparative studies. (B) Nuclear extracts (10 μg of protein) prepared from untreated (lane 1) and from 1-h (lane 2), 4-h (lane 3), and 24-h (lane 4) acute-phase-induced livers were incubated with a ^{32}P-labeled SAA promoter DNA fragment (−193 to −136), and the resulting DNA-protein complexes were resolved in a 6% native polyacrylamide gel. (C) Nuclear extracts (30 μg) prepared from untreated (lane 1) and from 1-h (lane 2), 4-h (lane 3), and 24-h (lane 4) acute-phase-induced rabbit livers were analyzed for C/EBP-α, C/EBP-β, and C/EBP-δ on Western immunoblots as described in Materials and Methods. Migration positions of the transcription factors are indicated.

nuclear extracts. This complex seems to be unrelated to C/EBP. In all cases, the same amounts of nuclear proteins of comparable quality were used (Fig. 2A). As translocation of the transcription factors from the cytoplasm to the nucleus occurs quite rapidly (usually within minutes), for example, translocation of NF-κB starts within 5 min (2) in response to stimuli, the absence of C/EBP-δ-specific activity even after 1 h of acute-phase induction (lane 2) suggests that unlike NF-κB, C/EBP-δ is not simply translocated to the nucleus. Likewise, the DNA-binding activity of C/EBP-β (formation of complex 3a), which was low at 4 h, also was induced significantly at the 24-h time point (compare lanes 1 to 4). It seems likely, therefore, that both C/EBP-β and C/EBP-δ are newly synthesized following acute-phase induction.

To determine whether the observed changes in the DNA-binding activities of C/EBP isoforms correlate with the actual protein content of the individual C/EBP, we performed Western immunoblot analyses (Fig. 2C). Consistent with the DNA-binding activity, no detectable amount of C/EBP-δ was seen in the uninduced and 1-h acute-phase-induced liver, but the level increased dramatically within 4 h (lane 3) and remained highly elevated at 24 h (lane 4). Surprisingly, we observed two closely migrating C/EBP-δ-specific bands in 4-h-induced nuclear extract and one band in 24-h-induced nuclear extract. This result suggested that C/EBP-δ might undergo some kind of posttranslational modification which was incomplete at the earlier stages of acute-phase induction. The protein level of C/EBP-α decreased considerably (about 50% over the control value) at the 4-h time period (Fig. 2C) and gradually increased thereafter. This finding is consistent with the observation of a reduction in the C/EBP-α-specific mRNA and an increase in the C/EBP-δ-specific mRNA in Hep3B2 liver cells following cytokine treatment (17). In the Western immunoblot assay, we did not observe any notable change in the level of C/EBP-β. This is probably due to the characteristics of the anti-C/EBP-β antiserum used, which functions well in the DNA-binding and immunoprecipitation assays, but is not very effective in Western immunoblot assays.

Response of the SAA promoter to the individual C/EBP isoforms. To assess the in vivo role of the C/EBP transcription factors that interact with the SAA promoter, we performed a series of cotransfection experiments using BNL liver cells. A reporter gene, pSAA-CAT, containing one copy of the promoter element of the SAA gene from −193 to −136, was transfected alone or with an expression plasmid containing the C/EBP-α, C/EBP-β, or C/EBP-δ gene. Differential induction by the various factors was observed in the CAT cotransfection assay (Fig. 3). In transfection assays, C/EBP-α transactivated the pSAA-CAT reporter gene at a relatively low dose (Fig. 3A). However, the level of the reporter gene expression did not increase with increasing amounts of C/EBP-α, nor did it alter much in the presence of CM, indicating that CM has no effect on the transactivation potential of C/EBP-α. In contrast, C/EBP-β and C/EBP-δ activated the reporter gene expression in a dose-dependent manner (Figs. 3B and C), and this effect was elevated significantly in the presence of CM in the medium. Comparison of the data in Fig. 3B and C indicated that C/EBP-β has a modest transactivation potential on the SAA promoter, while C/EBP-δ seems to significantly influence transcription from the same promoter region. To ensure that the increased transcription of the pSAA-CAT reporter gene is indeed due to the interaction of transfected C/EBPs with the C/EBP-binding elements of the SAA promoter, we constructed a reporter gene, pmtSAA-CAT, in which the native SAA promoter carrying the C/EBP-binding elements was replaced with one containing mutations in this region and used it in the transfection assays. The expression of pmtSAA-CAT was not induced by any of the three C/EBP isoforms, nor was it expressed at a significant level. Also, expression of the *tk* promoter of the vector pBLCAT2 remained unaffected by the transfected C/EBP plasmid DNAs. It is evident from these results that C/EBP-δ and C/EBP-β are able to transactivate the

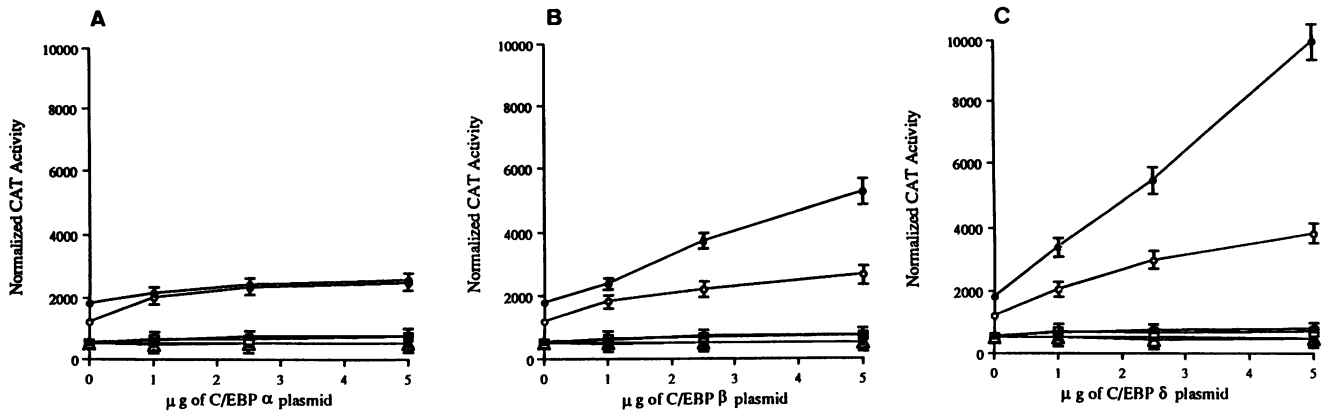

FIG. 3. Transactivation of the SAA-CAT reporter gene by the C/EBP isoforms. The reporter plasmid, pSAA-CAT, contained one copy of the SAA promoter element (−193 to −136) in front of the *tk* promoter in the pBLCAT2 vector. Identical experiments were performed with a reporter plasmid, pmtSAA-CAT, that was mutated at the C/EBP-binding sites within the SAA promoter (27) and with pBLCAT2, the parent vector plasmid. The reporter plasmids (5 μg) were cotransfected in BNL CL.2 liver cells with pSV-β-gal (2 μg) and either increasing amounts of the MSV-expressed C/EBP-α gene (A), increasing amounts of the MSV-expressed C/EBP-β gene (B), or increasing amounts of the MSV-expressed C/EBP-δ gene (C). The transfection assays using pSAA-CAT (○, ●), pmtSAA-CAT (□, ■), and pBL-CAT2 (△, ▲) were performed in the presence (filled symbols) or absence (open symbols) of CM. CAT activity was measured as radioactivity in [^{14}C]chloramphenicol acetate produced by an equivalent amount of transfected cell extracts. Transfections were normalized to the β-galactosidase control activity. Plotted values represent the means and standard errors of the means derived from three separate transfection assays.

SAA promoter in vivo and that for full potential, some CM-dependent modification or activation is required.

Dephosphorylation of the nuclear extract reduces the DNA-binding ability of endogenous C/EBP-δ. Since CM considerably increased the transactivating abilities of both C/EBP-δ and C/EBP-β, it seemed likely that in order to exert the full transactivating potential, some CM-dependent modulations of these two proteins are necessary. As many transcription factors are known to be regulated by phosphorylation (15, 31) and since the signal transduction pathway for activation of transcription factors induced by cytokines involves activation of protein kinases (33, 35), we wanted to examine whether the DNA-binding activities of C/EBP-δ and C/EBP-β are affected by a phosphorylation event. Normal and acute-phase-induced nuclear extracts were dephosphorylated with CIP and tested for their DNA-binding abilities (Fig. 4). The dephosphorylated nuclear extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue stained to ensure that proteins were intact and not degraded by any nonspecific protease present as a contaminant in CIP (data not shown). The two DNA-protein complexes 1 and 2 formed by C/EBP-δ were inhibited upon CIP treatment of the induced nuclear extract (Fig. 4, lanes 7 to 9 and 5′ to 6′) but remained unaffected when CIP treatment of the nuclear extracts were performed in the presence of phosphatase inhibitors (lane 7′). The other complexes (3a and 3b) formed by C/EBP-β and C/EBP-α were efficiently made even after CIP treatment of the nuclear extracts. Taken together, these data indicate that inhibition of formation of the C/EBP-δ-specific DNA-protein complex was not the result of breakdown of this protein by any protease contaminants present in CIP. These results demonstrate that covalent modification of C-EBP-δ by phosphorylation is essential for its DNA-binding ability. In contrast, dephosphorylation did not alter the DNA-binding abilities of C/EBP-α and C/EBP-β. Our finding is consistent with the observation that although C/EBP-β is activated by phosphorylation, this modulation does not affect its DNA-binding ability (36).

The DNA-binding activity of C/EBP-δ is increased in nuclear extracts prepared from cells grown in the presence of protein phosphatase inhibitors. To study whether phosphorylation indeed stimulates the DNA-binding activity of C/EBP-δ in vivo, we used the phosphatase inhibitors okadaic acid and sodium orthovanadate, which inhibit cellular serine/threonine phosphatases and tyrosine phosphatases, respectively (6, 14). BNL cells and C/EBP-δ-transfected BNL cells were grown either in the presence or in the absence of the phosphatase inhibitors. Nuclear extracts were prepared from the treated cells and used in the DNA-binding assay. As seen in Fig. 5A, incubation of cells with phosphatase inhibitors resulted in

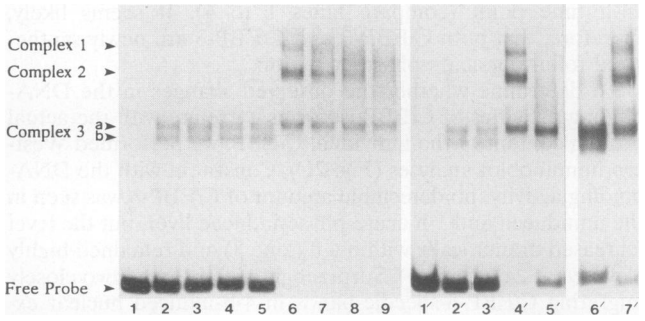

FIG. 4. Evidence that phosphorylation regulates the DNA-binding ability of C/EBP-δ. Nuclear extracts (10 μg of protein per lane) from normal rabbit liver (lanes 2 to 5, 2′, and 3′) and acute-phase-induced rabbit liver (lanes 6 to 9 and 4′ to 7′) were incubated with various amounts of CIP for 30 min at room temperature. Nuclear extracts were untreated (lanes 2, 6, 2′, and 4′) or treated with 0.5 U (lanes 3 and 7), 1.0 U (lanes 4 and 8), 1.5 U (lanes 5 and 9), or 2.5 U (lanes 3′, 5′, 6′, and 7′) of CIP. Lane 7′ also contained phosphatase inhibitors (NaF [50 mM], okadaic acid [5 μM], and sodium orthovanadate [1 mM]) during CIP treatment. Phosphatase-treated extracts were subsequently used in DNA-binding assays using ^{32}P-labeled SAA promoter DNA (−193 to −136) as the probe. Lanes 1 and 1′ contained the probe only. Resultant DNA-protein complexes were fractionated in a 6% native polyacrylamide gel.

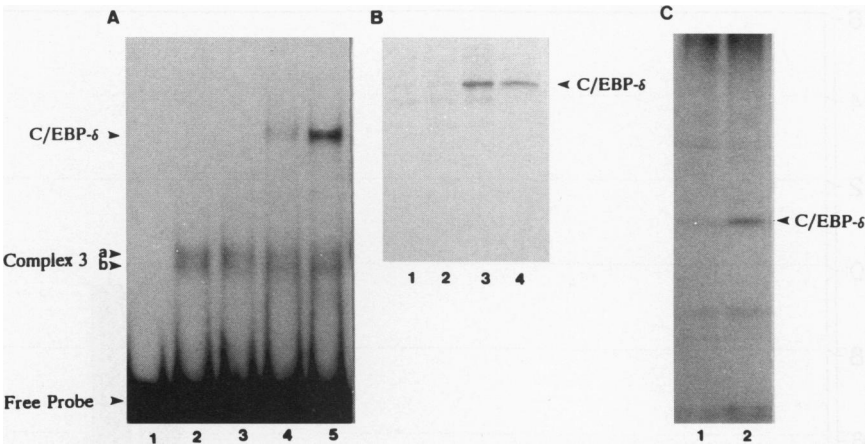

FIG. 5. Evidence that treatment of cells with cellular phosphatase inhibitors stimulates the DNA-binding activity of C/EBP-δ in vivo. (A) Nuclear extracts prepared from BNL cells (lane 2), BNL cells grown in the presence of 100 nM okadaic acid and 100 μM vanadate (lane 3), C/EBP-δ-transfected BNL cells (lane 4), and C/EBP-δ-transfected BNL cells grown in the presence of 100 nM okadaic acid and 100 μM vanadate (lane 5) were used in the DNA-binding assays using ^{32}P-labeled SAA promoter DNA (−193 to −136) as the probe. Resultant DNA-protein complexes were fractionated in a 6% native polyacrylamide gel. Lane 1 contains the probe only. (B) Western blot analysis of the nuclear extracts. Thirty micrograms of cellular proteins prepared from BNL cells (lane 1), BNL cells grown in the presence of 100 nM okadaic acid and 100 μM vanadate (lane 2), C/EBP-δ-transfected BNL cells (lane 3), or C/EBP-δ-transfected BNL cells grown in the presence of 100 nM okadaic acid and 100 μM vanadate (lane 4) was separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with the C/EBP-δ-specific antibody as described in Materials and Methods. The arrowhead indicates the position of C/EBP-δ. (C) Phosphorylation of C/EBP-δ in vivo. BNL cells were transfected with expression plasmid MSV-C/EBP-δ in duplicate. Prior to labeling, cells were grown by incubation in DMEM containing 5% fetal calf serum for 15 h. The cells were labeled with $^{32}P_i$ (0.5 mCi) in phosphate-free DMEM for 2 h either alone (lane 1) or in the presence of 100 nM okadaic acid and 100 μM vanadate (lane 2). ^{32}P-labeled cellular proteins were immunoprecipitated with the C/EBP-δ-specific antibody. The immunoprecipitated complexes were dissociated by heating at 95°C for 15 min in a buffer containing 1% SDS, and the phosphoproteins were fractionated by SDS-PAGE. The arrowhead indicates the position of C/EBP-δ.

higher levels of C/EBP-δ-specific DNA-binding activity (compare lanes 4 and 5) but had no effect on formation of endogenous C/EBP-α- and C/EBP-β-specific DNA-protein complexes (complexes 3a and 3b in lanes 2 to 5). Identities of these DNA-protein complexes were verified by using C/EBP-α-, C/EBP-β-, and C/EBP-δ-specific antibodies (data not shown). To ensure that this increased C/EBP-δ-specific DNA-binding activity is not due merely to an increased accumulation of C/EBP-δ protein in the transfected cells, we performed Western blot analysis to monitor the level of C/EBP-δ protein in these nuclear extracts (Fig. 5B). The levels of C/EBP-δ protein in the transfected cells (lanes 3 and 4) were found to be nearly same, indicating that phosphatase inhibitors did not promote increased synthesis of this protein in the liver cells. It is therefore likely that the increased C/EBP-δ-specific DNA-binding activity was due to the increased level of phosphorylation of this protein facilitated by okadaic acid and sodium orthovanadate.

To determine if indeed C/EBP-δ is hyperphosphorylated in the presence of okadaic acid and sodium orthovanadate, we labeled C/EBP-δ-transfected BNL cells with $^{32}P_i$ and immunoprecipitated the ^{32}P-labeled cellular proteins with antibody specific to C/EBP-δ. As seen in Fig. 5C, BNL cells transfected with a C/EBP-δ plasmid revealed a phosphorylated protein band (lanes 1 and 2), the intensity of which increased (hyperphosphorylation) when the cells were labeled in the presence of the phosphatase inhibitors (okadaic acid and sodium orthovanadate). This increase in phosphorylation (lane 2) was not due to the increased expression of C/EBP-δ protein in the presence of phosphatase inhibitors, since the level of C/EBP-δ expression did not change following this treatment (Fig. 5B).

Protein phosphatase inhibitors stimulate the transactivating potential of certain C/EBP isoforms. To determine if phosphorylation increases the transactivating abilities of the C/EBP isoforms under in vivo conditions, we used okadaic acid and sodium orthovanadate in transfection assays. The pSAA-CAT reporter gene, containing bp −193 to −136 of the SAA 5′ promoter region, was transfected together with either a C/EBP-α, C/EBP-β, or C/EBP-δ expression plasmid, and the transfected liver cells were incubated in the presence of either okadaic acid or sodium orthovanadate or both. All transfection assays included a constant amount of total plasmid DNA and also a β-galactosidase expression plasmid to monitor the transfection efficiency. As seen in Fig. 6, okadaic acid and sodium orthovanadate stimulated the transactivating ability of C/EBP-δ (compare bars 11 to 13), which was even more pronounced when both inhibitors were simultaneously used (bar 14). A similar effect of these two phosphatase inhibitors has been reported in a recent study on the activation of the mitogen-activated protein (MAP) kinase cascade (34). In this report, Samuels et al. (34) have shown that protein phosphatases can play a major role in the protein phosphorylation cascade by increasing the activity of MAP kinase and thus turning on the MAP kinase pathway, leading to oncogenic transformation. The effect of the phosphatase inhibitors okadaic acid and sodium orthovanadate in the C/EBP-δ-transfected BNL cells probably involves a similar phenomenon whereby the endogenous resident protein kinases are stimulated as a result of lowered protein phosphatase activity. Activated protein kinases subsequently increased the activity of C/EBP-δ to potentiate the transcription (compare bars 11 and 14) of the pSAA-CAT reporter gene. These two phosphatase inhibitors also augmented C/EBP-β-mediated SAA reporter gene transcription, although at a considerably lower level (compare bars 7 and 10), but did not have any effect on C/EBP-α-mediated transcription of the SAA gene (bars 3 to 6). These results provided further evidence that C/EBP-β and C/EBP-δ isoforms are activated via a phosphorylation event

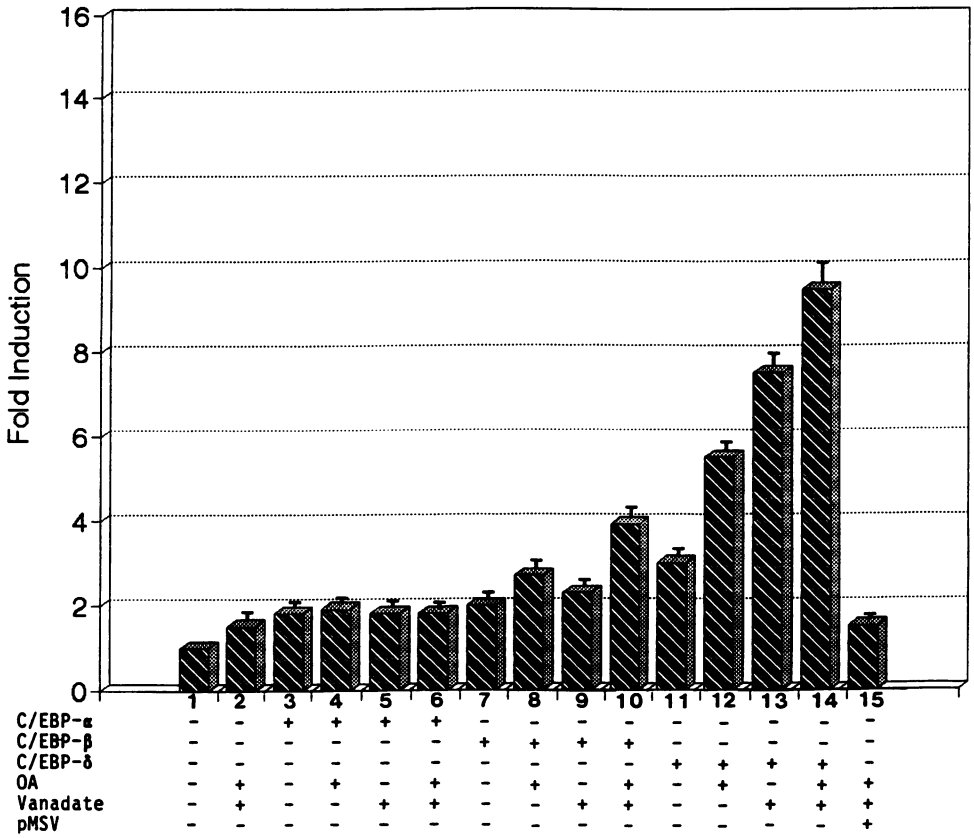

FIG. 6. Evidence that cellular phosphatase inhibitors stimulate the transactivating abilities of C/EBP-β and C/EBP-δ. The reporter construct, pSAA-CAT, consists of the 5′ flanking region (−193 to −136) of the rabbit SAA gene linked to the CAT gene. The reporter gene (10 μg) was cotransfected in BNL CL.2 liver cells together with pSV-β-gal (2 μg) and 5 μg of MSV-expressed C/EBP-α, C/EBP-β, or C/EBP-δ plasmid DNA. Transfected cells were incubated in the presence of CM, and where indicated, 100 nM okadaic acid (OA) or 100 μM sodium orthovanadate or both were added. The control cotransfection assay (bar 15) contained, in addition to the reporter plasmid pSAA-CAT (10 μg) plus pSV-β-gal (2 μg), 5 μg of pMSV (the plasmid vector for C/EBP genes). No increase in transcription indicates that the pMSV vector has no effect on potentiation of transcription. The fold induction was calculated by dividing the CAT activity of the reporter gene obtained under a given condition by the CAT activity of the reporter gene itself. Transfections were performed in triplicate, and the average CAT activity values with standard errors were used for the determination of fold induction.

involving both serine/threonine and tyrosine kinases. This is quite interesting because the signal transduction pathway involves both of these two classes of kinase in a phosphorylation cascade event (4). Incidentally, recent reports have shown that the transactivating ability of C/EBP-β is dependent on phosphorylation by the calmodulin-dependent protein kinase CamKII (36) and MAP kinase (24). An earlier report also implicated, in view of forskolin action, hyperphosphorylation of NF-IL6 (a homolog of C/EBP-β) and increased transactivation potential of this transcription factor (23). Our data indicate that C/EBP-δ is also activated by phosphorylation and that this posttranslational modification enhances its transactivation potential.

DISCUSSION

The turpentine-induced acute-phase reaction in rabbits is accompanied by an increase in the synthesis of SAA protein. The transcriptional induction process of the SAA gene under acute-phase conditions has been found to be controlled by the transcription factor C/EBP. The role of each member of this family (α, β, and δ) in the formation of the DNA-protein complexes with the SAA promoter elements has been investigated in this study. The data presented in Fig. 1 demonstrate that an inducible C/EBP isoform, called C/EBP-δ, is a major transcription factor which interacts with the SAA promoter by itself and also as a heteromer of C/EBP-α and -δ. In addition, binding of C/EBP-β is also increased under acute-phase conditions. The significance of such interactions of C/EBP isoforms with the SAA promoter region was evaluated by in vivo transactivation assays (Fig. 3). Our findings suggested that the involvement of C/EBP-β and -δ is important for the transcriptional induction of SAA in the liver. Interestingly, C/EBP-β was also present in the uninduced liver and capable of interacting with the SAA promoter element (Fig. 1). However, such an interaction is not sufficient to result in transcription because very little SAA mRNA has been found in the uninduced rabbit liver. Under acute-phase conditions, we have noted an induction of the C/EBP-δ isoform, which occurs within hours of the onset of the acute-phase reaction (Fig. 2); this isoform becomes a major transcription factor interacting with the SAA promoter. Some earlier studies reported that incubation of cells with cycloheximide and IL-1β markedly reduced the induction of SAA mRNA by IL-1β (37), and thus it was concluded that induction of SAA expression requires de novo protein synthesis. In light of these observations, we believe that

C/EBP-δ is that factor whose de novo synthesis is required for the transcriptional induction of the SAA gene. Recently, C/EBP-δ has been implicated in the transcriptional induction of the human complement C3 gene (17). In this report, Juan et al. (17) have shown an IL-1-mediated induction of C/EBP-δ in the human cultured hepatoma cell line Hep3B2 and its interaction with complement C3 gene. Using an animal exhibiting an acute-phase response, we have been able to detect the role of C/EBP-α (formation of complex 1 with C/EBP-δ), C/EBP-β (formation of complex 3a), and C/EBP-δ (formation of complexes 1 and 2) in SAA gene expression. Thus, a cumulative effect of these C/EBP isoforms is involved in SAA gene expression under acute-phase conditions.

One other important finding of this study is the phosphorylation-mediated activation of C/EBP-δ binding. Our results have demonstrated that C/EBP-δ can bind to SAA promoter DNA only as a phosphorylated form. In contrast, dephosphorylation of C/EBP-β did not alter its DNA-binding ability (Fig. 4, lanes 6 to 9, 4′, and 5′). Phosphorylation of C/EBP-β has been documented, and various protein kinases, including protein kinase A (23), CamKII (36), and MAP kinase (24), indirectly or directly have been implicated in its phosphorylation. Involvement of such diverse group of protein kinases in C/EBP-β phosphorylation is intriguing. It is possible that different kinases are activated in different cell types in response to different extracellular signals and exert diverse effects mediated by different phosphorylation events. In this report, we show that C/EBP-δ is phosphorylated and that such a posttranslational event is essential for SAA gene transcription under acute-phase conditions. This is evident from the finding that both sodium orthovanadate (an inhibitor of tyrosine phosphatases) and okadaic acid (a serine/threonine phosphatase inhibitor) enhanced the transactivation potential of C/EBP-δ and also, at a somewhat reduced level, of C/EBP-β. Also, it is noticeable that vanadate is more effective than okadaic acid, and together, they significantly increased the transactivating activity of C/EBP-δ. These results lead to the suggestion that activation of C/EBP-δ is regulated by a signal transduction pathway that includes both tyrosine and serine/threonine kinases. Our demonstration of a stimulatory role of phosphatase inhibitors in the transactivation potential of C/EBP-δ correlated with in vivo phosphorylation of C/EBP-δ indicates that a posttranslational modification by a member of the signal-transducing protein kinase cascade that involves both tyrosine and serine/threonine kinases might be a critical phenomenon in the acute-phase response. Interestingly, we have observed that cotransfection of BNL cells with C/EBP-δ and a MAP kinase expression plasmid (pCEP4 carrying the *erk1* gene) also enhances the transactivation potential of C/EBP-δ (unpublished observation). MAP kinase plays an important role in signal transduction and is a key enzyme in the signal-transducing protein kinase cascade (4). It is possible that phosphorylation of C/EBP-δ involves such a kinase cascade. It will be interesting to determine whether different protein kinases are capable of phosphorylating C/EBP-δ at different sites of this protein. Such an event may conceivably be regulated by different signaling processes and exert diverse effects in different cell types. Further studies on the phosphorylation of C/EBP-δ should reveal more information on its role in acute-phase synthesis of SAA in hepatic and extrahepatic tissues during inflammation.

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