The transcription factor serum amyloid A (SAA)-activating factor (SAF), a family of zinc finger proteins, plays a significant role in the induced expression of the SAA gene. Activity of SAF is regulated by a phosphorylation event involving serine/threonine protein kinase (Ray, A., Schatten, H., and Ray, B. K. (1999) J. Biol. Chem. 274, 4300–4308; Ray, A., and Ray, B. K. (1998) Mol. Cell. Biol. 18, 7327–7335). However, the identity of the protein kinase has so far remained unknown. Induction of SAA by phorbol 12-myristate 13-acetate, a known agonist of protein kinase C (PKC), suggested a potential role of the PKC signaling pathway in the activation process. The DNA binding activity of endogenous SAF was increased by agonists of PKC. In vitro phosphorylation of SAF-1 by PKC-β markedly increased its DNA binding ability. Consistent with these findings, treatment of cells with activators of PKC or overexpression of PKC-βII in transfected cells increased expression of an SAF-regulated promoter. Further analysis with a GAL4 reporter system indicated that PKC-mediated phosphorylation mostly increases the DNA binding activity of SAF-1. Together these data indicated that the PKC signaling pathway plays a major role in controlling expression of SAF-regulated genes by increasing the interaction between promoter DNA and phosphorylated SAF.

Serum amyloid A (SAA),1 an inflammation-responsive gene, is highly induced (100–1000-fold) by extracellular signals generated during periods of inflammation (1, 2). A higher level of plasma SAA is linked to the pathophysiology of many chronic inflammatory diseases including rheumatoid arthritis and secondary amyloidosis. In rheumatoid arthritis and osteoarthritis, autocrine induction of collagenase by SAA is shown to be critical for the destruction of connective tissues in the affected area (3–5). SAA is also identified as the precursor of amyloid A protein, one of the chief constituents of amyloid fibrils found in secondary and experimental amyloidosis (6). Induction of SAA is primarily regulated via transcriptional induction of this gene (7). Several different cytokines, IL-1, IL-6, and tumor necrosis factor-α alone or in combination, and inflammatory mediators like phorbol 12-myristate 13-acetate (PMA) can increase transcription of the SAA gene (2, 6, 8). In addition, corticosteroids have been shown to synergize the effects of IL-1 and IL-6 (9–11). Studies on the mechanism of SAA gene induction have shown that activation of SAA in many tissue types is regulated by the transcription factor SAF (12, 13). Many inflammatory agents that activate SAA, including interleukin-1 and -6, bacterial lipopolysaccharide, and minimally modified low density lipoprotein, also activate SAF and increase its transactivation potential (12–16). These studies also indicated that phosphorylation of SAF, by a serine/threonine protein kinase pathway, may be a critical step in regulating this process. For insight into mechanisms by which SAF is activated and thus regulates expression of SAA, analysis of its activation mechanism was undertaken.

Induction of SAA by PMA, a known agonist of protein kinase C (PKC), raises the possibility that a PKC-mediated phosphorylation event(s) may play an important role in activating SAF. PKCs play a crucial role in regulating many cellular functions including cell transformation, growth, and differentiation by modulating the activity of cellular target proteins and transcription factors (for reviews, see Refs. 17–19). Phosphorylation of many transcription factors by PKC is a crucial signal transduction event that results in an altered expression pattern of many genes. In the present study, we show that agonists of the PKC signaling pathway, including PMA, can increase expression of an SAF-dependent promoter as well as the DNA binding ability of endogenous SAF. Overexpression of PKC-βII in transfected cells without the addition of PKC-inducing agents produces a similar result. Taken together, these observations indicate, for the first time, a role for activated PKC in modulating the function of transcription factor SAF and expression of its target genes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—** Rabbit synoviocyte (HIG82) cells, obtained from the American Type Culture Collection, were derived from the interarticular soft tissue of the knee joint of a normal female New Zealand White rabbit. These cells maintain many of the features of normal rabbit synoviocytes and are activated by phorbol myristic acid and interleukin-1 (20). HIG82 cells were cultured in Dulbecco’s modified Eagle’s medium containing high glucose (4.5 g/liter) supplemented with 7% fetal calf serum. For induction, HIG82 cells were stimulated with 100 nM PMA, 4α-phorbol-12,13-didecanoate (4α-PDD), 1-oleoyl-2-acetyl-sn-glycerol (OAG), and 1,2-dioctanoyl-sn-glycerol (DOG). In some experiments, different concentrations of PMA and 4α-PDD, as indicated in the figure legends, were used. Myristoylated protein kinase inhibitor 19–27, a cell-permeable PKC-inhibitory peptide (PKC-I, 19–27), was added at concentrations of 10 and 50 μM as indicated in the figure legends. Calphostin C (21) and bisindolylmaleimide I (22), specific in-

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1 The abbreviations used are: SAA, serum amyloid A; SAF, SAA-activating factor; PKC, protein kinase C; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol-12,13-didecanoate; OAG, 1-oleoyl-2-acetyl-sn-glycerol; DOG, 1,2-dioctanoyl-sn-glycerol; RSV, Rous sarcoma virus; IL, interleukin.
2 The work was supported by National Institutes of Health Grant DK49205 (to A. R. and B. K. R.) and CA56769 (to A. P. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
3 To whom correspondence should be addressed: Dept. of Veterinary Pathobiology, University of Missouri, Columbia, Missouri 65211 and the Department of Pharmacology and the Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555
4 This article is available online at http://www.jbc.org
5 In this figure, the expression of SAF was measured by real-time PCR.
hhibitors of PKC, were added at a 100 nM concentration in the culture medium of HIG82 cells. These agents were obtained from Calbiochem.

Transient transfections of HIG82 cells were carried out by the calcium phosphate precipitation method (23) using a mixture of plasmid DNA containing 1 μg of reporter chloramphenicol acetyltransferase (CAT) gene (26) and an additional amount of phosphatase inhibitors (50 mM NaF, 1 mM sodium orthovanadate, and 5 μM okadaic acid). For antibody interaction studies, anti-FLAG antibody (Sigma) at 4 °C for 14–16 h in the immunoprecipitation buffer containing 2% SDS, 50 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol and heated at 100 °C for 10 min. These mixtures were fractionated in an SDS-10% polyacrylamide gel, and phosphorylated proteins were detected by autoradiography.

For use in DNA-binding assays, purified FLAG-SAF1 protein was incubated with 1.0 unit of PKC-β (Upstate Biotechnology) in a buffer system containing 10 mM HEPES, pH 7.9, 60 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM diethiothreitol, 0.1 mM ZnCl₂, 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol, 50 μg/ml poly(dI-dC), 0.2 mM sodium orthovanadate, 5 mM NaN₃, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml benzamidine, and 1 mM ATP in a 10-μl reaction mixture. Phosphorylated SAF-1 protein was further incubated with a radioactive SAF DNA-binding element and electrophoresed in a 6% native polyacrylamide gel. In some reactions, FLAG-SAF1 protein was preincubated with 1 μM of PKC-19–31 inhibitor peptide (Calbiochem) prior to the addition of PKC-β.

Diphosphorylation of FLAG-SAF1 protein was conducted by including 4 units of calf intestinal alkaline phosphatase during in vitro phosphorylation of FLAG-SAF1 protein. As a control, some reaction mixtures contained both PKC and phosphatase. The consequence of this action is the induction of expression of genes responsive to these transcription factors. SAA is one such gene shown to be activated by PMA (3–5, 33). To determine whether PMA treatment causes induction of SAA in rabbit synoviocyte HIG82 cells (ATCC) under the present culturing condition, HIG82 cells were cultured with PMA (100 nM) for 24 h, and the level of SAA mRNA was measured by Northern blot analysis (Fig. 1). PMA treatment caused marked induction of SAA mRNA level (compare lanes 1 and 2). The same blot was probed with an actin cDNA to measure the quantity and quality of input mRNA. These results showed that under our culturing condition, in HIG82 synoviocyte cells, the SAA gene is induced by PMA. Since PMA is a known agonist of protein kinase C, these data suggested the possibility of the involvement of PKC in SAA gene induction. Previous studies showed that induction of SAA in nonhepatic cells, including synoviocyte cells, is primarily regulated by the SAA transcription factor (12–14). We therefore investigated whether SAF is involved in the regulation of SAA induction in response to PMA treatment.

**RESULTS**

**Induction of SAA mRNA by PMA in HIG82 Synoviocyte Cells**—PMA is involved in many cellular processes including growth, differentiation, and development (reviewed in Refs. 29–32). These biological potentials of PMA are realized by its ability to activate multiple protein kinase cascades facilitating many cellular events. The underlying mechanism of these cellular events is the regulation of many transcription factors via phosphorylation triggered by PMA-stimulated protein kinases. The consequence of this action is the induction of expression of genes responsive to these transcription factors. SAA is one such gene shown to be activated by PMA (3–5, 33). To determine whether PMA treatment causes induction of SAA in rabbit synoviocyte HIG82 cells (ATCC) under the present culturing condition, HIG82 cells were cultured with PMA (100 nM) for 24 h, and the level of SAA mRNA was measured by Northern blot analysis (Fig. 1). PMA treatment caused marked induction of SAA mRNA level (compare lanes 1 and 2). The same blot was probed with an actin cDNA to measure the quantity and quality of input mRNA. These results showed that under our culturing condition, in HIG82 synoviocyte cells, the SAA gene is induced by PMA. Since PMA is a known agonist of protein kinase C, these data suggested the possibility of the involvement of PKC in SAA gene induction. Previous studies showed that induction of SAA in nonhepatic cells, including synoviocyte cells, is primarily regulated by the SAA transcription factor (12–14). We therefore investigated whether SAF is involved in the regulation of SAA induction in response to PMA treatment.

**Increase of DNA Binding Activity in PMA-stimulated Cells**—HIG82 cells were incubated in the presence of various concentrations of PMA for 30 min. Nuclear extracts prepared from these PMA-treated cells were subjected to a DNA-binding assay, using a radiolabeled SAF DNA-binding element of the SAA promoter (~254 to ~226) as the probe. While untreated cell nuclear extract (Fig. 2A, lane 1) formed several DNA-protein complexes (c–e), PMA treatment (Fig. 2A, lanes 2–4), led to the appearance of some additional complexes (a and b) in a concentration-dependent manner. At a higher concentration of PMA (lane 4), the levels of complexes c and d were also in...
Fig. 1. Induction of SAA mRNA by PMA. Rabbit synoviocyte HIG82 cells were grown in the presence of PMA (100 nM) for 24 h. Total RNA was prepared, and equal amounts (50 μg) were fractionated in formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized to SAA cDNA probe (top panel). Lane 1, RNA prepared from untreated cells; lane 2, RNA prepared from PMA-treated cells. For quantitative and qualitative evaluation, the membrane was reprobed with actin cDNA (bottom panel).

When SAF activation was induced by PKC, a nuclear factor unrelated to SAF was activated. The identity of this complex is not known. The induction of SAF by PMA is specific for the different nuclear proteins that can interact with the SAF DNA-binding element. To determine if induction of DNA binding activity is specific for PMA, HIG82 cells were stimulated with a phorbol ester 4α-PDD. In contrast to PMA, there was no change in the DNA binding activity in 4α-PDD-treated cells (Fig. 2A, lanes 5–7). Next, we determined the kinetics of induction of DNA-protein complexes by treating HIG82 cells with 100 nM PMA for different lengths of time. Time course studies showed that SAF treatment causes a rapid induction, within 30 min, of several complexes including a, b, and c (Fig. 2B, lanes 1–4). However, the intensity of these inducible complexes declined considerably at the 90-min time point (lane 4). Prolonged incubation, up to 24 h (data not shown) exhibited a pattern similar to the level seen at the 90-min time point. These results indicated that PMA rapidly induces activities of some nuclear proteins that can interact with the SAF DNA-binding element.

The specificity of PMA action was verified by monitoring the DNA binding activity of an unrelated transcription factor. Oct-1 DNA binding activity was assayed in various PMA-treated cells. Members of the Oct family of transcription factors specifically interact with an octamer motif, ATGCAAAT, a regulatory element important for tissue- and cell-specific transcription as well as for transcription of many housekeeping genes (34). No significant change in the level of Oct-1 DNA binding activity was noted in these PMA-treated cells (Fig. 2C, lanes 1–4). The kinetics of PMA action on these nuclear proteins is consistent with the mode of action of PMA in many other cell types. The accumulation of SAA mRNA begins early, but build up of a considerable pool of the mRNA that can be detected by Northern analysis employed in the present study (Fig. 1) takes at least several hours of treatment, and a large amount of accumulated SAA mRNA was detected at 24 h of treatment.

Characterization of the DNA-Protein Complexes—PMA-induced DNA-protein complexes were characterized by using wild type SAF DNA-binding oligonucleotide, nonspecific unrelated oligonucleotide, and a mutant SAF DNA-binding oligonucleotide (Fig. 3A, lanes 1–5). Competition with the molar excess of homologous SAF oligonucleotide inhibited all complexes but the complex c (lanes 2 and 3). No change in the DNA-protein complex formation was observed during competition with mutant SAF DNA-binding oligonucleotide (lane 4) or an unrelated nonspecific oligonucleotide (lane 5). An anti-SAF antibody (Fig. 3B, lanes 2 and 3) inhibited all complexes except the complex c. The anti-SAF antibody interacts with the zinc finger domain of the SAF family of proteins and thereby can neutralize multiple members of this family. From these results, we conclude that complexes a, b, d, and e are formed by SAF-related proteins. The identity of complex c, which is also slightly induced by PMA, presently is not very clear. Since complex c was not inhibited by a molar excess of competing homologous probe (Fig. 3A, lanes 2 and 3), we believe that this complex is formed by a nuclear factor unrelated to SAF and has a very low affinity toward the SAF DNA-binding element. Furthermore, when SAF-specific DNA binding was completely inhibited by the addition of higher levels of SAF antibody, there was a slight increase in the binding of this unrelated factor, which increased the level of complex c (Fig. 3B, lane 3). Taken together, these results showed that treatment of cells with PMA causes induction of some SAF-like DNA binding activity in HIG82 synovial cells.

Regulators of Protein Kinase C Induce SAF Activity—Since PMA is a potent activator of the PKC signaling pathway, we investigated whether PKC acts as a physiological regulator of SAF. If during PMA treatment SAF was activated by PKC, then other cellular activators of PKC should also activate SAF. OAG and DOG are known as potent stimulators of cellular PKC activity. As shown in Fig. 4A, like PMA, both OAG and DOG stimulated the DNA binding ability of SAF (lanes 2 and 3). Calphostin C is a highly specific inhibitor of PKC (21), which interacts with the protein’s regulatory domain by competing at the binding site of diacylglycerol and phorbol esters. Calphostin C inhibited the PMA-mediated increase of SAF DNA binding activity (lane 4). A similar result (lane 5) was obtained with a more specific, cell-permeable PKC inhibitor peptide 19–27 that inhibits PKC function by acting as a pseudosubstrate. From these findings, we conclude that PKC may be involved in a major way in the PMA-induced activation of SAF, which subsequently increases SAA expression.

We next asked whether inducers of PKC increase functional activity of SAF. To test this possibility, HIG82 cells were cotransfected with a reporter gene (SAF-CAT), containing three copies of SAF DNA-binding elements present in the SAF promoter, and an expression vector containing a full-length cDNA of SAF1. The cells cotransfected with the reporter plasmid and pCMV-SAF1 exhibited an approximately 4-fold higher level of SAF activity (Fig. 3B). The cells cotransfected with the reporter plasmid and an expression vector containing a full-length cDNA of SAF1 exhibited an approximately 4-fold higher level of SAF activity (Fig. 3B). The cells cotransfected with the reporter plasmid and a mutant reporter gene that contained three copies of mutated SAF DNA-binding elements. The mutant reporter gene did not respond to any of these agents. Together, these data indicate that agonists of PKC increase the expression of
SAF Activation by PKC

FIG. 2. PMA treatment causes activation of some nuclear proteins that bind to the SAF DNA-binding element of SAA promoter. A, HIG82 cells were incubated in the presence of various concentrations (as indicated) of PMA (lanes 2–4) and 4α-PDD (lanes 5–7) for 30 min. Nuclear extracts (10 μg of protein) prepared from these cells were incubated with a 32P-labeled SAF DNA-binding element (−254/−226) present in the SAA promoter. The reaction mixtures were fractionated in a native 6% polyacrylamide gel. Lane 1, equal amount of nuclear extract (10 μg of protein) prepared from untreated cells. B, HIG82 cells were incubated in the presence of 100 nM PMA for various lengths of time (as indicated in the figure). Nuclear extracts (10 μg) prepared from these cells were subjected to a DNA-binding assay with radiolabeled SAF DNA-binding element described above. Different DNA-protein complexes are designated as complexes a–e. C, nuclear extracts (10 μg) prepared from various PMA-treated cells (lanes 1–4) as used in B were subjected to a DNA-binding assay with a radiolabeled Oct-1 DNA-binding element as the probe. The Oct1-DNA complex is designated as Oct1.

FIG. 3. Characterization of DNA-protein complexes. A, nuclear extracts (10 μg) prepared from HIG82 cells treated with 100 nM of PMA for 30 min were subjected to a DNA-binding assay with radiolabeled SAF DNA-binding element (lanes 1–5). Lane 2, 50-fold molar excess of double-stranded wild type (wt) SAF DNA-binding oligonucleotide; lane 3, 100-fold molar excess of wild type SAF DNA-binding oligonucleotide; lane 4, 100-fold molar excess of mutant (mt) SAF DNA-binding element; lane 5, 100-fold molar excess of an unrelated (ur) oligonucleotide, Oct-1. B, nuclear extracts (10 μg) prepared from HIG82 cells treated with 100 nM PMA for 30 min were subjected to a DNA-binding assay with the radiolabeled SAF DNA-binding element described above. In addition to nuclear extract, lanes 2 and 3 contain 1 and 2 μl of anti-SAF antibody, respectively, and lane 4 contains 2 μl of preimmune serum. Different DNA-protein complexes are designated as complexes a–e.

SAF-regulated genes in HIG82 synovial cells.

PKC-mediated Phosphorylation Increases the DNA Binding Activity of Bacterially Expressed SAF Protein—In determining the role of PKC in SAF regulation, we first examined whether PKC can phosphorylate SAF. A full-length, bacterially expressed FLAG-SAF1 protein was affinity-purified over an anti-

FIG. 4. Inducers of PKC can increase SAF DNA binding activity and expression of a SAF-regulated promoter. A, HIG82 cells were incubated for 30 min in the presence of various activators and inhibitors of PKC activity. Nuclear extracts (10 μg) prepared from these cells were subjected to a DNA-binding assay with a 32P-labeled SAF DNA-binding element (−254/−226) of the SAA promoter. Lane 1, untreated cells; lanes 2–4, cells treated with OAG, DOG, and PMA plus calphostin C, respectively. Lane 5, PMA plus cell-permeable inhibitor peptide (PKC-I 19–27)-treated cells. Lane 6, PMA-treated cells. LANe 7, SAF1-expressing cells treated with 100 nM PMA, OAG, DOG, and calphostin C were added at 100 nM and PKC-I 19–27 was added at 50 μM. B, HIG82 cells were transfected with 1.0 μg of either wild type (wt) or mutant (mt) SAF-CAT reporter construct. In some transfection assay mixture, SAF1 expression plasmid, pCMV-SAF1 (1.0 μg), was included. Following transfection, cells were incubated with either PMA, OAG, calphostin C, or bisindolylmaleimide I. These PKC regulators were added at 100 nM concentrations of each. The CAT assay was performed as described under “Experimental Procedures.” Results represent averages of three separate experiments.

FLAG Sepharose column and in vitro phosphorylated, using purified PKC-β enzyme (Upstate Biotechnology) and [γ-32P]ATP. Phosphorylated radioactive SAF was readily detected before (Fig. 5A, lane 2) and after immunoprecipitation with anti-FLAG antibody (Fig. 5A, lane 4). These results showed that PKC-β can phosphorylate purified FLAG-SAF1 protein.

Next, we tested the effect of PKC-mediated phosphorylation on the DNA binding ability of SAF. Bacterially expressed FLAG-SAF1 protein was first in vitro phosphorylated with purified PKC-β enzyme and nonradioactive ATP and used in a DNA-binding assay. As seen in Fig. 5B, untreated FLAG-SAF1
protein interacted poorly with radiolabeled SAF DNA-binding element (lane 1), but upon phosphorylation with PKC-\(\beta\), the same protein interacted at a markedly higher level (lane 2). Prolonged exposure of the gel showed a band in lane 1 comigrating with the prominent band present in lane 2. No significant level of binding occurred when an inhibitor peptide was included in the phosphorylation reaction mixture (lane 3). The DNA-protein complex formed by PKC-phosphorylated SAF was supershifted by an anti-FLAG antibody (lane 4) and inhibited by an anti-SAF antibody (lane 5). In a reciprocal experiment, we monitored the effect of phosphatase and inhibitors of phosphatase on the DNA binding activity of \textit{in vitro} phosphorylated SAF (Fig. 5C, lanes 1–4). The addition of phosphatase severely reduced the DNA binding potential of \textit{in vitro} phosphorylated SAF protein (lane 3), while phosphatase inhibitors neutralized the action of phosphatase and restored its DNA binding ability (lane 4). Together, these results demonstrated that phosphorylation of SAF-1 by PKC-\(\beta\) is necessary for efficient DNA binding activity of SAF.

Overexpression of PKC-\(\beta\)II Can Enhance Expression of an SAF-regulated Promoter—We next asked whether overexpression of PKC-\(\beta\) in the absence of an inducer of PKC could increase expression of SAF-regulated genes. HIG82 cells were cotransfected with the GAL4-CAT reporter gene and pCMV-SAF-1 expression plasmid in the presence or absence of PKC-\(\beta\)II expression plasmid (Fig. 6). The addition of increasing concentrations of expression plasmid carrying PKC-\(\beta\)II in the transfected cells increased the transactivation potential of SAF1 in a dose-dependent manner. PKC-\(\beta\)II expression was monitored by Western immunoblot analysis and found to be higher as the amount of PKC-\(\beta\)II plasmid DNA was increased (data not shown). The empty vector had no enhancing effect, indicating the direct consequence of expressed PKC-\(\beta\)II in the transfected cells. The addition of the PKC inhibitor 19–27 peptide lowered PKC-stimulated SAF-1 transactivating ability.

PKC-mediated Phosphorylation Does Not Affect the Transactivation Potential of SAF—To define if PKC-mediated phosphorylation directly affects the transactivating potential of SAF, a full-length SAF-1 cDNA was fused in frame to the DNA-binding domain of yeast transcription factor GAL4 encoding amino acids 1–147. The resulting chimera, RSVGAL4DBD-SAF1, was tested for its ability to transactivate a reporter containing three GAL4 binding sites in front of a CAT gene. The RSVGAL4DBD-SAF1 construct considerably activated transcription of the GAL4-CAT reporter gene (Fig. 7, bar B). Interestingly, in contrast to results presented in Fig. 6, the addition of increasing concentrations of PKC-\(\beta\)II together with a constant amount of RSVGAL4DBD-SAF1 plasmid displayed virtually no additional effect on the expression of GAL4-CAT reporter gene (Fig. 7, bars C–E). As a control, we cotransfected the cells with GAL4-CAT and pCMVSAF-1 (bar I) or GAL4-CAT and RSVGAL4DBD (bar A) plasmid DNAs. In the absence of a transactivating domain, RSVGAL4DBD plasmid exhibited very little stimulating effect on the expression of the GAL4-CAT reporter gene. Likewise, in the absence of the GAL4 DNA-binding domain, pCMVSAF-1 had no effect on GAL4-CAT reporter gene expression. These results confirmed that the DNA-binding domains of SAF-1 and yeast GAL4 protein are different and that activation of GAL4-CAT reporter gene by RSVGAL4DBD-SAF1 is due to a combined effect of the GAL4 DNA-binding domain and the transactivating domain of SAF-1. Together, these data show that PKC-mediated phosphorylation increases only the DNA-binding potential of SAF-1 and has minimum effect on the transactivating domain of this protein.
SAF Activation by PKC

**FIG. 6. Overexpression of PKC-βII increases expression of SAF-regulated promoters.** HIG82 cells were transfected with 1.0 μg of SAF-CAT reporter gene with or without pCMV-SAF1 (1.0 μg) plasmid DNA, as indicated. In some transfection assays, an expression plasmid containing PKC-βII cDNA was included at amounts of 1, 2, and 3 μg, respectively. In the last two lanes, 3 μg of PKC-βII cDNA was used. As a negative control, empty vector (3 μg) lacking PKC cDNA sequences was used. Myristoylated PKC inhibitor peptide 19–27 (PKC-1 19–27) was added in some transfection assays at concentrations of 10 and 50 μM, respectively. These results represent an average of three separate experiments ± S.D.

**FIG. 7. PKC-βII does not alter the activation potential of SAF-1.** In the vector RSVGAL4DBD, GAL4 amino acids 1–147 encoding the DNA-binding domain and nuclear localization sequence of yeast transcription factor GAL4 are under the control of the RSV promoter. A full-length SAF-1 cDNA was fused in frame C-terminal to the DNA-binding domain (at the BamHI site) of GAL4 to prepare RSVGAL4DBD-SAF1 plasmid. HIG82 cells were transiently transfected with 1.0 μg of GAL4-CAT reporter DNA, which contains three copies of the GAL4 binding site. In some transfection mixtures, as indicated in the figure, RSVGAL4DBD plasmid DNA (1.0 μg) or RSVGAL4DBD-SAF1 DNA (1.0 μg) and increasing amounts (1, 2, and 3 μg) of an expression plasmid of PKC-βII were used. The results represent an average of three independent transfection experiments ± S.D.

**DISCUSSION**

In this paper, we have provided direct evidence for the activation of SAF by the PKC signaling pathway. The following novel findings were obtained: (i) agonists of PKC activation pathway or overexpression of PKC-βII can increase expression of a SAF-regulated promoter; (ii) in vitro phosphorylation of bacterially expressed SAF-1 by PKC-β markedly increases its DNA binding ability; and (iii) PKC-mediated phosphorylation increases the DNA binding ability of SAF-1 without affecting its transactivating potential.

SAF family of transcription factors was initially identified as a regulator of the SAA gene, and three members of this family were characterized by structural analysis (14). Multiple DNA-protein complexes in PMA-treated HIG82 cells that were detected by EMSA (Fig. 2) most likely are formed due to homomorphic and heteromeric interaction of these different SAF isoforms. Among the known SAF family members, SAF1 has been detected in most cell types, including HIG82 cells. For detailed analysis, we have therefore used SAF1 in the present investigation. The cloned cDNA of SAF1 exhibited considerable homology with human MAZ (35) and mouse Pur-1 (36) transcription factor cDNA, indicating that SAF1 is a member of this group of transcription factors. The SAF/MAZ/Pur-1 family of transcription factors function as regulators of SAA (12), c-myc (35), insulin (36), CD4 (37), and serotonin 1A receptor (38) genes. The c-myc proto-oncogene is an important factor in controlling both cellular proliferation and apoptosis, insulin is involved in stimulating cellular metabolism and proliferation, serotonin 1A receptor functions as a regulator of neuroendocrine function, CD4 protein is an important molecule in T-cell development and activation, and serum amyloid A is an inflammation-responsive protein. Understanding the induction mechanism of the SAF family of transcription factors thus has broad biological implications.

Protein kinase C family members are known to play important roles in mediating signal transduction, cellular differentiation, proliferation, and apoptosis. The PKC-mediated signaling pathway also regulates the inflammatory response mechanism initiated by many inflammatory agents. To date, there are at least 12 PKC members (reviewed in Refs. 17–19) that are classified into three groups: calcium-dependent or classic conventional PKC isoforms (α, β, δ, γ); calcium-independent novel PKC isoforms (ζ, ε, η, θ); and calcium- and diacylglycerol-independent, phosphatidylserine-dependent atypical PKC isoforms (ι, τ, λ, μ). It is believed that selected members may be involved in mediating diverse cellular responses. We found that phosphorylation of SAF-1 protein by PKC markedly increases its DNA binding activity (Fig. 5, B and C). The transactivating potential of pCMVSAF1-1 in the presence of agonists of PKC (Fig. 4B) or during overexpression of PKC-βII was also significantly higher (Fig. 6). However, the effect of PKC-mediated phosphorylation on SAF appears to be limited to increasing only its DNA binding activity. A GAL4-SAF1 construct, in which a full-length SAF-1 cDNA is fused in frame to the C-terminal of the GAL4 DNA-binding domain, considerably increased expression of a GAL4-CAT reporter gene, indicating that SAF-1 can confer transcriptional activation on a heterologous DNA-binding protein. The transactivating potential of GAL4-SAF1 was not altered when transfected cells were allowed to overexpress PKC-β protein (Fig. 7). These results suggested that PKC-mediated phosphorylation has little effect on the transactivating domains of SAF-1.

There are several consensus PKC phosphorylation sites in the SAF1 coding sequence. Some PKC sites are present at the amino-terminal region, and one potential PKC site is present.
within the fifth zinc finger domain, located at the carboxyl-terminal region of SAF1 protein. Although exact DNA-binding domains of SAF-1 are yet to be defined, it can be speculated that PKC-induced phosphorylation at the fifth zinc finger domain or at the other PKC phosphorylation sites may induce some conformational changes that facilitate the DNA binding ability of SAF1. The importance of individual PKC phosphorylation sites, however, remains to be determined via mutagenesis studies of all possible PKC phosphorylation sites. In conclusion, our study demonstrates that SAF1 protein, a member of the SAF/MAZ/Pur-1 family of transcription factors, becomes phosphorylated and activated by agonists of the PKC signaling pathway.

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REFERENCES

1. Kushner, I. (1982) Ann. N.Y. Acad. Sci. 389, 39–48
2. Sipe, J. D. (1994) Crit. Rev. Clin. Lab. Sci. 31, 325–354
3. Strissel, K. J., Girard, M. T., West-Mays, J. A., Rinehart, W. B., Cook, J. R., Brinckerhoff, C. E., and Fini, M. E. (1997) Exp. Cell. Res. 237, 275–287
4. Brinckerhoff, C. E., Mitchell, T. I., Karmilowicz, M. J., Kluve-Beckerman, B., and Benson, M. D. (1989) Science 244, 655–657
5. Mitchell, T. I., Coon, C. I., and Povrik, L. F., Taylor, R. S., and Grant, S. (1994) Cancer Res. 54, 1704–1714
6. Geckeler, V., Boer, R., Uberall, F., Isle, W., Schubert, C., Utz, I., Hoffman, J., Sanders, K. H., Schachtele, C., Kliem, K., and Grunicke, H. (1996) Br. J. Cancer 74, 897–905
7. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Luckow, B., and Schutz, G. (1987) Nucleic Acids Res. 15, 5490
9. Murray, N. R., Baumgardner, G. P., Burns, D. J., and Fields, A. P. (1993) J. Biol. Chem. 268, 5429–5433
10. Flemington, E. K., Speck, S. H., and Kaelin, W. G., Jr. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6914–6918
11. Blanar, M. A., and Rutter, W. J. (1992) Science 256, 1014–1018
12. Huberman, E., and Jones, C. A. (1985) Carcinog. Compr. Surv. 10, 263–273
13. Vandenbark, G. R., and Niedel, J. E. (1984) J. Immunol. 133, 681–685
14. Roman, D., Burt, D. W., Cheshire, J. K., and Woo, P. (1989) Mol. Cell. Biol. 9, 1908–1916
15. Urieli-Shoval, S., Meek, R. L., Hanson, R. H., Eriksen, N., and Sipe, J. D. (1997) Mol. Cell. Biol. 17, 7327–7335
16. Ray, B. K., Chatterjee, S., and Ray, A. (1999) Mol. Cell. Biol. 18, 7237–7243
17. Murray, N. R., Thompson, L. J., and Fields, A. P. (1997) in Protein Kinase C: Molecular Biology Intelligence Unit (Parker, P. J., and Dekker, L., eds) pp. 97–120, R. G. Landis Co., Austin, TX
18. Liu, W. S., and Hechman, C. A. (1988) Calcium Signal. 10, 529–542