Protein Kinase A Signaling Pathway Regulates Transcriptional Activity of SAF-1 by Unmasking Its DNA-binding Domains*

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Serum amyloid A (SAA) activating factor-1 (SAF-1) is an inducible transcription factor that plays a key role in the regulation of several inflammation-responsive genes including SAA and matrix metalloproteinase-1. Increased synthesis of SAA and matrix metalloproteinase-1 is associated with pathogenesis of several diseases including amyloidosis, arthritis, and atherosclerosis. Previously, we showed in vivo interaction of SAF-1 and protein kinase A (PKA) and presented evidence for induction of SAF-1-regulated genes by a PKA signaling pathway. Here we demonstrate a mechanism by which PKA increases functional activities of SAF-1. Site-directed mutagenesis and phosphorylation analyses revealed two sites in the SAF-1 protein, serine 187 and threonine 386, as the target of PKA. Interestingly, mutation of both PKA phosphorylation sites created a highly active SAF-1 protein with high DNA-binding ability. Furthermore, we found that terminal deletion of SAF-1 protein from either end creates SAF-1 isoforms that are highly transcriptionally active. Partial proteolysis experiments indicated that unphosphorylated and phosphorylated SAF-1 proteins are structurally distinct. Together these results suggest that under native condition, N and C termini of SAF-1 are engaged in an inhibitory intramolecular interaction. PKA-mediated phosphorylation increases transcriptional activity of SAF-1 by unmasking the DNA-binding domain.

Altered gene expression leading either to normal cell growth or pathological conditions, in response to extracellular and/or intracellular signaling, requires participation of activated transcription factors. Many transcription factors remain in an inactive form in the cell but become activated during cytokine- or growth factor-mediated signaling. Activation of transcription factors is therefore regarded as the final step in inducing or repressing specific gene transcription. The serum amyloid A activating factor (SAF), a family of zinc finger proteins was first identified as a transcription factor that is expressed in several tissues and cell types and is involved in regulating induction of serum amyloid A protein (SAA) during inflammation (1, 2). Persistent high levels of SAA are linked to various pathophysiological conditions, including amyloidosis, rheumatoid arthritis, and atherosclerosis (3, 4). In a recent study (5), SAF-1 is identified as a key regulator of increased matrix metalloproteinase 1 synthesis under osteoarthritic conditions. The DNA-binding element of SAF-1 and its human and mouse homolog MAZ/Pur-1 (6, 7) contains GC-rich sequences that are found in the promoter regions of many genes. Consequently, SAF-1/MAZ/Pur-1 is shown to regulate expression of a variety of genes, including SAA (1), c-myc (6), insulin (7), serotonin 1A receptor (8), adenovirus major late promoter (9), CD4 (10), γ-fibrinogen (11), PMNT (12), and CLC-K1 (13). The DNA-binding and transactivating ability of SAF-1 is induced in response to many inflammatory conditions (2, 14, 15), primarily via serine/threonine phosphorylation (2).

An understanding of how SAF-1 and its homologs regulate gene expression requires (i) identification of the signaling pathways that activates this transcription factor and (ii) detailed analyses of the involvement of regulatory partners. One powerful approach involves employing the yeast two-hybrid system, which identifies protein-protein interactions that occur in vivo in the course of regulating many cellular activities. Using the two-hybrid system, we recently identified (16) that the catalytic subunit of PKA interacts with SAF-1. The PKA signaling pathway is one of the prominent signaling pathways (17). An increase in the intracellular levels of cAMP, generated by activation of adenylate cyclase, results in the activation of PKA. PKA exists as tetrameric holoenzyme that contains two regulatory CAMP-binding (R-I) subunits and two catalytic (C) subunits. Increase of intracellular cAMP level causes binding of cAMP to the regulatory subunits, resulting in the release of the catalytic subunits of PKA (18). Activated PKA then mediates the effects of cAMP by phosphorylating various substrates, including transcription factors, and thereby affects both the regulation of key metabolic enzymes and expression of numerous target genes.

In a previous study (19) we noted that agonists of cAMP markedly increase the DNA-binding ability of endogenous SAF proteins. Furthermore, transactivation potential of SAF-1-regulated promoter was induced in response to ectopic expression of the catalytic subunit of PKA (19). The present study was initiated to understand the underlying mechanism through which PKA activates functional capacities of SAF-1. We identified two consensus phosphorylation sites in the SAF-1 protein, one at the N-terminal and the other at the C-terminal end, that are primary targets of PKA. We show that mutation of both of these PKA phosphorylation sites creates a high DNA-binding and transcriptionally active SAF-1 protein. When a single site is mutated, the C-terminal PKA phosphorylation site mutant of SAF-1 displayed virtual inactivity whereas the N-terminal PKA phosphorylation site mutant exhibited high transcriptional activity. Terminal truncation of SAF-1 from

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The abbreviations used are: SAF, serum amyloid A activating factor; SAA, serum amyloid A; PKA, protein kinase A; CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay; 8-Br-cAMP, 8-bromo adenosine 3′,5′-cyclic monophosphate; Rp-8-Br-cAMP, Rp-isomer of 8-bromo adenosine 3′,5′-cyclic monophosphate; PKI, PKA inhibitor; wt, wild-type; DMEM, Dulbecco’s modified Eagle’s medium.
either end created transcriptionally active SAF-1 proteins with high DNA-binding function. Limited proteolysis of unphosphorylated and phosphorylated SAF-1 consistently produced slightly different peptide fragments. Together these results indicate that phosphorylation of corresponding domains of SAF-1 by PKA is not directly involved in increasing the DNA-binding activity of this protein; instead phosphorylation by PKA induces a conformational change that exposes the DNA-binding domain of SAF-1 before transfection experiments were performed at least three times.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**H–82 rabbit synoviocyte 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). H–82 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose (4.5 g/liter) supplemented with 7% fetal calf serum. Cells were transfected with 8-Br-cAMP (0.4 mM), Rp-8-Br-cAMP (0.4 mM), and cell-permeable myristoylated PKA inhibitor 14–22 amide (PKI) (2 μM) as indicated in the text and figure legends. These agents were purchased from Calbiochem-Novabiochem.

**Transfection experiments** of H–82 cells were carried out by the calcium phosphate method (21). The amount of DNA in each transfection assay was kept same by using carrier DNA. Reporter plasmids and the eucaryotic expression vectors containing SAF-1 cDNA or PKA cDNA were transfected into 3T3 cells in amounts as indicated in the figure legends along with 1 μg of pSV-β-galactosidase (Promega). The pSV-β-galactosidase plasmid was included as an internal control for measuring transfection efficiency. Cells were harvested 24 h post-transfection, and CAT activity in the cell extracts was determined as described previously (21). β-Galactosidase activity was assayed with the substrate o-nitrophenyl-β-D-galactopranoside as described (21). Cell extracts containing equivalent amount of β-galactosidase activity were used for CAT assay. Prior to the CAT assay, each cell extract was heated at 60 °C for 10 min to inactivate endogenous acetylase activity. Different agents used in the transfection assay had no effect on β-galactosidase expression. All transfection experiments were performed at least three times.

**Plasmids—**The CAT reporter plasmids, wt-SAF-CAT and mutant SAF-1/CAT, were constructed by ligating three tandem copies of the wild-type SAF-binding element (1, 2) and mutated sequence, respectively into the pBLCAT2 vector (22). Sequence of the wild-type element and mutated sequence, respectively into the pBLCAT2 vector (22) are shown below. The resulting clones were verified by DNA sequencing. To assay for protein, the DNA-binding assay was kept same by using carrier DNA. Reporter plasmids and the CAT assay, each cell extract was heated at 60 °C for 10 min to inactivate endogenous acetylase activity. Different agents used in the transfection assay had no effect on β-galactosidase expression. All transfection experiments were performed at least three times.

**PKA Regulates SAF-1 Activity**

Construction of GALA/DSLAF-1 Plasmids—Plasmid pSV-GALAD (25), encoding the DNA-binding domain of yeast GAL4 gene, which is located within amino acids 1–147, was used to prepare the GALA/DSLAF-1 construct. Full-length SAF-1 cDNA and the mutant forms of SAF-1 were fused in-frame at the C-terminal to GALAD at the unique BamHI site. The resulting clones were verified by DNA sequencing.

**Electrophoretic Mobility Shift Assay (EMSAs)—**EMSAs was performed with equal protein amounts of bacterially expressed proteins according to the methods described previously (2). Protein concentrations were measured by Bradford’s method (26). Radiolabeled probe containing SAF-binding element of SSA promoter was prepared by using [α-32P]dCTP as the substrate to label the double-stranded oligonucleotide (22). Two complementary oligonucleotide pairs, 5'-ACTCTCCACACAGCGTCG-3' and 5'-CGAGGAGGAGGTTTGGTGGCGG-5', were annealed to prepare the double-stranded SAF-binding element. In some assays, bacterially expressed wild-type and mutant SAF-1 proteins were phosphorylated with 1.0 unit of the catalytic subunit of PKA (Calbiochem-Novabiochem) in a volume of 10 μl prior to the DNA-binding assay. Some assays contained calf intestinal alkaline phosphatase (4.0 units).

**In Vitro Phosphorylation—**The phosphorylation reaction was performed in 50 mM Tris-HCl (pH 7.5)/10 mM MgCl2/0.1 mM EDTA/1 mM diithiothreitol/0.1 mM ZnCl2/0.2 mM sodium orthovanadate/5 mM NaF/1 mM phenylmethylsulfonyl fluoride/0.5 mg/ml benzamidine/100 μM of unlabeled ATP/10 μCi of 1-32P]ATP (6000 Ci/mmol)/1 unit of PKA (Calbiochem-Novabiochem). The reaction reached a steady state at 45 min at 25 °C. In each reaction, 0.1 μg of purified wild-type or mutant SAF1 proteins or 20 to 50 ng of synthetic SAF-1 peptides was added. Phosphorylated protein was immunoprecipitated by incubating with an anti-FLAG antibody (Sigma) at 4 °C for 14–16 h in the immunoprecipitation buffer containing 50 mM Tris-HCl (pH 8.0)/150 mM NaCl/1% (v/v) Nonidet P-40/0.1% (v/v) SDS/2.5 mM phenylmethylsulfonyl fluoride/0.5 mg/ml benzamidine. Next, 50 μl of protein G-agarose slurry was added to the reaction mixture and incubated for 2 h at 4 °C with gentle shaking in a rotary shaker. After centrifugation, the pellet was washed five times with the immunoprecipitation buffer (5 min each) to remove any unbound radioactivity. The pellet was resuspended in 20 μl of buffer containing 2% (w/v) SDS/50 mM Tris–HCl (pH 8.9)/15 μM β-mercaptoethanol and heated at 100 °C for 10 min. These mixtures were fractionated in SDS–11% polyacrylamide gel, and phosphorylated proteins were detected by autoradiography.

**In Vivo Phosphorylation—**H–82 cells were transfected with pCMVFLAG-SAF-1(wt), pCMVFLAG-SAF-1(S187A,T386A), pCMVFLAG-SAF-1(S187A,T386A) and pCMVFLAG-SAF-1(S187A) mutant plasmids were prepared by subcloning two mutant cDNA's of SAF-1 in the pCMV3 vector. The pCMVFLAG-SAF-1 plasmid was constructed by first amplifying, in-frame, a FLAG-tagged sequence at the N terminus of the full-length SAF-1 cDNA; the resulting plasmid, pCMVFLAG-SAF-1, was further subcloned in the pCMV3 vector (Invitrogen). The pCMVFLAGSAF-1(1-386) and pCMVFLAGSAF-1(1-386) mutant plasmids were prepared by subcloning two mutant cDNA's of SAF-1 in the pCMV3 vector. The mutant cDNA's were generated by megaprimer PCR (24) using appropriate oligonucleotides ~35 bp in length. To amplify, effectively, the GC-rich sequences present in the SAF-1 sequence, GC-melt (Clontech) was added to the PCR mixture. The PCR products were subcloned in pTZ119 vector and sequenced for verification of mutations. A FLAG epitope was attached at the N-terminal end of mutant cDNA's prior to subcloning in pCMV3 vector. The full-length SAF-1 (1–477) and deletion constructs, SAF-1 (1–380) and SAF-1 (188–477) were prepared by subcloning the respective PCR-created fragments into pTZ119U vector. The primer sets were designed to phosphorylate SAF-1 (1–477), 5'-CTCATCTAGCTACAGCTACAGT-3' and 5'-GGTACGAGGCTACAGCTACAGT-3'; for SAF-1 (188–477), 5'-CTCAGCTACAGCTACAGT-3'; for SAF-1 (188-477), 5'-CTCAGCTACAGCTACAGT-3'; for SAF-1 (188–477), 5'-CTCAGCTACAGCTACAGT-3'; and 5'-GGTACGAGGCTACAGCTACAGT-3'.

**Limited Proteolysis—**Both native and phosphorylated SAF-1 proteins were subjected to partial proteolysis by V8 protease. SAF-1 proteins were phosphorylated as described above using non-radioactive ATP. The reaction mixtures were diluted 4-fold with sterile distilled water. Both the native and phosphorylated SAF-1 proteins (1 μg each) were then incubated with V8 protease (0.5 μg) in a total volume of 10 μl at 37 °C for different time periods as described in the figure legends. Protease reaction was stopped by adding 10 μl of a sample buffer containing 100 mM Tris–HCl (pH 6.8)/1% (v/v) glycerol/50 mM EDTA/15 μM β-mercaptoethanol and heating the mixture at 95 °C for 10 min. The samples were subsequently fractionated in a SDS-12% PAGE and transferred onto nitrocellulose membrane. The immunoblotting was performed as described earlier (27) with anti-FLAG (Sigma) antibody. Bands were detected by using a chemiluminescence detection system (Amersham Biosciences).

**Western Immunoblot Assay—**Proteins were separated by SDS-11% PAGE and transferred onto nitrocellulose membrane. The immunoblotting was performed as described earlier (27) with anti-FLAG (Sigma) antibody. Bands were detected by using a chemiluminescence detection system (Amersham Biosciences).
Activation of SAF-1 by PKA

—Previous studies employing yeast two-hybrid assay showed that SAF-1 associates with the catalytic subunit of PKA (16), and activation of PKA in response to cAMP-signaling increased the DNA-binding activity of endogenous SAF proteins in cAMP-treated cells (19). As PKA is one of the key enzymes involved in the regulation of many biological processes it is reasonable to predict that PKA may be a key effector in modulating expression of genes regulated by SAF-1. To gain further insights into the mechanism by which PKA activates expression of SAF-1-regulated genes, we undertook the following experiments. HIG-82 synovial cells that exhibit optimal response to SAF-1 expression were cotransfected with a reporter plasmid containing three copies of wild-type or mutant SAF DNA-binding elements. In some assays cells were cotransfected with pCMVSAF-1 (0.5 μg of DNA) and pCo-PKA (0.5 μg of DNA) expression plasmids as indicated. Following glycerol shock, the cells were incubated for an additional 24 h and then harvested. During this period, some cells were incubated with 8-Br-cAMP (0.4 mM), Rp-8-Br-cAMP (0.4 mM), or myristoylated cell-permeable PKI inhibitor 14–22 amide (2 μM). CAT activity was determined as described under “Experimental Procedures.” The results shown are averages of three separate experiments. B, HIG-82 cells were transfected with 1.0 μg of 0.6 SAA-CAT3 reporter plasmid alone or together with pCMVSAF-1 (0.5 μg of DNA) and pCo-PKA (0.5 μg of DNA) expression plasmids as indicated. Similar to that in A, cells in some transfection reactions were incubated with same concentrations of 8-Br-cAMP, Rp-8-Br-cAMP, or myristoylated cell-permeable PKI inhibitor 14–22 amide. C, PKA phosphorylates SAF-1 protein. Purified FLAG-SAF-1 protein (0.1 μg) either alone (lane 3) or with 1.0 unit of PKA (lanes 1 and 2) was used in a phosphorylation assay with [γ-32P]ATP, separated on 11% SDS-PAGE, and autoradiographed. Assay mixture in lane 2 contained alkaline phosphatase. Details of phosphorylation and immunoprecipitation reactions are described under “Experimental Procedures.” D, phosphorylation of SAF-1 by PKA increases its DNA-binding ability. Purified FLAG-SAF1 protein was phosphorylated by PKA and non-radioactive ATP prior to the use in the DNA-binding assay. Phosphorylated FLAG-SAF1 protein (0.5 μg) was incubated with a radiolabeled SAF DNA-binding element as the probe. Lane 1 contains FLAG-SAF1 protein alone, lanes 2 and 3 contain FLAG-SAF1 protein plus 1.0 unit of PKA, and lane 4 contains only PKA. In lane 3, alkaline phosphatase was added during phosphorylation of FLAG-SAF1 protein with PKA. E, Western blot analysis. Parallel phosphorylation reactions containing FLAG-SAF1 protein, as described for D, were prepared, and the reaction mixtures were separated by SDS-11%PAGE, transferred to a nitrocellulose membrane, and probed with anti-FLAG antibody (Sigma).
expression of SAF-CAT reporter indicating the specificity of PKA action. It is noteworthy that expression of the reporter gene was induced by both 8-Br-cAMP and PKA, which probably is because of activation of endogenous SAF-1 by these agents. Because the reporter used in these assays was constructed using tandem multiple copies of short SAF-1 DNA-binding elements, we further verified the inductive effects of PKA by using a natural promoter, 0.6 SAA-CAT3 reporter gene that contained a 600-bp upstream promoter region of rabbit SAA gene (2, 23). The HIG-82 cells were transfected with 0.6 SAA-contained a 600-bp upstream promoter region of rabbit SAA elements, we further verified the inducive effects of PKA by using tandem multiple copies of short SAF-1 DNA-binding elements, as indicated (Fig. 1B). Both 8-Br-cAMP and PKA increased expression of 0.6SAA-CAT reporter similarly. However, the rate of induction was more profound with the SAF-CAT reporter. Next, to assess how PKA potentiates SAF-1 action, we determined whether SAF-1 is phosphorylated by PKA. Bacterially expressed FLAG-SAF1 protein was incubated with purified catalytic subunit of PKA and [γ-32P]ATP and further immunoprecipitated with anti-FLAG antibody. A strong radioactive band comigrating at the predicted size of FLAG-SAF1 protein (Fig. 1C, lane 1) was seen but not in the lanes where phosphatase was included in the phosphorylation assay (Fig. 1C, lane 2) or where PKA was absent in the reaction mixture (Fig. 1C, lane 3). As cotransfection of cells with activators of PKA or PKA itself induced SAF-regulated promoters, we wondered whether phosphorylation by PKA alters the capacity of SAF-1 to interact with DNA. As seen in Fig. 1D, PKA-mediated phosphorylation markedly increased (about 10-fold as judged by the radioactivity in the DNA-protein complex) the DNA-binding activity of SAF-1 (compare lanes 1 and 2). Formation of no DNA-protein when phosphatase was included (lane 3) or when SAF-1 protein was omitted (lane 4) indicated the specificity of this assay. This result was in accord with previous observations that showed increased DNA-binding ability of endogenous SAF proteins in cAMP-treated cells (19). Western blot assay with anti-FLAG antibody was performed to verify the input of SAF-1 protein in each DNA-binding assay (Fig. 1E).

PKA Directly Affects Transactivating Ability of SAF-1—The observation that PKA-mediated phosphorylation markedly improves the DNA-binding ability of SAF-1 protein prompted us to investigate whether overall increase of transactivation potential of SAF-1 is because of just increased DNA-binding ability or because of a combination of increased DNA-binding and transactivating functions. To test these possibilities we fused, in-frame, the full-length SAF-1 DNA downstream of the DNA-binding domain of yeast GAL4 transactivation factor and tested the transactivation potential of this chimeric protein using a CAT reporter construct driven by highly specific GAL4 DNA-binding elements, known as the UAS, that drives transcription of the reporter gene, CAT. B, activation of transcription from a GAL4 binding site-driven CAT reporter construct. HIG-82 cells were transfected with GAL4CAT reporter, together with GAL4DBD-SAF1 or GAL4DBD plasmid. Some transfection assays contained pCo-PA plasmid or myristoylated PKI inhibitor 14–22 amide (2 µg) as indicated. Cells were harvested 24 h after glycerol shock, and CAT activity was measured. The results shown are averages of three separate experiments.

PKA Regulates SAF-1 Activity

PKA-activated transcription factor SAF-1 in HeLa cells increases transactivating potential of SAF-1. Together these results strongly suggested that SAF-1 transcription factor is a target of PKA-mediated signaling.

Mapping of the Major PKA Phosphorylation Sites in SAF-1—To map the sites in SAF-1 that are phosphorylated by PKA, we examined the primary amino acid sequence of SAF-1. The consensus PKA phosphorylation sites are identified as (R/K)(R/K)/S/T, RX(S/T), or RX(S/T) (X denotes any amino acid) in which the former one is the most potent phosphate acceptor site. Primary sequence of SAF-1 showed two major PKA phosphorylation sites, one at the KKRKS187 and the other at RAHT386 peptide sequence. The RAHT386 is located in the middle of the fifth zinc finger. Both of these sites are highly conserved and present in the human, rabbit, and mouse species (Fig. 3A). First, we examined whether these sequences are authentic PKA phosphorylation sites. Two short peptides, SAF-1(PKA1) and SAF-1(PKA2), containing sequences of these regions, were subjected to in vitro phosphorylation with PKA and [γ-32P]ATP. Both of these peptides were efficiently labeled (Fig. 3B, lanes 3 and 4) whereas the mutant peptides in which serine 187 and threonine 386 were replaced with alanine remained unlabeled (Fig. 3C). These results indicated that these amino acid sequences of SAF-1 are potential targets of PKA-mediated phosphorylation.

To determine whether the above mentioned sites are indeed PKA phosphorylation sites, as well as to understand the functional significance of these two putative PKA phosphorylation sites in the context of native SAF-1 protein, we opted to prepare mutants in which these sites are specifically mutated. A double mutant SAF-1(S187A,T386A) was prepared in which both Ser187 and Thr386 were replaced with non-phosphorylatable alanine (Fig. 4A). To understand the role of each site, we also prepared two single site mutants of SAF-1 (Fig. 4A). First, we verified by in vitro phosphorylation assay the effectiveness of these mutations in terms of resisting phosphorylation when these altered sequences are parts of SAF-1 protein. To determine, wild-type and mutant cDNAs of SAF-1 were cloned in bacterial expression vector; proteins were expressed and affinity-purified. Equal amounts of each protein were subjected to in vitro phosphorylation assay the effectiveness of these mutations in terms of resisting phosphorylation when these altered sequences are parts of SAF-1 protein.
**vitro** phosphorylation assay with purified PKA, and radiolabeled proteins were separated by SDS-PAGE. Coomassie Blue staining was performed to determine the amount and quality of each protein used in the phosphorylation assay (Fig. 4B). As seen in the Fig. 4C, lane 2 the double mutant SAF-1(S187A, T386A) containing two defective phosphorylation sites was very poorly labeled as compared with the wild-type SAF-1 protein (Fig. 4C, lane 1). Because Coomassie Blue staining of these proteins indicated the use of approximately equal amounts of the substrates in the kinase reaction, these data clearly showed that **in vitro** phosphorylation of SAF-1 is mainly dependent on serine 187 and threonine 386. The SAF-1(T386A) mutant protein also showed a comparable low level of 32P incorporation despite the presence of one intact phosphorylation site (Fig. 4C, lane 3). However, it was quite surprising to find high level of 32P incorporation in the SAF-1(S187A) mutant protein (Fig. 4C, lane 4). A higher level of 32P incorporation in SAF-1(S187A) mutant despite the use of similar amounts of protein was challenging to explain. The most simple interpretation of these results is that SAF-1(S187A) mutant gets phosphorylated at a much better rate than the wild-type SAF-1 protein because of some conformational changes resulting from the mutation. If we also consider that all wild-type SAF-1 molecules are not completely phosphorylated in the conditions provided in our phosphorylation assay, then it is possible that phosphorylation of increased number SAF-1(S187A).
PKA Regulates SAF-1 Activity

Fig. 5. Mutation of two PKA phosphorylation sites increases DNA-binding ability and transactivation potential of SAF-1. A, affinity-purified FLAGSAF-1(wt) and FLAGSAF-1(S187A,T386A) proteins (0.1 μg in each lane) were used in EMSA with 32P-labeled SAF-binding oligonucleotide probe as described under “Experimental Procedures.” In lanes 1 and 2, unphosphorylated proteins were used whereas in lanes 2 and 3, proteins were phosphorylated by PKA and non-radioactive ATP prior to the DNA-binding assay. B, Western immunoblot analysis of SAF-1 and SAF-1(S187A,T386A) proteins. Equal amount of SAF-1 and SAF-1(T386A) proteins as used in lanes 1–4 in A were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-FLAG antibody. C, HIG-82 cells were transfected with 0.5 μg of SAF-CAT reporter plasmid alone or together with 0.5 μg of pCMVFLAGSAF-1 or 0.5 μg of pCMVFLAGSAF-1(S187A,T386A) expression plasmids. Some transfection assays contained 0.5 μg of pCyc-PKA plasmid DNA. Cells were harvested 24 h after glycerol shock, and CAT activity was measured. The results shown are averages of three separate experiments. D, Western immunoblot analysis of transfected cells for determining expression pattern of transfected plasmids. 50 μg of protein from cells transfected with expression plasmids, as indicated, were separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and immunoblotted with anti-FLAG antibody. E, activation of transcription from a GAL4-binding site-driven CAT reporter construct. HIG-82 cells were transfected with GALACAT reporter, together with GAL4DBDSAF-1 or GAL4DBDSAF1(S187A,T386A) mutant plasmid. Some transfection assays contained pCyc-PKA plasmid as indicated. Cells were harvested 24 h after glycerol shock, and CAT activity was measured. The results shown are averages of three separate experiments.

protein molecules could result in more 32P incorporation despite the absence of one good phosphorylation site. Therefore more radioactivity in Fig. 4C, lane 4, could represent simply more molecules of phosphorylated SAF-1(S187A) protein at one site as compared with less molecules of wild-type SAF-1 phosphorylated at two sites (Fig. 4C, lane 1).

To test whether in vivo these two phosphorylation sites are responsive to PKA action and also to assess the relative phosphorylation capacity of the mutant SAF-1 proteins, we transfected HIG-82 cells with FLAG-tagged wild-type SAF-1, SAF-1(T386A), SAF-1(S187A), and SAF-1(S187A,T386A) expression plasmids and metabolically labeled the transfected cells with 32P, following addition of cAMP to activate the PKA signaling pathway. Ectopically expressed and radiolabeled SAF-1 phosphoproteins were immunoprecipitated with anti-FLAG antibody and separated by SDS-PAGE. In correlation with the in vitro phosphorylation assay, very little radioactivity was seen in the lanes containing SAF-1(S187A,T386A) and SAF-1(T386A) mutant proteins whereas SAF-1(S187A) protein was significantly more labeled as compared with the wild-type SAF-1. Together, these results indicated that serine 187 and threonine 386 are major PKA phosphorylation sites in SAF-1.

Mutation of Both PKA Phosphorylation Sites Increases Transcriptional Capacity of SAF-1—Having established that Ser187 and Thr386 are primary targets of PKA-mediated phosphorylation both in vivo and in vitro, we examined what effect these mutations convey on the biological activities of SAF-1. DNA-binding ability of SAF-1(S187A,T386A) protein was compared with wild-type SAF-1 protein by using equal amounts of both protein. Bacterially expressed and purified proteins were either left untreated or subjected to PKA treatment prior to the DNA-binding assays. It was surprising to find that compared with wild-type SAF-1, SAF-1(S187A,T386A) protein was much more active in terms of the DNA-binding ability (Fig. 5A, compare lanes 1 and 3). Quantitation of the DNA-protein complexes from a typical DNA-binding experiment showed 50-fold more count in the DNA-protein complex formed by SAF-1(S187A,T386A) protein over wild-type SAF-1. When SAF-1(S187A,T386A) protein was treated with PKA prior to the DNA-binding assay, there was virtually no increase in the DNA-binding activity of this protein (Fig. 5A, lanes 3 and 4). Under the same conditions, however, DNA-binding activity of wild-type SAF-1 was highly increased (Fig. 5A, lanes 1 and 2). This finding correlated well with earlier results, shown in Fig. 4, that demonstrated unresponsive characteristics of SAF-1(S187A,T386A) protein toward PKA. Western blot assay was performed to assess the levels of proteins used in the DNA-binding assay, which appeared to be at similar levels (Fig. 5B).

To determine overall transactivation potential of SAF-1(S187A,T386A) protein, this cDNA was subcloned in mammalian expression vector. It was quite surprising to note that although DNA-binding ability of SAF-1(S187A,T386A) protein was at least 50-fold higher than wild-type SAF-1, overall transactivating potential of this protein increased only by about 2-fold (Fig. 5C). Transactivation potential of SAF-1(S187A, T386A) was further increased although at a low level when cells were cotransfected with PKA expression plasmid. We believe this additional gain is resulting from PKA-activated endogenous SAF-1 action on SAF-CAT reporter. Western blot assay showed similar levels of expression pattern of each transfected plasmid (Fig. 5D). Next we examined transactivating function of SAF-1(S187A,T386A) protein by expressing it as a
DNA-binding levels of SAF-1(T386A) and SAF-1(S187A) mutants of wild-type SAF-1 were highly induced, but no change in the response to PKA-mediated phosphorylation. A Western blot analysis of FLAGSAF-1(T386A), FLAGSAF-1(wt), and FLAGSAF-1(S187A) proteins. Equal amounts of proteins as used in lanes 1–6 in A were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-FLAG antibody. C, HIG-82 cells were transfected with 1.0 μg of SAF-CAT reporter plasmid alone or together with 0.5 μg of pCMV-FLAGSAF-1, pCMV-FLAGSAF-1(S187A), or pCMV-FLAGSAF-1(T386A) expression plasmids as indicated. The latter plasmids are indicated as SAF-1(wt), SAF-1(S187A), and SAF-1(T386A), respectively. Some transfection assays contained 0.5 μg of pCo-PKA plasmid DNA. Cells were harvested 24 h after glycerol shock, and CAT activity was measured. The results shown are averages of three separate experiments. D, Western blot analysis of transfected cells to measure expression pattern of transfected plasmids. 50 μg of protein from transfected cells with expression plasmids, as used in C, were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-FLAG antibody. E, HIG082 cells were transfected with 1.0 μg of GAL4-CAT reporter plasmid alone or together with 0.5 μg of GAL4DBDSAF-1, GAL4DBDSAF-1(T386A), or GAL4DBDSAF-1(S187A) expression plasmids as indicated. Some transfection assays contained 0.5 μg of pCo-PKA plasmid DNA. Cells were harvested 24 h after glycerol shock, and CAT activity was measured. The results shown are averages of three separate experiments.

GAL4DBD fusion protein. This assay showed that transactivating function of SAF-1(S187A,T386A) protein is slightly higher than wild-type SAF-1 and is unresponsive to PKA action (Fig. 5E).

Above results showed that SAF-1(S187A,T386A) protein is functionally more active than wild-type SAF-1, although it lacks two active PKA phosphorylation sites and does not respond to PKA-mediated phosphorylation. This is a contradiction to the notion that phosphorylation by PKA is somehow necessary for the activation of SAF-1 as judged by the findings shown in Figs. 1 and 2. These apparently paradoxical findings suggest that the induction mechanism of SAF-1 by PKA is complex. In view of these unexpected results obtained from SAF-1(S187A,T386A) mutant, we examined the functional effects of single PKA phosphorylation site mutation on SAF-1.

Mutation at Thr1386 Phosphorylation Site Reduces Transcriptional Capacity whereas Mutation atSer187 Increases Transactivation Potential of SAF-1—First we assessed the DNA-binding abilities of single PKA site mutants of SAF-1, SAF-1(T386A), and SAF-1(S187A). Bacterially expressed, purified wild-type FLAGSAF-1, FLAGSAF-1(T386A), and FLAGSAF-1(S187A) proteins were subjected to PKA-mediated phosphorylation prior to their use in the DNA-binding assay. DNA-binding activity of unphosphorylated wild-type SAF-1 and SAF-1(T386A) mutant protein was comparable similar whereas SAF-1(S187A) mutant exhibited remarkably higher levels of DNA-binding ability (Fig. 6A, lanes 1, 3, and 5). In response to PKA-mediated phosphorylation, DNA-binding ability of wild-type SAF-1 was highly induced, but no change in the DNA-binding levels of SAF-1(T386A) and SAF-1(S187A) mutant proteins was seen (Fig. 6A, lanes 2, 4, and 6). Western blot assay with anti-FLAG antibody was done to ensure equal input of these proteins (Fig. 6B).

To examine overall transactivating abilities of SAF-1(T386A) and SAF-1(S187A) mutants, we cotransfected HIG-82 cells with SAF-CAT reporter and pCMV-FLAGSAF-1(T386A) or pCMV-FLAGSAF-1(S187A) expression plasmids (Fig. 6C). The results show that whereas SAF-1(T386A) acts poorly, SAF-1(S187A) is highly capable of promoting expression of SAF-CAT reporter gene. However, it was interesting to note that transactivating potential of SAF-1(S187A) did not increase as much as its DNA-binding ability was increased when compared with untreated SAF-1(wt). Furthermore, transactivating potential of SAF-1(S187A) was minimally enhanced in response to PKA-mediated phosphorylation. Western blot assay with anti-FLAG antibody showed no discrepancy at the expression level of these proteins (Fig. 6D) suggesting that differential activities of SAF-1(T386A) and SAF-1(S187A) mutants are not because of dissimilar levels of expression of these constructs.

We examined specific transactivating functions of SAF-1(T386A) and SAF-1(S187A) mutants by expressing them as GAL4DBD fusion proteins (Fig. 6E). This assay showed that SAF-1(T386A) has no transactivating function, because it was unable to induce GAL4-CAT reporter gene expression. In contrast, transactivating function SAF-1(S187A) protein was slightly higher than wild-type SAF-1. Transactivating functions of both mutants remained virtually unchanged in response to PKA-mediated phosphorylation.

Data obtained from three SAF-1 mutants further emphasized that induction mechanism of SAF-1 by PKA is far from being conventional. First, PKA vastly improves the DNA-binding and transactivating functions of wild-type SAF-1, yet the SAF-1(S187A,T386A) mutant containing two impaired PKA phosphorylation sites is highly active. Second, a single PKA phosphorylation site mutant, SAF-1(T386A) protein, does not get phosphorylated although it contains one intact PKA phosphorylation site. This protein is functionally inactive because of both poor DNA-binding and transactivation functions. Third,

PKA Regulates SAF-1 Activity

Fig. 6. Mutation of threonine 386 at RLRAHTVR site decreases whereas mutation of serine 187 at the EKKRKSKGP site increases transactivation potential and DNA-binding ability of SAF-1. A, affinity-purified FLAGSAF-1(T386A), FLAGSAF-1(wt), and FLAGSAF-1(S187A) proteins (0.1 μg in each lane) were used in EMSA with 32P-labeled SAF-binding oligonucleotide probe as described under “Experimental Procedures.” In lanes 2, 4, and 6 proteins were phosphorylated with PKA prior to their use in the DNA-binding assay. B, Western immunoblot analysis of FLAGSAF-1(T386A), FLAGSAF-1(wt), and FLAGSAF-1(S187A) proteins. Equal amounts of proteins as used in lanes 1–6 in A were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-FLAG antibody. C, HIG-82 cells were transfected with 1.0 μg of SAF-CAT reporter plasmid alone or together with 0.5 μg of pCMV-FLAGSAF-1, pCMV-FLAGSAF-1(S187A), or pCMV-FLAGSAF-1(T386A) expression plasmids as indicated. The latter plasmids are indicated as SAF-1(wt), SAF-1(S187A), and SAF-1(T386A), respectively. Some transfection assays contained 0.5 μg of pCo-PKA plasmid DNA. Cells were harvested 24 h after glycerol shock, and CAT activity was measured. The results shown are averages of three separate experiments. D, Western blot analysis of transfected cells to measure expression pattern of transfected plasmids. 50 μg of protein from transfected cells with expression plasmids, as used in C, were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-FLAG antibody. E, HIG082 cells were transfected with 1.0 μg of GAL4-CAT reporter plasmid alone or together with 0.5 μg of GAL4DBDSAF-1, GAL4DBDSAF-1(T386A), or GAL4DBDSAF-1(S187A) expression plasmids as indicated. Some transfection assays contained 0.5 μg of pCo-PKA plasmid DNA. Cells were harvested 24 h after glycerol shock, and CAT activity was measured. The results shown are averages of three separate experiments.
the SAF-1(S187A) mutant is much better phosphorylated than wild-type SAF-1 despite lacking one PKA phosphorylation site. SAF-1(S187A) protein exhibited high DNA-binding and transactivating functions, as well. What do these apparently conflicting results suggest? We hypothesized that PKA-mediated phosphorylation is not involved in improving the DNA-binding ability or transactivating ability of the respective domains; instead it is involved in improving the accessibility of these regions, possibly inducing a conformational change.

**SAF-1 Undergoes a Conformational Change in Response to PKA-mediated Phosphorylation**—The possibility that SAF-1 may undergo a conformational change in response to PKA-mediated phosphorylation was examined. Our hypothesis was that if SAF-1 undergoes a conformational change as a result of PKA phosphorylation then we might see a different proteolytic cleavage pattern with phosphorylated and unphosphorylated SAF-1. Bacterially expressed, unphosphorylated, and PKA-phosphorylated SAF-1 proteins were subjected to limited proteolytic digestion. The digested products were separated by SDS-PAGE and probed with anti-SAF-1 antibody. The V8 protease was used, because in preliminary experiments it generated a better ladder of peptide fragments than other proteases (data not shown). Although the cleavage pattern of unphosphorylated and phosphorylated SAF-1 looked similar, some additional bands were seen only with phosphorylated SAF-1 (Fig. 7). Appearance of these bands because of hypersensitive cleavage sites suggested that during phosphorylation, SAF-1 undergoes some structural changes, allowing easy access of these regions, possibly inducing a conformational change.

**Terminally Truncated SAF-1 Proteins, Lacking either One of the Two PKA Phosphorylation Sites, Are Functionally Active**—As we contemplated the idea that PKA, by phosphorylating SAF-1, unmasks its active domains, we decided to examine whether terminal truncation of SAF-1 will achieve similar results. This is not unprecedented as terminal deletion, internal deletion, or substitution of amino acids at one end is shown to increase the DNA-binding and transactivating potential of some of the members of the Ets-1 family of transcription factors (29–33). Two truncated SAF-1 proteins lacking either the C-terminal or N-terminal PKA phosphorylation site were constructed by PCR amplification (Fig. 8A). Bacterial expression of these proteins was verified by SDS-PAGE (Fig. 8B). DNA-binding activities of these truncated SAF-1 proteins were examined by EMSA and compared with wild-type SAF-1. As seen in Fig. 8C, equal amounts of unphosphorylated SAF-1 (1–380) protein formed much more DNA-protein complexes than full-length unphosphorylated SAF-1 protein (Fig. 8C, compare lanes 1 and 3). The second mutant protein, SAF-1 (188–477), exhibited even higher DNA-binding ability in its unphosphorylated form (Fig. 8C, compare lanes 1 and 5). When these proteins were incubated with PKA prior to the DNA-binding assay, there was some increase in their DNA-binding activities, but it was not as high as seen with wild-type SAF-1 (Fig. 8C, lanes 1–6). Lanes 7 and 8 of Fig. 8C show a short exposure of the complexes resolved in the lanes 5 and 6 to reveal the effect of PKA.

Next, we examined the transactivating function of these truncated SAF-1 constructs. Both of these constructs exhibited much higher levels of transactivation potential than wild-type SAF-1. Again, it was noticeable that transactivating ability of these mutant proteins does not exactly correlate with their high DNA-binding potentials. Furthermore, in the presence of PKA expression plasmid, their transactivation potentials did not increase as much as we noted with wild-type SAF-1. Nonetheless, it was clear that terminal truncation of SAF-1 from either ends creates SAF-1 proteins that are functionally superior to the wild-type form.

**DISCUSSION**

Previous investigations suggested that interaction between SAF-1 and PKA-Ca may regulate the functional activity of SAF-1 (16, 19). In the present report, we confirm these results by identifying the PKA phosphorylation sites in SAF-1 and provide a mechanism of activation of SAF-1 by the PKA signaling pathway. SAF-1 protein contains two consensus PKA phosphorylation sites that are highly conserved among several species. The effect of each PKA phosphorylation site in the context of whole protein was examined by preparing several constructs of SAF-1 containing altered phosphorylation sites and mutants lacking these regions. Our results indicate that PKA regulates functional activity of SAF-1 by changing the conformation of this protein and thereby unmasking its DNA-binding domains that in turn create a high DNA-binding protein with potent transactivating function.

In search of the mechanism by which PKA-mediated phosphorylation activates SAF-1 transcription factor, we created a mutant form of SAF-1, in which two major PKA phosphorylation sites were altered. It was verified by in vitro and in vivo phosphorylation assays that this mutant is indeed defective and therefore is suitable for examining changes of function induced by phosphorylation (Fig. 4). It was, however, very interesting to find that DNA-binding activity and overall transactivating potential of this dual PKA phosphorylation site mutant, SAF-1(S187A,T386A), was markedly higher than the wild-type SAF-1 protein (Fig. 5). This result apparently suggested that PKA-mediated phosphorylation has no influence in increasing functional activities of SAF-1. The phosphorylation pattern of two single PKA phosphorylation site mutants was also perplexing. The SAF-1(S187A) mutant containing one defective phosphorylation site was phosphorylated better than wild-type SAF-1 whereas SAF-1(T386A) mutant protein containing one defective phosphorylation site was phosphorylated at a negligible level. Functional properties of these two mutants were also strikingly different. Although SAF-1(S187A) protein was highly active, SAF-1(T386A) protein displayed virtual inactivity. Based upon these findings, we hypothesized that PKA-mediated phosphorylation perhaps changes the structural configuration of SAF-1 rather than improving the activities of its DNA-binding or transactivating domains. Consistent with this possibility, we showed that non-phosphorylated and phosphorylated forms of SAF-1 are structurally distinct (Fig. 7). That PKA-mediated phosphorylation is necessary...
PKA Regulates SAF-1 Activity

**FIG. 8.** Removal of C-terminal or N-terminal end enhances DNA-binding and transactivation potential of SAF-1. A, schematic illustration of domains contained in the full-length and truncated SAF-1 proteins. Each hatched box represents one zinc finger domain. The two PKA phosphorylation sites are identified. B, synthesis of each bacterially expressed protein was verified by SDS-PAGE and Coomassie Blue staining as indicated. C, DNA-binding assay. Affinity-purified FLAGSAF-1 (1–477), FLAGSAF-1 (1–380), and FLAGSAF-1 (188–477) proteins (0.1 μg in each lane) were incubated in the presence (+) or absence (−) of purified PKA and tested for DNA-binding ability using a 32P-labeled double-stranded SAF-binding oligonucleotide probe, as described under “Experimental Procedures.” D, HIG-82 cells were transfected with 0.5 μg of SAF-CAT reporter plasmid alone or together with pCMVFLAGSAF-1 (1–477), pCMVFLAGSAF-1 (1–380), pCMVFLAGSAF-1 (188–477), or PKA expression plasmids, as indicated. Cells were harvested 24 h after glycerol shock, and CAT activity was measured. The results shown are averages of three separate experiments.

For unfolding SAF-1 structure was also clear from the results obtained from terminal truncated SAF-1 constructs (Fig. 8). Together, these results pointed that unfolding of native structure of SAF-1 is a critical parameter that regulates functional activities of SAF-1. If the native structure is unfolded by any other means, for example by terminal deletion of this protein or mutation of a critical region as such seen in SAF-1(S187A) mutant, then SAF-1 protein becomes active, mostly because of high DNA-binding ability. For this reason, already unfolded SAF-1 proteins do not exhibit further activation by PKA. In the SAF-1(T386A) mutant, mutation of threonine at the 386 position, we believe, creates a structurally rigid form of SAF-1 that could not be unfolded and activated even when subjected to PKA-induced phosphorylation. However, it is noteworthy that overall transactivation potentials of terminally deleted or phosphorylation defective SAF-1 mutants do not exactly correspond with their very high DNA-binding abilities. We provide some possible explanations of this issue later. By accounting all facts we propose a model for how SAF-1 is activated by PKA (Fig. 9).

In this model we presume that native SAF-1 protein is folded in such a way that its active DNA-binding domains are buried within the structure and thereby remains unavailable for DNA interaction. PKA-mediated phosphorylation unfolds the native protein and exposes the active DNA-binding region of SAF-1. The active DNA-binding region of SAF-1, apparently consisting of several zinc finger domains, can also be completely unmasked when this protein is truncated at either side. Change of structural conformation leading to the activation of a transcription factor is not unprecedented. A case in point is Elk-1 protein where mitogen-activated protein kinase-mediated phosphorylation of the C-terminal transcriptional activation domain induces a conformational change of Elk-1 resulting in the stimulation of DNA-binding activity (29). Other examples are ERM and Ets-1 proteins. In ERM protein, PKA-mediated phosphorylation results in decreased DNA-binding but higher transactivating potential presumably through conformational change of this protein (30). In case of Ets-1 protein, deletion of either N-terminal, C-terminal, or internal coding sequences results in proteins that displayed higher DNA-binding activity because of conformational change of this protein (31–33).

**FIG. 9.** Model depicting how PKA-mediated phosphorylation increases the DNA-binding and transactivation potential of SAF-1. A, the zinc finger domains of SAF-1, represented by six oval boxes, are masked by the N- and C-terminal ends. B, phosphorylation causes local changes in the conformation resulting in the unmasking of the active domains. C, deletion of N- or C-terminal end of SAF-1 protein relieves intramolecular inhibition and causes unfolding of the protein, resulting in high DNA-binding and transactivating potential.

At present, little is known about the protein partners of SAF-1. Although highly speculative, several results of our investigation suggest that the amino acids at the terminal regions may be involved in interacting with some PKA-activated cofactors that may act as bridging molecules between SAF-1 and basal transcription machinery to further increase transactivation potential of SAF-1. In support of this hypothesis are two facts. First, we consistently noted that expression of SAF-CAT reporter increases at a remarkably high level when cells were cotransfected with just PKA expression plasmid or with agonists of PKA (Fig. 1, A and B). We explained this result by assuming that PKA activates endogenous SAF-1 protein, which in turn increases SAF-CAT reporter gene expression. However, induction of SAF-CAT gene expression was not as high; it was merely slightly higher than the additive value when cells were cotransfected with pCMVSAF-1 and PKA (Fig. 1, A and B). Similar results were obtained when cells were transfected with increasing concentrations of SAF-1 expression plasmid and...
PKA (data not shown). So the question remains, why exogenously added SAF-1 plasmid could not increase the expression of SAF-CAT at the same rate of endogenous SAF-1? Second, the mutants, SAF-1(S187A), SAF-1 (188–477), and SAF-1 (1–380), in their unphosphorylated form show very high DNA-binding activity but modestly higher transactivating functions as compared with wild-type SAF-1 (see Figs. 6 and 8). Moreover, DNA-binding and transactivating potentials of these mutants do not improve much in the presence of PKA. Again, why? We present a simple explanation of this complex issue. Two independent and parallel actions of PKA might be occurring. (i) PKA-mediated phosphorylation increases transactivating potential of SAF-1 by first unmasking its DNA-binding domain. (ii) PKA activates some as yet unidentified interacting partners of SAF-1, which upon interaction with SAF-1 at the terminal ends increase its transactivating function. Indeed, transcription factor Sp1 might be one such partner. Recent studies indicated that Sp1 is activated by PKA-dependent signal transduction pathway (34) and is phosphorylated by PKA (35). Furthermore, Sp1 has been shown to directly interact with SAF-1 (15). Such an interaction of SAF-1 can enhance juxtaposition of regulatory domains of these transcription factors with the components of basal transcription complex. Consistent with this possibility, Sp1 has been shown to recruit TFIID via its interaction with TAFII 130 (36, 37). Taken together, when cells are transfected with SAF-CAT reporter and PKA expression plasmid, endogenous SAF-1 protein is unmasked to become a highly DNA-binding transcription factor, which then upon interaction with some PKA-activated cofactors becomes fully transcriptionally active. As the endogenous cofactors become limiting, we do not see proportionately higher levels of SAF-CAT expression when cells are transfected with high levels of exogenous SAF-1 and PKA. This hypothesis also explains why SAF-1 (188–477) and SAF-1 (1–380) mutants have very high DNA-binding but modest transcriptional activities. Deletion of the N terminus in SAF-1 (188–477) mutant or C terminus in SAF-1 (1–380) mutant prevents the cofactor(s) from interacting with the unmasked, high DNA-binding SAF-1 protein, thereby leaving them modestly transcriptionally active. In case of SAF-1(S187A) mutant, we believe that mutation at serine 187, although it unmask the active DNA-binding region, prevents interaction of the cofactor(s). It must also be mentioned that the PKA-mediated activation of SAF-1 occurs similarly with 0.6SAACAT3 and SAF-CAT reporters, but this effect is more pronounced when HIG-82 synovial cells are used instead of BNL liver cells (data not shown). This data further suggested the participation of cell type-specific cofactors for obtaining full transcriptional response. In conclusion, we provide evidence that phosphorylation of SAF-1 by PKA leads to a conformational change of this protein, which by unmasking of its DNA-binding domains increases its functional abilities.

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Protein Kinase A Signaling Pathway Regulates Transcriptional Activity of SAF-1 by Unmasking Its DNA-binding Domains

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