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Catalytic Subunit of Protein Kinase A Is an Interacting Partner of the Inflammation-Responsive Transcription Factor Serum Amyloid A-Activating Factor-1

Bimal K. Ray, Jing Chen, and Alpana Ray

Serum amyloid A-activating factor-1 (SAF-1) is a zinc finger transcription factor that is activated by many mediators of inflammation including IL-1, IL-6, and bacterial LPS. However, the mechanism of activation is not fully understood. To identify possible activation partners for SAF-1, we used a yeast two-hybrid system that detected interaction between the catalytic subunit of cyclic AMP-dependent protein kinase (PKA-Cα) and SAF-1. Immunofluorescence and combined immunoprecipitation-Western blot analyses revealed colocalization and interaction between SAF-1 and PKA-Cα. In vivo evidence of SAF-1 and PKA-Cα interaction was further revealed by coinmunoprecipitation of these two proteins in cAMP-activated liver cells. We further show that SAF-1 is phosphorylated in vitro by PKA-Cα and that addition of cAMP markedly induces in vivo phosphorylation of SAF-1 and transcription of SAF-regulated reporter genes. These results showed that SAF1-PKA-Cα interaction is involved in functional activation of SAF-1.


Department of Veterinary Pathobiology, University of Missouri, Columbia, MO 65211
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2 Address correspondence and reprint requests to Dr. Alpana Ray, Department of Veterinary Pathobiology, University of Missouri, Columbia, MO 65211. E-mail address: rayal@missouri.edu
3 Abbreviations used in this paper: SAA, serum amyloid A; SAF, SAA-activating factor; PKA, protein kinase A; PKC, protein kinase C; CAT, chloramphenicol acetyltransferase; MAZ, myc-associated zinc finger protein (MAZ) (13) and murine Pur-1 (14). To date, SAF-1/MAZ/Pur-1 is identified as a regulator of c-myc, insulin, serotonin 1A receptor, CD4, γ-fibrinogen, phentanylolamine N-methyltransferase, and CLC-K1 genes (15-19). These findings suggest that the SAF-1/MAZ/Pur-1 family of proteins have a wide regulatory role in governing many cellular processes.

Materials and Methods

Yeast two-hybrid screen and strains

A truncated form of SAF-1 (aa 187–477), hereafter designated as SAF-1(aa187–477), was cloned into the pAS2-1 vector (CLONTECH Laboratories, Palo Alto, CA), and the resultant pAS2-1-SAF1(aa187–477) plasmid was used as a bait to screen a mouse brain MATCHMAKER cDNA library (CLONTECH Laboratories) following the manufacturer’s protocol. CG1945 yeast cells were transformed with pAS2-1-SAF1(aa187–477) plasmid DNA. A 200-μg sample of the cDNA library cloned in pACT2 plasmid was then transformed into CG1945 cells containing pAS2-1-SAF1(aa187–477). Transformed cells were plated onto medium lacking trytophan, leucine and histidine but containing 15 mM 3-amino-1,2,4-triazole. Growth on His⁻ plates indicated an interaction between SAF-1(aa187–477) and the protein coded by an unknown cDNA. Selected colonies were further assayed for β-galactosidase activity. After 3 rounds of selection, 20 colonies were chosen for further analysis by DNA sequencing.
Cell culture and transfection

BNL CL2 liver cells, a normal embryonic liver cell line derived from a BALB/c mouse, were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing high glucose (4.5 g/L) supplemented with 7% FCS. These cells retained many of the features of normal liver cells. Transient transfections were conducted by the calcium phosphate precipitation method (20). Reporter plasmids and expression vectors containing SAF-1 cDNA were transfected in duplicate, along with 1 μg pSV-β-gal (Promega, Madison, WI). The pSV-β-gal plasmid DNA was used as an internal control for measuring transfection efficiency. The amount of DNA in each transfection assay was kept same by using carrier DNA. Some cells were treated with 8-bromo (8-Br)-cAMP (0.4 mM), myristoylated cell-permeable PKA inhibitor 1–22 amide, (PKI) (5 μM), and H89 (5 μM). Cells were incubated for 48 h posttransfection, and cell extracts containing equivalent amounts of β-galactosidase activity were used for chloramphenicol acetyltransferase (CAT) assay as described previously (9–11). β-Galactosidase activity was assayed with the substrate O-nitrophenyl-β-D-galactopyranoside as described (20). All transfection experiments were performed at least three times.

Plasmid constructs

The CAT reporter plasmids wtSAF-CAT and mtSAF-CAT were constructed by ligating three copies of wild-type or mutant SAF DNA-binding element, −254 to −226 bp, of the SAA promoter (9) into pBlC2T plasmid. The sequences of the wild-type and mutant SAF DNA-binding elements are 5′-CCGTTCTCCTCCACACGCCTGATA-3′ and 5′-CCGTTCTCCTCCACACGCCTGATTGC-3′, respectively. Underlined bases represent altered sequences. The pCMV-SAIF expression plasmid was prepared by inserting a full length SAF-1 cDNA (10) under the control of cytomegalovirus (CMV) promoter in pCDNA3 vector (Invitrogen, San Diego, CA). Bacterially expressed SAF-1 protein was obtained by using FLAG-SAF1 plasmid constructed as described earlier (21) and affinity purified from anti-FLAG-agarose gel as described (21).

Immunoprecipitation, communoprecipitation, and Western blotting

Bacterially expressed FLAG-SAF1 protein (0.5 μg) was incubated with 1.0 U (1.3 μg) of a purified preparation of catalytic subunit of PKA (Calbiochem-Novabiochem, La Jolla, CA) in 20 μl 10 mM HEPES (pH 7.9), 10 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.1 mM ZnCl2, 0.5 mM sodium orthovanadate, 10 mM NaF, 1 mM PMSF, 0.5 μg/ml benzamide buffer at 25°C for 1 h. After incubation, 0.5 ml immunoprecipitation buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2.5 mM PMSF, 1% Nonidet P-40, 0.1% SDS, 0.5 mg/ml benzamide was added to the mixture, and proteins were immunoprecipitated with anti-PKA Ab (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 16 h. Next, 50 μl protein G-agarose slurry was added to the reaction mixture and incubated for 2 h at 4°C, and bound proteins were eluted and separated by SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with anti-FLAG Ab (Sigma, St. Louis, MO). Chemiluminescence reaction was performed with ECL detection kit using the manufacturer’s protocol (Amersham, Arlington Heights, IL).

For communoprecipitation analysis, BNL CL2 liver cells were grown in duplicate in 100-mm dishes. One set of cells was not treated with any agent, and the other set was treated with 8-Br-cAMP (0.4 mM, final concentration) for 4 h. After incubation, cells were harvested, washed in PBS, and resuspended in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 2.5 mM PMSF, 0.5 mg/ml benzamidine, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.2 mM sodium orthovanadate, and 100 mM NaF plus 1% SDS. After lysis, SDS concentration was reduced to 0.1% by adding lysis buffer without SDS. The FLAG-labeled SAF-1 protein was isolated by immunoprecipitation with anti-FLAG Ab, fractionated in an SDS–11% polyacrylamide gel, and detected by autoradiography.

Results

Yeast two-hybrid assay detected interaction between SAF-1 and PKA-Cα

To identify proteins that might in vivo interact with SAF-1 and therefore be involved in regulating its activity, we used the yeast two-hybrid system. We generated a bait that produced a fusion protein containing the GAL4 DNA-binding domain (GAL4-BBD) and a truncated form of SAF-1187 (0.4 nM). Before the labeling, cells were grown in DMEM plus 5% FCS for 24 h. The 3′-labeled cells were harvested, washed quickly in phosphate-free DMEM, and lysed by adding hot lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 2.5 mM PMSF, 0.5 mg/ml benzamidine, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.2 mM sodium orthovanadate, 100 mM NaF plus 1% SDS). After lysis, SDS concentration was reduced to 0.1% by adding lysis buffer without SDS. The 3′-labeled SAF-1 protein was isolated by immunoprecipitation with anti-SAF1 Ab, fractionated in an SDS–11% polyacrylamide gel, and detected by autoradiography.

In vivo phosphorylation of SAF-1

BNL CL2 liver cells were transfected with pCMV-SAF1-1 expression plasmid. The cells were metabolically labeled with [32P]orthophosphate (3P; 0.3 mCi/ml) in phosphate free DMEM for 6 h in the absence or presence of 8-Br-cAMP (0.4 mM). Before the labeling, cells were grown in DMEM plus 5% FCS for 24 h. The 3′-labeled cells were harvested, washed quickly in phosphate-free DMEM, and lysed by adding hot lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 2.5 mM PMSF, 0.5 mg/ml benzamidine, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.2 mM sodium orthovanadate, 100 mM NaF plus 1% SDS). After lysis, SDS concentration was reduced to 0.1% by adding lysis buffer without SDS. The 3′-labeled SAF-1 protein was isolated by immunoprecipitation with anti-SAF1 Ab, fractionated in an SDS–11% polyacrylamide gel, and detected by autoradiography.

In vitro phosphorylation and immunoprecipitation of SAF-1

The phosphorylation reaction was performed in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 100 μM unlabeled ATP, 10 μCi [γ-32P]ATP (6000 Ci/ mmol), 0.5 μg purified FLAG-SAF1 protein, 1.0 U (1.3 μg) of purified catalytic subunit of PKA (Calbiochem-Novabiochem) at 30°C for 30 min in a total volume of 50 μl. In some reactions, PKI inhibitor peptide (5 μM) was added (Calbiochem-Novabiochem). After incubation, one-half of in vitro phosphorylated reaction was immunoprecipitated with anti-FLAG Ab (Sigma).

Yeast two-hybrid assay detected interaction between SAF-1 and PKA-Cα

For coimmunoprecipitation analysis, BNL CL2 liver cells were grown in duplicate with pCMV-FLAG-SAF1 plasmid DNA. After transfection, one set of cells was stimulated with 8-Br-cAMP (0.4 mM) for 10 min, and permeabilized in PBS-0.2% Triton X-100 for 5 min at room temperature. After a washing in PBS, cells were blocked for 1 h in a blocking buffer (PBS-1% BSA-1% goat serum) and further incubated for another hour with anti-FLAG mouse IgG plus anti-PKA rabbit IgG in the blocking buffer. After three washings in PBS, cells were incubated with a mixture of goat anti-mouse IgG Alexa Fluor 546, goat anti-rabbit IgG Alexa Fluor 488, and DAPI (all three reagents were obtained from Molecular Probes, Eugene, OR) in the blocking buffer for 30 min in the dark and then washed three times in PBS. A few drops of Fluoromount-G (Fisher Scientific, Pittsburgh, PA) were added, the coverslips were placed, and the edges of the coverglasses were sealed with clear nail polish. Fluorescent-labeled cells were visualized in an epifluorescence microscope (Axioskop; Zeiss, Oberkochen, Germany).

Yeast two-hybrid assay detected interaction between SAF-1 and PKA-Cα

To identify proteins that might in vivo interact with SAF-1 and therefore be involved in regulating its activity, we used the yeast two-hybrid system. We generated a bait that produced a fusion protein containing the GAL4 DNA-binding domain (GAL4-BBD) and a truncated form of SAF-1187 (0.4 nM). Before the labeling, cells were grown in DMEM plus 5% FCS for 24 h. The 3′-labeled cells were harvested, washed quickly in phosphate-free DMEM, and lysed by adding hot lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 2.5 mM PMSF, 0.5 mg/ml benzamidine, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.2 mM sodium orthovanadate, 100 mM NaF plus 1% SDS). After lysis, SDS concentration was reduced to 0.1% by adding lysis buffer without SDS. The 3′-labeled SAF-1 protein was isolated by immunoprecipitation with anti-SAF1 Ab, fractionated in an SDS–11% polyacrylamide gel, and detected by autoradiography.
SAF-1 interacts in vitro with PKA-Cα

To test whether SAF-1 and PKA proteins might interact in vitro as well, we used a combined immunoprecipitation/Western blot analysis. Bacterially expressed FLAG-SAF1 protein was incubated with purified preparation of the catalytic subunit of PKA (Calbiochem-Novabiochem). After incubation, proteins were immunoprecipitated with anti-PKA Ab and further detected by anti-FLAG Ab. In the absence of PKA (Fig. 2, lane 3), SAF-1 could not be immunoprecipitated and thus remained absent in Western blot analysis. We show that anti-FLAG Ab is specific because it detects only FLAG-SAF1 protein (lane 4) but not PKA (lane 5). These results showed that SAF-1 interacts with PKA under in vitro conditions.

**PKA interacts with SAF in vivo**

To test whether PKA-Cα and SAF interact in vivo with each other, we used coimmunoprecipitation assay. Cellular proteins of BNL CL2 liver cells were treated with anti-SAF1 Ab to selectively immunoprecipitate intracellular SAF1 (Fig. 3). When anti-SAF1 immunoprecipitated proteins were probed with anti-PKA Ab, a band (Fig. 3, lane 2) was preferentially detected in cAMP-treated cells that comigrated with the catalytic subunit of PKA (Fig. 3, lane 3). This result suggested that in vivo SAF and PKA interact with each other. Similar to our finding, a recent report showed interaction between PKA-Cα and SOX-9, a transcription factor involved in chondrocyte differentiation (23). Surprisingly, the level of PKA coimmunoprecipitated with SAF1 protein was much less in untreated BNL cells (Fig. 3, lane 1) indicating that cAMP treatment of the cells is necessary either for optimal release of the catalytic subunit of PKA from its native form or for optimal interaction of SAF1 with PKA. In a reciprocal experiment, immunoprecipitation of cellular proteins with anti-PKA-Cα Ab pulled down SAF1 protein at a favorable level when cAMP was added to the medium (Fig. 3, compare lanes 4 and 5). The role of cAMP in SAF-PKA interaction in vivo is interesting and suggested that cAMP-mediated actions facilitate interaction between these two proteins.

**Colocalization of SAF-1 and PKA in the cells after cAMP stimulation**

For an interaction between two proteins to have a functional and physiological consequence, they must be expressed in the same cell. To address this, BNL CL2 cells were transfected with pCMV-FLAG-SAF1 plasmid and grown on coverslips. Next, one set of cells was stimulated with 8-Br-cAMP and processed for immunofluorescence assay using both anti-FLAG and anti-PKA Abs. As seen in Fig. 4A, SAF protein was seen in the cytoplasm but also at a lower level in the nucleus of unstimulated cells. In contrast, PKA

**FIGURE 3.** Cellular SAF-1 interacts with the catalytic subunit of PKA in vivo. BNL CL2 liver cells were treated with 8-Br-cAMP (0.4 mM) for 4 h. Cells were harvested and lysed as described in Materials and Methods. Lysates from both control and cAMP-treated cells were used in the immunoprecipitation reaction. As a control, the catalytic subunit of PKA (Calbiochem-Novabiochem) was used in lane 7 that was not immunoprecipitated. In a reciprocal experiment, purified SAF1 protein was used in lane 6 as a control. Proteins in lanes 1 and 2 were immunoprecipitated with anti-SAF1 Ab before their separation by 10% SDS-PAGE. Proteins in lanes 4 and 5 were immunoprecipitated with anti-PKA Ab (Santa Cruz Biotechnology) before their separation in a 10% SDS-PAGE. After transfer to a nitrocellulose membrane, proteins were probed with either anti-PKA (lanes 1–3) or anti-SAF (lanes 4–6) Abs.

**FIGURE 1.** SAF-1 interacts with the catalytic subunit of PKA in yeast. A, Bait plasmid used for screening. Sequences containing amino acids from 187 to 477 of SAF1 were cloned into pAS2-1 vector (CLONTECH Laboratories) in frame with the GAL4-DBD. B, Yeast colonies that were transformed with pAS2-1-SAF1187–477 and pACT2 alone or pAS2-1-SAF1187–477 and pACT2-PKA-Cα were streaked on selective plates with (−His) and without (+His) histidine.

**FIGURE 2.** Bacterially expressed SAF-1 protein interacts with catalytic subunit of PKA in vitro. FLAG-SAF1 fusion protein was incubated with the catalytic subunit of PKA (Calbiochem-Novabiochem). Lanes 1 and 4 contain 0.5 μg FLAG-SAF1 protein, lane 2 contains 0.5 μg FLAG-SAF1 protein plus 1.0 U (1.3 μg) PKA, and lanes 3 and 5 contain 1.0 U (1.3 μg) PKA. Proteins in lanes 1–3 were immunoprecipitated with anti-PKA Ab (Santa Cruz Biotechnology) before their separation by 10% SDS-PAGE. After transfer to a nitrocellulose membrane, all proteins were probed with anti-FLAG Ab.
The presence of 8-Br-cAMP (0.4 mM) for 4 h

was seen predominantly in the cytoplasm of unstimulated cells. However, in response to cAMP stimulation (Fig. 4B), both PKA and SAF proteins were present at a higher level in the nuclei. These results demonstrated that SAF and PKA colocalize under some in vivo conditions.

cAMP increases and inhibitors of PKA block expression of an SAF-regulated reporter gene

Interaction of SAF-1 with PKA provided a clue for the involvement of the cAMP/PKA signaling pathway in modulating SAF function. To that end, we sought to determine whether intracellular elevation of cAMP, an inducer of PKA activity in cells, has any effect on SAF activity. A CAT gene containing three tandem copies of SAF-binding elements present in the SAA gene was chosen as a reporter gene system to monitor the function of activated SAF. BNL CL2 liver cells were transfected in duplicate with SAF-CAT reporter constructs either alone or with 1.0 μg pCMV-SAF1 plasmid DNA. After transfection, one set of cells was incubated with 8-Br-cAMP (0.4 mM) or H89 (5 μM) or myristoylated PKI peptide amide (1 μM) (Calbiochem-Novabiochem). CAT activity was determined as described in Materials and Methods. The results represent averages of three separate experiments.

Regulators of PKA affect SAF-mediated reporter gene expression. BNL CL2 liver cells were cotransfected in duplicate, with 1.0 μg wild-type SAF-CAT reporter constructs either alone or with 1.0 μg pCMV-SAF1 plasmid DNA. After transfection, one set of cells was incubated with 8-Br-cAMP (0.4 mM) or H89 (5 μM) or myristoylated PKI peptide amide (1 μM) (Calbiochem-Novabiochem). CAT activity was determined as described in Materials and Methods. The results represent averages of three separate experiments.

FIGURE 4. Colocalization of SAF-1 and PKA-Cα in liver cells. BNL CL2 cells were transfected with FLAG-SAF-1 expression plasmid DNA in duplicate and grown on coverslips. One set of cells was incubated in the presence of 8-Br-cAMP (0.4 mM) for 4 h (B), and the other was left untreated (A). Fluorescent microscopy was performed as described in Materials and Methods. Cells were stained with 4',6'-diamidino-2-phenylindole (DAPI) for nuclear staining and probed with anti-FLAG and anti-PKA Ab.

FIGURE 5. Regulators of PKA affect SAF-mediated reporter gene expression. BNL CL2 liver cells were cotransfected in duplicate, with 1.0 μg wild-type SAF-CAT reporter constructs either alone or with 1.0 μg pCMV-SAF1 plasmid DNA. After transfection, one set of cells was incubated with 8-Br-cAMP (0.4 mM) or H89 (5 μM) or myristoylated PKI peptide amide (1 μM) (Calbiochem-Novabiochem). CAT activity was determined as described in Materials and Methods. The results represent averages of three separate experiments.

Phosphorylation of SAF-1 is increased by cAMP

To determine whether phosphorylation of SAF-1 in vivo is increased by cAMP, we labeled pCMV-SAF1-transfected BNL CL2 cells with 32P, in the presence or absence of 8-Br-cAMP. The 32P-labeled cellular phosphorylases were immunoprecipitated with anti-SAF-1 Ab and separated by SDS-PAGE. As seen in Fig. 6A, cells transfected with SAF-1 expression plasmid revealed a phosphorylated protein band, the intensity of which increased by severalfold when cells were labeled in the presence of cAMP (Fig. 6A, lane 2) and reduced in the presence of H89 (Fig. 6A, lane 3). This increase of phosphorylation was not due to any increase in the expression of the transfected SAF-1 gene, because the level of SAF-1 protein remained same during these different treatments, as determined by Western immunoblot analysis (data not shown). This suggested that although SAF-1 is present in the cells, its activity is regulated by PKA on activation by cAMP.

Discussion

Our data show for the first time that in vivo, SAF-1 transcription factor interacts with PKA-Cα and acts as a substrate for PKA.
PKA-activating agents. Reciprocally, specifically, Kozbor et al. (12) and minimally modified low density lipoprotein (28), we speculate that these diverse effects may be mediated by a combination of many signal transducing protein kinases. Further studies should reveal more about the regulatory mechanisms involving activation of SAF-1 protein.

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