Overexpression of Serum Amyloid A-Activating Factor 1 Inhibits Cell Proliferation by the Induction of Cyclin-Dependent Protein Kinase Inhibitor p21/WAF-1/Cip-1/Sdi-1 Expression

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*J Immunol* 2004; 172:5006-5015; doi: 10.4049/jimmunol.172.8.5006

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Overexpression of Serum Amyloid A-Activating Factor 1 Inhibits Cell Proliferation by the Induction of Cyclin-Dependent Protein Kinase Inhibitor p21\(^{\text{WAF-1/Cip-1/Sdi-1}}\) Expression\(^1\)

Alpana Ray,\(^2\) Arvind Shakya, Deepak Kumar, and Bimal K. Ray

Inflammation-responsive transcription factor, serum amyloid A-activating factor 1 (SAF-1), has been shown to regulate several genes, including serum amyloid A, \(\gamma\)-fibrinogen, and matrix metalloproteinase 1, whose abnormal expression is associated with the pathogenesis of arthritis, atherosclerosis, and amyloidosis. Prolonged high level expression of SAF-1 in cultured cells failed to produce any stable cell line that overexpresses SAF-1. To test the fate of SAF-1-overexpressing cells, the cells were monitored for growth and morphological changes over time. The cells that were programmed to overproduce SAF-1 were found to undergo growth arrest and reduce DNA synthesis within 3 days after transfection. These cells undergo marked morphological changes from typical fibroblasts to round morphology and gradually cease to exist. Microarray analysis for cell cycle-specific genes in SAF1-transfected cells identified several candidate genes whose expression levels were altered during SAF-1 overexpression. Cdk inhibitor protein p21 was significantly affected by SAF-1; its expression level was highly induced by cellular conditions where SAF-1 is abundant. The increased level of p21 in the cell drives it to a growth arrest mode, a condition previously found to be controlled by p53. In this study we provide evidence that, similar to p53, SAF-1 is able to activate p21 gene expression by promoting transcription directly via its interaction with the p21 promoter. Together these data indicate that SAF-1 controls cell cycle progression via p21 induction, and pathophysiological conditions that favor overexpression of SAF-1, such as an acute inflammatory condition, can trigger cellular growth arrest. The Journal of Immunology, 2004, 172: 5006 –5015.

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\({\text{1}}\) Abbreviations used in this paper: RA, rheumatoid arthritis; CAT, chloramphenicol acetyltransferase; Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor; GFP, green fluorescence protein; hr, humanized Renilla reniformis; IRES, internal ribosome entry site; MMP-1, matrix metalloproteinase 1; Rb, retinoblastoma protein; SAF, serum amyloid A-activating factor.

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Received for publication October 28, 2003. Accepted for publication February 5, 2004.

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\({\text{1}}\) This work was supported by U.S. Public Health Service Grant DK49205.

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senescent cells. In the cell, p21 is found in quaternary complexes that consist of cyclins, CDks, and proliferating cell nuclear Ag, a subunit of DNA polymerase δ (22). Formation of these complexes is essential for cell cycle progression. An increase in p21 along with other CKls, results in suppression of Cdks activity, allowing accumulation of hypophosphorylated Rb and cell cycle arrest in G1 phase (23, 24).

Although the single most remarkable feature of p21 is that it is induced by the tumor suppressor p53 (18), p21 gene expression is also regulated by p53-independent mechanisms. Some other inducers of p21 expression are TGF-β (25), MyoD (26), and Sp1 (27). TGF-β has been shown to inhibit cell cycle progression via induction of p21 (25). The myogenic transcription factor MyoD causes terminal withdrawal of cells from the cell cycle by concomitantly inducing p21 expression and myocyte differentiation (26). Phorbol ester- and okadaic acid-mediated induction of p21 is regulated by Sp1 (27). Activation of p21 under various physiologic conditions by different transcription factors underscores the regulatory role of this protein in maintaining cellular homeostasis. In the present study we demonstrate that one of the targets of SAF-1 transcription factor is the p21 gene. Increased synthesis of SAF-1 increases mRNA and protein levels of p21, which, in turn, blocks the proliferation of synovial cells. We show that SAF-1, by interacting at a GC-rich region of p21 promoter, increases its expression. These data suggest a novel role for SAF-1 that could be exploited as a therapeutic strategy for RA, a disease plagued by uncontrolled proliferation of synovial fibroblasts.

Materials and Methods

Cell culture and transfection

Rabbit synoviocyte (HIG82) cells, obtained from American Type Culture Collection (Manassas, VA), were derived from the intercartilaginous soft tissue of the knee joint of a normal female New Zealand White rabbit. These cells were cultured in DMEM containing high glucose (4.5 g/l) supplemented with 7% FCS. For IL-1β stimulation, HIG82 cells were incubated with IL-1β (200 U/ml) for 48 h. Unless otherwise mentioned, all transfection reactions were conducted in 24-well tissue culture plates using the calcium phosphate method with a mixture of DNAs containing 1 µg of chloramphenicol acetyltransferase (CAT) reporter plasmid, 1 µg of pSV-β-galactosidase plasmid (Promega, Madison, WI) as a control for measuring transfection efficiency, and carrier DNA so that the total amount of DNA in each transfection remained constant. In some transfection assays, in addition to the CAT reporter and pSV-β-galactosidase plasmids, varying concentrations of pcDSAF-1, pcDSAF-1(mt), or pCMV-p53 plasmids were included. In some experiments, HIG82 cells were transfected in 60-mm dishes with 5.0 µg of pcDSAF-1 for 48 h, and cells were harvested for the preparation of nuclear extract. To monitor green fluorescence protein (GFP) expression, transient transfection was performed with 1 µg each of pAAV-internal ribosome entry site (IRES)-GFP (Stratagene, La Jolla, CA) or pAAV-SAF1-IRES-GFP plasmids. To monitor cell growth, HIG82 cells were transfected for either 24 or 48 h with 1.0 µg of pcDNA3, pCDp21, or pcDSAF-1 plasmids. In some cotransfection assays, increasing concentrations (0.25, 0.5, and 1.0 µg) of pCDRev-p21 plasmid were included along with 1.0 µg of pcDSAF-1. At the completion of the transfection reaction, cells were incubated for 3 h with 10 µCi of [3H]thymidine. After harvesting the cells, DNA was precipitated, and incorporated radioactivity was measured using a scintillation counter.

Plasmid construction

Reporter CAT plasmids containing progressively deleted promoter regions of p21 were constructed by cloning various segments of the p21 promoter into the HindIII and BamHI sites of the promoterless pBLCAT3 plasmid vector (28). The progressively deleted segments were derived by PCR from a 2.4-kb p21 promoter DNA containing sequences from −2264 to −11, which was provided by Dr. B. Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD) (18). The p21−3.0ApalΔCAT plasmid was derived from the p21−0.3CAT by removal of a segment flanked by two Apal sites located at nt positions −131 and −66 of p21. Plasmid p21(−131/−66)CAT2 was constructed by ligating an Apal fragment of p21 promoter containing the region from −131 to −66 to the basal promoter region present in the pBLCAT2 vector (28). For ectopic expression of SAF-1 in cultured cells, the coding region of SAF-1 cDNA was cloned into the FLAG-epitope tagged pcDNA3 vector (Invitrogen, Carlsbad, CA) that contains a powerful CMV promoter for high level expression of the cloned genes. This plasmid construct is referred to as pcDSAF-1. Plasmid pcDSAF-1(mt) contains a SAF-1 cDNA sequence cloned into the pcDNA3 vector in opposite orientation, and it does not produce any functional SAF-1 protein. Plasmid pCMVp53 and pCDp21 containing the coding regions of p53 and p21 cloned into pcCMV4 and pcDNA3 vectors, respectively, were gifts from Dr. B. Vogelstein. Plasmid pCDRev-p21 contains a p21 cDNA sequence cloned into pcDNA3 vector in opposite orientation. This plasmid does not produce any functional p21 protein and reduces the expression level of p21 in pcDSAF-1-transfected cells, as determined by Western blot analysis (data not shown). For coexpression of SAF-1 and GFP, pAAV-ires-humanized Renilla reniformis GFP (hrGFP) vector (Stratagene) was used. Full-length SAF-1-coding sequence was cloned into the unique EcoRI and SstI sites of the pAAV-IRES-hrGFP vector. In this vector, SAF-1 and green fluorescent protein (GFP) are expressed from a CMV promoter as a single bicistronic mRNA. An internal ribosome entry sequence (IRES) located between SAF-1 and GFP allows both proteins to be translated from the single mRNA expressed in transfected cells. Full-length p21 coding sequence was also cloned into pAAV-IRES-hrGFP vector at the unique EcoRI and SstI sites. The identity of each construct was verified by DNA sequence analysis.

Immunofluorescence analysis

HIG82 cells, grown in eight-well chamber slides, were transfected with either pAAV-IRES-GFP or pAAV-SAF-1-IRES-GFP vectors. At different time points, up to 14 h after transfection, medium was removed, and cells were washed in PBS twice and then fixed in acetone-chloroform (1/1) at −20°C for 10 min. The cells were blocked for 1 h in a blocking buffer (PBS/1% BSA/1% goat serum) and further incubated for 1 h with SAF-1 antibody. After three washes in PBS, cells were incubated with goat anti-mouse IgG Alexa Fluor 546 in the blocking buffer for 30 min in the dark and washed three times in PBS. A few drops of Fluoromount-G (Fisher Scientific, Pittsburgh, PA) were added, and the cover glass was placed. Fluorescence-labeled cells were visualized in an epifluorescence microscope (Axioptoph; Zeiss, Oberkochen, Germany).

Protein preparation

To prepare SAF-1 protein, SAF-1 cDNA was subcloned into bacterial expression vector, pRSET A (Invitrogen). Highly expressed SAF-1 protein was purified using nickel-agarose (Invitrogen) column chromatography following the manufacturer’s protocol.

Western immunoblot assay

Cell extracts (50 µg of protein) were fractionated in SDS-PAGE and transferred onto a nitrocellulose membrane using an electrobetter. After transfer, relative amounts of proteins in each lane were determined by staining with Ponceau S solution (Sigma-Aldrich, St. Louis, MO). Immunoblotting was performed as described previously (29) with anti-SAF-1 Ab (3) or an anti-p21 mAb obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bands were detected using a chemiluminescence detection system (Amer sham Pharmacia Biotech, Arlington Heights, IL).

RNA isolation and analysis

Total RNA was isolated from unduced, IL-1β-induced, and pmsid-transfected HIG62 cells using guanidine thiocyanate (30). Fifty micrograms of RNA samples were fractionated in 10% agarose gel containing 2.2 M formaldehyde and transferred onto nylon membranes. The blot was subsequently hybridized in the presence of 50% formamide at 42°C with human p21 cDNA probe. After stripping the bound probe, the same membrane was further hybridized to an actin cDNA probe to ensure similar loading of each RNA sample in each lane.

Gene expression array analysis

To determine the expression of cell cycle-specific genes, a GEAray Q series human cell cycle gene array kit (Superarray Bioscience, Frederick, MD) was used. Total RNA was isolated from HIG82 cells transfected for 48 h in 100-mm dishes with 10.0 µg of either empty vector pcDNA3 or pcDSAF-1. Both RNA samples were reverse transcribed to produce [32P]-labeled probes following the manufacturer’s protocol. Cell cycle-specific genes in the nylon membranes were hybridized to heat-denatured radiolabeled probes at 55°C for 16 h in a hybridization buffer provided by the manufacturer, and after washing twice in 2× SSC/1% SDS, followed by two additional washes in 0.1× SSC/1% SDS at 55°C for 15 min each, the
membranes were exposed to x-ray film. The spots were detected by autoradiography, and the intensities of the corresponding spots in the two membranes were compared for two RNA populations used to generate the probe.

**EMSA and DNase I footprint analysis**

Nuclear extracts were prepared from uninduced, IL-1β-induced, and plasmid-transfected HIG82 cells as described previously (31). Protein content was measured by Bradford method (32). EMSA was performed with equal amounts of proteins using a method described previously (8). Radiolabeled probe containing SAF-binding element of p21 promoter was prepared by incorporating [α-32P]dCTP in the oligonucleotide probe. Two complimentary oligonucleotides, 5'-GCCGCACCGCTAAGGTAGCTGACAGAGCG-3' and 3'-GGGGCCCTCCAGCCAGGCGGCGGCGCCACCACCCTGGC-5', representing sequences from −131 to −88 of the p21 proximal promoter, were annealed to prepare the double-stranded SAF-binding element. Also, the double-stranded SAF-binding oligonucleotide of the SAA gene, the SAA SAF-1 oligonucleotide, a known binding site of SAF (33) with the core DNA-binding sequence 5'-GGGGCCCCTCCAGCCAGGCGGCGGCGGCGCCACCACCCTGGC-3', Underlined bases represent altered sequences. As a nonspecific oligonucleotide, Oct-1 binding element with the sequence 5'-TGTCGAATGC AAATCCTAGAA-3' was used. For Ab interaction studies, anti-SAF-1 Ab or a preimmune serum was added to the reaction mixture during a preincubation period of 30 min on ice. For the DNase I footprint assay, a DNA probe, carrying p21 promoter sequences from −299 to +11, was radiolabeled at one end with 32P. The labeled probe was incubated separately with either nuclear extract or affinity-purified SAF-1 protein. The DNase I-protected regions were determined following a method described previously (34).

**Results**

**Transiently expressed SAF-1 inhibits proliferation of HIG82 synovial cells**

To examine the fate of synovial cells due to overexpression of SAF-1, we transfected HIG82 cells with an expression plasmid containing full-length SAF-1 cDNA in pcDNA3 vector (8). Stably transfected cells were selected using G418. We noted that none of the G418-resistant clones isolated after prolonged (2-mo) drug selection overexpresses SAF-1 (data not shown). This surprising result prompted us to assess whether and at what level in the transiently transfected HIG82 cells SAF-1 is expressed. We used

**FIGURE 1.** Inhibition of synovial cell growth by overexpression of SAF-1. A, Synovial fibroblast cells, HIG82, were transiently transfected for 48 h with 1.0 µg of pcDSAF-1 (+) or empty vector pcDNA3 (−). Nuclear extracts (10 µg of protein each), prepared following the methods described in Materials and Methods, were used in bandshift assay with 32P-labeled SAF-binding oligonucleotide of SAA promoter, a known binding site of SAF (33). The DNA-protein complexes (designated a–e) were resolved in a 6% native polyacrylamide gel. B, Characterization of the DNA-protein complex. Nuclear extract from pcDSAF1-transfected HIG82 cells (10 µg of protein each) was used in a bandshift assay with 32P-labeled SAF-binding oligonucleotide, as described in A. In lanes 2 and 3, 25 and 50 ng, respectively, of competitor SAF-binding oligonucleotide were included in the binding reaction. Lanes 4 and 5 contain 1 and 2 µl respectively, of a 10-fold diluted anti-SAF-1 antibody. C, Western blot analysis of SAF-1 in nuclear extracts (50 µg of protein each) isolated from pcDNA3- and pcDSAF-1-transfected HIG82 cells (lanes 1 and 2, respectively). An arrow indicates the migration position of SAF-1. D, Coomassie Blue staining of proteins in the same amounts of nuclear extracts as those used in C. E, HIG82 cells were transfected, as described in Materials and Methods, with either empty vector pcDNA3 or pcDSAF-1. Twenty-four hours later, the cells were plated in a 12-well plate at a very low density and grown in the presence of G418 (100 µg/ml medium). The cells were viewed under microscope on days 1, 3, and 5 after plating, and colony growth was monitored by counting the number of cells in the individual cluster and the total number of clusters in each well. The data represent an average of six individual experiments performed in duplicate.
EMSA and Western blot analysis to measure the activity and the level of SAF-1 protein in the transiently transfected HIG82 cells. A higher level of DNA-binding activity was seen in pcDSAF-1 plasmid transfected cells compared with empty vector pcDNA3-transfected cells (Fig. 1A, lanes 1 and 2). Of the five DNA-protein complexes, designated a–e, four complexes, a, b, d, and e, are more abundant in the SAF1-transfected cells, whereas complex c remains unchanged. The identity of proteins in these complexes was determined using SAF-specific competitor oligonucleotide and anti-SAF-1 antibody. Complexes a, b, d, and e were inhibited by both the SAF-binding oligonucleotide (Fig. 1B, lanes 2 and 3) and anti-SAF-1 Ab (Fig. 1B, lanes 4 and 5). Complex c, in contrast, was not inhibited by anti SAF-1 Ab and was only partially affected by the competitor SAF oligonucleotide. Thus complex c is not specific to SAF-1. The specificity of the other complexes was further verified using a nonspecific oligonucleotide in the competition analysis and a nonspecific Ab in the ablation supershift assay (data not shown). Inhibition of complexes a, b, d, and e by anti-SAF-1 Ab indicated the involvement of SAF-1 in the formation of these complexes. The appearance of multiple SAF-1 complexes could be due to the heteromeric interaction of SAF-1 with other proteins such as Sp1 (35) or other members of the SAF family, e.g., SAF-2 (11). Western blot analysis with anti-SAF-1 Ab confirmed higher levels of SAF-1 protein in pcDSAF-1-transfected cells (Fig. 1C). Thus, SAF-1 is overexpressed in an early stage of transfection of cells. The absence of this protein in stably transfected cells raised the possibility that the transiently transfected cells ceased to grow. We, therefore, designed a colony growth assay to determine whether pcDSAF-1-transfected cells were growing at the same rate as the control empty vector-transfected cells. HIG82 cells were transfected with empty vector, pcDNA3, or SAF-1-expressing vector, pcDSAF-1. Twenty-four hours after transfection, cells were plated at a very low density and grown in presence of G418. Cell clusters that formed were monitored for an

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<th>Table 1. [^{3}H]thymidine incorporation into pcDSAF-1 and pcDNA3 transfected HIG-82 cellsa</th>
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a All experiments were performed in 12-well culture plates, and \[^{3}H\]thymidine incorporation was performed 24 and 48 h after transfection.

b \[^{3}H\]thymidine incorporation over a 3-h period was measured from the entire cell population in each dish. Data represent the mean ± SD for each construct.

c Number of plates assayed.
increase in the number of cells in each cluster on days 1, 3, and 5 after plating. Cells transfected with control pcDNA3 plasmid formed cell clusters containing more than two cells on days 3 and 5, indicating that these cells had undergone growth. However, very few cells in pDSAF1-transfected plates formed clusters of more than one cell even on day 5 (Fig. 1E). These initial results suggested that overexpression of SAF-1 is probably growth inhibitory and explained why it became difficult to isolate any stably transfected cells that express high levels of SAF-1.

**SAF-1 induces proliferation arrest of HIG82 cells**

To better understand the function of SAF-1 we prepared a construct in which SAF-1 cDNA was cloned in AAV-IRES-hrGFP vector (Stratagene). The AAV-IRES-hrGFP vector contains a dicistronic expression cassette in which the humanized *Renilla reniformis* GFP is expressed as a second open reading frame that is translated from a single mRNA, with the first open reading frame coding for the test gene. This vector served as a useful expression marker for the expression of SAF-1 gene in cells transfected with this plasmid, as both SAF-1 and GFP are translated from the same transcript and these cells can be easily monitored live as they are growing. HIG82 cells were transfected with AAV-IRES-GFP or AAV-SAF1-IRES-GFP expression plasmids, and at various time points GFP-expressing green cells were visualized by fluorescent microscopy. The morphology of cells expressing both SAF-1 and GFP proteins turned distinctively round over time, whereas cells expressing only GFP retained a normal stretched pattern (Fig. 2A). SAF-1- plus GFP-expressing cells retained the round morphology without any further growth for several days, and their number gradually declined upon prolonged incubation (>12 days; data not shown). The presence of a high level of SAF-1 in these cells was also verified by immunofluorescence analysis, which showed an abundance of this protein in pAAV-SAF1-IRES-GFP-transfected cells only (Fig. 2B). These results suggested that the expression of SAF-1 is associated with growth arrest. To determine the rate of DNA synthesis in SAF-1-overexpressing cells, we enriched GFP-positive cells from AAV-IRES-GFP- or AAV-SAF1-IRES-GFP-transfected HIG82 cells by flow cytometry to obtain homogenous populations of transfected cells. The GFP-positive cells were incubated in the presence of [3H]thymidine at different time points. In Table I, the results of three independent experiments are presented. There was a marked decrease in the incorporation of [3H]thymidine by the cells overexpressing SAF1 protein compared with vector-transfected cells, in all three experiments. At 48 h, the rate of [3H]thymidine incorporation in SAF-1-overexpressing cells was further reduced. In summary, round morphology, reduced rate of DNA synthesis, and slow colony growth revealed that overexpression of SAF-1 in transfected cells may cause growth arrest of the cells.

**SAF-1 induces p21 mRNA and protein levels**

As SAF-1 overexpression caused cell growth inhibition, the possibility of a change in cell cycle regulation is quite likely. To test this possibility we used microarray analysis to assess any change in the expression pattern of any of the known genes involved in cell cycle regulation. Total RNA from both control vector pcDNA3 and pDSAF1-transfected HIG82 cells were used to probe the expression of a cassette of genes involved in cell cycle regulation. One of the genes whose expression was significantly stimulated during SAF-1 overexpression was the CKI, p21. The results of microarray analysis were further verified by RNA blot analysis (Fig. 3). The induction of p21 mRNA in transiently SAF1-transfected cells was significantly higher (Fig. 3A, compare between lanes 1 and 2). Western immunoblot analysis revealed that the p21 protein level is also increased in SAF1-transfected cells (Fig. 3A, lanes 5 and 6). To assess whether p21 expression is also induced in a physiological condition that activates SAF, we treated HIG82 cells with IL-1β that was earlier shown to activate SAF (35). Nuclear extracts prepared from IL-1β-induced HIG82 cells...
exhibited increased DNA-binding activity compared with uninduced cells (Fig. 3B, lanes 1 and 2). DNA-protein complexes, a–e, were characterized using competition analysis and Ab ablation/supershift assay and were found to contain SAF-1 (Fig. 3B, lanes 3–5). Northern analysis of p21 transcript in RNA samples prepared from these cells showed increased p21 mRNA levels in the cytokine-treated cells (Fig. 3B, lanes 6 and 7). The level of p21 protein was also increased in cytokine-induced cells (Fig. 3B, lanes 8 and 9). Notably, some earlier reports demonstrated induction of p21 expression by inflammatory cytokines, including IL-1, IL-6, and oncostatin M, in human melanoma and osteoblast cells (36, 37).

**Overexpression of p21 gene induces growth arrest**

Overexpression of p21 in SAF-1-transfected HIG82 cells raised the possibility that growth arrest of these cells may be mediated by p21. To test this possibility, HIG82 cells were transiently transfected with an expression plasmid carrying the coding sequences for p21, and the cell growth pattern was monitored (Fig. 4). Cells transfected with p21 exhibited a significant reduction in thymidine incorporation compared with cells transfected with the vector alone or untransfected cells (Fig. 4A). This result indicated inhibition of synovial cell growth in response to p21. Similar inhibition was observed when the cells were transfected with expression plasmid carrying the coding region of SAF-1 (Fig. 4A). Interestingly, cotransfection of these cells with pcDSAF-1 and pcDRev-p21, a plasmid carrying p21 cDNA in reverse orientation, resulted in a dose-dependent recovery from SAF-1-mediated cell growth inhibition. Transfection of HIG82 cells with pcDRev-p21 reduces the expression level of p21 as determined by Western blot analysis (data not shown). This was further evident from the results shown in Fig. 4B. HIG82 cells transfected with pAAV-p21-IRES-GFP exhibited round morphology over time, and the number of cells gradually declined (Fig. 4B, compare a–c). Cotransfection of cells with pAAV-p21-IRES-GFP and pcDRev-p21 reversed cellular morphology to almost normal pattern (Fig. 4B, d). This effect was most likely due to antisense RNA synthesis by the pcDRev-p21.

**SAF-1 activates p21 promoter**

Induction of p21 mRNA in SAF-1-transfected HIG82 cells suggested a possible role of SAF-1 in the regulation of expression of this gene. To determine whether SAF-1 indeed stimulates p21 gene expression, we transfected HIG82 cells with a promoter construct (p21-2.4CAT) containing 2.4 kb of upstream promoter sequences of the p21 gene with or without pcDSAF-1 expression plasmid. As p53 is known to induce p21 promoter, as a control, we cotransfected p21-2.4CAT reporter plasmid with pcMVp53 expression

**FIGURE 4.** Overexpression of p21 induces growth arrest. A, HIG82 cells were transiently transfected with 1.0 µg of eukaryotic expression plasmid pcDNA3 (empty vector) or the same vector containing the full-length coding regions of p21 (pcDp21) and SAF-1 (pcDSAF-1). In some cotransfection assays, an expression plasmid containing p21 in reverse orientation (pcDRev-p21) was added at increasing concentrations (0.25, 0.5, and 1.0 µg of DNA, respectively). The cells were subsequently grown for 24 and 48 h, respectively. Transfected cells at each time point were incubated with [3H]thymidine for incorporation of radioactivity into DNA over a 3-h period. Data represent the mean ± SD for each transfection reaction performed in triplicate. B, HIG82 cells were cotransfected with 1.0 µg each of pAAV-p21-IRES-GFP plus pcDRev-p21 for 72 h. The cells were visualized under a fluorescent microscope at 48 and 72 h after transfection. Magnification, ×400.

**FIGURE 5.** SAF-1 induces p21 promoter activity. A, The reporter plasmid, p21-2.4CAT, contains the promoter region of p21 spanning sequences from −2264 to +11, with a TATA box located at the −46 nt position. B, HIG82 cells were transfected with 1.0 µg of p21-2.4CAT reporter plasmid DNA either alone or with increasing concentrations (0.25, 0.50, and 1.0 µg, respectively) of pcDSAF-1, pcDSAF-1(mt), or pcCMVp53 expression plasmids. Details of transfection and CAT assay procedures are described in Materials and Methods. Results represent an average of three separate experiments.
plasmid. The expression of p21–2.4CAT reporter was induced in a dose-dependent manner by SAF-1 expression plasmid, but no induction was seen when a mutant SAF-1 was used (Fig. 5). Moreover, induction of the reporter gene expression by SAF-1 was at a comparable level with that of p53. Western immunoblot analysis revealed that both SAF-1 and p53 proteins are expressed in a dose-dependent manner in the transfected HIG82 cells (data not shown). These results suggested that p21 gene expression is regulated by SAF-1.

For further definition of the p21 promoter element, several deletion constructs were prepared in which progressively deleted fragments of p21 promoter were ligated to the CAT reporter gene. From the panel of deletion mutants, the region still retaining SAF-1 responsiveness was determined to be within −299 to +11 (Fig. 6A). A significant loss of promoter activity was observed due to a deletion of the region −129 to −66, which is flanked by two Apol sites. This indicated that the promoter element responsive to SAF-1 might be located in this region. To further verify this possibility, the region −129 to −66 was ligated to pBLCAT2 vector, which contains a TATA box, but lacks any other promoter elements, and therefore the reporter gene cannot be induced without any additional promoter element. Using such a construct, we noted a very high level of induction when the cells were cotransfected with SAF-1, whereas the vector alone remained unresponsive (Fig. 6B). These results indicated that a functional SAF-1-responsive element is located within −129 and −66 of the p21 promoter region.

Mapping of the SAF-binding element of p21 promoter
To determine the precise location(s) of p21 promoter where SAF-1 binds, we performed a DNase I footprint assay using a p21 DNA that contains sequences from −299 to +11. Within this DNA, a GC-rich region spanning nt sequence from −88 to −132 of the p21 promoter was found to be protected by SAF-1-transfected nuclear extract of HIG82 cells (Fig. 7). Similar protection was achieved when purified SAF-1 protein was used in the footprint assay (Fig. 7, lane 4). These data indicated that SAF-1 interacts with the p21 proximal promoter at nt position −129 to −88. In the functional assay, described in Fig. 6B, a region spanning −131 to −66 showed responsiveness to SAF-1. Together these data suggest that SAF-1 action probably involves sequences from −129 to −88 of the p21 promoter.

Characterization of the SAF-binding element of the p21 promoter
To determine whether SAF-1 directly interacts with the p21 promoter, we used the minimum promoter region of p21 from −131 to −88 as a probe in the EMSA (Fig. 8A). Nuclear extracts prepared from HIG82 cells transfected with pcDSAF-1 bound to the p21 probe with higher avidity than untransfected HIG82 cell nuclear extract (Fig. 8B, lanes 1 and 2). Furthermore, bacterially expressed and affinity-purified SAF-1 protein bound to this p21-specific probe (lanes 3 and 4). The specificity of the DNA-protein complex formed by pcDSAF-1-transfected cell nuclear extract was further verified using competitor oligonucleotide. The DNA-protein complex was specific because it was not inhibited by an unrelated oligonucleotide (Fig. 8B, lane 5), but was competed by p21 oligonucleotide (Fig. 8B, lanes 7 and 8) and inhibited by anti-SAF-1 Ab (Fig. 8B, lane 9). The DNA-protein complex was also inhibited by the SAF-binding oligonucleotide of SAA promoter, a bona fide binding element of SAF-1 (Fig. 8B, lane 10). A mutant p21 oligonucleotide was unable to inhibit the complex formation (Fig. 8B, lane 11). In a reciprocal experiment, using radiolabeled SAA SAF-1 oligonucleotide as a probe in EMSA, p21 oligonucleotide (−131/−88) was found to compete as well as the bona fide binding element of SAF-1 (Fig. 8C, compare lanes 2 and 3), whereas the mutant p21 oligonucleotide was unable to compete (Fig. 8C, lane 4). Together these results characterized the proximal promoter region of the p21 gene, which is induced in SAF-1-transfected cells as well as in the cytokine-induced cells where SAF is
The DNase I footprint analysis, identification of a novel protein binding domain in p21 promoter. A single 5’ end-labeled p21 DNA fragment (−299 to +11) was incubated under conditions for EMSA with SAF-1-transfected synoviocyte (HIG82) nuclear extracts. Ten and 20 μg of proteins were used in the footprint assay shown in lanes 2 and 3, respectively. One microgram of affinity-purified SAF-1 protein was used in the assay shown in lane 4. In lane 1 the probe was incubated with no added nuclear extract. DNA-protein complexes were incubated with DNase I, and the resultant DNA fragments were fractionated in an 8% sequencing gel containing 8 M urea. Lane 5 contains nucleotide size markers that determine the nucleotide positions of the protected region. The DNA sequence of the p21 promoter from −132 to −88 is indicated.

activated. Furthermore, direct interaction of SAF-1 to the p21 promoter was established. As p21 expression is known to cause cell cycle arrest, the results reported above provide evidence for cellular fate when SAF-1 is overexpressed.

Discussion

The novel finding of this study is that overexpression of SAF-1, a zinc finger transcription factor, causes a growth-arresting signal that, in turn, stops cellular proliferation via activating p21 gene expression. Furthermore, this study reveals an unknown mechanism by which expression of the p21 gene is modulated.

As SAF-1 is a transcription factor, its growth inhibitory effect would be predicted to be attributable to the transcriptional activation of genes responsible for the inhibition. The microarray study pointed toward p21 gene expression as a potential candidate, which was further verified by RNA blot analysis. SAF-1-transfected HIG82 synoviocyte cells, which transiently overexpress SAF-1, exhibited moderate, but consistently elevated, p21 mRNA levels compared with control cells. To investigate the mechanism through which SAF-1 induces the expression of p21, we performed functional analysis of the p21 promoter. Using progressively deleted p21 promoter constructs, we identified the SAF-1-interacting region. It is interesting to note that this region of p21 promoter was identified to contain TGF-β-responsive elements as well as PMA and okadaic acid-responsive elements. Sp1 transcription factor, which is capable of binding to GGGCGG sequence, was shown to play critical role in PMA- and okadaic acid-induced expression of the p21 gene (27). For TGF-β-mediated induction of p21 expression, Sp1 as well as Sp3 and other Sp1-like factors were suggested to be involved, and a proximal promoter region located within −83 and −74 was shown to be the Sp1-binding element that drives inducible expression (38). The data presented in our study clearly show a role for SAF-1 in inducing p21 expression by its interaction through the adjacent region. It is noteworthy that SAF-1, being a Cys<sup>2</sup>His<sup>2</sup>-type, zinc finger transcription factor like Sp1, is capable of interacting with Sp1 and the heterodimer of SAF-1, and Sp1 is shown to synergistically activate the expressions of SAA (35) and serotonin 1A receptor (37) genes. It is quite likely that SAF-1 and Sp1 provide a similar cooperative effect in p21 gene expression. Further analysis will provide information for such a combinatorial effect. Although SAF-1 directly interacts with the p21 promoter, as described in this paper, we cannot rule out the possibility of the synergizing effect of Sp1 in the trans-activation function of SAF-1 in the induction of p21.

Hyperproliferation of synovial fibroblast cells is a prominent feature of rheumatoid arthritis. Hyperproliferative synovial cells, although nonmalignant, are described as transformed appearing synoviocytes because they share some common features of transformed cells, including irregular nuclei, rough endoplasmic reticulum, and changes in the normally spindle-shaped cell skeleton. Consistent with this cellular feature of the synoviocytes in RA, the cellular level of cell cycle inhibitor p21 has been found to be decreased (39). This finding led to the use of p21 as a potential therapeutic agent in RA. Overexpression of p21 or p16, another member of Cdk inhibitors, is shown to ameliorate experimental arthritis in multiple animal models by reducing the levels of IL-1β, TNF-α, and IL-6 mRNAs (40). More recent studies have shown that in RA-derived synovial fibroblast cells, overexpression of p21 reduces levels of IL-6 and MMP-1 proteins (41). Although the mechanism by which p21 exerts such inhibitory effects are not fully understood, it is speculated that inhibition of cellular proliferation through p21 overexpression may be one of the reasons for the reduced levels of synoviocytes and consequent down-regulation of these proteins synthesized by the cells.

The link between cell cycle activity in the synoviocyte cells and inflammation in the development of arthritis is further established by the present findings that demonstrate the role of the inflammation-responsive transcription factor SAF-1 in cell cycle regulation via p21 induction. Interestingly, SAF-1 is also shown to increase the expression of MMP-1 in both chondrocyte and synoviocyte cells (3), which contradicts the previous finding that p21 induction suppresses MMP-1 expression (41). How do we reconcile such apparent contradictory behaviors of the synoviocyte cells? We propose that the threshold level of SAF-1 in the cells is very important. A persistently higher than normal level of SAF-1 activity is implicated in increasing MMP-1 protein level during the slow progression of the disease state of RA. However, when the cellular level of SAF-1 becomes very high during the progression of the disease, the p21 gene is activated, culminating in inhibition of cellular proliferation. Although in the present study we have not measured the level of MMP-1 during increased p21 gene expression, it is conceivable that the cells in which proliferation is inhibited will show a natural reduction of the MMP-1 protein level.
FIGURE 8. Interaction of SAF-1 with the p21 promoter. A, Proximal promoter element of p21, containing sequences from −131 to −88, was used as a radiolabeled probe in the DNA-protein binding assay. B, Nuclear extracts (10 µg of protein) prepared from HIG82 cells transfected with either pcDNA3 (control; lane 1) or pcDSAF-1 (lanes 2 and 5–11) or affinity-purified SAF-1 protein (lanes 3 and 4) were incubated with radiolabeled p21 promoter element (−131/−88) under the conditions of EMSA, and the DNA-protein complexes were resolved in a 6% native polyacrylamide gel. In lanes 7 and 8, 25- and 50-fold molar excess (25 and 50 ng of DNA, respectively) of the unlabeled p21 oligonucleotide (−131/−88) was added as the binding competitor of the radiolabeled probe. In lane 6, a 50-fold molar excess (50 ng of DNA) of a nonspecific oligonucleotide, whose sequence is given in Materials and Methods, was added. In lane 9, 2 µl of 10-fold diluted anti-SAF-1 serum was added during a preincubation of 30 min on ice. In lanes 10 and 11, a 50-fold molar excess of the SAF-1-binding oligonucleotide of SAA and a mutant p21 (−131/−88) oligonucleotide were respectively added. DNA sequences of these oligonucleotides are described in Materials and Methods. C, EMSA was performed using a 32P-labeled SAF-binding oligonucleotide of SAA, described in Materials and Methods, as a probe. Nuclear extract (10 µg of protein) prepared from HIG82 cells transfected with pcDSAF-1 was incubated with the radiolabeled SAA-SAF-1 probe (lane 1). A 50-fold molar excess of three competitor oligonucleotides, SAA-SAF-1, p21-SAF-1, and mut p21, were included in some binding reactions (lanes 2–4, respectively).

Thus, SAF-1 might play a yang-ying role by initiating tissue destruction by inducing MMP-1 expression and later promoting repair process by promoting p21 expression. Further studies may provide a clue as to how such an intricate balance is maintained.

Acknowledgments
We thank Dr. B. Vogelstein for generous gifts of the p21 gene promoter DNA as well as p21 and p53 cDNA samples.

References


