Chemokine stromal cell-derived factor-1 induction by C/EBPβ activation is associated with all-*trans*-retinoic acid-induced leukemic cell differentiation

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Abstract: Stromal cell-derived factor-1 (SDF-1/CXCL12) is one of the essential chemokines, which mediates hematopoietic differentiations. However, the mechanism by which SDF-1 expression is regulated in granulocyte differentiation is poorly understood. Here, we suggest a novel mechanism by which all-trans-retinoic acid (ATRA) induces the expression of SDF-1 during the differentiation of promyelomonocytic leukemic U937 cells. Moreover, we also demonstrate that activation of transcription factor C/EBPβ by ATRA regulates SDF-1 expression in U937 cells. In addition, we show that the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and Pyk2 are up-regulated by SDF-1 and increased markedly by the costimulation of ATRA and SDF-1. Furthermore, ATRA and SDF-1α additively induce U937 cell differentiation. Indeed, silencing the expression of SDF-1 inhibits ATRA-induced granulocyte differentiation significantly. Taken together, these results indicate that SDF-1α is involved in granulocyte differentiation in response to ATRA, mediated by the activation of the transcription factor C/EBPβ. *J. Leukoc. Biol.* 82: 1332–1339; 2007.

Key Words: *SDF-1 · granulocyte · ATRA · p21*

INTRODUCTION

Chemokines are a group of small (8–14 KDa), mostly basic, structurally related molecules, which have several conserved cysteine residues. Generally, chemokines regulate leukocyte trafficking and play pivotal roles in the development, homeostasis, and function of the immune system [1, 2]. Despite the fact that chemokines usually bind to multiple receptors and that a chemokine receptor can be engaged by more than one chemokine, α-chemokine stromal-derived factor-1 (SDF-1) specifically binds to a specific receptor CXCR4 [3, 4], suggesting that the SDF-1/CXCR4 engagement plays an important biological role in cellular chemotaxis, angiogenesis, and cell proliferation [5–7].

The multistep processes leading to the differentiation of hematopoietic progenitor cells toward the erythroid, lymphoid, or myeloid lineages are regulated, at least in part, by a network of cytokines and various differentiation-inducing agents. Among hematopoietic growth factors, GM-CSF, G-CSF, and M-CSF have been suggested to regulate primary myelopoietic lineage [8, 9]. In addition, GM-CSF and TGF-β are able to differentiate human promyelocytic U937 cells into mature myeloid cells. Moreover, synergistic effects were suggested when retinoic acid (RA), vitamin D3, and TNF-α were used to stimulate differentiation of U937 cells [10, 11]. Among these, all-*trans*-RA (ATRA), a vitamin A metabolite, recognized by RA receptor (RAR)/retinoid X receptor (RXR), induces the granulocytic differentiation of U937 cells and contributes to granulopoiesis [12]. ATRA is also known to induce the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, resulting in cell cycle arrest at the G_1/S phase [13].

In this study, we demonstrated that ATRA induces the expression of SDF-1 regulated by transcription factor C/EBPβ activation during acute promyelomonocytic leukemic (APL) U937 cell differentiation. These results support that the ATRA-induced differentiation of U937 cells requires C/EBPβ-regulated SDF-1 gene expression. We also determined that a cyclin-dependent kinase inhibitor and differentiation marker, $p21^{WAF1/CIP1}$, is up-regulated by SDF-1α in U937 cells and is markedly increased by the costimulation of ATRA and SDF-1α, resulting in efficient induction of granulocyte differentiation.

MATERIALS AND METHODS

Plasmid construction of SDF-1 promoter and cDNA region

A 1.918-kb fragment of SDF-1 5′-flanking region was amplified by PCR from normal human spleen genomic DNA (BioChain Institute, Inc., Hayward, CA,

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USA) using the primer set, SDF-1-1918 sense and SDF-1 antisense, with *ExTaq* Hot Start DNA polymerase (Takara Shuzo, Otsu, Japan). A *Kpn*I site was added to the 5′ end of SDF-1-1918, and a *Hin*dIII site was added to the 5′ end of SDF-1-antisense. The PCR product was cloned into the pGL3 basic luciferase reporter plasmid (Promega, Madison, WI, USA), and the resulting reporter construct was designated pGL3B/SDF-1-1918. Then, five nested 5′-end deletion constructs were generated by PCR using the plasmid pGL3B/SDF-1-1918 as the template. Two nested 5′-end deletion constructs (pGL3B/SDF-1-546, pGL3B/SDF-1-432) were generated using the restriction enzymes *Sac*I and *Xma*I. Five constructs generated by PCR were amplified with the same 3′-oligonucleotide (SDF-1-antisense) and the corresponding, specific 5′-oligonucleotide: SDF-1-1918 (CCC GGT ACC TCA GGC TTC TGG GAC AGA TCC); SDF-1-1292 (CCC GGG TAC CCT AAT GCA GCC GCT GAC CG); SDF-1-767 (CCC GGG TAC CCT CAT TGA ATC TCC CTG CCC A); SDF-1-395 (CCC GGG TAC CTC AGT TCC CGC CAT CGA AAG G); SDF-1-213 (CCC GGG TAC CCT GAG AAG GTC AAA GGC CGG AG); SDF-1 antisense (CCC AAG CTT GCG GCT GAC GGA GAG TGA AAG TG).

The human (h)SDF-1 cDNA region (1–89 amino acids) was amplified using the primer set SDF-1 cDNA sense (5′-CCC GGA ATT CAT GAA CGC CAA GGT CGT-3′) and SDF-1 cDNA antisense (5′-CCC GCT CGA GCG GTT ACT TGT TTA AAG C-3′) from human liver cDNA (BioChain Institute). The PCR product was ligated into the pcDNA expression vector, and the resulting construct was renamed pcDNA/SDF-1.

Cell culture and evaluation of cell differentiation

U937 human promyelomonocytic leukemia cells were cultivated in RPMI 1640 with 10% heat-inactivated FBS (Gibco-BRL, Grand Island, NY, USA) and 1% (v/v) penicillin-streptomycin (Gibco-BRL) at 37°C in a humidified atmosphere containing 5% CO_2 and fed every 2 days. Differentiation of U937 cells was evaluated by morphology and nitroblue tetrazolium (NBT) reduction assay and flow cytometry analysis (Beckman Coulter, Fullerton, CA, USA). NBT reduction was measured by adding 1 ml cell suspension ($0.5–2\times10^6$ cells) to a solution containing 2 mg/ml NBT and 20 ng/ml PMA in PBS. The reaction was allowed to proceed for 1 h at 37°C and was stopped by the addition of 0.4 ml cold 2 M HCl. The formazan product was obtained by centrifugation of the sample at 700 *g* for 10 min. The supernatant was discarded, and the formazan was dissolved in 1 ml Me_2SO. The absorbance of the solution was measured at 595 nm. Data are expressed as absorbance units/10^6 cells [14].

Transient transfection and luciferase assay

Cultured cells were transiently transfected by standard electroporation in 24-well culture plates with the SDF-1 luciferase reporter plasmids and the p21 luciferase reporter plasmids and cotransfected with the pSV110/β-galactosidase reporter plasmid (Promega) and expression vectors for C/EBPα, C/EBPβ, liver-inhibiting protein (LIP), Creb-binding protein (CBP), p300, RARα, RXR, and hyaluronan (HA)/SDF-1. The total amounts of expression vectors were kept constant by adding pcDNA3.1/HisC (Invitrogen, Carlsbad, CA, USA). Relative luciferase activities were measured by Beetle luciferin (Promega) using the multilabel counter *VICTOR*^{3} (Perkin Elmer, Wellesley, MA, USA), and the results were normalized to the β-galactosidase activity. All transfection results represent the mean of at least three independent experiments.

RNA isolation and RT-PCR

Total RNA from U937 cells was prepared using Trizol (JBI, Korea), according to the manufacturer's recommendation. The cDNA was synthesized from 5 μg total RNA with avian myloblastosis virus RT (Promega) using a random hexamer at 42°C for 1 h. The PCR primers for SDF-1α gene amplification were: 5′-GCC ATG AAC GCC AAG GTC GT-3′ (sense), 5′-GGC TGT TGT GCT TAC TTG TTT AAA GC-3′ (antisense); for β-actin gene amplification: 5′-GAC TAC CTC ATG AAG ATC-3′ (sense), 5′-GAT CCA CAT CTG CTG GAA-3′ (antisense). The SDF-1α cDNAs were amplified by PCR under the following conditions: 38 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s in a thermal cycler. The PCR products were examined by electrophoresis on 2% agarose gels.

Chromatin immunoprecipitation (ChIP) assay

U937 cells were collected and cross-linked with 1% formaldehyde at 37°C for 10 min and then rinsed with ice-cold PBS twice and centrifuged for 5 min at 2000 *g*. Cells were then resuspended in 0.2 ml lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.1, 1 mM PMSF, protease inhibitor cocktail (Roche, Rotkreuz, Switzerland)] and sonicated nine times for 10 s each, followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1.1% Triton X-100, 0.01% SDS, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1) followed by immunoclearing with 60 μl protein A-agarose and 2 μg sheared salmon sperm DNA for 30 min at 4°C. Immunoprecipitation was performed overnight at 4°C with the C/EBPβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After immunoprecipitation, 60 μl protein A-agarose with 2 μg sheared salmon sperm DNA was added, and the incubation was continued for another hour. Precipitates were washed sequentially in the following three washing buffers for 5 min each: a low-salt immune complex washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), a high-salt immune complex washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and a LiCl immune complex washing buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were washed two times with Tris-EDTA buffer and then used in PCR assays to determine the binding sequences.

SDS-PAGE and Western blots

Equal amounts of cell lysates from every 1×10^6 cell in the presence or absence of ATRA or recombinant hSDF-1 (rhSDF-1) treatment were verified by the Bradford assay and then loaded and separated by SDS-PAGE. The gels were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). For Western blotting, the membranes were incubated with anti-SDF-1 (R&D Systems, Minneapolis, MN, USA), anti-β-tubulin, anti-Pyk2, anti-C/EBPβ, anti-HA, and anti-$p21^{WAF1/CIP1}$ (Santa Cruz Biotechnology) in TBST containing 1% nonfat dried skim milk for 2 h at room temperature. After washing three times with cold TBST, the blotted membranes were incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 30 min at room temperature. After washing three times with cold TBST, the protein bands were visualized using the ECL-Western blotting detection system from Amersham (Arlington Heights, IL, USA).

RNA interference and nucleofection

For small interfering (si)RNA-mediated down-regulation of SDF-1α, SDF-1α-specific siRNA and negative control siRNA were purchased from Bioneer (Daejeon, Korea). The siRNA experiments were performed with a final concentration of 700 pmol/L duplex siRNA. For nucleofection into U937 cells, Nucleofector Kit V was used (Amaxa, Gaithersburg, MD, USA). For nucleofection, 2 million low-passage cells were mixed with duplex siRNA directed against SDF-1α (target sequence, CUCAUUGAAUCUCCCUGCCCA), and the cell-specific nucleofection program V-01 (Amaxa) was used to deliver the siRNA duplex into the nuclei of the cells. Following nucleofection, cells were incubated for 48 h with ATRA or vehicle (DMSO). Differentiation was subsequently evaluated by the NBT reduction assay.

RESULTS

Expression of SDF-1 and C/EBPβ is up-regulated during ATRA-induced U937 cell differentiation

Although many studies have shown that SDF-1 is expressed in various tissues and cells, such as bone marrow, heart, brain, liver, and hematopoietic cells, it is not clear whether SDF-1 is associated with granulocyte differentiation. To verify the expression of SDF-1 during granulocytic differentiation, we evaluated the expression of SDF-1 by various approaches as described in Materials and Methods. As shown in **Figure 1, A** and **B**, ATRA stimulation in U937 cells increased the levels of SDF-1 mRNA and protein in a time-dependent manner. As shown in Figure 1, ATRA stimulation increased SDF-1 protein secretion over a 24-h period. In addition, SDF-1 mRNA ex-

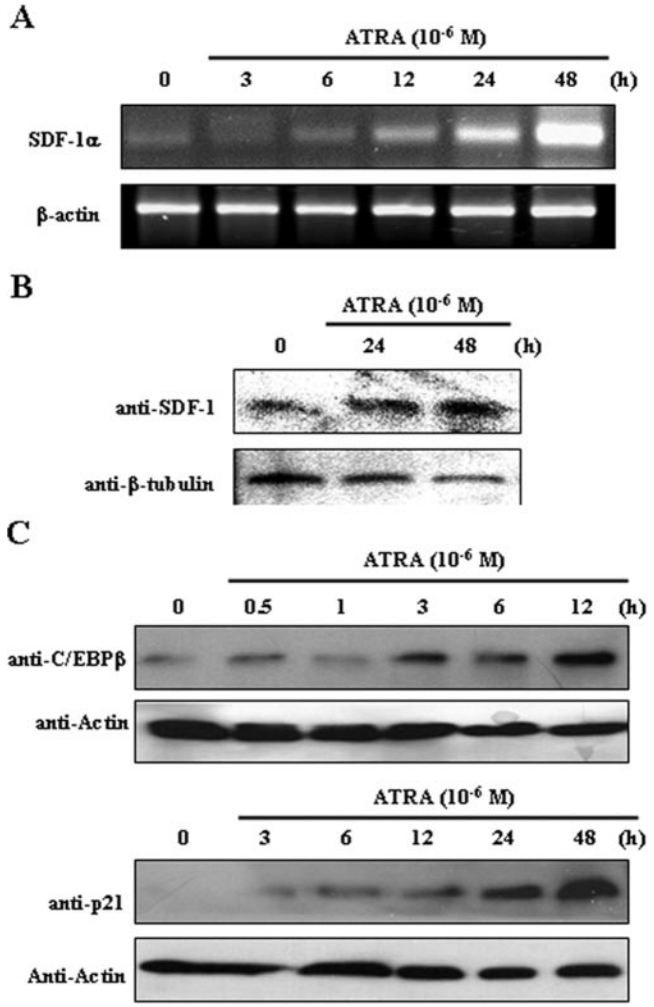

Fig. 1. Expression of SDF-1 and C/EBPβ increases during ATRA-induced U937 cell differentiation. (A) Equal amounts of total RNA from U937 cells were converted to single-stranded cDNAs by RT. These cDNAs were used as templates for PCR using SDF-1 primers; β-actin primers were used as control. Data shown are representative of three independent experiments. (B) U937 cells were treated with ATRA for 48 h. Equal amounts of protein were separated on 15% SDS-PAGE and transferred to PVDF membranes, which were subjected to Western blot analysis using antibodies for SDF-1 or β-tubulin as indicated. β-Tubulin expression from 1×10^6 cells is shown as a protein-loading control. Data shown are representative of three independent experiments. (C) Cell lysates from U937 cells, unstimulated or stimulated with 10^{-6} M ATRA for various time periods, were immunoblotted with anti-C/EBPβ and anti-p21 antibody or antiactin antibody. Western blot detection of actin was used to estimate protein loading for each lane. Data shown are representative of three independent experiments.

pression was enhanced in other leukemic cells, such as NB4 and HL60, in response to ATRA (data not shown). These results indicate that ATRA stimulation induces SDF-1 expression during granulocytic differentiation. Previous studies have shown that ATRA is able to induce granulocytic differentiation of U937 cells by inducing $p21^{WAF1/CIP1}$ expression [15, 16]. Several studies recently showed that C/EBPβ can also induce granulocyte differentiation [17, 18]. Moreover, ATRA stimulation has been suggested to up-regulate C/EBPβ expression, which in turn, enhances its transcriptional activity [19]. By analyzing the DNA sequence of the promoter region of the SDF-1 gene, we found a C/EBPβ-responsive element in the 5′-flanking region of the gene. These findings prompted us to examine the protein expression of $p21^{WAF1/CIP1}$ and C/EBPβ in U937 cells stimulated with ATRA. A time-course analysis revealed that ATRA stimulation induced C/EBPβ and $p21^{WAF1/CIP1}$ expression at 6 h after treatment (Fig. 1C), implying that ATRA stimulation regulates the expression of C/EBPβ protein as an immediate early response and that C/EBPβ induction by ATRA could be associated with the SDF-1 expression.

C/EBPβ regulates SDF-1 promoter activity in ATRA-stimulated U937 cells

To address the mechanism by which SDF-1 gene expression is regulated during granulocyte differentiation, we examined the promoter region of the SDF-1 gene. The 5′-flanking region of the hSDF-1 gene was obtained by PCR cloning from normal human spleen genomic DNA and cloned into the pGL3 basic luciferase reporter plasmid (Promega). DNA sequencing was performed to confirm the integrity of the sequence. Using the "MOTIF" database analysis program to identify any consensus sequences in the 5′-flanking region of the SDF-1 gene, we identified putative binding sites for transcription factors including C/EBPs. We also determined that there is no canonical TATA box in the SDF-1 promoter region, similar to other CXC chemokine promoters [20].

As ATRA stimulation increased the protein and mRNA levels of SDF-1 (Fig. 1), we tested whether ATRA stimulation is able to induce SDF-1 promoter activity. As shown in **Figure 2D**, ATRA treatment alone increased SDF-1 promoter activity up to 1.8-fold. ATRA is known to bind directly to the ligand-binding domain of RARs, members of the nuclear receptor superfamily [21–23], to induce transcriptional activation. Ligand binding also causes the release of corepressor complexes, followed by recruitment of coactivator complexes to the RAR/RXR dimer, leading to activation of gene transcription [24, 25]. RARα and RARγ are believed to be most important in regulating granulopoiesis [26]. Therefore, it is necessary to determine whether the RARs RARα and RXR are involved in transactivation of an ATRA-induced SDF-1 promoter. Therefore, we transiently cotransfected a RARα or RXR expression construct with the SPF-1 promoter reporter construct. Transfection of RARα, RXR, or both slightly increased the SDF-1 promoter activity, indicating that RARα or RXR may not mediate SDF-1 expression in response to ATRA in cells (data not shown). However, ectopic expression of C/EBPβ increased SDF-1 promoter activity significantly. These data suggest that these nuclear receptors do not play a major role in SDF-1 gene expression; rather, ATRA-induced SDF-1 gene expression is mediated largely by the transcription factor C/EBPβ. As shown in Figure 2A, C/EBPβ dose-dependently increased the luciferase activity derived from the SDF-1 promoter transactivation, and expression of C/EBPα, another member of the C/EBPs, did not up-regulate SDF-1 expression at the transcriptional level. These results indicate that C/EBPβ plays a specific role in SDF-1 up-regulation during ATRA-induced granulocyte differentiation.

The coactivator p300 and the homologous CBP appear to enhance transcriptional activity through interactions with a

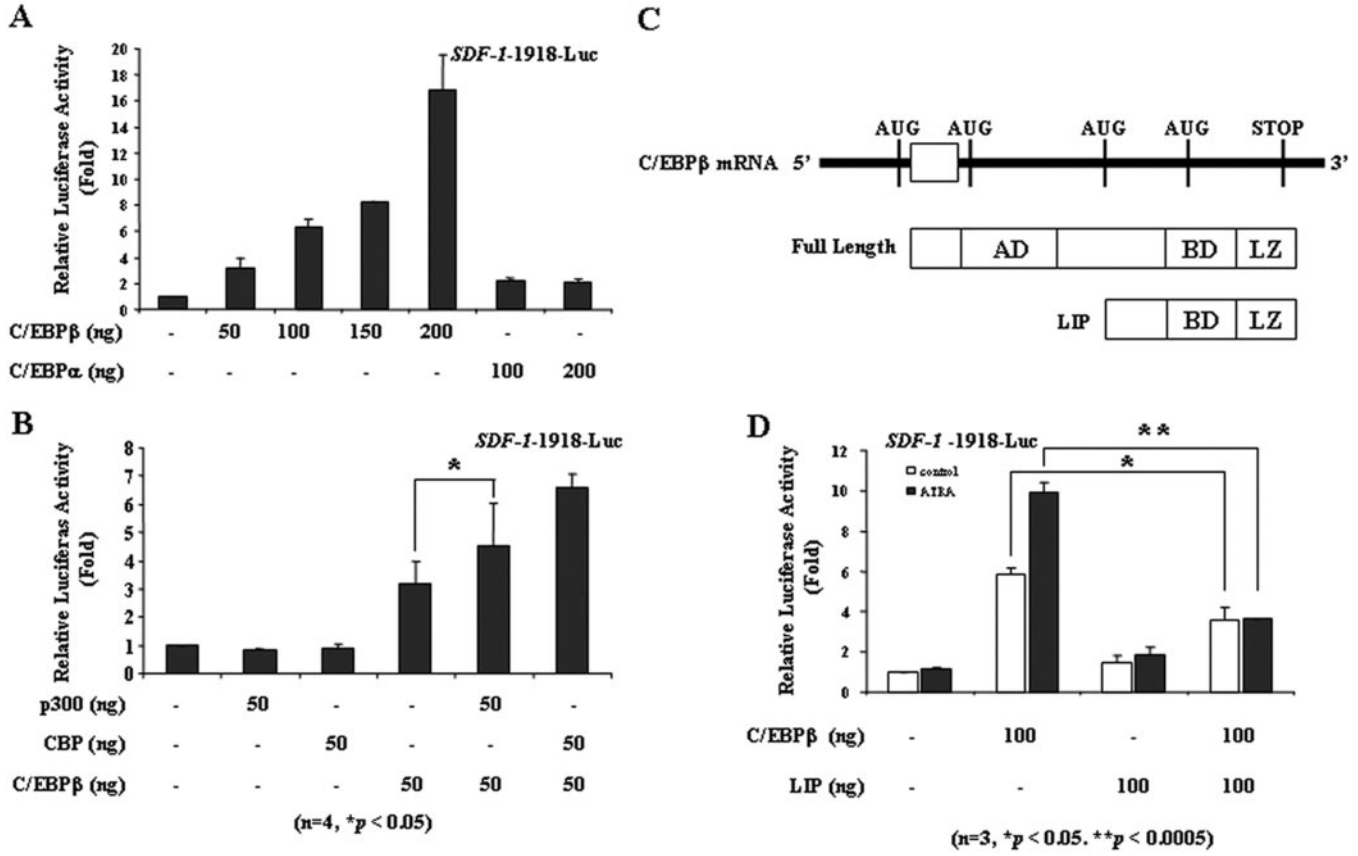

Fig. 2. C/EBPβ plays a role in transactivating the SDF-1 promoter. (A) Cells were transfected with the indicated amounts of expression vectors encoding C/EBPα and C/EBPβ along with the hSDF-1 promoter luciferase reporter. C/EBPα protein expression in the transfected cells was confirmed by Western blot using anti-C/EBPα antibody. Data are representative of four independent experiments. Values represent mean ± SD (n=4); (B) The mammalian expression plasmids encoding C/EBPβ, CBP, and p300 were transfected with the SDF-1 promoter reporter plasmid into cells as indicated. Data are representative of four independent experiments. Values represent mean ± SD (n=4); *, $P < 0.05$, compared with C/EBPβ transfectants. (C) Schematic structures of C/EBPβ and LIP. The C/EBPβ gene contains several AUG codons, which serve as translation start sites. The full-length isoform with activation domain (AD), binding domain (BD), and leucine zipper domain (LZ) is depicted. The N-terminally truncated isoform LIP lacks the activation domain. (D) The SDF-1 gene promoter was cotransfected into cultured cells with expression vectors for C/EBPβ and LIP as indicated in the presence or absence of 10^{-6} M ATRA. Luciferase activity of the cell lysates was measured. Data are representative of four independent experiments. Values represent mean ± SD (n=3); *, $P < 0.05$, compared with C/EBPβ transfectants; **, $P < 0.0005$.

variety of DNA-bound transcription factors, which lead to enhanced activation of the basal transcription machinery [27, 28]. As a previous study showed that C/EBP molecules might also be regulated by CBP and p300 [29], we next investigated whether C/EBPβ-dependent transactivation of the SDF-1 promoter is mediated by these coactivators. To address this, cells were transiently cotransfected with C/EBPβ or p300 expression construct and SDF-1 reporter construct. As shown in Figure 2B, these coactivators significantly stimulated transactivation of SDF-1 promoter activity with C/EBPβ (Fig. 2B), suggesting that p300 and CBP recruited by C/EBPβ may induce effective SDF-1 gene transcription in granulocyte differentiation.

The C/EBPβ RNA transcript, which does not contain the intron found in the gene, is post-transcriptionally regulated by leaky ribosomal scanning, resulting in the expression of four isoforms: the 38-kDa full-length isoform, the 35-kDa liver-activating protein, the 20-kDa LIP, and the 16-kDa truncated isoform [30, 31] (Fig. 2C). LIP can act as a dominant-negative inhibitor of C/EBPβ function by forming nonfunctional heterodimers with the other members [30]. Overexpression of LIP inhibited the SDF-1 gene promoter activity transactivated by C/EBPβ (Fig. 2D). These data provide more evidence supporting the important transcriptional role of C/EBPβ in SDF-1 gene expression.

ATRA stimulation causes binding of C/EBPβ to the SDF-1 promoter region

Next, to identify the C/EBPβ-responsive elements in the SDF-1 promoter region, we generated various deletion mutants of the SDF-1 promoter reporter construct by PCR cloning, as described in Materials and Methods. We cotransfected various deletion mutants of SDF-1 promoter reporter constructs into cells with the C/EBPβ expression construct and then measured luciferase activity. As shown in **Figure 3A**, transfection of the –213 SDF-1 promoter reporter construct reduced C/EBPβ-dependent promoter activity significantly (Fig. 3A). This result indicates that there could be C/EBPβ-responsive elements between residues –395 and –213 within the SDF-1 promoter region.

To examine whether the increased SDF-1 promoter activity induced by C/EBPβ expression results from the direct DNA-

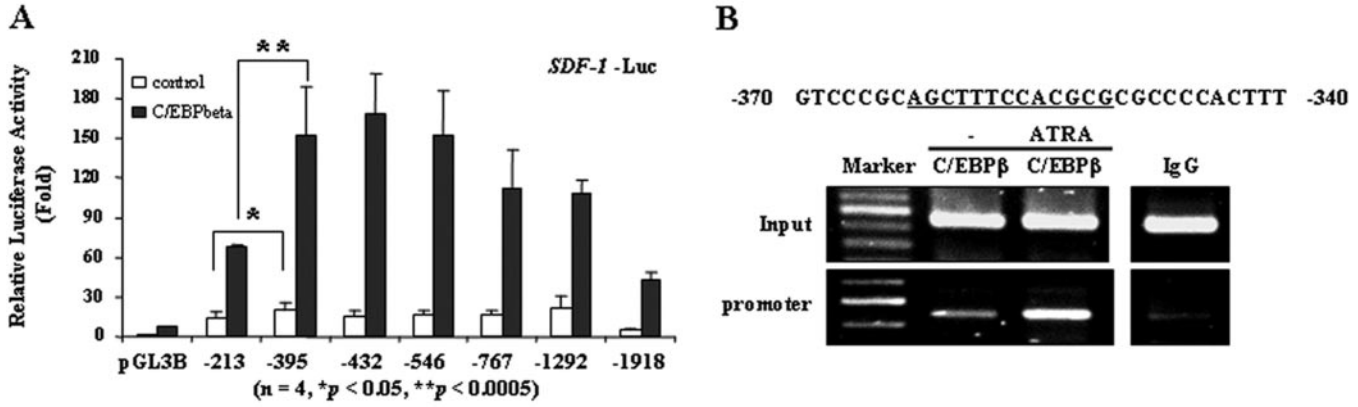

Fig. 3. Identification of the C/EBPβ-binding region in the SDF-1 promoter. (A) Luciferase activity of the promoter-deletion constructs in cells. Cultured cells were transfected with the indicated promoter-deletion constructs. Promoter activity was measured as luciferase activity normalized to β-galactosidase activity. Bars indicate the mean of five independent experiments; error bars indicate SD (n=4); *, $P < 0.05$; **, $P < 0.0005$, compared with the –213 promoter construct. (B) ChIP assay of C/EBPβ occupancy on the SDF-1 promoter in the absence or presence of 10^{-6} M ATRA. Following formaldehyde cross-linking, soluble chromatin was prepared. After immunoprecipitation with C/EBPβ antibody, precipitated DNAs were used in PCR analysis. The input lane shows the starting chromatin extracts. Data shown are representative of three independent experiments.

binding activity of C/EBPβ on the SDF-1 promoter, we performed a ChIP assay with C/EBPβ antibody. Isolated DNA was subjected to PCR before (input) and after ChIP using primer sets designed to amplify the region of the SDF-1 promoter harboring the C/EBPβ-binding site (–395 to –213). The C/EBPβ-binding site in mock-treated, cultured cells appeared to bind C/EBPβ weakly. However, the SDF-1 promoter and C/EBPβ complex on chromatin DNA were increased by ATRA stimulation for 48 h in U937 cells (Fig. 3B). These results indicate that C/EBPβ transactivates SDF-1 gene expression at the transcriptional level by directly binding to the promoter region of SDF-1 gene during ATRA-induced granulocyte differentiation.

SDF-1 stimulation promotes $p21^{WAF1/CIP1}$ and Pyk expression in U937 cells

Transcriptional control of $p21^{WAF1/CIP1}$ by p53-dependent and -independent mechanisms is critical for growth arrest and differentiation of various tumors [32, 33]. In addition, $p21^{WAF1/CIP1}$ is known as a target gene for ATRA in normal haematopoiesis [34]. To determine whether SDF-1 stimulation can regulate $p21^{WAF1/CIP1}$ expression, cells were cotransfected with the $p21^{WAF1/CIP1}$ promoter as a reporter and the HA-tagged SDF-1 expression plasmid. Ectopic expression of SDF-1 dose-dependently increased the $p21^{WAF1/CIP1}$ promoter activity (see Fig. 5A). The transfection efficiency and protein expression of HA-tagged SDF-1 were confirmed by Western blots using anti-HA antibody (**Fig. 4A**, lower). Likewise, $p21^{WAF1/CIP1}$ mRNA levels were strongly induced by 40 ng/ml rSDF-1α after 48 h treatment in U937 cells. We also observed that SDF-1 stimulation increased mRNA expression of another cyclin-cyclin-dependent kinase inhibitor, $p27^{KIP1}$ (Fig. 4B).

We next tested whether SDF-1 could regulate the expression of $p21^{WAF1/CIP1}$ protein in U937 cells. To study this, the expression of $p21^{WAF1/CIP1}$ and Pyk genes was evaluated in ATRA-induced granulocyte differentiation. Protein lysates were prepared from differentiating U937 cells followed by SDS-PAGE analysis. Western blotting was performed with antibodies against Pyk and $p21^{WAF1/CIP1}$. As shown in **Figure 5C**, $p21^{WAF1/CIP1}$ protein expression was low in mock-treated U937 cells, but its expression was up-regulated gradually by SDF-1 in a dose- and time-dependent manner (Fig. 4, C and D). In addition, Pyk2 was induced by rSDF-1 and might play a role in activating Pyk2-mediated signaling pathways, including adhesion molecules, during U937 cell differentiation (Fig. 4, C and D). Therefore, these results indicate that SDF-1 may play an important role in U937 cell differentiation through induction of differentiation-related gene expression including $p21^{WAF1/CIP1}$, p27, and Pyk.

SDF-1 and ATRA synergistically up-regulate $p21^{WAF1/CIP1}$ expression and induce U937 cell differentiation

We then studied whether ATRA and SDF-1 cooperate to induce p21 protein expression. As expected, the results showed that $p21^{WAF1/CIP1}$ protein levels were up-regulated by cotreatment with ATRA and rSDF-1 (Fig. 5A). Also, treatment with ATRA and rSDF-1 increased Pyk2 and C/EBPβ protein levels significantly. Next, we investigated the costimulatory effect of ATRA (10^{-6} or 10^{-7} M) and rSDF-1(10 ng/ml) in U937 cells. As shown as Figure 5B, cotreatment with ATRA and SDF-1 synergistically induced granulocyte differentiation as measured by the NBT reduction assay. It is interesting that our data showed that the cotreatment with 10^{-6} M ATRA and rSDF-1 increased the protein expression of $p21^{WAF1/CIP1}$ and Pyk2 more effectively than 10^{-6} M ATRA alone. These data indicate that ATRA signaling and SDF-1 production are sufficient to induce granulocyte differentiation through up-regulation of target gene expression.

Furthermore, to confirm the functional relevance of ATRA-induced SDF-1 in U937 cell differentiation, we performed SDF-1 knockdown studies using SDF-1 siRNA in ATRA-induced granulocyte differentiation of U937 cells. As shown in Figure 5C, SDF-1 siRNA inhibited ATRA-induced granulocyte differentiation significantly, as measured by NBT reduc-

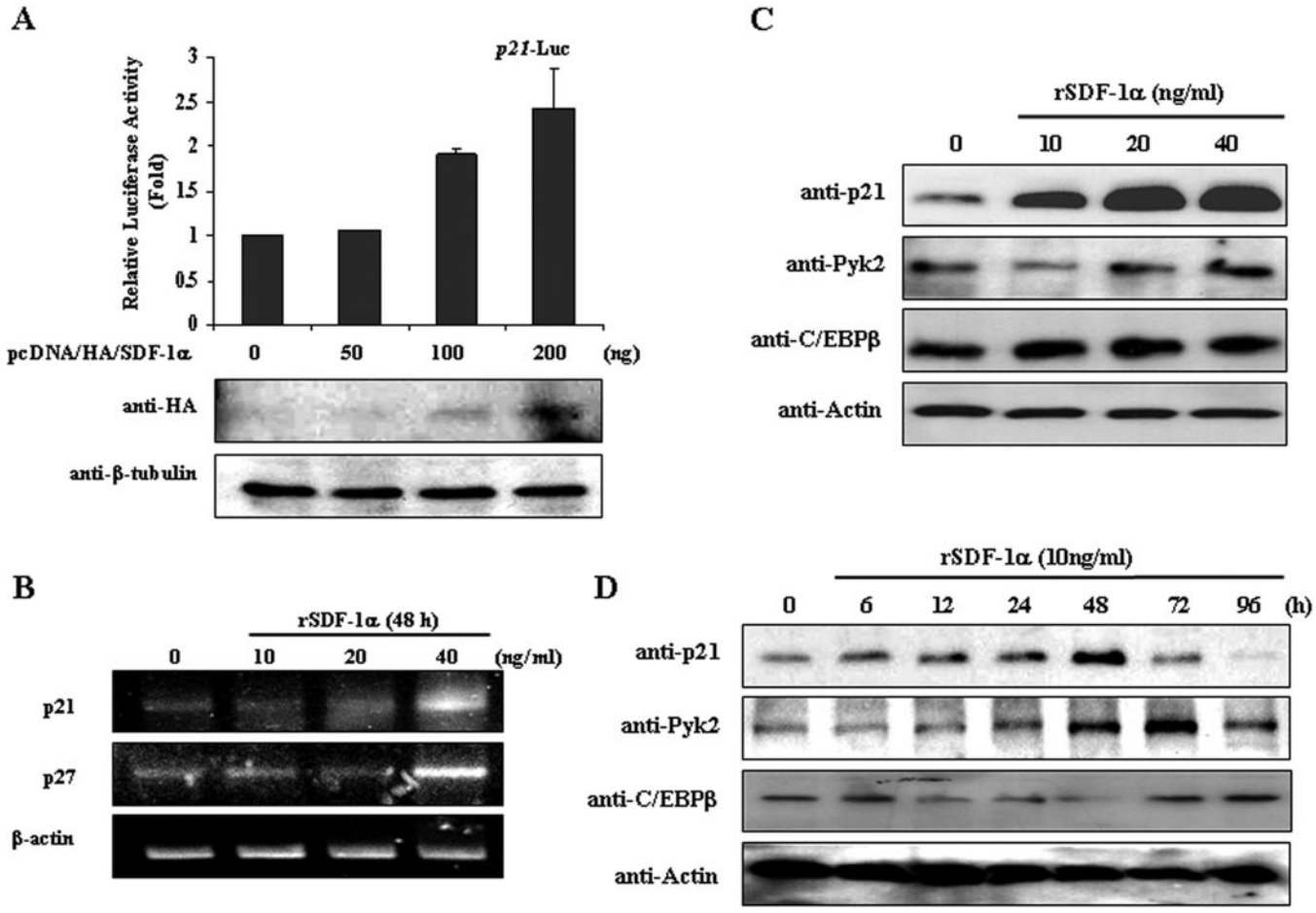

Fig. 4. SDF-1 promotes $p21^{WAF1/CIP}$ expression. (A, Upper) Cultured cells were transfected with the p21 promoter luciferase reporter gene together with a HA/SDF-1 expression vector. After harvesting the cells, the promoter activity was measured as luciferase activity normalized to β-galactosidase activity. All values represent the mean of duplicate samples; data shown are representative of three independent experiments. (Lower) The protein expression of HA/SDF-1α was determined by Western blotting with the HA-specific antibody. The amount of β-tubulin was determined with β-tubulin-specific antibody as a loading control. Values represent mean ± SD (n=4). (B) U937 cells were treated with 10, 20, and 40 ng/ml rSDF-1 for 48 h. The p21 mRNA levels were detected by RT-PCR, with β-actin as loading control. (C and D) U937 cells were cultured with complete growth medium containing rSDF-1 at the indicated concentration (C) and duration (D). Whole cell lysate from each sample was separated and transferred to PVDF membranes, which were subjected to Western blot analysis using antibodies specific for p21, Pyk2, and C/EBPβ as indicated. Western blot detection of β-tubulin was used to estimate protein loading for each lane. Data shown are representative of three independent experiments.

tion. This result provides direct evidence that SDF-1 expression plays an important role in granulocyte differentiation.

DISCUSSION

In this study, we demonstrated that ATRA stimulation elevated SDF-1 expression in granulocytic differentiation (Fig. 1). Also, to investigate the mechanism by which SDF-1 gene expression is regulated, the promoter region of the SDF-1 gene was examined, and transient transfection with various deletion mutants and ChIP assays revealed that the transcription factor C/EBPβ binds to the promoter region of the SDF-1 gene to up-regulate its expression in response to ATRA.

Although little is known about the ATRA target genes that regulate granulocyte differentiation, $p21^{WAF1/CIP1}$ and p27 cyclin-dependent kinase inhibitors have been shown to be involved in granulocyte differentiation. Ectopic expression of $p21^{WAF1/CIP1}$ and p27 in a human myeloblastic leukemia cell line resulted in the induction of granulocyte differentiation. Moreover, several inducers of myeloid cell differentiation up-regulate $p21^{WAF1/CIP1}$ expression [35]. Therefore, the $p21^{WAF1/CIP1}$ promoter appears to harbor multiple response elements for several differentiation signals, such as ATRA-mediated growth arrest and the subsequent differentiation, followed by G_1 arrest in the cell cycle. We showed that the $p21^{WAF1/CIP1}$ expression level was up-regulated during granulocyte differentiation of U937 cells. Ectopic expression of C/EBPβ also resulted in enhanced expression of $p21^{WAF1/CIP1}$, whereas $p21^{WAF1/CIP1}$ expression is reduced in cells transfected with dominant-negative C/EBPβ (data not shown). This suggests that transcriptional activation of $p21^{WAF1/CIP1}$ is involved in C/EBPβ-dependent granulocyte differentiation, by inducing cell cycle arrest, which might be necessary for the induction of terminal differentiation.

Hematopoietic cell development is controlled by lineage-specific and nonspecific transcription factors [36, 37]. C/EBPs belong to the basic region/leucine zipper class of transcription

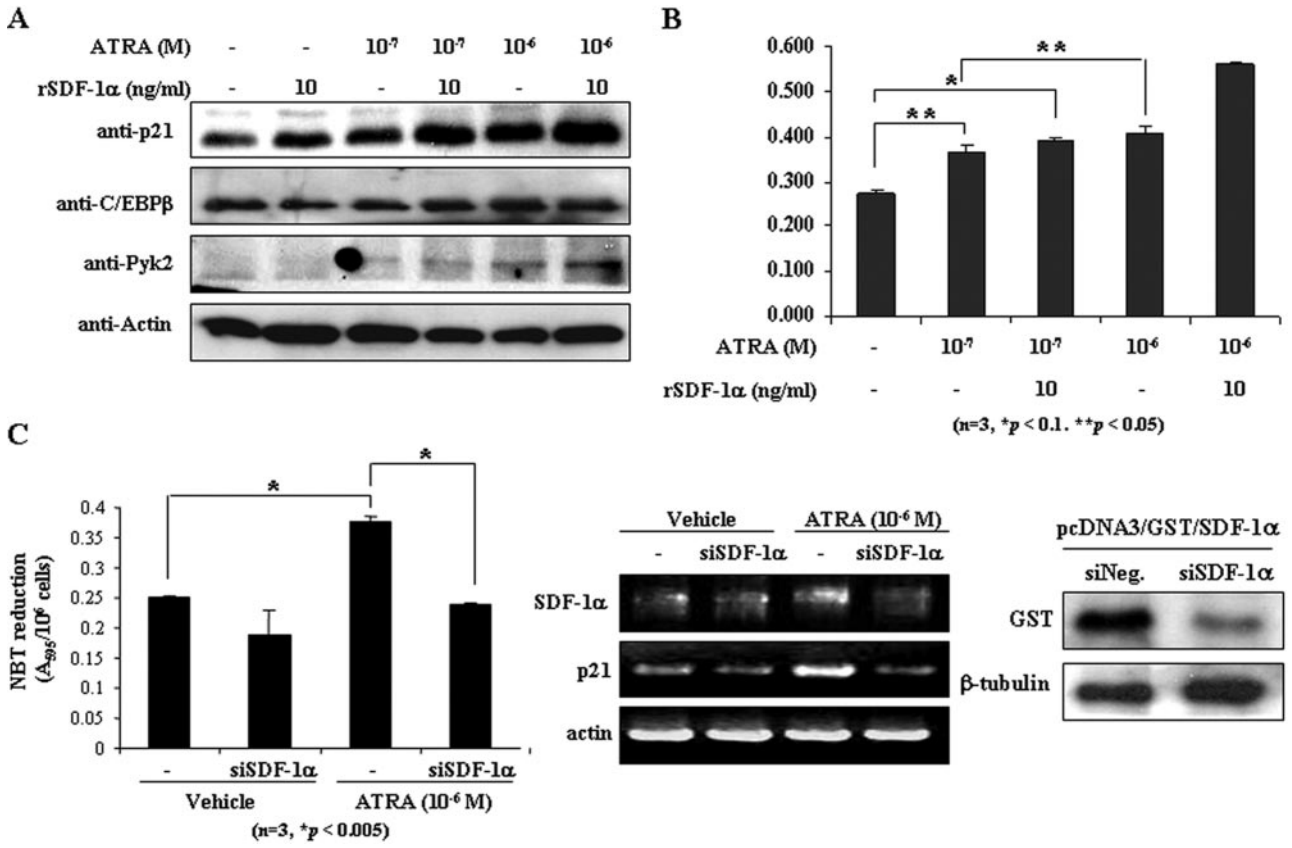

Fig. 5. SDF-1 and ATRA additively up-regulate p21 expression and induce U937 cell differentiation. (A) U937 cells were propagated with complete growth medium containing 10 ng/ml rSDF-1 and 10^{-7} or 10^{-6} M ATRA concurrently as indicated. Whole cell lysate from each sample was separated on 12% SDS-PAGE and transferred to PVDF membranes, which were subjected to Western blot analysis using antibodies specific for p21, Pyk2, and C/EBPβ as indicated. Western blot detection of β-tubulin was used to estimate protein loading for each lane. Data shown are representative of three independent experiments. (B) U937 cells were treated with ATRA and rSDF-1 for 48 h, and the differentiation yield was determined by scoring NBT reduction; the absorbance values are presented. Data shown are representative of three independent experiments. Values represent mean ± SD ($n=3$–4); *, $P < 0.1$; **, $P < 0.05$, compared with mock transfectants. (C) For siRNA-mediated down-regulation of SDF-1α, negative control siRNA or SDF-1α-specific siRNA was electroporated into U937 cells with Nucleofector Kit V (Amaxa); cells were then incubated with ATRA or vehicle (DMSO) for 48 h. (Left) Differentiation was evaluated by NBT reduction assay. Values represent mean ± SD ($n=3$); *, $P < 0.005$, compared with mock transfectants. (Right) Total RNA was prepared and analyzed for SDF-1α and p21 expression by RT-PCR as described. Actin was used as an internal control. The decreased protein expression of SDF-1α with specific siRNA for SDF-1α in the transfecton of pcDNA3/GST/SDF-1α was confirmed with Western blot analysis using anti-GST antibody.

factors and play a role in the differentiation of a broad range of tissues. In the hematopoietic system, C/EBP family members are expressed mostly in the macrophage and granulocytic lineage and participate in the regulation of macrophage and granulocyte-restricted genes, such as the M-CSF receptor, G-CSF receptor, and GM-CSF receptor genes [12, 38]. Targeted disruption of the C/EBPδ, C/EBPβ, or C/EBPε gene resulted in defects, which predominantly affected the granulocytic lineage [39]. Recently, C/EBPβ was shown to be enhanced and required in ATRA-induced differentiation of APL cells. Moreover, inhibiting C/EBP activity, specifically C/EBPβ expression, dramatically reduced the APL response to ATRA [19].

Since the successful introduction of ATRA for treatment of APL, a potentially less-toxic cancer therapeutic strategy known as "differentiation therapy" has been developed to induce cancer cells to undergo terminal differentiation of cancer cells to block their continuous proliferation. Therefore, understanding the mechanisms by which ATRA induces leukemia cell differentiation has attracted significant attention. Despite the success of ATRA in the treatment of APL, many patients relapse with ATRA-resistant disease. Initially, the possibility of using ATRA and SDF-1 in a combination treatment was investigated in vitro. ATRA and SDF-1 were found to increase $p21^{WAF1/CIP1}$ expression synergistically and induce differentiation when used in combination (Fig. 5).

Based on the data presented in this study, these results can be interpreted in the context of a model of ATRA-induced leukemic cell differentiation mediated by SDF-1. This model is consistent with the finding that strongly suggests that the mechanism involves SDF-1 in leukemic cell differentiation [40–43]. Thus, in addition to demonstrating the novel relationship between ATRA and SDF-1, this work provides an example of the physiological relevance of the differentiation-inducing agent and the chemokine cross-talk within each signaling pathway.

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