Mechanisms of SHP-1 P2 promoter regulation in hematopoietic cells and its silencing in HTLV-1-transformed T cells

Koichi Nakase, Jihua Cheng, Quan Zhu, and Wayne A. Marasco1

Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute and Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA

Abstract: The Src homology-2-containing protein-tyrosine phosphatase 1 (SHP-1), is a negative regulator of cell signaling. It is also considered a tumor suppressor gene because of its ability to antagonize the action of tyrosine kinases. Although SHP-1 is expressed strongly in hematopoietic cells, decreased expression has been observed in various hematological malignancies, which suggests a central involvement of SHP-1 in leukemogenesis. We have shown previously that human T cell lymphotropic virus type-1 (HTLV-1) Tax-induced promoter silencing (TIPS) is an early event causing down-regulation of SHP-1 expression, which is dependent on NF-κB. In this study, DNase I footprinting and EMSA also revealed binding of transcription factors, specificity protein 1 (Sp1) and octamer-binding transcription factor 1 (Oct-1) to the P2 promoter, and site-directed mutagenesis confirmed that these factors contribute to the basal P2 promoter activity. Chromatin immunoprecipitation (CHIP) assays showed that Sp1, Oct-1, NF-κB, CREB-1, and RNA polymerase II interacted with the core SHP-1 P2 promoter in CD4+ T cells and Jurkat cells but not in HTLV-1-transformed MT-2 and HUT102 cells when HTLV-1 Tax is present. Furthermore, bisulfite sequencing of the SHP-1 P2 core region revealed heavy CpG methylation in HTLV-1-transformed cells compared with freshly isolated CD4+ T cells and HTLV-1-noninfected T cell lines. A significant inverse correlation between degree of CpG methylation and expression of SHP-1 mRNA or protein was observed. Taken together, our data support the notion that in HTLV-1-transformed CD4+ T cells, TIPS causes dissociation of transcription factors from the core SHP-1 P2 promoter, which in turn leads to subsequent DNA methylation, an important early step for leukemogenesis.

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Key Words: DNA methylation · adult T cell leukemia · leukemogenesis

INTRODUCTION

Phosphorylation of tyrosine residues is important for cellular signal transduction, control of the mitotic cycle, and neoplastic transformation. Protein tyrosine kinases and protein tyrosine phosphatases control the phosphorylation status of tyrosine residues [1]. Src homology-2 (SH2)-containing protein-tyrosine phosphatase 1 (SHP-1), also called PTPN6, PTP1C, HCP, HCPH, SHP1, HPTP1C, and SH-PTP1, is a nontransmembrane phosphotyrosine phosphatase containing two SH2 domains and functions as an early negative regulator of cell signaling. The SH2 domains of SHP-1 form high-affinity complexes with activated receptor tyrosine kinases, such as epidermal growth factor receptor, stem-cell factor receptor, and other phosphotyrosine-containing proteins. Dephosphorylation of these proteins leads to negative regulation of receptor tyrosine kinase signaling [2]. The human SHP-1 gene maps to 12p13, a region commonly involved in leukemia-associated chromosomal translocations and deletions [3, 4]. The SHP-1 gene encodes at least two isoforms of SHP-1 protein as a result of different transcription initiation sites. SHP-1 expression in hematopoietic cells is regulated mainly by the P2 promoter [5] and functions at multiple stages of hematopoietic development [6].

Recently, the core promoter region of the SHP-1 P2 promoter was identified [7, 8]. Critical regions within the P2 promoter, defined as the large core (LC; –120–+157) and small core (SC; –120–+24), respectively, showed similar basal activity compared with the full-length promoter but differed in that only the LC was responsive to Tax-mediated suppression [7]. In addition, NF-κB p65 [7] and PU.1 [8] were shown to activate transcription of the SHP-1 gene in hematopoietic cells. However, the precise mechanisms of SHP-1 transcriptional regulation are not well understood. Although SHP-1 is expressed strongly in hematopoietic cells, decreased expression of SHP-1 has been reported in numerous hematological malignancies [9–16]. These findings, together with its negative regulation of the JAK/STAT signaling and growth inhibitory effects, have provided growing evidence that SHP-1

1 Correspondence: Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney St., Boston, MA 02115, USA. E-mail: wayne_marasco@dfci.harvard.edu

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functions as a tumor suppressor gene [17]. Although aberrant SHP-1 splicing has been observed in acute myelogenous leukemia [18], Jurkat cells [19], and T cells from leukemia and lymphoma cell lines [20], no mutations have been identified in the SHP-1 open-reading frame or P2 promoter [9, 11, 16, 21]. Methylation of putative CpG islands, which are located 150–300 bp upstream of the defined LC promoter, has been reported in various hematological malignancies [11, 12, 22–26]. DNA methylation of the P2 core promoter has also been reported in cutaneous T cell lymphoma (CTCL) tissues and cell lines [9, 27] as well as in T cell lymphoma cell lines [28] but was not observed in chronic myelogenous leukemia cells [16].

Human T cell lymphotropic virus type-1 (HTLV-1) is a retrovirus that encodes oncoprotein Tax and the etiologic agent of adult T cell leukemia (ATL), a hematologic malignancy with a poor prognosis [29]. Tax modulates the expression of many viral and cellular genes and impairs the cell cycle and growth control [30]. In HTLV-1-infected cells, acute-immortalized and chronic-transformed HTLV-1-positive cell lines, and cells freshly isolated from adult T cell leukemia patients, frequent loss of SHP-1 expression has been reported [11, 21, 31]. Based on a previous study reported by our group, Tax-induced promoter silencing (TIPS) was demonstrated as an important mechanism of SHP-1 P2 promoter silencing during HTLV-1 infection, and NF-kB was identified as the target of Tax inhibition but not CREB-binding protein (CBP)/P300 [7]. In this report, further studies were performed to define the basal mechanisms of SHP-1 P2 promoter regulation and its silencing in HTLV-1-transformed cells. Additional transcription factors were found to be involved in SHP-1 P2 promoter regulation. The methylation status of the SHP-1 core promoter in normal cells versus HTLV-1-transformed cells was also investigated. These studies suggest that upon initial inhibition through NF-kB [7], TIPS proceeds to extensive DNA methylation of CpG residues in the P2 core promoter, leading to further loss of critical transcription factor binding and complete promoter silencing.

MATERIALS AND METHODS

Transcription factor search programs

Transcription factor search programs MATCH (http://www.gene-regulation.com/pub/programs.html#match) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) were used to identify transcription factors binding to the promoter of SHP-1 and its silencing (TIPS) was demonstrated as an important mechanism of SHP-1 P2 promoter regulation and its silencing during HTLV-1 infection, and NF-kB was identified as the target of Tax inhibition but not CREB-binding protein (CBP)/P300 [7]. In this report, further studies were performed to define the basal mechanisms of SHP-1 P2 promoter regulation and its silencing in HTLV-1-transformed cells. Additional transcription factors were found to be involved in SHP-1 P2 promoter regulation. The methylation status of the SHP-1 core promoter in normal cells versus HTLV-1-transformed cells was also investigated. These studies suggest that upon initial inhibition through NF-kB [7], TIPS proceeds to extensive DNA methylation of CpG residues in the P2 core promoter, leading to further loss of critical transcription factor binding and complete promoter silencing.

EMSA

EMSA was performed using the gel-shift assay system by Promega (Madison, WI, USA). Sequences of the probes were shown in Table 1. To prepare double-stranded DNA probes, DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA, USA) were annealed by mixing 350 pmol each oligonucleotide in 100 μl 1X annealing buffer [20 mM Tris (pH 7.5), 2 mM MgCl2, 1 mM DTT, 50 mM NaCl]; incubated at 95°C for 5 min, and slowly cooled down to room temperature. The 110-bp SHP-1 P2 SC DNA fragment probe was prepared by digesting the plasmid pGL3-LC-P2 [7] with BglII and XhoI and gel-purified with QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). For a supershift experiment, nuclear extracts were incubated with 2 μg Sp1 (sc-14027x), CREB-1 (sc-106x), NF-κB p65 (sc-372x), PU.1 (sc-352x), IFN regulatory factor 1 (IFN-1; sc-195a), or IFN-2 (sc-496a) antibodies (Santa Cruz Biotechnology) for 10 min at room temperature before the binding reaction. The protein/DNA complexes were resolved on 4–6% polyacrylamide gels in 0.5 X Tris-boric acid-EDTA buffer for 1.5 h at 300 V and analyzed by autoradiography or Phosphorimager Storm 820 and Image Quant (Molecular Dynamics, Piscataway, NJ, USA).

In vitro DNase I footprinting

DNase I footprinting experiments were performed with the Core Footprinting system kit (Promega). The plasmids pGL3-LC-P2 and pGL3-F70R80 [7] were used to generate DNA probe. pGL3-F70R80 was digested with Smal and HindIII. The 156-bp fragment was gel-purified and named the SC probe. pGL3-LC-P2 was digested with BsmAl and MstI. The 301-bp band was gel-purified and named the LC probe. These DNA fragments were labeled with 32P-ATP and digested with XhoI (SC probe) or BsmAl (LC probe) to obtain the single-end-labeled probe, followed by phenol/chloroform extraction. Labeled probe was incubated with nuclear extracts on ice for 10 min. Ca2+/Mg2+ solution was then added to the binding reaction and incubated at room temperature (RT) for 1 min, followed by addition of DNase I and incubation at RT for 2 min. Upon addition of stop solution and phenol/chloroform extraction, samples were resolved on 6% polyacrylamide and 7 M urea sequencing gel and analyzed by autoradiography.

Bisulfite sequencing

Genomic DNA was purified with the DNeasy tissue kit (Qiagen). DNA (1 μg) was modified by sodium bisulfite using the EpiTect bisulfite kit (Qiagen). To analyze CpG methylation of SHP-1 P2 LC, PCR primers were designed as follows: 5’-ATG TTT TTG AGT TTT TGA TTG TAG A-3’ (bisulfite forward) and 5’-AAT AAC AAA CCC TTA CCT CAC CAT-3’ (bisulfite reverse). The PCR mixture consisted of 2 μl bisulfito-converted DNA, 45 μl Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA, USA), and 500 nM final concentration of the respective bisulfite forward and reverse primers each. Cycling conditions were 3 min at 95°C for initial denaturation, 40 cycles of 95°C, 30 sec; 60°C, 30 sec; and 72°C, 30 sec.

Table 1. Oligonucleotide Sequences of EMSA Probes

<table>
<thead>
<tr>
<th>Probes</th>
<th>Sequence of upper strands</th>
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<tbody>
<tr>
<td>Oct-1 BS1</td>
<td>5’-TGCTTCTCTCCTCCCTTCTGT-3’</td>
</tr>
<tr>
<td>Oct-1 BS2</td>
<td>5’-GTTACCGAAGTCGAGT-3’</td>
</tr>
<tr>
<td>SHP-1 Spl</td>
<td>5’-GGTCCGCGGCGGTCCTTCCT-3’</td>
</tr>
<tr>
<td>NF-κB 2nd</td>
<td>5’-CGTGTCGCGGCGGTCCTTCCT-3’</td>
</tr>
<tr>
<td>Oct-1 BS1 mutant</td>
<td>5’-TGCTTTGTCGTCCTTCTTCCT-3’</td>
</tr>
<tr>
<td>Oct-1 BS2 mutant</td>
<td>5’-GTTACCGAAGTCGAGT-3’</td>
</tr>
<tr>
<td>SHP-1 Spl mutant</td>
<td>5’-GGTCCGCGGCGGTCCTTCCT-3’</td>
</tr>
<tr>
<td>NF-κB 1st mutant</td>
<td>5’-CAAGTGAGTTCCCCCAAGGG-3’</td>
</tr>
<tr>
<td>NF-κB 2nd mutant</td>
<td>5’-CAAGTGAGTTCCCCCAAGGG-3’</td>
</tr>
<tr>
<td>AP2 consensus</td>
<td>5’-GATCGAATGGGGCGGCGGCGGCGG-3’</td>
</tr>
</tbody>
</table>

Preparation of nuclear extract

Jurkat + PMA nuclear extract was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other nuclear extracts were prepared with the nuclear extract kit from Active Motif (Carlsbad, CA, USA).
followed by a final extension at 72°C for 7 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen), cloned into the pCR4-TOPO vector (TOPO TA cloning kit, Invitrogen), and introduced into DH5α cells (Invitrogen). Ten clones were selected at random from each PCR product for sequencing analysis.

Site-directed mutagenesis

Site-directed mutagenesis was performed with the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), and pGL3-LC-P2 was used as a template. DNA sequencing was confirmed on the final mutant constructs prior to Maxiprep.

Luciferase reporter assays

Jurkat LT cells (1×10⁶) were transfected with 500 ng pGL3-LC-P2 plasmid or each mutant plasmid using SuperFect transfection reagent (Qiagen). Procedures for transfection and luciferase reporter assays were described previously [7].

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with a ChIP-IT Express kit (Active Motif) with modification. After removal of culture media, cells were fixed with 1% formaldehyde for 8 min, centrifuged, and supernatant was discarded. Glycine stop-fix solution was added to the pellet and incubated for 5 min. After wash with PBS, the cell pellet was resuspended in cold lysis buffer and incubated on ice for 30 min. Then, the supernatant was removed, and the pellet was resuspended in shearing buffer. The sample was sonicated 12 times for 30 s each with Sonifier 450 (Branson Ultrasonic, Danbury, CT, USA) so that the fragment became 200–1000 bp. The sample was centrifuged at 12,000 rpm, 4°C, for 12 min, and the supernatant was used for immunoprecipitation. Antibodies against NF-κB p50 (sc-7178x), NF-κB p65 (sc-372x), Oct-1 (sc-232x), Sp1 (sc-14027x), CREB-1 (sc-186x), PU.1 (sc-14027), and RNA polymerase II (sc-9001x) were from Santa Cruz Biotechnology, and Tax antibody (Tab172) was from National Institutes of Health (NIH)-AIDS Research and Reference Reagent Program (Bethesda, MD, USA). The chromatin DNA (4 μg) was incubated with 2 μg antibody overnight at 4°C. After three wash samples, were reverse-cross-linked and proteinase K-treated. The antibody-bound chromatin samples were analyzed by PCR with primers 5’-AGTGCCACCGTGCTTGCTTC-3’ and 5’-CAGTCTGGGCTGGCCACTGCA-3’ to amplify the SHP-1 P2 promoter SC. The PCR mixture consisted of 3.25 μl precipitated DNA, 45 μl Platinum PCR SuperMix High Fidelity (Invitrogen), 1.5 μl dimethyl sulfoxide, and 200 nM final concentration of the respective forward and reverse primers made up to a final volume of 50 μl. Cycling condition is: initial denaturation at 95°C, 5 min; 30 cycles at 95°C, 30 sec; 56°C, 30 sec; and 72°C, 30 sec; final extension at 72°C for 7 min. As a negative control, PCR was performed with primers that recognize exon 5 (5 kb downstream from the P2 promoter) of the SHP-1 gene 5’-GTACCATGGCCACATGTCTG-3’ and 5’-CATGACCTTGATGTGGGTGATA-3’. Diluted chromatin (100X) without immunoprecipitation was used as an input control. The PCR products were visualized in 2% agarose gels with ethidium bromide staining.

Western blot study

For Western blot, whole cell lysates (50 μg) were separated on 10% SDS-polyacrylamide gels and transferred to Piotran nitrocellulose transfer membrane (Whatman GmbH, Dassel, Germany). The membrane was incubated with blocking buffer (PBS with 0.1% Tween 20 and 5% skim milk) at 4°C overnight and incubated thereafter with antibodies from Santa Cruz Biotechnology including SH-PTP1 (SHP-1; sc-287) and β-tubulin antibody (sc-9104). After extensive washes with PBS containing 0.1% Tween 20, the membrane was stained.

Fig. 1. Identification of transcription factor binding sites in the SHP-1 P2 promoter. (A) Schematic organization of the human SHP-1 P2 promoter. Full-length promoter (–802 to +157 relative to the transcription initiation site), LC (–120 to +157), and SC (–120 to +24) are shown by arrows, and transcription factor binding sites are labeled. In vitro DNase I footprinting of the SHP-1 P2 promoter SC probe (B) and LC probe (C). The 32P-labeled probe was incubated with increasing amount of nuclear extracts or BSA as indicated above each panel. Numbers at the right of each panel indicate nucleotides upstream (+) of the major transcription initiation site (+1).
incubated with HRP-linked anti-rabbit antibody IgG (Cell Signaling Technology, Danvers, MA, USA) and developed using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA).

RESULTS

Identification of transcription factor-binding regions in the SHP-1 P2 promoter

To better understand the mechanism(s) of SHP-1 P2 promoter regulation, several methods were used to systematically identify the transcription factors involved. Based on our deletional analysis [7], an indispensable region for SHP-1 P2 basal promoter activity was the SC (–120 ~ +24; Fig. 1A). DNase I footprinting analysis was performed, first using the SC as a probe, and the DNA sequences between –40 ~ –30 and –100 ~ –55 were found to be protected (Fig. 1B). The footprint located at –100 ~ –55 was examined further with the LC probe (–120 ~ +157; Fig. 1A), and two footprints were found between –100 ~ –85 (FP1) and –80 ~ –55 (FP2; Fig. 1C). The –40 ~ –30 footprint was designated as FP3.

Evaluation of putative transcription factors binding to the SHP-1 P2 promoter

Two computer-aided programs (MATCH and TFSEARCH) were used to search for the candidate transcription factor-binding sites within the DNase I-protected SHP-1 P2 regions. The following putative transcription factors were predicted to bind FP1: ETS-like transcription factor 1 (Elk-1), Ikaros 1 (Ik-1), Oct-1, and PU-1; FP2: Elk-1, Oct-1, PU-1, and NF-κB; and FP3: Sp1 (Fig. 1A, and see Fig. 4B). The Pu.1 sites reported by Wlodarski et al. [8] overlap with each Oct-1 recognition sequence. The binding of NF-κB to the FP2 region was confirmed previously using EMSA and mutagenesis analysis [7].

To verify if the computer-predicted transcription factors indeed bind to the core promoter, the SHP-1 SC probe was used for EMSA and antibody supershift assay (Fig. 2A). Compared with the irrelevant IRF-1 antibody, addition of the Oct-1, NF-κB p65, or Sp1 antibodies either disrupted or supershifted specific protein-DNA complexes, thus revealing the presence of these factors within the complexes. Antibodies assays of different cells of hematopoietic origin. DNA fragments were prepared and immunoprecipitated (IP) with the indicated antibodies as described in Materials and Methods. Upon purification, the region containing the SHP-1 P2 promoter SC or the SHP-1 Exon 5 (a negative control) was amplified by PCR and separated by electrophoresis. DNA input and nontemplate reaction served as positive and negative controls of PCR, respectively. pol II, Polymerase II.

Fig. 2. Role of NF-κB, Oct-1, and Sp1 in SHP-1 P2 promoter activity regulation. (A and B) Binding of Oct-1, NF-κB, and Sp1 to the SHP-1 P2 promoter as determined by EMSA. (A) The 32P-labeled SHP-1 P2 SC probe was incubated with 1 μg Jurkat nuclear extract (NE). One hundred-fold molar excess of cold SHP-1 SC DNA was used to confirm the specificity. Oct-1, NF-κB, and Sp1 antibodies were used for supershift assay. IRF-1 antibody was used as an irrelevant control. (B) 32P-Labeled, 20 bp, double-stranded oligonucleotides encoding putative Sp1-binding sites of SHP-1 P2 were incubated with 8 μg Jurkat + PMA nuclear extract. One hundred-fold molar excess of unlabeled oligonucleotide was used in a competition assay to confirm the binding specificity. Sp1 antibody was used in indicated supershift analyses, and IRF-2 antibody was used as an irrelevant antibody control. Wt, Wild-type-binding site sequence; Mt, mutant probes. (C) Evaluation of the transcription factors binding to the SHP-1 P2 core promoter in vivo by ChIP assays of different cells of hematopoietic origin. DNA fragments were prepared and immunoprecipitated (IP) with the indicated antibodies as described in Materials and Methods. Upon purification, the region containing the SHP-1 P2 promoter SC or the SHP-1 Exon 5 (a negative control) was amplified by PCR and separated by electrophoresis. DNA input and nontemplate reaction served as positive and negative controls of PCR, respectively. pol II, Polymerase II.
against Elk-1 and Ik-1 failed to show any effects (data not shown).

Using a similar protocol as used previously with NF-κB binding analysis [7], 20 bp oligonucleotide probes corresponding to the two predicted Oct-1- and one Sp1-binding sites and their corresponding mutant sequences (Table 1) were synthesized and used to confirm binding of these transcription factors more precisely. As shown in Figure 2B, when incubated with Jurkat PMA + nuclear extract, the Sp1 probe formed a protein-DNA complex that could be competed away specifically by cold Sp1 oligonucleotides but not cold AP2 control oligonucleotide. While addition of the Sp1 antibody completely disrupted this protein-DNA complex, the irrelevant IRF-2 antibody showed no effect. Site-specific mutation at the Sp1 site not only eliminated Sp1 binding to the mutant extract, the Sp1 probe formed a protein-DNA complex that could be competed away specifically by cold Sp1 oligonucleotides but also could not compete with the Sp1-specific complex formed with the 32P-labeled wild-type Sp1 probe. These results suggest that Sp1 indeed binds to the predicted Sp1 site within the SHP-1 P2 promoter FP3 region. Although no binding was observed with a probe containing Oct-1 BS1 (within FP1) in the presence of Jurkat nuclear extract, EMSA and antibody supershift experiments did reveal putative Oct-1-containing protein-DNA complexes with the Oct-1 BS2 oligo probe within FP2 (data not shown).

Factors binding to the SHP-1 P2 promoter SC in vivo

To confirm transcription factor binding to the SHP-1 P2 promoter in vivo, ChIP assay was performed on chromatin isolated from fresh CD4+ T cells of a healthy donor and the Jurkat cell line (Fig. 2C, top two panels). As can be seen, Oct-1, Sp1, NF-κB, and CREB-1 bind to the SHP-1 P2 SC in CD4+ T cells and Jurkat cells. NF-κB p50 was the predominant NF-κB component binding to the promoter in CD4+ T cells, and NF-κB p65 was predominant in Jurkat cells. Interestingly, PU.1 bound to SHP-1 P2 SC only in Jurkat cells but not in CD4+ T cells. The fact that RNA polymerase II binding is observed indicates that the SHP-1 P2 promoter is actively transcribed in CD4+ T cells and Jurkat cells. Contrary to fresh CD4+ cells and Jurkat cells, binding of NF-κB, Sp1, Oct-1, CREB-1, PU.1, and RNA polymerase II to the SHP-1 P2 promoter in MT-2 and HUT102 cells (both are HTLV-1-transformed and express Tax protein) was barely detectable, while HTLV-1 oncprotein Tax protein was clearly present (Fig. 2C, bottom two panels). These results indicate that in addition to NF-κB [7] and PU.1 [8], Sp1, Oct-1, and CREB-1 are associated with the SHP-1 P2 promoter, and such association appears to be mutually exclusive with the binding of Tax to the promoter.

An alternative explanation for the above ChIP assay results is that the expression of the transcription factors tested was too low in HTLV-1-transformed cells to be detected by the ChIP assay; however, the observations of NF-κB activation in ATL [32], lymphoma, myeloid and lymphoid leukemia cells [33] made it unlikely. To explore further, the activity of NF-κB in HTLV-1-transformed and -noninfected cell lines was tested by EMSA using the 32P-labeled SHP-1 NF-κB second probe and Sp1 probe (Fig. 3, A and B). Quantification by Phospholmager analysis indicated that with equal amounts of proteins in each nuclear extract, HTLV-1-transformed MT-2 and HUT102 cells actually had more binding of NF-κB or Sp1 to the probe than that from HTLV-1-noninfected Jurkat cells, suggesting that mechanisms other than a limited quantity of NF-κB and Sp1, such as binding of Tax and subsequent modification of the SHP-1 P2 promoter, could be a major cause of decreased transcription factor binding and silencing of the SHP-1 P2 promoter in HTLV-1-transformed and ATL cell lines.

Role of Oct-1, NF-κB, and Sp1 in SHP-1 promoter activity

NF-κB [7] and PU.1 [8] were demonstrated previously to regulate basal P2 promoter activity of SHP-1. To determine if Sp1 and Oct-1 sites within the SHP-1 P2 promoter are transcriptionally functional, we performed a luciferase reporter assay with SHP-1 P2 LC, with or without mutations at Oct-1- and Sp1-binding sites. Mutation at Sp1 and at two sites each for Oct-1 and NF-κB (specific mutation listed in Table 1)
reduced luciferase reporter activity to 20%, 40%, and 50% of the wild-type LC promoter, respectively (Fig. 4A). These results demonstrate that like NF-κB and PU.1, Sp1 and Oct-1 are important for P2 basal promoter activity. Although no binding of Oct-1 to its BS1 oligo probe was observed in the EMSA studies, results of the reporter assay suggest that Oct-1 BS1 and -2 sites are required for basal P2 promoter activity. The involvement of Sp1 and Oct-1 in TIPS was also interrogated. Data in Figure 4A show that although Tax lost its ability to inhibit the NF-κB double mutant, the Sp1 and Oct-1 mutants were still repressed by Tax, which is consistent with our previous observation that NF-κB is an important target for TIPS [7].

DNA methylation of the SHP-1P2 promoter LC

DNA methylation of the SHP-1 P2 promoter core region has been reported in T cell lymphoma cells [9, 27, 28]. In addition to TIPS, DNA methylation might be responsible for the low level of SHP-1 mRNA expression observed in MT-2, MT-4, HUT102, and HUT78 cells [21]. Bisulfite sequencing was used to determine the DNA methylation state of the SHP-1 P2 LC. The methylation status of 13 CpG residues was analyzed, and 11 were located within the LC (Fig. 5A). With 10 clones per cell line tested (Fig. 5B), only one CpG methylation (0.8%) was detected in CD4+ T cells freshly isolated from healthy donor blood and MOLT-4 (acute lymphoblastic leukemia). SupT1 (T cell lymphoblastic lymphoma) also showed a low percentage (3.1%) of methylation, while Jurkat (acute lymphoblastic leukemia) and HUT78 (CTCL cell line) exhibited a higher percentage of CpG methylation, at 31.5% and 79.2%, respectively. In contrast, HTLV-1-transformed cell lines in general showed a much higher degree of methylation: MT-4 (adult T cell leukemia) cells at 65.4%, MT-2 (T cell lymphoblast) at 90%, B1 (acutely HTLV-1-transformed CD4+ T cell) at 90.8%, and HUT102 (adult T cell lymphoma) at 96.2%. It is also interesting to note that 88.5% of CpG within the SHP-1 P2 promoter core region in 293T human kidney epithelial cells were methylated.

Inverse correlation between SHP-1 P2 LC DNA methylation and SHP-1 mRNA/protein expression

To evaluate the effect of DNA methylation on SHP-1 expression, we compared levels of CpG methylation with
SHP-1 mRNA expression. Calculations based on the data presented in Figure 6A revealed that the Pearson product-moment correlation coefficient of the percentage of CpG methylation and level of SHP-1 mRNA is –0.71 (P<0.05), showing significant inverse correlation between SHP-1 P2 LC methylation and its mRNA expression (Fig. 6B).

Western blot analyses were performed to compare SHP-1 protein expression in HTLV-1-noninfected and -transformed cells. These studies showed that SHP-1 protein expressed in four out of five HTLV-1-noninfected cell lines but only one out of eight HTLV-1-transformed cell lines had detectable SHP-1 protein expression (Fig. 6C). By Fisher's exact test, HTLV-1-transformed cell lines had a significantly lower probability of SHP-1 expression compared with HTLV-1-noninfected cell lines (P=0.03). Combining this result with the data from bisulfite sequencing analysis, CpG methylation of the SHP-1 P2 LC region is observed frequently in HTLV-1-transformed cells but not fresh CD4+ T cells and is negatively correlated with SHP-1 mRNA and protein expression in hematopoietic cells.

DISCUSSION

In this study, the mechanisms of hematopoietic cell-specific SHP-1 P2 promoter regulation in normal and HTLV-1-transformed cells were investigated. In addition to previously demonstrated NF-kB [7] and PU.1 [8], Sp1 and Oct-1 were identified as transcription factors involved in regulating SHP-1 P2 basal promoter activity through DNase I footprinting, EMSA, ChIP assay, and mutational analyses. Furthermore, Sp1, Oct-1, NF-kB, CREB-1, and RNA polymerase II interacted with the core SHP-1 P2 promoter in CD4+ T cells and Jurkat cells but not in HTLV-1-transformed MT-2 and HUT102 cells when P2 promoter-bound HTLV-1 Tax was present. Heavy CpG methylation of the core P2 promoter was seen in HTLV-1-transformed cells but not fresh CD4+ T cells and was inversely associated with SHP-1 mRNA and protein expression.

The presence of RNA polymerase II on the SHP-1 P2 promoter in CD4+ T cells argues that the SC region indeed represents a transcriptionally active core promoter. Based on the results of site-specific mutants presented in Figure 4A, Sp1 appears to have an even more significant role in positive regulation of SHP-1 P2 basal activity than that of Oct-1 (PU.1) or NF-kB. ChIP assay results also indicate that CREB, an important transcription regulator in the CREB/activating transcription factor (ATF) pathway, is associated with the SHP-1 P2 SC in Jurkat and freshly isolated CD4+ T cells (Fig. 2C). As no direct binding of CREB to its predicted binding site located between +33 and +40 was observed (data not shown), involve-
ment of CREB in the SHP-1 P2 promoter complex may be through an indirect interaction. The role of CREB in regulating SHP-1 P2 promoter activity remains to be determined, although results from previous transient transfection analyses suggested limited, if any, involvement of CBP, p300, and CREB in activation of the SHP-1 P2 promoter basal activity and reversal of TIPS [7].

There are two PU.1-binding sites located within the SHP-1 P2 core promoter: CTTCTC overlapping with FP1 and GAGAAG in FP2, and each one is separately part of an Oct-1-binding site (Fig. 4B). Mutations in these sequences could affect the binding of Oct-1 and PU.1, which in turn, affects the SHP-1 promoter activity. This hypothesis is supported by the results presented in Figure 4A and the observations reported by Wlodarski et al. [8]. The ChIP assay data in Figure 2C clearly showed that PU.1 and Oct-1 were present on the SHP-1 P2 SC region in Jurkat cells, suggesting that both factors are components of a regulatory complex for transcription from the SHP-1 P2 promoter. However, PU.1 is not associated with SHP-1 P2 in CD4+ T cells in vivo (Fig. 2C), which is consistent with the previous report that expression of PU.1 decreases as T cells mature. PU.1 has only been reported to be expressed in a subset of Th2 cells [34]. Thus, the precise role of PU.1 in regulating the SHP-1 P2 promoter awaits further investigation.

As revealed by the ChIP assay (Fig. 2C), Sp1, Oct-1, NF-κB, CREB-1, and RNA polymerase II were absent from the SHP-1 P2 in HTLV-1-transformed MT-2 and HUT102 cells, where little expression of SHP-1 was detected (Fig. 6C), indicating that the P2 promoter became inactive and possibly transitioned to a heterochromatin state. Interestingly, HTLV-1 oncoprotein Tax was associated with the P2 promoter in both cases. We had reported previously that Tax could induce SHP-1 P2 promoter silencing (TIPS) through recruitment of an inhibitory complex (IC) that includes histone deacetylase 1 (HDAC-1), and NF-κB is the primary target for TIPS [7]. Data presented in Figure 4A again confirm our previous observation that only NF-κB mutations are able to abolish TIPS. We hypothesize that competitive binding or modification by the Tax-associated IC components may lead to dissociation of NF-κB [7], Sp1, and CREB from the SHP-1 P2 promoter (Fig. 7A). As SHP-1 inactivation would be favorable for proliferation of malignant cells, it would be beneficial for HTLV-1 to repress the expression of SHP-1 early during infection to facilitate its transformation of T cells and their progression to adult T cell leukemia.

DNA methylation could also cause dissociation of transcription factors from the SHP-1 P2 core region and transformation from euchromatin to heterochromatin, thus completing the progress of SHP-1 repression. Bisulfite sequencing revealed that the SHP-1 P2 promoter LC is heavily methylated, especially in HTLV-1-transformed cell lines (Fig. 5B). This observation is in accordance with the report by Oka et al. [11], where analyses of sequences 150–300 bp upstream of the P2 LC showed strong methylation in 65% of ATL samples as com-
pared with 25% in the case of the acute lymphoblastic leukemia cells. Regarding the SHP-1 P2 core region in HTLV-1-transformed cells, six CpG methylations located between −15 and +145 were observed in Figure 5, in addition to those reported by Zhang et al. [27] for CTCL cells and tissue. It is interesting to note that the human kidney epithelial 293T cells showed 88.5% of CpG methylation within the SHP-1 P2 region. This is the first report that the core region of the SHP-1 P2 promoter is methylated in nonhematopoietic cells and suggests that DNA methylation may contribute to the tissue-specific regulation of this promoter as well as SHP-1 silencing in other HTLV-negative, malignant T cell lines such as HUT-78.

Among the methylated CpGs, five are located directly within or near the transcription factor Oct-1, Sp1, and NF-κB-binding sites. Significant inverse correlation was observed between SHP-1 P2 LC methylation and SHP-1 mRNA expression (Fig. 6, A and B), as well as SHP-1 protein expression (Fig. 6C). This supports the notion that SHP-1 P2 LC is a pivotal region for transcription, as methylation of only a core region covering the transcription start site is associated consistently with gene silencing [35]. Interestingly, acutely HTLV-1-transformed B1 cells showed that 90.8% of CpG in the SHP-1 P2 promoter LC region is methylated. This observation suggests that HTLV-1 infection is an important step for the SHP-1 P2 promoter methylation and silencing. As shown in Figure 4A, Tax can repress SHP-1 P2 promoter activity as early as 60 h after transient transfection. On the other hand, DNA methylation of the SHP-1 P2 promoter was not yet observed by Day 7 after HTLV-1 proviral clone transfection to CD4+ T cells (data not shown). We thus believe that selective CpG methylation could be a sequential event following association of HTLV-1 Tax and HDAC-1 with the SHP-1 P2 promoter. This hypothesis is supported by the fact that HDAC1 is known to interact with DNA methyltransferases DNMT1, DNMT3a, and DNMT3b; [36], and HDAC-1 [7, 27] and DNMT1 [27] are associated with the SHP-1 P2 core promoter. Taken together with our previously published data [7], we propose an extended model for SHP-1 P2 promoter silencing in HTLV-1-infected cells that includes an initial Tax-mediated IC binding, followed by modification of the bound transcription factors/histones and DNA methylation (Fig. 7A), which leads to a transition from euchromatin to heterochromatin during the process of cell transformation (Fig. 7B). It should be noted that although Tax is known to be critical for cellular transformation in HTLV-1-infected cells, clinical samples from ATL patients often lack Tax expression. It is possible that the presence of Tax protein on the SHP-1 P2 promoter might not be necessary after initial silencing followed by completion of SHP-1 P2 promoter DNA methylation and cellular transformation. Careful time-course studies of the epigenetic changes on the SHP-1 P2 promoter during a longitudinal period after HTLV-1 infection of CD4+ T cells are required to confirm the proposed model.

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