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USF/c-Myc Enhances, While Yin-Yang 1 Suppresses, the Promoter Activity of CXCR4, a Coreceptor for HIV-1 Entry

Masako Moriuchi,1* Hiroyuki Moriuchi,1,2* David M. Margolis,† and Anthony S. Fauci*†

Transcription factors USF1 and USF2 up-regulate gene expression (i.e., HIV-1 long terminal repeats) via interaction with an E box on their target promoters, which is also a binding site for c-Myc. The c-Myc oncoprotein is important in control of cellular proliferation and differentiation, while Yin-Yang 1 (YY1) has been shown to control the expression of a number of cellular and viral genes. These two proteins physically interact with each other and mutually inhibit their respective biological functions. In this study, we show that USF/c-Myc up-regulates, while YY1 down-regulates the promoter activity of CXCR4, a coreceptor for T cell-tropic HIV-1 entry. We have identified an E box around −260 and a YY1 binding site around −300 relative to the transcription start site. Mutation of the E box abolished USF/c-Myc-mediated up-regulation of CXCR4 promoter activity, and mutation of the YY1 binding site was associated with unresponsiveness to YY1-mediated inhibition. These data suggest that USF/c-Myc and YY1 may play an important role in the HIV-1-replicative cycle, by modulating both the viral fusion/entry process and viral expression. The Journal of Immunology, 1999, 162: 5986–5992.

A number of cellular factors of the host have been shown to modulate replication of HIV-1 at various steps of the viral replication cycle (1). At the level of viral expression, numerous cellular transcription factors regulate transcription from the long terminal repeat (LTR),3 the promoter of HIV-1.

While many of these factors (i.e., nuclear factor-xB/Rel family proteins (2), C/EBP (NF-IL6) (3, 4), Sp1 (5), or USF (6)) enhance LTR activity (reviewed in Ref. 7), a few factors have been shown to directly reduce HIV expression at the promoter level. YY1, a multifunctional transcription factor, has been shown to repress a number of cellular and viral gene promoters (reviewed in Ref. 8), including the HIV-1 LTR (9, 10).

Host cell factors also affect HIV at earlier steps in its replication cycle. HIV-1 entry into cells requires the CD4 molecule as well as a fusion/entry cofactor (11–18). Several chemokine receptors serve as fusion/entry cofactors, among which CXCR4, a receptor for CXC chemokine stromal-derived factor (SDF-1) (19, 20), is a major fusion/entry cofactor for T cell-tropic HIV-1. Functional expression of CXCR4 is influenced by other host cell factors. SDF-1, a ligand of CXCR4, regulates cell surface expression of CXCR4 (21, 22), whereas IL-2 regulates the steady-state level of CXCR4 mRNA (23). We have recently cloned and analyzed the promoter region of CXCR4 (24), and found that two transcription factors, nuclear respiratory factor-1 (NRF-1) and Sp1, interact with the proximal region of the CXCR4 promoter, and that the former is critical for CXCR4 promoter activity. CXCR4 expression on monocytes has also been shown to be down-regulated upon differentiation of these cells into mature macrophages (25).

In this study, we characterize the further upstream region of the CXCR4 promoter. We report that USF/c-Myc and YY1 bind to the upstream region of the CXCR4 promoter, and positively or negatively regulate the CXCR4 promoter, respectively. Involvement of USF and YY1 at two critical steps of HIV-1 replication, viral fusion/entry and expression, suggests that these transcription factors may play a role in the pathogenesis of HIV disease.

Materials and Methods

Plasmids

Plasmid pGL-CXCR4(−357) contains the CXCR4 upstream sequence between −357 and +51 relative to the transcription start site (TSS), followed by the luciferase gene (24). Plasmids pGL-CXCR4ΔE box and pGL-CXCR4YY1 were constructed by PCR-based site-directed mutagenesis (26) to generate mutations on an E box around −260 and on a YY1 binding site around −300 relative to the TSS, respectively (see Table I and Fig. 1). Plasmid pBpuro-c-myc-ER (a generous gift of T. D. Littlewood, Imperial Cancer Research Fund, London, U.K.) encodes human c-Myc fused to the hormone-binding domain of the mutant murine estrogen receptor. Functional expression of a c-Myc fusion protein by the transfected plasmid is induced by 4-hydroxytamoxifen (10 nM; Sigma, St. Louis, MO) (27). Plasmids pCMV-Myc and pCMV-Max were gifts of D. Ayer (University of Utah, Salt Lake City, UT) (28) and used for in vitro synthesis of c-Myc and Max proteins. USF expression plasmids pSV-USF1 and pSV-USF2 were kindly provided by M. Sawadogo (University of Texas M. D. Anderson Cancer Center, Houston, TX) (29), and a YY1 expression plasmid pCMV-YY1 was a generous gift of T. Shenk (Princeton University, Princeton, NJ) (30).

Cells

PBMC were obtained from healthy volunteers, as described previously (31). A3.01 human CD4+ T cells were propagated as described previously (24, 32).

Transfection, and luciferase and β-galactosidase assays

Transient expression and luciferase and β-galactosidase assays were performed as described previously (32, 33).

DNase I footprinting

DNase I footprinting was performed as described previously (34). Probes used for DNase I footprinting were prepared as follows: a MluI-SphI fragment and a MluI-SphI fragment of pGL-CXCR4(−357) were labeled with [32P]dCTP using Klenow enzyme and gel purified. These fragments span the CXCR4 promoter sequence between −357 and −277, and between −283 and −46, respectively, relative to the TSS. Furthermore, the CXCR4
Table 1. Oligonucleotides used for gel mobility shift assays and site-directed mutagenesis

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>L-E box</td>
<td>5'-TGCTAGCTCCACACTGCTTCAGGCAGGCATC-3'</td>
</tr>
<tr>
<td>L-E box-m</td>
<td>5'-TGCTAGCTCCACACTGCTTCAGGCAGGCAAGAC-3'</td>
</tr>
<tr>
<td>E box</td>
<td>5'-GAACGGCATCACCTGCTGGACTCCCTGTCGTT-3'</td>
</tr>
<tr>
<td>L-YY1</td>
<td>5'-TACGGCAATGGAGACCCAGCAAGGAC-3'</td>
</tr>
<tr>
<td>L-YY1-m1</td>
<td>5'-TAGCAAGGATCCACGCGCCAAGAGAC-3'</td>
</tr>
<tr>
<td>L-YY1-m2</td>
<td>5'-TAGCAAGGATCCACGCGCCAAGAGAC-3'</td>
</tr>
<tr>
<td>YY1</td>
<td>5'-CAGCCTGGCGGCAATCTGGGCGGCGTG-3'</td>
</tr>
<tr>
<td>GATA</td>
<td>5'-CATCCTGATTACAAAGAAGTACCTCAGC-3'</td>
</tr>
<tr>
<td>STAT</td>
<td>5'-GTATTCCTACGAAAAGGAC-3'</td>
</tr>
</tbody>
</table>

* Sequences corresponding to cis-acting elements are underlined. Mutated nucleotides are shown in bold letters. Oligonucleotides E box, YY1, GATA, and STAT were purchased from Santa Cruz Biotechnology.

Results

Identification of putative cis-acting elements in the upstream region of the CXCR4 promoter

First, we assessed the binding of cellular transcription factors to the CXCR4 promoter region between −350 and −150 relative to the TSS in DNase I footprinting analysis. Among areas protected from DNase I digestion (Figs. 1 and 2), regions around −260 (FP-5) and −300 (FP-6) were found to share homology to an E box (a binding site for basic-helix-loop-helix-leucine zipper [bHLHZip] family proteins (35)) and a YY1 binding site (8), respectively.

bHLHZip proteins c-Myc, USF-1, and USF-2 bind to the CXCR4 promoter

To demonstrate that the putative E box in the CXCR4 promoter region actually serves as a binding site for bHLHZip family of transcription factors, gel-mobility shift assays were performed using in vitro synthesized proteins. Either c-Myc/Max, USF-1, or USF-2 protein formed a complex with an L-E box oligonucleotide corresponding to the FP-5 region (Fig. 3A); however, c-Myc/Max complex was detectable only after longer exposure (data not shown). c-Myc protein alone could not form a complex with the L-E box oligonucleotide (data not shown), most likely reflecting the fact that Max protein is an obligate heterodimeric partner for c-Myc (35). When A3.01 CD4+ T cell nuclear extracts were used instead of purified proteins, a DNA-protein complex, which was specifically competed by unlabeled probe as well as by an oligonucleotide containing the consensus E box, was formed (Fig. 3B). Supershift assays using specific Abs to c-Myc, USF1, or USF2 indicated that the major complex contained both USF1 and USF2, but not c-Myc (Fig. 3C). These results indicate that bHLHZip proteins c-Myc, USF1, and USF2 bind to the CXCR4 promoter E box; however, USF1 and USF2 have higher affinity for the E box than does c-Myc.
bHLHZip proteins c-Myc, USF-1, and USF-2 up-regulate CXCR4 promoter activity

To investigate whether bHLHZip proteins c-Myc, USF1, and USF2, which can bind to the CXCR4 promoter, are capable of regulating expression from the CXCR4 promoter, expression vectors for these proteins were cotransfected with CXCR4 promoter-luciferase reporter constructs. Transfection of c-Myc up-regulated expression from the CXCR4 promoter; this effect was dependent upon the presence of an intact E box (Fig. 4A). Cotransfection with the Max expression vector did not further enhance c-Myc-mediated transactivation of the CXCR4 promoter (data not shown), probably because Max is constitutively and abundantly expressed in most cell types (35, 36). USF1, and to a slightly lesser extent, USF2, also increased expression of the CXCR4 promoter reporter gene. Mutation of the E box ablated this effect (Fig. 4B).

YY1 binds to the CXCR4 promoter

To determine whether YY1 actually binds to the region, gel-mobility shift assays were performed using bacterially synthesized YY1 protein. Purified YY1 protein formed a complex with an oligonucleotide corresponding to the FP-6 region (Fig. 5A). A3.01 CD4^+ T cell nuclear extracts were found to contain two complexes binding to the FP-6 region, which were specifically competed by unlabeled probe; a fast migrating complex, but not a slowly migrating complex, was also specifically competed by an oligonucleotide containing the consensus YY1 binding motif (Fig. 5B; Table I). Gel-shift interference assays using anti-YY1 Ab confirmed that the fast migrating complex contains YY1 (Fig. 5C). Thus, these results clearly indicate that YY1 binds to the CXCR4 promoter. A slowly migrating complex was specifically competed by an oligonucleotide containing the consensus GATA binding motif (Fig. 5B); however, Ab to GATA1, GATA2, or GATA3 did not affect the mobility or formation of the complex (data not shown). In addition, the FP-6 region has only limited homology to...
GATA binding motif. Identification of this complex is currently in progress.

**YY1 suppresses CXCR4 promoter activity**

In the context of other promoters, YY1 has been shown to activate transcription, repress transcription, or initiate transcription (reviewed by Shrivastava and Calame, Ref. 8). To investigate how YY1 regulates activity of the CXCR4 promoter, we transfected A3.01 CD4⁺ T cells with a CXCR4 promoter/luciferase reporter construct along with a YY1 expression vector. The cells were either unstimulated (control) or treated (Myc) with 10 nM of 4-hydroxytamoxifen (Sigma) 24 h after transfection. 4-Hydroxytamoxifen at this concentration by itself had no effect on CXCR4 promoter activity (data not shown). Cells were harvested 48 h after transfection for luciferase assays. Fold induction indicates luciferase activity relative to basal promoter activity. Results were representative of four independent experiments. B, USF1 and USF2 up-regulate CXCR4 promoter activity. PBMC were transfected with 20 μg of either pGL-CXCR4(−357) or pGL-CXCR4ΔE box along with 20 μg of either pSV2-CAT, pSV-USF1, or pSV-USF2. Results were representative of four independent experiments.
activity 2 days later. Overexpression of YY1 suppressed the CXCR4 promoter in A3.01 cells up to 80% in a dose-dependent manner (Fig. 6A).

To determine whether the YY1 binding site on the CXCR4 promoter sequence involves YY1-mediated repression of the CXCR4 promoter, site-directed mutations were introduced on the YY1 binding site, and its activity was compared with that of wild-type promoter. An oligonucleotide with mutations on the YY1 binding site (L−YY1-m1 in Table I) lost its ability to disrupt the YY1 complex formation in gel-mobility shift assays (Fig. 5B). Introduction of mutations into the CXCR4 promoter/luciferase reporter construct resulted in approximately a 2-fold increase in the basal activity (Fig. 6B), and its activity was not markedly influenced by cotransfection with the YY1 expression vector (Fig. 6A). These results indicate that YY1 inhibits the expression of the CXCR4 promoter through its binding to a site around −300 relative to the TSS.

c-Myc and YY1 mutually inhibit the transregulatory functions on the CXCR4 promoter

YY1 has been shown to physically associate with c-Myc, and the interaction between the two proteins appears to result in mutual inhibition of their transregulatory functions (37). To investigate whether these two transcription factors mutually affect their functions related to CXCR4 promoter activity, expression vectors for YY1 and c-Myc were transfected into A3.01 cells individually or in combination along with pGL-CXCR4(−357). As shown above, overexpression of YY1 repressed, while c-Myc transactivated, the CXCR4 promoter activity; however, the net effect of the cotransfection of both expression vectors on the promoter was negligible (Fig. 7), implying their mutually inhibitory interaction.

Discussion

In this study, we have demonstrated that cellular YY1 transcription factor down-regulates CXCR4 expression through its interaction with the CXCR4 promoter. Previous studies demonstrated that T cell activation with IL-2 or anti-CD3 Ab increased CXCR4 expression (24, 36), and a cellular transcription factor, NRF-1, is critical for CXCR4 promoter activity (24). YY1 is the first cellular factor to be identified as a repressor of CXCR4 expression.

YY1 is widely expressed and highly conserved among mammalian species, and thus can be categorized as a ubiquitous transcription factor (8, 30). YY1 can directly interact with a wide array of cellular and viral proteins and regulates a number of cellular and viral promoters, depending on the gene as well as the cell type in question (reviewed in Ref. 8). For example, YY1 has been shown to associate with the c-Myc protein, and this association may lead to mutual inhibition of the transcription functions of both proteins (37). The c-Myc protein is a potent regulator of cell growth and differentiation, and c-Myc expression is down-regulated upon differentiation of cells of the monocyte/macrophage lineage into more mature phenotypes (38). Considering the relatively weak affinity of c-Myc protein to the CXCR4 promoter E box, we hypothesized that this oncoprotein may activate CXCR4 promoter primarily through the relief of repression mediated by YY1, as has been reported in other promoter contexts (30). Our hypothesis was supported by the results shown in Fig. 7. In contrast to c-Myc, other bHLHZip transcription factors, USF1 and USF2, which have higher affinity for the CXCR4 promoter E box than does c-Myc and are not known to interact with YY1, appear to act through their direct binding to the E box.

Other cellular proteins associated with YY1 include cyclophilin A (CyPA) and FK506-binding protein 12 (39). These two proteins specifically interact with YY1 and alter its transcriptional activity,
pression of HIV fusion/entry cofactors is regulated. Further characteriza-
tion of the promoter regions for these cofactors will help delineate the molecular mechanisms that control their expression.

Acknowledgments

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