Human immunodeficiency virus type 1 Tat prevents dephosphorylation of Sp1 by TCF-4 in astrocytes

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INTRODUCTION

Sp1 belongs to a zinc-finger family of transcription factors and was first identified based on its ability to interact with the GC-rich motif of simian virus 40 regulatory sequences (Dynan & Tjian, 1983; Jackson & Tjian, 1988). This protein plays a critical role in many cellular events by regulating expression of several other genes, including early embryonic development and the maintenance of terminal cell differentiation (Hasleton et al., 2003). The structure of Sp1 is composed of four domains, A–D, and a zinc-finger region, which is involved in DNA–protein interactions (Pascal & Tjian, 1991). Domains A and B are the two major glutamine-rich regions essential for transcriptional activation. Domain C has been shown to be involved in synergistic activation with steroid regulatory element-binding proteins (Athanikar et al., 1997). Domain D, by interacting with other activation domains, creates a configuration that maximizes their activity (Courry & Tjian, 1988). The DNA-binding and transcriptional activities of Sp1 are regulated by the state of phosphorylation that follows changes in the cell cycle (Dovat et al., 2002). Sp1 protein is stabilized by O-linked glycosylation, which confers resistance to proteasome-dependent degradation (Yang et al., 2001). Sp1 regulates gene transcription by cooperating with various signalling pathways (Milanini-Mongiat et al., 2002).

Sp1 associates physically and cooperates functionally with several cellular transcriptional activators including TFIID, YY1, GATA, NF-κB, Purz, E2F1, pRB and SREBP-1 (Seto et al., 1993; Perkins et al., 1994; Gregory et al., 1996; Karleseder et al., 1996; Naar et al., 1998; Chang et al., 2001; Hilton & Wang, 2003). Sp1 may also associate with several viral regulatory proteins including the human immunodeficiency virus type 1 (HIV-1) regulatory protein, Tat (Loregian et al., 2003). HIV-1 Tat stimulates viral gene transcription from the proviral long terminal repeat (LTR) by interacting with several cellular factors such as Sp1, TCF-4 and the Tat-associated kinase, cdk9/P-TEFb (Jeang et al., 1993; Wortman et al., 2002; Bres et al., 2005). cdk9/p-TEFb is recruited by Tat to the trans-activation response (TAR) element, located at the 5’ termini of all viral transcripts (Brady & Kashanchi, 2005). This complex stimulates transcriptional elongation by phosphorylating the RNA polymerase II C-terminal domain (Wei et al., 1998; Peruzzi et al, 2005). Tat has also been shown to induce the phosphorylation of Sp1 through dsDNA-dependent protein kinase (DNA-PK) (Chun et al., 1998).
TCF-4 and the other human T-cell factor proteins (TCF-1, -2 and -3) are sequence-specific HMG box transcription factors that function as the downstream effectors of Wnt/β-catenin signals (Giese et al., 1992; Korinek et al., 1997; Morin et al., 1997; Hurlstone & Clevers, 2002). In the absence of nuclear β-catenin, the TCF proteins strongly repress transcription through binding to the Groucho family of transcriptional co-repressors (Cavallo et al., 1998; Brantjes et al., 2001). Binding is mediated by a conserved N-terminal glutamine-rich region in the Groucho/TLE proteins and a region between the N-terminal β-catenin-binding site and HMG box in the TCF proteins. Stabilization of β-catenin by Wnt or other signals results in its nuclear accumulation and TCF binding, mediated by sites in the TCF N terminus and β-catenin Armadillo repeats (Rubinfeld et al., 1996; Graham et al., 2000; Poy et al., 2001). β-Catenin then serves to stimulate TCF transcriptional activity by recruiting multiple co-activator proteins such as CBP/p300, Brg1 and CARM1 (van de Wetering et al., 1997; Hecht et al., 2000; Miyagishi et al., 2000; Barker et al., 2001).

Previously, we examined the effect of TCF-4 on transcription of the HIV-1 promoter in human astrocytic cells (Wortman et al., 2002). We demonstrated that expression of TCF-4 decreased basal transcription of the HIV-1 LTR. We also showed that TCF-4 exerted its effect through the sequence spanning positions −80 to −68 of the LTR. This sequence encompasses the GC-rich domain, which is the binding site of Sp1. In this study, we focused on the transcriptional activity of TCF-4 and assessed the ability of TCF-4 to cooperate with Sp1 in regulating transcription of the HIV-1 promoter through the GC-rich motif. We demonstrated that the interaction of TCF-4 and Sp1 prevented Sp1–DNA association and decreased the transcriptional abilities of Sp1. However, the addition of Tat alleviated the effect of TCF-4 on Sp1. Taken together, these observations provide evidence for the cooperative interaction of TCF-4, the important transcription factor of the Wnt pathway, with Sp1; this interaction may determine the level of viral gene transcription in human astrocytic cells.

METHODS

Plasmids. The HIV-1 LTR–luciferase (luc) full-length reporter plasmid and its deletion mutants have been described previously (Amini et al., 2004; Sweet et al., 2005). The cytomegalovirus (CMV) promoter-driven CMV–Sp1 expression plasmid was kindly provided by R. Tjian (University of California at Berkeley, CA, USA). CMV–Sp1 deletion mutants and the 6 × Sp1–CAT reporter construct have been described previously (Amini et al., 2004). CMV–TCF-4 and GST–TCF-4 expression plasmids have been described previously (Wortman et al., 2002). The p21VAF luc reporter plasmid was obtained from B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA) (Waldman et al., 1995).

Cell culture, transfection, luciferase and CAT assays. Human glioblastoma (U-87 MG) and HeLa-derived (HL3T1) cells were maintained in DMEM containing 10% fetal calf serum and antibiotics. HL3T1 cells contain stably integrated, silent copies of the HIV-1 LTR promoter linked to the CAT gene. Cells were transfected with 0.5 μg of the reporter plasmids (full-length LTR or the deletion mutants) in the presence or absence of 2.5 μg of various expression cDNAs as described previously. The amount of DNA used for transfection was normalized with pcDNA3 plasmid. Each transfection was repeated multiple times with different plasmid preparations. Cell extracts were prepared 48 h after transfection and luciferase assays were performed as described previously (Amini et al., 2005).

Overexpression and purification of recombinant proteins. GST–TCF-4 fusion proteins were prokaryotically expressed and purified as described previously (Abraham et al., 2005). The integrity and purity of the GST fusion proteins were analysed by SDS-PAGE followed by Coomassie blue staining. Known amounts of BSA were included as controls on the same gel. Radiolabelled Sp1 deletion-mutant proteins were synthesized with the TNT-coupled wheatgerm extract system according to the manufacturer’s recommendations (Promega).

In vitro protein–protein interactions (GST pull-down assay). For in vitro binding assays, 4 μl 35S-labelled and translated Sp1 deletion mutants were incubated with 50-μg GST or GST–TCF-4 fusion protein coupled to glutathione–Sepharose beads in 300 μl lysis buffer (LB 150) for 2 h at 4 °C with continuous rocking (Amini et al., 2002). After incubation, the beads were pelleted and washed five times with LB 150. Bound proteins were eluted with Laemmli sample buffer, heated to 95 °C for 5 min and separated by SDS-PAGE.

Gel electrophoretic mobility-shift assay. A gel electrophoretic mobility-shift assay was performed as described previously (Amini et al., 2004). Oligonucleotides corresponding to the HIV-1 LTR promoter region were synthesized, annealed, labelled with [32P]ATP and incubated at 4 °C for 30 min with 10 μg nuclear extracts. For supershift assays, antibodies directed against Sp1 (Santa Cruz Biotechnology) were mixed with nuclear proteins for 1 h at 4 °C prior to addition of the probe. The sequences of the oligonucleotides used in these experiments were 5′−AGGGAGGCGTGGCCTGGGCGGGA−CTGG−3′ (−78/−52) and 5′−CCAGTCCCCGCCAGCCACGCTT−CCCT−3′ (−52/−78).

Kinase assays. Kinase assays were performed essentially as described previously (Graña et al., 1994). Briefly, 50 or 43 ng pure Sp1 was incubated with 50 ng DNA-PK (purchased from Santa Cruz) and with 0.5-μg TCF-4 produced by in vitro translation (IVT–TCF-4) in 10 μl reaction mixtures containing 50 mM Tris/HCl (pH 7.5), 10 mM MgCl2, 1 mM DTT, 100 μg BSA ml−1, 50 mM ATP and 2.5 μCi [32P]ATP. Note that kinase assays were also performed in the presence of 0.5 μg GST or GST–Tat and 250 ng dsDNA when needed. Reactions were incubated at 37 °C for 30 min, stopped with SDS loading dye, separated by 10% SDS-PAGE, fixed, stained and then allowed to dry before autoradiography.

Immunoprecipitation and Western blotting. U-87 MG cells were transfected with 5 μg Sp1 expression plasmid. Forty-eight hours after transfection, 300 μg cell extract was immunoprecipitated with anti-TCF-4 antibody or rabbit serum. Western blot analysis was carried out as described previously (Amini et al., 2004, 2005) using anti-Sp1 antibody.

Chromatin immunoprecipitation (ChiP) assay. HL3T1 cells were grown overnight in 100 mm dishes to 60–70% confluency; cells were then transfected with 1 μg Tat and/or TCF-4 expression plasmids using FuGENE 6 transfection reagent (Roche Applied Sciences). Plates were returned to the incubator for 40–48 h. Cells were crosslinked with formaldehyde and harvested and ChiP was performed. Only 5 × 106 cells were used for each immunoprecipitation reaction as the plasmid is present at a high copy number. The remainder of the procedure followed standard protocols for ChiP analysis as described by Wells & Farnham (2002). The resulting DNA was
analysed by PCR using the following HIV-1 LTR primers, which amplified the region between −120 and +66: forward, 5′-AAGCGTGTACCATGACACGTGGCT-3′, and reverse, 5′-TTGAGGATCCAGCAGTGCGGTTC-3′. Anti-Sp1 antibody and rabbit anti-mouse IgG were used in the procedure.

RESULTS

Modulation of the HIV-1 LTR by Sp1 and TCF-4

Previously, we demonstrated the ability of TCF-4 to modulate HIV-1 LTR transcription through potential binding sites for the Sp1 transcription factor in the GC-rich region within the LTR (Wortman et al., 2002). To assess the effect of Sp1 on transcription of the HIV-1 LTR in the presence and absence of TCF-4, U-87 MG cells were transfected with a reporter plasmid containing the LTR upstream regulatory sequence fused to a reporter gene along with 0·5 μg plasmids expressing Sp1 and/or TCF-4. As shown in Fig. 1(a), Sp1, but not TCF-4, enhanced LTR activity by 18·89-fold. This activation was altered when Sp1 and TCF-4 plasmids were coexpressed (~6·33-fold). The HIV-1 LTR has been shown to contain three GC-rich motifs localized between positions −78 and −42 (Jones et al., 1986).

Next, we utilized a mutant construct in which two Sp1-binding sites were removed, HIV-1 LTR Δ(−78/−58) (Sawaya et al., 1998a). As shown in Fig. 1(b), although weak, a partial mutation of the Sp1 sites did not affect the ability of Sp1 to activate HIV-1 LTR Δ(−78/−58) (~5·79-fold). Once again, this activation was altered when plasmids Sp1 and TCF-4 were coexpressed (~1·41-fold).

To demonstrate functional cooperativity between Sp1 and TCF-4 further, we utilized a synthetic promoter reporter construct containing six classical Sp1 binding sites (6 × Sp1–CAT) fused to a minimal promoter sequence from HIV-1 (Chun & Jeang, 1996). As anticipated, in the presence of Sp1, the level of transcription from the synthetic promoter was increased (~7·71-fold; Fig. 1c). Combined production of Sp1 and TCF-4, however, caused a significant decrease in the transcriptional activation of the test promoter (~3·14-fold).

The inhibitory effect of TCF-4 was studied further using the p21WAF1 promoter. To this end, U-87 MG cells were transfected with reporter plasmid containing the p21 upstream regulatory sequence fused to the reporter gene along with 0·5 μg plasmids expressing Sp1 and/or TCF-4. As shown in Fig. 1(d), TCF-4 (~1·20-fold) was able to inhibit Sp1-mediated activation (~6·53-fold) of p21WAF1 promoter activity (~2·67-fold). Note that the DNA sequence spanning −93 to +1 revealed the presence of four characteristic GC-rich motifs, probable binding sites for the Sp1 transcription factor, positioned upstream from the TATA box at position −44 (Waldman et al., 1995).

These results led us to conclude that the functional interplay between TCF-4 and Sp1 is not promoter specific.

![Fig. 1. Transcriptional regulation of the HIV-1 promoter by TCF-4 in the presence of Sp1. U-87 MG cells were transfected with plasmids encoding full-length LTR–luc (a), an LTR deletion mutant (b), 6 × Sp1–CAT (c) or the p21WAF1–luc deletion mutant (d) either alone or in combination with plasmids expressing TCF-4 and/or Sp1. Luciferase or CAT activities were determined after 48 h and are presented as fold activation. The basal levels of transcription were set at 1·0. Data represent the mean value of at least three separate transfection experiments. The fold activation is given above each lane.](http://vir.sgmjournals.org)

TCF-4 prevents Sp1–DNA binding

Next, we examined whether TFC-4 affected Sp1 transcriptional ability by preventing binding to its DNA cognate motif. We performed band-shift assays using U-87 MG cells, which we transfected with 5 μg Sp1 and/or TCF-4 expression plasmids. Ten micrograms of nuclear extract prepared from U-87 MG cells was incubated with labelled GC-rich dsDNA primer spanning nucleotides −78 to −52. As shown
in Fig. 2(a), the intensity of the band corresponding to the DNA–Sp1 complex was slightly enhanced in U-87 MG cells transfected with a plasmid expressing Sp1 (Fig. 2a, compare complex C1 in lanes 2 and 3). To examine whether proteins of the Sp1 family formed complex C1, we performed supershift experiments (Fig. 2a, lanes 4–6). The presence of the Sp1 protein in complex C1 was demonstrated, as the addition of anti-Sp1 antibodies (Fig. 2a, lanes 4 and 5), but not the addition of a non-immune serum (Fig. 2a, lane 6), led to an up-shift of the complex and the formation of a new complex, C2. Anti-Sp1 antibodies were unable to abolish binding of the complex C1 to the DNA completely, which led us to conclude that, in addition to Sp1, other proteins are present in the C1 complex (Fig. 2a, lanes 4 and 5). Competition using unlabelled wild-type or mutant DNA probes verified the specificity of the complex (data not shown). The level of association of Sp1 with the DNA probe was abolished in cells expressing Sp1 and TCF-4, suggesting that TCF-4 may prevent binding of Sp1 to its cognate motif (Fig. 2a, compare complex C1 in lanes 3 and 8). As expected, TCF-4 was unable to bind to the DNA (lane 7).

To investigate further the ability of TCF-4 to interact functionally with Sp1 and to affect Sp1 binding to the HIV-1 LTR, we performed a ChIP assay. The HL3T1 cell line, which contains integrated HIV-1 LTR–CAT, was transfected with TCF-4 expression plasmid. Forty-eight hours after transfection, cells were harvested and subjected to a Western blot or a ChIP assay. As a positive control, anti-TCF-4 antibody was used to show the efficiency of transfection in these cells.

**Fig. 2.** Functional interplay between Sp1 and TCF-4. (a) Band-shift assays were performed using γ-32P-labelled 27 bp synthetic dsDNA fragments containing the wild-type GC-rich motif of the HIV-1 promoter (−78/−52). Labelled DNA probes (∼10^5 c.p.m.) were incubated with 10 μg nuclear extract prepared from cells transfected with Sp1 and/or TCF-4, as indicated above each lane. Supershift experiments were performed with 10 μg nuclear proteins in the presence of antibodies directed against Sp1 as indicated. C1 indicates complexes formed upon addition of nuclear extracts. C2 indicates the complexes supershifted by Sp1 antibodies. NS identifies the position of a non-specific complex. Free probe is visible at the bottom of the gel. (b) HL3T1 cells were transfected with 1 μg TCF-4 expression plasmid. Nuclear proteins (50 μg) were subjected to Western blot analysis using anti-TCF-4 or anti-Grb2 antibodies. (c) HL3T1 cells were transfected (lanes 5–7) or not (lanes 1–4) with 1 μg TCF-4 expression plasmid. Interaction of endogenous Sp1 with HIV-1 LTR DNA was demonstrated by ChIP assays. The primers used in these experiments are described in Methods. Anti-Sp1 (lanes 4 and 7), pre-immune serum (NMS; lanes 3 and 6) or no antibody (lanes 2 and 5) was used in these experiments. Protein markers were loaded in lane 1. (d) Fifty nanograms of pure Sp1 (lanes 1–4) or DNA-PK (lanes 3 and 4) was incubated with 5 μl (∼0.5 μg) IVT-TCF-4 (lanes 2 and 4) or with IVT buffer (lanes 1 and 3) and [γ-32P]ATP and subjected to a kinase assay. The position of phosphorylated Sp1 is shown.
Next, we sought to identify the mechanisms used by TCF-4 to prevent Sp1–DNA binding. It has been shown that activation of the HIV-1 LTR is influenced by phosphorylation of Sp1, which is affected by Tat and DNA-PK (Chun et al., 1998). Furthermore, in normal liver, phosphorylation of Sp1 results in a decrease in its DNA-binding affinity (Leggett et al., 1995). Finally, in a more recent study, Fojas de Borja et al. (2001) demonstrated that phosphorylation of Sp1 increases its transcriptional activities and DNA-binding abilities. To that end, we performed in vitro kinase assays (Fig. 2d) by mixing 50 ng purified Sp1 and/or DNA-PK with IVT buffer or 0.5 µg IVT-TCF-4 and [γ-32P]ATP. dsDNA oligonucleotides containing the Sp1-binding motif were also added to the mixture. It has been reported that phosphorylation of Sp1 is enhanced by binding to its cognate DNA (Chun et al., 1998). After incubation at 37°C for 30 min, anti-Sp1 was added to the reaction for 2 h, followed by the addition of protein A-Sepharose beads and incubation for another 1 h. The reaction was stopped with SDS loading dye, separated by 10% SDS-PAGE, fixed, stained and allowed to dry before autoradiography. As shown in Fig. 2d, Sp1 was strongly phosphorylated when incubated with IVT buffer in the presence compared with in the absence of DNA-PK (Fig. 2d, compare lanes 1 and 3). This phosphorylation was abolished when incubated with IVT-TCF-4 (Fig. 2d, lanes 2 and 4). Note that the IVT solution contained several other kinases, which may have contributed to the weak phosphorylation of Sp1 in the absence of DNA-PK (Fig. 2d, lane 1).

The inhibitory effect of TCF-4 was studied further in the presence of wortmannin. Wortmannin has been reported to inhibit DNA-PK function potently (Hartley et al., 1995). Wortmannin addition affected phosphorylation of Sp1 in the presence of exogenous TCF-4 but not in its absence (data not shown). Our results led us to conclude that the endogenous levels of TCF-4 were not enough to prevent the phosphorylation of both endogenous and exogenous Sp1. Addition of excess TCF-4 led to inhibition of Sp1 phosphorylation (both endogenous and exogenous).

**Physical interaction between Sp1 and TCF-4**

Results from the functional interaction of TCF-4 and Sp1 provided a rationale to investigate the physical association of these two proteins and to identify the regions within Sp1 that are important for their cooperativity. To that end, U-87 MG cells were transfected separately with 5 µg Sp1 or TCF-4 expression plasmid. Forty-eight hours after transfection, cells were washed and whole-cell proteins were extracted. Isolated proteins (300 µg) were used for co-immunoprecipitation using anti-TCF-4 (Fig. 3a, lanes 2 and 5) or anti-Sp1 (Fig. 3b, lane 4) antibodies. As a negative control, extracts were mixed with normal rabbit serum (Fig. 3a, lanes 3 and 6; Fig. 3b, lane 3). Bound proteins were eluted and subjected to Western blot analysis using anti-Sp1 or anti-TCF-4 antibodies. HeLa cell extracts (Santa Cruz) were used as a positive control (Fig. 3b, lane 1). Note that the two Sp1 isoforms were expressed in U-87 MG cells (Fig. 3a, lane 4), whereas the exogenously added Sp1 increased only the 105 kDa Sp1 (Fig. 3a, lane 1). The results shown in Fig. 3(a) demonstrated the interaction of TCF-4 with the two forms of Sp1 (96 and 105 kDa) whether expressed exogenously (Fig. 3a, lane 2) or endogenously (Fig. 3a, lane 4). Normal rabbit serum used as a negative control demonstrated the specificity of these interactions (Fig. 3a, lanes 3 and 6).

Reciprocally, cell extracts were co-immunoprecipitated with a polyclonal anti-Sp1 antibody (Fig. 3b, lane 4) or with rabbit serum (Fig. 3b, lane 3). Cellular extracts isolated from HeLa or U-87 MG cells transfected with TCF-4 were used as a positive control (Fig. 3b, lanes 1 and 2). Anti-TCF-4 antibody was used in Western blot analysis (Fig. 3b), which again confirmed the existence of an association between TCF-4 and Sp1.

Next, we mapped the TCF-4-binding domain within Sp1. To this end, a series of N- and C-terminal deletion mutants of Sp1 was created and, after production by a cell-free translation system, these mutants were used in GST pull-down assays using GST or GST–TCF-4. As shown in Fig. 3(c), the region encompassing aa 266–350 within Sp1 is the region involved in binding with TCF-4. This region has been shown to contain one of the serine-/threonine-rich (S/T) domains that are positioned at the N terminus of the protein. Fig. 3(d) illustrates representative results from the GST pull-down assays. These observations suggested that interaction of TCF-4 with Sp1 may prevent phosphorylation of Sp1.

**Functional interplay between Sp1 and TCF-4 in the presence of HIV-1 Tat**

Chun et al. (1998) demonstrated that Sp1 phosphorylation by DNA-PK influences the levels of activation of the HIV-1 LTR. These authors also showed that Tat modified the conformation of Sp1, which allowed Sp1–DNA-PK interaction (Chun et al., 1998). These observations led us to hypothesize that Tat might play a pivotal role in Sp1 phosphorylation. To investigate this hypothesis further, we examined whether functional interplay between TCF-4 and Sp1 could be affected in the presence of Tat. U-87 MG cells were transfected with the HIV-1 LTR, HIV-1 LTR Δ(−78/−58) or 6×Sp1–CAT reporter plasmid alone or in the presence of Sp1, TCF-4 and Tat expression plasmids using various combinations. As shown in Fig. 4(a), Tat activated the LTR by 38-48-fold (Fig. 4a, compare lanes 1 and 2). This activation was enhanced further when plasmids Sp1 and Tat were
coexpressed (71-95-fold) (Fig. 4a, compare lanes 2 and 3). Addition of TCF-4 did not affect activation of the HIV-1 LTR by Tat or Tat plus Sp1 (Fig. 4a, compare lanes 2 and 4, and 3 and 5).

The functional interplay between Tat, Sp1 and TCF-4 was studied further in the absence of the Sp1-binding motif. U-87 MG cells were transfected with a mutant HIV-1 LTR construct where two of the three Sp1-binding sites had been

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**Fig. 3.** Association of TCF-4 and Sp1 proteins in vivo and in vitro. (a, b) Cell lysates were prepared from U-87 MG cells transfected with 10 μg plasmid expressing different Sp1 or TCF-4 cDNA constructs as indicated. Fifty micrograms was used for Western blot analysis to verify the expression of Sp1 (a, lanes 1 and 4) or TCF-4 (b, lanes 1 and 2). Approximately 300 μg cell extract was utilized in immunoprecipitations (IP) followed by Western blotting utilizing anti-TCF-4 (a, lanes 2 and 5) or rabbit serum (a, lanes 3 and 6), followed by anti-Sp1 antibody. As in (a), anti-Sp1 antibody (b, lane 4) or rabbit serum (lane 3) was used for IP followed by anti-TCF-4 antibody. Arrowheads depict the positions of the 95 and 105 kDa Sp1 (a) and the ~72 kDa TCF-4 (b). (c) Schematic presentation of Sp1 and its various deletion mutants. ZF1, ZF2 and ZF3 indicate zinc-finger domains 1–3. The binding ability of TCF-4 to various Sp1 mutants is shown on the right. (d) In vitro-synthesized 35S-labelled Sp1 mutant proteins were incubated with GST or GST–TCF-4. After incubation for 1 h at 4°C, bound proteins were eluted and analysed by SDS-PAGE.

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**Fig. 4.** Regulation of transcription of the LTR by TCF-4 and Sp1 in the presence of Tat. U-87 MG cells were transfected with plasmids of full-length LTR–luc (a) or its deletion mutant (b) or with the reporter plasmid containing six Sp1 binding sites at the 5′-end of the CAT DNA sequence (6 × Sp1) (c) either alone or in combination with plasmids expressing TCF-4, Sp1 or Tat using various combinations. Data represent the mean value of at least three separate transfection experiments. The fold activation is represented above each lane.
DNA in the presence of Tat. U-87 MG cells were transfected to correlate further the functional interplay among Tat, binding Tat alleviates the effect of TCF-4 on Sp1–DNA mobility-shift assays were performed using 10

Finally, to demonstrate further the functional cooperativity between Sp1 and Tat, we utilized the synthetic promoter 6 × Sp1–CAT. As shown in Fig. 4(c), Tat activated the synthetic promoter by 6-19-fold (Fig. 4c, lane 2). This activation was enhanced further in the presence of Sp1 (17-96-fold; Fig. 4c, compare lanes 2 and 3). Addition of TCF-4 did not affect Tat activation of the synthetic promoter (Fig. 4c, compare lanes 2 and 4). Combined expression of Sp1, Tat and TCF-4 did not cause any significant change/decrease in the transcriptional activation of the test promoter (~14-48-fold) (Fig. 4c, compare lanes 3 and 5).

**Tat alleviates the effect of TCF-4 on Sp1–DNA binding**

To correlate further the functional interplay among Tat, Sp1 and TCF-4, we examined the ability of Sp1 to bind to DNA in the presence of Tat. U-87 MG cells were transfected with 5 µg Sp1, Tat and/or TCF-4 expression plasmids. Gel mobility-shift assays were performed using 10 µg nuclear proteins. As shown in Fig. 5(a), the intensity of the band corresponding to the DNA–Sp1 complex was enhanced slightly in U-87 MG cells transfected with a plasmid expressing Sp1 (Fig. 5a, compare complex C1 in lanes 2 and 3). The level of this complex decreased in cells expressing Sp1 and TCF-4, suggesting that TCF-4 may prevent binding of Sp1 to its responsive DNA motif (Fig. 5a, compare complex C1 in lanes 3 and 5). Note that TCF-4 and/or Tat were unable to bind to the DNA (Fig. 5a, lanes 4 and 6). The intensity of the C1 complex did not change in the presence of TCF-4 and Tat (Fig. 5a, lane 7). Addition of Tat did not affect the intensity of the complex formed in the presence of overexpressed Sp1 (Fig. 5a, lane 8). Interestingly, the intensity of the band corresponding to binding of the DNA probe to Sp1 in U-87 MG cells transfected with a plasmid expressing Sp1 was not affected by TCF-4 in the presence of Tat (Fig. 5a, lane 9).

To investigate further the ability of Tat to alleviate the effect of TCF-4 on Sp1–DNA binding, we performed Western blot and ChIP assays. HL3T1 cells were transfected with TCF-4 (Fig. 5b, lane 2) and/or Tat (Fig. 5b, lane 3) expression plasmids. Forty-eight hours after transfection, cells were harvested and subjected to Western blot or ChIP analysis. As a positive control, anti-TCF-4 antibody was used to demonstrate the efficiency of transfection in these cells (Fig. 5b, compare lane 1 with lanes 2 and 3). Anti-Grb2 antibody was used to show equal loading (Fig. 5b). The ChIP assay was performed using anti-Sp1 antibody. Rabbit anti-mouse serum was used as a negative control. As shown in Fig. 5(c), Sp1 was unable to associate with HIV-1 LTR DNA in the absence of Tat (Fig. 5c, compare lanes 4 and 7).

Next, we sought to examine the phosphorylation status of Sp1 in the presence of Tat and TCF-4. We performed in vitro kinase assays by mixing 43 ng purified Sp1, with or without 50 ng (~30 U) DNA-PK and 0.5 µg IVT-TCF-4, with [γ-32P]ATP in the presence of 250 ng dsDNA oligonucleotides containing the Sp1-binding motif. The mixture was incubated with either GST or GST–Tat. After incubation at 37°C for 30 min, the reaction was stopped with SDS loading dye, separated by 10% SDS-PAGE, fixed, stained and allowed to dry before autoradiography. As shown in Fig. 5(d), Sp1 was phosphorylated when incubated with DNA-PK and the Sp1-binding motif (Fig. 5d, compare lanes 1 and 2). The addition of TCF-4 abolished the phosphorylation of Sp1 in the presence and absence of DNA-PK (Fig. 5d, compare lane 2 with lanes 3 and 4). The addition of GST–Tat strongly enhanced the phosphorylation of Sp1 only in the presence of DNA-PK (Fig. 5d, compare lanes 5 and 6). Interestingly, TCF-4 did not affect the phosphorylation of Sp1 in the presence of GST–Tat (Fig. 5d, lane 8).

**DISCUSSION**

In this study, we demonstrated that TCF-4 can form complexes with Sp1 in vitro and in vivo and that this interaction interferes with Sp1 recognition and binding to the GC-rich domain within the HIV-1 LTR. Deletion of the GC-binding domain did not affect the negative impact of TCF-4 on Sp1 function. We also demonstrated that TCF-4 prevents phosphorylation of Sp1 by DNA-PK and that the addition of Tat alleviates the effect of TCF-4 on the phosphorylation and function of Sp1. The mechanism of TCF-4 interference with Sp1 required the second S/T domain of Sp1 and this was necessary and sufficient to prevent Sp1 binding to its cognate sites. The S/T-rich domain is shared by several Sp-family members (Sp1, -2, -3 and -4; Bouwman & Philipsen, 2002). Several kinases including casein kinase II and DNA-PK phosphorylate the S/T-rich domain of Sp1 (Bouwman & Philipsen, 2002). It has been shown previously that phosphorylation of Sp1 by DNA-PK is necessary for Sp1–DNA interaction (Chun et al., 1998). Therefore, post-translational modifications of Sp1, such as phosphorylation, are critical for regulating Sp1–DNA binding ability and transcriptional activities (Bouwman & Philipsen, 2002). Note that phosphorylation is also important for TCF-4. It has been shown that Nemo-like kinase phosphorylates TCF-4 and that this phosphorylation prevents β-catenin–TCF-4 complexes from binding to DNA (Ishitani et al., 2003).

In addition to phosphorylation, Sp1 may lose its ability to bind DNA if competing with another protein for DNA binding or if the promoter to which it could bind is methylated. For example, Ding et al. (2004) showed that the SANT domain of human MI-ER1 protein interacts with Sp1.
and interferes with GC box recognition (Ding et al., 2004). In another example, the authors created an artificial zinc-finger protein, which could inhibit Sp1–DNA binding by interacting with incoming Sp1 and preventing target recognition (Kim et al., 2005). Finally, hypermethylation around Sp1-binding sites has been reported to reduce Sp1–DNA binding, thereby decreasing Sp1 transcriptional activities (Macleod et al., 1994; Zhu et al., 2003).

In addition to its interaction with DNA, Sp1 also interacts with cellular factors that may have a positive or negative impact on gene expression. For example, Sp1 was shown to interact physically via its DNA-binding domain with transcriptional activators such as the p65/RelA subunit of NF-κB, the erythroid factor GATA-1, YY1, the cell-cycle regulator E2F, c-Jun and YB-1 (Lee et al., 1993; Perkins et al., 1994; Gregory et al., 1996; Karlseder et al., 1996; Lin et al., 1996; Sawaya et al., 1998a). Reciprocally, several other factors interact with Sp1 and negatively affect Sp1–DNA associations. These factors include B-myb, p107, PML, FBI-1, TAF-1 and MDM2 (Datta et al., 1995; Vallian et al., 1998; Johnson-Pais et al., 2001; Lee et al., 2002; Suzuki et al., 2003; Cicchillitti et al., 2004). Therefore, TCF-4 has joined a set of several Sp1-interacting transcription factors that

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**Fig. 5.** Functional interplay between Sp1 and TCF-4 in the presence of Tat. (a) Band-shift assays were performed using GC-rich motif oligonucleotides of the HIV-1 promoter (−78/−52). Labelled DNA probes were incubated with nuclear extracts prepared from cells transfected with Sp1, TCF-4 or Tat using various combinations. C1 indicates the complexes formed upon addition of nuclear extracts. NS identifies the position of non-specific complexes. Free probe is visible at the bottom of the gel. (b) HL3T1 cells were transfected with 1-0 μg TCF-4 (lane 2) and/or Tat (lane 3) expression plasmids. Fifty micrograms of nuclear proteins was subjected to Western blot analysis using anti-TCF-4 or anti-Grb2 antibodies. (c) HL3T1 cells were transfected with 1-0 μg TCF-4 (lanes 2–4) or TCF-4 and Tat (lanes 5–7) expression plasmids as indicated. Interaction of endogenous Sp1 with HIV-1 LTR DNA was demonstrated by ChIP assays. The primers used in these experiments are described in Methods. Anti-Sp1 (lanes 4 and 7), pre-immune serum (NMS; lanes 3 and 6) or no antibody (lanes 2 and 5) was used. Protein markers were loaded in lane 1. (d) Pure Sp1 (43 ng) in the presence or absence of 50 ng (~30 U DNA-PK) and/or ~0-5 μg IVT-TCF-4 (as indicated) was incubated with GST (lanes 1–4) or GST–Tat (5–8) and with [γ-32P]ATP and subjected to a kinase assay. Sp1 phosphorylation was assessed by SDS-PAGE. The intensity of the bands (shown below the histogram) was measured by densitometry and the numbers are presented as a histogram; the basal level was set at 1-0.
prevent Sp1–DNA interactions and impair Sp1-mediated transcriptional activities. Sp1 has also been shown to interact with HIV-1 proteins, including Tat and Vpr (Jeang et al., 1993; Sawaya et al., 1998b). Jeang et al. (1993) demonstrated that Tat and Sp1 interact in vitro and in vivo. Interestingly, other studies have demonstrated that Tat does not bind directly to Sp1 and that their interaction is mediated by a third factor (Loregian et al., 2003). DNA-PK was identified as this third cellular partner that could bridge Tat and Sp1 (Chun et al., 1998). The same group demonstrated that Tat brings DNA-PK into the proximity of Sp1, which allows its phosphorylation and enhancement of Sp1–DNA interactions. Other studies have reported that the human TATA-binding protein (TBP) may play such a role because of its interaction with Tat and Sp1 (Emili et al., 1994; Kashanchi et al., 1994). However, TBP was not able to bridge Tat and Sp1, as demonstrated by yeast three-protein experiments. This could be explained by the fact that both Tat and Sp1 bind the C-terminal conserved domain of TBP, raising the possibility that they may compete to bind TBP (Emili et al., 1994; Kashanchi et al., 1994). Another study suggested the cyclin T1/cdk9 complex as a potential candidate for bridging Tat and Sp1. Cyclin T1 was reported to interact with Tat and with the protein kinase cdk9, enhancing the affinity of Tat for TAR–RNA binding (Wei et al., 1998). The Tat–cyclin T1/cdk9–TAR association ensures hyperphosphorylation of the C-terminal domain of RNA polymerase II holoenzyme by cdk9, thus promoting transcriptional elongation of the nascent viral mRNA (Wei et al., 1998). Thus, one could speculate that interaction between Tat and the cyclin T1/cdk9 complex has a role in bringing the protein kinase into the proximity of Sp1 bound to the HIV–1 LTR. Conversely, interaction of Sp1 with cyclin T1 and activation of the HIV–1 LTR in the absence of Tat (Kim et al., 2005) contradicts the suggestion that cyclin T1 is the bridging protein. Therefore, DNA-PK remains the only suitable protein that can bring Tat and Sp1 together, and any alteration in DNA-PK function may affect the transcription of HIV–1.

Our results illustrate a new direction in inhibiting HIV–1 gene expression by using the cellular protein TCF-4. These results also confirm the existence of three types of regulator of the HIV–1 LTR. Several studies have demonstrated that transcription of the HIV–1 LTR starts immediately after the virus infects the cell (immediate-early stage), which increases after the synthesis of Tat (early stage) and then develops further with the synthesis of Vpr (late stage). Each stage is controlled by a series of transcriptional regulators. Therefore, TCF-4 belongs to the first group of regulators, which negatively affect the transcription of the HIV–1 LTR (Fig. 1). Once Tat is synthesized, TCF-4 loses its ability to inhibit Sp1, which in turn allows the enhancement of transcription. Any extension of TCF-4 function might affect transcription of HIV–1, as well as its replication.

Note that the physical association of TCF-4 with Sp1 may not be the only pathway used by TCF-4 to prevent the phosphorylation of Sp1. Based on our results (Fig. 2d), we speculate that TCF-4 may interfere with the Sp1–DNA-PK association or with the association of Sp1 with other kinases, and this is the subject of ongoing experiments. Furthermore, our results have demonstrated that, in addition to endogenous TCF-4, excess TCF-4 is required to prevent phosphorylation of the S/T domain of Sp1. Therefore, any manipulation to increase the level of TCF-4 could have a negative effect on transcription of the HIV–1 LTR. Finally, since U–87 MG cells contain both forms of Sp1 (95 and 105 kDa; Fig. 3a), identification of the form inhibited by TCF-4 is necessary.

In summary, several therapeutic strategies such as inhibition of viral transcription (e.g. antisense oligonucleotides, small interfering RNA and ribozymes) have long been recognized as an important goal in HIV–1 therapy. In this study, based on the molecular mechanism of interaction among regulatory proteins and DNA, we identified TCF-4 as a new cellular protein that can inhibit HIV–1 transcription. Approaches aimed towards targeting the transcription mechanism involving Sp1 and GC boxes may represent a highly effective alternative therapy for HIV–1 infection.

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