C/EBPβ regulates human immunodeficiency virus 1 gene expression through its association with cdk9

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Transcriptional regulation of the human immunodeficiency virus type 1 (HIV-1) is a complex event that requires the cooperative action of both viral (e.g. Tat) and cellular (e.g. C/EBPβ, NF-κB) factors. The HIV-1 Tat protein recruits the human positive transcription elongation factor P-TEFb, consisting of cdk9 and cyclin T1, to the HIV-1 transactivation response (TAR) region. In the absence of TAR, Tat activates the HIV-1 long terminal repeat (LTR) through its association with several cellular factors including C/EBPβ. C/EBPβ is a member of the CCAAT/enhancer-binding protein family of transcription factors and has been shown to be a critical transcriptional regulator of HIV-1 LTR. We examined whether Tat–C/EBPβ association requires the presence of the P-TEFb complex. Using immunoprecipitation followed by Western blot, we demonstrated that C/EBPβ–cyclin T1 association requires the presence of cdk9. Further, due to its instability, cdk9 was unable to physically interact with C/EBPβ in the absence of cyclin T1 or Tat. Using kinase assays, we demonstrated that cdk9, but not a cdk9 dominant-negative mutant (cdk9-dn), phosphorylates C/EBPβ. Our functional data show that co-transfection of C/EBPβ and cdk9 leads to an increase in HIV-1 gene expression when compared to C/EBPβ alone. Addition of C/EBP homologous protein (CHOP) inhibits C/EBPβ transcriptional activity in the presence and absence of cdk9 and causes a delay in HIV-1 replication in T-cells. Together, our data suggest that Tat–C/EBPβ association is mediated through cdk9, and that phosphorylated C/EBPβ may influence AIDS progression by increasing expression of HIV-1 genes.

INTRODUCTION

Transcription of the integrated human immunodeficiency virus type 1 (HIV-1) proviral genome is an essential step in the retrovirus life cycle and is accomplished through a complex network of interactions between cis-elements and trans-acting factors. The DNA cis-acting regulatory sites that control HIV-1 gene transcription reside in the long terminal repeat (LTR) upstream from the transcription start site (nucleotide +1). A fundamental role in this process is exerted by the viral protein Tat, a powerful transactivator of viral gene expression. Tat stimulates the processivity of RNA polymerase II following initiation from the LTR via its interaction with the subunits of the positive transcription elongation factor (P-TEFb) (Peterlin & Price, 2006). In addition to its association with the P-TEFb complex, Tat is able to physically and functionally associate with several cellular transcription factors including p300, P/CAF and C/EBPβ (Brady & Kashanchi, 2005; Peruzzi, 2006).

The P-TEFb complex is composed of two subunits, cyclin T1 and its associated kinase, cdk9 (Yu et al., 2006). Cyclin T1 was first identified as a Tat-binding protein that mediates cooperative binding of the Tat–cyclin T1 complex to the transactivation response (TAR) region and in turn recruits cdk9 to RNA polymerase II (Garriga & Graña, 2004; Ghafouri et al., 2006). Cyclin T1 contains several domains including the N-terminal domain, a putative coiled-coil motif, a His-rich motif and a carboxy-terminal PEST sequence (Wei et al., 1998). Cyclin T1–Tat interaction requires the entire cyclin box and a key cysteine residue (C261) within the TRM (Tat–TAR recognition motif) (Garber et al., 1998). This residue is essential for zinc-dependent HIV-Tat binding. Upregulation of cyclin T1
correlates with hyperphosphorylation of RNA polymerase II and HIV replication in stimulated peripheral blood lymphocytes (Liou et al., 2004; Marshall & Graha, 2006).

The 42 kDa protein, cdk9, like many other cyclin-dependent kinases (CDKs), was identified during a cDNA screening intended to isolate novel regulators of the mammalian cell cycle (Graha et al., 1994). As no cyclin partner or cell cycle function was demonstrated at that time, cdk9 was temporarily designated PITALRE for its PSTAIRE-like sequence, a conserved motif found in CDC2 and related kinases (Pines, 1994; Jeffrey et al., 1995). cdk9 was shown to phosphorylate itself, as well as a variety of substrates and to be associated with various proteins including TRAF2 (MacLachlan et al., 1998), B-Myb (De Falco et al., 2000), the 75K small nuclear RNA (snRNA) (Nguyen et al., 2001), pRb (Simone et al., 2002), gp130 (Falco et al., 2002), Pch1/Pct1 (Pei et al., 2003) and p53 (Claudio et al., 2006). cdk9 also phosphorylates the molecular chaperone Hsp70 or a kinase-specific chaperone complex, Hsp90–Cdcd37, to form two separate chaperone–cdk9 complexes (Zhang et al., 2004). Depletion of cdk9 blocks Tat-dependent transactivation, indicating that cdk9 is a subunit of the cellular cofactor required for Tat to stimulate transcription (Zhu et al., 1997). Thus, cdk9 and cyclin T1, in addition to Tat, are key regulators of HIV-1 gene expression. In the nucleus, not every cyclin T1–cdk9 heterodimer displays P-TEFb transcripional activity. In fact, about half of the heterodimers in HeLa cells are sequestered in a complex with the 75K snRNA and the HEXIM1 protein (Chen et al., 2004; Yik et al., 2004). Whereas 75K serves as a molecular scaffold to mediate the interaction of HEXIM1 with cyclin T1–cdk9, HEXIM1 inhibits the cdk9 kinase activity and prevents P-TEFb from binding to transcriptional templates (Michels et al., 2004). HEXIM1 was also shown to inhibit Tat transcriptional activity (Fraldi et al., 2005).

C/EBPs comprise a family of basic region leucine zipper (bZIP) transcription factors that, as a prerequisite for DNA binding through their basic regions, dimerize through their adjacent C-terminal leucine zippers to form homodimers or heterodimers with other C/EBP family members (Romji & Foka, 2002). The C/EBP gene is transcribed into a single 1.4 kb mRNA (Cao et al., 1991; Descombes & Schibler, 1991). At the protein level, however, multiple C/EBP isoforms, varying in size from 14 to 38 kDa, have been reported (Descombes & Schibler, 1991). The C/EBP isoforms include two full-length LAP (liver-enriched activator protein) isoforms (38 and 34 kDa) and two truncated 14 and 21 kDa LIP (liver-enriched inhibitory protein) isoforms (Descombes & Schibler, 1991). Another member of the C/EBP family is called CHOP (C/EBP-homologous protein) and acts in most, but not all, circumstances as a dominant-negative inhibitor of DNA binding when it is heterodimerized to another C/EBP partner (Ron & Habener, 1992). While the C/EBPs are well conserved through their bZIP regions, they exhibit more limited conservation in their N-terminal activation and regulatory domains (Sebastian & Johnson, 2006). The functional importance of amino-terminal determinants in C/EBP-mediated transcriptional regulation is supported by the fact that C/EBPβ153–297 (LIP), a truncated form of C/EBPβ, can function as a repressor of C/EBP transcriptional activity (Ruocco et al., 1996; Kowenz-Leutz et al., 1994). C/EBPα, β and δ commonly function as strong transcriptional activators, while C/EBPγ and ζ (CHOP) generally act to inhibit C/EBP transcriptional activators (Lekstrom-Himes & Xanthopoulos, 1998; Gao et al., 2002). C/EBP-binding sites have been identified in the promoter regions of numerous genes, including HIV-1 LTR (Henderson et al., 1995), the genes for interleukin 6 (IL-6; Akira et al., 1990), tumour necrosis factor alpha (TNF-α; Pope et al., 1994) and monocyte chemoattractant protein-1 (MCP-1; Sekine et al., 2002). Moreover, the activity of C/EBPβ is influenced by various inflammatory stimuli, including lipopolysaccharide (An et al., 1996), IL-6 (Poli et al., 1990) and TNF-α (Yin et al., 1996).

We previously demonstrated the ability of HIV-Tat and C/EBPβ to physically and functionally associate (Coyle-Rink et al., 2002; Abraham et al., 2005). In this study, we examined whether the interplay between Tat and C/EBPβ is dependent on the Tat partners, cyclin T1 and cdk9.

**METHODS**

**Plasmids.** The cdk9, cdk9 dominant negative (cdk9-dn), cyclin T1, HIV-1 Tat, C/EBPβ and CHOP expression plasmids were previously described (Sawaya et al., 2000; Abraham et al., 2005; Claudio et al., 2006). pGEX-2T-cyclin T1 (wild-type and mutants), glutathione S-transferase (GST)–cdk9 and GST–C/EBPβ were previously described (Abraham et al., 2005).

**Cell culture and transfection assays.** The human U-87 MG glioblastoma cell line was maintained in Dulbecco’s modified Eagle’s medium + 10% FBS. Cells were transfected with 0.5 µg reporter plasmid [HIV-LTR-luc (full-length or mutant)] or co-transfected with 1.0 µg various expression cDNAs (Amini et al., 2004). The amount of DNA used for each transfection was normalized with pcDNA3 vector plasmids. Each transfection was repeated multiple times with different plasmid preparations. Cell extracts were prepared 48 h after transfection and luciferase or chloramphenicol acetyl transferase (CAT) assays were performed as previously described (Amini et al., 2002).

**Overexpression and purification of recombinant proteins.** GST–cdk9, GST–C/EBPβ and GST–cyclin T1 fusion proteins were expressed and purified as previously described (Rossi et al., 2006). The integrity and purity of the GST fusion proteins were analysed by SDS-PAGE followed by Coomassie blue staining. Radiolabelled cdk9 and C/EBPβ full-length and deletion mutant proteins were synthesized using the TntT Coupled Wheat Germ Extract system according to the manufacturer’s recommendations (Promega).

**In vitro protein–protein interactions (GST pull-down assay).** Four microlitres of 35S-labelled and in vitro-translated (IVT) C/EBPβ T1 was incubated with 5 µg GST or GST–cyclin T1 fusion proteins coupled to glutathione–Sepharose beads in 300 µl lysis buffer 150 (LB-150) for 2 h at 4°C with continuous rocking. After incubation, beads were pelleted and washed five times with LB-150 buffer. The same procedure was used with labelled and translated
p24 ELISA. p24 antigen-capture ELISA for tissue culture supernatants was performed as described by the manufacturer (Beckman Coulter). Each sample was assayed over a 10^4-fold range of dilution to ensure quantification was based on an absorbance value within the linear range of the standards.

Results

The ability of HIV-1 Tat to physically and functionally associate with cyclin T1/cdk9 as well as with C/EBPβ (Wei et al., 1998; Coyle-Rink et al., 2002) provides us with the rationale to examine whether addition of cyclin T1 or cdk9 affects Tat–C/EBPβ association. First, we investigated whether C/EBPβ associates with cyclin T1 in the absence of Tat and cdk9. U-87MG cells were transfected with plasmids encoding C/EBPβ or cyclin T1 (as indicated). As shown in Fig. 1(a), non-transfected (lane 1) or C/EBPβ-transfected cells (lane 4) were used as controls to mark the positions of C/EBPβ as well as the efficiency of transfection (endogenous vs overexpressed, respectively). Proteins were immunoprecipitated with a polyclonal anti-cyclin T1 antibody (lanes 2 and 5) or with non-immune rabbit serum (NIS) (lanes 3 and 6), followed by Western blot analysis using anti-C/EBPβ antibody. This experiment demonstrated the ability of endogenous (lane 2) and exogenous C/EBPβ (lane 5) to interact with endogenous cyclin T1. Specificity of cyclin T1–C/EBPβ interaction was confirmed using NIS. Interestingly, immunoprecipitation/
Western blot analysis performed with extracts prepared from C/EBPβ-transfected cells revealed that cyclin T1 associates with several C/EBP isoforms (45 and 38 kDa). Note that C/EBPβ migrates around 45 kDa as previously described (Roman et al., 1990).

Reciprocally, proteins were immunoprecipitated with a polyclonal anti-C/EBPβ antibody (Fig. 1b, lanes 2 and 5) or with NIS (lanes 3 and 6), followed by Western blot analysis using anti-cyclin T1 antibody. Non-transfected (lane 1) or cyclin T1-transfected cells (lane 4) were used as negative and positive controls, respectively. Specificity of C/EBPβ–cyclin T1 interaction was confirmed using NIS (lanes 3 and 6). Cyclin T1 was co-immunoprecipitated by anti-C/EBPβ antibody (lanes 2 and 5). These experiments demonstrated the ability of endogenous and exogenous cyclin T1 to interact with endogenous and exogenous C/EBPβ in the absence of Tat.

Cyclin T1 binding to C/EBPβ was also tested by GST pull-down assays using IVT-35S-labelled C/EBPβ. Various C-terminal cyclin T1 deletion mutants were fused to GST and incubated with purified recombinant C/EBPβ, Tat or C/EBPβ+Tat (Fig. 1d). Labelled proteins were incubated with GST or GST–cyclin T1 fusion proteins coupled to glutathione–Sepharose beads. As shown in Fig. 1(c), deletion of the C-terminal regions of cyclin T1 up to residue 300 did not affect its ability to bind to C/EBPβ. However, deletion of residues 300–250 abrogated the interaction of C/EBPβ with cyclin T1 (Fig. 1d). Interestingly, HIV-1 Tat protein was previously shown to interact with the same domain within cyclin T1 (Sawaya et al., 2000). Cyclin T1–Tat interaction requires the entire cyclin box and a key cysteine residue (C261) (Garber et al., 1998). In the light of this observation, the experiment was repeated using labelled Tat and/or C/EBPβ. Data obtained from incubation of labelled Tat protein with GST or GST–cyclin T1 full-length and deletion mutants corroborate the previously described data. Interestingly, formation of the C/EBPβ–cyclin T1 complex was not affected by the addition of Tat. These results led us to suggest that C/EBPβ–Tat–cyclin T1 co-exist in the same protein complex. Fig. 1(e) illustrates the size of GST (lane 2) and GST–cyclin T1 deletion mutants (lanes 3–8).

Next, we examined the ability of cyclin T1 to associate with C/EBPβ in the absence of the cyclin T1 partner, cdk9. It should be noted that cdk9 and cyclin T1, in addition to Tat, are key regulators of transcription of HIV-1 gene expression. U-87MG cells were transfected with C/EBPβ expression plasmid. Twenty-four hours later, total cell protein was isolated from these cells as well as untransfected controls and 500 μg was incubated with anti-cdk9 antibody. After continuous rotation for 12 h, 20 μl Sepharose A beads were added to the mixture and incubated for another 2 h. The beads were then removed by microcentrifugation and the cdk9-depleted extracts (supernatant) was subjected to Western blot analysis. Fifty micrograms of protein isolated from the Sepharose beads or from the supernatant was subjected to Western blot analysis to verify that the antibodies had immunodepleted cdk9. As shown in Fig. 2(a), depletion of cdk9 was complete in the supernatant of extracts incubated with anti-cdk9 antibody but not with NIS (compare lane 3 to lane 2). Extracts prepared from non-transfected or cdk9-transfected cells were used as negative and positive controls, respectively (lanes 1 and 4).

Next, we examined the ability of C/EBPβ to physically associate with cyclin T1 in the presence and absence of cdk9. Three hundred micrograms of extracts prepared from non-transfected or C/EBPβ-transfected cells was incubated with anti-cyclin T1 antibody (Fig. 2b, lanes 2 and 5) or with NIS (lanes 3 and 6). Extracts prepared from cdk9-depleted extracts were also immunoprecipitated with anti-cyclin T1 antibody (lane 8). As a control, 50 μg extract prepared from non-transfected, C/EBPβ-transfected or cdk9-depleted cells was subjected to Western blot analysis using C/EBPβ antibody (lanes 1, 4 and 7). As shown in Fig. 2(b), endogenous, as well as overexpressed, C/EBPβ physically associates with endogenous cyclin T (lanes 2 and 5). Interestingly, C/EBPβ lost its ability to interact with cyclin T1 in the absence of cdk9 (compare lane 8 to lanes 2 and 5). These results led us to conclude that cdk9 associates with C/EBPβ and therefore C/EBPβ was depleted with cdk9. Note that C/EBPβ was not detected in cdk9-depleted extracts (lane 7), which may be due to the association of C/EBPβ with cdk9.

Reciprocally, the cells were immunoprecipitated with anti-C/EBPβ antibody and subjected to Western blot analysis using anti-cyclin T1 antibody. As shown in Fig. 2(c), endogenous and overexpressed C/EBPβ remain associated with cyclin T1 (lanes 2 and 5) in the presence of cdk9 but not in its absence (lane 8). Interestingly, cyclin T1 was not detected in cdk9-depleted extracts (lane 7). The observed associations are specific because no bands were observed when the immunoprecipitations were performed using NIS (lanes 3 and 6). These results led us to conclude that association of C/EBPβ with cyclin T1 is cdk9-dependent.

To further demonstrate the specificity of these interactions, and to confirm the dependence of C/EBPβ–cyclin T1 association on the presence of cdk9, similar experiments were performed using non-transfected and cdk9-transfected U-87MG cells where cyclin T1 was depleted. As shown in Fig. 2(d), non-transfected (lane 1) and cdk9-transfected cells (lane 4) were used as negative and positive controls, respectively. Specificity of C/EBPβ–cdk9 interaction was shown with NIS (lanes 3 and 6). The C/EBPβ was specifically co-immunoprecipitated by anti-cdk9 antibody (lanes 2 and 5). Extracts prepared from cyclin T1-depleted extracts were also immunoprecipitated with anti-C/EBPβ antibody (lane 8). As a control, 50 μg extract prepared from cyclin T1-depleted cells was subjected to Western blot analysis using cdk9 antibody (lane 7). Interestingly, cdk9 lost its ability to interact with C/EBPβ in the absence of cyclin T1 (compare lane 8 to lanes 2 and 5). These results can be explained by the
fact that cdk9 is not stable in the absence of cyclin T1 (Garriga & Graña, 2004).

Next, we sought to examine the physical association between C/EBPβ and cdk9 in the presence of Tat and in the absence of cyclin T1 using non-transfected and cdk9-transfected U-87MG cells. Note that Tat expression plasmid was transfected in all the samples. As shown in Fig. 2(e), extracts prepared from non-transfected (lane 1) or cdk9-transfected (lane 4) U-87MG cells were used as negative and positive controls, respectively. Specificity of C/EBPβ–cdk9 interaction was shown with NIS (lanes 3 and 6). The C/EBPβ was specifically co-immunoprecipitated by anti-ckd9 antibody (lanes 2 and 5). Extracts prepared from cyclin T1-depleted cells were also immunoprecipitated with anti-C/EBPβ antibody (lane 8). As a control, 50 μg extract prepared from cyclin T1-depleted cells was subjected to Western blot analysis using cdk9 antibody (lane 7). Surprisingly, cdk9 remained associated with C/EBPβ in the absence of cyclin T1 (compare lane 8 to lanes 2 and 5). These results led us to conclude that Tat helped the stabilization of cdk9 in the absence of cyclin T1 and therefore allowed cdk9–C/EBPβ association.

Fig. 2. C/EBPβ associates with Tat via cdk9. (a) Five hundred micrograms of total cell proteins was prepared from U-87MG cells and immunoprecipitated (IP) with anti-cdk9 (lane 3) antibody or with NIS (lane 2). Fifty micrograms of the depleted supernatant was then used for Western blot analysis along with protein extracts prepared from non-transfected (lane 1) or cdk9-transfected (lane 4) U-87MG cells. The arrow head depicts the position of cdk9 protein. (b, c) Cell lysates were prepared from cdk9-depleted U-87MG cells [(b) and (c), lanes 7 and 8]. As a control, the cells were transfected with C/EBPβ expression plasmid [(b) and (c), lanes 4–6]. Three hundred micrograms of cell extract was immunoprecipitated followed by Western blot analysis utilizing anti-C/EBPβ (b) or anti-cyclin T1 (c) antibody. NIS was used as negative control [(b) and (c), lanes 3 and 6]. Fifty micrograms of control, cdk9-depleted or cdk9-transfected [(b) and (c), lanes 1, 4 and 7, respectively] extract was utilized for Western blot assays. The arrowhead indicates the position of C/EBPβ and cyclin T1 proteins. (d, e) Cell lysates were prepared from cyclin T1-depleted U-87MG cells [(d) and (e), lanes 7 and 8]. The cells were transfected with cdk9 expression plasmid [(d) and (e), lanes 4–6] as control. Three hundred micrograms of cell extract was immunoprecipitated using anti-C/EBPβ antibody followed by Western blot analysis utilizing anti-cdk9 antibody. NIS was used as negative control [(d) and (e), lanes 3 and 6]. Fifty micrograms of control, cdk9-transfected and cyclin T1-depleted [(d) and (e), lanes 1, 4 and 7, respectively] extract was utilized for Western blot assays. The arrowhead depicts the position of cdk9 protein. (f) Cell lysates were prepared from cdk9-depleted U-87MG cells (lanes 4 and 5). As a control, the cells were transfected with Tat expression plasmid (lanes 2–5). Three hundred micrograms of cell extract was immunoprecipitated using anti-Tat antibody followed by Western blot utilizing anti-C/EBPβ antibody. NIS was used as negative control (lanes 3 and 5). Fifty micrograms of control extracts (lane 1) was utilized for Western blot assays. The arrowhead depicts the position of C/EBPβ protein.
To further investigate the dependence of C/EBPβ–Tat association on the presence of cdk9, immunoprecipitation/Western blot assays were performed using cdk9-depleted extracts. U-87MG cells were transfected with Tat expression plasmid. Twenty-four hours post-transfection, the cells were washed and cellular proteins were prepared from non- or Tat-transfected cells. As shown in Fig. 2(f), extracts prepared from non-transfected cells were used as negative controls (lane 1). Specificity of C/EBPβ–Tat interaction was shown using NIS (lanes 3 and 5). C/EBPβ was specifically co-immunoprecipitated with anti-Tat antibody (lane 2). Extracts prepared from cdk9-depleted extracts were also immunoprecipitated with anti-Tat antibody (lane 4). Interestingly, Tat lost its ability to associate with C/EBPβ in the absence of cdk9 (compare lanes 2 and 4). These results confirmed our hypothesis regarding the ability of Tat to help the stabilization of cdk9 in the absence of cyclin T1 observed in Fig. 2(e).

Note that physical association between cyclin T1 and C/EBPβ was also studied in the presence of Tat and absence of cdk9. Our results demonstrated the inability of cyclin T1 to associate with C/EBPβ in the absence of cdk9, regardless of the presence or absence of Tat (data not shown).

C/EBPβ–cdk9 association was also tested in vitro by GST pull-down assays using IVT-35S-labelled cdk9 or IVT-32P-labelled C/EBPβ. Labelled proteins were incubated with GST, GST–C/EBPβ or GST–cdk9 fusion protein coupled to glutathione–Sepharose beads. As shown in Fig. 3(a), C/EBPβ was retained by the GST–cdk9 fusion protein but not by GST alone (compare lanes 2 and 3). Reciprocally, 35S-labelled cdk9 was incubated with GST or GST–C/EBPβ coupled to beads. As shown in Fig. 3(b), cdk9 was retained by GST–C/EBPβ but not by GST alone (compare lanes 2 and 3). Fig. 3(c) illustrates the sizes of GST (lane 2), GST–cdk9 (lane 3) and GST–C/EBPβ (lane 4), respectively.

The ability of cdk9 to phosphorylate other proteins gave us a rationale to investigate whether the C/EBPβ–cdk9 association leads to C/EBPβ phosphorylation. Further, it has been shown that phosphorylation of C/EBPβ contributes to cell survival and inhibition of pro-caspases, which leads to C/EBPβ induction (Buck et al., 2001). Kinase assays were performed using 300 μg cell proteins prepared from U-87MG cells and incubated with GST or GST–C/EBPβ. A kinase assay was performed as described in Methods. As shown in Fig. 3(d), endogenous cdk9 phosphorylates endogenous C/EBPβ and GST–C/EBPβ (lane 2) but not GST (lane 1).

The specificity of C/EBPβ phosphorylation by cdk9 was further confirmed using a cdk9 dominant-negative mutant (cdk9-dn) (Gran˜a et al., 1994) using IVT-cdk9 proteins. cdk9 or cdk9-dn was incubated with GST or with GST–C/EBPβ and the kinase assay was performed. As shown in Fig. 3(e), cdk9 phosphorylates GST–C/EBPβ (lane 2) but not GST alone (lane 1). IVT-cdk9-dn was unable to phosphorylate GST (lane 3) or GST–C/EBPβ (lane 4). These results demonstrated the ability of endogenous, immunoprecipitated and pure cdk9 to phosphorylate C/EBPβ; they also confirmed the specificity of this phosphorylation.

Phosphorylation of C/EBPβ by cdk9 was functionally studied using transient transfection assays. In addition, it was previously demonstrated that CHOP forms heterodimers with C/EBPβ and inhibits its DNA-binding activity and its function as a transcriptional regulator (Shirakawa et al., 2006). U-87MG cells were transfected with 0.5 μg HIV-LTR reporter plasmid alone or in the presence of 1.0 μg Tat, cdk9, cdk9-dn, C/EBPβ or CHOP expression plasmids using various combinations. The amount of DNA used for each transfection was normalized with pCDNA3 or pCEP-4 vector plasmids. Forty-eight hours after transfection, the cells were washed and processed for luciferase assay. As shown in Fig. 4(a), HIV-1 LTR was activated by C/EBPβ, cdk9 and Tat expression plasmids, respectively (compare lanes 3, 5 and 11 to lane 1). As expected, addition of CHOP or cdk9-dn did not appreciably affect HIV-1 gene expression (compare lanes 2 and 8 to lane 1). Activation of HIV-LTR by C/EBPβ was further enhanced in the presence of cdk9 (lane 6), Tat (lane 12) or cdk9 + Tat (lane 14) expression.
plasmids. This effect decreased when C/EBPβ was co-transfected with CHOP (lane 4), cdk9-dn (lane 9) or Tat + cdk9-dn (lane 16) expression plasmids. Therefore, we concluded that phosphorylation of C/EBPβ by cdk9 is important for C/EBPβ to perform its transcriptional activity. Note that the cells were transfected in triplicate in which one dish was used to verify the efficiency of transfection, which was examined by Western blot analysis as shown in Fig. 4(b). Grb2 was used to verify the equal loading of cellular proteins.

The ability of C/EBPβ to activate HIV-1 LTR through its direct binding to the region spanning nt −115 to −107 (Lee et al., 2001; Ruocco et al., 1996) prompted us to study its effect on HIV-1 LTR in the absence of this region (−105 to +66). U-87MG cells were transfected with 0.5 μg HIV-LTR (full-length and mutant) reporter plasmids alone or in the presence of 1.0 μg Tat or C/EBPβ expression plasmids. The amount of DNA used for each transfection was normalized with pcDNA3 vector plasmid. Forty-eight hours after transfection, the cells were washed and processed for CAT assay. As shown in Fig. 4(c), C/EBPβ activates full-length (−456 to +66) (lane 2) and mutant (−105 to +66) (lane 5) HIV-1 LTR by approx. 11- and seven-fold, respectively. As a positive control, the cells were transfected with Tat expression plasmid (lanes 3 and 6). These results further confirm our observation regarding the functional and physical interplay between C/EBPβ and cdk9. Our results also corroborate previously published results where transcription from HIV-1 LTRs lacking C/EBP sites was shown to be induced following addition of C/EBP (Henderson et al., 1995).

Finally, we confirmed a role for C/EBPβ in HIV-1 transcription and replication, using a replication assay. U937 cells were infected with HIVJR-FL after being transfected with 2.5 μg CHOP expression plasmid. Empty pcDNA3 vector was used as a negative control (Fig. 4b). The progression of infection was visually observed by the increase in syncytium formation in the infected cells. The cells and the supernatants were collected every 48 h. At the indicated times, using ELISA assays against p24, we observed that inhibition of C/EBPβ activity by CHOP leads to a delay in HIV-1 replication in human T-cells.

**DISCUSSION**

Previous studies have shown that the transcriptional activator C/EBPβ activates several cellular and viral genes including the HIV-1 LTR and that C/EBPβ is necessary for HIV-1 replication in monocytes (Henderson et al., 1995; Henderson & Calame, 1997). C/EBPβ does not influence HIV-1 infection, but can affect HIV-1 replication by regulating virus transcription in part through its association with viral proteins and the recruitment of co-activators to LTR (Lee et al., 2001). Based on these findings, we, as well as others, were able to demonstrate the ability of C/EBPβ to physically and functionally interact with HIV-1 Tat and Vpr proteins (Coyle-Rink et al., 2002; Hogan et al., 2003). Here, we investigated the relationship between C/EBPβ and Tat’s partners, cyclin T1 and cdk9. We demonstrated the ability of C/EBPβ to interact with cyclin T1 in the presence of cdk9 but not in its absence. We also showed that C/EBPβ physically and functionally associates with cdk9, which in turn has the ability to phosphorylate C/EBPβ.
The HIV-1 C/EBP binding site overlaps with a consensus sequence (E box), which is a binding site for proteins of the B class of basic helix–loop–helix leucine zipper (b-HLH-Zip) family (Dang et al., 1992). Members of this family include c-Myc, Max, Mad, TFE3, TFEB, and Mxi1 (Murre et al., 1989; Blackwood & Eisenman, 1991; Ayer et al., 1993), which form homo- and heteromultimers and are potentially able to associate with C/EBP or nuclear factor (NF)-κB transcription factors. These factors play a positive role in the regulation of HIV-1 promoter. The HIV-1 LTR contains at least two C/EBP sites (491–483 and 300–292), which could positively contribute to promoter activation (Zhang et al., 1995), suggesting that C/EBP sites can function as positive or negative regulators of gene expression depending on their location relative to the TATA box of the LTR. The biological relevance of the HIV-1 C/EBP/β binding site is suggested by the fact that the sequence appears to be conserved in primary isolates from AIDS patients over several years (Michael et al., 1994). C/EBP/β was also shown to impact HIV-1 replication through the recruitment of co-activators to the LTR (Lee et al., 2001). This latest observation is in accord with our earlier data, where we demonstrated that depletion of cdk9 prevents association between C/EBP/β and Tat. Further, it has been shown that the depletion of cdk9 blocks Tat-dependent transactivation and Tat-associated kinase activity, indicating that cdk9 is a subunit of a cellular cofactor and a co-activator required for Tat to stimulate viral transcription (Zhu et al., 1997).

The interplay between Tat and C/EBP/β is not limited to HIV-1 LTR. We previously reported the ability of both proteins to affect MCP-1 gene expression (Abraham et al., 2005). Other studies have also previously described such interplay in regulating the IL-6 promoter (Sponser et al., 2007). Tat was shown to activate IL-6 gene expression through its cooperation with C/EBP transcription factors. In this study, Tat increased the nuclear levels of C/EBP transcription factors by inducing post-translational modifications of C/EBP factors through the activation of specific kinases, which phosphorylate C/EBP/β and render it more transcriptionally active (Nakajima et al., 1993). The above data are consistent with our findings where we demonstrated the ability of cdk9 to phosphorylate C/EBP/β and enhance its transcriptional activity. These data suggest that Tat may promote HIV-1 gene expression by upregulating the cellular levels of transcription factors acting on the viral LTR. The association of Tat with phosphorylated C/EBP on the IL-6 promoter increases the DNA-binding activity of C/EBP dimers by enhancing their affinity for the target DNA. In this regard, the identity of the specific phosphorylated residue within C/EBP/β and whether phosphorylation of C/EBP/β by cdk9 leads to an increase of C/EBP/β-binding activity remain to be clarified.

Our results also show that CHOP, a member of the C/EBP family, which was known as an inhibitor of C/EBP, can function as an inhibitor of HIV-1 gene expression and replication. In this regard, one may suggest that interaction of C/EBP/β with CHOP might prevent cdk9–C/EBP/β association. Finally, it is possible that cdk9 facilitates cross-communication of C/EBP with Tat or cellular transcription initiation factors. This observation is in accord with studies describing the ability of C/EBP/β to interact with components of the basal transcriptional machinery such as the TATA-binding protein and transcription factor IIB (Nerlov & Ziff, 1995) and other transcription factors, including NF-κB and the cAMP response element-binding protein (Hohaus et al., 1995; Westendorf et al., 1998). These proteins may cooperate in the recruitment of co-activators to the HIV-1 LTR in cells.

In summary, we demonstrated that C/EBP/β is necessary for establishing HIV-1 infection of T cells and influencing HIV-1 gene expression through its interaction with cdk9. Further, our data also suggest that phosphorylated C/EBP/β may affect the expression of inflammatory cytokines and, if so, could have an indirect influence on HIV-1 disease progression. Finally, our results provide insight into the general mechanisms by which C/EBP/β regulates HIV-1 transcription through the recruitment of Tat activators to the LTR (e.g. cdk9).

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