Cooperative Interaction between HIV-1 Regulatory Proteins Tat and Vpr Modulates Transcription of the Viral Genome*

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The virion-associated protein of human immunodeficiency virus, type 1 (HIV-1), Vpr, is a small protein with 96 amino acid residues that has the ability to modulate transcription of HIV-1 long terminal repeat (LTR) promoter activity and affects several cellular functions. In this study we have employed molecular approaches to further investigate the mechanism by which Vpr exerts its regulatory effect upon the LTR. We show that by structural and functional interaction with Tat, a potent viral regulatory protein, Vpr synergistically enhances the transcriptional activity of the HIV-1 LTR. Because Tat utilizes cyclin T1 and its partner, CDK9, to elevate the level of transcription from the LTR, we examined the cooperativity between Vpr, Tat, and cyclin T1/CDK9 on viral gene transcription. Results from co-transfection studies indicated superactivation of LTR by Tat and cyclin T1/CDK9 in the presence of wild type Vpr. This activation was not observed with the R73S mutant of Vpr, which contains arginine to serine transition at residue 73. Interestingly, expression of R73S mutant in cells exerts a negative effect on the observed superactivation of the LTR by Tat, cyclin T1/CDK9, and wild type Vpr. Results from protein-protein interaction studies indicated that Vpr is associated with both Tat and cyclin T1 in cells expressing these proteins. Use of deletion mutant proteins in binding studies revealed that the binding sites for Tat and Vpr within cyclin T1 are distinct and that association of these two viral proteins with cyclin T1 is independent from each other. These observations suggest a working model on the cooperative interaction of Vpr with viral and cellular proteins and its involvement in control of viral gene transcription and replication. Moreover identification of R73S mutant of Vpr provides a new therapeutic avenue for controlling HIV-1 gene transcription and replication in the infected cells.

Vpr represents one of the HIV-1 accessory gene products which is composed of 96 amino acid residues. Because of its association with the p6 region of the p55<sup>env</sup> precursor (1), Vpr is efficiently packaged into virions (2). Vpr has been considered a late gene product, although recent studies have implicated a functional role at the immediate-early phase during HIV-1 infection (3).

Vpr enhances viral replication in nondividing macrophages and causes proliferating human cells to undergo an arrest or delay in the G<sub>2</sub> phase of the cell cycle (4–6). This latter property of Vpr serves to amplify the activity of the viral promoter and enhances the replication of HIV-1 (7–9). The mechanisms by which Vpr modulates progression of the cell cycle and transcriptional activation of HIV-1 are not fully understood. Many of the determinants of cell cycle arrest reside in the arginine-rich COOH-terminal portion of the Vpr protein (10, 11). This COOH-terminal region most closely resembles a canonical nuclear localization signal; however, this region can be deleted with no impairment of nuclear localization (10).

The activation of the HIV-1 LTR by Vpr was shown to be mediated, at least in part, through its direct interaction with the cellular transcription factor Sp1, which binds to the GC-rich DNA sequence of LTR (12, 13). Direct interaction of Vpr with DNA and RNA has been reported (14). Vpr also associates with a number of cellular factors including p300 and TFIIIB and modulates the activity of the HIV-1 LTR (13, 15, 16). Interestingly, some of these factors also cooperate with the viral potent transcriptional activator, Tat (17, 18). Earlier studies have shown that Tat activation of LTR transcription is mediated through its binding to TAR RNA sequence located at the 5′ end of nascent viral transcripts (19). Subsequent studies have demonstrated that Tat has the ability to enhance HIV-1 LTR activity in the absence of TAR in cells derived from the central nervous system as well as in lymphoid cells under certain physiologic conditions (20, 21). Further studies have revealed that TAR-independent activation of the LTR can be mediated by NF-κB proteins (21, 22).

Tat also binds with high affinity and specificity to cyclin T1, an event that is believed to induce the elongation process of RNA polymerase II (23, 24). The 87-kDa cyclin T1 binds to the cyclin-dependent kinase, CDK9 (PITALRE) to form a complex called p-TEFb (positive transcription elongation -factor b) (25). According to one model, although Tat/p-TEFb complexes bind to TAR, CDK9 phosphorylates the carboxyl-terminal domain of RNA polymerase II, and that enhances the level of viral gene transcription (26).

Because Tat and Vpr are involved in the activation of the HIV-1 LTR through either the interaction with several common cellular regulatory proteins (13, 15–18) and/or binding to an RNA molecule (14, 27), we focused our attention on the structural and functional interplay between these two viral proteins. In this study we provide evidence for the physical interaction of Vpr with Tat and demonstrate that cooperativity between Tat,
Vpr, cyclin T1, and CDK9 is functionally significant because it leads to up-regulation of viral gene transcription.

**MATERIALS AND METHODS**

*Plasmids—* HIV-1 LTR Luciferase reporter plasmid was a gift from Nicole Israel (Pasteur Institute). To construct CMV-HA-Tat, a 300-bp Tat cDNA from pcDNA3-Tat was excised upon treatment of the DNA with BamHI-EcoRI, and the fragment was inserted into HindIII-BamHI sites of pcDNA3-HA. The plasmid pE BV-His B-Vpr contains the coding region of the Vpr gene cloned downstream of a histidine epitope tag. HIV-1 glutathione S-transferase (GST)-Tat expression vectors (1–86), (1–72), (1–48), and (Δ2–36) were obtained from the AIDS Research and Reference Reagent Program of the National Institutes of Health (Bethesda, MD). GST-Tat (1–67), (1–50), (1–40), (20–72), (30–72), (40–72), and (50–72) were a gift from K.T. Jeang (Laboratory of Molecular Microbiology, NIAID, National Institutes of Health, Bethesda, MD). PGEX-2T-Cyclin (wild type and deletion mutants) were described by Fujinaga et al. (28). pcDNA3-C3DK9 and GST-CDK9 were a gift from A. Giordano (Thomas Jefferson University, Philadelphia, PA). CMV-Cyclin T1 and CMV-HA-Cyclin T1 full-length constructs were a gift from D. H. Price (University of Iowa, Iowa City, Iowa) and K. Jones (The Salk Institute for Biological Studies, La Jolla, CA). To create a chimeric reporter plasmid of the wild-type Tat DNA fragment from CMV-Tat R73S was inserted in place of EcoRI-SalI sites of the wild type proviral pNL4–3. To construct EYFP-Tat, a 300-bp cDNA was excised with BamHI-EcoRI from pcDNA3-Tat and inserted into BglII-EcoRI sites of EYFP. To generate ECFP-Vpr wild type, a 300-bp cDNA was excised with BamHI-HindIII from pGEX-2T-Vpr (gift from Joseph Kulkowski, Thomas Jefferson University, Philadelphia, PA) and inserted into BglII-HindIII sites of ECFP.

**Cell Culture, Transfection, and Luciferase Assay—** Purified primary human fetal central nervous system cultures were prepared from 12–16-week-old human fetal brain tissue (purchased from Advanced BioScience Resources, Inc, Alameda, CA) by a modified procedure based on the methods of Cole and de Vellis (29) and Yong and Antel (30). Human astrocytic glial cell line U-87MG was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). Human lymphocytic T-cells, CEMX174, were maintained in RPMI 1640 supplemented with 10% fetal calf serum. Cells (5 x 10^6) cultured on 60-mm plates and grown overnight were transfected by the Lipofectamine method for primary human fetal central nervous system cultures or by DEAE-dextran method for CEMX174. 1-ml aliquots of reporter plasmid (LTR-luciferase) or co-transfected with reporter DNA and 0.5–0.75 μg of various expression vectors. To control for transfection efficiency, plasmids encoding HA-Tat (CMV-HA-Tat), GST-Tat (1–67), (1–50), (1–40), (20–72), (30–72), (40–72), and (50–72) were transfected with pcDNA-HA-Tat 86. 48 h post-transfection, cell extracts were prepared 48 h after transfection, and Luciferase assays were performed (Promega, Madison, WI).

**In Vivo and in Vitro Production of Proteins—** HIV-1 GST-Tat, GST-Vpr, and GST-cyclin T1 fusion proteins were prokaryotically expressed and purified as described (31). The integrity and purity of the GST fusion proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. Known amounts of bovine serum albumin were included on the same gel for determination of the yield of the full-length proteins. Radiolabeled Vpr, cyclin T1, and Tat proteins were synthesized with the TNT-coupled wheat germ extract system according to the manufacturer’s recommendations (Promega, Madison, WI).

**In Vitro Protein-Protein Interactions (GST Pull-down Assay)—** For in vitro binding assays, 3 μl of radioactively labeled cDNA translated Vpr, cyclin T1, or Tat were incubated with 5 μg of GST or fusion proteins GST-Tat (full-length or deletion mutants), GST-Vpr (wild type or mutant), or GST-cyclin T1 (full-length or deletion mutants), coupled to glutathione-Sepharose beads in 300 μl of LB 150 for 1 h at 4 °C with continuous rocking. After the incubation, the beads were pelleted and washed five times with LB 150 buffer. The bound proteins were eluted with Laemmli sample buffer, were heated to 95 °C for 10 min, and were separated by SDS-polyacrylamide gel electrophoresis.

**Co-immunoprecipitation and Western Blotting—** U-87MG cells were transfected with 5 μg of a plasmid encoding wild type pE BV-His B-Vpr and/or 5 μg of a plasmid encoding pcDNA-HA-cyclin T1 and/or 5 μg of a plasmid encoding pcDNA-HA-Tat 86. 48 h post-transfection, cell extracts (300 μg/immunoprecipitated sample) derived from the transfected cells were subjected to co-immunoprecipitation with a T7-antibody, which recognizes the His portion in pE BV-His B-Vpr, or with anti-HA (12CA5, from Roche Molecular Biochemicals), which recognizes the HA portion of either pcDNA-HA-cyclin T1 or Tat. The procedure used in Western blots has been described previously (12). p24 ELISA—p24 antigen capture ELISA for tissue culture supernatants was performed as described by the manufacturer (Coulter-Immunotech). Each sample was assayed over 10,000-fold range of dilution to ensure quantitation was based on an OD value within the linear range of the standards.

**RESULTS**

**Functional Interaction of Tat and Vpr—** We utilized primary culture of human microglial cells to investigate the transcriptional activity of the HIV-1 LTR. As shown in Fig. 1, A, expression of Tat significantly enhanced transcription of the viral promoter in these cell cultures (compare lane 1 with lane 2). Similarly, transfection of these cells in the presence of Vpr alone caused a modest activation of HIV-1 LTR (lane 3). Co-expression of Vpr and Tat led to a synergistic activation of viral transcription (lane 4), suggesting that these two viral proteins cooperate with each other to enhance the function of the HIV-1 LTR.

We determined whether enhanced activation of HIV-1 LTR by Tat and Vpr is mediated through direct interaction between Tat and Vpr proteins. First, we examined the ability of these two proteins to interact with each other. When Tat and Vpr were radiolabeled with 35S by in vitro translation and incubated with either GST, GST-Vpr, or GST-Tat fusion protein coupled to glutathione-Sepharose beads, we found that 35S-labeled Tat was retained by the GST-Vpr fusion protein (Fig. 1B), and 35S-labeled Vpr was retained by the GST-Tat fusion protein (Fig. 1C). The control GST alone was unable to retain either Tat or Vpr (Fig. 1B and C). These data permit the conclusion that these two proteins interact with each other in an in vitro system.

A similar binding assay was performed with deletion mutants of Tat, and the results revealed that the region responsible for binding of Vpr is located between amino acids 50–67 of the Tat protein (data not shown). This domain has been shown to serve as the binding site for several cellular factors including Sp1 and PurA (18, 32) and is important for Tat nuclear localization (27, 33).

Previous studies have shown that ectopic expression of Vpr in cells results in an accumulation of this protein in the nucleus and the nucleolus (33), whereas wild type Vpr localizes to the nucleus and, to a lesser extent, to the cytoplasm (34). To determine subcellular localization of these two proteins in the cells expressing both proteins, human astrocytic glial cells, U-87MG were transfected with various DNA plasmids expressing Tat or Vpr fused to either the yellow or cyan fluorescent proteins, respectively (EYFP and ECFP). As shown in Fig. 1D, EYFP-Tat was found only in the nucleus (top panel), whereas ECFP-Vpr is localized in the nucleus as well as in the cytoplasm (middle panel). As shown in the bottom panel, Tat and Vpr fusion proteins were detected in the same cell compartment. The control EYFP and ECFP proteins exhibited no evidence of subcellular localization (data not shown).

**Involvement of p-TEFb Complex and Vpr in Activation of HIV-1 LTR—** As described earlier, cyclin T1 binds to CDK9 to form the pTAEf-b complex (23, 35). As a part of this complex, cyclin T1 is involved in the phosphorylation of the carboxyl terminus of RNA polymerase II, which is important in the elongation process of LTR transcription mediated by Tat (23, 36). Because the later event requires the interaction between Tat and cyclin T1, we determined whether this binding is affected by Vpr. Plasmids encoding HA-Tat (CMV-HA-Tat),
HA-cyclin T1 (CMV-HA-cyclin T), or His-Vpr (EBV-His-Vpr) were introduced into U-87MG cells. Extracts of transfected cells were obtained 48 h after transfection and were co-immunoprecipitated with either T7-antibody, which recognizes the His-tag (Fig. 2A), or with monoclonal anti-HA antibody (Fig. 2B). Preimmune serum was used as a control for nonspecific binding (Fig. 2, A and B, lanes 2 and 5). The specificity of the antibodies was confirmed by the use of nuclear extracts from nontransfected cells (Fig. 2, A and B, lane 3) or nuclear extracts from cells transfected with either Vpr (Fig. 2A, lane 9) or cyclin T1 (Fig. 2B, lane 9). As anticipated, Tat was associated with cyclin T1 (Fig. 2A, lane 7), and surprisingly, Vpr was also able to bind Tat and cyclin T1 (Fig. 2, A, lanes 6 and 8, and B, lanes 6–8). These results permit the conclusion that Vpr, in addition to its already established functions, plays a role in the formation of a ternary complex, Tat-Vpr-cyclin T1, and might act as an anchor between Tat and cyclin T1. Furthermore, Vpr is required for optimal transactivation by Tat and therefore might play a role at the early phase of infection. This is in part supported by previous observations, suggesting a potential role for Vpr in the early phase of infection (3).

The formation of a ternary complex prompted us to investigate the implication of these observations in the regulation of LTR transcription. As before, Tat significantly stimulated transcription of HIV-1 LTR in human primary microglial cells (Fig. 2C, lane 2). Transfection of microglial cells in the presence of plasmids expressing Tat and cyclin T1 or expressing Tat and CDK9 enhanced the level of HIV-1 LTR transcription about 125- and 100-fold, respectively (Fig. 2C, lanes 4 and 5). In the presence of three expressor plasmids, Tat, cyclin T1, and CDK9, LTR activity was further elevated (135-fold) (Fig. 2C, lane 6).

Vpr stimulated, albeit modestly, transcription of the HIV-1 LTR in human primary microglial cells (Fig. 2C, lane 3). Transfection in the presence of the plasmids expressing Vpr and cyclin T1 or expressing Vpr and CDK9, augmented the HIV-1 LTR activity about 100- and 150-fold, respectively (Fig. 2C, lanes 10 and 11, respectively). The activity of the HIV-1 LTR was superenhanced (about 900-fold) when all the expression vectors for Tat, Vpr, cyclin T1, and CDK9 were co-introduced into the cells (lane 12). These data support the conclusion that these proteins work together to modulate the function of HIV-LTR. Evidently, functional interaction of Vpr with either cyclin T1 or CDK9, but not cyclin T1 plus CDK9, results in stimulation of Vpr-mediated transcription of LTR in microglial cells. In the presence of Tat,
transfected with plasmids expressing wild type Vpr fused to T7-His or Tat fused to HA or Cyclin T1 fused to HA. Approximately 300 μg of extract were utilized in immunoprecipitations followed by Western blot utilizing anti-HA (lanes 1–9) and anti-T7 (lanes 1–9) antibodies. In parallel, 100 μg of extracts were utilized by direct Western blot assay (lanes 1 and 4 of each panel). Preimmune serum was used to verify the specificity of binding (lanes 2 and 5 of each panel). Arrows depict the positions of the 87-kDa Cyclin T1 and 14-kDa Vpr. The immunoprecipitation (I.P.) and Western blot analyses were carried out according to the procedure described previously (12). The asterisks in A and B depict the positions of the nonspecific bands. C, transcriptional activity of Vpr, CDK9, Cyclin T1, and Tat upon the HIV-1 LTR promoter. The human primary microglial cells were transfected with 0.1 μg of the reporter LTR-Luciferase plasmid (LTR-Luc) alone or combined with 0.5 μg of either Vpr, CDK9, cyclin T1, or Tat expression plasmids in various combinations. Luciferase activity was determined 48 h after transfection. The values shown on the top of each bar represent the fold activation over the basal HIV-1 LTR promoter arbitrarily set at one. The data represent the mean values of at least three separate transfection experiments.

however, Vpr has the ability to cooperate with both cyclin T1 plus CDK9 and synergizes the stimulatory effect of these proteins upon LTR transcription.

Mapping of Vpr-binding Site within Cyclin T1—To gain more information regarding the interaction between Vpr, Tat, and the cyclin T1/CDK9 complex, we performed binding experiments to determine whether Vpr and Tat share a common binding site on cyclin T1. The region encompassing amino acids 250–300 within the cyclin T1 protein has been previously shown to be important for complexation between Tat and cyclin T1 (28). Full-length cyclin T1 protein and its deletion mutant proteins were utilized in GST pull-down assay. We found that 35S-labeled Tat was retained by the GST-cyclin T1 (1–726), (1–479), and (1–300) fusion proteins and not by GST-cyclin T1 (1–250) fusion protein (Fig. 3). Further, 35S-labeled Vpr was retained by the GST-cyclin T1 (1–726) and (1–479) fusion proteins and not by GST-cyclin T1 (1–300) or (1–250) fusion proteins (Fig. 3). GST alone was unable to retain either Tat or Vpr. Reciprocal experiments utilizing radiolabeled cyclin T1 and its mutant variants in GST pull-down assay using GST-Tat and GST-Vpr confirmed these observations and indicated that the region encompassing amino acids 300–479 is important for cyclin T1-Vpr interaction. As seen, the binding region of Vpr-cyclin T1 is different from the one described previously for Tat-cyclin T1 (28). Therefore, we conclude that Tat and Vpr may bind to cyclin T1 separately and independent of each other.

Role of Vpr (R73S) Mutant—In previous studies we demonstrated that mutant R73S of Vpr can function as a transdominant protein and inhibit the effect of wild type Vpr on either the regulation of the cell cycle or the activation of HIV-LTR (37, 38). Here, we evaluated the ability of R73S in cooperating with cyclin T1, CDK9, and Tat in modulating HIV-1 LTR activity and viral replication.

First, we determined the ability of this mutant to bind either Tat or cyclin T1. Tat was radiolabeled with 35S by in vitro translation and incubated with either GST or GST-Vpr (R73S) fusion protein coupled to glutathione-Sepharose beads. The GST-Vpr (R73S) fusion protein retained 35S-labeled Tat (Fig. 4A, lane 4); GST alone failed to retain 35S-labeled Tat (Fig. 4A, lane 3). Reciprocally, R73S was radiolabeled with 35S by in vitro translation, and incubated with GST, GST-CDK9, GST-cyclin T1, or GST-Tat fusion proteins, coupled to glutathione-Sepharose beads. As shown in Fig. 4B, The GST-cyclin T1 and GST-Tat fusion proteins retained 35S-labeled mutant Vpr (Fig. 4B, lanes 5 and 6) but not GST alone or GST-CDK9 (Fig. 4B, lanes 3 and 4).

We then determined the ability of mutated Vpr (R73S) to affect the activation of the HIV-1 LTR. A series of transient transfection assays were performed with primary cultures of human microglial cells (Fig. 4C). The cells were transfected with LTR-luciferase alone or with expression vectors for wild type Vpr, Tat, cyclin T1, CDK9, and Vpr mutant, separately or in various combinations. Cell extracts were prepared 48 h after transfection, and luciferase assays were performed. Surprisingly, mutant R73S was able to inhibit transcription of the HIV-1 LTR in every case (lanes 6, 11, 12, 13, 15, and 16). This is an interesting observation in light of the protein binding data shown in A and B of Fig. 4, suggesting that although mutant R73S retains its ability to interact with Tat and cyclin T1, unlike the wild type Vpr, this mutant may not positively cooperate with these activators and synergize their activities. In contrast, it is evident that the functional interaction of R73S with Tat and its partner cyclin T1 abrogates their stimulatory effect upon the LTR transcription.

Finally, we determined the effect of R73S on the expression of the HIV-1 LTR in infected cells. The three proviral genomes, pNL4–3 wild type, pNL4–3-Vpr (R73S), and pNL4–3 ΔVpr,
were introduced into lymphocytic T-cells, CEMx174, along with LTR-luciferase plasmid. The effects of wild type or mutant Vpr were evaluated through either luciferase (Fig. 4D) or a p24 antigen ELISA (Fig. 4E). As expected the proviral DNA pNL4–3 lacking Vpr failed to activate expression of the HIV-1 LTR to the same level as pNL4–3 (Fig. 4D, compare lanes 1 and 2). Viruses released into the medium were collected 3, 5, 7, 9, 11, 13, and 15 days post-transfection and quantitated by a p24 antigen ELISA (Fig. 4E). The chimeric virus pNL4–3 Vpr mutant (R73S) reduced the level of p24 by 75% (Fig. 4E). This reduction was observed for as long as 15 days post-transfection.

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post-transfection and quantitated by p24 antigen ELISA (Fig. 4E). The chimeric virus pNL4–3 Vpr mutant (R73S) reduced the level of p24 by 75% (compare open circles to solid symbols). This reduction was observed for as long as 15 days post-infection.

**DISCUSSION**

In this work, we show that Tat can interact with Vpr and that their complexation may be physiologically important for optimal replication of HIV-1. Interestingly, Vpr also interacts with the cellular protein, cyclin T1, which plays a role in Tat-induced transcription of the viral LTR. Moreover, our results have shown a contribution of wild type Vpr and cyclin T1 toward Tat transactivation activity. Further, the activation of HIV-1 LTR by Vpr appears stronger in the presence of cyclin T1 and Tat. By binding to cyclin T1, Vpr adds a new role to its functions. As a part of the p-TEFb complex, cyclin T1 has been shown to be involved in phosphorylation of the carboxyl-terminal functions. As a part of the p-TEFb complex, cyclin T1 has been shown to be involved in phosphorylation of the carboxyl-terminal functions. As a part of the p-TEFb complex, cyclin T1 has been shown to be involved in phosphorylation of the carboxyl-terminal functions. As a part of the p-TEFb complex, cyclin T1 has been shown to be involved in phosphorylation of the carboxyl-terminal functions. As a part of the p-TEFb complex, cyclin T1 has been shown to be involved in phosphorylation of the carboxyl-terminal functions.