**HIV-1 Tat Elongates the GI Phase and Indirectly Promotes HIV-1 Gene Expression in Cells of Glial Origin**

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**Abstract**

Human immunodeficiency virus type-1 (HIV-1) infection of the central nervous system (CNS) gives rise to many of the neurological complications in patients with AIDS. Infection of microglial cells and astrocytes in the brain promotes the release of HIV-1 Tat and other candidate neurotoxins that may be associated with the widespread neuropathology. To examine the contribution of HIV-1 Tat to the interplay between virus and CNS cells, the human astrocytic cell line, U-87MG, was treated with recombinant Tat protein. Fluorescence-activated cell sorting analysis indicated that Tat induces a GI arrest in these cells. Consistent with this observation, lower levels of cyclin E-Cdk2 kinase activity and phosphorylated Rb were detected in the Tat-treated cells compared with the control cells. Interestingly, our observations indicate that the underphosphorylated form of Rb that is prevalent in Tat-treated cells promotes HIV-1 transcription by a mechanism involving the NF-kB enhancer region. Taken together, the data presented here provide the first evidence that the HIV-1 regulatory protein, Tat, may manipulate the host cell cycle to promote viral gene expression.

These findings relate to the current hypothesis that indirect effects of HIV-1 infection of the CNS may contribute to the neurological complications associated with AIDS dementia complex.

Neuropathological features of HIV-1 infection include reactive astrogliosis, neuronal loss, widespread myelin pallor, subtle alteration of neocortical dendritic processes, and formation of multinucleated giant cells (1–3). The magnitude of the clinical dysfunction and CNS pathology associated with HIV-1 infection is difficult to reconcile with the small number of HIV-1-infected macrophages and resident microglia, as well as infected astrocytes in the brain (7–12). Earlier studies indicated that Tat may be released from infected cells, be taken up by uninfected neighboring cells, and exert its regulatory action (13, 14). Tat is an accessory protein that stimulates HIV-1 expression and has a pleiotropic effect on cells, ranging from stimulating cell proliferation to inducing apoptosis, depending on the cell type (14–25). Astrocytes secrete many supporting growth factors and are involved in neurotransmitter uptake and in maintaining the integrity of the blood-brain barrier. Thus, biological agents that alter the proliferation and activation state of these cells may lead to a broad spectrum of abnormalities and dysfunction (26).

Earlier observations showed that overexpression of Tat in astrocytic cells and treatment of cells with extracellular Tat can stimulate expression of several important cellular genes, including cytokines and extracellular matrix proteins (27–30). These observations led us to the hypothesis that Tat may alter the activation and proliferation state of astrocytes and contribute to the pathogenesis of AIDS-associated dementia.

The reciprocal nature of the interaction between virus and host is expected, since HIV-1 is susceptible to regulation by cellular factors and therefore by the state of the host cell. There is evidence to suggest that cellular factors, including B-myb, E2F-1, and p53, which are involved in the control of cellular proliferation, may play a role in modulation of HIV-1 gene expression (31–33). Normal cellular proliferation occurs through an orderly progression of positive and negative regulatory events and is orchestrated by the activity of complexes consisting of cyclins and their associated catalytic partners, the cyclin-dependent kinases (34–37). During the G1/G2 phase, the decision of cells to commit to the cell cycle is partly dependent on the appropriate activation of G1 cyclin-Cdk complexes by extracellular stimuli. One of the most well characterized targets of these G1 cyclin-Cdk complexes is the retinoblastoma protein, Rb (38–40). Early in the G1 phase, Rb exists primarily in an underphosphorylated state, at which time it interacts with the transcription factor, E2F-1, and inhibits its growth-promoting function (41–44). Phosphorylation of Rb in late G1 by the G1 cyclin-Cdk complexes results in the release of free E2F-1, which activates transcription of several genes involved in S phase (45). In this study, we sought to further examine the interplay between virus and host cell cycle progression. We demonstrate that the viral transactivator protein, Tat, is able to arrest human astrocytic cells, U-87MG, in the G1 phase of the cell cycle by dysregulating the expression and activity of cyclin E and Cdk2. This results in accumulation of Rb in its underphos-
phorylated form, which in turn augments transcription directed by the HIV-1 LTR. We propose that in addition to its ability to directly transactivate the HIV-1 LTR, Tat alters the proliferation state of astrocytes and facilitates expression and replication of the HIV-1 genome.

EXPERIMENTAL PROCEDURES

Fluorescence-activated Cell Sorting Analysis—Low passage human astrocytic cells, U-87MG, obtained from ATCC were maintained at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Life Technologies, Inc.). Cells were synchronized in G0/G1 by incubating in serum-free media for 60 h and subsequently stimulated by the addition of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 μM chloroquine, and 200 ng/ml glutathione S-transferase (GST) or GST-Tat (27). GST fusion proteins were prepared as described previously (46). The transactivating potential of GST-Tat preparations was verified by the addition of Tat into the media of cells transfected with the HIV-LTR-CAT construct (data not shown) prior to use in experimental assays. At various time points after stimulation, cells were harvested by trypsinization, washed in PBS, and stored in 70% ethanol/PBS. Samples were treated with RNase (Sigma) and stained with propidium iodide before analysis with a fluorescence-activated cell sorter (Multicycle-Phoenix Flow System) using the Cycle Test Plus Kit (Coulter).

Northern Blot Analysis—Total cellular RNA was extracted from cells by the guanidinium-isothiocyanate method described previously (47). 20 μg of total RNA was fractionated by electrophoresis through a 1% formaldehyde-agarose gel and transferred onto nitrocellulose membranes (Hybond N, Amersham Pharmacia Biotech). The membranes were probed with the 1.2-kilobase pair of cyclin E and the 0.9-kilobase pair of cyclin H1. The blots were hybridized by overnight incubation at 42 °C in 5× SSC, 1× Denhardt’s solution, 300 μg/ml denatured salmon sperm DNA, 50% deionized formamide (pH 6.0), and 0.5% SDS with 1× 106 cpm/ml denatured radiolabeled DNA probe. After the membranes were washed once in 2× SSC and 0.1% SDS for 15 min at room temperature and twice in 0.2× SSC and 0.1% SDS at 42 °C, they were exposed to X-Omat AR (Eastman Kodak Co.) or XAR-Paji film at −70 °C with an intensifying screen. Quantitative evaluation of the transcripts was carried out by densitometric scanning of the band corresponding to cyclins and Cdkks and after normalizing to the control GAPDH RNA levels expressed in arbitrary densitometric units.

Histone H1 Kinase Assay—Kinase activity was assayed as described previously (60). 300 μg of nuclear extract was incubated overnight at 4 °C with rabbit polyclonal antibodies specific for human cyclin E (sc-198, Santa Cruz Biotechnology, Inc.) and human Cdk2 (sc-163, Santa Cruz). Protein A-Sepharose (Sigma) was used to precipitate the (-117/+3 CAT construct and ligating the fragment into the Xbal–HindIII sites of the pGL3 vector (Promega). Luciferase activity was measured according to the manufacturer’s instructions using a scintillation counter (Perkin-Elmer). The following expression plasmids were used: CMV-neo, CMV-Rb, pSVE, pSVE-Rb WT, and pSVE-Rb Mut (Δ775–814) (51). The pSVE-derived plasmids were kindly provided by Dr. D. Templeton (Case Western Reserve University).

RESULTS

Tat Promotes Elongation of the G1 Phase in Glial Cells—HIV-1 Tat is an 86-amino acid viral protein that activates HIV-1 transcription by binding to the transacting response region in the HIV-1 LTR and promoting efficient elongation of viral transcripts (52). Tat is also a potent transactivator of cellular gene expression. Earlier studies indicated that Tat can stimulate the expression of extracellular matrix proteins, including fibronectin and collagen and cytokines, including transforming growth factor-β, tumor necrosis factor-α, IL-1, and IL-6 (27, 29, 30, 53–59). Transforming growth factor-β is a pleiotropic cytokine that can inhibit proliferation of astrocytes and induce morphological changes characteristic of hypertrophy (60–62). IL-1 and tumor necrosis factor-α stimulate proliferation of astrocytes (63–65) and can counteract growth-promoting effects of other cytokines and inhibit the proliferation of human glioblastoma cells (61, 66, 67). Since Tat can induce the expression of cytokines that can exert positive and negative effects on the growth of astrocytic cells, we decided to examine the effect of Tat on astrocyte proliferation. Fluorescence-activated cell sorting analysis of synchronized U-87MG cells harvested at various intervals after stimulation with 10% serum and 200 ng/ml GST revealed that the normal length of the U-87MG cell cycle is approximately 24 h, with the majority of cells (greater than 60%) at S phase within 20 h (Fig. 1A). By contrast, U-87MG cells treated with highly purified, biologically active recombinant Tat (GST-Tat) failed to advance into S phase, and nearly 62% of cells were retained in G1 phase at 24 h poststimulation (Fig. 1B). While 27% of untreated cells were at G1 phase, 58% of Tat-treated cells were found in this phase, suggesting that Tat can delay or perhaps inhibit the progression of cells from G1 into S phase. Under similar conditions, mutant Tat fusion protein, which contains the first 48 amino acid residues of the protein, showed no effect on cell cycle progression of the treated cells,2 indicating that the observed effect is specific and mediated by full-length Tat protein. Although the primary block occurs in G1, Tat may have additional effects in S phase. In addition, a peak suggestive of apoptosis appeared at 24 h poststimulation in Tat-treated cells, reminiscent of previous observations indicating that HIV-1 Tat can induce apoptosis in uninfected lymphoid cells (19, 20, 22, 24).

Tat Dysregulates Expression and Activity of Cyclin E and Cdk2—Normal transit of cells from the G1/G0 into S phase relies on appropriate expression and activation of the G1 cyclins and their associated catalytic subunits (34–37). To define the mechanism by which Tat perturbs the cell cycle, we examined the effect of Tat on the expression of various cyclins and Cdkks that are normally active during the G1 and S phases. Northern blot analysis indicated abnormal accumulation of both cyclin E and Cdk2 mRNA in Tat-treated cells, as compared with untreated cells. Consistent with previous reports (68), the level of cyclin E mRNA peaked sharply during mid-G1 (8–10 h) in control cells (Fig. 2A, open bars). By contrast, Tat-treated cells exhibited a slow but progressive increase in

2 S. Ansari and K. Khalili, unpublished observations.
the level of cyclin E transcripts (Fig. 2A, solid bars). The expression of Cdk2 was also drastically altered in Tat-treated cells, as compared with untreated cells. The levels of Cdk2 mRNA in Tat-treated cells peaked within 2–4 h after stimulation and then diminished (Fig. 2B, solid bars), instead of peaking in mid-G1 phase (8–10 h) and then remained relatively constant (Fig. 2B, open bars). The differential expression of other cyclins, including cyclins A, B, and D was not significantly altered by the addition of Tat into the media (data not shown). Considering the abnormal expression of cyclin E and Cdk2 upon treatment with recombinant Tat protein, it was of interest to examine the effect of Tat on the kinase activity associated with cyclin E and Cdk2 at various intervals after stimulation of the G0/G1-synchronized U-87MG cells. The kinase activity of immunocomplexes from control cells obtained by antibodies against both cyclin E (Fig. 3A) and Cdk2 (Fig. 3B) peaked in mid-G1 (8 h) and disappeared by the time the cells were primarily in G2/M (24 h) (lanes 1, 2, 4, and 6). The cyclin E-associated kinase activity isolated from Tat-treated cells exhibited no drastic changes at 8 h poststimulation and remained virtually at a constant level through 24 h poststimulation (Fig. 3A, lanes 1, 3, 5, and 7). The Cdk2-associated kinase activity derived from Tat-treated cells, however, followed a pattern of expression similar to that of the control cells, although the levels of activity were noticeably lower than in untreated cells (Fig. 3B, lanes 1, 3, 5, and 7). We found no drastic alterations in the kinase activity of other cyclins including cyclin A and Cdk4 upon Tat treatment of the cells.\(^3\)

Tat Prevents Phosphorylation of Rb—The regulated expression of cyclins and Cdkks is crucial for normal cell division to occur. Overexpression of cyclin E, for example, can promote transformation of immortalized cells and has been associated with the incidence of gastric and breast carcinomas (69, 70). At the same time, inhibition of the catalytic activity of the cyclin E-Cdk2 complex by overexpression of Cdk inhibitors, including p21 and p27 can promote cell cycle arrest (71). One function of the cyclin E-Cdk2 complex that directly influences its role in regulation of the cell cycle is phosphorylation and inactivation of the tumor suppressor protein, Rb (39). In light of the observation that Tat causes aberrant expression of cyclin E and Cdk2, we sought to examine the effect of Tat treatment on phosphorylation of Rb. Results from Western blot analysis of nuclear extracts from control cells indicated that the levels of hyperphosphorylated form of Rb, ppRb, increased by 8 h after stimulation with serum (Fig. 4, lanes 1, 2, 4, and 6). The presence of a low level of phosphorylated Rb in serum-starved cells prior to stimulation may stem from the fact that the synchronization process is not absolute and a percentage of cells are not in G0 or early G1. In Tat-treated cells, however, a progressive decrease in ppRb was detected at 8, 16, and 24 h poststimulation (Fig. 4, lanes 1, 3, 5, and 7). These observations are in accord with the hypothesis that G1 arrest may result from decreased activity of the cyclin E-Cdk2 complex, which allows Rb to remain in its active underphosphorylated form.

**HIV-1 Transcription Is Activated by Rb through the NF-κB Enhancer Sequence**—When Rb is underphosphorylated, it interacts with several proteins involved in regulating cell proliferation and differentiation, including E2F-1 (41–44). Earlier studies indicated that E2F-1 represses HIV-1 gene expression by binding to a site embedded within the NF-κB enhancer region of the HIV-1 promoter and interacting with the p50 subunit of NF-κB (32).\(^4\) Since treatment of glial cells with Tat prevents appropriate phosphorylation of Rb and consequently promotes its association with E2F-1, it was of interest to examine the role of Rb in modulating HIV-1 transcription. Overexpression of Rb in U-87MG cells by transient transfection resulted in a mild increase in the activity of the full-length HIV-1 LTR. Of interest, sequential 5' and 3' deletions of the promoter enhanced responsiveness to Rb activation (Fig. 5A). A significant (6-fold) activation of the HIV-1 promoter was observed using a promoter deletion construct containing only the

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\(^3\) S. Sharma and K. Khalili, unpublished data.

\(^4\) Kundu, M., Guermah, M., Roeder, R. G., Amini, S., and Khalili, K. (1997) J. Biol. Chem. 272, 29468–29474.
NF-κB enhancer sequence and the three Sp1 binding sites (Fig. 5A). Removal of the NF-κB binding sites abolished the ability of Rb to activate transcription, suggesting that these sites are important for mediating response to Rb. To determine whether the NF-κB sites could maintain responsiveness to Rb in a heterologous context, we examined the effect of Rb overexpression on the activities of the κB-WAPCAT and GC-WAPCAT constructs, which contain the HIV-1 enhancer sequence (two NF-κB binding sites) or the HIV-1 GC-rich region (three Sp1 binding sites) inserted upstream of the heterologous promoter derived from whey-acidic protein gene. As shown in Fig. 5B, the NF-κB binding sites, but not the Sp1 binding sites, conferred responsiveness to Rb. Furthermore, when cells were cultured under serum-free conditions after transfection, an increase in NF-κB-mediated activation by Rb was observed, suggesting that the phosphorylation state of Rb may influence its ability to activate transcription (Fig. 5B).

Phosphorylation of Rb Is Not Required for Activation of the HIV-1 LTR—To investigate the significance of Rb phosphorylation in modulating its transcriptional activity further, we used a construct encoding a variant of Rb that lacks four putative C-terminal Cdk phosphorylation sites in transient transfection experiments. The mutated version of Rb transactivated the HIV-1 LTR to a greater extent than wild-type Rb (Fig. 6A). Western blot analysis indicated that in addition to hypophosphorylated (pRb) forms of Rb, the exogenously expressed wild-type Rb can be phosphorylated and form a slower migrating band (ppRb) (Fig. 6B, lane 2). As anticipated, the mutant version of Rb showed no evidence for phosphorylation of pRb, as indicated by the absence of ppRb, suggesting that the mutations effectively prevent its modification (Fig. 6B, lane 3). Fig. 6C indicates that ectopic expression of Rb also promotes HIV-1 transcription in SAOS-2, a cell line that does not express endogenous Rb protein, and cannot phosphorylate exogenously expressed Rb (72). Thus, it appears that underphosphorylated Rb is transcriptionally active with respect to the HIV-1 promoter and that phosphorylation may attenuate the transactivating potential of Rb.

Activity of the HIV-1 LTR Peaks during the G1 Phase—Since the phosphorylation state of Rb is regulated in a cell cycle-dependent manner, we reasoned that the activity of the HIV-1 promoter may be accordingly regulated. Toward this end, cells were transfected with a luciferase-reporter construct containing the minimal Rb-responsive HIV-1 LTR sequences and synchronized in Gα/G1. Cells were subsequently released by the addition of serum. Luciferase assays performed at 4-h intervals beginning at the time of stimulation indicated that the activity of the HIV-1 LTR is highest early in the G1 phase (0–8 h) (Fig. 7, A and B). The increased binding activity of NF-κB during G1 is at least partly responsible for the increased transcriptional activity observed early in G1. The activity of the HIV-1 LTR diminishes as cells progress through G1 and enter S phase (12–20 h) (Fig. 7). This decline in HIV-1 LTR activity occurs during the period when Rb is phosphorylated and releases E2F-1 (41–43). Considering that NF-κB activation of the HIV-1 LTR is repressed by E2F-1, it is possible that Rb modulates the extent of activation by the NF-κB subunits through its association with E2F-1 during the cell cycle. It should be emphasized
that Rb does not modulate NF-κB directly but enables the NF-κB “binding region” by interaction with E2F-1.

**DISCUSSION**

AIDS dementia complex is one of the most prevalent neurological complications of HIV-1 infection of the central nervous system. ADC affects almost 10% of AIDS patients (73). Productive infection in the CNS occurs primarily in macrophages and resident microglia (74, 75). However, there is evidence of a “restricted” infection in astrocytes (76). The term “restricted” is used to describe the restriction of viral gene expression to the regulatory proteins, Tat, Rev, and Nef, which are derived from the multiply spliced viral mRNA species commonly found in infected astrocytes (62). To account for the discrepancy between the small infected cell population and the widespread pathology, the current models regarding the neuropathogenesis of ADC propose a major role for indirect effects of HIV-1 infection (77). In this respect, the infection of astrocytes may play a central role in the pathogenesis of ADC. Infected astrocytes and microglial cells can release the viral protein Tat. Tat can be taken up by neighboring cells in a biologically active form that can stimulate the expression of cytokines, including IL-1, IL-6, tumor necrosis factor-α, and transforming growth factor-β and several extracellular matrix proteins in the CNS (25, 27, 29, 53–59, 78). Tat has also been shown to induce neuronal death in culture (79–81). When injected into the brains of mice, Tat can stimulate edema and gliosis (82). Here, we provide the first evidence that Tat inhibits the proliferation of glioblastoma cells, which are similar in many respects to activated astrocytes. By altering the cellular pathways involved in regulating astrocyte proliferation, Tat has the potential to disrupt the supportive function of astrocytes and contribute to the neuronal loss associated with ADC.

Cellular proliferation is regulated by a series of positive and negative phosphorylation events, many of which involve cyclins and cyclin-dependent kinases. The progression of cells from G1 to S phase relies primarily on the cyclin D-Cdk4, cyclin D-Cdk6, and cyclin E-Cdk2 complex subunits (34–37). In this study, we demonstrate that Tat may block cells in the G1 phase by inhibiting the kinase activity of Cdk2 in astrocytic cells. The dissociation between the cyclin E- and Cdk2-associated kinase...
activity may also contribute to the disruption in the cell cycle. Other groups have demonstrated that Tat may inhibit the proliferative response of T-lymphocytes to antigenic and mitogenic stimuli (16, 18, 20, 23). This negative response is associated with decreased IL-2 production (21). IL-2 decreases the proliferative response of T-lymphocytes to antigenic and mitogenic stimuli.

Phosphorylation of Rb is not required for activation of the HIV-1 LTR. A, U-87MG cells were transfected with 1 μg of the −117/+3 HIV-1 LTR reporter construct and 5 μg of the indicated pSVE expression plasmid. CAT activity is indicated as -fold activation with respect to basal activity derived from cells transfected with pSVE. Data represent an average of three independent experiments. B, whole cell extracts were prepared from cells transfected with pSVE (lane 1), pSVE hemagglutinin-tagged wild type Rb (lane 2), and pSVE hemagglutinin-tagged mutant Rb (D775–817) (lane 3). Extracts were analyzed by Western blot using an antibody directed toward the hemagglutinin-epitope. The phosphorylated (ppRb) and underphosphorylated (pRb) forms of Rb are indicated. C, SAOS-2 cells were transfected with 3 μg of the indicated reporter plasmid and 5 μg of CMV-neo or CMV-Rb. CAT activity is indicated as percentage of acetylation and represents an average of two independent experiments.

FIG. 6. Phosphorylation of Rb is not required for activation of the HIV-1 LTR. A, U-87MG cells were transfected with 1 μg of the −117/+3 HIV-1 LTR reporter construct and 5 μg of the indicated pSVE expression plasmid. CAT activity is indicated as -fold activation with respect to basal activity derived from cells transfected with pSVE. Data represent an average of three independent experiments. B, whole cell extracts were prepared from cells transfected with pSVE (lane 1), pSVE hemagglutinin-tagged wild type Rb (lane 2), and pSVE hemagglutinin-tagged mutant Rb (D775–817) (lane 3). Extracts were analyzed by Western blot using an antibody directed toward the hemagglutinin-epitope. The phosphorylated (ppRb) and underphosphorylated (pRb) forms of Rb are indicated. C, SAOS-2 cells were transfected with 3 μg of the indicated reporter plasmid and 5 μg of CMV-neo or CMV-Rb. CAT activity is indicated as percentage of acetylation and represents an average of two independent experiments.

stimulates HIV-1 transcription. When Rb exists in this form, it can interact with E2F-1 and prevent it from modulating transcription (41–44). Earlier studies demonstrated that the cell cycle regulatory protein, E2F-1, represses the activity of the HIV-1 promoter by binding to a site within the HIV-1 enhancer region and interacting with the 50-kDa subunit of NF-κB (p50) (32). Since the same region of the promoter is targeted by Rb, it is possible that Rb modulates HIV-1 transcription by binding to E2F-1 and inhibiting its repressive activity. It appears that by arresting cells in the G1 phase, Tat is able to maintain cells in a state that is favorable for HIV-1 transcription (Fig. 6).

The observations presented in this study provide the first evidence that HIV-1, akin to the DNA tumor viruses, encodes regulatory proteins that manipulate host cell proliferation to promote viral advantage. Both HIV-1 and the DNA tumor viruses encode proteins that target the activity of the retinoblastoma protein, although to different ends. By preventing Rb phosphorylation, Tat blocks cellular proliferation at the G1 phase. By contrast, E1A, T-antigen, and E7 bind Rb and disrupt its interaction with E2F-1, which promotes entry of cells into the S phase (84). The phase of the cell cycle favored by HIV-1 versus the DNA tumor viruses reflects one of the fundamental differences between these viruses: the nature of the genome. HIV-1 relies on the host cell transcription machinery for replication, because its genome consists of RNA. The genomes of the adenovirus, Simian virus 40, and human papilloma virus consist of DNA, so these viruses rely on the host cell DNA synthesis machinery for replication. Another interesting parallel is that both HIV-1 and adenovirus modulate the function of E2F-1 on their respective promoters to promote viral
transcription. E1A promotes the release of E2F-1, which activates transcription of the adenovirus E2 promoter (44, 85). Tat, on the other hand, promotes sequestration of E2F-1, a negative regulator of HIV-1 transcription (32, 4). Taken together, our data suggest that the complex interplay between virus and host, with respect to host cell cycle and HIV-1 replication, may promote HIV-1 gene expression in astrocytic cells. Furthermore, these interactions, by altering the state of astrocytes and stimulating the production of neurotrophic factors, could contribute to the pathogenesis of ADC.

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