The C Terminus of HIV-1 Tat Modulates the Extent of CD178-mediated Apoptosis of T Cells*

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HIV infection and the progression to AIDS are characterized by the depletion of CD4⁺ T cells through apoptosis of the uninfected bystander cells and the direct killing of HIV-infected cells. This is mediated in part by the human immunodeficiency virus, type 1 Tat protein, which is secreted by virally infected cells and taken up by uninfected cells and CD178 gene expression, which is critically involved in T cell apoptosis. The differing ability of HIV strains to induce death of infected and uninfected cells may play a role in the clinical and biological differences displayed by HIV strains. We chemically synthesized the 86-residue truncated short variant of Tat and its full-length form. We show that the trans-activation ability of Tat at the long terminal repeat does not correlate with T cell apoptosis but that the ability of Tat to up-regulate CD178 mRNA expression and induce apoptosis in T cells is critically dependent on the C terminus of Tat. Moreover, the greater 86-residue Tat-induced apoptosis is via the extrinsic pathway of CD95-CD178.

The induction of apoptosis in uninfected bystander cells has been postulated as a mechanism of CD4⁺ T cell depletion and immune dysregulation during the clinical course of HIV² infection (1, 2), leading to the loss of immune competence (3, 4). Apoptotic cell death is a process characterized by cell shrinkage, loss of membrane integrity, DNA fragmentation, and formation of apoptotic bodies (5). The key apoptotic effectors in mammals are a family of cysteine-containing, aspartate-specific proteases called caspases (6). The CD95/CD178 (Fas/Fas ligand) system also has a well known role in mediating apoptosis and has been shown to play a possible role of variable significance in the induction of apoptosis in bystander cells in in vitro HIV systems (7) but not in the apoptosis of activated primary CD4⁺ T cells (8). CD95-mediated apoptosis is irreversible and is, thus, tightly regulated both pre- and post-CD95 engagement (9). For T cells, one of the major forms of regulation is exerted at the level of CD178 expression. CD178 mRNA is not expressed in resting T cells but is induced shortly after an activating stimulus (10). In HIV-infected individuals, both CD4⁺ and CD8⁺ T cells are more susceptible to CD178-induced apoptosis, and this is related to the regulation of surface levels of both CD95 and CD178. It has been shown that T cells from HIV-infected individuals overexpress CD178 (11), the proportion of these T cells increases with disease progression (12–14), and the rate of apoptosis is correlated with disease progression (15). The HIV-1 trans-acting regulatory protein (Tat) has been shown to up-regulate CD178 expression in infected (16) and non-infected bystander cells (17, 18). However, it has also been shown that Tat does not require the CD178-CD95 interaction to induce apoptosis and that Tat binds to tubulin and LIS1, perturbing microtubule dynamics, leading to apoptosis through the intrinsic mitochondrial pathway (19–21). Tat is an 86–101 residue regulatory protein (9–11 kDa) produced early in HIV-1 infection whose primary role is in regulating productive and processive transcription from the HIV-1 long terminal repeat (LTR) (22–24). Tat is secreted from infected cells (25–27) and has been detected using weak avidity anti-Tat antibodies in the sera of HIV-1-infected patients at concentrations of up to 40 ng/ml. However, current estimates of Tat concentrations are thought to be an underestimation as Tat is rapidly taken up by cells, local concentrations of Tat in lymphoid tissues might be higher, the antibody used for detection has weak avidity, and Tat in vivo might be sequestered by endogenous anti-Tat antibody and/or by glycosaminoglycans (28). Extracellular Tat has a variety of effects on a number of different cell types (for review, see Ref. 29). The viral mRNA for Tat is composed of two exons (30). The first exon codes for 72 amino acids (residues 1–72) and contains three important functional regions; they are the cysteine-rich region (amino acids 22–37), the basic region (amino acids 49–57), and the glutamine-rich region (amino acids 58–72). The second exon codes for a variable number of amino acids (residues 14–31) that contribute to viral infectivity and other functions (31).

Two different forms of Tat are found in clinical isolates; they are an 86-residue form and a longer, more predominant 101-residue form (32). In research to date, the most widely used form of Tat is Tat HXB2 (86), which is a truncated 86-residue form from a laboratory-passaged subtype B viral strain (32, 33). Indeed, a single nucleotide change at the putative residue 87 allows the translation of the full-length 101-amino acid form (HXB2 (100)). Therefore, the extreme C terminus of Tat has not been frequently considered in research even though it has been shown to be significant in several biological assays (34–36) and is the predominant form present in clinical isolates. In this study we compared the ability of these two forms of Tat to trans-activate the HIV-1 LTR and assessed their ability to induce apoptosis in T cells. We show that the last 14 residues at the C terminus of Tat present in the full-length 100-amino acid form are implicated in both a greater trans-activation ability and in a reduced ability to up-regulate CD178 expression and induce apoptosis via the extrinsic pathway.
**Materials and Methods**

Protein synthesis, purification, and characterization—The Tat proteins were synthesized in solid phase using Fast Fmoc chemistry according to the method of Barany and Merrifield (37) using HMP (4-hydroxymethyl-phenoxymethyl-copolystyrene, 1% divinylbenzene)-preloaded resin (0.5 mmol) (PerkinElmer) on an automated synthesizer (ABI 433A, PerkinElmer) and purified as described elsewhere (18, 38, 39). High pressure liquid chromatography (HPLC) analysis was carried out on a Beckman Coulter HPLC apparatus using a Merck Chromolith™ Performance RP-8e (4.6 x 100 mm) column (Merck). Buffer A was water with 0.1% (v/v) trifluoroacetic acid (Sigma-Aldrich), and Buffer B was acetonitrile (Merck) with 0.1% (v/v) trifluoroacetic acid. The gradient was buffer B from 10 to 50% in 15 min with a 1.8-ml/min flow rate. Amino acid analyses were performed on a model 6300 Beckman analyzer, and mass spectrometry was carried out using an Etant™ matrix-assisted laser desorption ionization time-of-flight (Amersham Biosciences).

Trans-activation with HIV LTR-transfected Cells—HeLa P4 cells containing the bacterial lacZ gene under the control of the HIV-1 LTR, as described by Clavel and Charneau (40), were used in this study. The activity of the synthetic Tat protein was analyzed by monitoring the production of β-galactosidase after activation of lacZ expression mainly as previously described with some modifications (38). Briefly, 2 x 10⁵ cells/well were incubated in 24-well flat-bottomed plates (Falcon) at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen) and 100 μg/ml neomycin (Invitrogen). After 24 h, cells were washed with phosphate-buffered saline (PBS). Tat protein was dissolved in phosphate buffer at pH 6 (to avoid precipitation that occurs at neutral pH) and was directly mixed with Dulbecco’s modified Eagle’s medium supplemented with 0.01% (w/v) protease (Sigma-Aldrich) and 0.1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) and added to the cells. After 16 h at 37 °C, 5% CO₂ cells were washed with PBS, lysed, the β-galactosidase content was measured with a commercially available antigen capture enzyme-linked immunosorbent assay (β-galactosidase enzyme-linked immunosorbent assay, Roche Diagnostics), and absorbance values (B₄₅₀) were measured at 405 nm. Results were normalized using the Bradford reagent (Sigma-Aldrich). B₄₅₀ corresponds to the background β-galactosidase expressed by HeLa P4 cells in Dulbecco’s modified Eagle’s medium supplemented with 0.01% (w/v) protease (Sigma-Aldrich) and 0.1% (w/v) BSA (Sigma-Aldrich) without Tat. Concentrations of Tat used are noted in Fig. 1.

Entry of Tat into the Cells—HeLa P4 cells were cultured and treated with Tat as for the trans-activation assay. After treatment with Tat, the culture medium was removed, and cells were washed with cold PBS and lysed for 30 min at 4 °C in radioimmunoprecipitation assay buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1% Triton, 1 mM EDTA, and protease inhibitors) without BSA. We obtained a cytoplasmic supernatant and a pellet corresponding to the nuclear extract by centrifuging the lysates at 4000 x g for 15 min at 4 °C. All supernatants were stored at −80 °C. After a brief sonication, the lysates were clarified by centrifugation at 10,000 rpm, and protein content was measured by the Bradford method (Bio-Rad). 40 μg/sample was then heated at 100 °C for 5 min in Laemmli sample buffer containing reducing agent, separated by 15% SDS-polyacrylamide gel, and blotted onto Protran® nitrocellulose membranes (Schleicher & Schuell). Nonspecific sites were blocked by 1 h of incubation at room temperature with PBS, 0.1% (v/v) Tween 20 (Sigma-Aldrich), and 10% (w/v) dried nonfat milk (10% MPBS). Membranes were then incubated with anti-HXB2 (100) and anti-HXB2 (86) rabbit sera raised as previously described (39) at 1:5000 dilution in 10% MPBS overnight, washed 4 times, and incubated with an anti-rabbit horseradish peroxidase-labeled secondary antibody at 1:1000 dilution (Sigma-Aldrich) for 1 h at room temperature in 10% MPBS. The bound horseradish peroxidase was revealed with H₂O₂, 0.1% (w/v) diamino-benzidine tetrahydrochloride (Sigma-Aldrich) in PBS as substrate. The intensity of immunoblot bands was analyzed by densitometric imaging using the freely available Scion Image (Scion Corp., Frederick, MD) on a personal computer.

Apoptosis assay—Human peripheral blood mononuclear cells were isolated from heparinized blood of healthy donors by using density centrifugation over Ficoll-Hypaque (Amersham Biosciences). After 2 washes with PBS supplemented with 10% (v/v) autologous serum and 2 mM EDTA (Sigma Aldrich), CD4⁺ cells were then isolated using the CD4⁺ isolation kit using MidiMACs with LS columns (Miltenyi Biotech, Auburn, CA) and cultured in AIM-V™ medium with human serum albumin (Invitrogen) until used.

The Jurkat clone I9.2 cell line was purchased from American Type Culture Collection and cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin (all from Invitrogen). The Jurkat I9.2 cell line is a clone that is functionally defective for Fas-associated death domain and is completely resistant to both caspase-8- and CD178-induced apoptosis (41).

To induce apoptosis cells were cultured in 24-well plates in RPMI 1640 (Invitrogen) 0.1% (w/v) BSA (Sigma Aldrich) for 19 h without sera in the presence or not of Tat. Tat was prepared as for the trans-activation assay.

To differentiate between early apoptosis and late apoptosis/necrosis, cells were harvested at 19 h post-treatment and immediately stained with annexin V/fluorescein isothiocyanate and propidium iodide according to the protocol provided by the manufacturer (BD Pharmingen). The harvest and staining took 1 h, and then stained cells were immediately analyzed by flow cytometry (FACScan, BD Biosciences).

To determine caspase-8 and caspase-9 activity, cells were harvested at 14 h post-treatment and immediately stained with annexin V/fluorescein isothiocyanate and propidium iodide according to the protocol provided by the manufacturer (BD Pharmingen). The harvest and staining took 1 h, and then stained cells were immediately analyzed by flow cytometry (FACScan, BD Biosciences). Cytogram analysis was performed with Cell Quest Pro™ software (BD Bioscience).

Real-time PCR—CD178 mRNA expression in CD4⁻ T cells was measured by real time PCR. 1 x 10⁶ cells/ml were incubated in 24-well flat-bottomed plates (Falcon) at 37 °C with 2 μM Tat for 14 h. Total cellular RNA was prepared with the RNeasy Mini kit in accordance with the manufacturer’s directions (Qiagen, Valencia, CA). 1 μg of total RNA was used for reverse transcription with random primers. CD178 mRNA expression in relation to β2 microglobulin expression (internal standard) was determined using the LightCycler System and the FastStart DNA master SYBR Green I. Primers used were as follows: CD178 sense, 5'-ATCTTCAAACCTGTTAAA-3'; CD178 antisense, 5'-ATCTTCAAACCTGTTAAA-3'. Primers were used as follows: CD178 sense, 5’-GTAGGATTGGGGCTGGGAT-3’, and antisense, 5’-AGTTGGAATTCGGCTTTAA-3’; β2 microglobulin sense, 5’-CGCATTGGAATTCGCTTAA-3’, and antisense 5’-ATCTTCAAACCTGTTAAA-3'.
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**RESULTS**

**Full-length HXB2 Has a Higher Trans-activation Activity than the Truncated 86-Residue Form**—In this study we have used the full-length form of Tat HXB2 (HXB2 (100)) as described by Jeang (32) and the 86-residue-truncated form of Tat HXB2 (Tat HXB2 (86)). Both proteins were obtained using Fast Fmoc chemistry and, after purification using HPLC, gave a good HPLC homogeneity (Fig. 1A). The 86-residue truncated form is 9.84 kDa, and the full-length form is 11.54 kDa. These proteins were synthesized by solid phase Fmoc chemistry. A, HPLC analysis data of the two variants of Tat. Solvent gradient is as described under “Materials and Methods.” HXB2 (86) showed just one peak at 9.3 min (34.8% acetonitrile, 0.1% (v/v) trifluoroacetic acid), and HXB2 (100) showed just one peak at 9.9 min (36.4% acetonitrile, 0.1% (v/v) trifluoroacetic acid). B, mass spectrum of HXB2 (86) and HXB2 (100).

We assessed the ability of these Tat variants to cross the cell membrane of HeLa P4 cells and trans-activate the stably transfected HIV-1 LTR in these cells as used previously by ourselves and others (18, 38, 40, 42–46). Initially, HeLa P4 cells were incubated with 1 μM Tat, which was added to the culture medium. This is the minimum amount of Tat that can be used due to the avidity of the two specific anti-Tat antibodies used. After immunoblotting, we analyzed the blot using Scion Image. We compared the densitometric ratio between T:C and T:N (T, Tat; C, cytoplasmic fraction; N, nucleic fraction) for each Tat. Using this semiquantitative methodology, we found that both the truncated and the full-length form were taken up in equivalent quantities by the transfected cells and were found predominantly in the cytoplasmic region. Only a small portion of either Tat reached the nucleus to activate transcription, which agrees with previous data (18, 26) (Fig. 2A). Importantly, both the truncated and the full-length form demonstrated a similar efficiency in their cytoplasmic and nuclear uptake. Thus, the C terminus does not play a role in the cellular uptake of Tat, which is again in agreement with previous data (47).

When we tested for the trans-activation activity of the two variants, we found that at almost all concentrations tested there was a significant difference, with HXB2 (100) having significantly more trans-activation activity than HXB2 (86) (Fig. 2B), which agrees with previously published data (45, 48). Only at 2 μM was there no statistical difference, but this could be due to a saturation of this system (p = 0.065). We also observed a dose-dependent response in the levels of β-galactosidase being quantified.

Tat HXB2 (100) Induces Less Apoptosis than Tat HXB2 (86)—It is known that the addition of extracellular recombinant Tat HXB2 (86) to cultures of both primary CD4+ T cells and Jurkat T cell lines induces apoptosis and increases their sensitivity to apoptotic signals, thus contributing in part to the progressive loss of T cells associated with HIV-1 infection (17, 49–51). We investigated the ability of Tat HXB2 (100) and Tat HXB2 (86) to induce apoptosis in primary CD4+ T cells. We used an assay in which primary CD4+ T cells were incubated with the Tat proteins at 2 μM, and the apoptotic effects were evaluated by flow cytometry after labeling the cells with propidium iodide and fluorescein isothiocyanate-conjugated annexin V (Fig. 3). Compared with the control, both HXB2 (100) and HXB2 (86) induced a significant level of apoptosis in these cells (p = 0.0005 and 0.0001, respectively) (Fig. 3B). There was also a significant difference between the levels of apoptosis induced by these two variants, with Tat HXB2 (86) inducing almost four times the level of apoptosis compared to Tat HXB2 (100) (Fig. 3B).

**CCATGATG-3’**. The reaction mixture was initially incubated at 95 °C for 10 min to denature the cDNA. Amplification was performed for 45 cycles, with the following cycle parameters: 10 s of denaturation at 95 °C, 10 s of primer annealing at 65 °C, and 15 s of fragment elongation at 72 °C. Quantification and melting curve were analyzed with LightCycler analysis software, RelQuant (Roche Diagnostics). All results were expressed as the ratio between the copy number of the target gene and the copy number of B2 microglobulin and normalized so that CD178 expression in the non-treated cells equals 1.00.

**Statistics**—All p values correspond to two-sample t tests assuming unequal variances, unless indicated otherwise.

**FIGURE 1.** HXB2 HIV-1 strain was isolated in France and corresponds to B subtype (Ratner et al. (33)). The 86-residue truncated form is 9.84 kDa, and the full-length form is 11.54 kDa. These proteins were synthesized by solid phase Fmoc chemistry. A, HPLC analysis data of the two variants of Tat. Solvent gradient is as described under “Materials and Methods.” HXB2 (86) showed just one peak at 9.3 min (34.8% acetonitrile, 0.1% (v/v) trifluoroacetic acid), and HXB2 (100) showed just one peak at 9.9 min (36.4% acetonitrile, 0.1% (v/v) trifluoroacetic acid). B, mass spectrum of HXB2 (86) and HXB2 (100).

**FIGURE 2.** Trans-activation assay with HeLa P4 cells transfected with the HIV-1 long terminal repeat lacZ construct (40). A, entry of Tat into HeLa P4 cells. HXB2 (86) and HXB2 (100) enter the cells with the same efficiency. HeLa P4 cells were incubated with 1 μM Tat. Whole cell lysates were prepared 4 h post-treatment and analyzed by anti-Tat immunoblotting. This blot is representative of three independent experiments. T, Tat; C, cytoplasmic fraction; N, nucleic fraction. The analysis of this blot is discussed in the text. B, trans-activation was measured with the different Tat variants at six concentrations. HXB2 (100) is represented by the light gray bars, and HXB2 (86) is represented by the dark gray bars. Without Tat, β-galactosidase was expressed at a basal level, which was used as a control (white bar). B, absorbance values measured at 405 nm; Bg, background β-galactosidase expressed by HeLa P4 cells in Dulbecco’s modified Eagle’s medium supplemented with 0.01% (w/v) proline and 0.1% (w/v) BSA without Tat. HXB2 (100) had higher trans-activation activity at all concentrations. p values are displayed above each pairing and are the result of three independent experiments carried out in triplicate.
apoptosis of Tat HXB2 (100) ($p = 0.0001$) (Fig. 3B). We also observed a significant difference in the numbers of cells that lost membrane integrity as a result of very late-stage apoptosis and early apoptotic cells between the two Tat variants as well as an overall difference (Fig. 3A). When the cells were treated with HXB2 (86), the percentage of cells in late-stage apoptosis was always significantly higher compared with HXB2 (100), which suggests that HXB2 (86) induces apoptosis faster than the full-length form.

Tat HXB2 (86) Has a Greater Up-regulation of Caspase-8 Activity than HXB2 (100)—The hallmark of apoptosis is the activation of specific caspases, which initiate and propagate the apoptotic cascade. The extrinsic pathway was examined by detecting cleavage and subsequent activation of caspase-8 by flow cytometry. Caspase-8 cleavage in the death-inducing signaling complex (DISC) occurs in two consecutive steps. The first cleaves p55/p53 precursors into p43, p41, and p12 subunits. The p12 subunit is processed to an active p10 fragment. Second-step cleavage of p43/p41 releases an active p18 fragment (52). In the apoptotic signal transduction pathway, mitochondria play an essential role by releasing apoptogenic factors such as cytochrome c and apoptosis-inducing factor (53). Cytochrome c binds to Apaf-1, thus recruiting and activating one of the major caspases, caspase-9, that resides in the cytoplasm. Therefore, the intrinsic mitochondrial pathway was examined by detecting the active form of caspase-9. CD4$^+$ T cells were treated with 2 $\mu$M Tat in RPMI 1640, 0.1% BSA for 14 h and examined for caspase-8 and caspase-9 cleavage by flow cytometry.

Both HXB2 (86) and HXB2 (100) up-regulated the active form of caspase-8, with HXB2 (100) inducing twice as much activation as the untreated control. However, this was not found to be significant ($p = 0.206$). HXB2 (86) up-regulated the activation of caspase-8 to 4 times that of the untreated control and twice that of the HXB2 (100)-treated cells and was found to be significant in both cases ($p = 0.0047$ and 0.0001, respectively) (Fig. 4A). Both HXB2 (86) and HXB2 (100) induced significantly more caspase-9 activity than the untreated control ($p = 0.0001$ and 0.0012, respectively). As with the caspase-8 activity, HXB2 (86) also induced significantly more caspase-9 activity than HXB2 (100) ($p = 0.0001$) (Fig. 4B).

Tat HXB2 (86) Induces a Greater Up-regulation of CD178 mRNA than Tat HXB2 (100)—To further elucidate the role that the extrinsic pathway plays in the difference in apoptosis induced by these two variants, we next analyzed the expression of CD178 in treated cells. Previous reports have suggested that a pathway by which Tat induces apoptosis is by up-regulating the expression of CD178 (17). To investigate the contribution of this pathway in Tat HXB2-induced apoptosis, quantitative analysis of CD178 mRNA up-regulation in primary CD4$^+$ T cells treated with 2 $\mu$M Tat HXB2 (86) and Tat HXB2 (100) was carried out over 14 h. Treatment of cells with Tat HXB2 (86) significantly up-regulated the expression of CD178 3-fold higher than that of Tat HXB2 (100) ($p = 0.012$) (Fig. 4C). Therefore, the expression of CD178 expression correlates with the level of apoptosis observed and does not correlate with trans-activation activity. This suggests that the extrinsic pathway may be important in the differences in apoptosis levels observed between the two variants.
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The Difference in the Apoptotic Potential of the Two Forms Is Dependent upon the Up-regulation of CD178 and Caspase-8 Activity—To investigate the contribution of the CD95-CD178 pathway in Tat-induced apoptosis, the apoptotic activity of Tat was examined in the human Jurkat T cell line I9.2, which has a mutation in the cysteine protease caspase-8 that renders it functionally defective for Fas-associated death domain and is completely resistant to CD178-induced apoptosis (41). As with the CD4+ T cells, both HXB2 (100) and HXB2 (86) induced a significant level of apoptosis in Jurkat I9.2 cells (p = 0.0039 and 0.0219, respectively). Although the truncated form of Tat induced slightly more apoptosis in this cell line than the full-length form of Tat, this was not statistically significant (p = 0.074) (Fig. 4D). This suggests that the greater HXB2 (86)-induced apoptosis observed in the primary CD4+ T cells was via the extrinsic pathway of CD95-CD178 and caspase-8.

DISCUSSION

A hallmark of HIV-1 infection is the progressive loss of CD4+ T cells. Apoptosis and activation-induced cell death are known to be involved in this process (1, 2, 8). Co-culture experiments of HIV-1-infected and uninfected cells have shown that whereas HIV-infected cells are resistant to HIV-induced death, uninfected bystander CD4+ T cells undergo apoptosis (49). It is well known that HIV infection increases the expression of CD178 and may thereby contribute to the apoptosis of T cells in AIDS (13). However, the mechanism by which HIV-1 Tat plays a role in this up-regulation is yet to be finalized. Some studies have suggested that Tat only induces activation-induced cell death and has no effect on resting T cells (17, 51, 54), whereas others have shown that activation is not necessary and that Tat can directly induce apoptosis in resting T cells (18, 55, 56). We demonstrate that Tat can induce apoptosis in resting T cells, and we also show another important function of the C terminus of HIV-1 Tat. In this study, we found a significant difference both in the apoptotic and in the trans-activation activities of the two forms of HIV-1 Tat HXB2. The sequence of these two proteins is identical apart from the presence of 14 amino acids at the C terminus of HXB2 (100); therefore, any variation in the function of these two proteins is as a result of the additional 14 amino acids present at the C terminus.

One pathway by which Tat has been shown to induce apoptosis is by up-regulating CD178 expression (17). The ability by which Tat up-regulates this expression and its correlation with the activity of caspase-8 and the level of apoptosis observed in our assays indicates that this receptor-mediated pathway is important in Tat-induced apoptosis. In this study we observed that the truncated form of Tat was more efficient in up-regulating CD178 mRNA and caspase-8 activity than the full-length form of Tat. Another pathway by which Tat induces apoptosis is by directly targeting the microtubule cytoskeleton, preventing depolymerization and liberating Bim, which leads to apoptosis through the mitochondrial pathway and involves the release of cytochrome c and the activation of caspase-9 and is independent of the CD95/CD178 pathway (19). It has previously been shown that Tat HXB2 is able to bind to tubulin in vitro (19, 20) and that Phe64 and the glutamine-rich region of Tat are both important in this binding and in the ensuing apoptosis (18, 19). The N terminus and the C terminus of Tat are both important in this binding and in the apoptosis of T cells. One pathway by which Tat can directly induce apoptosis is by targeting the microtubule cytoskeleton, preventing depolymerization and liberating Bim, which leads to apoptosis through the mitochondrial pathway and involves the release of cytochrome c and the activation of caspase-9 and is independent of the CD95/CD178 pathway (19). It has previously been shown that Tat HXB2 is able to bind to tubulin in vitro (19, 20) and that Phe64 and the glutamine-rich region of Tat are both important in this binding and in the ensuing apoptosis (18, 19). The N terminus and the C terminus of Tat are not implicated in this interaction (20). We have found that the extra 14 residues at the C terminus of HXB2 (100) also has an effect on the activation of caspase-9, with the truncated form more efficient in activating this caspase. However, when we used cells that were functionally defective for Fas-associated death domain, the difference in apoptotic activity observed between these two proteins was vastly reduced and was not statistically
It is interesting to note that individuals infected with subtype D progress more rapidly to AIDS than other subtypes (57, 58), and of the 122 subtype D Tat sequences found in the Los Alamos database, 83% of those have a stop codon at putative residue 87, whereas less than 20% are found with this mutation in all other subtypes. Furthermore, recent NMR studies of a full-length 100 residue Tat protein have placed the extra residues at the C terminus of Tat over the glutamine-rich region, masking the α-helix in this region from solvent.3 Previous studies have shown that the glutamine-rich region of Tat and, in particular, the α-helix formed in that region is important in up-regulating CD178 mRNA expression and in binding to tubulin (18). The role that this plays in disease progression is yet to be elucidated.

Tat targets the microtubule cytoskeleton to induce apoptosis and up-regulates CD178 mRNA expression, but apoptosis does not occur in the very cells where Tat is produced (59). It is unlikely that HIV has evolved a highly specific mechanism by which Tat kills off only uninfected bystander cells; thus, it is possible that two HIV-encoded proteins control the activities of the main regulators of the mitochondria-dependent apoptotic pathway in infected cells. HIV-1 Nef activates the phosphatidylinositol-3-phosphate-activated kinase, which inactivates the pro-apoptotic protein Bad by phosphorylation (60) and HIV-1 Vpr down-modulates the pro-apoptotic Bax and up-regulates the levels of the anti-apoptotic Bcl-2 (50). Thus, Nef and Vpr possibly counteract the effects of Tat in infected cells and protect the infected cells from apoptosis. In summary, the data presented here show a functional importance of the 14 amino acids present at the C terminus of HXB2 (100) in Tat-mediated apoptosis. It is possible that such mutations lead to functional differences that may be related to disease progression. These results emphasize an important role that Tat may play in the progression to AIDS in HIV-infected persons and point to the importance of developing therapies that target Tat.

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REFERENCES

1. Ameisen, J. C., and Capron, A. (1991) Immunol. Today 12, 102–105
2. Finkel, T. H., Tudor-Williams, G., Banda, N. K., Cotton, M. F., Curiel, T., Monks, C., Banda, N. K., Curiel, T., Monks, C., Tiu, Y. W., and Liu, Y. (2000) EMBO J. 19, 5121–5129
3. Sauvage, B., Coudert, F., Lepont, K., Vallade, P., Grun, W., and Mora, A. (2001) Nature 411, 557–561
4. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) J. Biol. Chem. 271, 1469–1476
5. Noraz, N., Goulas, J., Corbel, J., Brunner, T., and Spector, S. A. (1997) AIDS 11, 1671–1680
6. Tschopp, J., Irmler, M., and Thome, M. (1998) Curr. Opin. Immunol. 10, 552–558
7. J. D. Watkins, A. Malmendal, N. C. Nielsen, and E. P. Loret, manuscript in preparation.
