Cutting Edge: A Short Polypeptide Domain of HIV-1-Tat Protein Mediates Pathogenesis


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HIV-1 encodes the transactivating protein Tat, which is essential for virus replication and progression of HIV disease. However, Tat has multiple domains, and consequently the molecular mechanisms by which it acts remain unclear. In this report, we provide evidence that cellular activation by Tat involves a short core domain, Tat21–40, containing only 20 aa including seven cysteine residues highly conserved in most HIV-1 subtypes. Effective induction by Tat21–40 of both NF-kB-mediated HIV replication and TAR-dependent transactivation of HIV-long terminal repeat indicates that this short sequence is sufficient to promote HIV infection. Moreover, Tat21–40 possesses potent angiogenic activity, further underscoring its role in HIV pathogenesis. These data provide the first demonstration that a 20-residue core domain sequence of Tat is sufficient to transactivate, induce HIV replication, and trigger angiogenesis. This short peptide sequence provides a potential novel therapeutic target for disrupting the functions of Tat and inhibiting progression of HIV disease. The Journal of Immunology, 1999, 163: 15–20.

The transactivator Tat of HIV-1 (1) is an 86-aa protein released by infected cells and plays a critical role in the progression of HIV disease (1, 2). Transactivation of the HIV-long terminal repeat (LTR)2 promoter by the Tat protein is essential for both viral gene expression and virus replication. Extracellular Tat released by infected cells during the acute phase of infection enters noninfected cells and disrupts many host immune functions by activating a wide variety of genes regulated by specific viral and endogeneous cellular promotors (3, 4). We and others have previously shown that Tat mimics many of the effects of HIV infection of monocytes including increased matrix metalloproteinase-9 and cytokine production, and collagen expression in glioblastoma cells (5–7). These observations correlate with high levels of cytokines such as IL-1, IL-6, and TNF found in sera from HIV-infected individuals that leads to an increase of the level of HIV replication. These reports suggest a role of extracellular Tat in promoting viral pathogenesis. However, Tat has multiple domains, and consequently how Tat induces these diverse effects is not clearly understood. In the present study, we have dissected the sequence of Tat and identified a domain that mediates the cellular and viral effects of extracellular Tat protein. These findings are potentially important for understanding the progression of HIV pathogenesis and in the development of potential therapeutic applications.

Materials and Methods

Tat protein

The HIV-1-Tat protein used in these experiments was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergies and Infectious Disease, National Institutes of Health, from Dr. Andrew Rice or Dr. John N. Brady (6). HIV-Tat was dissolved at 10 μg/ml in treatment buffer (PBS containing 1 mg/ml BSA and 0.1 mM DTT) and frozen in aliquots at −80°C. Tat preparations were screened and found to be negative for endotoxin contamination.

Synthesis and purification of Tat peptides

Tat peptides were synthesized by solid phase synthesis on an Applied Biosystems peptide synthesizer Model 430A (Foster City, CA) (8). After an initial HPLC purification of the crude cysteine-containing peptides, they were redissolved in 0.1 M Tris acetate buffer (pH 8.3) and air-oxidized overnight. Peptides were then subjected to desalting and purification by reverse-phase HPLC, lyophilized, and stored at −70°C. Peptide identities were confirmed by amino acid compositional analysis and plasma desorption mass spectroscopic analysis.

Acknowledgments

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2 Abbreviations used in this paper: LTR, long terminal repeat; CAM, chorioallantoic membrane; CAT, chloramphenicol acetyltransferase.
Monocyte isolation and infection with HIV

Monocytes were isolated from the PBMC of donors seronegative for HIV and hepatitis after leukapheresis and purification by countercurrent centrifugal elutriation (9). Primary monocytes cultured for 5 days were exposed to HIV-1HXB1, a monocytotrophic HIV strain (Advanced Biotechnologies, Columbia, MD), at a multiplicity of infection of 0.01 infectious virus particles/target cell (10).

Electroporation of cells

Cells were electroporated as previously described (11). CEM cells (12D7) were cultured at a density of 0.5–0.8 × 10^6 cells/ml with daily media additions. Typically, 5 × 10^6 cells were electroporated with 5 μg of either purified plasmid or Tat protein and 5 μg of reporter plasmid. Tat peptides or Tat protein and the reporter HIV LTR-chloramphenicol acetyltransferase (CAT) or the TAR mutant HIV TM26 LTR-CAT were mixed with cells and electroporated using a cell porater apparatus (Life Technologies/TRL, Gaithersburg, MD). Cell mixtures were electroporated at 800 V in RPMI 1640 medium without serum. Following electroporation, cells were plated in 10 ml complete medium, and samples were collected 24 h later for CAT assays.

EMSA for NF-κB

Monocytes (1 × 10^7/ml) were treated with Tat protein or Tat peptides at 37°C for 15 min. Nuclear extracts were then prepared and analyzed by EMSA as previously described (12).

CAM assay

The chick CAM assay was conducted as described (13) to determine the angiogenic activity of rTat and its derived peptides. Briefly, salt-free aqueous solution (5 ml) containing 5.3 pmol of rTat or its derived peptides (Tat21–40, Tat33–68, or Tat41–52) was loaded onto a 1/4 piece of 15-mm Thermonox disk (Nunc, Naperville, IL), and the sample was dried under sterile air. The disk loaded with sample was placed on the CAM of a 10-day-old chick embryo. After 72 h incubation, negative or positive responses were scored under a microscope. A positive response was characterized as the appearance of a typical radiating network (spokewheel) pattern of new blood vessels around the loaded samples. Assays for each test sample were conducted in two sets of eggs, and each set contained 12–15 eggs.

Results and Discussion

To identify Tat-specific sequences responsible for cellular dysfunction, we synthesized overlapping peptides from various subdomains of consensus-B and other HIV-1 subtypes (Fig. 1). Using these peptides, we have identified a novel domain that can mediate viral transactivation. We found that, like rTat, the 20-aa core domain Tat21–40 containing seven cysteine residues, all of which are strongly conserved in various subtypes, enhanced HIV replication by greater than 4-fold (Table I). A peptide derived from the basic domain (Tat33–68) induced a lesser increase in viral replication compared with Tat21–40. In contrast, Tat41–52, a peptide sequence located between the core and the basic domains, and a variety of peptides from other positions in the Tat sequence, had no significant effect on HIV replication.

Consistent with its enhancement of viral replication, Tat21–40 treatment produced a marked increase in HIV-associated cytopathic effects in monocytes as indicated by formation of multinucleated giant cells (Fig. 2c); the effects were similar to those induced by rTat protein itself (Fig. 2b). The effect of Tat33–68 was substantially less than that of Tat21–40 (Fig. 2d). Tat41–52, the peptide between core and basic domains, and peptides from other Tat domains did not alter HIV-associated cytopathic effects (Fig. 2e, and data not shown). Thus, a major active site for stimulating HIV replication and monocyte dysfunction can be localized to the 20-residue peptide Tat21–40.

Table 1. Effect of various Tat peptides on HIV replication in monocytes

<table>
<thead>
<tr>
<th>Residue Numbers</th>
<th>Tat Sequence</th>
<th>p24 (pg/ml) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>471 ± 10</td>
</tr>
<tr>
<td>Tat21–40</td>
<td>rTat</td>
<td>1866 ± 26</td>
</tr>
<tr>
<td>Tat33–68</td>
<td>MEPEVDPLRLEPKHGSGQKPT</td>
<td>421 ± 3</td>
</tr>
<tr>
<td>Tat41–52</td>
<td>PKWHGSPQKPTACTNCYCKKCCFHCQVCF</td>
<td>378 ± 4</td>
</tr>
<tr>
<td>Tat21–40</td>
<td>ACTNCYCKKCCFHCQVCF</td>
<td>1958 ± 101</td>
</tr>
<tr>
<td>Tat33–68</td>
<td>CFHQCQVFPTKGLGISYGRK</td>
<td>429 ± 24</td>
</tr>
<tr>
<td>Tat41–52</td>
<td>RRQRKRAQHNSQTHQAS</td>
<td>969 ± 75</td>
</tr>
<tr>
<td>Tat21–40</td>
<td>CYCKKCCFHCQVFCTT</td>
<td>836 ± 123</td>
</tr>
<tr>
<td>Tat33–68</td>
<td>KGLGISYGRKKR</td>
<td>370 ± 2</td>
</tr>
<tr>
<td>Tat41–52</td>
<td>LDPWNHPGSQPT</td>
<td>360 ± 9</td>
</tr>
<tr>
<td>Tat21–40</td>
<td>LEPWNHPGSQPK</td>
<td>376 ± 2</td>
</tr>
</tbody>
</table>

Monocytes cultured for 5 days were treated with rTat or Tat peptides for 18 h. Cells were then infected with HIVHXB1 strain as described in Materials and Methods. On day 5, cells were harvested, and concentrations of p24 gag Ag in culture supernatants were determined using a DuPont (Wilmington, DE) p24 ELISA test kit. Data are representative of two separate experiments and expressed as mean ± SEM of triplicate determinations.

* Recombinant full-length Tat.
One of the mechanisms by which HIV-Tat potentiates HIV replication involves transactivation of the HIV-1 LTR via its binding to the TAR sequence along with other cellular factors, resulting in increased viral transcription initiation and elongation (14). To characterize further the mechanism of Tat transactivation of the HIV-LTR, CEM lymphoid cells were transfected with wild-type promoter in the presence of various Tat peptides, and the extent of transactivation was determined using CAT assays (14). These analyses revealed a 9-fold induction of HIV-LTR by the Tat21–40 peptide (Fig. 3, A and B); full-length rTat produced a 25-fold induction. The actual effectiveness of induction by Tat21–40 might be greater than observed due to the low solubility of this complex hydrophobic peptide in aqueous buffers.

We found that the presence of Cys22 in core domain Tat21–40 (and three adjacent residues) was critical for viral activation, because deletion of these residues substantially reduced the ability of Tat21–40 to activate HIV infection (Tat25–40 in Fig. 3 and Table I). Because rTat activation of the HIV-LTR promoter is required for productive HIV replication (15), our demonstration of induction by the Tat21–40 sequence conserved in most HIV-1 subtypes further confirms a functional role of Tat21–40 in HIV infection. In contrast, transfection of CEM cells with a TAR mutant (HIV TM26 LTR-CAT) construct in the presence of various Tat peptides, and the extent of transactivation was determined using CAT assays (14). These analyses revealed a 9-fold induction of HIV-LTR by the Tat21–40 peptide (Fig. 3, A and B); full-length rTat produced a 25-fold induction. The actual effectiveness of induction by Tat21–40 might be greater than observed due to the low solubility of this complex hydrophobic peptide in aqueous buffers.

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Although the precise mechanism of virus regulation by host factors is not clear, it is generally believed that in addition to other unknown factors, Tat and cytokines play a key role in the pathogenesis of HIV infection. Extracellular HIV-Tat causes activation of intracellular signal transduction pathways that culminate in the production of various cytokines (5, 20). Therefore, because of its ability to induce host factors, Tat is believed to be a key factor for viral enhancement. HIV-Tat activates both viral and host cell genes, and the host NF-κB transcription factor contributes to immune dysregulation during HIV infection (21, 22). Because macrophages are a well-known reservoir for HIV in vivo, we examined the ability of Tat peptides to activate the expression of NF-κB in these cells. Monocytes were treated with rTat and other peptides, nuclear extracts were prepared, and NF-κB activity was examined by gel shift assay using an NF-κB consensus oligonucleotide. Our results show that the ability of HIV-Tat to activate NF-κB was retained in core peptide Tat21–40 and to a lesser extent Tat53–68 (Fig. 4). Treatment of monocytes with the Tat21–40 peptide rapidly activated NF-κB (within 15 min after exposure) by greater than 9-fold as compared with 3-fold induction by Tat53–68. Interestingly, despite inducing NF-κB activity, Tat53–68 had little effect on transactivation of HIV-LTR as shown in Fig. 3. These observations delineate two distinct mechanisms for viral activation by HIV-Tat: 1) TAR-dependent transactivation of HIV-LTR involving Tat21–40 domain, and 2) TAR-independent activation of virus replication involving the host factor NF-κB by an intracellular signal transduction pathway. Our results are complementary to those recently reported by Mayne et al. (23), who have demonstrated the involvement of protein kinase A, phospholipase C and protein tyrosine kinase in Tat-mediated induction of NF-κB and cytokine production by monocytes.

Tat is released by HIV-infected cells into the extracellular milieu, and has been implicated as a cofactor in the pathogenesis of
Kaposi’s sarcoma (24), an angioproliferative disease frequently seen in HIV-infected individuals. There is increasing evidence that HIV-Tat induces endothelial cell migration, invasion, and angiogenic processes in vivo (25). To test for potential angiogenic activity of the core domain implicated above in viral pathogenesis, we examined the ability of Tat peptides to induce neovascularization using the CAM assay. Our results indicate that picomol quantities (5.2 pmol/egg) of Tat21–40 can induce neovascularization (Fig. 5). Recombinant Tat alone was less effective in inducing an angiogenic response, as reported by others (25). No significant angiogenic response was observed using vehicle alone or the control peptide Tat41–52 containing sequence between the core and basic domains (Fig. 5). Interestingly, Tat53–68 from the Tat basic domain also had substantial activity; as noted above, this peptide had either partial or minimal activities in assays for HIV replication, cytopathic effects, and transactivation of the HIV-LTR promoter. The exact mechanism of neovascularization in vivo is not clear. However, one scenario is that Tat-induced cytokines stimulate endothelial cells, degrade basement membrane matrix by local enhancement of matrix metalloproteinase-9 secretion and migrate into adjacent tissue to form new blood vessel networks.

Detectable levels of Tat have been reported in HIV-infected individuals (26), suggesting the presence of extracellular HIV-Tat protein in certain phases of HIV infection. It has also been shown that high levels of anti-Tat Abs are directly related to low viral load (27, 28) in seropositive nonprogressor patients. Therefore, a strategy targeting a required site(s) in Tat might provide a novel therapeutic modality to reduce disease progression in HIV-infected individuals. In this paper we have provided evidence that a short core domain of the Tat protein, Tat21–40 consisting of 7 cysteine residues and only 13 other amino acids, is a potent inducer of HIV transactivation and replication. This domain is highly conserved in various HIV-1 subtypes, including the newly discovered group O. Some of these results are complementary to those demonstrating the involvement of Tat and the core domain in the process of monocyte chemotaxis in response to Tat (29, 30), which may contribute to altered immunoregulation in HIV-infected individuals. It is important to note that monocytes differentiate into tissue-resident macrophages, which are nonrecirculating cells. Therefore, HIV-infected macrophages could continue to infect neighboring normal cells and contribute to the tissue damage typically seen after HIV infection. Thus, the active domain Tat21–40 possibly in combination with Tat53–68 provides a novel candidate target for a potential therapeutic vaccine or a dominant-negative strategy to reduce Tat-mediated progression of disease in individuals with HIV infection.

Acknowledgments

We thank Dr. Hira Nakhasi for constant support, suggestions, and critical review of the manuscript.

<table>
<thead>
<tr>
<th>HIV WT LTR-CAT</th>
<th>HIV TMBB LTR-CAT</th>
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<tr>
<td><strong>Fold Induction</strong></td>
<td><strong>Percent Conversion</strong></td>
</tr>
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<td>1</td>
<td>24.7</td>
</tr>
</tbody>
</table>

**FIGURE 3.** Transactivation of HIV-LTR by rTat and Tat peptides by CAT assay. A, Transfection of CEM cells with wild-type HIV-LTR Tat construct. B, Quantitative analysis of HIV-LTR transactivation by Tat and Tat peptides. C, Transfection of CEM cells with a TAR mutant construct as a control in the presence of the indicated peptides.

**FIGURE 4.** Effect of Tat or Tat peptides on activation of NF-κB in monocytes. Monocytes were treated with Tat or Tat peptides at 37°C for 15 min. Nuclear extracts were then prepared, and NF-κB activity was assessed by gel-shift assay.
FIGURE 5. Induction of angiogenesis by rTat or Tat-derived peptides in the chick CAM assay. A, rTat or its derived peptides (Tat53–68, Tat53–52) was loaded (5.3 pmol/egg) on CAMs of 10-day-old embryos. After 72 h incubation at 37°C, fat emulsion (whipping cream) was injected under the CAMs to help visualize the vascular networks. Disks and surrounding CAMs were photographed: vehicle (a), rTat (b), Tat21–40 (c), Tat53–68 (d), or Tat51–52 (e). B, Incidence of angiogenic response induced by rTat or Tat peptides. Each bar represents the average percentage of positive eggs from two independent sets of assays consisting of 12–15 eggs. Positive responses involved eggs which showed a spokewheel pattern of new blood vessels around the loaded samples. p values were calculated by using the Student’s paired t test, based on comparisons with water control samples tested at the same time, *, p < 0.02. p values vs control were as follows: rTat, 0.104; Tat21–40, 0.006; Tat53–68, 0.017; and Tat51–52, 0.313.

References

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