Selective CXCR4 antagonism by Tat: Implications for in vivo expansion of coreceptor use by HIV-1

Hua Xiao*,†, Christine Neuveut,*‡, H. Lee Tiffany*,†, Monsef Benkirane†, Elizabeth A. Rich*,§, Philip M. Murphy*,∥ and Kuan-Teh Jeang*‡¶

Laboratories of *Molecular Microbiology and ‡Host Defenses, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892-0460

Chemokines and chemokine receptors play important roles in HIV-1 infection and tropism. CCR5 is the major macrophage-tropic coreceptor for HIV-1 whereas CXC chemokine receptor 4 (CXCR4) serves the counterpart function for T cell-tropic viruses. An outstanding biological mystery is why only R5-HIV-1 is initially detected in new seroconvertors who are exposed to R5 and X4 viruses. Indeed, X4 virus emerges in a minority of patients and only in the late stage of disease, suggesting that early negative selection against HIV-1–CXCR4 interaction may exist. Here, we report that the HIV-1 Tat protein, which is secreted from virus-infected cells, is a CXCR4-specific antagonist. Soluble Tat selectively inhibited the entry and replication of X4, but not R5, virus in peripheral blood mononuclear cells (PBMCs). We propose that one functional consequence of secreted Tat is to select against X4 viruses, thereby influencing the early in vivo course of HIV-1 disease.

Materials and Methods

Competitive Ligand Binding. For 125I-labeled chemokine binding to whole cells, 1 × 10⁶ cells were incubated for 1 h at room temperature in buffer [HBSS plus 1% BSA and 0.1% sodium azide (pH 7.4)] containing 0.1 nM 125I-labeled chemokines (NEN) in the presence or absence of unlabeled chemokines or Tat. Specific activity for each chemokine used was 2200 Ci/mmol, as indicated by the manufacturer. Cells were centrifuged through Hanks' balanced salt solution (HBSS)/1% BSA containing 0.5 M NaCl, the supernatants were removed, and pellets were counted in a gamma counter. Each condition was tested in triplicate. Data are presented as unadjusted cell-associated counts. Where indicated, binding was competed with a 40-aa Tat peptide corresponding to residues 11 to 50. SDF-1 binding to CXCR4 was performed using human PBMCs; RANTES or MIP-1β binding to CCR5 was performed using HEK293 cells stably expressing transfected hCCR5 (17). Specific competition used an excess (100 nM) of unlabeled cognate chemokine.

Calcium Mobilization Assays. Calcium-mobilization was measured by Fura-2 fluorescence as previously described (18).

Purification of Proteins. 1-Exon (1–72) and 2-exon (1–101) Tat or mutant derivatives were fused to the maltose-binding protein in Escherichia coli expression vector pMAL-c2. Recombinant proteins were purified according to the manufacturer’s protocol (New England BioLabs). 1-exon and 2-exon Tat fusion proteins were checked to be bioologically active for cellular uptake and transactivation of an unintegrated HIV-long terminal repeat (LTR)-reporter.

Cell Culture, HIV-1 Infection, and Reverse Transcriptase (RT) Assays. PBMCs were stimulated with phytohemagglutinin 3 days before infection and were maintained in medium containing IL-2. Infections were performed in duplicate. Cells (1 × 10⁶) were infected with 100 tissue culture ID₅₀ (TCID₅₀) of X4-HIV-1 strain NL4-3 with the indicated concentrations of soluble pro-

Abbreviations: RANTES, regulated upon activation, normal T cell expressed and secreted; SDF, stromal cell-derived factor; CCR5, CC chemokine receptor 5; CXCR4, CXC chemokine receptor 4; PBMC, peripheral blood mononuclear cell; MIP, macrophage inflammatory protein; LTR, long terminal repeat; RT, reverse transcriptase; PVDF, polyvinylidene difluoride; MBP, maltose-binding protein; GST, glutathione S-transferase; MAGI, multinuclear activation of a galactosidase indicator.

*H.X., C.N., and H.L.T. contributed equally to this work.

†Deceased July 10, 1998.

‡To whom reprint requests should be addressed at: Building 4, Room 306, 9000 Rockville Pike, National Institutes of Health, Bethesda, MD 20892-0460. E-mail: kj7e@nih.gov.

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teins. Fresh proteins were replenished into culture medium every 4 days, and culture supernatants were sampled for RT activity as previously described (19). All HIV-1 infections were repeated a minimum of 3 times.

**Immunodetection of Tat in Serum.** Poly(vinylidene difluoride) (PVDF) (Immobilon P, Millipore) membrane was activated with methanol for 30 s and washed four times (1 min each) with TN buffer [50 mM Tris-HCl (pH 8.0)/50 mM NaCl]. Membrane was placed on a sheet of Whatman 3M paper lightly soaked with TN buffer and positioned atop a dry pad of absorbent paper. Serum samples (0.5 ml) were mixed with an equal volume of 2x TN buffer. Samples were spotted 50 ml at a time onto PVDF membrane and allowed to slowly air dry. Membranes were then treated with blocking buffer [0.2% 1-block reagent (Tropix)/1/x PBS/0.1% Tween 20] followed by incubation with a mixture of two polyclonal rabbit anti-Tat sera (raised to Tat peptide residues 1–25 and 62–86). Parallel assays were performed in which 5 μg of maltose-binding protein (MBP)-Tat (1–101) protein was added to anti-Tat sera for 1 h at room temperature before incubation with filters. Washing and chemiluminescent detection were according to manufacturer’s protocol (Tropix, Bedford, MA).

**PCR Analyses.** Infected cells (1 x 10⁶) were washed and resuspended into 100 ml of lysis buffer [10 mM Tris-HCl (pH 8.0)/0.5 mM EDTA/0.001% Triton X-100/0.001% SDS]. PCR was performed with HIV-1-specific primers and actin primers as detailed in the legends to Fig. 4 and Fig. 5. Each 100-μl PCR reaction contained 5 μl of cell lysate. Specific PCR products were confirmed by Southern hybridization to 35P-labeled HIV-1 LTR probe (Fig. 4).

**RESULTS**

**HIV-1 Tat Binds CXCR4.** Tat is selectively secreted from HIV-1-infected cells (20, 21), suggesting potential extracellular functions (22, 23) in addition to its well-characterized nuclear transcriptional role. Fortuitously, we noted amino acid similarities between Tat and chemokines. When Tat was compared with 14 CC-chemokines (including I-309, RANTES, MIP-1α, MIP-1β, eotaxin, and others), several conserved elements, including a CC-motif (at Tat amino acids 30 and 31) and a high density of basic amino acids were revealed (data not shown; alignment comparisons available on request). Related features including a CXC-motif (at Tat amino acids 25 and 27) were also seen between Tat and CXC-chemokines (SDF-1, MIP-2α, MIP-2β, NAP-2, IL-8, GCP-2, and ENA-78; data not shown). Despite these similarities, the overall sequence identities between Tat and chemokines remain, at best, modest; and the three-dimensional structures are completely different.

Chemokines regulate leukocyte trafficking by activating specific G protein-coupled receptors. Many chemokine receptors, including CXCR4 (24) and CCR5 (2–4), have been characterized as G protein (co)-factors for HIV. Ligands for these chemokine receptors can affect HIV-coreceptor activity (2–4, 25, 26). The weak sequence-relatedness between Tat and chemokines prompted us to ask whether Tat affects HIV coreceptor activity. We first checked whether Tat is an authentic ligand for chemokine receptor(s) by using a ligand-receptor competition assay (Fig. 1a). Cells expressing either CXCR4 or CCR5 were separately equilibrated with 125I-labeled ligands (SDF-1α, RANTES, or MIP-1β) with or without escalating amounts of Tat or 100 nM unlabeled chemokine. Tat competed efficiently (as effective as unlabeled SDF-1) for 125I-SDF-1 binding to CXCR4+ cells (Fig. 1a Left), but did not compete for binding of either 125I-RANTES (Fig. 1a Center) or 125I-MIP-1β to CCR5 (Fig. 1a Right). Consistent with this, protein affinity chromatography results showed direct binding of glutathione S-transferase (GST)-Tat (1–101) to CXCR4 without significantly detectable binding to CCR8, CCR5, or CCR4 (Fig. 1b). A similar binding specificity by Tat for CXCR4 protein was also verified in yeast two-hybrid assays (data not shown).

**Tat Antagonizes CXCR4-Function.** The physical interaction between Tat and CXCR4 (Fig. 1 and data not shown) directed us to consider functional significance (i.e., would extracellular Tat perturb SDF-1/CXCR4 signaling?). To ensure that any observed biological effect(s) emanated from Tat and not from endotoxins that might copurify in minute quantities with recombinant protein expressed in E. coli, we synthesized Tat and mutant polypeptides bearing alanine substitutions in the CC or CXC motifs, which were verified for purity by mass spectroscopy (J. Coligan, unpublished data). Stimulus-induced calcium flux occurred when PBMCs were exposed to SDF-1 (Fig. 2, panel 1, top recording); however, pretreatment with Tat peptide completely abolished this flux (Fig. 2, panel 1, bottom recording). Mutant Tat polypeptide changed in the CXC motif failed to...
Tat inhibits CXCR4-dependent infection of cells by HIV-1 NL4-3. Phytohemagglutinin-stimulated PBMCs were infected with 100 TCID<sub>50</sub> of NL4-3 in the presence of indicated proteins at either 2 mg/ml (a) or 20 ng/ml (b). Fresh proteins were replenished into cultures at days 4, 8, and 12, and media supernatants were sampled for RT activity. Representative day 4 RT values (similar profiles were also seen on days 8 and 12; not shown) are presented; results were replicated three times. (c) Tat and SDF-1 synergistically inhibit NL6-3 infection of PBMCs. PBMCs with MBP alone, MBP + SDF-1, or MBP + SDFTat101 were infected with NL6-3. SDFTat concentration was 800 ng/ml; MBP or MBPTat72 was at 2 μg/ml. RT average values from three independent experiments are from day 8 after infection.

affect SDF-1-signaling (Fig. 2; panel 1, middle recording). Collectively, these results show a functional interaction between Tat and CXCR4 that requires an intact CXC motif.

Specificity of Tat for CXCR4 was compared with effects on PBMCs, monocytes, or a CCR5-expressing HEK293 cell line (hCCR5/293; Fig. 2; panels 2–5) by CCR5 agonists, RANTES, MIP-1α, and MIP-1β. In contrast with SDF-1-signaling (Fig. 2, panel 1), Tat did not affect signaling by these CCR5 agonists (Fig. 2; panels 2–5; lower recordings).

Tat Inhibits CXCR4- but Not CCR5-Tropic Infection of Cells by HIV-1. The above findings suggest that soluble Tat might selectively affect HIV-1 envelope–CXCR4 interaction. To address this issue, biologically active forms of 1-exon (Tat 72) and 2-exon (Tat 101) Tat proteins fused to MBP were expressed and purified. MBPTat72, MBPTat101, and MBP alone were tested at concentrations of 2 mg/ml and 20 ng/ml (Fig. 3 a and b) in X4-tropic infection of PBMCs. Compared with MBP alone, MBPTat101 at 20 ng/ml significantly inhibited infection; curiously, the same protein at 2 mg/ml slightly promoted infection (Fig. 3a). Opposing concentration-dependent findings have been described for several cytokines (27). A possible explanation for our findings here is that two separate Tat activities, a chemokine receptor-blocking role at low concentration and a dominant nuclear trans-activation function secondary to cellular uptake of Tat in monocytes, and that both inhibit HIV-1 at ng/ml concentrations (28). Hence, anti-HIV-1 potencies of Tat and β-chemokines are similar, with the latter being selective for R5- and the former for X4-HIV-1.

SDF-1 inhibits X4 virus infection of PBMCs (29). We wondered whether Tat potentiates this SDF-1 inhibition. At concentrations of 20 ng/ml, 200 ng/ml, and 800 ng/ml, SDF-1-treated cells showed 6-, 8.5-, and 10-fold inhibition of HIV-1 infection, respectively, when compared with mock-treated PBMCs (data not shown). When SDF-1 (800 ng/ml) was incubated with 1-exon Tat (2 mg/ml) or with the wild-type peptide used in Fig. 2 (data not shown), X4 infection of PBMCs was reduced by an additional 10-fold over that of SDF-1 alone (Fig. 3c). Because both Tat and SDF-1 can bind to CXCR4 (Figs. 1 and 2), we interpret these results as Tat contributing to further occupy CXCR4, which might otherwise be vacant despite 800 ng/ml of SDF-1. However, we cannot exclude that there could be additional complex interactions between HIV-1 envelope/SDF-1/Tat with CD4 and CXCR4.

Tat Inhibits HIV-1 Infection at the Step of Entry. The above findings suggest, but do not prove, that Tat inhibits entry of X4 virus into cells. To test this hypothesis directly, we measured virus entry by using PCR (Fig. 4 a and b) and multinuclear activation of a galactosidase indicator (MAGI)-based (Fig. 4c) assays. Tat's interference with CXCR4-dependent entry was checked by comparing infection of HOS-CXCR4 cells by X4-NL4-3 with HOS-CCR5 cells by R5-NLAD8 (30; Fig. 4b). Paired infections incubated with either MBP alone (Fig. 4b Left) or MBPTat72 (Fig. 4b Right) were analyzed by PCR at 0 (Fig. 4b; lanes 1, 2, 7, and 8), 3 (Fig. 4b; lanes 3, 4, 9, and 10), and 6 h (Fig. 4b; lanes 5, 6, 11, and 12) after virus inoculation. MBPTat72 was found to inhibit X4- (Fig. 4b; lanes 4 and 6) but not R5-dependent (Fig. 4b, lanes 10 and 12) virus infection. Similar results were achieved by using wild-type Tat peptide (Fig. 2; data not shown). We also checked Tat's CXCR4-specific activity by using the widely accepted MAGI-entry assay (31). Here, MBPTat72 inhibited X4-tropic NL4-3 infection of U373-MAGI-CXCR4 cells, but did...
not affect R5-tropic NLAD8 infection of U373-MAGI-CCR5 cells (Fig. 4c).

Soluble Tat Selects Against CXCR4-Tropic Env Residue. We asked next whether Tat's selective effect at CXCR4 influences evolution of viral tropism. Several residues in the V3 loop of gp120 are known to influence tropism; changes at one or two of these positions can sufficiently alter coreceptor specificity (9, 10, 11). One corollary, amongst many, on coreceptor usage by HIV-1 is that the charge of V3 residue 25 exerts an important, albeit only partial, contribution to tropism.

Detection of Soluble Tat in HIV-1 Seropositive Individuals. If Tat functions in vivo as an X4 restriction factor, then circulating Tat protein in HIV-1-infected individuals perhaps could be verified. Meaningful in vivo measurements of Tat have been difficult for several reasons. First, existing Tat antibodies are weak in avidity and are poorly standardized from laboratory to laboratory (ref. 32; K.T.J., unpublished data). Quantitative values from different investigators are thus difficult to compare. Second, the relevant body compartment (blood, mucosa, lymph node?) to sample for Tat is not known. Third, Tat most likely is a locally acting cytokine that can be trapped by molecules such as heparin sulfates becoming sequestered on extracellular matrices. Recognizing these caveats, we attempted to measure Tat protein in 80 anonymous HIV-1+ patient sera from the National Institutes of Health Clinical Center.

More than 33% of the 80 sera had humoral reactivity to Tat (data not shown). We next checked for soluble Tat in these sera by using a mixture of two hyperimmune rabbit antibodies raised by us to synthetic Tat peptides. The antisera at 1:1000 dilution...
easily detected 0.1 ng of membrane-bound MBPTat101 (Fig. 6, top row, left). When HIV-1+ patient sera were individually spotted onto 1 × 1 cm² PVDF filters and were reacted with rabbit anti-Tat followed with detections by chemiluminescence, 5 of 80 samples showed clear reactivity to anti-Tat (Fig. 6, rows 4 and 5). These signals were Tat specific because competitions with excess MBPTat101 protein eliminated detection (Fig. 6, rows 2 and 3). Extrapolating from Tat standards, the five positives approximated concentrations from 2 ng/ml to 40 ng/ml. These values could be underestimations because local concentrations of Tat in lymphoid tissues might be higher and Tat in vivo might be sequestered by endogenous anti-Tat and/or by glycosaminoglycans. Until more reliable methods can be developed to measure in vivo amounts of viral protein, the full biological implications of circulating Tat protein cannot be entirely determined.

Discussion

Current findings on neutralizing antibodies and cytotoxic T cell responses insufficiently explain the slow appearance of X4 viruses in HIV-1-infected individuals. The relatively late emergence of X4-HIV-1 (>7 y) after infection has prompted several investigators to suggest the existence of an early negative selection against X4 virus (6, 16). Here, we propose the virally encoded Tat protein as an early selective factor against X4-HIV-1. We show that Tat binds CXCR4 (but not CCR5; Fig. 1), abolishes SDF-1/CXCR4 (but not β-chemokine/CCR5) signaling (Fig. 2), inhibits X4- (but not R5-) mediated viral infection/entry of cells (Figs. 3 and 4), and selects against X4-HIV-1 tropism in infections of PBMC (Fig. 5).

Why might HIV-1 encode a factor against X4 tropism? Chronologically, appearance of X4-HIV-1 in vivo coincides with a rapid decline of CD4+ T cells and onset of clinical immunodeficiency (29, 33, 34). From several perspectives X4-HIV-1 is considered more pathogenic than its R5 counterpart (34). Thus, if moderate (i.e., R5 virus) pathogenicity benefits HIV-1 for purposes of transmission and maintenance in host populations, then X4 antagonism through Tat may represent a means for the virus to achieve host-independent moderation of virulence.

Currently, we do not understand how selectivity for CXCR4 is achieved by Tat. Our experiments do support that Tat's CXC motif is important (Figs. 1 and 2). Additional mechanistic insights emerge from three recent reports characterizing X4-specific small molecule inhibitors (AMD3100, ref. 35; T22, ref. 36; and ALX40-4C, ref. 37). A common concept that emerges from these three diverse inhibitors is a principle that positively charged molecules show strong binding for CXCR4 (38). Thus, AMD3100 is highly cationic under physiological conditions (39); ALX40-4C is a nonapeptide of 9 arginines; and T22 is an 18-mer with 8 positively charged (lysine or arginine) amino acids. Interestingly, CXCR4's extracellular surface is extremely acidic whereas CCR5's surface is more neutral to basic; these charge properties are also consistent with the Tat/coreceptor specificity described here. Accordingly, the antiviral potency of Tat [~90% inhibition of X4 viruses at 20 ng/ml (Fig. 3) and ~50% inhibition at 2 ng/ml; data not shown] is similar to that of AMD3100 (35) and T22 (36) and is slightly superior to ALX40-4C (37).

Our hypothesis of selective X4-antagonism must be evaluated cautiously in view of recent success in vaccinating subhuman primates with Tat (41). Should it act in vivo to slow R5 to X4 transition, then a potential consequence of immunization with Tat might be to accelerate emergence of X4 virus when the vaccinee is subsequently infected with HIV-1. Whereas it is unclear what early emergence of X4 virus in humans might mean, recent observations suggest that this change would not benefit disease course (29, 33, 34). Balanced against this prediction are recent reports that immunization with Tat in macaques partially protected the host against subsequent challenge with a highly pathogenic simian-HIV (SHIV; refs. 42 and 43). However, because monkeys naturally do not use CXCR4 as a coreceptor for infection by simian immunodeficiency virus (SIV; ref. 44), it is unclear whether loss of Tat's CXCR4 antagonism holds the same implication for SHIV/macaques as for HIV-1/humans. Proposed and ongoing studies on vaccinating humans with Tat (41) should yield clarifying information.

Our findings here are somewhat at odds with two recent reports that suggested that Tat up-regulates both CXCR4 and CCR5 (45) or only CXCR4 (46) expression. In our experiments, we consistently have failed to observe either CXCR4 or CCR5 expression being modulated by Tat. However, we cannot exclude that cell surface antagonism of CXCR4 by Tat might lead to compensatory up-regulation of expression. Indeed, as noted in Fig. 3, several competing effects of Tat likely coexist with optimal suppression of X4 virus requiring a complex interaction of Tat + SDF-1 at CXCR4. In this regard, despite suppression from Tat + SDF-1, in a minority of cases X4-HIV-1 does eventually emerge in vivo. Possibly, this emergence is a consequence of progressive degradation of lymphoid architecture by R5 viruses leading to loss of SDF-1 production (16) coupled with the contribution to virus replication, at this stage, by Tat's induction of CXCR4 expression (45, 46).

Note Added in Proof. After submission of this work, we noted that similar findings were reported by Ghezzi et al. (47). We thank I. Quinto, T. Murakami, S. Smith, and B. Berkhourt for critical readings; M. Van and L. Lin for preparation of manuscript; C. Lane for anonymous sera; M. Emerman for cells; J. Coligan for help with peptides; and AIDS Targeted Antiviral Program from the Office of the Director, National Institutes of Health for funding.


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