Activity of synthetic peptides from the Tat protein of human immunodeficiency virus type 1

(Received Immunodeficiency Syndrome/Transactivator Protein)

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*Communicated by David Baltimore, July 10, 1989*

**ABSTRACT** To determine which of the 86 amino acids in the Tat protein of human immunodeficiency virus type 1 (HIV-1) are important for transactivation, peptides from Tat were synthesized and their activity was measured in cells containing a chloramphenicol acetyltransferase reporter gene under control of the HIV long terminal repeat promoter. Although the Tat sequence contains arginine- and cysteine-rich stretches that are difficult to synthesize, it was possible to prepare pure peptides in good yield by using fluoren-9-ylmethylcarbonyl (Fmoc) chemistry. A peptide containing residues 1-58 had 5-10% of the activity of full-length Tat. Deleting 4 amino acids from the N terminus of this peptide further reduced activity, while peptides with more extensive N-terminal deletions and peptides missing the basic region at the C terminus had no detectable activity. A peptide previously reported to transactivate, Tat-(37-62), was completely inactive in our assays. Inactive peptides were also tested as possible inhibitors of transactivation. Tat-(21-38), which contains the cysteine-rich region and can form heterodimers with intact Tat, is more sensitive to inhibition by Tat-(37-62) than are Tat-(1-40) or Tat-(21-38). Thus, these results indicate that the cysteine-rich region of Tat is required for transactivation.

**MATERIALS AND METHODS**

**Synthesis of Tat Peptides.** Syntheses were performed using Fmoc chemistry on a Milligen/Biosearch model 9600 peptide synthesizer with a peptide amide linkers-norleucine-4-methylbenzylidramine (PAL-Nle-MBHA) polystyrene resin (Milligen/Biosearch; 0.5 g). The benzotriazololoxypyridis(di- methylaminophosphonothiofluorophosphate/1-hydroxybenzotriazol (BOP/HOBr) coupling method (24) was used with coupling times of 1-4 hr and with double coupling of His-33. Protecting groups were tert-butyl ester (for Glu and Asp), 2,2,5,7,8-pentamethylichroman-6-sulfonyl (Arg), tert-butylcarbonyl (Lys), trityl (His and Cys), tert-butyl (Ser, Thr, and Tyr), and trimethoxybenzyl (Asn and Gin). All peptides were synthesized as their C-terminal amides. After synthesis was completed, protecting groups were removed and the peptide chains were cleaved from the resin with trifluoroacetic acid/ethanedithiol/thioanisole/anisole (90:3:5:2, vol/vol). The mixture was filtered and the products were obtained by addition of cold anhydrous diethyl ether to the filtrate. The precipitate was collected by filtration, thoroughly washed with ether, and dried.

**Purification and Analysis of Tat and Peptides.** Peptides were treated with 0.5 M dithiothreitol at 37°C for 30 min to ensure complete reduction of the cysteines and were purified on a C4 HPLC column (Vydac) using an acetonitrile gradient in 0.1% trifluoroacetic acid. Amino acid composition was determined by hydrolysis in 6 M HCl containing 0.5% phenol at 110°C and analysis on an LKB Alpha Plus analyzer. Peptide purity (>90%) was determined by HPLC using a linear gradient of <0.5% per min. Tat-(37-62)-peptide (0.6 nmol) was sequenced (27 cycles on a model 470A Applied Biosys-

Abbreviations: CAT, chloramphenicol acetyltransferase; FAB, fastatom bombardment; Fmoc, fluoren-9-ylmethylcarbonyl; HIV, human immunodeficiency virus; LTR, long terminal repeat.

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tems protein sequencer; P. Matsudaira, Whitehead Institute) and found to be correct. Tat-(37–62) was also analyzed by fast-atom bombardment (FAB) mass spectrometry (T. D. Williams and J. A. Leary, University of California, Berkeley). A VG ZAB2-EQ instrument was used with a primary FAB beam energy of 30-keV cesium ions (1 eV = 1.602 × 10−9 J) at 3-μA emission current. Peptide was dissolved in dilute acetic acid before addition to the 3-nitrobenzyl alcohol liquid matrix, and mass assignments were made by calibration against cesium iodide cluster ions. The measured molecular weight of the MH+ ion was 3060 (calculated, 3059.8).

Tat-(1–72) and Tat-(1–86) bacterial proteins were purified as described (20, 22). Chymotryptic fragments of Tat (residues 1–47 and 48–86) were prepared by partial proteolysis of Tat-(1–86) with chymotrypsin (20) followed by C8 HPLC purification.

Transactivation Assays. Transactivation was assayed by introducing Tat proteins or peptides into HeLa cells containing an integrated HIV-1 LTR–chloramphenicol acetyltransferase (CAT) gene construct (HL3T1 cells; ref. 25). For most experiments, cells (50–70% confluent) were scrape-loaded with peptide in 25-mm wells in the presence of 100 μM chloroquine (see ref. 22). The combination of scrape-loading and chloroquine treatment gives about 10-fold higher transactivation than either method alone. Cells were incubated at 37°C for 24 hr and assayed for CAT activity (26). Some plasmids (pHIV-CAT, pSV2cat72, or pSV2cat) were transiently transfected into HeLa cells by lipofection for 4 hr (27) before introduction of proteins or peptides. In these cases, CAT activity was assayed 24 hr after peptide treatment. All cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

RESULTS

Peptide Synthesis. To determine which regions of the 86-amino acid Tat protein are important for transactivation, several Tat fragments were synthesized using Fmoc chemistry (Fig. 1). Peptides corresponding to Tat residues 1–58, 5–58, 10–58, 15–58, 21–58, and 38–58 were synthesized by starting with the C-terminal Pro–58 and removing aliquots of the resin at appropriate points in the synthesis. Similarly, peptides corresponding to residues 1–38, 4–38, 10–38, 15–38, and 21–38 were synthesized by starting with the C-terminal Phe–38. An additional peptide containing residues 37–62 was synthesized. After cleavage and deprotection, each peptide was reduced with 0.5 M dithiothreitol, purified by reverse-phase HPLC (to >90% purity by HPLC analysis), and characterized by amino acid analysis. The identity of Tat-(37–62) was further confirmed by peptide sequencing and FAB mass spectrometry. The n–58 series of peptides was synthesized twice, and the final products from each synthesis were identical. Overall yields of Tat-(1–58), (1–38), and (37–62) were estimated to be 20%, 30%, and 60%, respectively.

Ultraviolet absorption spectroscopy was used to estimate the amount of each peptide and to monitor metal binding to the cysteine-rich region (residues 22–37) (20, 21). Peptides containing the cysteine-rich region were titrated with CdCl2 and showed the expected stoichiometry of 2 metal ions per peptide monomer (refs. 20 and 21; data not shown). The reduced Tat peptides were also analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions (Fig. 2). Each peptide showed the expected migration based on size and charge, and, as seen with the intact protein (20), peptides that contained the cysteine-rich region oxidized to form Tat oligomers during electrophoresis.

Activity of Tat Peptides. Each Tat peptide was tested for its ability to transactivate the HIV-1 promoter. Peptides were scrape-loaded into HeLa cells containing an integrated LTR–CAT construct (HL3T1 cells) (22). Cells were also incubated with chloroquine, which inhibits Tat proteolysis (22) and increases the signal about 10-fold. Bacterially synthesized Tat-(1–58) and Tat-(1–72) transactivated the HIV-1 promoter at least 35-fold (Fig. 3). Of the synthetic peptides with the C terminus at residue 58, Tat-(1–58) showed reasonable transactivation (about 35-fold; Fig. 3). Tat-(5–58) also showed some transactivation (about 10-fold), but no other peptide had significant activity. No activity was seen with any peptide ending with its C terminus at Phe–38. Chymotryptic fragments containing residues 1–47 or 48–86 were also assayed and showed no transactivation (data not shown). These results suggest that the N-terminal region, the cysteine-rich region, which oxidizes during electrophoresis to form dimers and higher oligomers.
region, and the basic region are all necessary for Tat transactivation.

It was recently reported (28) that a Tat peptide containing only residues 37–62 retained most of the activity of the full-length protein. However, a similar peptide, Tat-(38–58), was completely inactive in our assays (Fig. 3). To test directly whether the Tat-(37–62)-peptide has transactivation activity, we resynthesized Tat-(37–62) and confirmed its identity by amino acid analysis, peptide sequencing, and FAB mass spectrometry (see Materials and Methods). Tat-(37–62) was introduced into HL3T1 cells by (i) adding peptide to the medium, (ii) scrape-loading, (iii) incubating cells with peptide and chloroquine, or (iv) scrape-loading in the presence of chloroquine. The peptide had no detectable activity, even at molar concentrations 100-fold higher than Tat-(1–72) (Fig. 4). Cellular uptake of radiiodinated Tat-(37–62) was also measured (see ref. 22). The amount of peptide taken up from the medium was about 10-fold less than for Tat-(1–72), but the amount of internalized peptide could be increased by scrape-loading (data not shown). Thus, even though the Tat-(37–62)-peptide was present inside cells, it showed no transactivation activity.

Inhibition of Transactivation. It was shown previously that Tat could form metal-linked dimers in vitro and that an 18-residue peptide containing just the cysteine-rich region (residues 21–38) could mimic Tat dimerization (20, 21). The peptide also formed heterodimers with the intact protein, suggesting that if the dimer is important for Tat transactivation, Tat-(21–38) might inhibit the activity of Tat by forming inactive heterodimers (21). To test this possibility, various amounts of Tat-(21–38) were scrape-loaded into HL3T1 cells along with the intact protein. Transactivation was inhibited >90% at high Tat-(21–38) concentration (200 μg/ml; data not shown). To test whether inhibition was specific for Tat and the HIV-1 promoter, pHIV-CAT was cotransfected with a Tat expression vector, pSV2tat72 (22), or pSV2cat was transfected into HeLa cells. After 4 hr, cells were scrape-loaded with various amounts of Tat-(21–38)-peptide. Inhibition by Tat-(21–38) was nonspecific: both the HIV-1 promoter and the simian virus 40 early promoter were inhibited at high peptide concentrations (Fig. 5). Similar inhibition was seen when pHIV-CAT was transfected into HeLa cells and the transfectants were scrape-loaded with bacterial Tat-(1–72) protein and synthetic Tat-(21–28)-peptide (data not shown). Inhibition by other Tat peptides (residues 5–58,
It has been reported that a synthetic peptide containing only Tat residues 37–62 has significant transactivation activity (28). This seemed inconsistent with our results and with the results of mutagenesis studies showing that many residues outside this region, and in particular the cysteines located between residues 22 and 37, are essential for Tat activity (18, 29, 31). The cysteines are highly conserved among all isolates of both HIV-1 and HIV-2 (the only difference being one additional amino acid between the first pair of cysteines in HIV-2 Tat), and this region mediates metal-linked dimerization of Tat in vitro (20, 21). To resolve the apparent discrepancy, we synthesized Tat-(37–62) and found that it has no detectable activity under four different assay conditions (Fig. 4). We cannot explain the difference in results.

Tat has been shown to form metal-linked dimers in vitro, with four metal ions bridging the cysteine-rich regions from each monomer (20). A peptide containing just the cysteine-rich region [Tat-(21–38)] formed heterodimers with intact Tat in vitro (21), leading to the suggestion that this peptide might inhibit transactivation if dimerization is important for activity. Tat-(21–38) was found to inhibit Tat transactivation at high peptide concentrations, but the effect was nonspecific since it also blocked expression from the simian virus 40 early promoter at similar concentrations (Fig. 5). This inhibition may be caused by the seven peptide thiol groups, since other thiol reagents, such as 2-mercaptoethanol or dithiothreitol, can inhibit cell growth (A.D.F., unpublished data). Other Tat peptides were also tested for inhibition and no specific effects were seen. Although there are many possible explanations for why these peptides do not show trans-dominant inhibition in our assays, it seems unlikely that they can be used to specifically block Tat function in vivo.

**Note Added in Proof.** In a recent paper (32), it was suggested that Tat peptides may be unstable during the time course of our assays. However, it has been shown (22) that transactivation can be easily detected after only 1 hr of Tat treatment (35-fold). Transactivation increases to more than 1000-fold after 8 hr and reaches a plateau by 24 hr. It seems unlikely that treatment times can explain any differences in the activity of Tat-(37–62) or other Tat peptides.

We thank Carl Pabo for discussions and support during the early stages of this work, Hiroki Morizono and Beishan Liu for technical assistance, Todd Williams and Julie Leary for mass spectrometry, and Peter Kim for helpful discussions. This work was supported in part by a grant from the Lucille P. Markey Charitable Trust.


**FIG. 5.** Effect of Tat-(21–38) on transactivation of the HIV-1 promoter and the simian virus 40 early promoter. HeLa cells were transfected with pHIV-CAT plus pSV2tat72 or with pSV2cat by lipofection for 4 hr (27). One milliliter of fresh medium was added, and Tat-(21–38) was scrape-loaded in the presence of chloroquine. CAT activity was determined 24 hr after scrape-loading.

10–58, 15–58, 21–58, 38–58, and 1–38) was also tested but none showed any inhibition at 20 μg/ml.

**DISCUSSION**

A quantitative analysis of synthetic HIV-1 Tat peptides and bacterially produced Tat proteins has shown that most of the Tat sequence is required to achieve high levels of transactivation. This analysis was made possible by recent advances in Fmoc chemistry that allow synthesis of the complex cysteine- and arginine-rich sequences found in Tat. The major advantages of Fmoc chemistry for such sequences are the relatively mild cleavage conditions, which help minimize irreversible cysteine modifications, and the complete deprotection of cysteine and arginine side chains achieved by using trityl and pentamethylchroman-6-sulfonyl protecting groups, respectively.

The full-length Tat protein is 86 amino acids long and is encoded by two exons; the N-terminal 72 residues are encoded by the first exon and the C-terminal 14 residues are encoded by the second exon. In the unspliced mRNA, there is a highly conserved termination codon immediately after residue 72, allowing translation of just the 72-amino acid exon. The 72- and 86-amino-acid peptides have similar activities (Fig. 3), consistent with previous studies suggesting that the second exon is dispensable for activity (8, 9, 17). A synthetic peptide corresponding to Tat residues 1–58 has 5–10% of the activity of the 1–86 or 1–72 bacterial protein (Fig. 3), suggesting that residues between 58 and 72 also contribute to transactivation. Other studies have shown that a Tat deletion mutant containing residues 1–60 and Tat fusion proteins containing residues 1–56 or 1–58 have somewhat reduced activity (9, 29, 30).

Deleting further from the C terminus, to residue 47 in the chymotryptic fragment or to residue 38 in the synthetic peptides, completely eliminates transactivation. These peptides lack the basic region of Tat, which seems to be important for nuclear localization (18, 19), and mutations in this region have been shown to reduce transactivation (18, 19). Residues at the N terminus are also important for activity; a peptide with the first 4 amino acids deleted [Tat-(5–58)] has reduced activity, and peptides with more extensive deletions [Tat-(10–58), (15–58), and (21–58)] are completely inactive (Fig. 3). Garcia et al. (29) reported that an N-terminal deletion mutant containing residues 10–86 also had little activity. Tat-(21–38), a small peptide containing just the cysteine-rich region, had no activity.

**Table 1.** Relative CAT activity of Tat-(21–38) (μg)