The N-terminal Structure of HIV-1 Tat Is Required for Suppression of CD26-dependent T Cell Growth*

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Evidence exists that the human immunodeficiency virus-1 (HIV-1) transactivator Tat occurs extracellularly and is involved in the immunosuppression of non-HIV-1-infected T cells of acquired immunodeficiency syndrome (AIDS) patients. The mechanism of this immunosuppressive activity of Tat has been controversially discussed. Interestingly, Tat binds to the T cell activation marker CD26, which has been shown to play a key role in the regulation of growth of lymphocytes and to inhibit its dipeptidyl peptidase IV (DP IV) activity. Here we show that the N-terminal nonapeptide MDPVDPNIE of Tat is a competitive inhibitor of DP IV and suppresses DNA synthesis of tetanus toxoid-stimulated peripheral blood mononuclear cells. Amino acid exchanges at positions 5 and 6 strongly weaken these effects. 1H nuclear magnetic resonance and molecular dynamics simulations of Tat(1–9), I5-Tat(1–9), and L6-Tat(1–9) suggest a similar backbone conformation for Tat(1–9) and L6-Tat(1–9). The solution conformation of I5-Tat(1–9) considerably differs from the other two. However, Tat(1–9) fits into our previously proposed active site model of DP IV in contrast to I5-Tat(1–9) and L6-Tat(1–9). Conformational alterations with regard to the parent peptide and spatial hindrances between these both compounds and DP IV can explain the loss of inhibitory activity. Our data suggest that the N-terminal residues of HIV-1 Tat do interact directly with the active site of DP IV and that DP IV does mediate Tat’s immunosuppressive effects.

The human immunodeficiency virus-1 (HIV-1) Tat protein is a transactivator regulating the transcription of the viral genes and is essential for viral replication in vitro (1, 2). In addition, Tat exerts a variety of effects on cell growth and metabolism. Interestingly, HIV-1-infected T cells release Tat into the culture supernatant (3, 4). Extracellular Tat suppresses antigen-, anti-CD3-, and mitogen-induced activation of human T cells (5–7). Moreover, Westendorp et al. (8) have recently described that Tat sensitizes T cells to Fas-mediated apoptosis. One possible pathway of mediation of the immunosuppressive effects by extracellular Tat is the interaction with cell surface proteins. Some investigators demonstrated that Tat interacts with dipeptidyl peptidase IV (DP IV), an exopeptidase, which cleaves N-terminal dipeptides from oligopeptides with proline or alanine in the penultimate position and which is important for the growth of lymphocytes (6, 9). Specific inhibitors of DP IV suppress mitogen- and alloantigen-induced T cell proliferation, B and natural killer cell growth, as well as immunoglobulin secretion and modulate cytokine production (10–16).

However, the molecular mechanism of the function of DP IV in the proliferation and activation processes has not been resolved yet. Interestingly, the viral protein Tat is a natural inhibitor of DP IV and evidence exists that the immunosuppressive effects of HIV-1 are mediated at least in part by Tat-DP IV interactions (17). Recently, we have demonstrated the special importance of the N terminus of Tat. Modification of the N-terminal amino group with rhodamine prevented inhibition of DP IV as well as suppression of DNA synthesis in pokeweed mitogen-stimulated human T cells (7). In the present study, we examined the structural requirements of the N-terminal part of Tat for the effects on DP IV activity and cell proliferation.

EXPERIMENTAL PROCEDURES

Synthetic DP IV Inhibitor and Peptides—The DP IV inhibitor Lys(ZNO3)4-thiazolide was synthesized as described previously (12). Human IL-2(1–12) and Tat(1–9) peptides were synthesized by solid phase peptide synthesis with Fmoc (9-fluorenylmethoxycarbonyl) technique using the peptide synthesizer 431A (Applied Biosystems) (7). HIV-1 Tat(1–86) and Tat(1–72) were synthesized on polyoxyethylene-N-methylmorpholine. The synthetic peptides were purified by reversed-phase high performance liquid chromatography and characterized by mass spectrometry.

Preparation of Peripheral Blood Mononuclear Cells and Proliferation Assay—Peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood of healthy donors as described by Reinhold et al. (16). PBMC (105 cells/100 ml) were stimulated in serum-free CG medium with tetanus toxoid (100 ng/ml; Calbiochem-Novabiochem, Bad Soden/Ts., Germany) in the presence of effectors in the concentrations indicated. After 6 days, cultures were pulsed for an additional 16 h with [3H]methylthymidine (0.2 µCi/well; Amersham, Braunschweig, Ger-
many). Cells were harvested on glass fiber filters, and the incorporated radioactivity was measured by scintillation counting.

**DP IV-catalyzed Hydrolysis of IL-2(1–12)—**After preincubation (30 min, 37 °C) of 550 femtomoles of porcine kidney DP IV in 1 μl of 10 mM sodium phosphate assay buffer, pH 7.4, with 2 μl of effector in assay buffer, the enzymatic reaction was started by addition of 2 μl of 1 mM IL-2(1–12) substrate in assay buffer. Samples were incubated for 30 min at 37 °C. Thereafter, the reaction was stopped by addition of 2 μl of 30 mM phosphoric acid. Degradation of DP IV peptide substrate was measured by the method of free zone capillary electrophoresis using Biofocus 3000 system of Bio-Rad (München, Germany). Separations were performed as described by Reinhold et al. (17).

**Results**

3H NMR Spectroscopy—**1H NMR spectra were measured at 303 K at 6 mM peptide concentration on Bruker ARX500 and Varian UNITY 500 spectrometers. Chemical shifts were calibrated with respect to 3-(tri-methylsilyl)-1,3,3,2,2-tetrafluoroethyltrimethylsilane (sodium salt). Water suppression was achieved by using presaturation during relaxation delay. Torsion angles ϕ were calculated using Karplus-type equations (19). For determination of temperature coefficients spectra were recorded from 303 to 323 K in steps of 2 K. Standard methods were used to perform COSY, TOCSY, and ROESY experiments (20–22). Pulse programs were taken from Bruker and Varian software libraries. TOCSY and ROESY spectra were recorded in the phase-sensitive mode using the TIPPI or States-Haberkorn-Ruben methods (23–25). The TOCSY experiment used the MLEV-17 sequence for spin-lock (mixing time, τm = 80 ms) (26). The ROESY experiment (τm = 300 ms) at 303 K was used for observation of NOEs. For both TOCSY and ROESY, 512 FIDs of 2000 data points, 24 scans each, were acquired. In both dimensions, the data were processed using n/2-shifted, squared sinebell weighting functions. Zero filling to 2000 was applied in ω1.

**Energy Calculations and Molecular Dynamics Studies—**The compounds were energy-minimized to a convergence of energy gradient less than 0.001 kcal/mol Å using the TRIPOS force field included in the SYBYL MAXIMIN2 module (27). The partial atomic charges were computed using the Gasteiger method (28, 29). A distance independent dielectric constant ε = 78 was used in calculations to mimic the experimental NMR conditions (water solution). The manually built nonapeptides were optimized and used as an input for 200 cycles of simulated annealing dynamics runs. Each cycle included 1000 fs simulation at 1000 K and cooling to 300 K within 1000 fs. The resulting low energy conformations at 200 K were optimized by energy minimization. Starting with the most stable conformations found in the first run the procedure was repeated. Those conformations that fit the NMR data best were selected and solvated by water using a precomputed water box of 1400 solvent molecules. Molecular dynamics simulations at 300 K using periodic boundary conditions were carried out to test the stability of the conformations in solution. During the whole simulation time the overall structure of the three nonapeptides kept in equilibrium with the starting conformation except for the frequently observed breaking and forming of the hydrogen bonds between the side chains of Asn7 and Asp5. In the dynamics simulations we did not take into consideration any restraints based on NOEs of Tat(1–9), I5-Tat(1–9), and L6-Tat(1–9). Nevertheless, the average structures agree very well with all observed NMR data such as NOEs, dihedral angels f derived from Karplus equations, and temperature coefficients of the amide protons. Interactions of the Tat peptides with the model of the active site. To prove the specificity of the Tat-DP IV interaction, those replacements of Asp5 by Ile and Pro5 by Leu strongly influenced DP IV inhibition (Fig. 1A). I5-Tat(1–9) and L5-Tat(1–9) exerted 22 ± 6% and 9 ± 10% inhibition of IL-2(1–12) cleavage, respectively, compared with 62 ± 6% for P5-Tat(1–9) (Fig. 1B). Similar effects were observed on DNA synthesis of tetanus toxoid-stimulated PBMC (Fig. 1B). P5-Tat(1–9) or M5-Tat(1–9) reduced [3H]methyImidine incorporation as did Tat(1–9), whereas I5-Tat(1–9) and L5-Tat(1–9) had no effect.

The replacement of Pro5 by the slightly more hydrophobic Leu and the replacement of Asp5 by Ile generated Tat(1–9) peptide analogues which are ineffective in inhibition of IL-2(1–12) cleavage as well as in suppression of DNA synthesis suggesting differences in the preferred conformation of Tat(1–9), I5-Tat(1–9) and L5-Tat(1–9). Therefore, we performed 1H NMR studies in H2O/D2O (90:10) and molecular dynamics simulations on these three compounds.

**Results**

Recently, we observed that Tat(1–86) suppresses DNA synthesis in pokeweed mitogen- (2 μg/ml) and tetanus toxoid-stimulated PBMC in the same concentration range as Lys[2(NO2)]-thiazolidide, a highly specific, synthetic inhibitor of DP IV (IC50 = 4.5 μM), does (7). This is in striking accordance with the inhibitory effects of Tat(1–86) and Lys[2(NO2)]-thiazolidide on DP IV-catalyzed IL-2(1–12) cleavage. N-terminal-modified rhodamine-Tat(1–72) did not inhibit DP IV activity and had no effect on DNA synthesis (7).

**Interaction of the N-Terminal Structure of Tat with CD26**
L<sup>5</sup>-Tat(1–9) are similar, but the solution conformation of I<sup>5</sup>-Tat(1–9) considerably differs from the other two (Fig. 2). Docking of the determined solution conformations of the three peptides to the active site model of DP IV, proposed by us earlier, results in a marked difference: Tat(1–9) forms several attractive interactions to the amino acids of the active site, in contrast to both I<sup>5</sup>-Tat(1–9) and L<sup>5</sup>-Tat(1–9). Some of these interactions are missing in the I<sup>5</sup>-Tat(1–9) and L<sup>5</sup>-Tat(1–9) derivatives (compare Fig. 3, A and B) that leads to reduced affinities to the active site of DP IV in comparison to the parent peptide Tat(1–9). Therefore, the model helps to explain the low competitive inhibitory activity of both I<sup>5</sup>-Tat(1–9) and L<sup>5</sup>-Tat(1–9).

In more detail, from the <sup>1</sup>H chemical shifts, particularly αH shifts derived from the one-dimensional <sup>1</sup>H NMR spectra of the three compounds in water, it appears that all three peptides are relatively flexible, and a range of conformations is likely to exist in equilibrium with each other. Absence of NOEs between adjacent C<sup>α</sup>H protons and absence of chemical exchange cross peaks in TOCSY and ROESY spectra indicated that the average preferred solution conformation of the three peptides is characterized by all-trans peptide bonds. The <sup>3</sup>J<sub>HN-CαH</sub> homonuclear coupling constants for both peptides were in a range of 7.4 ± 0.8 Hz typical for conformational averaging of linear peptides. With a few exceptions no NOEs between NH(<i>i</i>) and NH(<i>i</i>+1) protons in the ROESY experiments were observed, suggesting that the amide protons are in general not directed to one side of the backbone but alternately oriented.

In Tat(1–9) the amide protons of Asp<sup>2</sup>, Val<sup>4</sup>, Asp<sup>5</sup>, and Glu<sup>9</sup> showed temperature coefficients −Δδ/ΔT from −7.15 to −8.55 ppm/K indicating intermolecular hydrogen bond formation with another solute or a solvent molecule. Smaller temperature coefficients in the range of −5.00 to −5.50 ppm/K expected for protons shielded from the surrounding solvent were observed.
for the protons N™H, N™H™, N™H™ of Asn™ and the amide proton of Ile™.

Theoretical conformational energy calculations were carried out using the TRIPPOS force field implemented in the SYBYL molecular modeling package (27). Of the roughly 400 obtained low energy conformations the preferred solution structure in water was selected by the comparison of all conformations with the NMR data. All vicinal coupling constants 3JHN-CaH and corresponding torsion angles ϕ of L5-Tat(1–9) as well as the temperature coefficients of the amide protons and relevant NOEs resembled those of Tat(1–9) suggesting a similar overall backbone conformation for L6-Tat(1–9) (Fig. 2A). The amino acids Met¹ to Val⁴ adopt an extended structure. Furthermore, two inverse γ-turns appear to exist both between Asp⁵ and Asn⁷ and Pro⁶ (or Leu⁶) and Ile⁸. The conformations of both peptides Tat(1–9) and L⁵-Tat(1–9) are additionally characterized by a hydrogen bond formed between the carboxyl group of Asp⁵ and its own NH group (Fig. 2A). With the hydrophobic side chain of Ile in L⁵-Tat(1–9) this interaction is not possible. The NMR data of I⁵-Tat(1–9) (NOEs and vicinal coupling constants) indicated alterations in the backbone torsion angles in particular in position 5 but also in positions 2, 4, 7, and 9. Altogether, a completely different solution conformation is favored for this peptide (Fig. 2B).

Recently, we proposed a model of the active site of DP IV on the basis of a multitude of experimental findings concerning the substrate specificity as well as results from site-directed mutagenesis experiments (30). The determined solution conformations of the N-terminal Tat fragments were used to dock to this model. Indeed, we found attractive interactions of several residues of Tat(1–9) with the active site of DP IV (Fig. 3A). The only slight alteration of the conformation that appeared to be necessary is the twist of the dihedral angle Ψ¹ (Met¹) by about 180°. In the solution conformation the N-terminal protonated

Fig. 3. Stereo representation of the docking of Tat(1–9) (magenta) into the pocket of the active site of DP IV (atom typed) (A) and of I⁵-Tat(1–9) (B). The steric hindrance between the Leu⁶ side chain of L⁵-Tat(1–9) (orange carbon atoms) and Trp⁶⁰ is shown. Dashed lines indicate hydrogen bonds.
amino group forms a salt bridge with the side chain of Asp^2, which may be removed when the N terminus of Tat(1–9) interacts with the negatively charged side chain of Asp^2 that is involved in the active site of rat-DP IV. The requirement of such a type of interaction for the inhibitory activity of Tat was proved by the substitution of the positively charged N terminus by rhodamine which led to an inactive compound. Hydrogen bonds are formed between the carbonyl oxygen atom of Val^4 of Tat(1–9) and the amide groups of catalytically active Ser^631 and Trp^630. Interestingly, a number of attractive hydrophobic interactions occur between the Tat(1–9) and the active site of DP IV. Thus, the side chains of Met^1 and Asp^2 interact with Trp^628, the pyrrolidine ring of Pro^3 with the tyrosine side chain of Tyr^632, Val^4 with His^740 and Pro^5 with Trp^630. Altogether, these interactions might explain the competitive inhibition of DP IV.

If Pro^6 in Tat(1–9) is substituted with Leu the affinity to DP IV is considerably reduced. Based on the energy minimized docking of Tat(1–9) into the active site we tried to dock the L^6-Tat(1–9) in the same manner. However, because of the more bulky side chain of Leu a strong steric hindrance between its side chain and the side chain of Trp^630 occurs (see Fig. 3A, orange highlighted side chain of Leu^6). An energy optimization of this starting conformation led to a conformation of L^6-Tat(1–9) where the Leu^6 residue may perform attractive interactions with Trp^630 but simultaneously other interactions were considerably weakened particularly those of Pro^6 and Val^4 including the hydrogen bonds of these residues to DP IV. This effect can explain the very low competitive inhibitory activity of L^6-Tat(1–9) despite the similarity of its solution conformation to that of Tat(1–9).

In the case of the I^5-Tat(1–9) derivative both important hydrophobic interactions between Pro^5 and Trp^630 and Pro^6 with the side chain of Tyr^632 are almost completely removed in comparison to the native nonapeptide. This is caused by the considerable different conformation at Ile^5 which leads to spatial hindrances between the side chain of this residue and Gly^20 of the active site of DP IV (Fig. 3B). This repulsion prevents the diving of this derivative into the binding pocket of DP IV.

**DISCUSSION**

Even early in infection, T lymphocytes from HIV-1-positive individuals have a defect in mounting an antigen-specific response (31). Several groups have shown that the HIV-1 Tat protein occurs extracellularly and exerts immunosuppressive effects (4, 5, 32). Interestingly, Tat interacts with the T cell activation marker CD26 and inhibits its dipeptidyl peptidase IV and suppresses DNA synthesis in pokeweed mitogen-stimulated human T cells as synthetic DP IV inhibitors do (4, 7, 9). This suggests that the defects in the immune response of HIV-1-infected individuals are mediated at least in part by Tat-DP IV interactions. In previous examinations we demonstrated the importance of the charged N terminus of Tat. N-terminal modification with rhodamine prevented inhibition of DP IV as well as suppression of T cell proliferation whereas the short N-terminal nonapeptide Tat(1–9) inhibited DP IV activity (7).

With our DP IV assay using the N-terminal dodecapeptide of IL-2 as substrate, we observed a competitive inhibition mechanism for Tat(1–9), suggesting specific binding to or at least near by the active site, although Tat(1–9) is not a putative DP IV substrate because it contains neither Pro nor Ala in penultimate position. To determine the amino acids necessary for the interaction with DP IV we synthesized nonapeptides derived from the N terminus of Tat containing amino acid substitutions. The peptide with Pro instead of Asp in the penultimate position slightly enhanced the inhibitory action. Val in position 4 was also not crucial for DP IV inhibition. However, the exchange of the negatively charged hydrophilic Asp with the uncharged hydrophobic Ile generated a peptide with strongly reduced inhibitory activity, indicating that Asp in position 5 is important for inhibition of IL-2(1–12) cleavage. Most strikingly, the replacement of Pro in position 6 by the slightly more hydrophobic Leu generated a peptide being ineffective in inhibition of DP IV as well as in suppression of DNA synthesis. These data suggest that the active site of DP IV would interact not only with the amino acids flanking the cleavage site but also with the amino acids in positions 5 and 6.

The N-terminal regions of the well known DP IV substrates neuropeptide Y and pancreatic polypeptide adopt extended polyproline-like helical structures stabilized by having proline as every third residue (proline in positions 2, 5, and 8). The polyproline II helix is an extended structure with three residues per turn. It is found prominently in collagen. Short sequences adopting this conformation have been identified on the surface of proteins in a surprisingly large number of cases. Although very few of these sequences consist entirely of proline residues, the majority contain at least one proline. Many proline-rich sequences function as a “sticky arm” binding rapidly and reversibly to other proteins (33). Tat(1–9) has two XXP repeats as neuropeptide Y, suggesting that it could form a polyproline-like conformation. The exchange of Pro^6 by Leu^6 in L^5-Tat(1–9) could lead to a change in the conformation and be responsible for the lost inhibitory action of this peptide on DP IV.

NMR studies and molecular dynamics simulations of Tat(1–9) and L^5-Tat(1–9) resulted in similar overall backbone conformations for both peptides without the characteristics of a polyproline II helix and could not explain the reported effects. Using our unique active site model of DP IV developed on the basis of comparative molecular field analysis of competitive inhibitors and by force field calculations (30) we demonstrated that Tat(1–9) fits well and builds a number of attractive interactions. In contrast, the bulky side chain of Leu^6 in L^5-Tat(1–9) exerts strong steric hindrances to the side chain of Trp^630 of DP IV, whereas in the interaction with Tat(1–9), the flat aromatic ring of Trp^630 binds well to the flat hydrophobic surface of Pro^5 implicating the low competitive inhibitory activity of L^5-Tat(1–9) compared with that of natural Tat(1–9). The conformation of I^5-Tat(1–9) considerably differs from that of the other two peptides, which leads to reduced affinities to the active site of DP IV in comparison to the parent peptide Tat(1–9).

Furthermore, the model can also explain why P^2-Tat(1–9) exerted the highest effect of all investigated compounds in inhibition of DP IV and why none of these compounds is a substrate of DP IV. In the case of P^2-Tat(1–9) the pyrrolidine ring of Pro^2 adopts a conformation that allows attractive hydrophobic interactions with the side chain of Trp^628 and disturbs the formation of a salt bridge between Asp^5 and the N terminus in Tat(1–9). It has been shown that DP IV substrates must adopt a γ-turn (Cγ) conformation around the peptide bond to be cleaved (30). Only in this case the catalytically active oxygen atom of Ser^631 can attack the peptide bond perpendicular to the plane of the peptide bond. As shown in Figs. 2 and 3, Pro^3, which is near to Ser^631, forms an extended conformation (Ψ = 160°) instead of a γ-turn structure (Ψ = 80°). This would prevent an attack of the active serine to the carbonyl carbon atom of Pro^3 and therefore no peptide bond cleavage would be possible.

The high similarity of efficiency of the Tat(1–9)-derived peptides in the inhibition of DP IV activity and in the suppression of cell proliferation implies that immunosuppressive effects of
PBMC suggests that HIV-1 Tat mediates antiproliferative effect suppression of DNA synthesis in cultures of stimulated CD4+ T cells, leading to spatial hindrances preventing the docking to the active site of DP IV. Further studies revealed that position 6 at the Tat N terminus is altered to Leu6, to determine whether this has any consequences on DP IV-mediated regulation of lymphocyte proliferation. Interferon-γ contains proline in position 6 and IL-11, granulocyte/macrophage colony-stimulating factor, lymphotixin, monocyte chemotactic protein I, and macrophage inflammatory protein-1β release peptides containing proline in position 6 after cleavage of the N-terminal X-Pro-dipeptide. Experiments with two U937 cell clones gave evidence that control of cell proliferation via DP IV could be a general mechanism not only in T cells but also in other CD26 positive cells, since DP IV inhibitors decreased DNA synthesis of the DP IV expressing U937 cell clones but not of U937 cell clones expressing very low levels of DP IV (35).

Based on data obtained from biochemical studies, NMR, and molecular modeling we propose that the N-terminal sequence of the HIV-1 Tat protein behaves as a competitive inhibitor and that this protein interacts directly with the active site of DP IV. Tat(1–9)-derived peptides with amino acid substitutions in positions 2 or 4 enhanced the inhibition of IL-2(1–12) only slightly whereas substitutions in positions 5 or 6 led to peptides with strongly reduced inhibitory activity. The bulky side chain on Leu6 disturbs the interaction with DP IV hence explaining on the molecular level why the replacement of Pro6 to Leu6 generates a peptide with totally lost inhibitory effect on DP IV although the overall backbone conformation is similar for both analogues. Different conformation at Ile6 in Ile6-Tat(1–9) leads to spatial hindrances preventing the docking to the active site of DP IV.

The striking correlation of the action of the different Tat(1–9)-deduced peptides on DP IV-catalyzed IL-2(1–12) cleavage and on suppression of DNA synthesis in cultures of stimulated PBMC suggests that HIV-1 Tat mediates antiproliferative effects via specific DP IV interactions. Thus, substances interfering in the interactions of Asp5 and Pro6 of Tat and the active site of DP IV/CD26, e.g. by inhibition or competition, might be important for the therapy of HIV-1-induced AIDS. Furthermore, it could be very interesting to construct a recombinant virus wherein position 6 at the Tat N terminus is altered to Leu, to determine whether this has any consequences on infectivity.

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