

## Inhibition of HIV-1 virus replication using small soluble Tat peptides

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### Abstract

Although the introduction of highly active antiretroviral therapy (HAART) has led to a significant reduction in AIDS-related morbidity and mortality, unfortunately, many patients discontinue their initial HAART regimen, resulting in development of viral resistance. During HIV infection, the viral activator Tat is needed for viral progeny formation, and the basic and core domains of Tat are the most conserved parts of the protein. Here, we show that a Tat 41/44 peptide from the core domain can inhibit HIV-1 gene expression and replication. The peptides are not toxic to cells and target the Cdk2/Cyclin E complex, inhibiting the phosphorylation of serine 5 of RNAPII. Using the Cdk2 X-ray crystallography structure, we found that the low-energy wild-type peptides could bind to the ATP binding pocket, whereas the mutant peptide bound to the Cdk2 interface. Finally, we show that these peptides do not allow loading of the catalytic domain of the cdk/cyclin complex onto the HIV-1 promoter *in vivo*.

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### Introduction

Human immunodeficiency virus (HIV) is the etiological agent of AIDS. The pathogenesis of HIV-induced disease is complex and multifactorial (Fauci, 1996). Following infection, the reverse transcriptase synthesizes a double-stranded DNA molecule, which is then incorporated into the host genome. A robust cellular and humoral response inhibits viral production within weeks; however, a chronic persistent infection in lymphoid tissue persists throughout the life of the infected individual. Several key HIV and cellular proteins are necessary for this course of infection including the transactivator Tat. Viral clones deficient in Tat do not effectively replicate *in vitro*

or *in vivo*, and infected cells quiescent at the G<sub>0</sub> phase of cell cycle, lacking cytokine signals, do not produce infectious virus (Garza and Carr, 1995).

Tat not only stimulates the HIV long terminal repeat (LTR) promoter but also modulates and induces a number of cellular genes. Historically, the mechanism of action by Tat is at the level of initiation and elongation (Bohan et al., 1992; Feinberg et al., 1991; Kato et al., 1992; Laspia et al., 1989; Marciniak and Sharp, 1991; Marciniak et al., 1990). The effect of Tat on pre-initiation, initiation, and elongation has been observed through a number of biochemical interactions, including physical binding to SP1 (Chun et al., 1998), stabilization of the TFIID/TFIIA complex on the HIV-1 TATA box (Kashanchi et al., 1994), recruitment of a functional TBP or TFIID (Chiang and Roeder, 1995; Garcia-Martinez et al., 1997; Kashanchi et al., 1994; Veschambre et al., 1995), and direct binding to RNA polymerase II (RNAPII) (Blau et al., 1996; Cujec et al., 1997). In recent years, Tat has been shown to bind a number of other factors that regulate chromatin and enzymes that phosphorylate the C-terminal domain (CTD) of the large subunit of RNAPII

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(Herrmann and Rice, 1995; Parada and Roeder, 1996; Pumfery et al., 2003; Roebuck et al., 1997; Zhou et al., 2004), resulting in efficient transcription elongation. Finally, when using in vivo chromatin immunoprecipitation assays, Tat, like typical activators, stimulates the transcription complex formed on the HIV-1 LTR which contains TBP but not TBP-associated factors (Raha et al., 2005).

Activation of HIV-1 LTR transcriptional elongation occurs following the recruitment of Tat to the transcription machinery via a specific interaction with an RNA regulatory element (TAR), a 59-nucleotide RNA leader sequence that folds into a specific stem-loop structure. After binding to TAR RNA, Tat stimulates a specific protein kinase called TAK (Tat-associated kinase), resulting in hyperphosphorylation of the CTD of the large subunit of the RNAPII. The kinase subunit of TAK, Cdk9, is analogous to a component of a positive acting elongation factor isolated from *Drosophila* called pTEFb (Wu-Baer et al., 1995). pTEFb acts to stimulate promoter-paused RNAPII to enter into productive elongation (Chen et al., 1999; de Falco and Giordano, 1998; Karn, 1999; Majello et al., 1999; Wei et al., 1998). pTEFb is composed of Cdk9 and the C-type cyclins T1 (CycT1), CycT2a, CycT2b, or CycK (Herrmann and Mancini, 2001; Zhou et al., 1998). Cdk9 is a Cdc2-related kinase protein, previously named PITALRE, and is a serine–threonine kinase involved in many physiological processes. Cdk9 and Cyclin T1 are ubiquitous factors that affect many cellular processes, including cell differentiation and apoptosis (O’Keeffe et al., 2000; Romano et al., 1999; Yang et al., 2001). TAR and Tat become entirely dispensable for activation of the HIV-1 LTR promoter when the CycT1/pTEFb complex is artificially recruited to a heterologous promoter proximal RNA target (Fujinaga et al., 1998). Finally, over-expression of a dominant negative mutant form of Cdk9 specifically inhibits Tat transactivation and HIV-1 replication (Bieniasz et al., 1999). pTEFb is also critical in regulating transcriptional elongation by SPT4 and SPT5. SPT5 domains that bind SPT4 and RNAPII are critical for in vitro transcriptional repression by the CDK9 pharmacological inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) and activation by the Tat protein. Furthermore, SPT5 is a substrate for pTEFb phosphorylation, which suggests that the C-terminal repeats in SPT5, like those in the RNAPII CTD, are sites for pTEFb phosphorylation and function in modulating its transcription elongation properties (Fong and Zhou, 2000; Foskett et al., 2001; Garber et al., 2000; Ivanov et al., 2000; Kim et al., 1999; Ramanathan et al., 1999; Zhou et al., 2000).

The Tat core domain is the most conserved part of the protein and contributes to the binding of Tat to the TAR element (Bannwarth and Gatignol, 2005; Seelamgari et al., 2004). In the absence of a crystal structure, biophysical studies have provided little detail on the Tat structure. However, the structure of two regions was identified by NMR: a hydrophobic core (residues 32–47) and the C-terminal glutamine-rich domain (residues 60–76), surrounded by the highly flexible cysteine-rich and basic domains (Athanasios et al., 2004; Davis et al., 2004; Montebault et al., 2004). NMR/CD studies on Tat peptides suggest an  $\alpha$ -helical conformation for the

stretch of basic residues constituting the nuclear localization/RNA-binding domain and an amphipathic  $\alpha$ -helix for the core region (Calabro et al., 2005; Hakansson and Caffrey, 2003).

A number of laboratories have attempted to reduce viral transcription with various chemical and/or genetic inhibitors that restrain Tat-activated transcription (Bourgeois et al., 2002; Maniatis and Reed, 2002; Turpin et al., 1998). Other methods of inhibition also include RNA decoys, peptides, and RNAi inhibitory molecules (Filikov et al., 2000; Hamy et al., 1998; Jackson et al., 1998). Finally, synthetic small interfering RNAs (siRNAs) have been shown to induce degradation of specific mRNA targets in human cells by inducing RNA interference. Lately, very promising results have been shown with RNAi against HIV-1 accessory genes and cellular genes that control HIV-1 gene expression (Hwang et al., 1999; Jacque et al., 2002; Lawrence, 2002; Lee et al., 2002; Marozzi et al., 1998).

We previously have shown that soluble peptide analogs of the Tat core domain (amino acids 36–50) were able to effectively block HIV-1 LTR transactivation (Kashanchi et al., 1997). Using various Tat peptide analogs, with different amino acid substitutions in the core domain, we found that several minimal 15 aa Tat peptide analogs with double amino acid substitutions (41/44, 41/46, 41/47) exhibited varying degrees of inhibition of HIV transactivation. Most notably, the peptide analog 41/44 (Tat 41/44) exhibited an 87-fold suppression of Tat transactivation. These Tat peptide analogs had no effect on other promoters including HTLV-I, CMV, PTHR<sub>P</sub>, IgH, RAS, RSV, and SIV CAT, indicating that the Tat peptide analog 41/44 had a preferential effect on the HIV-1 promoter. Furthermore, the Tat peptide 36–72 (41/44) inhibited virus replication by approximately 30%, whereas analog 36–50 (41/44) inhibited replication by approximately 75%. Finally, we demonstrated that the 36–50 (41/44) peptide decreased virus production 85%, with no sign of cellular toxicity (Kashanchi et al., 1997).

In this study, we extended these findings and demonstrated that a smaller Tat 41/44 peptide from the HIV-1 Tat core domain can inhibit HIV-1 gene expression and replication. The advantage of this system is that peptides derived from Tat are presumably more specific in inhibiting Tat-mediated activation in vivo. Our studies in cell lines and peripheral blood mononuclear cells (PBMCs) indicate that these peptides are not toxic to cells at concentrations that normally inhibit HIV-1 replication, indicating that inhibition of HIV-1 activated transcription and viral progeny formation is possible with these peptides. The inhibition by Tat 41/44 is specific to the Tat/TAR complex and therefore specific for HIV-1. Our data further suggest that Tat 41/44 inhibits the kinase activity of Cdk2/Cyclin E in vitro and serine 5 phosphorylation of RNAPII that is in the context of the promoter proximal region of the HIV-1 LTR, and not cellular genes such as GAPDH. Furthermore, we show that these peptides do not allow loading of the catalytic domain of the Cdk/Cyclin complex onto the HIV-1 promoter in vivo. Finally, our results demonstrate that the Tat 41/44 peptide can inhibit HIV-1 infection of PBMCs in culture, as well as in PBMCs isolated from patients.

## Results

### Shorter Tat peptides inhibit transactivation

In recent years, the use of peptidomimetics has emerged as a powerful means for overcoming the limitations inherent in the physical characteristics of peptides, thus improving their therapeutic potential. In search of new molecular entities for discovering new drugs and materials, organic chemists have long been looking for innovative approaches that try to imitate nature in assembling quickly large numbers of distinct and diverse molecular structures from ‘nature-like’ and yet unnatural designer building blocks using a combinatorial approach overcoming barriers such as peptide bioavailability after oral administration, intestinal membrane permeability, size limitations, intestinal and hepatic metabolism, and, in some cases, solubility limitations. A number of successful attempts to date have included Saquinavir, which is a peptidomimetic inhibitor of HIV protease, reversible inhibitors of cysteine proteases and renin, interleukin-1 beta-converting enzyme, neutral endopeptidase, herpes simplex virus protease, thrombin, Ras farnesyl-transferase, the RGD motif, Factor Xa, and various aspartic proteases (Krvacic, 2001; Martin et al., 1998; Ripka and Rich, 1998; Tossi et al., 2000).

In order to use HIV-1 Tat peptide inhibitors for possible peptidomimetic studies, we attempted to shorten the 15 aa Tat 41/44 peptide. Here, the goal was to obtain a fully functional

inhibitor of at least 6 to 8 aa in length. Once a shorter peptide with biological activity is obtained, it will then be possible to design peptidomimics for better in vivo stability and cell penetration. A series of sequentially shorter peptides were synthesized by truncation from both N and C-termini of the Tat 36–50 (41/44) template. The rationale behind designing these peptides came from the available NMR studies of EIAV Tat. A similar core sequence of HIV-1 Tat is present in EIAV Tat, where the NMR coordinates were available from the Protein Data Bank (PDB ID: 1TVT). Based on the NMR coordinates of EIAV Tat, the structure of double mutated HIV-1 Tat core peptide (K41A/G44S) was modeled by an ab initio protein-folding program called RosettaABI (Simons et al., 1999). The conformations of both 15 aa and 10 aa Tat 41/44 peptides are shown in Figs. 1A and B. Upon close examination of the predicted structure, it was found that Phe38 may interact with Tyr47 (8 Å in distance), and this interaction may give rise to a stable structure in water. Therefore, the close proximity of Phe38 to Tyr47 may allow a loop structure of 10 aa to be made independent of the N-terminal cysteine or C-terminal basic domains of Tat. Even though this observation motivated our studies of the truncated 10 aa 41/44 peptide, the formation of such a loop structure may not be essential since peptides of such short length by themselves are in general rather flexible. Indeed, as discussed below, an even shorter 5 aa peptide was created with both Phe38 and Tyr47 removed and tested for its activity (Fig. 1C).

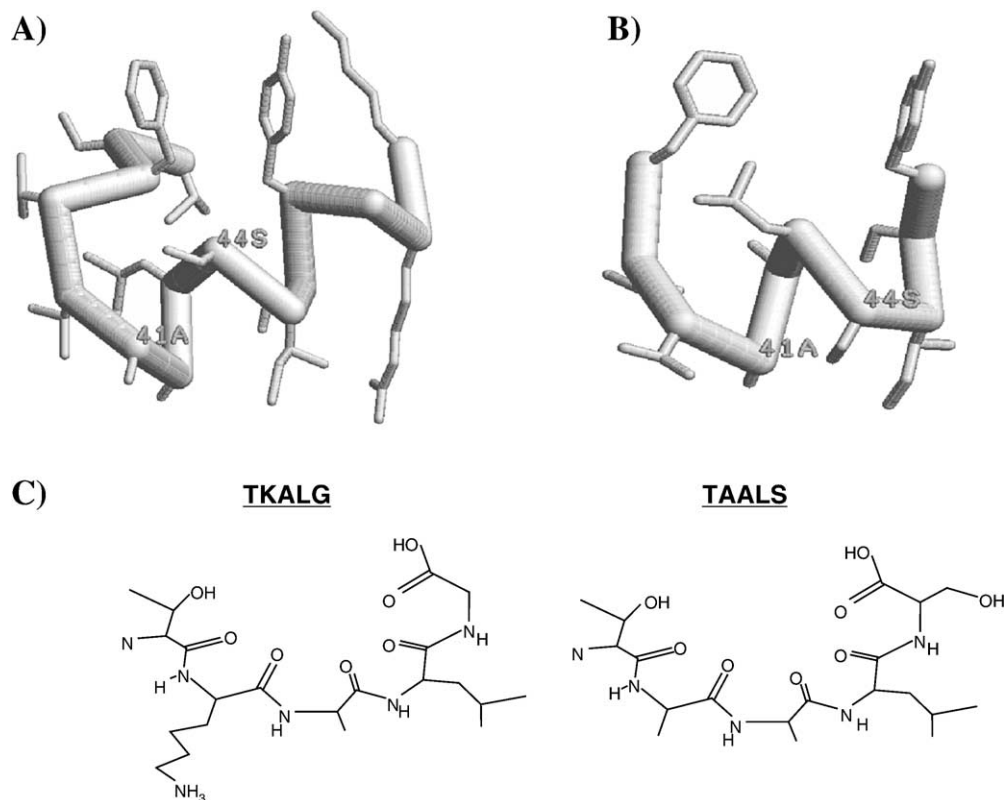


Fig. 1. Predicted structure of 15, 10, and 5 aa Tat peptides. Structural data were generated using the EIAV Tat NMR coordinates from Brookhaven Protein databank. The ‘‘Rasmol’’ and ‘‘Molecules R Us’’ programs were used to generate the Tat structures. (A) Structure of 15 aa Tat peptide, Tat 36–50 (41/44): V-C-F-T-T-A-A-L-S-I-S-Y-G-R-K. (B) Structure of 10 aa Tat peptide, Tat 38–47 (41/44): F-T-T-A-A-L-S-I-S-Y. (C) Structure of 5 aa Tat peptides, wild-type and 41/44 derivative: WT (TKALG) and 41/44 (TAALS).

Table 1  
Effect of various Tat peptide derivatives on HIV-1 replication

| Treatment                             | HIV p24 (pg/ml) |
|---------------------------------------|-----------------|
| –TNF                                  | 2.9 ± 0.03      |
| +TNF                                  | 86.1 ± 0.09     |
| TNF + Tat 40–44 (WT): TKALG           | 76.3 ± 0.18     |
| TNF + <b>Tat 40–44 (41/44): TAALS</b> | 2.5 ± 0.05      |
| TNF + Tat 1–86: (WT)                  | 109 ± 0.14      |
| TNF + <b>Tat 1–86: (Mut, 41/44)</b>   | 3.7 ± 0.04      |

Next, we tested various Tat peptide derivatives and their N- and C-terminal truncations in latent U<sub>1</sub> cells as well as determined the IC<sub>50</sub> in PBMCs. Briefly, 5 × 10<sup>6</sup> U<sub>1</sub> cells per sample were treated with or without TNF (5 µg/ml) and electroporated with various Tat peptides (2.5 µg/sample). A schematic of the structures of the 5 aa peptides is shown in Fig. 1C. Supernatants were harvested 48 h post-transfection for HIV-1 p24 antigen capture assay. As can be seen in Table 1, either wild-type full-length Tat protein (1–86) or the wild-type Tat 40–44 peptide did not suppress viral production. However, a mutant Tat [1–86 (41/44)] or Tat peptide [40–44 (41/44)] significantly suppressed the level of HIV-1 p24 production. These results indicate that the initial 15 aa peptide [36–50 (41/44)] can be trimmed both from the N- and C-terminal ends to create a small 5 aa analog that still efficiently inhibits HIV-1 transcription and propagation *in vivo*.

Peptidomimetic modification of active peptides can provide biostable analogs. Moreover, cyclization of linear peptides is frequently used as an attractive venue to provide both conformationally more restricted as well as more biostable analogs. Therefore, we attempted to cyclize our Tat peptides to increase structural stability and possibly their half-life. Cyclic

structures, including various cycloalkane, bicyclic, and heterocyclic analogs, that lead to conformational restriction have been used to generate biologically active molecules, particularly in the field of RGD mimetics and excitatory amino acid agonists and antagonists (Stefanic and Dolenc, 2004). We synthesized conformationally constrained analogs of the prototype 15, 10, and 5 aa peptides by preparing cyclic peptide analogs that would preserve the general predicted conformation of the peptide. Cyclization was achieved by peptides backbone, construction of a redox-stable thioether bridge, or through a side-chain/side-chain covalent junction (Borgatti et al., 1998; Lung et al., 1996; Nomizu et al., 1994; Oligino et al., 1997). Unfortunately, we were never able to obtain enough material to go beyond *in vitro* kinase inhibitory assays (see below). However, recently, we have been successful in synthesizing and cyclizing the small 5 aa peptides with two glycines (for flexibility) and adding nuclear signal sequences from either the SV40 or Tat basic domains for peptide delivery (see Materials and methods). As shown below, these peptides have now been used in *in vitro* kinase inhibitory assays, cell toxicity assays, chromatin immunoprecipitation assays, and PBMC studies with success.

Next, we asked whether the cyclized 5 aa peptide 41/44 could indeed inhibit replication of HIV-1 in PBMCs. Results in Fig. 2 show that the Tat 41/44 peptide was able to inhibit HIV-1 NL4-3 replication in cells. A positive control (3TC) was used in two independent experiments. The IC<sub>50</sub> for 3TC was determined to be 25 nM, and the IC<sub>50</sub> for the 41/44 peptide was 50 nM. The WT peptide showed no significant inhibition in these cells. Finally, to determine whether the Tat 41/44 peptide could also inhibit HIV-1 field isolates, we utilized two SI and NSI strains that were previously used by our laboratory

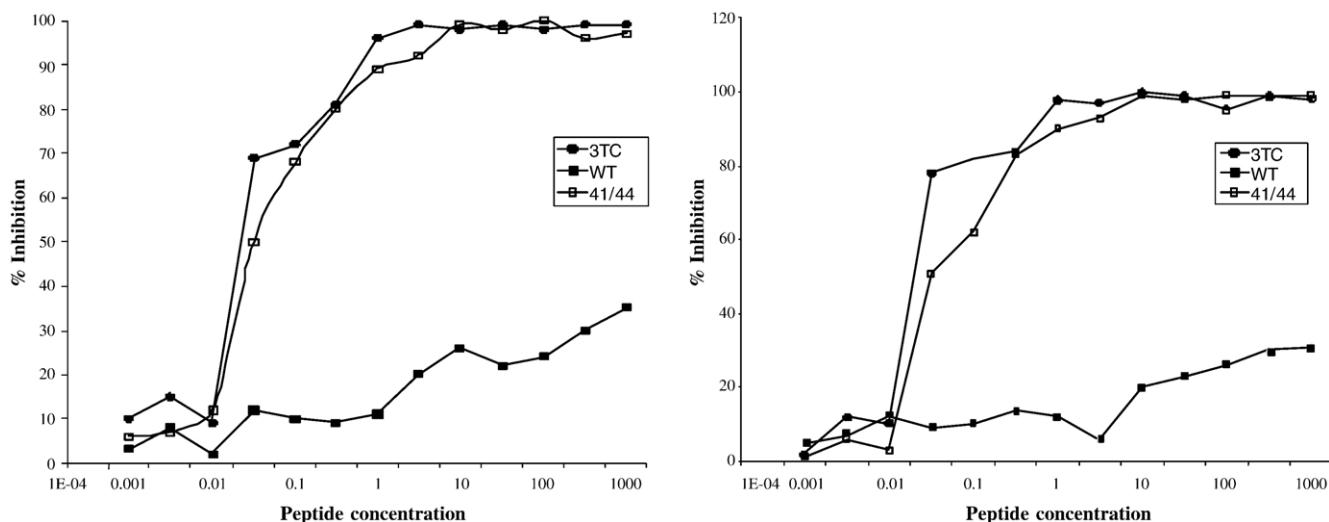


Fig. 2. Antiviral determination of Tat peptides in PBMC. Phytohemagglutinin (PHA)-activated PBMCs were placed in a 75-cm<sup>2</sup> flask at a density of 2 × 10<sup>6</sup> cells per ml and cultured for 48 h in complete RPMI 1640 medium with human lymphocyte IL-2. On the day of assay, PBMCs were inoculated with HIV-1 NL4-3 at an MOI of 0.005 for 2 h at 37 °C (500 TCID<sub>50</sub> per well). Unadsorbed virus was removed by centrifugation, cells were resuspended at 2 × 10<sup>6</sup> cells per ml in complete media, and 100,000 cells were added to triplicate wells of a round-bottom 96-well plate. The wells then received 100 µl of serially diluted Tat peptide [either WT or 41/44, cyclized 5 mer in 5% DMSO, (200 µl total)], and the plates were incubated at 37 °C. After 7 days, supernatants were collected and assayed for p24 antigen at a 1:800 dilution in HIV p24 antibody-coated microplates by quantitative ELISA. The 50% inhibitory concentrations (IC<sub>50</sub>) were calculated by use of a 4-parameter fit (SOFTmax PRO 3.0, Molecular Devices, Mountain View, California). A positive antiviral control drug, 3TC, was prepared and tested at a final concentration range of 1000 to 3.2 × 10<sup>-4</sup> µM. Concentrations are shown in micromolar from two independent experiments.

(Wang et al., 2001). Activated PBMCs were infected with two independent HIV-1 viral strains: SI (UG/92/029, subtype A envelope) and NSI (THA/92/001, subtype E envelope). After 8 h of infection, cells were washed, and fresh media plus various cyclized peptides (100 nM) were added to the media (Fig. 3). Ten microliter samples were obtained for p24 analysis every 6 days. Results in Fig. 3 show that the Tat 41/44 peptide, and not the WT peptide, effectively blocked both SI and NSI strains in PBMCs.

*Target(s) of the minimal 5 aa Tat 41/44 peptide*

We next utilized an immobilized biotin pull-down assay to determine the targets of the minimal Tat peptide. We and others have previously utilized this strategy on wild-type and acetylated Tat peptides to determine target proteins that bind at the initiation and elongation stages of HIV-1 transcription (Deng et al., 2000). We synthesized biotin-labeled peptides of wild-type and 41/44 derivatives, which were used for in vitro binding to nuclear extracts. Complexes were washed prior to SDS/PAGE and Western blotted for various transcription factors. As can be seen in Fig. 4, both wild-type and mutant peptides can bind to DNA-PK, Cdk9, and Cdk2 after a 100 mM salt wash. However, upon more stringent wash conditions at 600 mM salt, only the mutant peptide as well as the full-length Tat protein mutated at positions 41/44 bound to DNA-PK, Cdk9, and Cdk2. Therefore, the mutant 41/44 Tat peptide or protein can bind tightly to these three complexes either directly or indirectly. Two other control Westerns, TBP and CBP/p300, were also used in these assays. Wild-type Tat, but not the K41A mutant, can bind to TBP as has been shown previously (Kashanchi et al., 1994, 1996), while p300 binds to wild-type Tat at the acetyl-CoA binding motif (<sup>41</sup>KGXG<sup>44</sup>) but not to the mutated Tat (Deng et al., 2001). These results indicate that the 5 aa Tat 41/44 peptide is capable of tightly

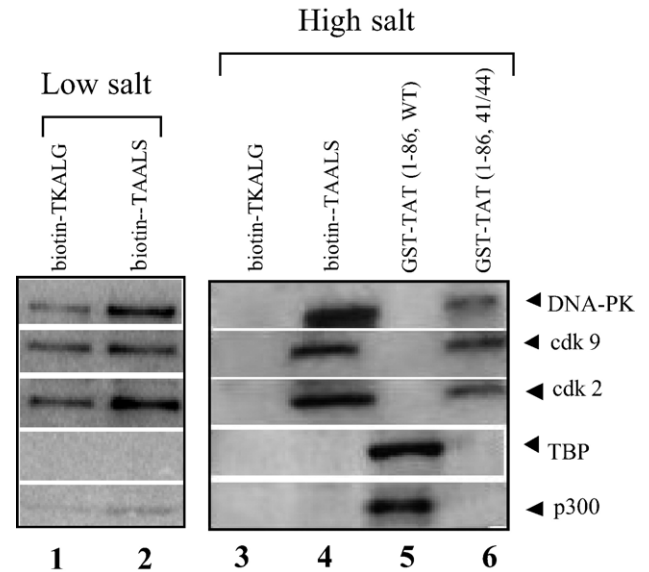


Fig. 4. Binding partners of wild-type and 41/44 mutant Tat peptides and proteins. Biotin-labeled Tat peptides (TKALG, and TAALS) or GST-Tat were mixed with CEM nuclear extracts. Samples were incubated overnight at 4 °C and the next day were pulled down with streptavidin or glutathione beads and washed three times with either TNE<sub>100</sub> + 0.1% NP-40 (lanes 1 and 2, low salt wash) or TNE<sub>600</sub> + 0.1% NP-40 (lanes 3–6, high salt wash). All bound complexes were then washed with TNE<sub>100</sub> + 0.1% NP-40 prior to loading on a 4–20% SDS/PAGE and Western blotted for DNA-PK, Cdk9, Cdk2, TBP, and p300, as indicated. Lanes 1–4 are the peptide pull-down results, and lanes 5 and 6 are GST-Tat full-length pull-downs.

binding to DNA-PK and cyclin-dependent kinases, all of which are active participants in transcription of the HIV-1 LTR.

*Target of DNA-PK and cyclin/cdk complexes*

The HIV-1 Tat protein activates viral gene expression through promoting transcriptional elongation by phosphoryla-

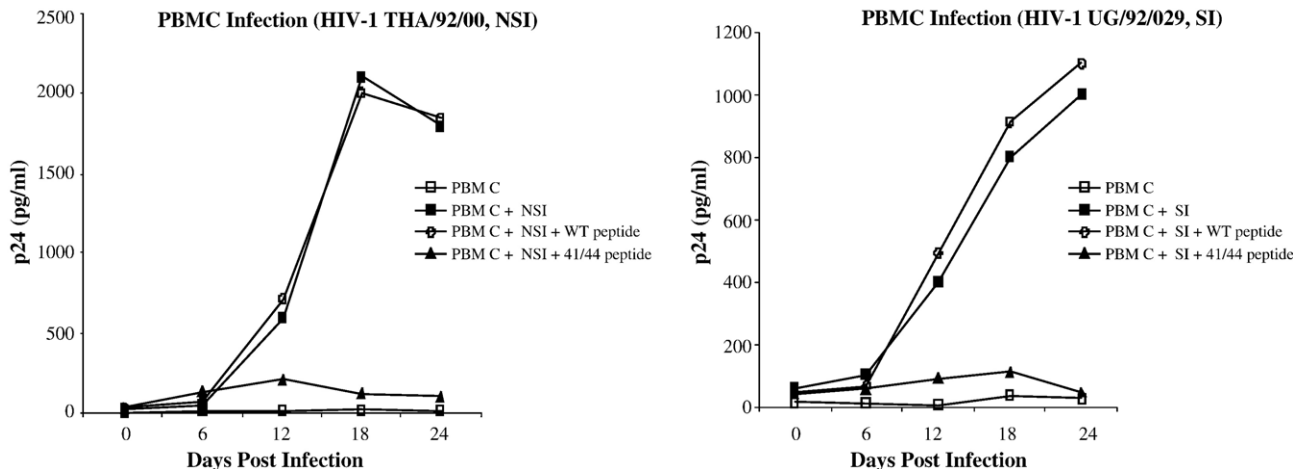


Fig. 3. Effect of Tat peptides in PBMCs infected with HIV-1 SI and NSI strains. PHA-activated PBMCs were cultured for 2 days prior to infection. Isolation and treatment of PBMCs were performed as described in Fig. 2. PBMCs were infected with either an SI (UG/92/029 Uganda strain, subtype A envelope, 5 ng of p24 gag antigen) or an NSI (THA/92/001, Thailand strain, subtype E envelope, 5 ng of p24 gag antigen) strain of HIV-1. After 8 h, cells were washed, and fresh media plus cyclized peptides, WT or 41/44 (100 nM), were added to the media. Plates were then incubated at 37 °C. Aliquots were taken for determination of p24 levels on days 6, 12, 18, and 24. Fresh media and peptides were added to the cultures when samples were taken, and peptide concentrations were maintained during the study period as described in Materials and Methods. All samples were processed for the presence of Gag p24 together after the completion of the experiment.

ting RNAPII. In this process, Tat enhances phosphorylation of the CTD of RNAPII by activating cyclin-dependent kinases (Cdks) associated with general transcription factors. The conserved CTD repeats of RNAPII are important sites of transcription regulation. To date, four cyclin/cdk complexes have been shown to phosphorylate the CTD, Cyclin C/Cdk8, Cyclin H/Cdk7 (subunits of transcription factors TFIIH), Cyclin T/Cdk9, and Cyclin K/Cdk9 complexes. Other protein kinases have also been described as able to phosphorylate the CTD. DNA-PK<sub>cs</sub> also acts as a CTD kinase only when stimulated by linear double-stranded DNA and by several transcriptional activators. Furthermore, we have recently reported that Cyclin E/Cdk2 could phosphorylate the CTD. Recombinant Cyclin E/Cdk2 stimulated Tat-dependent HIV-1 transcription in a reconstituted transcription assay, and immunodepletion of Cyclin E/Cdk2 from HeLa nuclear extracts blocked Tat-dependent transcription (Deng et al., 2002).

In recent years, a more complex picture of transcription has emerged where the RNAPII CTD functions both as an assembly platform for and a regulator of transcription and pre-mRNA processing machineries, including 5' capping, elongation, splicing, and polyadenylation (Cheng and Sharp, 2003; Chiu et al., 2001; 2002; Cho et al., 1998; Fong and Bentley, 2001; Ho and Shuman, 1999; Moteki and Price, 2002; Pei and Shuman, 2002; Proudfoot et al., 2002; Schroeder et al., 2000; Wen and Shatkin, 1999). Almost all of these events are triggered during transcription initiation by phosphorylation of CTD serine 5. In general, phosphorylation of CTD serine 5 is concentrated near the transcription initiation site (+1 area), while CTD serine 2 phosphorylation is observed in the transcription elongation complex. Therefore, we asked whether the Tat 41/44 peptide was able to inhibit phosphorylation of serine 2 or 5 of the RNAPII CTD present on the HIV-1 promoter. We first designed experiments to detect any possible inhibition of Cdk2/Cyclin E kinase activity in vitro, in host cells, and finally on RNAPII CTD serine 2 and 5 phosphorylation on the HIV-1 promoter in vivo using a Chromatin Immunoprecipitation (ChIP) assay. ChIP is a powerful approach that allows one to define the interaction of factors with specific chromosomal sites in living cells, thereby providing a snapshot of the native chromatin structure and factors bound to genes in different functional states. ChIP involves treating cells briefly with formaldehyde to cross-link proteins to DNA. An antibody against a protein suspected of binding a given *cis*-element is then used to immunoprecipitate chromatin fragments. Polymerase chain reaction analysis of the immunoprecipitate with primers flanking the *cis*-element reveals whether a specific DNA sequence is recovered in an immune-specific manner and, therefore, whether the protein contacted the site in living cells (Das et al., 2004; Weinmann and Farnham, 2002). Results are shown in Figs. 5 and 6. As can be seen in Fig. 5A, the Tat 41/44 peptide, but not the wild-type peptide (Lanes 8–10), can efficiently inhibit Cyclin E/Cdk2 kinase activity in vitro. The Tat 41/44 peptide inhibited the kinase reaction at an IC<sub>50</sub> of 20 nM. We next asked whether an over-expressed and purified Cyclin E/Cdk2 complex had a similar inhibition profile as compared to the complex isolated

from HIV-1-infected cells. Fig. 5B shows that recombinant baculavirus HA-tagged Cdk2/Cyclin E can also be inhibited by the Tat 41/44 peptides (both linear or cyclized).

To determine whether these peptides were toxic to cells, we utilized DNA labeling experiments after peptide treatment in CEM, U937, CaCo-2, PBMC, and ACH2 cells. As can be seen in Fig. 5C, the Tat peptides, either wild-type or the 41/44 derivative, had no apparent effect on uninfected cells up to 24 h. Similar experiments were performed at a later date and carried up to 7 days without any apparent toxicity (Fig. 5D). As expected, the Tat 41/44 peptides were inhibitory in HIV-1-infected cells, as evident by the p24 results (Fig. 5C, right hand insert). These results clearly indicate that, even though Tat 41/44 targets DNA-PK, Cdk2, and Cdk9, these interactions are not functionally significant in uninfected cells. Similar results with knockout of these genes have also been obtained by others in cell lines and KO mouse systems (Berthet et al., 2003; Geng et al., 2003; Mendez, 2003; Ortega et al., 2003). However, DNA-PK, Cdk2, and Cdk9 are critical in HIV-1-infected cells, especially when these proteins are concentrated on the HIV-1 promoter.

#### *Inhibition of CTD phosphorylation on the HIV-1 promoter*

To address whether Tat peptides in ACH2 cells were inhibiting HIV-1 LTR transcription, we devised a series of in vivo ChIP assays followed by PCR with specific primers to HIV (experimental) and GAPDH (control) genes. We used ACH2 cells after treatment with Tat peptides (described in Fig. 5C) and used the total DNA for a ChIP assay using two different antibodies. The antibodies were specific for phosphorylated RNAPII serine 5 (H14) and RNAPII serine 2 (H5). Following ChIP, samples were amplified with either HIV-1 LTR or GAPDH primers. As seen in Fig. 6, both linear and cyclized Tat 41/44 peptides efficiently inhibited serine 5 phosphorylation but not the serine 2 phosphorylation of the RNAPII CTD. Consistent with inhibition of serine 5, both levels of HIV RNA capping and elongation by SPT-5 were reduced with the Tat 41/44 peptide (data not shown). This is consistent with the notion that serine 5 phosphorylation recruits subsequent enzymes for capping, elongation, and splicing machineries. These peptides did not affect the RNAPII, capping, or elongation of the cellular gene GAPDH. These results imply that the mechanism of inhibition of the HIV-1 LTR by Tat 41/44 is via inhibition of serine 5 phosphorylation of an RNA polymerase that is specifically associated with the HIV-1 promoter and not a cellular promoter.

The experiments described above (Fig. 6) clearly show that the Tat 41/44 peptide was able to inhibit RNAPII CTD phosphorylation on HIV-1 DNA but does not address whether any of the three kinases was the target in the observed inhibition. We therefore determined whether we could observe these kinases on the HIV-1 promoter and then subsequently asked whether the Tat 41/44 peptide could inhibit any of these enzymes on the HIV-1 genome in vivo. Similar experiments as described in Fig. 5C were performed in ACH2 cells, and various antibodies were used for the initial ChIP assays.

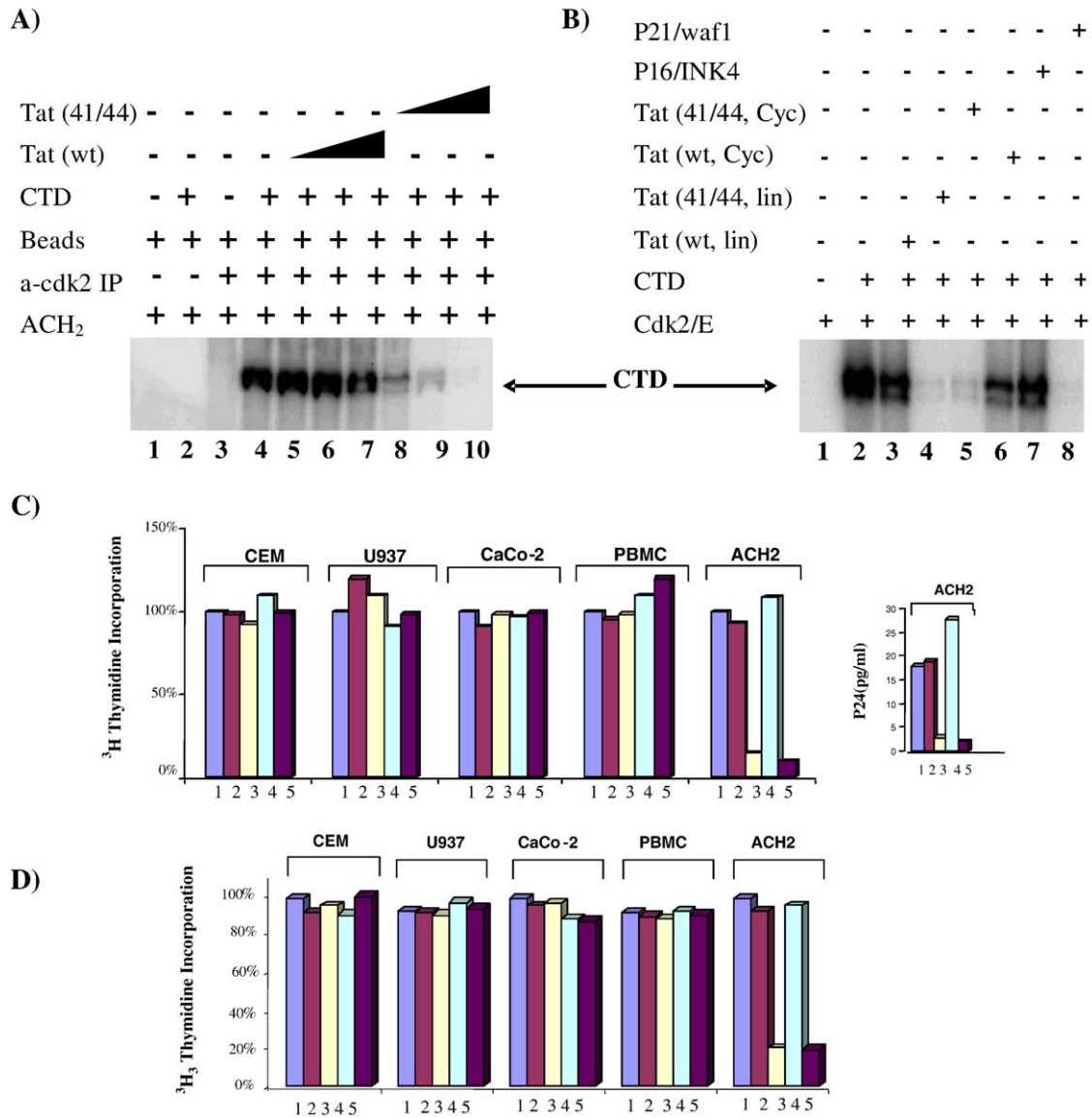


Fig. 5. Phosphorylation of RNAPII CTD by Cyclin E/Cdk2 complexes and the effect of the Tat 41/44 peptides in uninfected hosts. (A) HIV-1-infected cells (ACH2) were treated with TNF and Cyclin E/Cdk2 complexes were immunoprecipitated using an anti-Cdk2 antibody. The washed complexes were incubated with GST-CTD, and in vitro kinase assays were performed as described previously (Deng et al., 2002; Wang et al., 2001, 2002). Three concentrations (10, 100, 1000 ng) of 5 aa wild-type Tat (wt) or mutant (41/44) peptides were added to the reaction mixture in lanes 5–7 (wt) and lanes 8–10 (41/44). (B) Recombinant baculavirus Cyclin E and HA-Cdk2 were co-infected into Sf9 cells, and the active complex was purified on a 12CA5 antibody column. Excess HA peptide (300-fold) was used to purify the Cyclin E/Cdk2 complex. A CTD kinase assay was performed with Cyclin E/Cdk2 (0.5 unit/ml) and GST-CTD. Five aa Tat (wt, lin) and Tat (41/44, lin) peptides were added at 100 ng/reaction. Tat (wt, cyc) and Tat (41/44, cyc) are the 5 aa cyclized peptides described in Materials and Methods. (C) Proliferation studies were performed in various cell lines and uninfected PBMCs as described previously (Beales, 2002). Uninfected (CEM, U937, CaCo-2, PBMC) and infected cells (ACH2) were grown to mid log phase, treated with 5 ng TNF for 2 h (to induce virus in ACH2 cells), washed, and Tat peptides were electroporated into cells at 1 µg/2.5 × 10<sup>6</sup> cells (Dimitrov and Wolffe, 1997). DNA synthesis was estimated by measurement of [<sup>3</sup>H]-thymidine incorporation into the trichloroacetic acid (TCA) precipitable material, as described in Materials and Methods. Lanes 1—Mock; 2—Tat (wt, lin); 3—Tat (41/44, lin); 4—Tat (wt, Cyc), and 5—Tat (41/44, Cyc). The right hand insert is p24 ELISA results from ACH2 treated cells. (D) Similar experiment as described in panel C, except cells were carried up to 7 days prior to [<sup>3</sup>H]-thymidine incorporation and TCA precipitation.

Protein/DNA complexes were subsequently processed for PCR reactions using LTR/Gag-specific primers (+1/+300). The results in Fig. 7A show that the three kinases were present on the HIV-1 promoter after the cells were treated with TNF. The control antibodies, Cdk4 and Cdk6, did not bind to the HIV-1 promoter. Interestingly, and as expected, Cdk9 was also present on the HIV-1 promoter in the absence of TNF treatment. This is consistent with the results from the Karin

laboratory demonstrating that the Cyclin T/Cdk9 complex is present on the HIV-1 promoter before any activated transcription takes place (Kim et al., 2002). We then determined if the Tat 41/44 peptide could disrupt the interaction between the cyclin and its cdk partner in infected cells. Our rationale for this came from our modeling experiments (see Fig. 8 below), where the Tat 41/44 peptide showed that the highest probability of binding was at the interface between the cyclin and its cdk

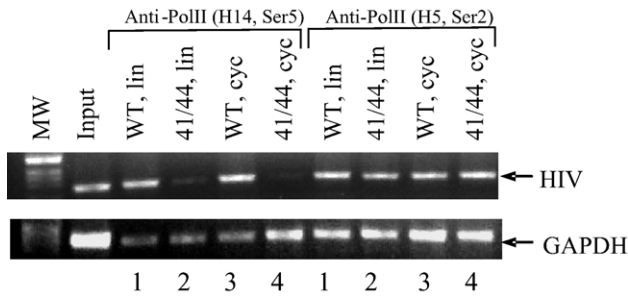


Fig. 6. Chromatin immunoprecipitation (ChIP) assay following Tat peptide treatment in ACH2 cells. Cells were treated with TNF for 2 h, washed, and electroporated with 1  $\mu$ g of peptide. Cells were resuspended in 10 ml of complete media and, 24 h later, cross-linked by addition of 1% HCHO to the medium. A modified chromatin immunoprecipitation (ChIP) assay (Braunstein et al., 1993; de la Fuente et al., 2000) was performed as described in Materials and Methods. Chromatin was immunoprecipitated using antibodies to Pol II Ser 2, Pol II Ser 5 overnight at 4 °C. Immunoprecipitated DNA was amplified using primers to the HIV LTR (top panel) or GAPDH (lower panel).

partner, thereby disrupting the interaction between these two components (cdks alone in the absence of their cyclin partner are not kinase active). The results in Fig. 7B demonstrate that very little Cyclin E and Cyclin T, and no Cdk2 or Cdk9, were present on the HIV-1 promoter after Tat 41/44 peptide treatment (lanes 9–12). This is consistent with our hypothesis that the Tat 41/44 peptide inhibits phosphorylation of RNAPII elongation by disrupting the cyclin/cdk complex in vivo.

#### Site of cdk2 binding to Tat peptide derivatives

The results from Fig. 4 indicated that the Tat 41/44 peptide interacts with cdk, but this interaction may be either direct or indirect. In the latter case, for example, the peptide may interact with a cyclin that in turn interacts with cdk. This scenario of indirect interaction may first appear rather natural since the peptide was derived from the Tat core region that is known to bind to cyclins (i.e., Cyclin T1), even though the 5 aa Tat 41/44 peptide was mutated in two out of five residues from the wild-type 5 aa Tat peptide. However, Fig. 7B clearly shows that Tat can recruit Cyclins E and T to the HIV-1 LTR promoter but not their cdk partners in the presence of the Tat 41/44 peptide. Taken together, the results shown in Figs. 4 and 7 strongly suggest that the Tat 41/44 peptide interacts with cdk directly. To further verify this hypothesis and to identify both the binding sites on Cdk2 and the mode of binding, such as potentially induced conformational changes of Cdk2 upon its binding to the Tat 41/44 peptide, we used computer docking methods as described in Materials and Methods.

From the X-ray crystallography studies of Cdk2 structures, it is known that Cdk2 possesses a very flexible region, the T-loop, which locks into a “closed” position towards cyclin only in the cyclin/cdk complex (PDB ID: 1FIN) but otherwise remains somewhat “open” (PDB ID: 1E1X). To investigate the potential effects of these two forms of cdk in binding to the Tat 41/44

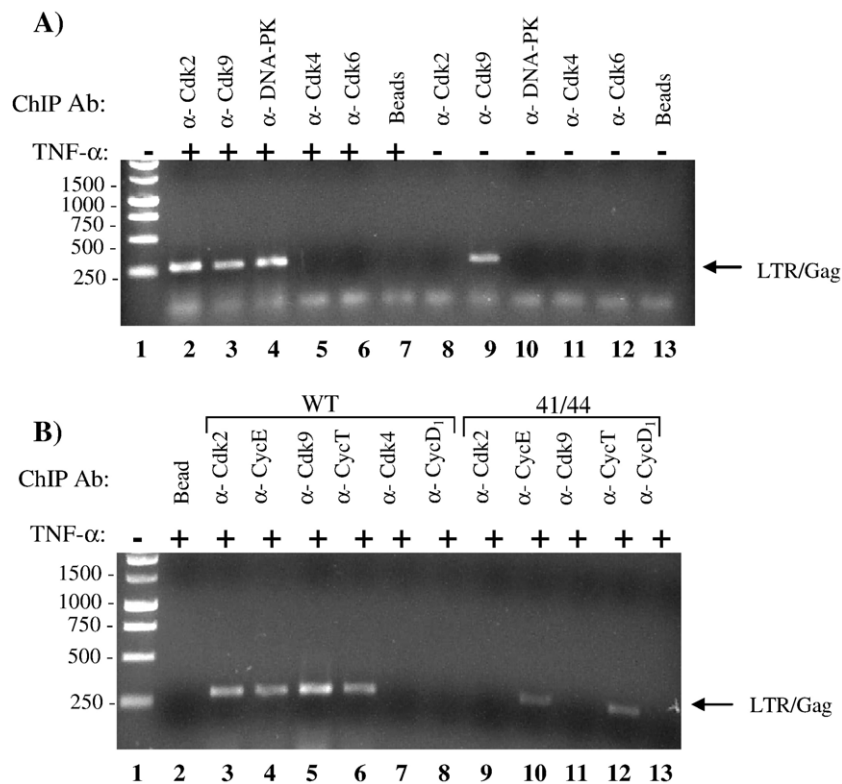


Fig. 7. Presence of various kinases on an activated HIV-1 promoter and disruption of the Cyclin/Cdk complex by the Tat 41/44 peptide in vivo. (A) Chromatin immunoprecipitation (ChIP) assays were performed in ACH2 cells before and after TNF treatment as described for Fig. 6. Immunoprecipitations were performed with anti-Cdk9, Cyclin T, Cdk2, Cyclin E, DNA-PK (experimental), Cdk4, Cdk6, and Cyclin D1 (controls) antibodies. Specific DNA sequences in the immunoprecipitates were detected by PCR using primers that span +1 to +300 of the HIV genome (LTR/Gag). (B) Similar design as in panel A in the presence of either Tat WT or 41/44 peptide (1  $\mu$ g peptide/2  $\times$  10<sup>6</sup> cells/10 ml media).



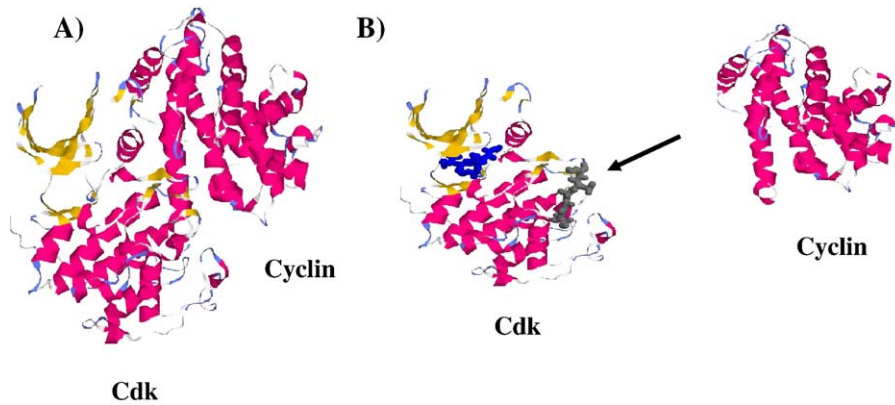


Fig. 8. Computational docking of Tat wild-type and 41/44 mutant peptides with Cdk2. (A) The Cdk2/Cyclin complex obtained from X-ray crystallography (Protein Data Bank ID: 1FIN). (B) Computational docking results of the wild-type peptide (TKALG, blue) and the mutant peptide (TAALS, gray) with Cdk2 (now separated from Cyclin) using AutoDock 3.0 with the simulated annealing algorithm.

peptide, we obtained 1188 docked conformations using the Cdk2 structures taken from 1E1X and 1FIN. Fig. 8A illustrates the structure of the closed cdk–cyclin complex (1FIN). In both closed and open cases, we consistently found that the low-energy wild-type peptides bound at the ATP binding pocket (Fig. 8B, blue molecule). However, for the mutant peptide, in addition to the ATP binding site, we also identified a second site of binding at the interface of Cdk2 and its cyclin partner. Fig. 8B shows the wild-type peptide (blue) binding at the ATP site and the mutant peptide (gray) binding at the interface of Cdk2 and its cyclin partner. Thus, computer docking studies suggest that the 5 aa Tat 41/44 peptide, TAALS, is capable of disrupting the binding of Cdk2 and its cyclin partner.

To identify more precisely the binding sites, we broke down the binding energies for each Cdk2 residue for the low-energy mutant docked conformations and ranked the residues according to energies averaged over the conformations (see Materials and methods). For the “open” Cdk2, the top ranking sites of Cdk2 at the interface are Tyr180, Asp235, Arg126, Ile173, Glu208, and Lys178. For the “closed” Cdk2, we found that the T-loop blocks the access to Arg126. To ensure these computational predictions, we designed a number of point mutations in the Cdk2 protein using a standard two-step PCR method (Kwon and Nordin, 1998; Wei et al., 2003). Mutations with alanine substitutions at positions 126, 134, 150, 178, 180, and 234 were readily available. These new mutations were cloned into a pGEX GST backbone, transformed, and expressed as GST fusions in *E. coli*. Following purification, the various Cdk2 proteins were bound to Tat peptides and detected by Western blotting against Tat. The results in Fig. 9 show that the Tat 41/44 peptide still retained better binding to Cdk2 (lane 3). However, among all the point mutants tested experimentally, two Cdk2 constructs lost either 100% binding or 50% binding to the Tat 41/44 peptide. These mutations were at positions 180 and 178 (lanes 8 and 9). Similar levels of Cdk2 were recovered after Tat binding to Cdk2, as evident by the anti-Cdk2 Western blot shown in Fig. 9. Collectively, these results further confirm our computational modeling approach indicating that the Tat 41/44 peptide preferentially binds to Cdk2 at the interface and specifically to Tyr180 and Lys178. Moreover, binding may

induce a closing of the T-loop so that Arg126 no longer participates in binding.

#### *Effect of excess ATP in Tat peptide competition*

As indicated above, our computational results also suggest that the Tat 41/44 peptide may bind at or near the ATP binding pocket of Cdk2. To examine whether this is the case, we used a simple batch-wise affinity chromatography approach linked to either the Tat peptide or purine derivatives to capture purified Cdk2 *in vitro*. This method has previously been used to detect specific kinase complexes binding to ATP analog ligands (Knockaert et al., 2000). We used an immobilized biotin–purvalanol B (an ATP analog that specifically binds to Cdk2) or biotin–Tat 41/44 peptide derivative on an agarose matrix to bind to purified Cdk2 (Fig. 10, top panel) or the Cyclin E/Cdk2 (Fig. 10, bottom two panels) complex. Subsequently, we titrated ATP to compete out the binding to Cdk2 or to the Cyclin E/Cdk2 complex and Western blotted the remaining bound complexes for the presence of Cdk2 or Cyclin E. The results in Fig. 10 indicated that cold excess ATP can efficiently compete out Cdk2 binding from the biotin–purvalanol B beads at an apparent  $IC_{50}$  of 10  $\mu$ M (lane 8). However, only at 100  $\mu$ M concentrations did we observe a 40% loss of Cdk2 from the biotin–Tat 41/44 peptide complex (lane 5), indicating that the Tat peptide did not preferentially bind to the ATP pocket. This was apparent in experiments using either Cdk2 alone or the Cyclin E/Cdk2 complex, indicating that the ATP site was accessible in either case to exogenously added ATP (Fig. 10, top and middle panels). Finally, a much more interesting pattern emerged when Western blotting for Cyclin E. Lanes 1–5 clearly show that Cyclin E was absent from the biotin–Tat 41/44 peptide at any concentration of exogenously added ATP; however, Cyclin E initially bound to the biotin–purvalanol B beads could be subsequently competed out with excess cold ATP (lanes 6–10). These results further indicate that the Tat 41/44 peptide was able to disrupt the Cyclin E/Cdk2 complex and bind only to Cdk2. On the other hand, ATP analogs, such as purvalanol B, are able to bind to the Cdk2 ATP pocket and not necessarily disassemble the Cyclin E/Cdk2 complex.

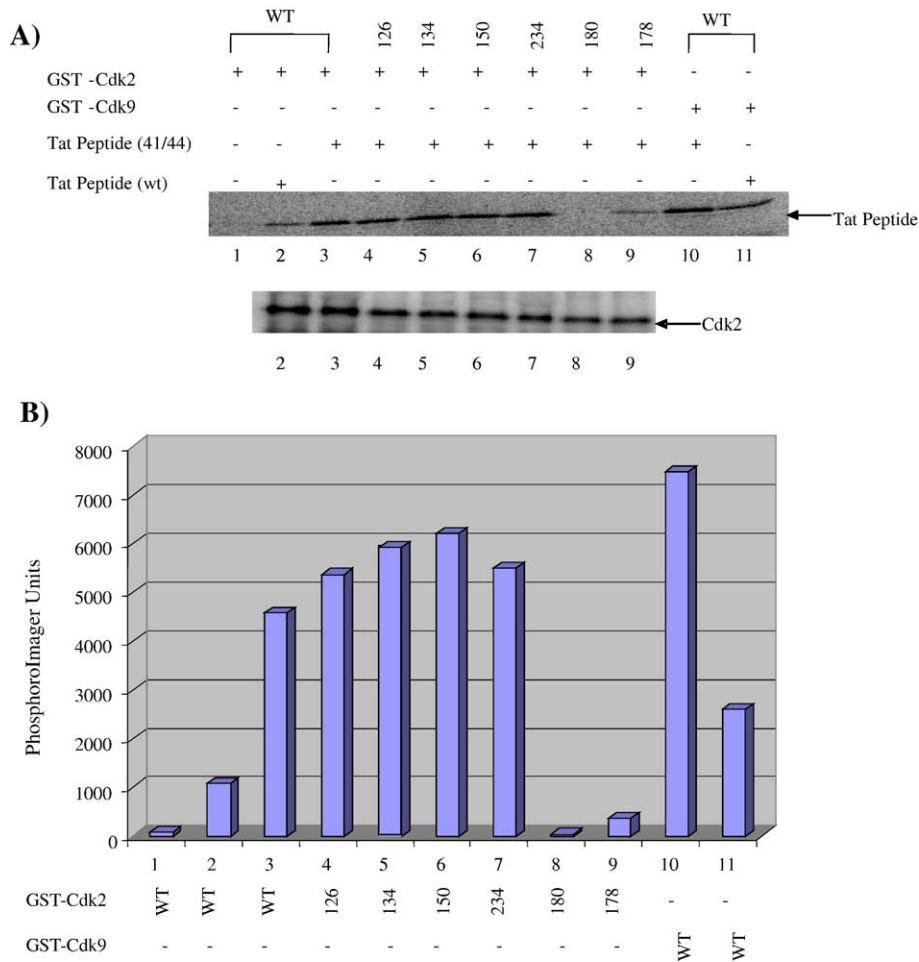


Fig. 9. Binding site for Tat 41/44 peptide to Cdk2. (A) GST-Cdk2 point mutants were generated by a two-step PCR method (Ho et al., 1989; Kwon et al., 1996) using primers against both the N- and C-terminus of Cdk2 (Rosenblatt et al., 1992; Tsai et al., 1991) (Acc #: AAX32258), which included *EcoRI* and *BamHI* sites for subcloning into the pGEX-3X *BamHI/EcoRI* site. The GST-Cdk2 clones were transformed and expressed as GST fusions in *E. coli*. Following purification, the various GST-Cdk2 proteins (3  $\mu\text{g}/\text{reaction}$ ) were used for binding to either wild-type or the Tat 41/44 peptide (10  $\mu\text{g}$ ). Bound Tat peptides were washed in  $\text{TNE}_{50}$  + 0.1% NP-40, electrophoresed through an 18% SDS/PAGE, and detected by Western blotting using polyclonal anti-Tat antibody (upper panel). Wild-type Cdk2 (lanes 1–3) and Cdk9 (lanes 10, 11) were used as positive controls. One-half of the samples were used for Western blotting against Cdk2 after the Tat peptide binding (lower panel). (B) Peptide bands from panel (A) were counted on a Molecular Dynamics software for both Cdk2 and Cdk9, and values were graphed.

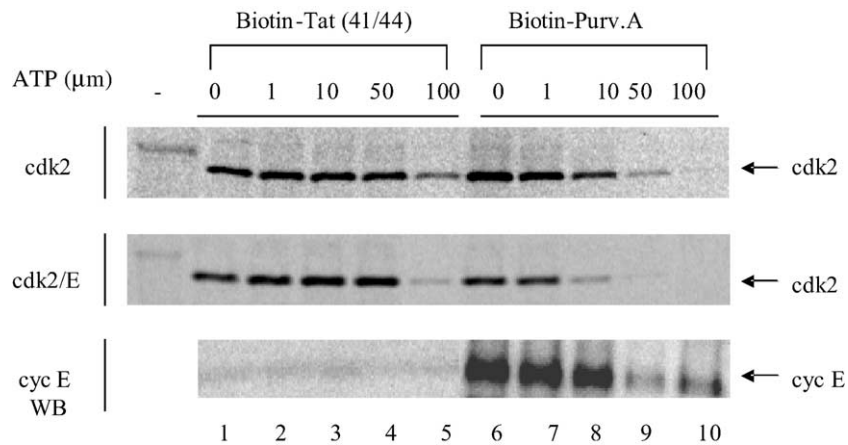


Fig. 10. Effect of excess ATP in Tat peptide competition. Batch affinity chromatography linked to either Tat biotin peptide (10  $\mu\text{g}/100$   $\mu\text{l}$  packed beads) or a purine derivative [biotin-purvalanol A, (Simons et al., 1999)] was used to capture purified Cdk2 or Cdk2/Cyclin E in vitro. After 4 h at 37  $^{\circ}\text{C}$ , cold ATP was added to compete out the binding to Cdk2 or the Cyclin E/Cdk2 complex, and the remaining bound complexes were Western blotted for the presence of Cdk2 or Cyclin E. Lanes 1–5: biotin-Tat 41/44 peptide; lanes 6–10: positive control ATP analog, biotin-purvalanol A.

## Discussion

Highly active antiretroviral therapy (HAART) dramatically changed the course of HIV treatment. Currently, this therapy involves the use of agents from at least two distinct classes of antivirals: a protease inhibitor (PI) in combination with two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) in combination with NRTIs (Barbaro et al., 2005). Recently, a third family of antivirals is now used clinically, with the advent of Enfuvirtide, the first fusion inhibitor (FI). Several pharmacological agents from these classes of antivirals include NRTIs, NNRTIs, PIs, and FIs. Compounds inhibiting HIV integrase, the third enzyme of HIV, and inhibitors of the gp120–CD4 interaction also hold great promise. Finally, compounds interacting with Tat/TAR have also been studied which inhibit HIV replication in low micromolar range (EM2487, tamacrazine, CGP 64222, CGA 137053, or siRNA against Tat among others) (Barbaro et al., 2005). However, in most cases, use of such treatment gives rise to resistant viruses, the mechanisms of inhibition are poorly defined, and only a few of these studies have explored a wide range of inhibition for various viral strains.

In the current study, we extend our initial findings that the Tat 41/44 transdominant peptide from the HIV-1 Tat core domain can inhibit HIV-1 gene expression. The advantage of this system is that peptides derived from Tat are presumably more specific in inhibiting Tat-mediated activation *in vivo*. We previously have shown that soluble peptide analogs of the Tat core domain (amino acid 36–50) were able to effectively block HIV-1 LTR transactivation, and the activity was specific to activated transcription of HIV-1 and not other promoters, including HTLV-I, CMV, PTHrP, IgH, RAS, RSV, and SIV CAT (Kashanchi et al., 1997). Sequentially shorter peptides were synthesized by truncation from both N- and C-termini of the 36–50 (41/44) template. We consistently observed a significant drop in viral titers in U<sub>1</sub> cells with one of the peptides, Tat 41/44. The effect was apparent not only when using the mutant peptide, but also when using a full-length Tat mutated at positions 41/44. Furthermore, our studies in cell lines and PBMCs indicated that these peptides were not toxic to cells at concentrations that normally inhibit HIV-1 replication, indicating that inhibition of HIV-1 activated transcription and viral progeny formation is possible with these peptides. The inhibition by Tat 41/44 was specific to the Tat/TAR complex and, therefore, specific for HIV-1.

When performing Tat peptide biotin pull-down experiments, we observed binding of a number of kinases that target RNA Pol II CTD. We concentrated on Cdk2/Cyclin E mainly because KO animals for either Cdk2 or Cyclin E are viable, making this cdk/cyclin complex an attractive target for HIV therapy. In 2003, the Barbacid laboratory first reported that embryonic fibroblasts lacking Cdk2 proliferate normally and become immortal after continuous passage in culture. Additionally, Cdk2<sup>-/-</sup> mice were viable and survived for up to 2 years, indicating that Cdk2 was also dispensable for the proliferation and survival of most cell types (Ortega et al.,

2003). At the same time, others reported similar findings demonstrating that the E type cyclins were also largely dispensable for development. Cyclin-E-deficient cells proliferated actively under conditions of continuous cell cycling (Berthet et al., 2003; Geng et al., 2003; Mendez, 2003). Therefore, Cdk2 and Cyclin E are not essential for the growth of normal, non-cancerous cells, and use of inhibitors against the Cdk2/Cyclin E complex may pose a viable option to inhibit HIV-1 in infected cells.

We have recently reported that Cyclin E/Cdk2 could also phosphorylate the RNA Pol II CTD, that recombinant Cyclin E/Cdk2 stimulated Tat-dependent HIV-1 transcription in a reconstituted transcription assay, and that immunodepletion of Cyclin E/Cdk2 from HeLa nuclear extracts blocked Tat-dependent transcription (Deng et al., 2002). These are significant findings since RNA Pol II CTD functions both as an assembly platform for and a regulator of the transcription and pre-mRNA processing machineries. During transcription initiation, serine 5 of the CTD heptad repeat is phosphorylated. This phosphorylation triggers a cascade of events beginning with the dissociation of transcription initiation factors from the CTD followed by the recruitment of the capping machinery and the allosteric activation of the capping enzyme. HIV-1 capping takes place during the transition from transcription initiation to elongation when the nascent pre-mRNA is only 20–40 nucleotides long (Zhou et al., 2004). Serine 5 is subsequently dephosphorylated, resulting in release of the capping machinery. Serine 2 of the heptad repeat is then phosphorylated, leading to the recruitment of factors (hSPT5 and TAT-SF1) involved in subsequent steps of HIV-1 RNA processing. Our current data suggest that Tat 41/44 inhibits the kinase activity of Cdk2/Cyclin E *in vitro* and serine 5 phosphorylation of RNAPII in the context of the promoter proximal region of the HIV-1 LTR and not cellular genes such as GAPDH. Therefore, down-regulation of serine 5 phosphorylation by the Tat 41/44 peptide may result in control of all pre-mRNA processing of HIV-1 RNA, including RNA processing, mRNA export, nonsense-mediated decay (NMD), and RNA degradation.

It is also important to note that we currently have no evidence suggesting which of the serine 5 phosphorylation sites in the 52 CTD repeats (conserved or variable regions) are phosphorylated by the Cdk2/Cyclin E complex. Therefore, it is possible that there is a partial loss of phosphorylation of some of the serine 5 sites when treating with the Tat 41/44 peptide. This would give rise to fewer HIV transcripts, which may be sufficient to halt the next round of infection (only 10–100 particles out of ~100,000 are infectious). Along these lines, Zhou et al. (2001) have previously correlated phosphorylation of serine 5 in HIV transcription initiation and elongation complexes with TFIIF. Therefore, an important question would be why does HIV need so many cyclin/cdk complexes for its transcriptional activity. Although we do not have a complete answer at this point, we do speculate that various cyclin/cdk complexes may be phosphorylating various CTD (conserved or variable) substrates, different stages of the cell cycle may determine which cyclin/cdk is used (Agbottah et al., 2005), and the status of the chromatin structure may determine

which cyclin/cdk is used efficiently, that is, Cdk2/Cyclin E has a better activity on the native integrated virus as compared to the naked or disorganized LTR DNA (Ammosova et al., 2005).

We also tested the mode of action for the Tat 41/44 peptide using computer simulation models. As in all molecular simulation programs, the energy function is a very important aspect of these analysis. For our analysis, we included electrostatic, van der Waals, and hydrogen bond terms. Because of the large number of energy calculations required for the thousands of the Metropolis steps for each temperature, we used the grid method that pre-stores the interaction energies. For our binding site search problem, we used a large grid that covers the entire protein and the multiple starting positions of the ligand peptide. A probe atom is placed at each point on the grid, and its energy with the receptor protein is calculated and stored. Such energy tables for all types of atoms in the ligand (carbon, oxygen, nitrogen, and hydrogen) served as our energy look-up tables. When the ligand moves in the grid during a simulated annealing run, the energy of a ligand atom is obtained by interpolation of the pre-stored table values at the eight surrounding grid points. We also modified the widely used AutoDock 3.0 program by writing an automated computer package to use a parallel computer cluster to carry out the multiple starting points docking calculations (Fig. 8). We observed that the wild-type peptide consistently preferred binding near the ATP binding site, while the mutant peptide interacted with a cavity of Cdk2 interfacing the cyclin, thus appearing to be capable of blocking the binding of Cdk2 with cyclin. Furthermore, in our calculation, the mutant peptide consistently had lower binding energy than the wild-type peptide. Therefore, based on experiments performed with the ChIP assay and modeling on cdk, we propose the following model for the mechanism of inhibition when using the Tat 41/44 peptide derivative (Fig. 11). The model predicts that Cdk2/Cyclin E is present on the HIV-1 promoter, is regulated by Tat (in the presence of TAR), and ultimately phosphorylates important substrates in the transcription machinery including the RNAPII CTD. In the presence of the Tat 41/44 peptide, cdk dissociates away from the cyclin component making the kinase inactive. The remaining cyclin complex on the HIV-1 promoter may ultimately be ubiquitinated and degraded, although

currently we have no formal proof of this event. However, there is high likelihood of cyclin degradation since this is one of the normal patterns of oscillating cyclins including Cyclins D, E, A, and B. Finally, in the current study, we have not addressed whether the free cyclin can bind to other cdk, a phenomenon that is common in many cyclin over-expressing cells, including breast cancer and HTLV-1 infection. Future experiments will address these critical issues, along with peptide feasibility studies (SCID-hu implants) and in vivo availability of peptides in animals.

## Materials and methods

### *PBMC infection and peptide treatment*

#### *Antiviral determination of Tat peptides in PMBC*

Phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) were thawed from liquid nitrogen, placed in a 75-cm<sup>2</sup> flask at a density of  $2 \times 10^6$  cells per ml, and cultured for 48 h in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 5 U/ml human lymphocyte IL-2 (Boehringer-Mannheim) (complete cell culture medium). On the day of assay, PBMCs ( $7 \times 10^6$ ) were inoculated with HIV-1 NL4-3 at an MOI of 0.005 in bulk in a 15-ml conical tube for 2 h at 37 °C. Unadsorbed virus was removed by centrifugation at  $400 \times g$  for 5 min, cells were resuspended in medium at  $2 \times 10^6$  cells per ml in complete cell culture medium, and 100  $\mu$ l (100,000 cells) was added to triplicate wells of a round-bottom 96-well plate. The wells then received 100  $\mu$ l of serially diluted Tat peptide (either WT or 41/44, cyclized 5 aa in 5% DMSO; 200  $\mu$ l total), and the plates were incubated at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere for 7 days. Plates were then centrifuged at  $400 \times g$  for 5 min, and supernatants were collected and assayed for p24 antigen at a 1:800 dilution in HIV p24 antibody-coated microplates (NEN Life Science Products, Inc.) by quantitative ELISA using the p24 standard supplied by the manufacturer. The 50% inhibitory concentration (IC<sub>50</sub>) was calculated by use of a 4-parameter fit (SOFTmax PRO 3.0, Molecular Devices, Mountain View, California). A positive antiviral control drug, 3TC (NIH AIDS

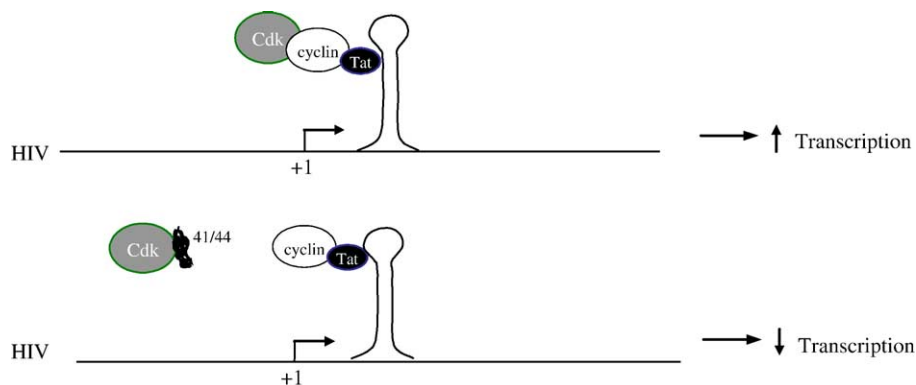


Fig. 11. Proposed model of the mechanism of Tat 41/44 peptide inhibition of the HIV-1 promoter. The model relies on experiments presented in Figs. 5–7, where the Tat 41/44 peptide, and not the wild-type peptide, was able to disrupt the interaction between Cdk and its Cyclin partner at the interface where these two molecules interact. The model is supported by computational docking results and by the ChIP assays shown in Fig. 7B.

Research and Reference Program), was prepared and tested at a final concentration range of 1000 to 0.00032  $\mu\text{M}$ . Concentrations are shown as micromolar from two independent experiments.

#### *Effect of Tat peptides in PBMC infected with HIV-1 SI and NSI strains*

Phytohemagglutinin-activated PBMCs were kept in culture for 2 days prior to each infection. Isolation and treatment of PBMCs were performed as described above. PBMCs were infected with either a syncytium-inducing (SI) (UG/92/029 Uganda strain, subtype A envelope, 5 ng of p24 gag antigen) or a non-syncytium-inducing (NSI) (THA/92/001, Thailand strain, subtype E envelope, 5 ng of p24 gag antigen) strain of HIV-1. Both viral isolates were obtained from the NIH AIDS Research and Reference Reagent Program. After 8 h of infection, cells were washed, and fresh media plus two cyclized peptides WT and 41/44 (100 nM) were added to the media. Plates were then incubated at 37 °C (5% CO<sub>2</sub>, incubator). On day six post-infection, 10  $\mu\text{l}$  supernatant samples were taken from each well, frozen, and stored (−20 °C) for determination of p24 levels. Cells were resuspended in complete media, and 50  $\mu\text{l}$ /well of the cell suspension was transferred to new 96-well plates containing 150  $\mu\text{l}$ /well fresh medium with peptides. The final peptide concentrations were the same as on day zero. Plates were incubated at 37 °C (5% CO<sub>2</sub>, incubator). On day 12, again, 10  $\mu\text{l}$  p24 samples were taken from each well, frozen, and stored at −20 °C. This time, 150  $\mu\text{l}$  of the culture supernatants was removed and replaced by 150  $\mu\text{l}$  of fresh medium with peptides. Again, the final concentrations of the peptides were the same as on day zero. Plates were incubated at 37 °C (5% CO<sub>2</sub>, incubator). On day 18, 10  $\mu\text{l}$  samples were taken from each well, frozen, and stored at −20 °C, and the experiment was terminated on day 24. All samples were processed for the presence of Gag p24 at the completion of the experiment. Tat peptides used throughout these studies were:

Tat 36–50 (WT): V-C-F-T-T-K-A-L-G-I-S-Y-G-R-K  
 Tat 36–50 (41/44): V-C-F-T-T-A-A-L-S-I-S-Y-G-R-K  
 Tat 38–47 (WT): F-T-T-K-A-L-G-I-S-Y  
 Tat 38–47 (41/44): F-T-T-A-A-L-S-I-S-Y  
 Tat 40–44 (WT): T-K-A-L-G  
 Tat 40–44 (41/44): T-A-A-L-S  
 Cyclized WT-Tat: CTKALGC-**GG**-YGRKKRRQRRR  
 Cyclized 41/44-Tat: CTAALSC-**GG**-YGRKKRRQRRR  
 Cyclized WT-Tat: CTKALGC-**GG**-PKKKRKV

#### *Peptide synthesis*

All peptides including those that were biotin-labeled were prepared on a PAL-PEG-polystyrene resin by continuous flow solid phase synthesis on a PerSeptive Biosystems Pioneer synthesizer (Framingham, MA) using HBTU-activated Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) amino acids. Peptides were purified using conventional reversed phase HPLC on Vydac C18 (Hesperia, CA) with an overall yield of 25–30%, based on starting resins. The purity of the peptides was confirmed further

by analytical reversed phase HPLC, capillary zone electrophoresis, and matrix-assisted laser absorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The quantity of each peptide was determined by Bio-Rad protein assay as well as running small aliquots on 4–20% or 15% SDS/PAGE followed by silver staining (Silver Stain Plus, Bio-Rad). Small 5 aa WT and 41/44 Tat peptides were synthesized using 9-fluorenylmethoxy carbonyl chemistry with *N,N*-dicyclohexylcarbodiimide-*N*-methylpyrrolidone/1 hydroxybenzotriazole esters on an automated ABI synthesizer, model 433. A set of Cys 2 compounds was also synthesized with two glycines for flexibility and peptide domains for delivery. These peptides were purified using C18, acetonitrile-in-water gradient chromatography, with a Waters high-performance liquid chromatograph prior to use. Sequences of the purified peptides were verified using an ABI automated sequencer. The peptides were lyophilized and stored at 4 °C prior to use.

#### *Phosphorylation of RNAPII CTD and the effect of Tat peptides in cells*

CTD phosphorylation was performed using anti-Cyclin E immunoprecipitates from HIV-1-infected cells (ACH2) treated with TNF or with a recombinant purified Cyclin E/Cdk2 complex. Anti-Cdk2 antibody (10  $\mu\text{g}$ ) and ACH2 protein extract (3 mg) were used in immunoprecipitations. The washed complex was incubated with 1  $\mu\text{g}$  of GST-CTD, which was expressed in *E. coli* and purified as previously described (Deng et al., 2002). In vitro kinase assays with Cyclin E/Cdk2 have previously been described (Deng et al., 2002; Wang et al., 2001, 2002). Three concentrations (10, 100, 1000 ng) of wild-type Tat 5 aa (wt) or the mutant (41/44) peptides were added to the reaction mixture. Recombinant baculavirus Cyclin E and HA-Cdk2 were co-infected into Sf9 cells, and the active complex was purified on a 12CA5 antibody column. Excess HA peptide (300 fold) was used to purify the Cyclin E/Cdk2 complex (Deng et al., 2002; Wang et al., 2001, 2002). A CTD kinase assay was performed with Cyclin E/Cdk2 (0.5 unit/ml) and 1  $\mu\text{g}$  of GST-CTD, and subsequently Tat (wt, lin) and Tat (41/44, lin) 5 aa peptides were added at 100 ng per reaction. The total kinase reaction volume was 20  $\mu\text{l}$ . Toxicity/Proliferation studies of Tat peptides were performed in various cell lines and uninfected PBMC as described previously (Beales, 2002). Uninfected cells (CEM, U937, CaCo-2, PBMCs) and infected cells (ACH2) were grown to mid log phase. Cells were first treated with 5 ng of TNF for 2 h (to induce virus in ACH2 cells) and washed. Tat peptides were electroporated into cells at 1  $\mu\text{g}/2.5 \times 10^6$  cells (Kashanchi et al., 1992). DNA synthesis was estimated by measurement of [<sup>3</sup>H]-thymidine incorporation into the trichloroacetic acid (TCA) precipitable material. [<sup>3</sup>H]-thymidine (0.1  $\mu\text{Ci}/\text{ml}$ , 10 Ci/mmol) was added 2 h before the end of a 24 h treatment period, the cells were washed, and DNA was TCA precipitated and counted as previously described (Beales, 2002). Some of the experiments were carried up to 7 days prior to [<sup>3</sup>H]-thymidine incorporation and TCA precipitation.

### Chromatin immunoprecipitation (ChIP) assay following Tat peptide treatment in ACH2 cells

Cells ( $2 \times 10^6$ ) were treated with TNF for 2 h, washed, and electroporated with 1  $\mu$ g of peptide. Cells were put in 10 ml of complete media, and, 24 h later, they were cross-linked by addition of 1% formaldehyde to the medium for 10 min. The chromatin immunoprecipitation (ChIP) method used was a modification from Braunstein et al. (1993) and de la Fuente et al. (2000). Crude nuclei prepared by hypotonic lysis were resuspended in 100  $\mu$ l SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1), sonicated under conditions that reduced DNA length to between 200 and 1000 base pairs, and debris removed by centrifugation. The chromatin solution was diluted 10-fold in IP buffer and pre-cleared for 45 min at 4 °C on protein A beads pre-adsorbed with sonicated salmon sperm DNA. The chromatin solution was then incubated with 10  $\mu$ g of various antibodies including Pol II Ser 2, Pol II Ser 5, SPT-5, cap (monoclonal anti-<sup>2,2,7</sup>-trimethylguanosine), Cdk9, Tat-SF1, Tat, Pol II large subunit, and phospho-histone H3 overnight at 4 °C. Immune complexes were collected with protein A beads pre-adsorbed with sonicated salmon sperm DNA. Following washes and elution, cross-links were reversed by heating at 65 °C for 4–5 h, and DNA was recovered by phenol extraction and ethanol precipitation. Specific DNA sequences in the immunoprecipitates were detected by PCR under conditions in which product yield was dependent on input DNA dose, using specific primers. Primers used were: LTR: 5'ACTTTTCCGGGGAGGCGCGATC3' (Forward), 5'GCCACTGCTAGAGATTTCCACACTG3' (Reverse), GAPDH: 5'TACTAGCGGTTTTACGGGCG3' (Forward), 5'TCGAACAGGAGGAGCAGAGA3' (Reverse).

### Computer modeling of the Tat (41/44) 5 aa peptide binding site on Cdk2

Among the three experimentally determined binding partners of the Tat 41/44 5 aa peptide (DNA-PK, Cdk9, and Cdk2), only the structure of Cdk2 is known. It is also important to note that we have attempted to crystallize both DNA-PK and Cdk9 enzymes in the presence of ATP analogs and have not had much success in obtaining uniform crystals. Therefore, we took advantage of the existing crystal structure and carried out computational docking studies of the wild-type 5 aa Tat peptide, TKALG, and the mutant peptide, TAALS, with Cdk2. The Cdk2 structure used was obtained from the Protein Data Bank ID 1E1X (resolution 1.85 Å). The structure is Cdk2 with an inhibitor bound to the ATP binding pocket, and the inhibitor was removed prior to docking. Docking was performed using the software AutoDock 3.0 (130) with the simulated annealing search algorithm. The receptor protein (Cdk2) was taken as rigid and the ligands (the wild-type and mutant peptides) as flexible. All torsion angles were allowed to change (except those about the peptide bond), giving 19 rotatable bonds for the wild-type peptide and 16 for the mutant. The annealing temperature was reduced with a geometric scheme, and each docking run took about 2 h on a 2.8 GHz

processor. Because we do not know the binding site, our docking runs were carried out from multiple starting points covering the surface of the receptor protein. A Linux computer cluster with 64 nodes was employed for our simulation.

For each docked ligand conformation, computer scripts were written to breakdown the total ligand–receptor interaction energy by receptor protein residues (this calculation determines binding site on the receptor protein) or by ligand atoms (this helps in ligand optimization). To determine the binding site of the wild-type and mutant peptides on Cdk2, binding energy per residue was calculated for the low-energy docked conformations, and each Cdk2 residue was ranked by average energy for these conformations.

It is well known from X-ray crystallography that the structure of Cdk2 changes when it binds with Cyclin A and when the Cyclin A/Cdk2 complex is phosphorylated at Thr160 (and thus activated) (Pavletich, 1999). In particular, the binding of Cyclin A causes the T loop of Cdk2 to move rather significantly. It has been suggested that Cdk2 possesses “an intrinsic conformational flexibility” (Pavletich, 1999), and there is a question on which structure of Cdk2 should be used as a design template (Davies et al., 2002; Johnson et al., 2002). Accordingly, both the wild-type and the mutant peptides were docked with the Cdk2 structure taken from a Cyclin A/Cdk2 complex (PDB id: 1FIN) using the methodology and the parameters described above.

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