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Effect of transcription peptide inhibitors on HIV-1 replication

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ABSTRACT

HIV-1 manipulates cellular machineries such as cyclin dependent kinases (cdks) and their cyclin elements, to stimulate virus production and maintain latent infection. Specifically, the HIV-1 viral protein Tat increases viral transcription by binding to the TAR promoter element. This binding event is mediated by the phosphorylation of Pol II by complexes such as cdk9/Cyclin T and cdk2/Cyclin E. Recent studies have shown that a Tat 41/44 peptide derivative prevents the loading of cdk2 onto the HIV-1 promoter, inhibiting gene expression and replication. Here we show that Tat peptide analogs computationally designed to dock at the cyclin binding site of cdk2 have the ability to bind to cdk2 and inhibit the association of cdk2 with the HIV promoter. Specifically, the peptide LAALS dissociated the complex and decreased kinase activity *in vitro*. We also describe our novel small animal model which utilizes humanized Rag2$^{-/-}\gamma_c^{-/-}$ mice. This small peptide inhibitor induces a decrease in HIV-1 viral transcription *in vitro* and minimizes viral loads *in vivo*.

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Introduction

Human immunodeficiency virus (HIV-1) is the etiological agent of acquired immunodeficiency syndrome (AIDS). Approximately 40 million people are currently infected with HIV-1 and 4 million new cases will be identified this year alone. When administered properly, the currently available HIV-1 medications are capable of controlling infection, however they do not represent a cure. Highly active antiretroviral therapy (HAART) is currently the most effective strategy in HIV-1 treatment and reduction in AIDS-related mortality. HAART is a multidrug therapy which has proven to be effective in lowering viral loads and improving the host immune response to HIV-1 infection. While the drugs merely prevent infection of new cells, latently infected cells continue to produce varying levels of wild type and mutant viruses. Additionally, the currently available drugs specifically target viral proteins, such that small polymorphisms largely affect the efficiency of drug action. The high rate of mutation caused by the viral RT enzyme, for example, and the large numbers of new virus produced during each round of infection allow the virus to select variants with mutations that make them resistant to currently available therapies.

Current therapies in HIV-1 are insufficient due to their inability to cure the disease and the proficiency of the virus to become resistant to the treatment over time. New therapies must be developed which target novel mechanisms important to the viral life cycle. HIV encodes only nine genes; a fact which forces the virus to interact with and subvert cellular processes for its own benefit. Identifying these interactions and their effect on both the virus and the cell will reveal a new range of targets for future therapeutics. Alternative preventative measures such as topical microbicides and vaccines are in development, however the rate at which HIV-1 can replicate and mutate forces the need for an in depth understanding of the viral/host cell relationship.

Tat is the HIV-1 viral transactivator protein responsible for transcriptional activation and elongation. Tat has been known to not only stimulate the HIV long terminal repeat (LTR) promoter, but also to

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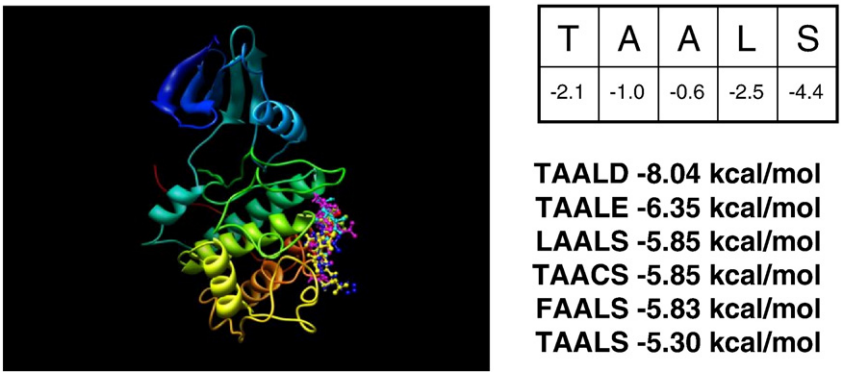

Fig. 1. Computational modeling of Tat analogs in the cyclin binding site of cdk2. An example of a three dimensional ligand binding model of a Tat peptide analog in the cdk2 binding site. Cdk2 is shown as a ribbon model where the secondary structure elements are indicated by arrows (β-sheets) and spirals (α-helices) and is colored from the N-terminus (blue) to the C-terminus (red). A Tat peptide is shown bound to cdk2 in a ball and stick configuration and colored based on amino acid residue. Computational modeling was performed to predict the binding affinities of the Tat derived analogs to the cyclin binding site of Cdk2. Peptides designed for the purpose of this study were chosen based on stronger binding affinities than the TAALS peptide published previously (Agbottah et al., 2006). The binding energies are expressed in kcal/mol.

modulate and induce cellular genes. Historically, the mechanism of action by Tat has been assigned to the level of initiation and elongation (Bohan et al., 1992; Brady and Kashanchi, 2005; Feinberg et al., 1991; Kato et al., 1992; Laspia et al., 1989; Marciniak et al., 1990; Marciniak and Sharp, 1991). Tat primarily activates the HIV LTR by binding to the TAR element, an RNA stem-loop structure, which facilitates elongation and recruitment of cellular factors. One of these cellular factors, p-TEFb, a protein kinase composed of cdk9 and Cyclin T_1, is activated by Tat, resulting in hyper-phosphorylation of the large subunit of the RNA polymerase II CTD and activation of transcription elongation (Chiang and Roeder, 1995; Dal Monte et al., 1997; Garcia-Martinez et al., 1997; Jeang et al., 1993; Kashanchi et al., 1996, 1994; Kim et al., 2002; Richter et al., 2002; Roebuck et al., 1997; Veschambre et al., 1995; Wei et al., 1998; Yu et al., 1995). In recent years Tat has also been shown to bind a number of other factors which regulate chromatin structure at the HIV promoter as well as enzymes that phosphorylate the large subunit of RNA Pol II resulting in efficient elongation of transcription, such as CBP/p300 (Bieniasz et al., 1999; Deng et al., 2000, 2001; Herrmann and Mancini, 2001; Herrmann and Rice, 1995; Yang et al., 1997; Zhu et al., 1997). In addition to controlling the functional modifications associated with viral transcription, Tat also undergoes post-translational modifications by host cellular proteins. For instance, it has been shown that Tat is acetylated at lysine 28 and 50, ubiquitinated at lysine 71, and methylated at arginine residues (Boulanger et al., 2005; Bres et al., 2003, 2002).

Cyclin-dependent kinases (cdks) and their corresponding catalytic Cyclin subunits are crucial regulators of eukaryotic cell cycle progression. These cdk/Cyclin complexes positively assist the cell cycle by aiding the progression through the G_1/S and G_2/M cell cycle checkpoints. Specifically, the progression through the G_1/S checkpoint is managed by the phosphorylation of the retinoblastoma (Rb) protein by the cdk2/Cyclin E complex (Athanassiou et al., 2004). The G_1/S checkpoint plays a key role in HIV-1 viral replication and in the transcription of proliferative genes, in that immediately following this cell cycle stage viral replication is dramatically increased (Athanassiou et al., 2004). This suggests that the regulation of kinase activity of complexes such as cdk2/Cyclin E complex play a role in Tat activated transcription. Along these lines, Tat recruits the p-TEFb and cdk2/Cyclin E complexes to the viral promoter allowing phosphorylation of RNA Pol II CTD (Herrmann and Rice, 1995; Nekhai et al., 2002; Parada and Roeder, 1996; Pumfery et al., 2003; Roebuck et al., 1997; Zhou et al., 2004). Deng et al. have shown that Tat stimulates the phosphorylation of the CTD by interacting with cdk2/Cyclin E. Moreover, the depletion of the cdk2/Cyclin E complex from HeLa nuclear extracts completely blocked Tat-dependent viral transcription (Deng et al., 2002). Therefore, cdk2/Cyclin E is necessary for the phosphorylation of the CTD and consequently for Tat-activated viral transcription (Ammosova et al., 2006; Nekhai et al., 2000, 1997, 2002). Additionally, it was observed that cdk2/Cyclin E exhibited an increase in kinase activity in latently infected cells due to the loss of the cdk inhibitor, p21/waf1. Further evidence that cdk2 is an important regulator of HIV-1 transcription was reported when Tat itself was found to be

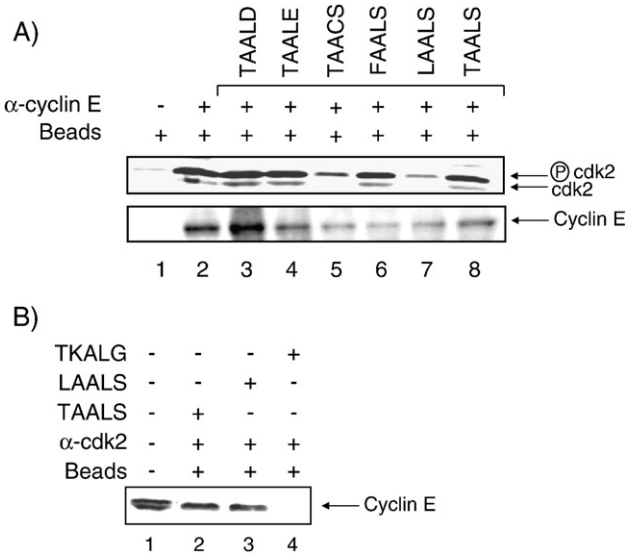

Fig. 2. Immunoprecipitation of Cyclin E and Western blot for cdk2 and phosphorylated cdk2 in the presence of Tat derived peptides. A) C81 fractionated cell extracts containing cdk2/Cyclin E were incubated with α-Cyclin E antibody in the presence of Tat derived peptides. Following pull-down samples were probed for both unphosphorylated and phosphorylated cdk2. Various competing peptides at 10 μg/μl/reaction were added at the time of immunoprecipitations. The wild type Tat sequence TKALG and IgG were included as negative controls (data not shown). B) cdk2/Cyclin E were purified from lysates of SF9 insect cells infected with baculoviruses producing cdk2 and Cyclin E as described in Materials and methods. Purity of cdk2/Cyclin E (Sephadex H200 column) was checked on 12% PAGE followed by Coomassie staining. cdk2/Cyclin E complex were used in immunoprecipitation with anti-cdk2 antibody (10 μg) and the pulled-down complex were incubated with various peptides (10 μg) for 4 h at 37 °C. Following the incubation, samples were spun down and supernatants were used for Western blot with anti-cyclin E antibody (1:1000 dilution). C) Purified GST-cdk2 and GST-cdk4 (2 μg of each double purified protein) were bound to the LAALS peptide (50 μg) overnight and subsequently washed with a low stringent buffer (TNE50+0.01% NP-40). Any bound peptides were then eluted using the same binding buffer plus 0.01% SDS. Eluted peptide samples were combined with MS washing buffer and injected into an ESI-MS LCQ Deca XP^{Plus} Thermo Finnigan mass spectrometer at 10 μl/min and read through an API source. Spectra were normalized to the highest abundant mass peak and were averaged over the course of injection. Data acquisition and peak analysis were performed through the Xcalibur software platforms. Data is shown zoomed in on relevant m/z ranges.

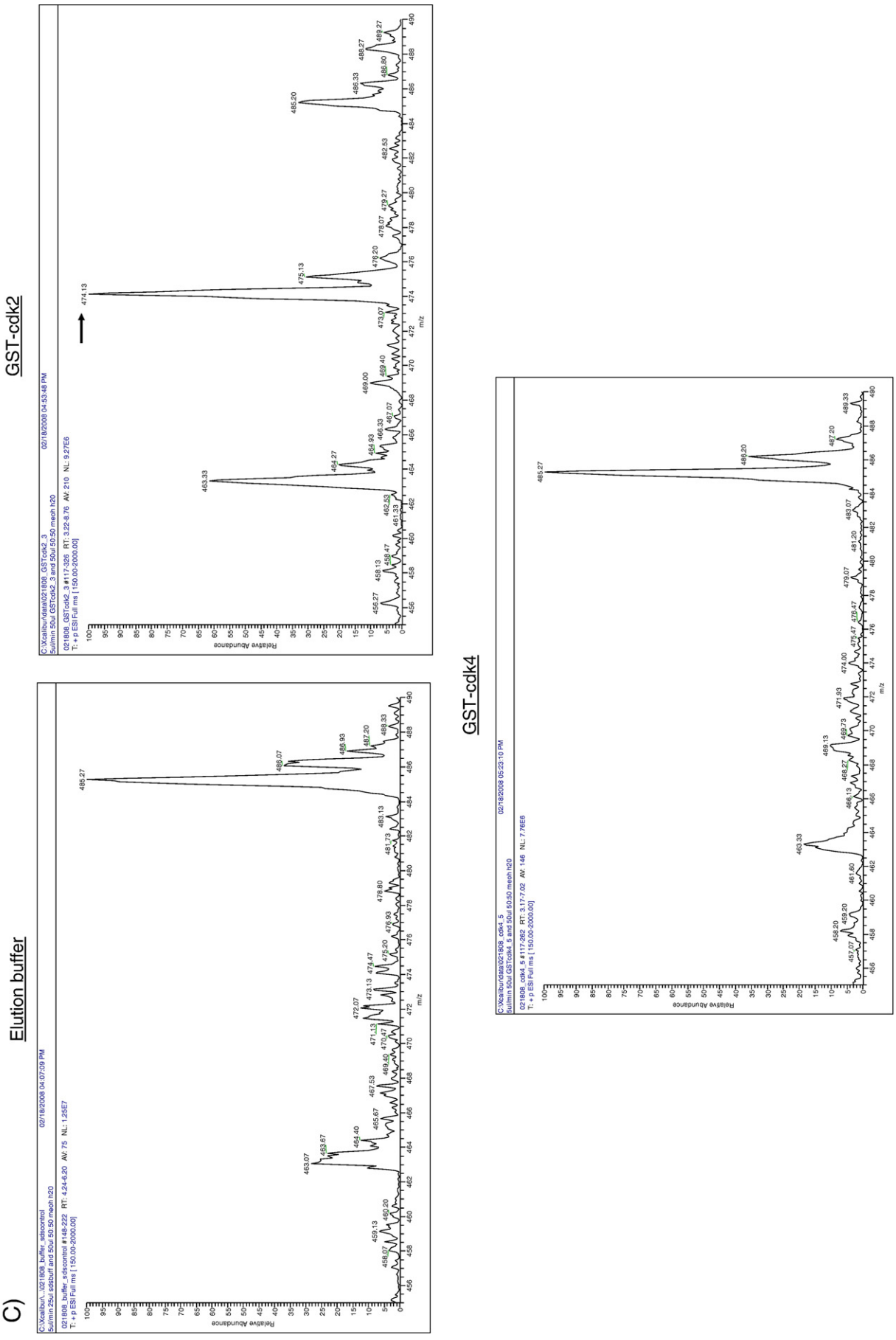

Fig. 2 (*continued*).

phosphorylated by cdk2 *in vitro* (Deng et al., 2002; Nekhai et al., 2000). siRNA against cdk2 was also found to inhibit Tat-induced HIV-1 transcription and viral replication indicating that cdk2 is crucial for viral progression (Ammosova et al., 2005). Finally, cdk2 was found to be non-essential for normal host cell proliferation as evident by the viability of cdk2 knock-out mice (Malumbres et al., 2003). Therefore, the inhibition of the cdk2/Cyclin E complex is an appealing target for HIV-1 anti-viral therapies.

Previously, we have shown that a Tat peptide (41/44) from the HIV-1 core domain can inhibit HIV-1 gene expression and act as a replication inhibitor (Agbottah et al., 2006). This Tat peptide was synthesized by truncation of the sequences from both the N- and C-terminus of Tat and included specific double mutations. This condensed analog retained the specificity necessary to inhibit HIV-1 viral transactivation, and not other viral promoters from CMV or HTLV-1. Furthermore this peptide was capable of inhibiting the kinase activity of cdk2/Cyclin E *in vitro* (Agbottah et al., 2006; Deng et al., 2002). Here we show a further refinement of these peptides using computational docking studies with the cdk2 structure, therefore characterizing the cdk2/Cyclin E binding interface and localizing potential dissociation sites both *in vitro* and *in vivo*. We identified a new set of peptides that have better inhibitory effect on activated transcription. These peptides also inhibit virus replication in a new humanized stem cell animal model *in vivo*.

Results

Predicted binding energies of Tat peptide analogs

To better design peptides that could possibly bind or compete for the Cyclin E and cdk2 interface, we first utilized computational docking models. Ligand candidates based on the Tat 41/44 mutant peptide from the previous study were computationally designed as indicated (Agbottah et al., 2006). Ligand candidates were prepared as single mutations of TAALS, therefore, not straying too far from the original Tat 41/44 sequence. Fig. 1 shows an example of a three dimensional computational docking model of a small peptide and cdk2. Here the structure of cdk2 is depicted in "ribbon" view such that the secondary structure elements α-helices and β-sheets are depicted as spirals and parallel arrows respectively. The cdk2 sequence is also colored from blue to red to correspond to the N- and the C-termini respectively. The small ligand in Fig. 1 is docked to the Cyclin binding site of cdk2 and is represented in a "ball and stick" view. From energy minimization studies, the affinities of each ligand to the Cyclin binding site of cdk2 were also determined. Each amino acid substitution provides different bonding opportunities where each binding energy is different. All of the newly proposed peptides have a higher predicted binding affinity than the Tat 41/44 peptide previously described (Agbottah et al., 2006) with TAALD having the highest. This suggests that these peptides may provide a higher percentage of dissociation of the cdk2/Cyclin E complex.

Specificity of Tat peptide in possibly dissociating cdk2/Cyclin E complex

In order to detect any possible dissociation of the cdk2/Cyclin E complex in the presence of the Tat peptides, immunoprecipitations of Cyclin E were performed. Cell extracts were first separated through a size-exclusion column into various fractions (total of 80). A representative amount of fractions were probed for cdk2 in order to determine a size range in which the complex was likely to elute (data not shown). This chromatographic step also minimized the chance that non-specific binding interactions would occur with cdk/Cyclin complexes. Also, in the previous Agbottah 2006 paper, we used immunoprecipitations from HIV-infected whole cell extracts, where the extracts contained two distinct populations of cdk2 complexes. When using a sizing column, one population that is fairly large comes out at 440 kDa size and a second population elutes from 67 kDa up to 150 kDa. The smaller size complexes (67 kDa–150 kDa) are active cdk2 complexes whereas the large 440 kDa complex is largely inactive in kinase activity and contains as yet unpublished proteins and RNA bound molecules (Kashanchi and Nekhai, unpublished data; Deng et al., 2002). Therefore, the smaller cdk2/Cyclin E containing fractions (29–32) were pooled together and the extract (250 μg) was used in immunoprecipitations with antibodies against Cyclin E in the presence or absence of the Tat peptides. Immunoprecipitates (IPs) were washed and Western blotted for the presence of cdk2 and Cyclin E. Results in Fig. 2A show the addition of six Tat derived peptides to the IP reaction. Lanes 1 and 2 serve as controls for non-specific binding and control IP reaction, respectively. There is a varying degree of dissociation amongst the various Tat peptide competitors. For instance, the LAALS peptide in lane 7 appears to have the highest level of dissociation followed by TAACS. Based on the computational binding energy prediction, it would have been assumed that TAALD would have the highest efficiency of dissociation due to the strong binding affinity (lane 3), but the actual biochemical test (IP followed by competition) showed a different result. Interestingly, the levels of detectable phosphorylated cdk2 in these IP/competition experiments vary depending on which peptide is used. For instance, LAALS and TAACS peptides appear to have lower recovery levels of phosphorylated cdk2, while the other peptides appear to have no effect on the basal recovery levels of phosphorylated cdk2. Also, the lack of efficiency of competition of the other peptides for cdk2 indicates that both unphosphorylated and phosphorylated cdk2 can equally bind to Cyclin E as seen in the immunoprecipitates.

We next asked if the new peptide derivatives could indeed dissociate the cdk2/Cyclin E complex *in vitro*. We therefore used a purified system where Baculovirus expressed cdk2/Cyclin E complexes (Ammosova et al., 2006) were used in immunoprecipitation with anti-cdk2 antibody and the pulled-down complexes were incubated with various peptides for 4 h at 37 °C. Following the incubation, samples were spun down and supernatants were used for Western blots with anti-Cyclin E antibody. Results in Fig. 2B show that two peptides (TAALS and LAALS), but not the wild type peptide (TKALG), were able to dissociate the Cyclin E away from the cdk2 by about ~50% (compare input level to lanes 2 and 3). These results further indicate that the new LAALS peptide is able to at least partially dissociate the cdk2/Cyclin E complex. Finally, we asked if we could indeed detect binding of the LAALS peptide to the cdk2 protein in the absence of its cyclin partner.

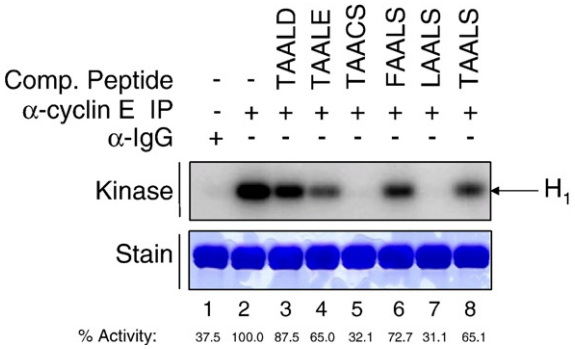

Fig. 3. Loss of kinase activity of cdk2/Cyclin E in the presence of competing peptides. Immunoprecipitation of Cyclin E in the presence of Tat peptides was performed as described in Materials and methods. Immunoprecipitated Cyclin E samples in the presence of Tat peptides were assessed for kinase activity by washing the beads with kinase buffer to equilibrate the reaction. Histone H1 (5 μg/reaction) was added to each reaction tube along with 2 μl of (γ-^{32}P) ATP (3000 Ci/mmol). Reactions were incubated at 37 °C for 30 min and stopped by the addition Laemmli buffer. The samples were separated on a 4–20% Tris–Glycine gel. Top panel represents samples ran on gel, dried and exposed to a PhosphorImager cassette and analyzed utilizing Molecular Dynamic's ImageQuant Software. Bottom panel represents samples that were stained with Coomassie blue, destained, and then dried for 2 h. Lane 2 served as a positive control (100%) activity without any competing peptides.

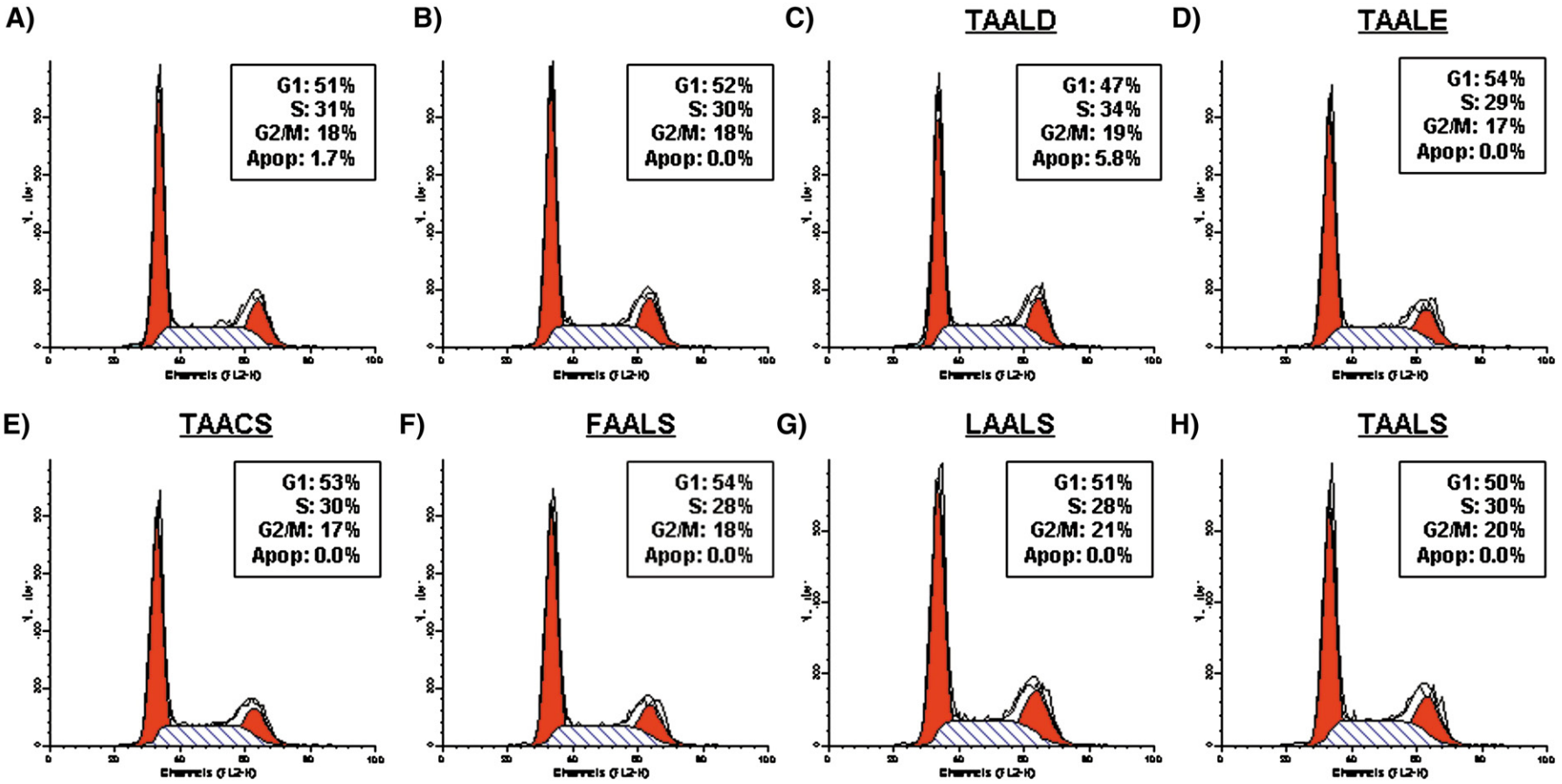

Fig. 4. Cell cycle analysis of cells in the presence of Tat derived peptides. Cell cycle analysis was performed on CEM cells either untreated or treated with WT or Tat derived peptides. Cells were collected by low speed centrifugation and washed with PBS without Ca^{2+} and Mg^{2+} and fixed with 70% ethanol. For fluorescence-activated cell sorting (FACS) analysis, cells were stained with a mixture of propidium iodide (PI) solution for 30 min at room temperature prior to analysis. The cells were acquired and analyzed using CELLQuest software (BD Biosciences). Acquired FACS data were analyzed by ModFit LT software (Verity Software House, Inc.). Various panels represent: A) Untreated CEM cells, B) CEM cells treated with wild type control peptide, C) TAALD, D) TAALE, E) TAACS, F) FAALS, G) LAALS, and H) TAALS. Percent of each cell populations in G1, S, G2/M, and apoptosis are indicated.

We designed an experiment where 2 μg of purified GST-cdk2 (experimental), and GST-cdk4 (control) were bound to the peptide and subsequently washed with a low stringent buffer (TNE50+0.01% NP-40). Any bound peptides were then eluted using the same binding buffer plus 0.01% SDS. Eluted peptides from the supernatant were then subjected to LCQ MS analysis (Thermo Finnegan) for detection. Results of the experiment are shown in Fig. 2C, where the SDS buffer alone shows a certain set of background molecular weight peaks (non-specific peaks, i.e. 463.07, and 485.27). A similar set of peaks also showed up (although with varying intensity) in the GST pull down experiment (data not shown). However, the specific LAALS peptide appeared only in the GST-cdk2 but not in the GST-cdk4 eluates, exhibiting a parent peak of 474.3 Da. Other control experiments, including the use of wild type peptides or Rb peptides which aren't normally phosphorylated by cdk2 exhibited no binding affinity using this assay, however cdk9 bound equally well to this peptide (data not shown). Collectively, these results imply that the LAALS is capable of binding to the cdk2 component of the kinase complex.

Inhibition of cdk2/Cyclin E kinase activity in vitro

As seen previously, at least two of the Tat derived peptides have been shown to dissociate the cdk2/Cyclin E complex as observed in the Western blot analysis. Next, the functional kinase activity of cdk2/Cyclin E in the presence of the inhibitors was measured. A radioactively labeled kinase assay was utilized to measure the levels of phosphorylation of Histone H1 in the presence of these peptides. Cyclin E immunoprecipitations were performed (similar to Fig. 2) and the pull-downs were washed and used in a kinase reaction. Substrate and radioactively labeled ATP were both added to the sample, and the

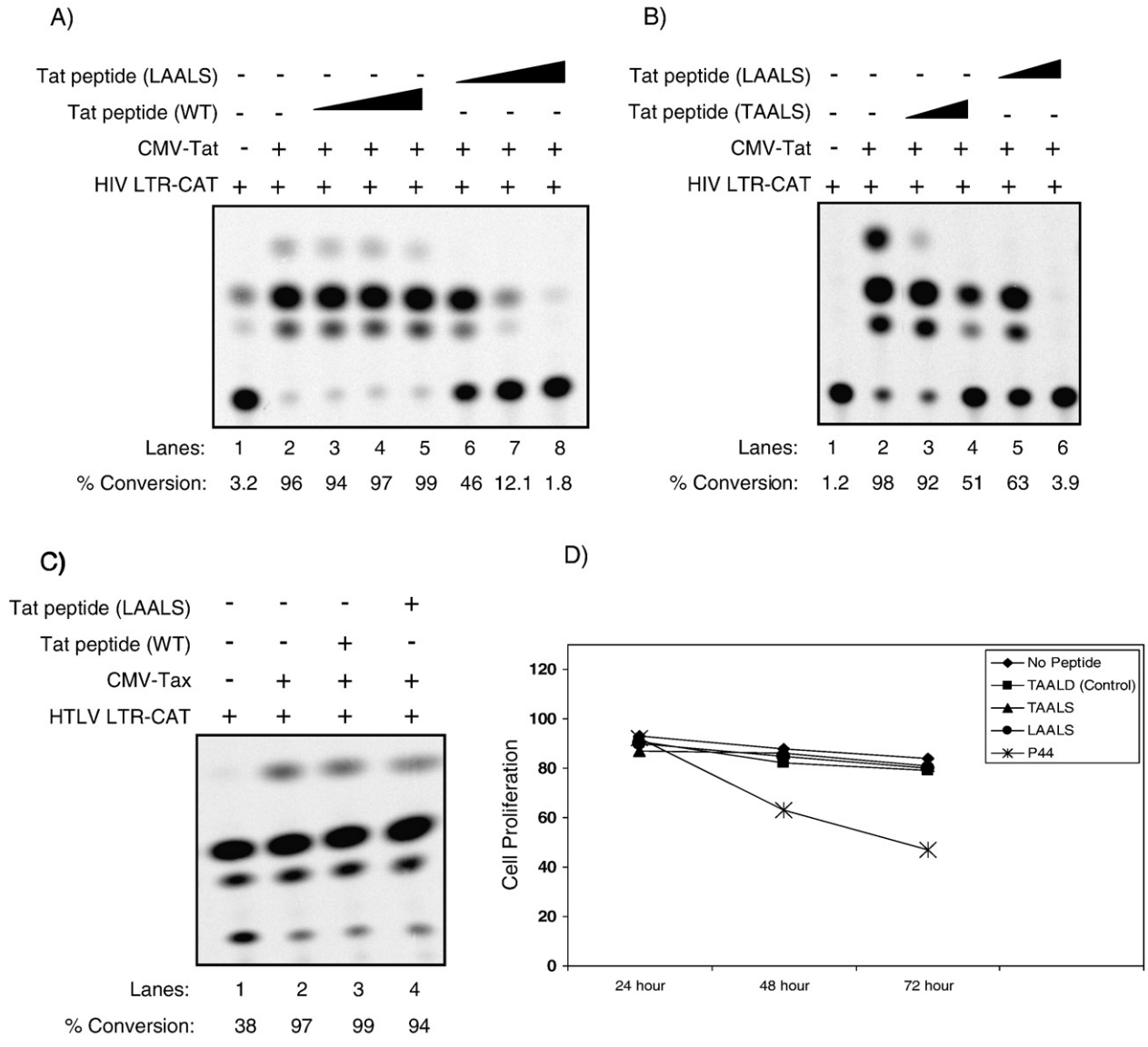

Fig. 5. Inhibition of HIV-1 transcription activity using transient transfection assay. Two plasmids namely reporter HIV-1 LTR-CAT (3 μg) as well as CMV-Tat (1 μg) plasmids were transfected either alone or with various concentrations of wild type or mutant peptides (1, 5, and 10 μM). Transfected CEM cells were kept in complete media for 48 h followed by total protein extraction and CAT assay. Panel A shows wild type LTR activity by itself (lane 1) and with Tat which activates the LTR promoter by more than 95% (lane 2). Wild type peptide (lanes 3–5) and mutant peptides (lanes 6–8) were all added at the time of transfection (electroporation). Panel B shows comparison of the old 41/44 peptide (TAALS) and the new derivative (LAALS) in CAT assay on HIV-1 LTR-CAT. Lane 3 and 5 were transfected with 1 μM and lanes 4 and 6 were transfected with 5 μM of each peptide. Panel C represents a similar experiment using HTLV-1 LTR-CAT (3 μg) and its transactivator CMV-Tax (1 μg) with either wild type peptide (lane 3) or LAALS peptide (lane 4) at 10 μM. Percent activity represents acetylated product over total ^{14}C counts in each lanes. Panel D represents trypan blue exclusion assay from cells transfected with 10 μM of each peptide for 72 h. Peptide p44 was used as positive control since it is known to modulate the p53 pathway and drive cells to apoptosis (Jalota-Badhwar et al., 2007).

reaction mixture was incubated at 37 °C for 1 h. Finally, reactions were terminated by the addition of SDS-loading buffer and loaded on a 4–20% SDS/PAGE. Kinase activity was detected in the positive control (Lane 2) and not the negative pulldown (Lane 1) in Fig. 3. Addition of the peptides had varying effects on kinase activity. The lowest levels of kinase activity can be seen where peptides LAALS and TAACS (Lanes 5 and 7) were added to the reaction as competitors. This indicates that the peptides were able to possibly dissociate the cdk2/Cyclin E complex enough to significantly inhibit the catalytic activity on Histone H1. It is important to note, however, that none of the peptides ever completely dissociated the complex so that there was always some residual kinase activity associated with the cdk2 in our assays. Collectively, these data indicate that both LAALS and TAACS are able to inhibit cdk2 kinase activity *in vitro*.

Cell cycle analysis of cells in the presence of inhibitory Tat peptides

Results from Fig. 3 indicated that there is high possibility that the Tat peptides could dissociate the cdk2/Cyclin E complex *in vitro* and therefore might interfere with cell cycle progression *in vivo*. To address the possible cytotoxicity effects of these Tat peptides, we utilized electroporation of peptides into CEM cells and cultured the cells for 4 days. Treated CEM cells were then harvested, stained for cell cycle, and analyzed by flow cytometry (Fig. 4). Similar concentrations of the peptides used in the kinase assay were used in electroporation transfection assays. Interestingly, there is no real inhibitory effect on cell cycle progression of these peptides in CEM cells as indicated by similar populations of cells at G1, S, or G2/M phases. None of the peptides induced a significant amount of apoptosis or build up in any one cell cycle stage. Therefore, the Tat peptides that compete and inhibit kinase activity of the cdk2 *in vitro* are not toxic in an *in vivo* functional assay.

Effect of Tat peptide inhibitor on HIV-1 transcription

Tat utilizes a number of complexes including cdk2/Cyclin E (Agbottah et al., 2006; Deng et al., 2002) to activate the HIV-1 LTR. We therefore used an initial CAT reporter assay to determine the effects of Tat peptides on Tat activation of the HIV-1 LTR. CEM cells were transfected, kept in complete media for 48 h, lysed and used for CAT assay. Results in Fig. 5A show that Tat activates the LTR promoter by more than 95% (lane 2). When using the wild type unmodified Tat peptide at 1, 5, and 10 μM no change in Tat activated transcription was seen (lanes 3–5). However, increasing concentration of LAALS peptide suppressed Tat activated transcription (lanes 6–8). When compared to the parent 41/44 mutant, the new peptide LAALS showed better overall inhibition (panel B). Using similar transfection experiments, the TAALS peptide at 5 μM inhibited activity by 50% (lane 4) whereas the same concentration of LAALS peptide inhibited the Tat activated transcription by more than 90% (lane 6). Finally, the inhibition was specific to Tat activation, since using HTLV-1 Tax and each of the peptides at high concentration (10 μM) did not inhibit Tax activated HTLV-1 promoter activity (panel C). More importantly neither of the peptides at 10 μM concentration shows any signs of toxicity in CEM cells up to 72 h (panel D). A positive control P44 peptide which reactivates p21/waf1 (Jalota-Badhwar et al., 2007) inhibited cell cycle progression and apoptosis as observed in a trypan blue assay. Therefore, these results collectively indicate that Tat peptide derivative LAALS inhibits HIV-1 activated transcription by Tat in transient transfected assays.

Effect of peptide inhibitor on HIV-1 infected latent cells

Next, we asked if the LAALS sequence was capable of suppressing integrated latent virus. We used OM10.1 cells since they carry a latent copy of HIV-1 and can be induced with the addition of TNF or other cytokines (Agbottah et al., 2006). We transfected OM10.1 cells with either TAALD or LAALS peptides (0.1, 1, 3 μM) and then treated cells with TNF-α for 2 h. Samples were kept at 37 °C for 4 days prior to RT assay. As controls, we used no peptide treatment, just TNF-α treatment or increasing concentration of cdk2 inhibitor Cyc202. Results are shown in Fig. 6A where increasing concentration of LAALS, but not TAALD, resulted in more than 90% suppression of HIV-1 in a concentration dependent manner (lanes 6–8). Importantly, these peptides had no appreciable toxicity effect even up to 5 μM in a trypan blue assay in CEM cells after 4 days (panel B). As expected, positive control reagents such as a Nocodazole or Hydroxyurea treatment of the cells inhibited cell growth, ultimately leading to cell death

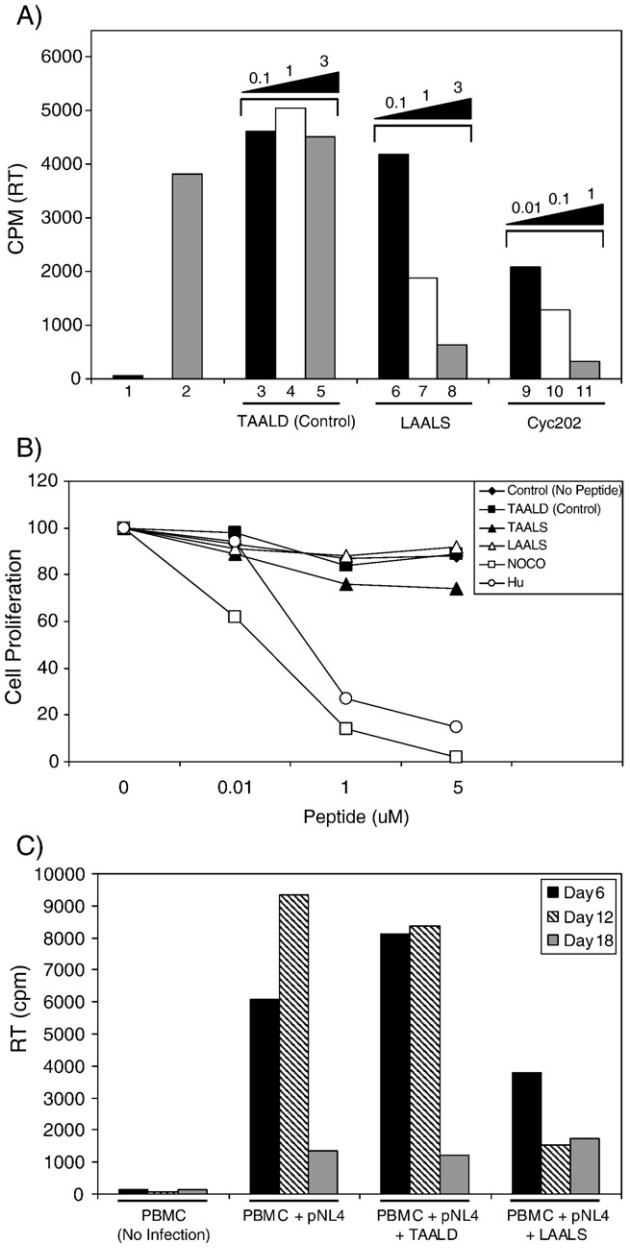

Fig. 6. Effect of peptide inhibitor on HIV-1 infected latent cells. OM10.1 cells were first transfected with either TAALD or LAALS peptides (0.1, 1, 3 μM) and then treated cells with TNF-α for 2 h. Samples were kept at 37 °C for 4 days prior to RT assay. Controls included no peptide treatment, just TNF treatment or increasing concentration of cdk inhibitor Cyc202 (panel A). Panel B represents trypan blue exclusion experiments that utilized 5 μM of each reagent for up to 4 days. Two cell cycle inhibitors namely Nocodazole (100 ng/ml) and hydroxyurea (5 mM) treatments were used as positive controls for apoptosis after 4 days. Panel C represents Activated PBMCs that were transfected with pNL4 with or without the two peptides. Cells were carried for up to 18 days and samples were collected at days 6, 12, and 18 for RT assay.

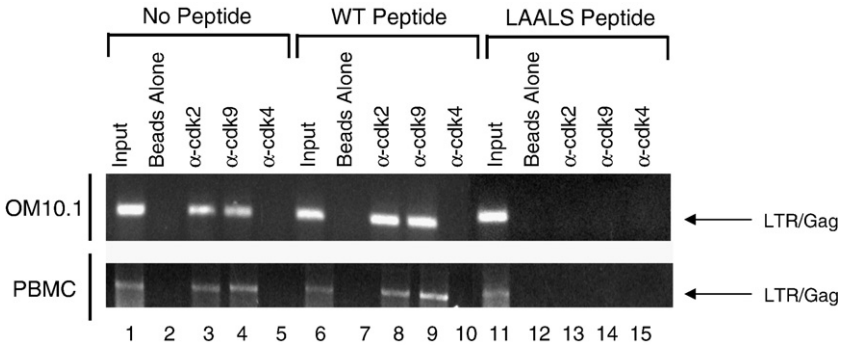

Fig. 7. Presence of various cdk complexes on HIV-1 LTR. Chromatin-immunoprecipitation (ChIP) was used to detect presence of cdk2, 4 or 9 on the HIV-1 promoter in the presence of either wild type or LAALS peptide inhibitors. Both OM10.1 (day 4) and infected PBMCs (day 12) cells were processed for ChIP assay. Specific anti-cdk antibodies (10 μg/reaction) were used to immunoprecipitate the chromosomal DNA followed by PCR for presence of LTR/Gag sequence. Lane 1 is the input DNA prior to IP, and lane 2 serves as negative control without antibody treatment. Lanes 3, 8 and 13 are PCR products from cdk2 IP; lanes 4, 9, and 14 are from cdk9 IP; and lanes 5, 10 and 15 are from cdk4 IP.

(panel B). Finally, we asked if these peptides could have an inhibitory effect on HIV-1 in PBMC infected cells. Activated PBMCs were transfected with pNL4 with or without the two peptides. As seen in panel C, infection resulted in active viral production by Day 6 and 12. Most cells did not grow or support viral replication by Day 18. Importantly, the LAALS (1 μM), but not TAALD, peptide was capable of suppressing viral activity in these PBMC infected cells. Collectively, these results suggest that the LAALS peptide is capable of inhibiting HIV propagation in both cell lines and primary cells.

Possible dissociation of cdk from HIV-1 promoter in peptide treated cells

We have previously shown that one mechanism of peptide inhibition in HIV-infected cells is to physically dissociate cdk away

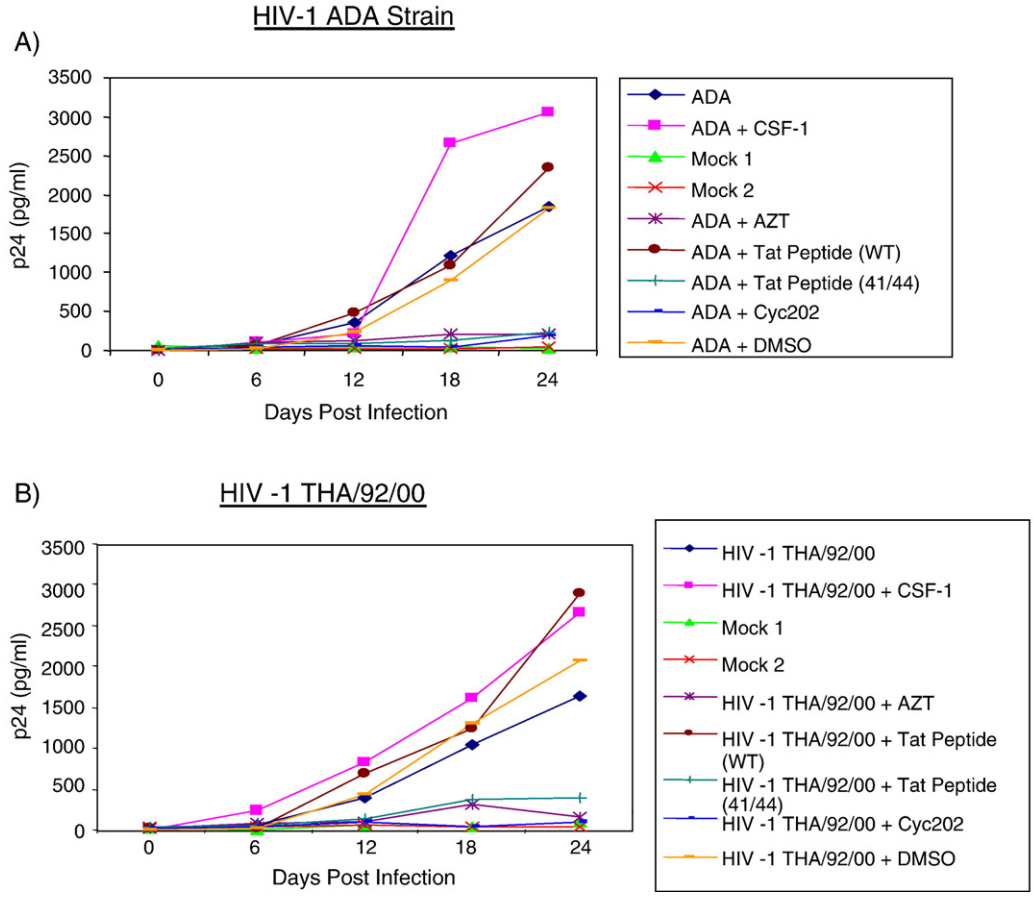

Fig. 8. Replication of HIV-1 ADA and THA primary isolate in PBMCs isolated from Rag2$^{-/-}\gamma_c^{-/-}$ mice. Peripheral blood samples from sixteen week animals were collected, washed and plated in a 96 well plate for viral infection. Cells were cultured at in RPMI 1640 medium containing FBS and treated with a mixture of streptomycin and penicillin antibiotics and L-glutamine (Invitrogen). Two strains of virus were used for infection. HIV-1_{ADA} a macrophage-tropic CCR5-using (R5) strains and THA/92/001, an NSI primary strain were used for infections. Samples were either treated with CSF-1, AZT, wild type or mutant Tat peptides, or Cyc202. Mock 1 was from animals that did receive stem cells but no virus, and Mock 2 was from animals that did not receive stem cells but virus added *in vitro*. Supernatants were subsequently collected and used for the p24 assay.

from the LTR promoter (Agbottah et al., 2006). We used chromatin immunoprecipitation (ChIP) assays to define molecules that are present or absent on the HIV promoter after peptide treatment. We therefore decided to perform similar experiments in the presence of the newly modified peptide. Both OM10.1 (Day 4) and infected PBMCs (Day 12) were cross linked and used for ChIP with anti-cdk2, anti-cdk9, and anti-cdk4 (control) antibodies. Following the ChIP assays, the recovered DNA was used in PCR with primers that span the LTR/Gag region (Agbottah et al., 2006). Results in Fig. 7 indicate that when cells were treated with no peptides or the control peptide (0.1 μM), both cdk2 and cdk9 were present on the HIV promoter region in both cell line and PBMC infected cells (lanes 3, 4, 8 and 9). Importantly, both cdk2 and cdk9 were dissociated from the HIV promoter when using LAALS (0.1 μM) peptide (lanes 13 and 14). Negative control cdk4 was not present on the HIV promoter (lanes 5, 10 and 15). Collectively, these results point to dissociation of the cdk2 and cdk9 away from the HIV promoter in peptide treated cells.

Effect of peptide inhibitors on HIV-1 RNA production in a newly developed mouse model

We have developed a mouse model where human cord blood stem cells are implanted into immunodeficient mice and allowed to re-constitute an immune system *in vivo*. The double-mutant mice with deleted recombinase activating gene 2 (RAG)-2 and common cytokine receptor gamma chain ($Rag2^{-/-}\gamma c^{-/-}$) has been seen by us and others to be a viable option in reconstitution experiments. These $Rag2^{-/-}\gamma c^{-/-}$ mice exhibit an alymphoid phenotype with no spontaneous tumor formation and can be implanted with hematopoietic stem cells that can be further enhanced by administration of exogenous human cytokines (Kosco-Vilbois, 2004). A number of recent publications using this strain as well as reconstitution in NOD/SCID mice with human stem cells show a very promising path for a feasible HIV/AIDS animal model (Baenziger et al., 2006; Berges et al., 2006; Gorantla et al., 2007; Watanabe et al., 2007a,b; Zhang et al., 2007). In our preliminary experiments, we found that mice tolerated a sub-lethal dose of 3.5 Gy (to destroy any residual endogenous immune system) and despite infertility, transplanted and non-transplanted animals showed no obvious defects compared with non-irradiated controls. We first reconstituted newborn $Rag2^{-/-}\gamma c^{-/-}$ mice intrahepatically (*i.h.*) with $CD34^+$ cord blood cells and subsequently analyzed between four and twenty-six weeks of age for presence of human $CD45^+$ hematopoietic cells. An increase in splenic and thymic cellularity was detectable, and as expected all mice beyond eight weeks developed mesenteric lymph nodes (Traggiai et al., 2004). The spleen, lymph node, and blood $CD19^+$ cells expressed the IgM antigens. Mice beyond eight weeks developed mesenteric lymph nodes, and showed enlarged spleens whereas lymph nodes and enlarged spleens were never detected in non-transplanted controls (data not shown).

We next asked whether cells from the transplanted animals could indeed allow replication of HIV-1 *in vitro*. PBMCs from implanted animals were used for *in vitro* infection of HIV-1 using the lab adapted macrophage-tropic strain ADA and a primary isolate called THA/92/00. This particular primary strain has previously been used by us for infection of human PBMCs *in vitro* (Agbottah et al., 2005). Sixteen week peripheral blood samples were initially collected, processed to lyse red cells and stored in liquid N_2. After confirmation that these animals contained human hematopoietic cells, frozen mononuclear cells (5×10^5) were thawed, washed and plated in a 96 well plate for viral infection. Cells were cultured in RPMI medium containing FBS and treated with a mixture of streptomycin and penicillin antibiotics and L-glutamine. Infected cells were washed and subsequently treated with either wild type or mutant Tat peptides (experimental), AZT, or Cyc202 (controls). Supernatant samples were collected for the presence of Gag p24 for up to 24 days. As a control, cells were allowed to differentiate into mature macrophages in the presence of CSF-1 (M-CSF). Figs. 8A and B shows that ADA could effectively replicate in these cells and that CSF-1 can slightly increase the viral titers in cells obtained from animals. Also, these three different inhibitors including Tat peptide (our initial 41/44 peptide), AZT and Cyc202 inhibited virus replication when infecting with the ADA or the THA strain. The effects of Tat peptide inhibitors and Cyc202 have previously been shown by us (Agbottah et al., 2005). Collectively, these results imply that transplantation of human $CD34^+$ cells into $Rag2^{-/-}\gamma_c^{-/-}$ mice allows differentiation of cells into competent human cells necessary for HIV-1 infection *in vitro* and the virus can be inhibited with Tat peptides or drugs that target pre- or post-integration events.

Inhibition of HIV-1 RNA production with Tat peptide derivative in transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice in vivo

To address whether HIV-1 could indeed replicate in these animals, we used the ADA viral stock with ~100,000 TCID50/ml for our initial infections into animals. Animals received virus alone and/or ~1000 μg/ml of AZT at the time of injection. A volume of 0.3 ml was injected into the intra-peritoneal region of anesthetized $Rag2^{-/-}\gamma_c^{-/-}$ mice with a blunt-tipped 30-gauge needle. Animals were infected twelve weeks post-transplantation. Animals that received HIV-1 and stem cells alone (Table 1; # 4, 5 and 6), showed a presence of viral RNA and integrated DNA. Animals treated with inhibitors including AZT and Tat peptide 41/44 showed a dramatic drop in viral RNA (Table 1; #7, 14 and 15). Both animals #3 and #20 served as negative controls and showed no HIV-1 RNA or DNA in these animals. We also observed similar inhibitory effects using LAALS peptide (animals #18 and 19). It is important to note that, although we were not able to successfully detect significant p24 levels in the serum of these animals, we co-cultivated (1:10 ratio) the infected PBMCs with human monocytes (U937 and THP) and human T-cells (CEM, H9, Jurkat) cells *in vitro* and found varying ng/ml quantities of HIV-1 Gag p24 in the supernatants of both monocytes and T-cells (data not shown).

To further confirm that the human stem cell transplantation could indeed support HIV-1 ADA growth and inhibition *in vivo*, and that these results were not an artifact of using this particular animal strain, we decided to use another strain of mice for similar subsequent studies. NOD/SCID mice (generous gift of Dr. Leonard Schultz, the Jackson Lab) were irradiated three weeks after birth and transplanted

Table 1
Effect of HIV inhibitors on *in vivo* replication of HIV-1 ADA in mice

Mouse ID no.	HIV-infected (ADA)	Treatment	Viral RNA copies	Viral DNA copies
3*	+	–	<100	<100
4*	+	–	1,237,925	855,109
5*	+	–	836,676	401,664
6*	+	–	574,730	988,111
7*	+	AZT	14,379	168,450
8^	+	AZT	26,892	190,347
9^	+	AZT	80,587	177,420
10*	+	Peptide (WT)	935,250	804,510
11*	+	Peptide (WT)	743,099	938,553
12^	+	Peptide (WT)	1,359,688	1,209,553
13^	+	Peptide (WT)	1,690,319	1,857,336
14*	+	Peptide (41/44)	58,477	640,653
15*	+	Peptide (41/44)	28,105	750,772
16^	+	Peptide (41/44)	103,266	1,800,841
17^	+	Peptide (41/44)	87,392	1,736,217
18*	+	Peptide (LAALS)	36,921	1,548,770
19*	+	Peptide (LAALS)	48,750	1,254,929
20*	–	–	<100	<100
21^	–	–	<100	<100

Animal #3 did not receive any human stem cells, and animals #16 and 17 did not receive HIV, but RPMI media as mock. (*) denotes Rag/γc animals and (^) denotes NOD/SCID animals.

with stem cells and treated either with AZT or peptide inhibitors. Results of these studies showed a consistent pattern of viral inhibition in AZT and Tat peptide treated animals (Table 1, #16 and 17). Interestingly, even though the transplantation efficiency at the time of cell and serum harvest was fairly high (27–61%) for these animals, still no reproducible levels of Gag p24 antigen could be detected in the serum of these animals after two weeks. However, both cell associated HIV-1 DNA and RNA was detectable in these animals (Table 1).

Discussion

The development of alternative antiretroviral therapies is crucial to the progression of HIV-1 treatment. Currently, this therapy involves the use of agents from distinct classes of antivirals: protease inhibitors, transcriptase inhibitors, fusion inhibitors, and integrase inhibitors. Recently, compounds interacting with Tat/TAR (or siRNA against Tat) have also been studied which inhibit HIV replication in a low micromolar range (Ammosova et al., 2006; Christensen et al., 2007; Mukerjee et al., 2007). However, most current antiretroviral therapies result in more drug resistant viral strains, poor inhibition, and low efficiency.

Pharmacological cdk inhibitors (PCIs) are an attractive concept for the next phase of antiretroviral therapies. Drugs that would target various cdk/Cyclin complexes which are vital in controlling viral transcription, replication, and progeny formation may allow for the inhibition of virally-induced cell cycle progression and result in the inhibition of viral transcription and replication. Artificial peptide inhibitors have been developed, and have shown selectivity for cdks through binding near the active site, however, in depth knowledge of both substrate and kinase is needed in order to be effective.

Previously, we have shown success in mimicking the core domain of the HIV-1 viral protein Tat sequence, and making simple alterations in order to impart an inhibitory effect on HIV-1 gene expression (Agbottah et al., 2006). The 41/44 peptide analog was found to act upon the cdk2/Cyclin E interface, which through a series of biochemical assays, was determined to be present on the HIV-1 promoter. In previous studies, a model for the interaction of cdk2/Cyclin E on the viral promoter was established where in the presence of TAR, Tat regulates this complex and is essential in phosphorylating transcriptional machinery, including the RNAPII CTD. A model for the peptide analog induced inhibition was also established, where the binding of the peptide to cdk2 causes it to dissociate from the Cyclin subunit, therefore inactivating the kinase and preventing activation of transcription factors through phosphorylation. Here, we continued these studies including additional peptides with varying efficacies for cdk2 inhibition and evaluate the inhibitory effects into an *in vivo* model.

In this study, the initial Tat 41/44 double-mutant, TAALS, was modified to include one additional point mutation to generate the peptides indicated in Fig. 1. Given the computationally derived binding affinities, the initial inhibition assay performed was meant to test the *in vitro* inhibitory effects of each peptide. The cdk2/Cyclin E IP Western data in Fig. 2 indicated that peptides TAACS and LAALS showed the highest degree of possible dissociation, and subsequently the greatest inhibition of the kinase activity (Fig. 3). This *in vitro* data suggests that not only are there varying degrees of specificity of each peptide for binding to cdk2, as predicted, but the dissociation seen is also indicative of loss of kinase activity. Interestingly, the two peptides shown to dissociate the complex most successfully did not have the highest predicted binding affinity energy as compared to all the predicted ligands. It is also important to mention that the two peptides specifically bound to the unphosphorylated form of cdk2, implying that the phosphorylated subunit has an induced conformational change, therefore closing the ligand binding site. These *in vitro* studies were carried out using immunoprecipitated complexes, and it is important to note that we have never observed a complete dissociation of the complex when using these Tat peptides. We transfected these inhibitory peptides into proliferating cells to monitor the level of apoptosis induced by inhibition of the cdk2/Cyclin E complex. Complementary concentrations of inhibitors were used in the cell cycle analysis as in the *in vitro* activity studies, and consequently proved that the amounts of peptides needed to inhibit the complex for HIV-1 transcription were not toxic to the cells.

The inhibitory peptide LAALS has thus far been the most adept at dissociating and inhibiting cdk2/Cyclin E as well as conferring little toxic effect on non-infected cells. We tested LAALS inhibitory effects on a simulated viral promoter. The effectiveness of LAALS in suppressing transcription of the HIV-1 LTR was monitored in Fig. 5. Increasing concentrations of LAALS in the presence of Tat resulted in a dramatic decrease in the LTR-CAT reporter activity as compared to the wild type peptide. Fig. 5C indicates that the activity of LAALS is specific for the HIV-1 promoter and not for HTLV (in the presence of Tax).

We tested the efficacy of LAALS in inhibiting viral replication in a latently infected cell line. Results in OM10.1 cells showed a decrease in viral replication correlating with an increase in concentration of LAALS treatment. This treatment exhibited an effect comparable to the well known cdk inhibitor drug Cyc202, and more importantly, the control peptide TAALD did not result in reduced viral replication. Again, cell proliferation was still not affected by this treatment. Fig. 6C further confirmed the drop in viral load by looking at the treatment of infected PBMCs with LAALS and a control peptide. The PBMCs treated with LAALS showed an overall decrease in viral load as compared to untreated and control treated cells.

The data thus far has indicated and implied a change in protein–protein interactions resulting in a lower amount of viral transcription in the presence of the peptide LAALS. This has been inferred by activity levels at the viral LTR as well as monitoring the levels of virus present.

It is well known that key cellular factors (i.e., NF-kB, Sp1 and cdk9) interact with the viral LTR resulting in Tat activated transcription which can be detected using ChIP assays. Fig. 7 shows a ChIP assay where latently infected cells and PBMC infected cells were either untreated, treated with control or LAALS peptides and screened for the presence of cdk2, cdk9, and cdk4 at the LTR. No changes were seen when cells were treated with the wild type peptide, however, in the presence of LAALS, there was no pulldown of cdk2 or cdk9 in either latent cells or PBMCs. Loss of cdk9 from the LTR in the presence of LAALS is very interesting and may prove to be significant. It is possible that the inhibition of cdk2/Cyclin E prevents a phosphorylation event which is otherwise necessary for the recruitment/modification of p-TEFb or that the peptide has some homology to the cdk9/Cyclin T interface site. Along these lines we have recently found that cdk9/Cyclin T is a substrate for cdk2/Cyclin E complex and may in fact enhance the auto-phosphorylation of cdk9 that is normally seen prior to Tat activated transcription (S. Nekhai and F. Kashanchi, unpublished data).

Finally, the current work utilizes a novel animal model that uses reconstitution of the human immune system when implanting irradiated mice with human $CD34^+$ stem cells. A previous pioneering report by the Manz group has indicated that the human T-cells were observed in mouse secondary lymphoid organs which underwent a number of post-thymic cell divisions as measured by TCR-rearrangement excision circles (TRECs) (Traggiai et al., 2004). Inspired by these results, and more recent similar publications (Baenziger et al., 2006; Berges et al., 2006; Gorantla et al., 2007; Watanabe et al., 2007a,b; Zhang et al., 2007) we injected $CD34^+$ cord blood cells into sub-lethally irradiated newborn $Rag2^{-/-}\gamma_c^{-/-}$ mice and observed a significant number of human $CD45^+$ hematopoietic cells when using FACS analysis. Our attempts to infect these cells *ex-vivo* and *in vivo* were successful using either T- or lab adapted macrophage-tropic viruses. When using the ADA and THA/92/00 viruses, we observed a healthy infection *in vitro* and were able to follow virus replication up to 24 days. These results were encouraging as they indicated that the *in vivo*

differentiated human cells could in fact be infected through normal HIV receptor/co-receptor pathway. However, to date we have not expanded our virus repertoire and do not know if virus replication is unique to these particular strains or can they be infected by a broad range of HIV-1 clades. Future studies will determine the efficiency of infection, level of pathogenesis, and whether certain viruses can replicate better in the *ex-vivo* system as compared to the *in vivo* model.

We were also encouraged by the inhibitor studies using AZT, Cyc202, and Tat peptide inhibitors as these compounds reduced detectable virus in the mouse model. Our *in vivo* infection studies in the transplanted animals are the most exciting results. Our initial injection of HIV or HIV + AZT utilized an *i.p.* route and seems to facilitate viral entry into cells. As such, we were able to observe HIV infection in these animals and a marked decrease of Gag DNA and RNA in AZT treated cells, indicating that the virus may be able to enter the cells and go through the RT process followed by integration and subsequent transcription. However, many factors, including better titration of virus, peptides, and drugs, will have to be performed to obtain valid viral kinetic data *in vivo*. Finally, we were not able to successfully detect p24 antigen levels in the serum of these animals; however upon co-cultivation of infected cells, we were able to observe ng/ml quantities of HIV-1 Gag in a tissue culture setting (data not shown). We believe that this may be due to many reasons including possible low viral production *in vivo*, rapid clearance of the virus within 24–48 h of release, possible accumulation of virus in specific organs as opposed to PBMCs, few rounds of viral replication, possible depletion of human T- and monocytic cells, or differing viral replication kinetics between tissue culture isolates and primary virus isolated from patients. Ongoing future experiments will clearly define the limits of this animal model and will determine the extent that the model could be used to study peptide inhibitors, drugs, siRNA, or vaccine candidates.

Materials and methods

Computational modeling

Computational docking studies were carried out as previously described by Agbottah et al. (2006). We took advantage of the existing crystal structure of cdk2 to mimic binding of each of the Tat peptide analogs to the cdk/Cyclin binding site. The cdk2 structure used was obtained from the Protein Data Bank ID 1FIN (resolution 1.85 A°). Docking was performed using the software AutoDock 3.0 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 TAALS. The annealing temperature was reduced with a geometric scheme, and each docking run took about 2 h on a 2.8 GHz processor. 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 ligand atoms (this assists 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. To further exploit this mechanism of cdk inhibition, we screened for other peptides that can potentially target this pocket more effectively with stronger binding affinities. To this end, we performed molecular docking simulations for all 95 possible single mutants of TAALS while restricting the allowed configurational space of these peptides to the local region around this new binding pocket of cdk2 by TAALS. The simulations resulted in 15 mutant variants of TAALS that have stronger binding affinities than that of TAALS, the top five of which are listed in Fig. 1 and are selected for further experimental studies.

Peptide synthesis

All peptides used for this study were commercially synthesized (SynBioSci, Livermore, CA) with the following sequences:

NH_2-T-A-A-L-D-**OH**
NH_2-T-A-A-L-E-**OH**
NH_2-L-A-A-L-S-**OH**
NH_2-T-A-A-C-S-**OH**
NH_2-F-A-A-L-S-**OH**
NH_2-T-A-A-L-S-**OH**

The purity of each peptide was analyzed by HPLC to greater than 98%. Mass spectral analysis was also performed to confirm the identity of each peptide as compared to the theoretical mass (Applied Biosystems Voyager System 1042). Peptides were resuspended in dH_2O to a concentration of 1 mg/ml and stored at −70C. Peptides were only thawed once prior to use for biochemical or *in vivo* experiments.

Cell culture

C81 is an HTLV-1-infected T-cell line that expresses Tax protein, and CEM (12D7) is an uninfected human T-cell line established from patients with T-cell leukemia. C81 and CEM cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (Quality Biological) and were incubated in a 5% CO_2 incubator at 37 °C. Cells were cultured to confluency and pelleted at 4 °C for 15 min at 3,000 rpm. The cell pellets were washed twice with 25 ml of phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (Quality Biological) and centrifuged once more. Cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM DTT, one complete protease cocktail tablet/50 ml) and incubated on ice for 20 min, with a gentle vortexing every 5 min. Cell lysates were transferred to Eppendorf tubes and were centrifuged at 10,000 rpm for 10 min. Supernatants were transferred to a fresh tube where protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Size-exclusion chromatography

C81 cell lysate (30 mg/ml) was fractionated on a Superose 6 HR 10/30 column (Amersham Biosciences, Piscataway, NJ) in Buffer D (20 mM HEPES (pH 7.9), 0.05 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.05 DTT, and 20% Glycerol). Flow-through was collected at 0.5 ml for 50 fractions. Every 10th fraction was analyzed by immunoblotting for cdk2 in order to determine the elution location of the cdk2/Cyclin E complex.

Immunoprecipitation

Cdk2 containing chromatography fractions (28–32) were pooled together for immunoprecipitation. The pooled C81 extracts (250 μg each) were combined with 10 μg/μl of each respective peptide. Cyclin E antibody (Santa Cruz, sc-198) was added to each reaction tube (10 μl, 2 μg), the reaction mixture was brought up to 500 μl with TNE_{50} + 0.1% NP-40 (100 mM Tris, pH 8.0; 50 mM NaCl; 1 mM EDTA, 0.1% Nonidet P-40) and was allowed to incubate while rotating overnight at 4 °C. α-IgG was added to extract as a negative control, and an IP was performed in the absence of competing peptide, acting as a positive control. The following day, 30 μl of a 30% Protein A & G bead slurry (CalBioChem, La Jolla, CA) was added to each reaction tube and allowed to incubate while rotating for 2 h at 4 °C. Samples were spun and washed 2× with TNE_{300} + 0.1% NP-40 (100 mM Tris, pH 8.0; 300 mM NaCl; 1 mM EDTA, 0.1% Nonidet P-40) and 1× with TNE_{50} + 0.1% NP-40 to remove non-specifically bound proteins. 2× Laemmli buffer was added to each sample and heated at 95 °C for

3 min. Samples were loaded and run on a 4–20% Tris–Glycine SDS/PAGE gel to be used for both Western blots and kinase assays.

Cdk2 and Cyclin E were purified from lysates of SF9 insect cells infected with baculoviruses producing cdk2 and Cyclin E. Briefly, 1 ml of cells (from 250 ml of culture) was lysed with 16 ml of lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM 2-mercaptoethanol, 10% glycerol and PMSF), homogenized on ice and centrifuged at 45,000 ×g for 1 h at 4 °C. Supernatant was loaded on Mono-Q 10/10 column (Amersham, USA). Two separate cell cultures, one infected with cdk2-expressing baculovirus and the other one infected with Cyclin E-expressing baculovirus were used for purification. The Mono-Q fractions containing cdk2 or Cyclin E were mixed 1:1 and loaded onto Superdex column (Sephadex H200, Amersham, USA). Purity of cdk2/Cyclin E was checked on 12% PAGE followed by Coomassie staining. We also analyzed the Superdex fractions by immunoblotting with anti-cdk2 antibodies and assayed their enzymatic activity using histone H1 and purified Tat proteins as substrates. Fractions containing cdk2/Cyclin E were concentrated using Microcon tubes (Amicon, USA).

Western blots

Immunoprecipitated Cyclin E samples in the presence of Tat peptides were probed for cdk2. IP samples separated on SDS/PAGE gels were transferred to a nitrocellulose membrane via a constant current of 70 mA overnight. The membrane was blocked with a 3% BSA solution in PBS containing 0.1% Tween-20, rocking for 2 h at 4 °C. A 1:1000 dilution of α-cdk2 antibody (Santa Cruz, sc-163) was added to the blocking solution and incubated rocking overnight at 4 °C. The membrane was washed with a fresh PBS+0.1% Tween-20 solution in order to wash off any residual primary antibody solution. A 1:1000 dilution of α-rabbit secondary antibody was added to a fresh 3% BSA solution in PBS+0.1% Tween-20 and incubated with the membrane, rocking for 2 h at 4 °C. The membrane was washed 2× with PBS+0.1% Tween-20 and 1× with PBS to remove any residual antibody. The membrane was exposed to chemiluminescence reagent (Pierce) in the dark for 5 min., and was developed using a Kodak Imager.

GST-cdk binding assay to peptide in vitro

GST-cdk2, cdk4, cdk9 and GST alone were expressed and purified twice over Glutathione Sepharose beads. The elution and re-binding dropped the non-specific background significantly. Peptides (50 μg) were allowed to bind various purified bead-GST proteins (2 μg) overnight at 4 °C. Next day they were washed with TNE50+0.01% NP-40 and eluted with 20 μl of elution buffer (TNE50+0.01% NP-40+0.01% SDS). Following centrifugation eluates were used for further analysis. Eluted peptide samples were combined with MS washing buffer (50:50 Methanol: Water+0.1% TFA) and loaded onto a Hamilton Gastight 250 μl syringe. Samples were manually injected into an ESI-MS LCQ Deca XP^{Plus} Thermo Finnigan mass spectrometer at 10 μl/min and read through an API source. Spectra were normalized to the highest abundant mass peak and were averaged over the course of injection. Data acquisition and peak analysis were performed through the Xcalibur software platforms. Data is shown zoomed in on relevant m/z ranges.

Kinase assay

Immunoprecipitated Cyclin E samples in the presence of Tat peptides were assessed for kinase activity. After the final TNE_{50}+0.1% NP-40 wash, beads were washed with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 50 mM NaF, 0.2 mM Na_3VO_4 and one complete tablet of protease cocktail inhibitor/50 ml buffer) to equilibrate the reaction. The indicated amount of Histone H1 was added to each reaction tube along with the indicated amount of (γ-^{32}P) ATP (3000 Ci/mmol). Reactions were incubated at 37 °C for 30 min and stopped by the addition of 15 μl Laemmli buffer. The samples were separated by reducing SDS-PAGE on a 4-20% Tris–Glycine gel. Gels were stained with Coomassie blue, destained, and then dried for 2 h. Following drying, the gels were exposed to a PhosphorImager cassette and analyzed utilizing Molecular Dynamic's ImageQuant Software.

Flow cytometry

Cell cycle analysis was performed on CEM cells either untreated or treated with WT or Tat derived peptides. Cells were collected by low speed centrifugation and washed with PBS without Ca^{2+} and Mg^{2+} and fixed with 70% ethanol. For fluorescence-activated cell sorting (FACS) analysis, cells were stained with a mixture of propidium iodide (PI) solution (PBS with Ca^{2+} and Mg^{2+}, 10 μg/ml RNase A, 0.1% Nonidet P-40, and 50 μg/ml PI) for 30 min at room temperature prior to analysis. The cells were acquired and analyzed using CELLQuest software (BD Biosciences). Acquired FACS data were analyzed by ModFit LT software (Verity Software House, Inc.).

CAT assay

Wild type LTR constructs from both HIV and HTLV-1 were electroporated into CEM cells. Extracts were prepared for chloramphenicol acetyltransferase (CAT) assay. The cells were harvested, washed once with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, pelleted, and resuspended in 150 μl of 0.25 M Tris (pH 7.8). The cells were freeze-thawed three times with vortexing and then incubated for 5 min at 68 °C followed by centrifugation. The supernatants were transferred to 1.5-ml Eppendorf tubes. After one final spin, the supernatant was again transferred to 1.5-ml Eppendorf tubes, and the protein concentration was determined. CAT assays were performed with 25 μg of protein as described in Kashanchi et al., 2000.

ChIP assays

Ten million OM10.1 cells in log phase were first electroporated with peptides and then incubated for 2 h with or 5 μg/ml TNF-α to induce transcription of latent proviral DNA. After 4 days, cells were cross linked (1% formaldehyde, 10 min at 37 °C) and samples were sonicated to reduce DNA fragments to 200–800 nt lengths for ChIP assays. Infected PBMCs (Day 12) were also treated for ChIP analysis. Specific cdk complexes were immunoprecipitated with appropriate antibodies (10 μg/reaction). DNA sequences in the immunoprecipitates were detected by PCR using primers specific for the HIV-1 LTR/Gag (Agbottah et al., 2006).

Mice

A set of male and female Rag2$^{-/-}\gamma_c^{-/-}$ mice were graciously donated by Dr. Anton Berns (The Netherlands Cancer Institute). Subsequent experiments utilized similar animals obtained from Dr. Ramesh Akkina (Colorado State University). Both sets of animal have previously been described and are identical to animals described in the Traggiai et al. manuscript (Traggiai et al., 2004). A breeding colony was successfully established at the GWUMC. All procedures and practices associated with the use of Rag2$^{-/-}\gamma_c^{-/-}$ mice was been approved by the GWUMC Committee on Human Research and Committee on Animal Research (IACUCC #007-4,5A1 and animal assurance #A-3205-01). The GWUMC Committee on Animal Research was charged with carrying out the regulations of the federal government's Animal Welfare Act governing the care and use of animals in research and instruction. The animals are housed in an AAALAC-approved vivarium GWUMC (BSL3 facility). Animal housing consisted of a Techniplast caging system and all changes were completed in a biosafety hood. Bedding, food and water were all autoclaved. Animals in the vivarium were monitored almost daily and project protocols were reviewed and approved by the University

Animal Use Care Committee. Data in Table 1 are also from Balb/c Rag2$^{-/-}\gamma_c^{-/-}$ animals.

The NOD/SCID mice (NOD.Cg-Prkdc^scid Il2rg^tm1Wjl/SzJ; Stock Number: 005557) were kindly donated by Dr. Leonard Schultz from the Jackson Laboratory Animal Resource Department. These mice were maintained in an AAALAC-approved vivarium housed within GWUMC's BSL-3 suite at all times. Ten three week old NOD/SCID pups were sub-lethally irradiated at a dose of 3.5 Gy using a CS-137 irradiator. Twenty-four hours later, the mice were given a 100 μl intraperitoneal (IP) injection of 100,000 human CD34$^+$ cord blood stem cells. These stem cells were allowed to differentiate for three weeks followed by infection with HIV-1. Each animal was given an IP injection of virus, virus plus drug, peptides or RPMI media. Two weeks after infection, the mice were sacrificed and blood was collected through cardiac puncture for further DNA and RNA analysis.

Human Stem cells

CD34 cells were either obtained from Cambrex Bio Science Walkersville, Inc. (Gaithersburg, MD) or prepared by us using the following method. Cells were separated using the CD34 MicroBead Kit (Miltenyi Biotec, Auburn, CA). This kit formerly termed Direct CD34 Progenitor Cell Isolation Kit is a single-step labeling system which allows for a fairly fast and easy isolation of CD34$^+$ cells. By using direct MicroBeads instead of an indirect labeling system, the washing step during the labeling procedure is abolished and resulting cell loss is avoided. CD34$^+$ cells were isolated from pooled cord blood samples. The CD34 MicroBead Kit contained FcR blocking reagent and MicroBeads which were conjugated to the monoclonal mouse anti-human CD34 antibody, QBEND/10. The CD34 antigen is a single transmembrane glycoprotein which is expressed on human hematopoietic progenitor cells and most endothelial cells. Fluorescent control staining of magnetically labeled cells requires a monoclonal CD34 antibody recognizing an epitope other than QBEND/10, e.g. clone AC136. All cells were washed with PBS prior to implantation.

FACS analysis for cells from animal studies

Samples (i.e, twenty-four weeks post-transplantation) were collected from euthanized animals and blood, thymus, bone marrow, mesenteric lymph nodes and spleen were collected from both transplanted and non-transplanted animals. In most cases to obtain peripheral blood cells, mice were bled from retro orbital venous sinus under anesthesia. After euthanasia, single cell suspensions were prepared from various organs and red cells were lysed in cold ACK lysis buffer (NH4Cl 8.29 g/l, KHCO3 1.00 g/l, disodium EDTA 2H2O 0.0372 g/l, membrane filtered, pH: 7.4±0.2, Osmolality: 290±5% mOsm/kg H2O). Remaining cells were then spun, washed once in 2 ml PBS and pelleted again. Samples were cross linked with 1 ml of 4% paraformaldehyde to fix cells and virus at room temperature for 10 min. Subsequently they were washed in 1 ml PBS/BSA/10% NMS (normal mouse serum), spun, washed in 100 μl PBS/BSA/NMS and added 20 μl of each antibody to cells. Samples were incubated in dark at RT, added 1 ml PBS/BSA/NMS, spun, and resuspended in 500 μl of PBS/BSA/NMS.

Lymph nodes, spleens and thymuses were cut in small pieces and homogenized on a 70 μ strainer with a syringe plunger. Cell homogenates were washed once with 30 ml of RPMI 1640 supplemented with 10% FCS, and once with RPMI 1640 alone, centrifuged and stained with antibodies for flow cytometric analysis. The following surface markers were used: CD45–FITC, CD3–FITC, CD19–FITC, CD34–FITC and CD14-APC and CD33-Cy5. Antibodies were purchased from e-Bioscience except for CD34 (BD Bioscience, San Jose, CA). These antibodies were chosen to identify subsets of hematopoetic cells that may have derived from the transplant. Cells were analyzed using a FACScalibur (Becton Dickinson Immunocytometry Systems). At least 200,000 cells were acquired from each sample for FACS analysis.

In vitro infection of human cells

Sixteen weeks peripheral blood samples from animals were collected, washed and platted for viral infection. After confirmation that these animals contained human hematopoietic cells, frozen mononuclear cells (5×10^5) were thawed, washed and platted in a 96 well plate for viral infection. Cells were cultured at 37 °C in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and treated with a mixture of 1% streptomycin, penicillin antibiotics, and 1% L-glutamine (Invitrogen, Carlsbad, CA). Two strains of virus were used for infection for 2 h *in vitro*. HIV-1_{ADA} is a macrophage-tropic CCR5-using (R5) strains (Jacque et al., 2002; Schmidtmayerova et al., 1997) and THA/92/001 is an NSI primary strain (Agbottah et al., 2005). ADA (2×10^5 cpm of RT activity) and THA/92/001 (Thailand strain, subtype E envelope, 5 ng of p24 Gag antigen) were used for these initial infections. Both viruses have previously been used by our group with successful infection rates. Washed cells were subsequently either treated with 2 ng/ml CSF-1 (Sigma, St. Louis, MO), AZT (100 μg/ml), wild type or mutant Tat peptides (100 nM), or Cyc202 (0.36 μM). Mock 1 was for animals that did receive stem cells but no virus, and Mock 2 was for animals that did not receive stem cells but virus added *in vitro*. Supernatants were collected and used for detection of p24 antigens (Agbottah et al., 2005).

In vivo infection of HIV-1 in Rag2$^{-/-}\gamma_c^{-/-}$ mice

Sixteen weeks post-transplantation animals were infected with ADA strain (~100,000 TCID50/ml). Various animals would receive virus alone, virus plus ~1000 μg/ml of AZT, or various peptides at 3 mg/kg using intrapertenial administration. To detect viral DNA in these animals (data not shown) four weeks later, chromosomal DNA was isolated from PBMCs and examined for presence of HIV-1 DNA. A total of 0.1, 0.01, and 0.001 mg of total DNA were used in PCRs to amplify both HIV and human HLA-specific sequences in parallel. The primers used were SK38 (59-ATA ATC CAC CTA TCC CAG TAG GAG AAAT-39) and SK39 (59-TTT GGT CCT TGT CTT ATG TCC AGA ATG C-39), which amplifies a 115-bp region of the HIV-1 *Gag* gene, and GH26 (59-GTGCTG CAG GTG TAA ACT TGT ACC AG-39) and GH27 (59-CAC GGA TCCGGT AGC AGC GGT AGA GTT G-39), which amplifies a 242-bp region of HLA-DQ-a. Mutant Gag primers included SK38 M (59-ATA ATC CAC GAC TTT GAG TAG GAG AAAT-39) and SK39M (59-TTT GGT CCT CGA TTT GAG TCC AGA ATG C-39). Amplification conditions were 1 min of denaturation at 95 °C, 2 min of annealing at 55 °C, and 3 min of extension at 72 °C for 35 cycles; followed by 7 min at 68 °C. Following PCR, samples were run on 2% agarose gels and EtBr stained for presence of DNA products.

For virological analysis from these animals, both viral RNA and DNA amplification was used as previously described (Watanabe et al., 2007a,b). Cell associated viral RNA (from 5×10^4) copy numbers were measured using a real-time quantification assay based on the TaqMan system (Applied Biosystems, Foster City, CA). Cell associated viral RNA was extracted and purified using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). This kit can be used to isolates plasma RNA, cell culture RNA, and cellular RNA. The RNA was subjected to reverse-transcription and amplification using a TaqMan one-step RT-PCR Master Mix Reagents Kit (PE Biosystems) with HIV-1 Gag consensus primers (forward, 5′-GGACA TCAAGCAGCCATGCAA-3′, and reverse, 5′-TGCT-ATGTCACTTCCCCTTGG-3′) and an HIV-1 Gag consensus TaqMan probe (FAM-5′-ACCATCAATGAGGAAGCTGCAG AA-3′-TAMRA). Probed products were quantitatively monitored by their fluorescence intensity with the ABI7300 Real-Time PCR system (PE Biosystems). To obtain control RNA for quantification, HIV-1 Gag RNA was synthesized using T7 RNA polymerase and pKS460. Viral DNA was extracted and purified

using a QIAamp DNA Mini Kit (Qiagen). Determination of HIV-1 DNA copy numbers was performed by real-time PCR assay with TaqMan Master mixture (PE Biosystems). Primers (forward, 5′-GGCTAAC-TAGGGAACCCACTG-3′; reverse, 5′ CTGCTAGAGATTTTCC ACACT-3′) and probes (FAM-5′ TAGTGTGTGCCCGTCTG TTGTGTGAC-3′-TAMRA) were designed for targeting the HIV-1 long terminal repeat region, R/U5. The viral DNA was quantified using LightCycler (Roche). Viral RNA and DNA were calculated based on the standard curve of control RNA and DNA. All assays were carried out in triplicate.

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