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Recruitment of Phosphatidylinositol 3-Kinase to CD28 Inhibits HIV Transcription by a Tat-Dependent Mechanism[1]

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Activation through the TCR and the costimulatory molecule CD28 influences the susceptibility of T cells to HIV-1 infection and regulates proviral gene expression. Signaling events initiated by CD28 that directly impact HIV-1 transcription have not been fully characterized. T cell lines expressing CD8α/28 chimeric receptors containing a mutation in tyrosine 173 to phenylalanine, which inhibits the recruitment of phosphatidylinositol 3-kinase (PI3K) to CD28, expressed higher levels of HIV-1 following T cell activation. Whereas constitutively active PI3K decreased provirus transcription, inhibiting endogenous PI3K with specific inhibitors or by overexpressing PTEN phosphatase enhanced HIV-1 expression. PI3K-dependent inhibition required the viral Tat protein and a *trans* activation response region element. Tat pull-down and coimmunoprecipitation experiments indicate that PI3K affects the formation of the Tat-associated kinase *trans*-activating complex. These studies demonstrate that PI3K negatively impacts HIV-1 transcription and that Tat activity is sensitive to T cell signaling events. *The Journal of Immunology*, 2002, 169: 254–260.

Productive infection of $CD4^+$ T cells by HIV-1 requires cell activation through the TCR and costimulatory molecules, including CD28 (1–5). CD28 signaling can serve as both a positive and negative regulator of infection and virus replication (3–9). For example, signaling through CD28 before infection prevents HIV-1 entry; however, signaling through the receptor after infection enhances virus transcription (10–12).

CD28 signaling is mediated by four tyrosine residues in its cytoplasmic tail that, when phosphorylated, recruit and activate various kinases and adapter molecules that are signaling intermediates (13). Deletions within the cytoplasmic tail and site-specific mutations have demonstrated that tyrosines at positions 188, 191, and 200 (Y188, Y191, and Y200) are necessary for the regulation of IL-2 production (3, 14), whereas the role of tyrosine 173 (Y173) remains controversial (15–21). Y173 interacts with both growth receptor-bound factor 2 (Grb-2)[3] and phosphatidylinositol 3-kinase (PI3K) (15, 20–25), with the role of the latter unclear. Whether any of the signaling events initiated by the tyrosines influences HIV-1 transcription has not yet been examined.

Signaling events initiated by the TCR and CD28 lead to increases in intracellular calcium, changes in cytoskeletal organization, and triggering of kinase cascades that can potentially target transcription factors such as NF-κB, NF-AT, AP-1, Sp1, and Ets-1 (26–31). Some of these factors are induced by CD28 signaling, and can bind sites within the HIV-1 long terminal repeat (LTR) and activate HIV-1 transcription (29, 32–34). Furthermore, the viral transcriptional *trans* activator Tat is necessary for efficient transcription. Tat binds an RNA stem loop structure in the *trans* activation response region (TAR) and recruits the Tat-associated kinase (TAK), which includes cyclin-dependent kinase 9 (Cdk9) and cyclin T1 complex to the LTR. This Tat-TAK complex phosphorylates RNA polymerase II C-terminal domain (RNA pol II CTD) activating transcriptional elongation (35–39). TAK has also been shown to be up-regulated during T cell stimulation, suggesting a possible mechanism by which CD28 enhances HIV-1 transcription (40).

Using Jurkat T cell lines expressing chimeric CD8α/28 receptors with mutations in critical tyrosines located in the cytoplasmic tail, we show that CD28 signaling is required for efficient HIV-1 transcription. However, recruitment of PI3K by Y173 inhibits HIV-1 transcription. We also show that PI3K negatively impacts HIV-1 transcription by blocking the formation of the Tat-TAK complex. These studies demonstrate that CD28-dependent signaling directly influences HIV-1 replication by targeting the activity of the viral factor Tat.

Materials and Methods

Cell lines

Jurkat clone E6-1 obtained from American Type Culture Collection (Manassas, VA) was cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2 M L-glutamine, and 0.5% Fungizone. The 293T human embryonic kidney and Chinese hamster ovary (CHO) expressing Fc receptors (CHO-Fc) (gift from I. Mellman, Yale University, New Haven, CT) were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2 M L-glutamine, and 0.5% Fungizone. Jurkat cell lines overexpressing CD8α/28 chimeric receptors 8WT, F173, and Δ167 (Fig. 1*A*) were described previously (13, 14). Expression of receptors was confirmed by Western blot (data not shown) and flow cytometry (Fig. 1*B*).

Generation of HIV-1 infectious titers and infections

Vesicular stomatitis virus glycoprotein-pseudotyped HIV-1 was generated by transfecting 293T cells with 15 μg of either T-tropic pNL4-3-Luc^+ Env^- Nef^- (HIV-*luc*) DNA (41) or pHXBnPLAP$^+$$Nef^-$ (HIV-placental alkaline phosphatase (PLAP)) (42) (obtained from National Institutes of

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[3] Abbreviations used in this paper: Grb-2, growth receptor-bound protein 2; Cdk9, cyclin-dependent kinase 9; CHO, Chinese hamster ovary; CTD, C-terminal domain; GFP, green fluorescent protein; LTR, long terminal repeat; PI3K, phosphatidylinositol 3-kinase; PLAP, placental alkaline phosphatase; pol II, polymerase II; TAK, Tat-associated kinase; TAR, *trans* activation response region.

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Health AIDS Research and Reference Reagent Program), 3 μg Rev in a Rous sarcoma virus expression construct DNA, and 3 μg LTR vesicular stomatitis virus glycoprotein DNA (43) by $CaPO_4$ transfection (44). Transfection efficiency was assessed by determining luciferase activity using the Promega luciferase kit (Madison, WI). Supernatants were collected and filtered through a 0.45-μm disc before infection. Typically, the multiplicity of infection of infectious supernatants was ~0.1–0.5. Jurkat cell lines were infected by culturing cells in the presence of virus stock for 12–24 h before replacing with fresh media. Cells were cultured for an additional 24 h before measuring luciferase activity to assess virus transcription.

CD3 and CD28 activation of T cells

Jurkat cells were washed and serum starved for 4 h before activation. CHO-Fc cells were plated at 2×10^5 cells/well in a 24-well plate, incubated 12 h to allow adherence, treated with mitomycin-C (Sigma-Aldrich, St. Louis, MO) at 10 μg/ml, and incubated in the absence of serum for 2 h before using to activate the Jurkat cell lines. A total of 1×10^6 Jurkat cells was activated by coculturing cells with the CHO-Fc and mouse anti-human CD3 (0.1 μg/ml) and/or CD28 (1.0 μg/ml) or CD8α (1.0 μg/ml) Abs (BD PharMingen, San Diego, CA). For some experiments, the inhibitors LY294002 (Biomol, Plymouth Meeting, PA) and wortmannin (Sigma-Aldrich) were added to cells at the time of stimulation. Human rIL-2 was added to some samples at concentrations ranging from 500 to 1500 pg/ml. Following 12-h stimulation, Jurkat cells were harvested and luciferase activity was measured.

Transient transfections

Jurkat cells suspended in 5% FCS/RPMI 1640 were transiently transfected with MFG retroviral expression construct encoding green fluorescent protein (GFP), HIV-*luc*, LTR-*luc*, or LTRΔTAR DNA (45) with pCI vector control, p110-CAAX (gift of J. Downward, Imperial Cancer Research Fund) or FLAG-PTEN (gift of M. Georgescu, The Rockefeller University, New York, NY) using Lipofectamine 2000 reagent (Life Technologies, Rockville, MD). Cells were then incubated for 12 h before harvesting to assay for luciferase activity. MFG-GFP was used to control for transfection efficiency, and was assayed 12 h posttransfection by flow cytometry.

Immunoprecipitation and immunoblots

Jurkat cells were either left uninfected or infected as described above with HIV-*luc* or HIV-PLAP. Forty-eight hours postinfection, cells infected with HIV-PLAP were sorted using CELLection Pan Mouse IgG kit (Dynal, Lake Success, NY) along with anti-human PLAP Abs (Sigma-Aldrich). The infected Jurkat cells were then serum starved 4 h before stimulation with mouse anti-human CD3 (0.1 μg/ml), CD28 (1.0 μg/ml), and CD8α (1.0 μg/ml) Abs (BD PharMingen), and cross-linked using rabbit anti-mouse Ab (3.0 μg/ml; New England Biolabs, Beverly, MA) for 12 h. Protein extracts were prepared by lysing cells in lysis buffer (10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8.0), 2 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Nonidet P-40, 1 mM PMSF, 1 mM pepstatin). Protein A/G (Santa Cruz Biotechnologies, Santa Cruz, CA) were incubated with whole cell extracts for 2 h to preclear the lysates. Protein A/G beads (20 μl) were precoated with 1 μg anti-Tat Ab (46) (the NT3 2D1.1 Ab obtained from AIDS Research and Reference Reagent Program, National Institutes of Health), washed twice in lysis buffer before adding to precleared whole cell extracts (5×10^6 cells/sample to 20 μl beads) for 15 h at 4°C. For in vitro GST-binding assays, Gst-Tat cloned into pGEX-2T (provided by J. Workman, Pennsylvania State University) was purified from m15 bacteria cells (Qiagen, Valencia, CA) by probe sonication and bound to beads by incubating for 12–24 h. Protein concentrations of extracts were measured using the Bradford assay (Bio-Rad, Hercules, CA). For some experiments, Western blots for β-actin were used as a control for protein loading (data not shown). Protein extracts from uninfected Jurkats were incubated with the GST-Tat-bound beads for 1.5 h at 4°C. Protein-bound beads were washed three times in lysis buffer before adding 1× SDS loading buffer containing DTT, boiled, and resolved by 15% SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and associated Cdk9 or cyclin T1 detected by primary Abs, goat anti-human cyclin T1, or Cdk9 Abs (Santa Cruz Biotechnologies). Filters were either stripped 45 min at 55°C using 100 mM 2-ME, 62.5 mM Tris-HCl (pH 6.7), and 2% SDS, or poisoned for 15 min at room temperature in 0.015% sodium azide in PBS before probing for Tat. Tat was detected using mouse anti-Tat mAb. Blots were developed using Amersham (Arlington Heights, IL) ECL kit.

Flow cytometry

To detect receptor expression on Jurkat cells, 1×10^6 cells were incubated in 100 μl staining medium (1% FCS in PBS) with 1 μg mouse IgG isotype control (Sigma-Aldrich), anti-CD3, anti-CD28, or anti-CD8α for 45 min on ice. Cells were washed three times in staining medium, resuspended in 100 μl staining medium and 1 μg FITC-conjugated anti-mouse secondary Ab (Sigma-Aldrich), and incubated on ice for 45 min. Cells were washed three times in staining medium, and fluorescence was measured using a Corixa (Seattle, WA) flow cytometer at Pennsylvania State University flow cytometry core facility.

Results

CD28 signaling enhances HIV-1 proviral transcription

To investigate how CD28 influences HIV-1 transcription, we used a series of Jurkat cell lines that, in addition to endogenous CD28, expressed chimeric CD8α/28 receptors (Fig. 1), allowing us to compare mutant receptors to wild type within the same cell line. The chimeric receptors were generated by fusing the extracellular and transmembrane domains of human CD8α to the human CD28 cytoplasmic domain, which is sufficient for CD28-mediated IL-2 induction and does not affect endogenous CD28 function or signaling (14). Furthermore, deletions and specific mutations were engineered into the CD28 cytoplasmic tail. In this study, we specifically examined the effect of Y173 on HIV-1 transcription and used three chimeric receptors: 8WT, which contains the wild-type CD28 cytoplasmic tail; Δ167, in which all the tyrosines have been deleted; and F173, in which Y173 was mutated to phenylalanine (Fig. 1*A*). Expression of these chimeric receptors in the different cell lines was approximately equivalent (Fig. 1*B*). In addition, ectopic expression of the CD8α/28 chimeric receptors had no effect on endogenous CD3 or CD28 levels (data not shown, Fig. 1*B*).

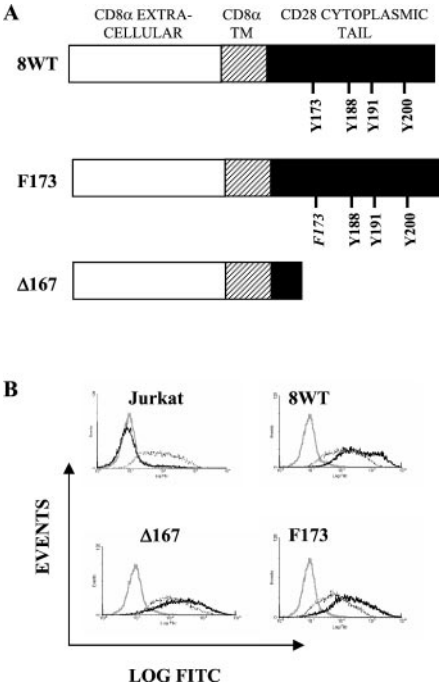

FIGURE 1. Expression of CD8α/28 chimeric receptors in Jurkat cells. *A*, Structure of chimeric constructs containing CD8α extracellular and transmembrane domains linked to CD28 cytoplasmic domain used in these experiments. *B*, Expression of CD8α/28 chimeric receptors in Jurkat cells. Cells were stained with FITC-conjugated isotype control (gray line), anti-CD8α (black line), or anti-CD28 (broken thin line) and analyzed by flow cytometry. Mean fluorescent intensities for the individual cell lines were as follows: WT anti-CD28 87.1, anti-CD8 10.5; 8WT anti-CD28 130, anti-CD8 411; F173 anti-CD28 64.7, anti-CD8 220; Δ167 anti-CD28 114, anti-CD8 227.

To monitor proviral expression during T cell activation, cells were infected with a replication-incompetent HIV-*luc* recombinant virus. This virus undergoes a single round of infection, and proviral expression is monitored by luciferase gene expression (43). The infected cells were activated with Abs against CD3, CD28, or CD8α presented by CHO-Fc cells to cross-link the receptors, mimicking cell to cell surface receptor interactions. As shown in Fig. 2, activation through CD28 or CD8α alone is not sufficient to activate HIV-1 transcription. Furthermore, stimulation of infected cells with anti-CD3 alone demonstrated only a modest induction of HIV-1 transcription. Activation of the cell lines with both anti-CD3 and anti-CD28 resulted in synergistic activation of HIV-1 transcription. No differences were observed in the ability of the different cell lines to respond to anti-CD3 plus anti-CD28 stimulation; therefore, this was used as an internal standard to normalize anti-CD3 plus anti-CD8α responses. More importantly, activation of HIV-1 transcription observed in cells stimulated through the 8WT chimeric receptor and CD3 showed comparable induction to the CD3 plus CD28 stimulation, demonstrating that signaling through the chimeric receptor is equivalent to that of endogenous CD28.

Cell lines expressing the Δ167 receptor, which lacks most of the cytoplasmic tail including the four tyrosine residues necessary for CD28 signaling, were unable to costimulate HIV-1 transcription upon stimulation with anti-CD3 and anti-CD8α. HIV-1 transcription in cells expressing this truncated receptor was approximately the same as that observed following activation through CD3 alone (Fig. 2). These results demonstrate that HIV-1 proviral expression is responsive to CD28 signaling and requires the four tyrosine residues in the cytoplasmic tail.

Cell lines expressing the F173 chimeric receptor consistently had a 2-fold increase in HIV-1 transcription following T cell activation with anti-CD3 plus the mutant chimeric receptor (Fig. 2). Also, a slight increase in CD8 stimulation alone was consistently observed in the F173 cells. The enhancement of transcription was not due to indirect effects of IL-2 since addition of exogenous IL-2 did not alter HIV-1 transcription (data not shown). However, CD3 plus CD28 induction of virus transcription in the F173 cells was comparable with 8WT, suggesting that ectopic expression of the F173 receptor did not lead to a general enhancement of HIV-1 expression (data not shown). These data suggest that tyrosine residue Y173 recruits factors that negatively regulate HIV-1 transcription following T cell activation.

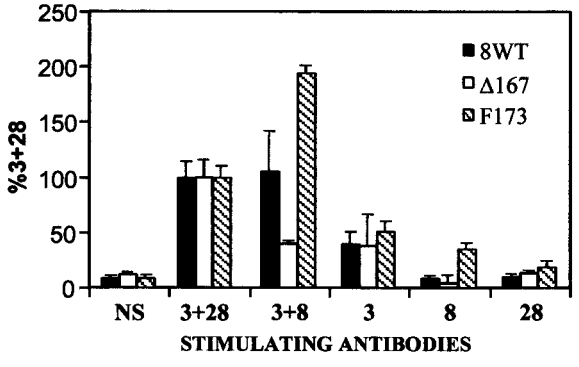

FIGURE 2. Mutation of F173 within CD28 enhances HIV-1 transcription. Jurkat cell lines expressing CD8α/28 chimeric receptors were infected with HIV-*luc*. Cells were not stimulated (NS) or stimulated with mAbs against CD3 (0.1 μg/ml), CD8α (1.0 μg/ml), and CD28 (1.0 μg/ml) individually or in combination, as described in *Materials and Methods*, for 12–24 h before lysing and measuring luciferase activity. Data are shown as percentage of endogenous CD3 + CD28 response for the respective cell lines. Each data point represents three independent stimulations, and error bars show the SD of these replicates. Data are from a single experiment, which is representative of a total of three experiments.

PI3K activation inhibits HIV-1 transcription

Previous studies have shown that Y173 within CD28 recruits and activates PI3K, and that mutating this residue to phenylalanine disrupts the interaction between PI3K and CD28 (data not shown) (15, 20, 21, 23, 24, 47). The increase in HIV-1 transcription observed in cell lines expressing the F173 mutant receptor suggests that PI3K negatively regulates virus transcription. To confirm an inhibitory role for PI3K on HIV-1 transcription, we used two chemically distinct PI3K inhibitors, LY294002 and wortmannin. When infected Jurkat cells expressing the chimeric 8WT receptor were stimulated in the presence of PI3K inhibitor LY294002, a 7-fold increase in HIV-1 transcription was observed (Fig. 3*A*). In contrast, no significant difference in virus expression was observed when F173-expressing cell lines were stimulated in the presence of inhibitor, consistent with the inability of this mutant receptor to recruit PI3K (Fig. 3*A*). To ensure that the increase in transcription was not due to inappropriate expression of chimeric receptors, parental Jurkat cells were infected and stimulated in the presence and absence of the PI3K inhibitors. As shown in Fig. 3*B*, addition of LY294002 or wortmannin to these cells resulted in 6-fold greater induction of HIV-1 transcription overstimulation through CD3 plus endogenous CD28 in the absence of PI3K inhibitors.

To confirm that PI3K negatively impacts HIV-1 transcription, we used p110CAAX, a constitutively active version of the PI3K catalytic subunit, and PTEN, a phosphatase that negatively regulates PI3K signaling. Eukaryotic expression constructs for these

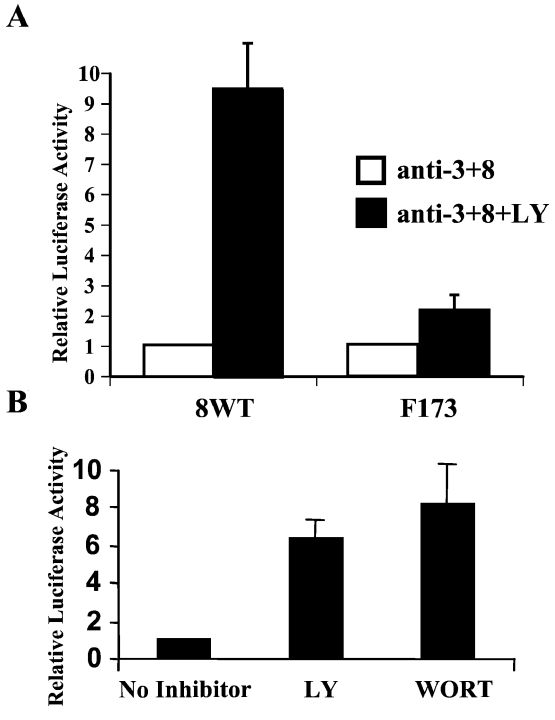

FIGURE 3. PI3K inhibitors enhance HIV-1 transcription. *A*, Cell lines expressing the indicated chimeric receptors were infected with HIV-*luc* and stimulated with anti-CD3 + anti-CD8α in the presence and absence of 50 μM LY294002. Data are shown as fold increase of stimulation in absence of inhibitor. *B*, Jurkat cells were infected with HIV-*luc* and stimulated with anti-CD3 + anti-CD28 in the presence and absence of 20 μM LY294002 or 100 nM wortmannin. In both *A* and *B*, luciferase activity was measured 18–24 h poststimulation, and values were normalized to controls stimulated in the absence of inhibitor. Data shown are one representative experiment of three, and error bars are the SE for three independent stimulations.

proteins were cotransfected with the HIV-*luc* viral cDNA into Jurkat cells, and luciferase activity was determined as an indicator of HIV-1 transcription. Overexpression of p110CAAX decreased virus transcription consistent with PI3K activation inhibiting HIV-1 transcription (Fig. 4*A*). Conversely, transfecting cells with PTEN resulted in a 3-fold increase in transcription of HIV-1 similar to the results obtained using specific inhibitors (Fig. 4*B*). Furthermore, ectopic expression of the constitutively active p110CAAX in the F173 cell line inhibited HIV-1 transcription upon activation through the mutant chimeric receptor and CD3 (Fig. 4*C*). These data provide additional evidence that PI3K acts as a negative regulator of HIV-1 transcription.

PI3K inhibits HIV-1 transcription through a Tat-dependent mechanism

To gain insight into mechanisms by which PI3K inhibits HIV-1 transcription, we performed transient transfections using an HIV-1 LTR luciferase reporter (LTR-*luc*) construct. However, unlike the results obtained with the HIV-*luc* viral construct, the dominant active and negative regulators of PI3K did not influence LTR activity (Fig. 5*A*). In addition, the LTR-*luc* construct was insensitive to LY294002 treatment (Fig. 5*B*). Since the LTR reporter construct lacks the ability to express viral proteins, these data suggest that HIV-1-encoded viral proteins are required for the ability of PI3K to inhibit virus transcription.

HIV-1 accessory proteins were systematically added to transient transfections with the HIV-1 LTR-*luc* in an attempt to rescue the PI3K-dependent effects seen with HIV-*luc* infection and transfections. Cotransfection of LTR-*luc* with HIV-1 Tat resulted in a 2.5-fold enhancement of transcription in the presence of PI3K inhibitor LY294002 (Fig. 5*B*). The effect of PI3K on Tat-dependent transcription required the TAR element since no significant induction of transcription was observed in LTR reporters lacking this element (Fig. 5*B*). In contrast, other accessory proteins, including Vpr and Nef, did not alter HIV-1 LTR transcription in a PI3K-dependent manner (Fig. 5*B*, data not shown). These data provide evidence that Tat or cellular regulators of Tat are downstream targets of PI3K signaling.

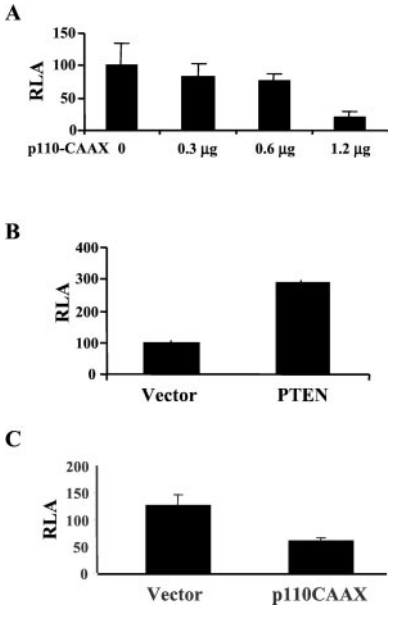

FIGURE 4. Constitutively active PI3K inhibits HIV-1 transcription. *A*, Jurkat cells were transiently transfected with HIV-*luc* (0.6 μg) plus indicated amounts of p110CAAX or empty expression construct pCI. *B*, HIV-*luc* (0.6 μg) was transiently transfected into cells along with PTEN (0.6 μg), or vector control (0.6 μg). For *A* and *B*, luciferase activity was measured 18–24 h posttransfection, and values were normalized to vector control (100) and presented as relative luciferase activity (RLA). *C*, F173 was transiently transfected with HIV-*luc* (0.5 μg) with p110CAAX (0.4 μg) or empty expression vector control (0.4 μg) and stimulated with CD3 plus CD28 or CD8α. Luciferase activity was measured 18–24 h poststimulation. Vector control was used to keep total DNA concentration at 2 μg for *A* and *B* and 10 μg for *C*. Transfection efficiency was measured using an independent reporter containing GFP and analyzed by flow cytometry. No significant differences in transfection efficiencies were observed (data not shown). Data shown are one representative experiment of three, and error bars are the SD of three individual transfections.

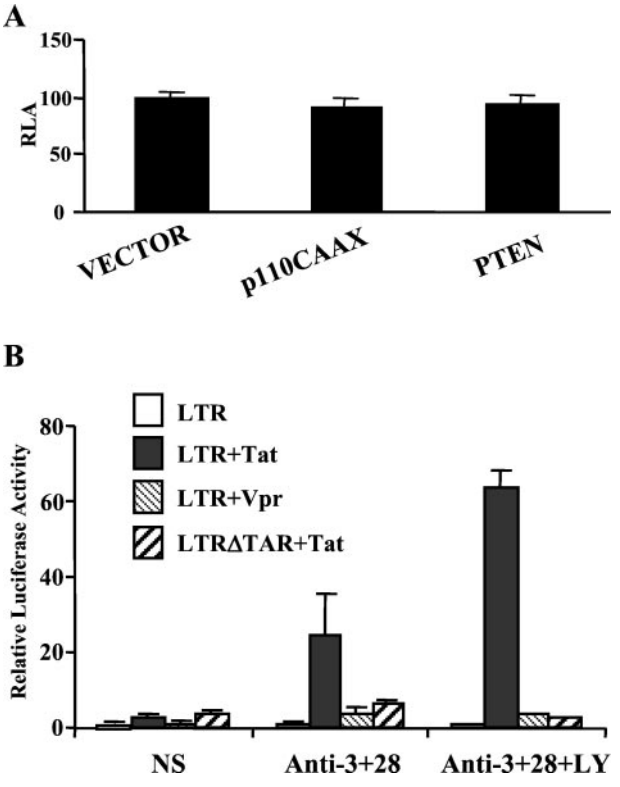

FIGURE 5. Tat is necessary for PI3K-mediated effects on HIV-1 transcription. *A*, Jurkat cells were transiently transfected with LTR-*luc* (0.6 μg) along with p110CAAX (0.6 μg), PTEN (0.6 μg), or pCI expression vector (0.6 μg). Luciferase activity was measured 18–24 h posttransfection, and data were normalized to vector control (100). *B*, LTR-*luc* (1.0 μg) or LTRΔTAR were transiently transfected with Tat (0.6 μg) or Vpr (0.6 μg). Cells were stimulated with CD3 + CD28 in the presence or absence of 20 μM LY294002 for 12–24 h before lysing cells and measuring luciferase activity. Empty expression vector was added to all transfection samples to assure that the final DNA concentration was at 2 μg. Values were normalized to LTR-*luc* in unstimulated cells (1.0). Data are shown as one representative experiment of three, and error bars represent SD for three individual stimulations.

PI3K targets Tat activation complex

Tat activation of transcription requires association with cellular protein complexes, including TAK (36–39). The Tat-TAK complex increases transcription by phosphorylating RNA pol II CTD, thus increasing the processivity of this transcription complex (36, 37, 40, 48). Therefore, a possible target of PI3K is the assembly of the Tat transcriptional activation complex. This was specifically tested using a Tat pull-down assay. Nuclear extracts from Jurkat cells stimulated in the absence or presence of PI3K inhibitors were incubated with bacterially expressed GST-Tat, and the ability of Tat to physically interact with cyclin T1, which mediates the recruitment of TAK to Tat, was determined by immunoblotting. As

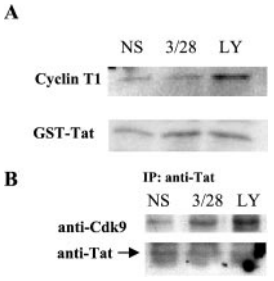

FIGURE 6. PI3K inhibits formation of Tat-TAK complex. *A,* Nuclear extracts prepared from cells unstimulated, anti-CD3 plus anti-CD28, or anti-CD3 plus anti-CD28 plus 20 μM LY294002-treated Jurkat cells were incubated with GST-Tat fusion protein. Associated cyclin T1 was detected by immunoblotting. Filters were stripped and reprobed with anti-Tat. *B,* Jurkat cells were infected with HIV-PLAP and positively selected using magnetic beads coated with anti-PLAP Abs (see *Materials and Methods*). Whole cell extracts were prepared from these HIV-infected cells and immunoprecipitated with anti-Tat. Association with TAK was determined by immunoblotting with anti-Cdk9 Ab. Filters were treated with sodium azide and reprobed with anti-Tat.

shown in Fig. 6*A*, inhibiting PI3K enhanced the ability of cyclin T1 to physically interact with Tat in vitro.

To demonstrate the interaction between Tat and TAK was regulated by PI3K in vivo, immunoprecipitations were performed with extracts from Jurkat cells infected with HIV-PLAP, which contains the PLAP surface marker that allows for positive identification and sorting of infected cells (42). Infected cells were stimulated in the presence or absence of PI3K inhibitors, and complexes were coimmunoprecipitated with anti-Tat Abs. Immunoblots demonstrated an increase in complex formation between Tat and Cdk9, a subunit of TAK, in the presence of PI3K inhibitor LY294002 (Fig. 6*B*), confirming that PI3K signals inhibit formation of the Tat transcriptional activation complex and suggesting a mechanism by which PI3K represses HIV-1 proviral transcription.

Discussion

T cell activation in response to TCR plus CD28 engagement results in the enhancement of HIV-1 transcription. However, how specific signal transduction pathways from CD28 directly impact HIV-1 expression has not been examined in detail. In this study, we show that HIV-1 transcription is regulated both positively and negatively by CD28 signals. In particular, the recruitment of PI3K to CD28 results in a decrease in HIV-1 transcription. Furthermore, the PI3K-dependent decrease in HIV-1 transcription is in part regulated by inhibiting the formation of the Tat-TAK transcriptional activation complex, demonstrating that Tat activity is responsive to T cell signaling events.

The ability of Tat to activate transcription through the TAR RNA element depends on its ability to recruit TAK, which is responsible for phosphorylation of the RNA pol II CTD increasing transcriptional processivity of the polymerase machinery. Tat-TAK interactions are regulated by both posttranslational and transcriptional mechanisms (36, 39). The interaction of the Tat-TAK complex with RNA has been shown to require acetylation by the coactivator PCAF and is negatively regulated by a second acetylation event mediated by p300 (35, 49). Furthermore, Cdk9 transcription and formation of TAK complexes are induced by a variety of agents that activate T cells, including PMA, PHA, cytokines IL-2, IL-6, plus TNF-α, or CD3 plus CD28 stimulation (38, 40, 50). Our data suggest that this complex may also be subject to negative regulation by cellular signaling cascades, including PI3K, which represses induction of HIV-1.

The role of PI3K during T cell activation is still controversial, and it has been implicated as both a positive and negative regulator of T cell responses. Our data show that CD28-dependent recruitment of PI3K initiates negative signals that impact formation of Tat *trans* activation complex, thus identifying the initial signal and end point for this signaling pathway. However, critical intermediate signal transduction events require further study. Candidate signaling intermediates include inducible T cell kinase, which may act both upstream and downstream of PI3K; AKT, which influences cell survival through up-regulation of Bcl-x_L in an NF-κB-dependent mechanism; and $p27^{KIP}$, a negative regulator of cell cycle progression (51–60). Furthermore, HIV-1 expression correlates with increased T cell apoptosis and disruption of the cell cycle. Multiple HIV-1 accessory proteins have been suggested to target these cellular processes (55, 61–65). Therefore, by blocking cell survival and cell cycle progression through PI3K activity, HIV-1 may promote a cellular state most compatible for high level expression.

It has been suggested that Nef alters PI3K-dependent signaling (61, 62, 64). DNA microarray analysis has shown that cells ectopically expressing Nef have elevated Cdk9 transcripts (64). Since the recombinant viruses used in these studies lack Nef, a detailed analysis of Nef function in the context of HIV-1 infection was not possible. However, cotransfecting Nef with the LTR-*luc* reporter did not have a significant effect on PI3K-dependent HIV-1 transcription (data not shown).

CD28 initiates multiple signaling events that lead to an overall activation of HIV-1 transcription. Therefore, other tyrosine residues in the CD28 cytoplasmic tail may counteract negative signals resulting from the recruitment of PI3K to CD28. Furthermore, Y^{173} has been shown to interact with Grb-2; however, its role in CD28 signaling is unclear (16). Although our results with chemical inhibitors, constitutively active PI3K, or PTEN would suggest that it is the recruitment of PI3K to Y173 that is predominately influencing HIV-1 transcription, it is possible that Grb-2 and PI3K may compete for binding at Y173 and have differential affects on T cell activation. The F173 mutant does not bind to either PI3K or Grb-2 (16). Studies assessing how Grb-2 and other signaling cascades recruited by $Y^{188,191,200}$ impact HIV-1 transcription will provide further insight into the mechanisms that contribute to the induction of HIV-1 transcription.

Previous studies characterizing the induction of HIV-1 transcription by T cell activation have relied heavily on transient transfections of LTR reporters and usually did not include viral proteins (66–68). Our results indicate that the Tat transcriptional activation complex is critical for PI3K-dependent inhibition of HIV-1 transcription and demonstrate the importance of examining the effects of signaling cascades on the expression of provirus in addition to reporter systems. This may also provide an explanation for difficulties in identifying a specific CD28 response element within the LTR. Whether Tat or other viral proteins are required for the overall CD28-dependent enhancement of transcription is currently being examined.

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