H. saimiri tyrosine-kinase interacting protein inhibits Tat function: A prototypic strategy for restricting HIV-1-induced cytopathic effects in immune cells

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Abstract

Herpesvirus saimiri (HVS)-transformed human T cells become permissive for X4 and R5 strains of human immunodeficiency virus type 1 (HIV-1), evidence that HVS-encoded proteins associated with T cell transformation enhance HIV-1 replication. Analyzing the contribution of transformation-associated bicistronic HVS open reading frames (ORF) to HIV-1 replication revealed expression of the second ORF saimiri transformation-associated protein type C (StpC) conferred the permissive phenotype to T cells. In contrast, expression of the first HVS ORF tyrosine-kinase interacting protein (Tip) in the absence of StpC enhanced restriction of HIV-1 replication in T cells and peripheral blood mononuclear cells. Understanding the mechanism whereby Tip enhanced restriction of HIV-1 replication may uncover unique pathways that could be targeted therapeutically. Here we report that Tip restricts HIV-1 replication in a monocyte-derived cell line and restricts reactivation of replication of HIV-1 in a T cell line harboring provirus. In this report, we begin to unravel the molecular underpinnings of Tip-mediated restriction. Tip mediates both lymphocyte-cell-specific kinase (Lck)-dependent and -independent effects on HIV-1 replication. We also provide evidence that Tip-mediated restriction is in part due to inhibition of Tat transactivation of the HIV-1 long terminal repeat (LTR). Expression of Tip in T cells increased activation of Stat1 and Stat3, as well as activation of protein kinase RNA-dependent (PKR/p68) and interferon-γ production. Taken together, these results provide evidence that Tip restricts HIV-1 replication and reactivation by inhibiting HIV-1 transcription while inducing an intercellular antiviral state. We propose that genetically engineered vectors driving Tip expression could provide a prototypic strategy for restricting HIV-1 replication and reactivation in diverse cell lineages.

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Introduction

Human immunodeficiency virus type 1 (HIV-1) replication can be restricted by innate immunity as well as by intrinsic or cell autonomous species-specific defense mechanisms. Innate mechanisms include interferon-induced proteins, two of which are PKR, a double-stranded RNA-dependent kinase that phosphorylates the α-subunit of the eukaryotic initiation factor 2 (eIF2-α) inhibiting translation, and 2′–5′ oligoadenylate synthetase (2′–5′OAS), which synthesizes 2′–5′ oligoadenylates that bind and allosterically activate the ribonuclease L (RNaseL) (Dong and Silverman, 1995; Dong et al., 1994; Kerr and Brown, 1978; Samuel, 1979). RNaseL, once activated by 2′–5′ oligoadenylates degrades mRNA, disrupting viral gene expression. Innate restriction mechanisms target a variety of viruses, many of which have evolved evasion strategies. Intrinsinc or cell autonomous species-specific defense mechanisms restricting retroviral replication before or after integration of proviral DNA have been identified. These defenses include restriction factor-1 (Ref1) (in humans) (Lv1/Lv2/TRIM5-α in African green monkeys) and APOBEC3G/CEM15 are members of the family of species-specific cell autonomous restriction

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factors that inhibit retroviral infections at a pre-integration stage. The viral capsid protein (gag/p24) is targeted and bound by Ref1, the gag Ref1 interaction prevents viral uncoating and subsequent steps in replication (Hatziannou et al., 2004; Schmitz et al., 2004; Stremlau et al., 2004). APOBEC3G/CEM15, a cytidine deaminase, produces uracil from cytidine on newly synthesized negative stranded DNA, resulting in G-to-A transition mutations on the subsequently synthesized positive DNA strand (Douaisi et al., 2004; Harris et al., 2003; Mangeat et al., 2003; Sheehy et al., 2002). The resulting G to A transitions give rise to nonsense mutations within the viral genome and a nonproductive infection. Host cytosine DNA methyltransferases methylate CpG sequences within the integrated proviral genome triggering transcriptional silencing (Bednarki et al., 1990). RelA-associated inhibitor (RAI) blocks proviral transcription by blocking the DNA-binding capacity of transcription factors NF-kB and Sp1, such that transcription of the long terminal repeat (LTR) is dramatically diminished (Takada et al., 2002). The zinc-finger antiviral protein, an mRNA exporter, restricts nuclear export of viral mRNA, preventing cytoplasmic accumulation and subsequent translation (Samuel, 2001). Tumor susceptibility gene 101 (TSG101) is a member of the Class E family of vacuolar sorting proteins (VSPs) that interacts with the late domain (p6) of HIV-1 Gag protein via a ubiquitin conjugating (E2)-like domain (Babst et al., 2000; Garrus et al., 2001). TSG101 has also been shown to associate with the endosomal sorting complex (ESCRT-I) important for sorting of ubiquitinated products into endosomes/multivesicular bodies (MVBs) (Katzmann et al., 2001). TSG101 interaction with Gag may target Gag to endosomal compartments for degradation restricting HIV-1 assembly or egress. More recently, it has been discovered that endogenous micro RNAs can interfere with translation of retroviral transcripts after retrotransposition and may represent an intrinsic host cell defense mechanism against viral replication (Lecellier et al., 2005).

HIV-1 replicates to high titers in human cells transformed in vitro by Herpesvirus saimiri (HVS) (Vella et al., 1997), evidence that HVS-encoded proteins associated with T cell transformation block one of the above intrinsic/innate restriction factors. Two HVS open reading frames (ORF) transcribed from a bicistronic message, tip and StpC, are necessary for transformation and are detected in HVS-transformed T cells. Expression of Tip and/or StpC in the absence of other HVS proteins had dramatic and canonical effects on replication of R5 and X4 strains of HIV-1 (Henderson et al., 1999; Raymond et al., 2004). StpC expression with or without tip resulted in a permissive phenotype for HIV-1 replication in T cell lines and peripheral blood mononuclear cells (PBMCs). StpC expression levels associated with permissive phenotype are StpC levels detected in HIV-transformed T cells (Henderson et al., 1999). On the other hand, Tip expression independent of StpC restricted HIV-1 replication and cytopathic effects in T cell lines and PBMCs. Tip expression levels associated with the restricted phenotype are higher than levels of Tip detected in HIV-transformed T cells (Henderson et al., 1999). Understanding the mechanism of Tip-mediated restriction of HIV-1 replication could identify new therapeutic approaches for HIV-1 infection. Tip associates with plasma membrane of transformed cells and increases activity of lymphocyte-cell-specific kinase (Lck), a member of the Src kinase family, and signal transducers and activators of transcription (STAT) 1 and 3 (Hartley and Cooper, 2000; Kjellen et al., 2002; Lund et al., 1995, 1997a). Two unique domains of Tip interact with Lck–Lck binding domain 1 (LBD) and Lck binding domain 2 (LBD2), while the consensus sequence YXPQ within Tip binds SH2 domains of Stat1 and Stat3 (Biesinger et al., 1995; Hartley and Cooper, 2000; Hartley et al., 1999; Lund et al., 1997a). In an effort to elucidate the mechanism(s) underlying Tip-mediated restriction of HIV-1 replication, we examined whether Tip-induced activation of these proteins altered HIV-1 replication. First, we show that Tip restricts HIV-1 replication in U937, a monocoyte-derived cell line, and HIV-1 reactivation in ACH2, a T cell line latently infected with replication competent HIV-1. Second, we provide evidence of a novel mechanism whereby Tip restricts HIV replication. Specifically, we show that expression of HVS-encoded Tip alters HIV-1 replication by (1) inhibiting HIV-1 Tat-mediated transactivation of the HIV LTR and (2) augmenting innate anti-viral immunity, specifically inducing phospho-tyrosine activation of Stat1, Stat3, and PKR. These results support the concept that Tip inhibition of Tat-mediated transactivation of the LTR in concert with Tip augmenting innate antiviral immunity may be developed as a prototypic therapeutic strategy to protect susceptible immune cells from HIV-1-associated cytopathic effects.

**Results**

**Tip restricts HIV-1 replication in the monocyte-derived cell line U937**

Tip restricts replication of X4 and R5 strains of HIV-1 in SupT1, MOLT4, and primary lymphocytes (Raymond et al., 2004). To determine whether Tip restricted HIV-1 replication in monocytes the monocyte-derived cell line, U937 was transduced with lentiviral vectors driving the expression of Tip and a neomycin resistance gene (Fig. 1A). Jurkat, a T cell-derived cell line, was transduced in parallel as a control. DNA extracted from neomycin-resistant U937 and Jurkat were shown to harbor the Tip transgene by Tip-nested PCR (Figs. 1B and C). Analysis of HIV-1 replication measured by accumulation of p24 demonstrated that Tip restricted HIV-1 strain IIIB replication approximately 7-fold in Jurkat and of HIV-1 strain SF162 replication 5-fold in U937 cells (Figs. 1D and E). Levels of Tip-mediated restriction of HIV-1 replication in Jurkat and U937 were consistent with restriction levels seen in SupT1 and MOLT4 (Henderson et al., 1999).

**Transduction with Tip post-infection influences HIV-1 replication in T cells, but not in monocyte-derived cell lines**

Tip expression prior to HIV-1 infection restricted HIV-1 replication in T cell and monocyte-derived cell lines and primary lymphocytes, evidence that Tip-mediated restriction is effective when Tip is expressed prior to infection. To determine whether Tip expression post-HIV-1 infection restricts HIV-1 replication, MOLT4 or U937 was transduced with pCTPN encoding Tip before or post-HIV-1 infection. As expected, Tip expression before
HIV-1 infection restricted HIV-1 replication 4-fold in MOLT4 (Fig. 2A) and 5-fold in U937 cells (Fig. 2B) as measured by p24 accumulation. Replication of IIIB strain of HIV-1 in MOLT4 was not restricted significantly when transduction with pCTPN driving the expression of Tip was post-infection (Fig. 2C). In contrast, transduction with pCTPN post-HIV-1 infection restricted replication of the SF162 strain of HIV-1 in U937 cells 4-fold, comparable to Tip expression before infection (Fig. 2D).

Reactivation of replication competent HIV-1 following Tip or StpC expression

ACH2, a T cell line latently infected with replication competent HIV-1, was stably transduced with pCTPN encoding Tip or pCSPP encoding StpC (Fig. 3A). Reactivation of replication competent HIV-1 in ACH2 transduced with pCTPN measured by the accumulation of p24 was reduced 3.3-fold 5 days post-transduction compared to that in ACH2 transduced with pCPN alone (Fig. 3B). HIV-reactivation 5 days post-transduction was up-regulated 1.8-fold in ACH2 transduced with pCSPP compared to that in cells transduced with pCPP alone (Fig. 3D). HIV-1 reactivation in ACH2 transduced with pCTPN as measured by the accumulation of p24 in the supernatants after 20 days was restricted 10-fold compared to that in cells transduced with pCPN alone (Fig. 3C). In contrast, HIV-1 reactivation was enhanced 3-fold in ACH2 transduced with pCSPP compared to in ACH2 transduced with pCPP (Fig. 3E). These results show that StpC expression can induce reactivation of replication competent HIV-1 in ACH2 comparable to TNF-α and phorbol esters (Folks et al., 1989; Pomerantz et al., 1990).

Role of Lck in Tip-mediated restriction of HIV-1 replication

To better understand Tip restriction of HIV-1 replication, we focused on cellular factors interacting with Tip. Analysis of DNA from neomycin-resistant JCaM1.6 cells (Jurkat-derived clone in which Lck is not active) transduced with the pCTPN by PCR indicated the presence of Tip transgene (Fig. 4A). In
contrast to Jurkat, transduction with pCTPN failed to restrict HIV-1 replication in JCaM1.6 cells (Fig. 4B) suggesting that Lck is essential for Tip-mediated restriction of HIV-1 replication. The role of Lck in Tip-mediated restriction of HIV-1 replication was further analyzed using JCaM-C1, a JCaM-derived cell line expressing Lck under control of a tetracycline repressible promoter. Lck is expressed in the absence of tetracycline, whereas Lck expression is repressed in JCaM-1 within 24 h following tetracycline treatment (Fig. 5A). Transduction with pCTPN resulted in a 4-fold restriction of HIV-1 replication in JCaM-C1 in the absence of tetracycline (presence of Lck) as measured by the accumulation of p24 (Fig. 5B, left panel). In contrast, Tip-mediated restriction of HIV-1 replication was lost in the presence of tetracycline (absence of Lck) (Fig. 5B, right panel). These experiments are consistent with Lck being essential for Tip-mediated restriction of HIV-1 replication in Jurkat-derived cell lines as measured by accumulation of p24.

**Tip-mediated restriction of HIV-1 replication occurs at the transcriptional level**

Tip-mediated restriction of HIV-1 replication occurs after retrotransposition by preventing the production of infectious virus, evidence that transcription of provirus might be restricted by Tip (Henderson et al., 1999; Raymond et al., 2004). To determine if expression of integrated proviral DNA is restricted by Tip, effects of Tip on Tat-mediated transactivation of chloramphenicol acetyl transferase (CAT) reporter gene expression under the control of the HIV-1 LTR were analyzed. Lck expression (Fig. 6A) and activity (Fig. 6B) were measured in Jurkat, MOLT4, and JCaM1.6, which lack Lck expression and activity. Transfection of LTR/CAT with pSVTat72 resulted in increased CAT expression in Jurkat (8-fold), MOLT4 (100-fold), and JCaM1.6 cells (14-fold) compared to transfection with LTR/CAT alone (Fig. 6C). Tat was able to transactivate the LTR/CAT reporter gene independent of Lck expression levels. However, co-transfection with LTR/CAT and pSVTat72 along with pCep4-Tip inhibited Tat-mediated LTR-driven reporter gene expression in Jurkat (1.5-fold), MOLT4 (7.3-fold), and JCaM 1.6 lacking Lck activity (14-fold) (Fig. 6C), evidence that Lck activity is not necessary for Tip-mediated inhibition of Tat transactivation. To further analyze whether Tip-mediated inhibition of Tat transactivation of LTR is Lck-independent, Lck expression was knocked down in Jurkat with siRNA. Transfection with siRNA reduced Lck expression in Jurkat 1.8-fold on day 1, 2.3-fold on day 4, and on day 7. Lck expression returned to nominal levels (Fig. 7A). Tip inhibited Tat-mediated transactivation of LTR CAT in Jurkat cells with reduced Lck expression (Fig. 7B). JCaM-C1 transfected with the LTR-CAT reporter construct and pCep4-Tip was used to confirm that Tip-mediated inhibition of Tat-mediated LTR-driven reporter gene expression is Lck-independent. Tip inhibited LTR-driven reporter gene expression in the absence (Fig. 8A) or presence of Lck (Fig. 8B), evidence that Tip inhibition of Tat-mediated LTR transactivation is Lck-independent, while Tip-mediated restriction of HIV-1 production is Lck-dependent.
Stats 1 and 3 are activated in cells transduced to express Tip

Tip binds and activates Stat1 and Stat3 in an Lck-dependent manner. Stat1 activation results in the expression of genes promoting antiproliferative and antiviral responses, whereas activation of Stat3 would promote growth (Hartley and Cooper, 2000; Lund et al., 1997b). We examined the effect of Tip expression on the activation of Stat1 and Stat3 in JCaM-C1 cells and role of Lck. In the presence of Lck, transfection with pCep4-Tip and pEGFP up-regulated the activation of Stat1 4.2-fold at 48 h (Fig. 9A) as measured by the accumulation of p24 in culture supernatants was monitored using p24 ELISA.

Fig. 3. Reactivation of replication competent HIV-1 is restricted in T cells transduced to express Tip. (A) Schematic representation of vectors driving the expression of StpC as described in Hasham and Tsygankov, 2004. StpC-PGK-Pac and PGK-Pac fragments from pMSCV were cloned into the multiple cloning site (MCS) of pH’CMV-MCS to generate pCSPP (StpC/Pac) and pCPP (Pac), respectively. ACH2 was transduced with pCPP, pCSPP, pCPN, or pCPTN, respectively. Cells were placed on puromycin or neomycin selection. Culture supernatants harvested either 5 days post-transduction (dpT) (B and D) or 20 days post-transduction (C and E). Reactivation as measured by the accumulation of p24 in culture supernatants was monitored using p24 ELISA.

Fig. 4. Tip-mediated restriction of HIV-1 replication is blocked in JCaM1.6 lacking Lck activity. JCaM1.6 was transduced with pCPN or pCPTN and placed on neomycin selection for 7 days. Surviving clones expanded and DNA extracted for nested PCR. (A) Photograph of ethidium-bromide stained agarose gel (1%) of the nested PCR product confirmed the presence of the Tip transgene in JCaM1.6 transduced with pCPTN. Lane 2(+) positive control MOLT4 transduced with Tip/Neo. (B) Surviving clones were infected with HIV-1 IIIB. Culture supernatants harvested 7 days post-infection were analyzed by p24 ELISA.
2.5-fold at 24 h (Fig. 9B), evidence that Stat1 activation by tyrosine phosphorylation is Lck-independent. In the presence of Lck transfection with pCep4-Tip and pEGFP, Stat3 activation was up-regulated 8-fold after 48 h (10A). Transfection with pCep4-Tip did not activate Stat3 in the absence of Lck (Fig. 10B), suggesting in JCaM-C1 that Stat3 tyrosine phosphorylation is Lck-dependent (Fig. 10B).

PKR expression and activation are up-regulated in Jurkat cells transduced with pCTPN

Phosphorylated Stat1 forms homodimers that bind interferon-α (IFN-α)-activated sequences (GAS) within the pkr promoter stimulating pkr transcription (Darnell et al., 1994; Horvath and Darnell, 1996; Shuai et al., 1994). Increased expression of PKR results in autoactivation, PKR-mediated inhibition of translation, and subsequent induction of apoptosis (Gil and Esteban, 2000; Meurs et al., 1990). To determine if Tip-induced Stat1 activation is biologically relevant, we analyzed PKR expression and activation in T cells expressing Tip. Jurkat cells transduced with pCTPN (Tip) showed increased total PKR expression levels (2.7-fold) (Figs. 11A and B). PKR activation was significantly increased in uninfected and HIV-1-infected Jurkat transduced with pCTPN (Tip) (Figs. 11A [lanes 5 and 6] and C).

IFN-γ production in PBMC and JCaM-C1 transduced with pCTPN

Increased expression of PKR in cells transduced with Tip suggests that expression of Tip activates the interferon signaling cascade. Since Stat1 and Stat3 are known to be activated by Tip and Stat1 is a mediator of the IFN-α response, we examined the
effect of Tip on IFN-γ production. HIV-1 has been shown to impair the production of Th1 cytokines such as IFN-γ and IL-12 (Chehimi et al., 1994; Meyaard et al., 1996), and increased levels of Th1 cytokines are detected in HIV-1-infected long term nonprogressors (Ullum et al., 1997). We determined whether Tip-induced activation of Stat1 resulted in the expression of IFN-γ production and whether Lck is essential. Transduction with pCTPN (Tip) dramatically increased IFN-γ production (approximately 25-fold) in HIV-1-infected JCaM-C1 without Lck (Fig. 12A) and in uninfected and HIV-1-infected PBMC (Fig. 12B) compared to cells transduced with pCPN (Neo). This is consistent with Tip-mediated activation of Stat1 being Lck-independent (Fig. 9B).

Discussion

Several viruses influence HIV-1 replication or HIV-1-induced disease progression (reviewed in Kannangara et al., 2005). Human Herpesvirus-8 (HHV-8) enhances HIV-1 replication in acutely infected cells and induces HIV-1 reactivation in latently infected cells (Caselli et al., 2005). Human T cells transformed by closely related HVS become highly permissive for HIV-1 replication (Vella et al., 1997). Paradoxically, Tip, one of two HVS ORFs associated with T cell transformation, was shown to restrict replication of X4 strains of HIV-1 in permissive T cell lines (Henderson et al., 1999). Tip was subsequently shown to restrict replication of R5 strains of HIV-1 in T cells and peripheral blood lymphocytes (Raymond et al., 2004). Here we demonstrate Tip restricts replication of R5 strains of HIV in a monocyte-derived cell line U937 (Fig. 1). HIV-1 infection of monocytes is important in pathogenesis, contributes to HIV-1-induced disease progression, and may constitute a reservoir for replication competent HIV-1 (Ancuta et al., 2006). Our results support the concept that strategies based on Tip-mediated restriction of HIV-1 replication could target monocytes as well as T cells. The antiviral properties of Tip may be in part related to pro-apoptotic effects of Tip during HIV infection (Hasham and Tsygankov, 2004).

Tip expression prior to viral infection restricted HIV-1 replication in T cells and a monocyte-derived cell line (Fig. 2). In
contrast, Tip expression post-HIV-1 infection restricted HIV-1 replication in monocytes, but to a much lesser degree in T cells. A plausible explanation for these time-dependent effects of Tip-mediated restriction is that the HIV-1 SF162 strain replicates more slowly in U937 than the IIIB strain in MOLT4 (Fig. 2). Transduction of ACH2 with pseudotyped virus encoding Tip reduced reactivation of HIV-1 replication compared to transduction of ACH2 with pseudotyped virus encoding Neo alone (Figs. 3B and D). HIV latency is maintained in ACH2 due to a transcriptional block (Emiliani et al., 1996). Reactivation of latent HIV-1 replication is dependent on Tat in concert with cellular transcription factors such as NF-κB (Brooks et al., 2003). Our finding that Tip inhibits Tat transactivation of reporter gene under control of the HIV-1 LTR is consistent with reactivation being controlled at the level of transactivation. This supports the notion that Tip inhibits transcription factors needed for reactivation of replication competent HIV-1 in ACH2. Consistent with this notion, we show that StpC, which enhances NF-κB activation, significantly increases reactivation of replication-competent HIV-1 in ACH2 (Figs. 3C and E). These studies suggest that Tip and StpC alter the intracellular environment such that the transcription factors necessary for replication are either inhibited or activated, respectively. Tip-mediated restriction of HIV-1 reactivation in ACH2 cells provides proof of the concept that therapeutic strategies based on Tip could potentially prevent HIV-1 replication during acute infection and prevent reactivation of replication-competent reservoirs of HIV-1 in T cells and monocytes. HIV-1 replication in JCaM1.6 cells lacking Lck activity was not restricted by Tip (Fig. 4). These results provide evidence that Lck is integral to the pathway whereby Tip restricts HIV-1 replication in T cells. Consistent with a role for Lck in Tip-mediated restriction of HIV-1 replication, Tip-mediated restriction of HIV-1 replication is restored in JCaM-C1 expressing Lck (Fig. 5B). Tip-induced alterations in signal transduction pathways activated by HIV-1 gp120 have been proposed to be involved in Tip-mediated restriction (Raymond et al., 2004). Lck is integral to T cell receptor signaling pathways and is associated with CD4 the primary receptor for HIV-1 (Weiss, 1993; Weiss and Littman, 1994). The level of Lck expression appears to influence Tip-
mediated inhibition of Tat transactivation (Fig. 6C). Tat is a multifunctional protein with transcriptional and translational effects on the HIV-1 life cycle (Bieniasz et al., 1999; Brand et al., 1997; Feinberg et al., 1991; McMillan et al., 1995). Levels of Lck were inversely related to the ability of Tip to inhibit Tat-mediated transactivation of the LTR (Fig. 6C). Jurkat cells expressing the highest levels of Lck had the least Tip-associated inhibition of Tat-mediated transcription activation of LTR-driven CAT expression. In contrast, JCaM1.6 with the lowest Lck expression and activity had the highest Tip-associated inhibition of Tat-mediated LTR-driven reporter gene expression. The fact that Tip failed to restrict HIV-1 replication in JCaM1.6 yet inhibited LTR-driven reporter gene expression suggests that Tip restricts HIV-1 replication via Lck-dependent and -independent pathways integral to HIV-1 replication. Taken together, these studies show Tip-mediated inhibition of Tat results in inhibition of HIV-1 replication post-integration.

In summary, we demonstrate that independent expression of HVS-encoded Tip restricts HIV replication and reactivation by (1) inhibiting Tat function and (2) activating Stat1 and Stat3 leading to IFN-γ like-antiviral state. The salient point of the data presented here is that Tip restriction of HIV-1 replication through inhibition of Tat and activation of IFN-γ like response may be developed as a prototypic therapeutic strategy to protect immune cells from HIV-1-induced cytopathic effects associated with disease progression.

Materials and methods

Cells

Human T cell lines Jurkat, JCaM1.6, and MOLT4, as well as U937, a monocyte-derived cell line, and the 293T cell line derived from a human renal embryo carcinoma, were obtained from American Tissue Type Culture (ATCC). JCaM1-C1 cells (Lck/JCaM1.6), an engineered variant of JCaM1.6 reconstituted to constitutively express Lck under the control of a tetracycline repressible promoter, were provided by Dr. D. Strauss (Virginia Commonwealth University, Richmond, VA). ACH2, is a T cell clone derived from A3.01 harboring replication competent HIV-1 obtained from the AIDS Research and Reagent Program (Rockville, MD). ACH2, Jurkat, JCaM1.6, JCaM1-C1, MOLT4, and U937 were maintained in RPMI 1640 containing 10% fetal bovine serum, 1% penicillin-streptomycin (CellGro), and gentamicin (1 μg/ml) (GIBCO-BRL) (R10 media). Tetracycline (100 ng/ml) was added to the media of JCaM-C1 to repress Lck expression in the absence (Tet−) or presence (Tet+) of tetracycline (100 ng/ml) was transfected with LTR-CAT, pSVTat72, and pCep4-Tip and harvested 48 h post-transfection. CAT activity in lysates (200 μg total protein) determined by ELISA. *P value < 0.05, Student’s t test.
expression. 293T was grown in DMEM supplemented with 10% fetal bovine serum, and antibiotics as described above for RPMI 1640. Peripheral blood mononuclear cells (PBMCs) were prepared from whole blood and cultured as described (Raymond et al., 2004).

Generation of Tip/neo pseudo-virus

Pseudotyped lentiviral vectors driving the expression of Tip/neo genes were produced as previously described (Hasham and Tsygankov, 2004) and either used immediately or stored at −80 °C. Briefly, 293T was transfected with the appropriate transfer vector (pCTPN or pCPN), packaging and envelope plasmids using calcium phosphate (Promega, Madison WI). Media were replenished 16 h after transfection, and cells were cultured for an additional 60 h, at which time culture supernatants were collected, filtered (0.45 μ), and stored at −80 °C.

Retroviral transduction of CD4+ T cell and monocytic lines to stably express Tip

JCaM1.6-C1 Strauss cells (Straus and Weiss, 1992). Cells were retrovirally transduced as previously described (Hasham and Tsygankov, 2004). Briefly, HIV-1-based retroviral vectors promoting Tip/neo (pCTPN) and corresponding control Neo (pCPN) vectors were transfected into the packaging cell line 293T to generate pseudoviruses containing either the selection marker alone or the Tip transgene along with an antibiotic selection marker. Cells were cloned by antibiotic selection and neomycin-resistant clones subsequent to limiting dilution. Clones expressing neo or Tip/neo were selected using G418 (250 μg/ml). Studies were performed with heterogeneous populations of Tip-positive cells.

Source and propagation of X4 and R5 strains of HIV-1

Transductions, HIV-1 propagation, and infections were carried out in the Biological Safety Level (BSL) 2 facility at Temple University School of Medicine using standard operating precautions. T-tropic (X4) strains (IIIB and RF) and the M-tropic (R5) strains (Ada-M, Ba-L, JRFL, and SF162) were obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, Rockville, MD). X4 strains were propagated in MOLT4 4 cells, whereas primary cultures of normal PBMC were used to propagate R5 strains. Viruses were purified from culture supernatants by ultracentrifugation at 33,000 rpm for 1 h at 4 °C in a Ti60 rotor. Pellets were collected, gently washed in PBS, and suspended in 1 ml complete medium per 50 ml of supernatant. Supernatants

Fig. 9. Phospho-tyrosine Stat1 activation is enhanced in Tip-expressing cells. Western blots and accompanying densitometry of JCaM-C1 cultured in the absence (A) or presence (B) of tetracycline (100 ng/ml) were transfected with pEGFP alone or pCEP4-Tip and pEGFP. JCaM-1 lysates were prepared 24, 36, and 48 h post-transfection for western blot analysis. Nitrocellulose membranes were blotted with tyrosine phospho-specific anti-Stat1 antibody, analyzed, stripped and re-probed with an antibody against total Stat1. Densitometry performed on resulting film using Image J software represents phospho-Stat1 compared to total Stat1. *P value < 0.05, Student’s t test.
were filtered through a 0.8-μm filter and then stored at −70 °C. X4 HIV-1 was titered on SupT1 cells by syncytia formation. Stocks ranged from 10^6 to 10^7 syncytia-forming units per 1 ml. R5 strains were titered by limiting dilutions followed by p24 analysis to determine the highest dilution that resulted in p24 values above background.

Quantification of HIV-1 replication in cell lines transduced to express Tip

Aliquots of infected cell culture supernatants were collected at 7, 10, and 15 days post-infection and stored at −80 °C. Experiments were performed three to five times and the data we present are the mean plus and minus the standard error of the mean. Viral concentrations were determined by p24 capture enzyme-linked immunosorbent assay (ELISA) kit (SAIC-Frederick, Frederick, MD). ELISA performed according to SAIC-Frederick protocol. Briefly, viral particles within infected culture supernatants were lysed with 0.1% Triton X-100 solution for 1 h at 37 °C. Lysates were then placed in wells pre-coated with a monoclonal anti-p24 antibody and incubated for 2 h at 37 °C. Wells were then washed and incubated at 37 °C with anti-p24 antibody (polyclonal) solution (100 μl/well) for 1 h. After washing, a horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody solution was added to each well, and the plates were incubated for 1 h at 37 °C. After incubation, wells were washed, the chromogenic substrate TMB (KPL, Gaithersburg, MD) was added, plates were incubated for approximately 30 min at room temperature, and absorbance at 450 nm determined.

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting of Tip were performed as previously described (Merlo et al., 1998). Briefly, cells expressing Tip were lysed in Tris/NaCl/EDTA (TNE) buffer containing 1% NP40 and 10 μM of aprotonin and leupeptin protease inhibitors. Tip was detected in 293T cells by the immunoblotting of whole cell lysates (100 μg of total protein per lane). Lysates were separated using SDS-PAGE, transferred to nitrocellulose membrane (BioRad, Hercules, CA), and the membrane probed with anti-Tip sera followed by an HRP-labeled anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Tip was then visualized with chemiluminescence using an ECL Plus kit (Amersham). For Lck detection, the monoclonal antibody 3A5 was used (Santa Cruz Biotechnology, Santa Cruz, CA).

Kinase assay

Effect of Tip expression on Lck activity in lymphoid cell lines was detected using the in vitro kinase assay. Cell lysates
containing 0.5–1 mg of total protein were subjected to immunoprecipitation using anti-Tip sera and PANSORBIN. Resulting precipitates were extensively washed and then incubated in kinase buffer containing [γ-32P] ATP (NEN, Boston, MA) for 20 min. Reactions were stopped by adding SDS-PAGE sample buffer. Phosphorylated proteins were separated using SDS-PAGE and visualized by autoradiography.

Expression and activation of Stat1, Stat3, and PKR

Whole cell lysates from transfected cells were prepared as described above. Briefly, cells were lysed in 1% Triton X-100 in Tris/NaCl/EDTA buffer with sodium vanadate (Sigma, St. Louis, MO), NaF, and a protease inhibitor cocktail (Sigma). Equal amounts of total protein (100 μg) measured by DC protein assay (BioRad) were analyzed by western blot. Phosphorylated proteins were separated using SDS-PAGE and visualized by autoradiography.

Expression and activation of protein kinase RNA-dependent (PKR) are increased in cells expressing Tip. (A) Jurkat cells were retrovirally transduced with pCPN (Neo) or pCTPN (Tip) to express the neomycin-resistant gene alone or with Tip. Cells were placed on neomycin selection for 7 days and then infected with RF strain of HIV-1. Culture supernatants and cells were harvested 15 days post-infection. Whole cell lysates prepared, total protein measured by BioRad DC protein assay (100 μg). Total PKR expression as analyzed by western blot. (B) Total PKR expression as determined by densitometry of 11A using ImageJ software. (C) Activated PKR as measured by densitometry of phospho-specific PKR Immunoblot 11A. *P value < 0.05, Student’s t test compared to selection (Neo) control.
SDS-PAGE gels, membranes were also probed with anti-GAPDH (Santa Cruz Biotechnology).

Expression plasmids used for reporter construct studies

Tip and Lck expression plasmids were constructed as previously described (Merlo et al., 1998). Tat expression vector (pSVTat72) and reporter constructs in which expression of the chloramphenicol acetyl transferase (CAT) is driven by the HIV long terminal repeat (LTR-CAT) were obtained from the AIDS Research and Reference Reagent Program (cat#294 and cat#2619, respectively). 293T, Jurkat, JCAM1.6, MOLT4, and JCaM-C1 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or Jurkat Trans-IT (Mirus). Transfection efficiency was normalized by co-transfecting with Luciferase plasmid (pGL3) (Promega) and measuring Luciferase levels in the whole lysates using the Luciferase assay system (Promega, Madison, WI).

Reporter assays—chloramphenicol acetyl transferase (CAT) ELISA

Whole cell lysates of transfected cells were analyzed for CAT concentration using a CAT ELISA performed according to the manufacturer’s (Roche Diagnostics, Nutley, NJ) protocol. Briefly, total protein concentration of whole cell lysates was measured using DC protein assay (BioRad). Cell extracts (200 µg total protein) were added to wells of a CAT ELISA plate coated with anti-CAT antibody covered and incubated at 37 °C for 1 h. Solutions were then removed and the wells washed with 250 µl of washing buffer (five times). Anti-CAT-DIG antibody was then added to each well and the plate covers, and the plates were incubated for 1 h at 37 °C. After incubation, plates were again washed five times with wash buffer, and an anti-DIG-POD antibody solution was added to each well. Plates were incubated at 37 °C for 1 h, after which the anti-DIG-POD antibody solution was removed and the plates were washed five times with wash buffer. POD substrate was added to each well, plates were incubated at room temperature for approximately 10 min, and then absorbance of the samples was measured at 405 nm.

Statistical analysis

Data expressed as the mean ± standard deviation. The difference in HIV replication of cells transduced with either empty (pCPN or pCPP) or Tip/StpC (pCTPN/pCSPP) was assessed using parametric paired Student’s t test comparison. Groups were determined to be different statistically when the P value <0.05. Cells transduced with selection marker gene Neo (pCPN) were directly compared to cells with Tip (pCTPN) while cells transduced with StpC (pCSPP) were compared to cells with only the selection marker Pac (pCPP).

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References


