Herpesvirus Saimiri Terminal Membrane Proteins Modulate HIV-1 Replication By Altering Nef and Tat Functions

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Abstract: Herpesvirus saimiri (HVS)-transformed human T cells expressing terminal membrane proteins (TMPs) tyrosine kinase interacting protein (Tip) and saimiri transformation associated protein strain C (StpC) are highly permissive for R5 and X4 strains of HIV-1. StpC expression enhances replication of R5 and X4 strains of HIV-1 and induces latent reservoirs of replication competent HIV-1 in cell lines derived from T cells or monocytes. Paradoxically Tip expression restricts replication and cytopathic effects of R5 and X4 strains of HIV-1 in T cells and monocytes post-retrotransposition. Understanding the canonical pathways whereby Tip and StpC alter HIV-1 replication may uncover novel therapeutic approaches to HIV-1 infection. Here we show Tip inhibits Tat-mediated transcriptional activation of the long terminal repeat (LTR). Tip mediated inhibition of Tat transactivation is reversed by Nef. Tip also mediates restriction of late-stage replication of HIV-1 by disrupting Nef interaction with lymphocyte-specific protein-tyrosine kinase (Lck) in lipid rafts. Specifically, in the presence of Tip, Lck does not localize to lipid rafts reducing Nef interaction with Lck within the lipid rafts. Finally, the permissive phenotype conferred by StpC is the result of synergy with Tat during transcriptional activation of the HIV-1 LTR. This transcriptional synergy between StpC and Tat requires Lck and NF-κB consensus binding sequences. These findings demonstrate that the HVS TMPs influence transcriptional and post-transcriptional stages in HIV-1 replication. We propose that HVS-encoded TMPs associated with T cell transformation have evolved ability to modulate the replication of competing retroviruses. Gene based approaches utilizing Tip and StpC may provide therapeutic models for treating acute and latent HIV-1 infections, respectively.

Keywords: HVS, HIV-1, StpC, Nef, Tat, TMPs.

INTRODUCTION

Central to AIDS pathogenesis is progressive loss of CD4+ T-lymphocytes in peripheral blood and lymphoid tissues, leading to severe immune dysfunction, opportunistic infections, and neoplasm associated with viral infection. The mechanism underlying CD4+ T cell depletion is unclear, evidence suggests that HIV-1 encoded negative effector of infection (*N*ef*)* plays an integral role. During HIV-1 infection the *nef* gene is transcribed early and abundantly, comprising over 80 percent of viral transcripts [37]. *nef* encodes a 25- to 32-kDa myristoylated protein found within the virion t localizing to plasma membranes, cytosol and nucleus of infected cells [13, 27]. Nef lacks known enzymatic activity; therefore, Nef-associated functions are likely due to interactions with host-cell proteins [16]. Four distinct functions are associated with Nef. First, Nef inhibits apoptosis of infected macrophages and dendritic cells contributing to reservoirs of replication competent HIV-1 [15]. Second, during HIV-1 infection of macrophages Nef activation of Hck kinase induces the production of cytokines and T cell chemoattractants resulting in the attraction, activation and priming of T cells for infection [38, 43, 44]. Virion-associated Nef increases T-cell activation prior to retrotransposition [50] through activation of Lck [5, 17, 40] and IL-2 signaling [20]. Third, Nef down regulates cell surface molecules, CD4, and major histocompatibility complexes (MHC) I and II preventing anti-viral T cell priming [1, 14, 39]. Lastly, Nef down regulates co stimulatory molecules CD28, CD80 and CD86 further preventing T cell priming [10]. Nef also enhances virion infectivity by modulating the lipid composition of the viral envelope and increasing the efficiency of reverse transcription [38, 52]. Overall, the role of Nef in HIV-1 induced pathogenesis is to prolong life of the infected cell, increase viral infectivity, and reduce immunosurveillance.

HVS encodes transforming proteins Tip and StpC, designated terminal membrane proteins (TMPs) to reflect localization of their respective open reading frames (ORF) near viral genomic terminal repeats [8]. Previously, we have demonstrated that the cytopathic effects (CPE) of R5 and X4 strains of HIV are enhanced by StpC expression while paradoxically CPE is restricted by Tip expression [25, 35]. The modification of HIV replication and CPE by Tip and StpC suggests that they interact with host restriction mechanisms or HIV-1-associated virulence factors. In this regard, Tip interacts with Lck, signal transducers and activators of transcription Stats 1 and 3, and Tip associated protein (Tap) [6, 22, 49]. Since Lck and Stats play key roles in T cell signaling and gene expression, restriction of HIV-1 replication may involve Tip interactions with these host proteins. Tip is a type II membrane protein containing a hydrophobic domain located in the C-terminus with a cytoplasmic N-terminus. Tip contains

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two functional Lck-binding domains (LBD) -LBD1 and LBD2, also referred to as the SH3-binding site and the Src-family regulatory site homology regions, respectively [4, 26]. Nef has also been shown to interact with SH3 and SH2 domains of Lck [11]. Lck kinase activity is increased by Tip [21, 30, 31], while several studies have indicated that Nef inhibits Lck kinase activity [11, 18, 19]. StpC, also a type II membrane protein interacts with the Ras GTPase, TRAF-2 and -6 and leads to increase NF-κB activation (reviewed in Brinkmann and Schulz [8]; Tsygankov [45]). Since Tip and StpC interact with or influence host factors integral to HIV-1 replication we designed experiments to determine the role interactions between TMPs and host proteins have in the paradoxical effects of Tip and StpC on HIV-1 replication and reactivation. In this report, we show that Tip restricts late stage HIV replication by disrupting Nef-Lck interaction in lipid rafts resulting in an accumulation of Lck in non-raft fractions. StpC enhances HIV-1 replication by synergizing with Tat to increase transcriptional transactivation of the LTR. The results reported here contribute fundamental knowledge regarding the molecular mechanisms underlying the paradoxical effects of Tip and StpC on HIV-1 replication.

MATERIALS AND METHODS

Cells

Human T cell line Jurkat and the 293T cell line derived from a human renal embryo carcinoma were obtained from American Tissue Type Culture (ATCC). JCaM-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 generously provided by Dr. D. Strauss (Virginia Commonwealth University, Richmond, VA). Jurkat and JCaM-C1 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100μg/ml streptomycin (Mediatech, Herndon, VA), and gentamicin (1μg/ml) (GIBCO-BRL, Carlsbad, CA) (R10 media). Tetracycline (1 μg/ml) was added to the media of JCaM-C1 cells to repress Lck expression 48 hr prior to use. 293T cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics as described above for RPMI 1640.

Generation of Tip/Neo Lentiviral Vectors

Pseudotyped lentiviral vectors encoding the Tip/neo genes were produced as previously described [17] and either used immediately or stored at -80ºC. Briefly, using calcium phosphate (Promega, Madison WI) 293T cells were transfected with the appropriate transfer vector (pCTPN or pCPN), packaging and envelope plasmids. Culture media were replenished 16 hours after transfection, and cells were cultured for an additional 60 hours. Culture supernatants were collected, filtered (0.45μ), and stored at -80ºC.

Transduction of CD4+ T Cell Lines with pCTPN

JCaM-C1 expressing Lck from a tetracycline repressible promoter [42] were retrovirally transduced as previously described [24]. Briefly, HIV-1-based retroviral vectors driving the expression of Tip/neo (pCTPN) and corresponding control Neo (pCPN) vectors were transfected into the packaging cell line 293T to generate lentiviral containing either the selection marker alone or the Tip transgene along with an antibiotic selection marker. JCaM-C1 clones expressing neo or Tip/neo were selected using G418 (250 μg/ml), and resistant clones were obtained by subsequent limiting dilution. Studies were performed with heterogeneous populations of Tip-positive cells.

Source and Propagation of X4 and R5 and Nef Minus 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 cells, whereas primary cultures of normal peripheral blood mononuclear cells (PBMC) were used to propagate R5 strains. Viruses were purified from culture supernatants by ultracentrifugation at 33,000 rpm for 1 h at 4ºC in a Ti60 rotor. Pellets were collected, gently washed in PBS, and re-suspended in 1 ml complete medium per 50 ml of supernatant. Supernatants were filtered through a 0.8-μ filter and then stored at -70ºC. X4 strain was titered on SupT1 cells by syncytia formation. Stocks ranged from 10^6 to 10^7 syncytia-forming units per 1 ml. R5 strain was titered by limiting dilutions followed by p24 analysis to determine the highest dilution resulting in p24 values above background. Nef minus HIV-1 was propagated by transfection of 7.5×10^6 293T cells in 15-cm dishes with 25 μg of pNL4-3 lacking a functional *nef* gene.

Quantification of HIV-1 Replication in Cell Lines Transduced To Express Tip

Infected cell culture supernatants were collected 7 days post-infection and stored at -80ºC. Viral concentrations determined by a p24 capture enzyme-linked immunosorbent assay (ELISA) kit (SAIC-Frederick, Frederick, MD). ELISA performed in accordance with the SAIC-Frederick protocol. Briefly, viral particles within infected culture supernatants were lysed with 0.1% Triton X-100 solution for 1 h at 37ºC. Lysates were placed in wells pre-coated with a monoclonal anti-p24 antibody and incubated for 2 hours at 37ºC. Wells were washed and incubated with anti-p24 antibody (polyclonal) solution (100 μl/well) for 1 hour at 37ºC. Horse radish peroxidase (HRP)-labeled anti-rabbit secondary antibody solution was added to each well after washing and wells were incubated for 1 h at 37ºC. After incubation, wells were washed, the chromogenic substrate TMB (KPL, Gaithersburg, MD) was added to each well, and the plates were incubated for approximately 30 min at room temperature. Absorbance of samples read at 450 nm.

Immunoprecipitation and Western Blot

Immunoprecipitation and Western blotting of Tip were performed as previously described [33]. 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 immmunoblotting of whole cell lysates (100 μg of total protein per lane). Lysates were separated using SDS-PAGE and transferred to nitrocellulose membrane (BioRad, Hercules, CA), and the membrane was probed with anti-Tip sera [33]

followed by an HRP-labeled anti-rabbit secondary antibody (Amersham, Piscataway, NJ). Tip was then visualized using chemiluminescence using an ECL Plus kit (Amersham). For Lck detection the monoclonal antibody 3A5 was used (Santa Cruz Biotechnology, Santa Cruz, CA).

Isolation of Lipid Rafts

Lipid rafts were isolated from 10^8 293T cells lysed in 0.5% Triton X-100-containing buffer as described [2]. Briefly, 1 ml of lysates was adjusted to 40% sucrose and layered with 2 ml of 30% and 1 ml of 5% sucrose and centrifuged at 200,000g for 16 h at 4°C. Raft isolation, immunoprecipitation, and western blot analysis performed on fractions. Fraction #3 and Fractions # 9 and 10 were used for lipid raft and non-raft fractions, respectively. This was confirmed by anti-Flotillin-1 (Santa Cruz Biotechnology, CA) dot blot (not shown). Fractions from the entire gradient were collected, solubilized with 2% Triton X-100 at room temperature for 15 min and were either immunoprecipitated with a Lck polyclonal antibody [33] prior to separation by SDS-PAGE or applied directly to an SDS gel. After SDS-PAGE, samples were analyzed by Western blotting with a rabbit polyclonal for Nef (AIDS Reference and Reagent Program) and Tip [33] and monoclonal anti-Lck antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Expression Plasmids

Tip and Lck expression plasmids were constructed as previously described [25]. To generate a Nef expression plasmid the *nef* gene was excised from transfer vector, pLconnefSN (AIDS Research and Reference Reagent Program, cat# 3297) using EcoR1 and then inserted into a EcoR1 linearlized pCDNA3.1 (Invitrogen, Carlsbad, CA) backbone to yield pNef-Ex. Orientation of *nef* in pNef-Ex was confirmed by Xho1 digest, inappropriate orientation yield a 100bp product while the correct orientation results in a 500 bp product. Tat expression vector (pSVTat72) and reporter constructs in which expression of the chloramphenicol acetyl transferase (CAT) was driven by the complete HIV long terminal repeat (pHIV-CAT) or with mutated NF-κB sequences linked to the CAT gene (pDκB-HIV-CAT) were obtained from the AIDS Research and Reference Reagent Program (cat#294, cat#2619 and cat#2618 respectively). Transient transfection of 293T, Jurkat, JCAM1.6, MOLT4, and JCaM-C1 cells were performed as described [25]. Transfection efficiency was normalized by co-transfecting with luciferase plasmid (pGL3) (Promega, Madison WI) and measuring luciferase levels in the whole lysates using the Luciferase assay system (Promega, Madison, WI).

Reporter Gene Expression Assays - Chloramphenicol Acetyl Transferase (CAT) ELISA

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

Statistical Analysis

Statistical significance ($P < 0.05$) was determined using unpaired Student's *t*-Test comparisons between cells transduced with either empty or Tip/StpC vectors.

RESULTS

Replication of an HIV-1 Nef Deletion Mutant is Not Restricted by Tip

Tip expression restricts HIV-1 replication post-integration in T cells [25, 35]. A HIV-1 *nef* deletion mutant was used to determine Nef's role in Tip-mediated restriction of HIV-1 replication. JCaM-C1, which expresses Lck under control of a tetracycline repressible promoter, was transduced with pCPN (Neo) or pCTPN (Tip/Neo). Replication of wild-type pNL4-derived HIV-l, but not pNL4 with *nef* deleted was restricted in JCaM-C1 transduced with pCTPN in the presence of Lck (Fig. **1A**). In the absence of Lck, Tip did not restrict replication of pNL4-derived wild-type or pNL4 with *nef* deleted (Fig. **1B**). These results suggest that Nef and Lck are integral to Tip-mediated restriction of HIV-1 replication. Interestingly, the effects of Tip on the replication of wild-type and Nef-deficient virus were opposite (Fig. **1A**). It is possible, therefore, that Tip has a potential to increase HIV-1 replication, for example, through activation of Lck [6, 31], Stats [32] and/or NF-AT [34], but in the presence of Nef, the major effect of Tip on HIV-1 is Nef-dependent.

Tip Inhibits Nef-Mediated Lck Translocation to Lipid Rafts

Tip and Nef have been shown to interact with Lck and affect it's kinase activity [11, 18, 31, 47]. To determine whether Tip would disrupt Nef interaction with Lck vectors driving the expression of Nef and Tip were used to co-express Nef and Tip (Fig. **2A**, lanes 1-3). Transfection of 293T with pLck, pNef-Ex, and pCep4-Tip expression plasmids followed by anti-Lck immunoprecipitation demonstrate that Tip and Lck interact directly in the whole cell lysates (Fig. **2A**, lane 9). As expected from these results anti-Tip immunoprecipitation also showed interaction with Lck (Fig. **2A**, lane 10). In contrast, anti-Nef immunoprecipitation clearly showed that Nef does not interact with either Tip or Lck in the whole cell lysate (Fig. **2A**, lane 8). Analysis of lipid rafts from 293T transfected with pLck and pNef-Ex or with pNef-Ex, pLck, and pCep4-Tip show that Nef-Lck interaction occurs within the lipid rafts in the absence of Tip (Fig. **2B**, lane 3). In non-raft fractions, Tip and Lck interact as observed in whole cell lysates (Fig. **2B**, lane 6). Interestingly, Lck localization to lipid rafts is dramatically reduced

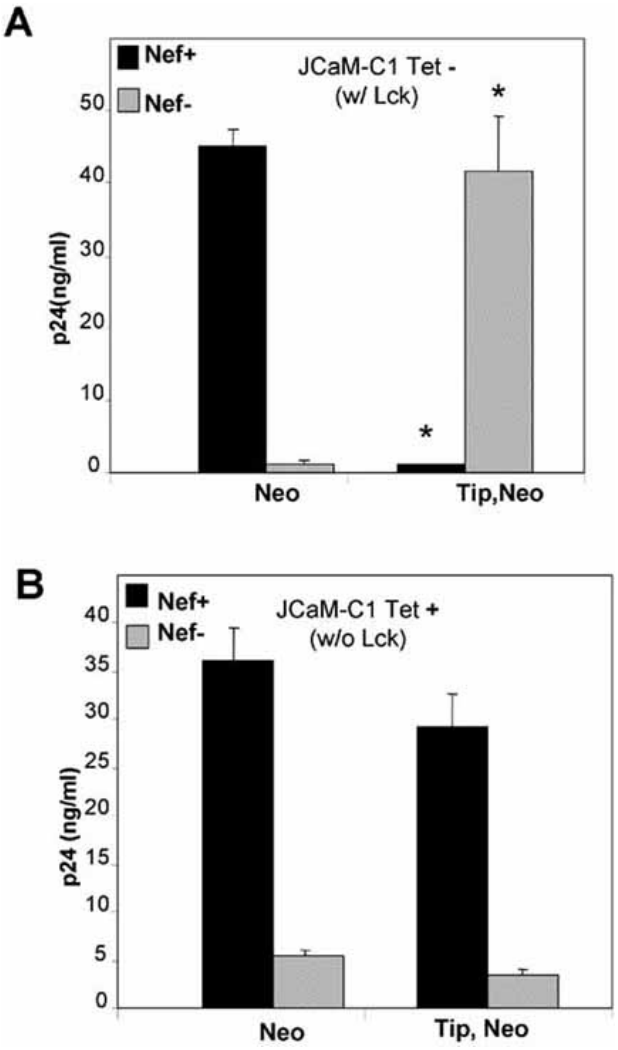

Fig. (1). Tip rescues *nef* minus HIV-1 as measured by release of virus. JCaM-C1 expressing Lck under the control of a tetracycline repressible promoter cells were retrovirally transduced to express Tip/Neo(pCTPN) or neo(pCPN) alone, placed on selection for 7 days and then infected with pNL4-derived wild type strain of HIV-1(solid bar) or a pNL4-derived *nef* deletion mutant (hatched bars) in the absence (A) or presence (B) of tetracycline. Culture supernatant were collected 7 days post infection and viral infection monitored by p24 capture ELISA. **p-value* < 0.05, Student's T-test.when Tip is expressed interfering with Nef ability to interact with Lck in lipid rafts (Fig. **2B**, lanes 1-3). These results show that Tip abrogates Lck-Nef interaction in lipid rafts resulting in retention and accumulation of Lck in non-raft fractions (Fig. **2B**, lanes 5 and 6).

Nef Reverses Tip-Mediated Inhibition of Tat HIV-1 LTR Transactivation

Tip restricts Tat-mediated transactivation of the LTR [36]. Since Nef has been shown to indirectly up-regulate HIV-1 replication [46, 51], we wanted to examine the effect of Nef on Tip inhibition of Tat transactivation of the HIV LTR. We co-expressed Nef and Tip in 293T cells containing a pHIV-CAT construct. Cells co-transfected with either pSV*tat*72 and pCEP4-*tip* or pSV*tat*72 alone show that Tip inhibits Tat-mediated transactivation of the LTR (Fig. **3**, # 1-3). In cells co-transfected with pNef-ex the ability of Tip to inhibit Tat transactivation of the LTR was reduced (Fig. **3**, # 4). These results provide evidence that Nef reverses Tip-mediated inhibition of Tat transactivation of the LTR (Fig. **3**, lane 5).

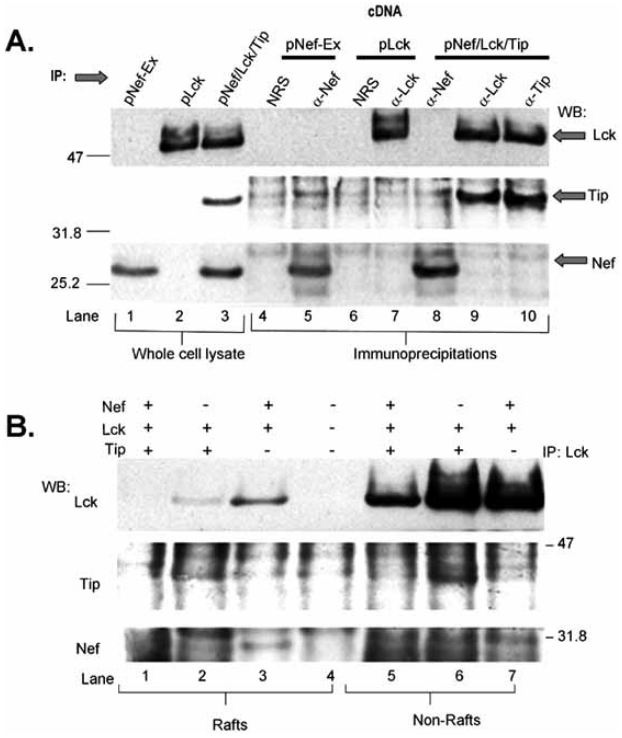

Fig. (2). Nef does not form a complex with Lck in cytosol. (A) Immunoblotting of 293T cells transfected with Nef (lanes1, 4 and 5) or Lck(lanes 2, 6 and 7) expression plasmids, or all three plasmids Nef, Tip, and Lck (lanes 3, 8, 9 and 10) harvested 48 hours post transfection and lysates subjected to immunoprecipitation and western blot analysis. (B**) Tip prevents Nef and Lck interaction in lipid rafts.** WB-Western Blot, IP-Immunoprecipitation. Raft isolations and Lck immunoprecipitation were performed on lysates from 293T cells transiently transfected with Nef, Tip and Lck expression plasmids. Cells were harvested 48 hours post transfection. Raft isolation, immunoprecipitation, and western blot analysis performed on fractions. Fraction #3 (raft) and Fraction # 9 and 10 (non-raft) used in analysis raft confirmed by anti-Flotillin-1(Santa Cruz Biotechnology, CA) dot blot (not shown).

NF-κB Consensus Binding Sequences within the HIV-1 LTR are Required to Enhance HIV-1 Replication

StpC activates the transcription factor NF-κB [29, 34], and the HIV-1 LTR contains NF-κB consensus binding sequences suggests the presence of a mechanism whereby the HVS encoded type II TMP could enhance HIV-1 replication. To determine the effects of StpC on HIV-1 LTR-driven transcription and the role of NF-κB, Jurkat cells were co-transfected with CAT reporter construct linked to an HIV-1 LTR with (pHIV-CAT) or without (pDκB-HIV-CAT) NF-κB consensus sequence binding sites, along with (pSVTat72)(Tat) and pSIC2 (StpC). As expected Tat enhances LTR-driven reporter gene transcription independent

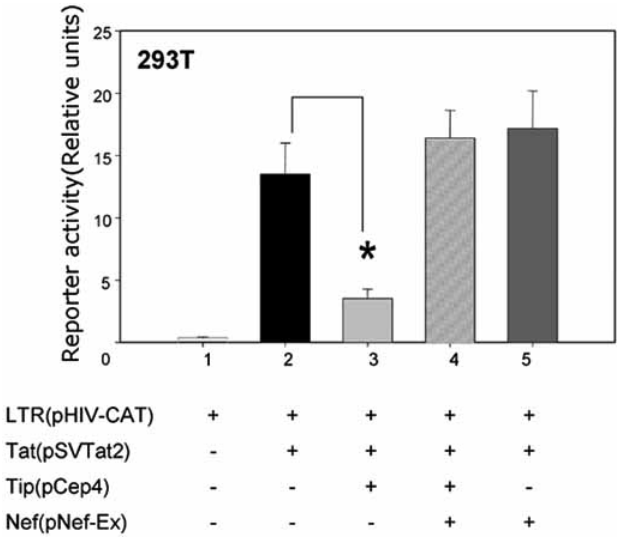

Fig. (3). Nef reverses Tip-mediated inhibition of Tat transactivation. Chloramphenicol acetyltransferase activity (CAT) of 293T cells transfected with PHIV-CAT reporter construct and pGL3 (Luciferase for normalization of transfection efficiency), Tat (pSVTat72), Tip (pCEP4-Tip) and Nef (pNef-EX) expression plasmids. Cells were harvested 48 hours post transfection and lysates assayed for CAT concentration using CAT ELISA and CAT values normalized to Luciferase activity. *p-value < 0.05, Student's t-Test.

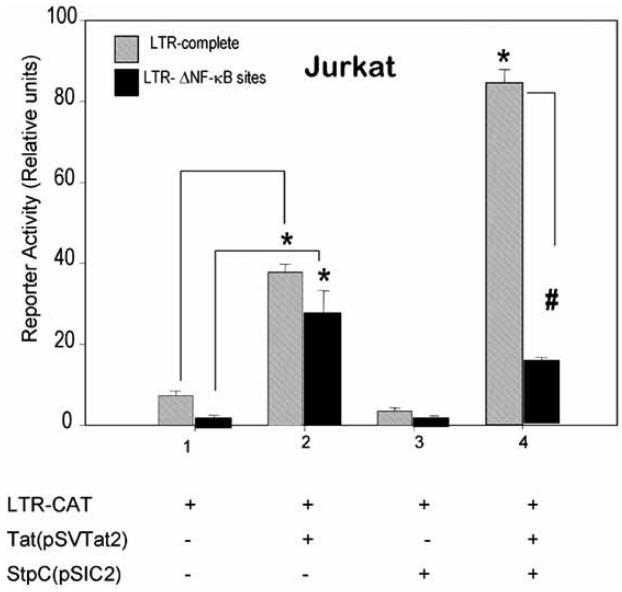

Fig. (4). StpC enhancement of Tat transactivation of the HIV LTR is NF-κB binding sequence dependent. Jurkat cells were transfected with PHIV-CAT reporter construct with or without NF-κB sites along with expression plasmids for StpC (pSIC2) and Tat (pSVTat72). Cells were harvested 48 h post-transfection and CAT activity determined by ELISA and normalized to Luciferase activity. Hatched bars complete LTR solid bars LTR without NF-kB consensus binding site *p-value<0.05, Student's T-test.

of the NF-κB binding site (Fig. **4**, # 2). Interestingly StpC synergized with Tat to transactivate the LTR containing a complete NF-κB binding site (pHIV-CAT) (Fig. **4**, # 4). However, in the absence of a complete LTR NF-κB binding site (pDκB-HIV-CAT) StpC failed to synergize with Tat to transactivate the LTR (Fig. **4**, # 4).

Synergy of StpC and Tat in Enhancing HIV-1 LTR Transactivation is Lck-Dependent

The role of Lck in StpC-mediated effects on LTR-driven transcription was analyzed in JCaM-C1 (a cell line expressing Lck under a tetracycline repressible promoter) transfected with pHIV-CAT along with expression plasmids for Tat (pSVTat72) and StpC (pSIC2). StpC enhanced Tat mediated transcription in the presence of Lck (Fig. **5A**, # 4). In contrast StpC does not enhance Tat mediated transactivation of the LTR in T cells in the absence of Lck expression, suggesting that synergy between StpC and Tat to enhance LTR-driven transcription is Lck-dependent (Fig. **5B**, # 4).

DISCUSSION

HVS-encoded Tip and StpC have paradoxical effects on HIV-1 replication [25]. T cells that have been transduced to express Tip produce significantly lowered levels of HIV-1 and exhibit reduced CPE [35]. Previously we have shown Tip restricts HIV-1 replication by inhibiting Tat transactivation of the LTR [36]. Here we show (1) Tip does not restrict HIV-1 replication as measured by release of virus in T cells without Lck; (2) since progeny virus is produced primarily from transcribed provirus, this suggests that Tip may also restrict a post-transcriptional step that is Lck-dependent and that this effect of Tip is more important in its overall effect. Here we demonstrate that replication HIV-1 with *nef* deleted is not restricted by Tip, but is actually enhanced (Fig. **1A**) providing evidence for a role for Nef in Tip-mediated restriction of late-stage replication. The HIV-1-encoded *nef* gene is one of the crucial determinants of disease progression [7, 9, 12, 28]. Tip targets an important molecular marker for HIV-1-induced disease progression and may as a result occupy a unique position as a therapeutic strategy.

Tip [23, 31, 48] and Nef [3, 11, 18] interact with SH2 and SH3 domains of Lck kinase, suggesting that Tip and Nef could compete for Lck binding. We show that Nef-Lck and Tip-Lck interactions occur in different cellular compartments (lipid rafts vs. whole cell lysates) and expression of Tip abrogates Nef-Lck lipid interactions in lipid rafts (Fig. **2A** and **B**). Nef controls the activation state of the infected cell, improves efficiency of reverse transcription, and down-regulates expression of key target molecules including CD4 and MHC I to increase infectivity, pathogenicity and to evade immunosurveillance. It would be predicted that Tip-mediated inhibition of Nef interaction with Lck would restrict HIV-1 replication and cytopathic effect. We show that Nef has the capacity to compensate for Tip-mediated inhibition of the Tat-transactivation of the LTR (Fig. **3**). Tip ability to antagonize Nef suggests that these gamma herpes virus TMPs may have evolved a secondary role to restrict replication of competing non-complementary viruses. The functions of Tip could be developed as a therapeutic strategy to restrict acute HIV infection and suggest that targeting Nef may significantly restrict HIV-1 replication. Since R5 strains of

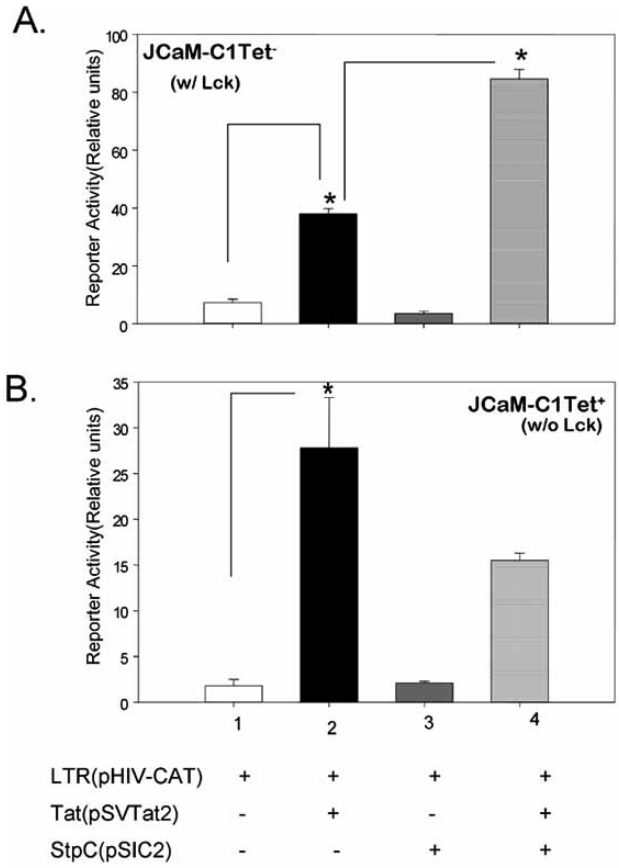

Fig. (5). StpC ability to synergize with Tat to transactivation the HIV-1 LTR is Lck dependent JCaM-C1 cells cultured in the absence (A) or presence (B) of tetracycline, were transfected with PHIV-CAT reporter construct and expression plasmids for StpC (pSIC2), and Tat(pSVTat72). Cell were harvested 48 hours post-transfection and CAT concentrations in lysates were measured by CAT ELISA. *p-value<0.05, Student's T-test.

HIV-1 are responsible for transmission and dominate during the early stages of infection and give rise to X4 syncytia inducing HIV-1, the ability of Tip to restrict replication of R5 strains of HIV-1 adds to the attractiveness of Tip as a gene based therapy. StpC, on the other hand, promotes HIV-1 replication and CPE. In this report, using reporter construct studies in Jurkat cells we have shown that increase in HIV-1 replication by StpC is due to an increase in Tat-mediated transcription of the HIV-1 LTR that is both NF-κB-consensus binding site and Lck-dependent (Figs. **4** and **5**). It is likely that StpC-mediated activation of NF-κB transcription factor is responsible for StpC-Tat synergy since the deletion of NF-κB binding sites within the LTR abrogated the ability of StpC to synergize with Tat to increase LTR-driven transcription. We have previously shown that StpC enhanced HIV replication independently of viral tropism in T cells and macrophages [35]. Therefore, these studies suggest that two distinct mechanisms, one dependent on NF-κB and the other dependent on Lck, may mediate the effect of StpC on viral replication. Alternatively, Lck may be essential for the effect of StpC on NF-κB in T cells, but it may be substituted by another specific protein tyrosine kinase in monocytes. Our previous results indicating that the effect of StpC on NF-κB activity in 293T cells is greatly up-regulated by Lck, but not by Src [41] argues in favor of the second explanation.

Overall, the results presented in this study suggest that Tip and StpC, in addition to inducing a T cell environment conducive to HVS survival, may restrict retroviral infection and latency in the host cell, preventing detrimental retroviral parasitism. This feature of Tip and StpC may eventually be harnessed for practical purposes. Highly active anti-retroviral therapy (HAART) eventually becomes ineffective due to resistance caused by the high mutation rate of HIV-1. Furthermore only actively replicating virus is targeted by HAART, allowing latent reservoirs of replication competent virus to persist. The ability of StpC to up-regulate HIV-1 replication *via* enhancing proviral transcription in T cells and macrophage suggests that it could be developed as a therapeutic strategy to reactivate latent HIV-1, improving the ability of HAART to eliminate reservoirs of replication competent HIV-1. In summary this study reveals aspects of the molecular mechanism(s) involved in the dichotomous effects of HVS-transforming proteins Tip and StpC on HIV-1 replication and points to new viral targets to treat acute and latent HIV-1 infection.

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REFERENCES

[1] Aiken C, Konner J, Landau NR, Lenburg ME, Trono D. (1994). Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell. 76: 853-864.

[2] Alexander M, Bor YC, Ravichandran KS, Hammarskjold ML, Rekosh D. (2004). Human immunodeficiency virus type 1 Nef associates with lipid rafts to downmodulate cell surface CD4 and class I major histocompatability complex expression and to increase viral infectivity. Journal of Virology. 78: 1685-1696.

[3] Arold S, O'Brien R, Franken P, Strub MP, Hoh F, Dumas C, Ladbury JE. (1998). RT loop flexibility enhances the specificity of Src family SH3 domains for HIV-1 Nef. Biochemistry. 37: 14683-14691.

[4] Bauer F, Hofinger E, Hoffmann S, Rosch P, Schweimer K, Sticht H. (2004). Characterization of Lck-binding elements in the herpesviral regulatory Tip protein. Biochemistry. 43: 14932-14939.

[5] Baur AS, Sawai ET, Dazin P, Fantl WJ, Cheng-Mayer C, Peterlin, BM. (1994). HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. Immunity. 1: 373-384.

[6] Biesinger B, Tsygankov AY, Fickenscher H, Emmrich F, Fleckenstein B, Bolen JB, Broker BM. (1995). The product of the Herpesvirus saimiri open reading frame 1 (tip) interacts with T cell-specific kinase p56lck in transformed cells. Journal of Biological Chemistry. 270: 4729-4734.

[7] Brambilla A, Turchetto L, Gatti A, Bovolenta C, Veglia F, Santagostino E, Gringeri A, Clementi M, Poli G, Bagnarelli P, Vicenzi E. (1999). Defective nef alleles in a cohort of hemophiliacs with progressing and nonprogressing HIV-1 infection. Virology. 259: 349-368.

[8] Brinkmann MM, Schulz TF. (2006). Regulation of intracellular signalling by the terminal membrane proteins of members of the Gammaherpesvirinae. Journal of General Virology. 87: 1047-1074.

[9] Carl S, Greenough TC, Krumbiegel M, Greenberg M, Skowronski J, Sullivan JL, Kirchhoff F. (2001). Modulation of different human immunodeficiency virus type 1 Nef functions during progression to AIDS. Journal of Virology. 75: 3657-3667.

[10] Chaudhry A, Das SR, Hussain A, Mayor S, George A, Bal V, Jameel S, Rath S. (2005). The Nef protein of HIV-1 induces loss of cell surface costimulatory molecules CD80 and CD86 in APCs. Journal of Immunology. 175: 4566-4574.

[11] Collette Y, Dutartre H, Benziane A, Ramos M, Benarous R, Harris M, Olive D. (1996). Physical and functional interaction of Nef with Lck. HIV-1 Nef-induced T-cell signaling defects. Journal of Biology Chemistry. 271: 6333-6341.

[12] Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellett A, Chatfield C, Lawson VA, Crowe S, Maerz A, Sonza S, Learmont J, Sullivan JS, Cunningham A, Dwyer D, Dowton D, Mills J. (1995). Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science. 270: 988-991.

[13] Franchini G, Robert-Guroff M, Ghrayeb J, Chang NT, Wong-Staal F. (1986). Cytoplasmic localization of the HTLV-III 3' orf protein in cultured T cells. Virology. 155: 593-599.

[14] Garcia JV, Alfano J, Miller AD. (1993). The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. Journal of Virology. 67: 1511-1516.

[15] Geleziunas R, Xu W, Takeda K, Ichijo H, Greene WC. (2001). HIV-1 Nef inhibits ASK1-dependent death signaling providing a potential mechanism for protecting the infected host cell. Nature. 410: 834-838.

[16] Geyer M, Fackler OT, Peterlin BM. (2001). Structure--function relationships in HIV-1 Nef. EMBO Reports. 2: 580-585.

[17] Greenway A, Azad A, McPhee D. (1995). Human immunodeficiency virus type 1 Nef protein inhibits activation pathways in peripheral blood mononuclear cells and T-cell lines. Journal of Virology. 69: 1842-1850.

[18] Greenway A, Azad A, Mills J, McPhee D. (1996). Human immunodeficiency virus type 1 Nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity. Journal of Virology. 70: 6701-6708.

[19] Greenway AL, Dutartre H, Allen K, McPhee DA, Olive D, Collette Y. (1999). Simian immunodeficiency virus and human immunodeficiency virus type 1 nef proteins show distinct patterns and mechanisms of Src kinase activation. Journal of Virology 73: 6152-6158.

[20] Greenway AL, McPhee DA, Grgacic E, Hewish D, Lucantoni A, Macreadie I, Azad A. (1994). Nef 27, but not the Nef 25 isoform of human immunodeficiency virus-type 1 pNL4.3 down-regulates surface CD4 and IL-2R expression in peripheral blood mononuclear cells and transformed T cells. Virology. 198: 245-256.

[21] Hartley DA, Amdjadi K, Hurley TR, Lund TC, Medveczky PG, Sefton BM. (2000). Activation of the Lck tyrosine protein kinase by the Herpesvirus saimiri tip protein involves two binding interactions. Virology. 276: 339-348.

[22] Hartley DA, Cooper GM. (2000). Direct binding and activation of STAT transcription factors by the herpesvirus saimiri protein tip. Journal of Biology Chemistry. 275: 16925-16932.

[23] Hartley DA, Hurley TR, Hardwick JS, Lund TC, Medveczky PG, Sefton BM. (1999). Activation of the lck tyrosine-protein kinase by the binding of the tip protein of herpesvirus saimiri in the absence of regulatory tyrosine phosphorylation. Journal of Biology Chemistry. 274: 20056-20059.

[24] Hasham MG, Tsygankov AY. (2004). Tip, an Lck-interacting protein of Herpesvirus saimiri, causes Fas- and Lck-dependent apoptosis of T lymphocytes. Virology. 320: 313-329.

[25] Henderson EE, Tsygankov AY, Merlo JJ, Romano G, Guan M. (1999). Altered replication of human immunodeficiency virus type 1 (HIV-1) in T cell lines retrovirally transduced to express Herpesvirus saimiri proteins StpC and/or Tip. Virology. 264: 125-133.

[26] Jung JU, Lang SM, Friedrich U, Jun T, Roberts TM, Desrosiers RC, Biesinger B. (1995). Identification of Lck-binding elements in tip of herpesvirus saimiri. Journal of Biology Chemistry 270: 20660-20667.

[27] Kaminchik J, Bashan N, Itach A, Sarver N, Gorecki M, Panet A. (1991). Genetic characterization of human immunodeficiency virus type 1 nef gene products translated *in vitro* and expressed in mammalian cells. Journal of Virology. 65: 583-588.

[28] Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. (1995). Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. New England Journal of Medicine. 332: 228-232.

[29] Lee H, Choi JK, Li M, Kaye K, Kieff E, Jung JU. (1999). Role of cellular tumor necrosis factor receptor-associated factors in NF-kappaB activation and lymphocyte transformation by herpesvirus Saimiri STP. Journal of Virology. 73: 3913-3919.

[30] Lund T, Medveczky MM, Geck P, Medveczky PG. (1995). A herpesvirus saimiri protein required for interleukin-2 independence is associated with membranes of transformed T cells. Journal of Virology. 69: 4495-4499.

[31] Lund T, Medveczky MM, Medveczky PG. (1997). Herpesvirus saimiri Tip-484 membrane protein markedly increases p56lck activity in T cells. Journal of Virology. 71: 378-382.

[32] Lund TC, Garcia R, Medveczky MM, Jove R, Medveczky PG. (1997). Activation of STAT transcription factors by herpesvirus Saimiri Tip-484 requires p56lck. Journal of Virology. 71: 6677-6682.

[33] Merlo JJ, Romano G, Gordon SS, Feshchenko EA, Peng G, Henderson EE, Tsygankov AY. (1998). Human T cells transduced by a retroviral vector to express Herpesvirus saimiri proteins TIP and STPC. Anticancer Research. 18: 2389-2396.

[34] Merlo JJ, Tsygankov AY. (2001). Herpesvirus saimiri oncoproteins Tip and StpC synergistically stimulate NF-kappaB activity and interleukin-2 gene expression. Virology. 279: 325-338.

[35] Raymond AD, Hasham MG, Tsygankov AY, Henderson EE. (2004). Herpesvirus saimiri-encoded proteins Tip and StpC modulate human immunodeficiency virus type 1 replication in T-cell lines and lymphocytes independently of viral tropism. Virology. 324: 60-66.

[36] Raymond AD, Hasham MG, Tsygankov AY, Henderson EE. (2006). H. saimiri tyrosine-kinase interacting protein inhibits Tat function: A prototypic strategy for restricting HIV-induced cytopathic effects in immune cells. Virology. 352: 253-267.

[37] Robert-Guroff M, Popovic M, Gartner S, Markham P, Gallo RC, Reitz MS. (1990). Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. Journal of Virology. 64: 3391-3398.

[38] Schwartz O, Marechal V, Danos O, Heard JM. (1995). Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. Journal of Virology. 69: 4053-4059.

[39] Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. (1996). Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nature Medicine. 2: 338-342.

[40] Simmons A, Aluvihare V, McMichael A. (2001). Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. Immunity. 14: 763-777.

[41] Sorokina EM, Merlo JJ, Tsygankov AY. (2004). Molecular mechanisms of the effect of herpesvirus saimiri protein StpC on the signaling pathway leading to NF-KappaB activation. Journal of Biological Chemistry. 279: 13469-13477.

[42] Straus DB, Weiss A. (1992). Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell. 70: 585-593.

[43] Swingler S, Brichacek B, Jacque J-M, Ulich C, Zhou J, Stevenson M. (2003). HIV-1 Nef intersects the macrophage CD40L signaling pathway to promote resting-cell infection. Nature. 424: 213-219.

[44] Swingler S, Mann A, Jacque J, Brichacek B, Sasseville VG, Williams K, Lackner AA, Janoff EN, Wang R, Fisher D, Stevenson M. (1999). HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nature Medicine. 5: 997-103.

[45] Tsygankov AY. (2005). Cell transformation by Herpesvirus saimiri. Journal of Cell Physiology. 203: 305-318.

[46] Varin A, Manna SK, Quivy V, Decrion AZ, Van Lint C, Herbein G, Aggarwal BB. (2003). Exogenous Nef protein activates NF-kappa B, AP-1, and c-Jun N-terminal kinase and stimulates HIV transcription in promonocytic cells. Role in AIDS pathogenesis. Journal of Biological Chemistry. 278: 2219-2227.

[47] Weiss A. (1993). T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. Cell. 73: 209-212.

[48] Wiese N, Tsygankov AY, Klauenberg U, Bolen JB, Fleischer B, Broker BM. (1996). Selective activation of T cell kinase p56lck by Herpesvirus saimiri protein tip. Journal of Biological Chemistry. 271: 847-852.

[49] Williams BJ, Boyne JR, Goodwin DJ, Roaden L, Hautbergue GM, Wilson SA, Whitehouse A. (2005). The prototype gamma-2 herpesvirus nucleocytoplasmic shuttling protein, ORF 57, transports viral RNA through the cellular mRNA export pathway. Biochemistry Journal. 387: 295-308.

[50] Wu Y, Marsh JW. (2001). Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. Science. 293: 1503-1506.

[51] Zheng YH Plemenitas A, Fielding CJ, Peterlin BM. (2003). Nef increases the synthesis of and transports cholesterol to lipid rafts and HIV-1 progeny virions. Proceedings of the National Academy of Sciences, United States of America. 100: 8460-8465.

[52] Zhou J, Aiken C. (2001). Nef enhances human immunodeficiency virus type 1 infectivity resulting from intervirion fusion: evidence supporting a role for Nef at the virion envelope. Journal of Virology. 75: 5851-5859.

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