Selective targeting of ITK blocks multiple steps of HIV replication

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Edited by Stephen P. Goff, Columbia University College of Physicians and Surgeons, New York, NY, and approved March 19, 2008 (received for review October 10, 2007)

Treatment for HIV has relied on the use of antiretroviral agents that can be subject to the development of resistant viruses. The study of inhibitors directed against cellular proteins required for HIV replication is therefore of growing interest. Inducible T cell kinase (ITK) is a Tec family tyrosine kinase that regulates T cell receptor (TCR)-induced activation of PLCγ-1, Ca^{2+} mobilization and transcription factor activation, and actin rearrangement downstream of both TCR and chemokine receptors. Because productive infection of T cells with HIV requires T cell activation, chemokine receptors and actin reorganization, we asked whether ITK affects HIV infection using ITK-specific siRNA, a kinase-inactive ITK mutant or an ITK inhibitor. We demonstrate that loss of ITK function resulted in marked reductions in intracellular p24 levels upon HIV infection. Loss of ITK function after establishment of HIV infection also decreased virus spread within the culture. Inhibition of ITK did not affect expression of the HIV coreceptors CD4 or CXCR4 but partially blocked HIV viral entry, an effect that correlated with decreased actin polarization to gp120. Additionally, ITK was required for efficient HIV transcription, and overexpression of ITK increased both viral transcription and virus-like particle formation. Our data suggest that inhibition of ITK blocks HIV infection by affecting multiple steps of HIV replication.

T cell signaling | transcription | tyrosine kinase | viral entry | kinase inhibitors

The establishment and persistence of HIV infection depend on events associated with T cell activation. T cell activation influences integration and transcription of the HIV genome (1) and expression of HIV in latent cellular reservoirs. Furthermore, the redistribution of CD4 and the chemokine receptors CXCR4 and CCR5 and the rearrangement of the cytoskeleton via Rac-dependent pathways are required for efficient virus entry and egress (2–5). It may therefore be expected that signaling events that coordinate these different cellular processes would be required for efficient viral replication. Targeting these pathways could provide potential therapeutic strategies for repressing HIV-1 replication in different cellular reservoirs.

Inducible T cell kinase (ITK) is a Tec family tyrosine kinase that plays a critical role in integrating pathways important for HIV replication (6). ITK is expressed in a limited number of cell types, including T cells, NK cells, and mast cells, and is important for TCR-mediated activation of T cells, where it participates in regulation of PLCγ-1, Ca^{2+} mobilization, and downstream activation of transcription factors (6). T cells from ITK-deficient mice show decreases in IL-2 production and proliferation and defects in T_H2 cytokine production (6). Recent data have also implicated ITK in the regulation of actin rearrangement downstream from both the TCR and chemokine receptors (6–8). Intriguingly, the effects of ITK on TCR-induced actin polarization appear to be kinase-independent and instead require the function of the Src-Homology 2 (SH2) protein interaction domain and the pleckstrin homology (PH) domain, which is important for interactions with phospholipids and membrane targeting (9). ITK-deficient cells also have defects in migration, actin polarization, and activation of Rac1 and Cdc42 in response to chemokines including SDF1α, the ligand for CXCR4, a coreceptor for HIV (7, 8). Nonetheless, ITK-deficient mice are still able to mount immune responses to viral infections, albeit delayed, suggesting that many immune functions are intact in the absence of ITK (10).

The ability of ITK to coordinate cytoskeleton reorganization and signaling downstream from the TCR and chemokine receptors suggests that ITK could be a critical factor in regulating HIV infection and replication in T cells. To evaluate this possibility, we blocked ITK expression or function using siRNA, a chemical inhibitor, or a dominant-negative mutant and examined the effects on HIV replication. Our results suggest that inhibition of ITK blocks HIV replication by affecting multiple stages of the HIV life cycle, including viral entry, transcription from the viral LTR, and virion assembly/release.

Results

ITK Is Required for HIV Infection. To assess whether ITK affects HIV replication, we transfected activated primary human $CD4^+$ T cells with siRNA specific for ITK (siITK) (9) or a control siRNA directed against GFP (siGFP). Twenty-four hours after siRNA transfection, when ITK expression was decreased, cells were infected with replication-competent NL43 virus, and infection was monitored by flow cytometry of intracellular p24 (Fig. 1*a*). Treatment with siITK did not alter cell viability (data not shown). However, siITK-treated cells exhibited decreased intracellular p24 compared to siGFP-treated cells. Similar reductions in virus replication were obtained in siITK-treated Jurkat cells [supporting information (SI) Fig. S1].

Because the effects of siITK are transient, we synthesized a published chemical inhibitor of ITK, BMS509744 (11), to eval-

Author contributions: J.A.R. and G.M.S. contributed equally to this work; J.A.R., G.M.S., J.-K.J., C.J.T., A.A., A.J.H., and P.L.S. designed research; J.A.R. and G.M.S. performed research; J.-K.J. and C.J.T. contributed new reagents/analytic tools; J.A.R., G.M.S., A.J.H., and P.L.S. analyzed data; and J.A.R., G.M.S., A.J.H., and P.L.S. wrote the paper.

Conflict of interest statement: A patent has been filed for ITK (patent application: E-151-2006/0-US-01; NIHA-0291, filed March 27, 2007).

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0709659105/DCSupplemental.

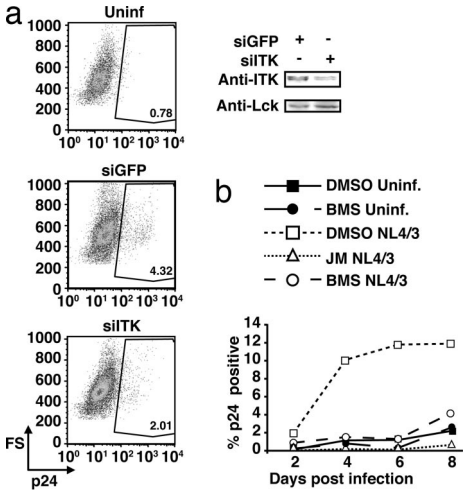

Fig. 1. ITK is required for efficient HIV replication. (*a*) Activated primary human CD4$^+$ T cells were transiently transfected with siRNA against GFP or ITK. Twenty-four hours after transfection, cells were infected with replication-competent NL43 virus. Two days later, infection was measured by flow cytometry, staining for intracellular p24. Immunobloting was performed on whole-cell lysates 24 h after transfection. Data are one representative of three individual experiments. (*b*) Primary CD4$^+$ T cells were stimulated with PMA (10 ng/ml), PHA (2 μg/ml), and IL-2 (20 units/ml), 2 d before assay, then incubated with DMSO (vehicle control) (1 μM) JM2987, or (10 μM) BMS509744 (ITK inhibitor) for 30 min before addition of replication competent NL4/3. Cells were incubated for the indicated number of days and infection measured by intracellular p24. Data are from one representative experiment of three individual experiments using cells from three separate donors.

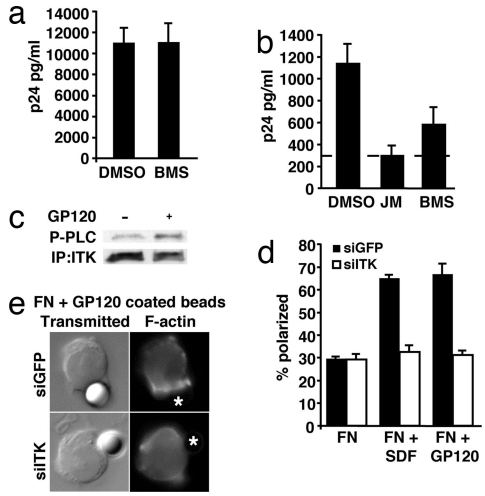

Fig. 2. Loss of ITK affects virus entry. Activated primary human CD4$^+$ T cells incubated with DMSO (1 μM) JM2987, or (10 μM) BMS509744 for 30 min were infected with replication competent NL4/3. (*a*) Binding was measured at 3 h postinfection by washing cells extensively, then lysing and analyzing p24 by ELISA. (*b*) Entry was measured at 5 h after infection for CD4$^+$ T cells by trypsinizing cells and then washing an additional five times in PBS before lysing and measuring intracellular p24 protein levels by ELISA. Values +/− SEM are from one representative experiment with $n = 3$ of three individual experiments from three separate donors. (*c*) Jurkat cells were treated with 5 μg/ml gp120 and lysed after 1 min at 37°C. ITK was immunoprecipitated and assayed for *in vitro* kinase activity using a GST-PLC-γ substrate. Data are from one of three similar experiments. (*d* and *e*) Jurkat cells were transiently transfected with siRNA against GFP or ITK for 24 h. Cells were conjugated to beads coated with fibronectin (FN), FN and SDF (20 nM), or GP120 (200 nM) for 5 min at 37°C, stained for F-actin, and scored for increased actin accumulation at the bead:cell interface. (*d*) Percentage of cells where actin is polarized to the contact site of the bead. Values +/− SEM represent the average over three experiments, scoring 50 conjugates each. (*e*) Representative pictures are shown for fibronectin plus GP120 coated beads.

uate the effects of ITK on HIV replication over longer periods of time. The activity of this compound was confirmed in an ITK *in vitro* kinase assay (data not shown). Inhibition of ITK with BMS509744 markedly reduced HIV infection of primary CD4$^+$ T cells for up to 8 days, the longest time point examined (Fig. 1*b*). For the first 6 days, levels of p24 in cells treated with BMS509744 were similar to background levels in uninfected cells or cells treated with the CXCR4 antagonist JM-2987 (Fig. 1*b*). Thus, inhibition of ITK blocked the establishment of HIV infection.

ITK Affects Viral Entry and gp120-Induced Actin Reorganization. To understand how ITK activity affects HIV replication, we examined sequential steps of HIV replication. Treatment of cells with siITK or BMS509744 did not affect expression of CD4 or CXCR4, which are required for HIV binding and entry into cells (Fig. S2 and data not shown). Consistent with these data, binding of HIV to activated primary CD4$^+$ T cells, as measured by ELISA of cell-associated p24Gag protein, was not affected by BMS509744 (Fig. 2*a*). To assay for virus entry, we examined levels of trypsin-resistant (intracellular) p24Gag associated with cells exposed to virus for 3–5 h. Although control cells showed increased intracellular p24 over the course of 5 h, treatment with the ITK inhibitor decreased HIV entry into primary CD4$^+$ T cells (Fig. 2*b* and Fig. S3): similar results were obtained with siITK-treated Jurkat cells (data not shown). Treatment of cells with the CXCR4 antagonist JM-2987 provided a measurement for nonspecific background in these assays (as indicated by the dotted line, Fig. 2*b*).

We recently found that ITK is activated in response to stimulation of CXCR4 by SDF1α and is required for actin cytoskeleton polarization and Rac activation in response to SDF1α (7, 8). The HIV envelop protein also induces actin reorganization and activation of Rac, which are required for entry of HIV into cells (12). To determine whether gp120 activates pathways involving ITK, Jurkat cells were treated with gp120 and ITK activity was determined by *in vitro* kinase assay. Treatment with gp120 led to a small but reproducible increase in ITK kinase activity (Fig. 2*c*). To determine whether ITK affects HIV-induced cytoskeletal changes, Jurkat cells were treated with siITK or siGFP and stimulated with beads coated with fibronectin, fibronectin plus SDF1α, or fibronectin plus HIV gp120-LAV, then fixed and stained for F-actin. Although control cells increased F-actin polarization to the site of contact with either SDF1α- or gp120-coated beads, siITK-treated cells showed impaired actin polarization to these stimuli (Fig. 2 *d* and *e*) (8). Similarly, primary human CD4$^+$ T cells treated with ITK inhibitor BMS509744 or JM2987 showed decreased F-actin polymerization to gp120 stimulation as determined by flow cytometry (Fig. S4). Thus, ITK is required for gp120-induced actin cytoskeleton rearrangement, a process essential for efficient HIV entry into host cells.

ITK Does Not Affect HIV Reverse Transcription or Proviral DNA Integration. To evaluate whether ITK affected other stages of HIV replication, we examined the steps immediately after virus entry. To bypass defects in viral entry, we pseudotyped replication-competent virus with vesicular stomatitis virus (VSV)-G, allowing for receptor-independent virus entry. Low-molecular-weight unintegrated DNA was isolated 4 h after infection by the method of Hirt, and strong-stop DNA levels were measured by PCR by using R5-U3 primers to evaluate reverse transcription of the viral genome and amplification of mitochondrial DNA for a control. No differences in the early products of reverse transcription were observed between infected siITK and siGFP-

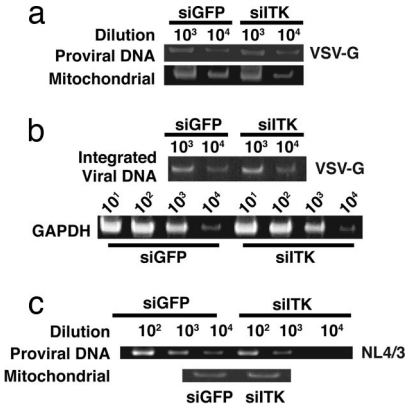

Fig. 3. ITK does not affect reverse transcription or integration. Jurkat cells were transiently transfected with siRNA to ITK or GFP, then infected with (*a* and *b*) NL4/3 pseudotyped with VSV-G envelop or (*c*) replication-competent intact NL43 virus. Data represent one of three individual experiments. (*a* and *c*) HIRT DNA was isolated after 4 h of infection for PCR analysis of preintegration strong-stop DNA from cells infected with (*a*) VSV-G pseudotyped virus or (*c*) NL-43. Mitochondrial DNA was amplified as a control. (*b*) Genomic DNA was isolated 48 h after infection with VSV-G pseudotyped virus and assayed for integrated viral DNA by PCR.

transfected Jurkat cells (Fig. 3*a*) or BMS vs. control-treated primary CD4 cells (Fig. S5*a*). Similar results were seen when integrated viral DNA was evaluated by nested PCR, amplifying products between Alu repeat- and viral-specific primers, compared with GAPDH controls (Fig. 3*b*, Fig. S5*b*). In contrast, when the virus entered by a receptor specific route, we observed a decrease in reverse-transcribed proviral DNA (Fig. 3*c*) and integration (Fig. S5*c*) in siITK-treated cells, consistent with their partial defect in viral entry. Similar results were obtained in activated primary human $CD4^+$ T cells (Fig. S5*d*). These data suggest that expression and/or function of ITK does not alter viral reverse transcription or proviral DNA integration.

ITK Is Required for Efficient Transcription from the HIV LTR. T cells from $ITK^{-/-}$ mice have impaired TCR-driven activation of the transcription factors NFAT, NF-κB, and AP-1, all of which bind to the HIV long terminal repeat to activate viral transcription in T cells (13–16). To evaluate whether ITK affects HIV transcription, Jurkat cells were transfected with an HIV-luciferase reporter, then transfected with siITK or siGFP and luciferase expression evaluated in response to anti-CD3 plus anti-CD28 stimulation (Fig. 4*a*). Alternatively, cell lines overexpressing a kinase-inactive ITK mutant (K390R) or control cell lines were transfected with the same HIV-luciferase reporter, and luciferase activity was assessed at baseline and in response to stimulation (Fig. 4*b*). In either case, reduction of ITK expression or expression of the kinase-inactive ITK mutant reduced expression of luciferase activity upon T cell activation by 60–80%. Conversely, transient transfection of a WT ITK expression vector increased expression of the HIV-luciferase reporter 4-fold, supporting that ITK is a positive regulator of HIV transcription (Fig. 4*c*).

ITK Enhances Virion Production. To determine whether ITK affects additional stages of the viral life cycle, we examined whether ITK influenced the late stages of virus assembly and egress. Because overexpression of Gag is sufficient for the formation of virus-like particles (VLPs) (17), we cotransfected Gag and ITK expression constructs into 293T cells. VLPs were assayed by ELISA for p24Gag in culture supernatants pelleted through 20% sucrose. Although ITK induced a modest increase in Gag expression from the CMV-based expression construct in 293T

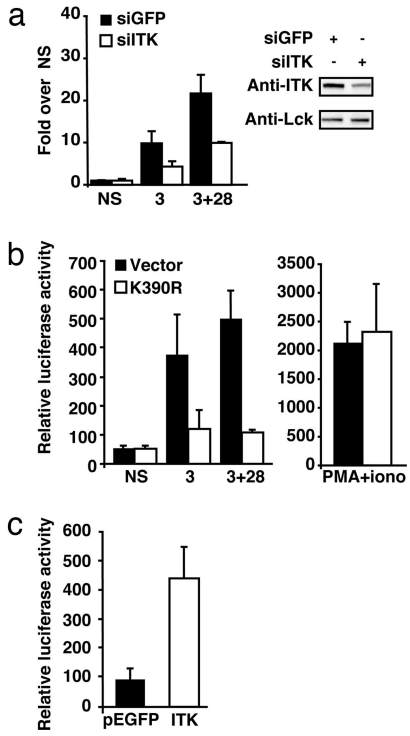

Fig. 4. Loss of ITK activity decreases HIV transcription. (*a*) Jurkat cells were transiently transfected with 1 μg of siRNA to ITK or GFP along with an HIV-luciferase reporter. Cells were left unstimulated (NS) or stimulated with plate bound purified anti-human CD3 (5 μg/ml) and anti-human CD28 (10 μg/ml) or PMA (10 ng/ml) and ionomycin (1 μM). Luciferase activity was measured 24 h after stimulation. Data are presented as fold induction over baseline luciferase activity and are from one representative of five individual experiments with $n = 3$. Error bars represent +/− SD. (*b*) Jurkat cell lines expressing kinase-inactive ITK (K390R) under control of the tet activator or control cells expressing the Tet activator alone were transiently transfected with NL4/3 luciferase and incubated with 1 μg/ml doxycycline for 24 h. After induction, K390R was expressed at ≈10-fold higher levels than endogenous ITK. Luciferase activity was assayed as in *a*. Data are from one representative of three individual experiments with $n = 3$. Error bars represent +/− SD. (*c*) Jurkat cells were transiently transfected with pEGFP or WT ITK and a HIV-luciferase reporter and 24 h later assayed for luciferase expression. Values +/− SD are from one representative experiment with $n = 3$ of 5 individual experiments.

cells, as determined by immunoblotting cell lysates, we consistently observed greater increases in VLP release in cells expressing ITK (Fig. 5 and Fig. S6). Enhancement of VLP release by ITK did not depend on kinase activity; however, mutating either the SH2 or the Pleckstrin homology domain of ITK reduced the ability of ITK to increase VLP production (Fig. 5). Thus, in addition to facilitating HIV entry and transcription, ITK potentiates virion assembly and release in a manner dependent on protein interactions and membrane recruitment.

siITK Blocks Viral Replication After Infection. To determine whether ITK also influences HIV replication after the establishment of infection, Jurkat cells were infected with replication-competent VSV-G-pseudotyped HXB2-PLAPnef+ (HIV-PLAP) for 24 h before introducing siITK or a control siRNA. The HIV-PLAP clone expresses placental alkaline phosphatase (PLAP) on the surface of infected cells, providing an additional marker for positive identification of infected cells. Treatment of infected cells with siITK decreased p24 release by 68% compared with siControl-treated cells 48 h after siRNA treatment (Fig. 6*a*). Furthermore, reduction of ITK led to fewer infected cells (6%

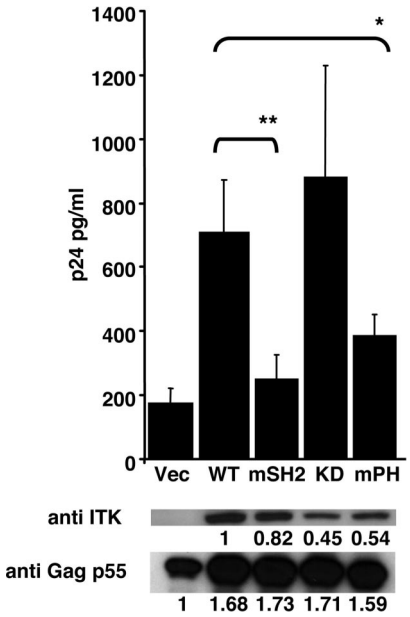

Fig. 5. ITK increases the release of virus-like particles. 293T cells were transfected with Gag (p96ZM651gag-opt) along with WT ITK, mSH2 (R265A), KD (K390R), mPH (XID), or pEGFP vector control via calcium phosphate. Seventy-two hours after transfection, supernatents were collected, spun on 20% sucrose cushion, and pelleted VLPs collected and analyzed for p24 via ELISA. Values +/− SD are from one representative experiment (with $n = 3$) of four individual experiments. *, $P = 0.05$, **, $P \leq 0.05$ Whole-cell lysates were analyzed for protein expression of ITK and Gag, and relative levels of Gag were determined by densitometry.

of siITK treated cells after 72 h vs. 18% of siControl-treated cells) that expressed lower levels of virus-associated PLAP (PLAP MFI 416 on infected siITK cells vs. 577 on siControl cells, Fig. 6 *b* and *c*). The siITK-mediated decrease in virus replication and spread persisted for up to 72 h, corresponding with the transient effects of the siITK. Thus, inhibition of ITK either before or postinfection suppresses HIV replication.

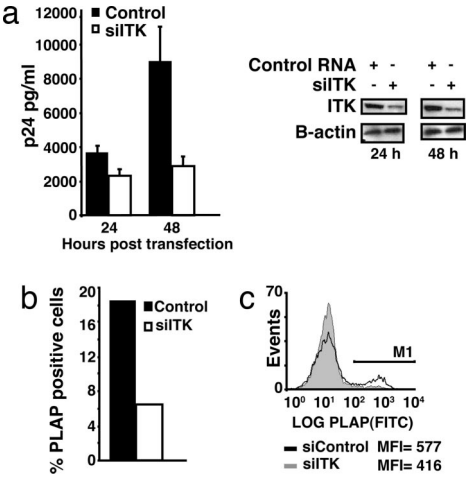

Fig. 6. Inhibition of ITK expression postinfection blocks viral production and spread. (*a*) Jurkat cells were infected with VSV-G-pseudotyped HXB-PLAPnef + virus for 24 h. Cells were then transiently transfected with siITK or control RNA. Supernatents and whole-cell lysate samples were taken at several time points after siRNA transfection and assayed for extracellular p24 by ELISA or ITK protein by immunoblot, respectively. (*b* and *c*) Jurkat cells were taken at 72 h after siRNA treatment, stained for surface PLAP expression and analyzed via flow cytometry. Data are from one representative of three individual experiments.

Discussion

Our results suggest that ITK is a positive regulator of HIV infection that affects multiple stages of HIV replication, including viral entry, transcription from the viral LTR, and virion assembly and release. These data were confirmed in both primary human $CD4^+$ T cells and the Jurkat leukemic T cell line, using either siRNA or chemical inhibition, suggesting that our findings are not cell-line-specific or due to off-target effects of the siRNA or chemical inhibition. Our findings thus provide insight into a cellular protein required for HIV replication.

ITK is required for full T cell activation *in vitro*, including transcription factor activation and T cell proliferation (6). It is therefore perhaps not surprising that ITK affected transcription from the HIV LTR. However, in addition to its effects on HIV transcription, ITK influenced both HIV entry and viral particle production/release. These events have been coupled with membrane polarization and redistribution of receptors, including CD4, CXCR4, and CCR5, to membrane rafts via actin-dependent processes (4, 5, 12). Our observation that ITK-deficient cells have impaired gp120-induced F-actin recruitment suggests that decreasing ITK may compromise the dynamic cytoskeletal reorganization required for virion entry. We have shown that ITK is important for TCR-induced recruitment of VAV1, a guanine nucleotide exchange factor for Rac, Rho, and Cdc42 that is required for TCR-induced actin and raft recruitment. Whether ITK increases virion particle production via effects on actin and raft dynamics or via other processes will require further investigation; however, it is intriguing that the effects on virion production appear to be kinase-independent, similar to ITK's role in TCR-induced VAV recruitment and actin regulation. It will also be of interest to evaluate whether ITK affects other cellular factors, such as moesin or filamin A, that also influence both cytoskeletal regulation and HIV infection (2, 18). Moreover, ITK may influence other cellular processes that modulate HIV infection in T cells, including protein interactions such as those of Gag with cyclophilin (19), a protein that also interacts with ITK (20). Finally, it is also of note that a recent article demonstrated increased VLP production associated with increased intracellular Ca^{2+}, a process influenced in T cells by ITK (21).

Although ITK is required for full T cell activation *in vitro* (6), *in vivo*, $Itk^{-/-}$ mice mount responses to viral infections and clear all viral infections tested to date, including VSV, vaccinia, and lymphocyte choriomeningitis virus (LCMV) (10). Indeed, the major immune defects observed *in vivo* in $Itk^{-/-}$ mice are T_H2 responses, leading to interest in ITK as a therapeutic target for treatment of T_H2-mediated diseases including asthma and hypersensitivity (22). It is, therefore, of interest that some report that T_H2 responses are associated with poor progression in the development of AIDS (23). Although other data argue that T helper cell polarization does not affect HIV progression (24), potential anti-T_H2 effects of ITK inhibitors may provide additional beneficial effects in limiting HIV replication. Moreover, data demonstrating that tyrosine kinase expressed in hepatocellular carcinoma (TEC) and Bruton's tyrosine kinase (BTK) are required for chemokine responses and activation of other cells (25) suggest that inhibition of Tec kinases may affect HIV infection in macrophages and monocytes.

Current treatment of HIV relies on antiretroviral agents that are subject to the development of resistant viruses. Because inhibitors directed against cellular proteins required for HIV replication may be less prone to this problem, the use of such inhibitors is of growing interest (38). It is therefore of note that ITK affects virus replication at multiple stages, including virion particle production, which is not currently targeted by antiret-

roviral therapy. Our results suggest that ITK inhibition provides a model for the study of cellular protein targets that affect HIV infection, which may be useful as part of a multidrug regimen directed against HIV.

Methods

Cells Culture Conditions. Jurkat clones E6–1 (American Type Culture Collection) were cultured in RPMI medium 1640 supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 M L-glutamine, and 20 mM Hepes. 293T human embryonic kidney cells were cultured in DMEM plus 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M L-glutamine. Human peripheral blood mononuclear cells were isolated from buffy coats with Ficoll/Histopaque 1077 gradient (Sigma). $CD4^+$ T cells were isolated by either negative selection (Miltenyi) or positive selection via magnetic bead separation (Dynal). Cells were stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 2 μg/ml phytohemagglutinin (Sigma), with or without 20 units/ml of IL-2 (Preprotech).

Generation of HIV-1 Infectious Virus. 293T cells were transfected by using either calcium phosphate or fugene 6 (Roche) with pNL43-luc (+) env (−) Nef(−) along with 3 μg of RSV-REV and 3 μg of DNA encoding either the HXB2 or VSV-G envelop proteins (26). For replication-competent viruses, 293T were transfected as described above with DNA encoding for HXB2, NL4/3, or pHXBnPLAP-IRES-N+ [AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health (27)]. Viral titers were assessed by ELISA of p24 in the supernatant of transfected 293T cells (Beckman Coulter or Perkin–Elmer). Supernatants were collected and filtered through a 0.45-μm disk before infection of T cells.

siRNA. Ten million cells were transiently transfected with 1 μg of siRNA against ITK (siITK) (9), GFP (siGFP, Qiagen), or a scrambled sequence (siControl) using a BTX T820 electroporator. Cells were washed in antibiotic free media and resuspend in RPMI medium 1640 plus 1% FCS and 20 mM Hepes. Cells were electroporated at either 300 V, 1 pulse, 20-ms gap, or 215 V, 1 pulse, 65-ms gap, placed immediately into prewarmed complete RPMI, and recovered for 24 h.

Generation of BMS509744. The synthesis of BMS509744 was performed in a manner similar to published methods (11). ^{1}H NMR (400 MHz, d_6-DMSO) δ8.00 (d, J_{HH} = 8.2 Hz, 2H), 7.74 (s, 1H), 7.49 (d, J_{HH} = 8.2 Hz, 2H), 7.06 (s, 1H), 7.05 (s, 1H), 3.90 (d, J_{HH} = 14.8 Hz, 1H), 3.77 (s, 3H), 3.68 (d, J_{HH} = 14.8 Hz, 1H), 3.40–3.58 (m, 4H), 3.22–3.38 (m, 2H), 3.00–3.16 (m, 2H), 2.46 (s, 3H), 2.14 (q, J_{HH} = 6.4 Hz, 1H), 1.99 and 1.91 (rotameric) (s, 3H), 0.92 (d, J_{HH} = 6.4 Hz, 3H), 0.83 (s, 9H). Mass analysis (TOFMS) m/z = 624.2681 ($M+H^+$) (theoretical 624.2678). Purity analysis was achieved by C_8 reversed phase LCMS using a linear gradient of water containing increasing amounts of CH_3CN (0 → 7 min, linear gradient from 10% → 70% CH_3CN at a flow rate of 1 ml/min: Rt 6.8 min) and revealed a purity of >90%.

Intracellular p24 Assays. Twenty-four hours after transfection, cells were infected with virus stock (equivalent to 200–300 ng of p24) for 4 h at 37°C, and then the media were replaced. For infections with inhibitors, cells were preincubated for 30–60 min with 1 μM JM2987 [AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (28–30)], 10 μM BMS509744 (11), or DMSO equivalent before infection. Infections were monitored at the indicated times by staining for intracellular p24 as follows: cells were washed in PBS, incubated with BD Permafix for 20 min, then washed in BD permawash buffer and incubated with anti p24 (KC57 RD1, Beckman Coulter). Flow cytometry was performed by using a FacsCalibur and analyzed with Flowjo software. Measurement of virus released into the culture supernatants was assayed by p24 ELISA. Cell-surface PLAP levels were measured by flow analysis using anti-PLAP (Sigma) and FITC-IgG2a (BD Pharmingen) antibodies.

Binding and Entry. Cells were infected as above for the indicated times. Assays were performed as described (31). Briefly, for binding assays, cells were washed 10 times in PBS and then lysed. Lysates were assayed for p24 by ELISA. For virus entry, cells were incubated for 5 min with trypsin and then washed five times with PBS before lysis and assaying for p24 by ELISA.

Kinase Assays. Jurkat cells were stimulated with 5 μg/ml gp120 LAV (Protein Sciences) for 1 min at 37°C. Cells were lysed and *in vitro* kinase assays performed by using a GST-PLC-γ construct as a synthetic substrate as described (32).

Actin Polarization. Jurkat cells were transiently transfected with siRNA and allowed to recover 24 h. Latex beads (Invitrogen) were coated with fibronectin 20 μg/ml alone or along with SDF1α 20 nM or 200 nM GP120 LAV protein at 10^7 beads/ml for 2 h at 37°C. Beads were washed three times with 3% BSA in PBS. Cells were incubated with 1% serum for 1 h before activation. Two million cells were mixed with 1.25 million beads and stimulated at 37°C for 5 min. Conjugates were stained with Alexa594-phalloindin (Molecular Probes) and analyzed on an inverted Zeiss Axiophot microscope. Cells were scored as positive if increased F-actin staining was observed at the site of bead contact, with one cell per bead being a conjugate and a total of 50 conjugates scored per condition per experiment for a total of three experiments (8).

Reverse Transcription and Integration. Viruses were pseudotyped with VSV-G, and cells were infected and assayed as described in (33, 34). Alternatively, NL4/3 was used. Briefly, for reverse transcription samples, low-molecular-weight DNA was extracted by the method of HIRT and strong-stop DNA amplified by using R5-U3 primers (35). Amplification of mitochondrial DNA was used for sample normalization. Integration samples were measured by isolating total cellular DNA and analyzed by nested PCR using Alu- and viral-specific primers (34, 36). GAPDH was used to normalize DNA concentration. GAPDH primers: 5′ ACCACAGTCCATGCCATCAC 3′ and 5′ TCCACCACCCTGTTGCTGTA 3′. GAPDH PCRs were performed at 94°C 5 min, 29 cycles of 94°C 30 s, 60°C 30 s, 72°C 30 s, followed by 72°C 10 min.

Transcription Assays. Jurkat E6.1 cells or primary human $CD4^+$ T cells were transiently electroporated with plasmid expression vectors and siRNAs for indicated conditions. Cells were immediately recovered after transfection with 20% FCS RPMI media for 24 h. Cells were then lysed, and luciferase activity was detected by a luminometer using Promega Luciferase assay kit. Alternatively, 24 h posttransfection, cells were stimulated on plate-bound anti-human CD3 (5 μg/ml), anti-human CD3 (5 μg/ml) plus anti-human CD28 (10 μg/ml) (NA/LE, PharMingen) or 10 ng/ml PMA and 1 μM ionomycin (Sigma) for 16–20 h before measuring luciferase activity.

Antibodies/Immunoblots. Cells were lysed in lysis buffer [1% Triton X 100, 20 mM Hepes, 50 mM B-glycerophsophate, 2 mM EGTA, 10% glycerol, 10 mM sodium fluoride, complete miniprotease inhibitor mixture (Roche)]. Proteins were separated on a 10% polyacrylamide gel, transferred to nitrocellulose or PVDF membrane and immunoblotted with anti-ITK (2F12) (Upstate), anti-LCK (Santa Cruz), or beta-actin (Sigma). Gag expression was assayed by immunoblotting with anti-HIV p24 (183-H12–5C from the National Institutes of Health AIDS Research and Reference Reagent Program).

VLPs. 293T cells were cotransfected with 2 μg of p96ZM651gag-opt [AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (37)] and 2 μg of pEGFP vector, WT ITK, mSH2 ITK (R265A), kinase inactive ITK (K390R), or mPH ITK using calcium phosphate (6). The media were replaced 16 h after transfection, and cells were cultured for 48 h. Supernatants were spun over 20% sucrose at 100,000 $\times g$ for 1 h at 4°C, pelleted VLPs resuspended in 1 ml of PBS, and p24 levels measured by ELISA.

ACKNOWLEDGMENTS. We thank J. Mascola for use of the Vaccine Research Center P3 facility; M. Louder for technical advice; and S. Anderson, M. Kirby, and M. Kensinger for technical support. pHXBnPLAP-IRES-N+, JM2987, and NIHp967m601 gag opt were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health. This work was funded in part by intramural funding of the National Human Genome Research Institute and the Intramural AIDS Targeted Antiviral Program (J.A.R. and P.L.S.), by funding from Penn State Tobacco Formula Funds (A.J.H. and A.A.), and by National Institutes of Health Grant AI62467 (to A.J.H.).

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