Selective targeting of ITK blocks multiple steps of HIV replication

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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 PLCγ1, Ca2+ 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 PLCγ1, Ca2+ 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 Tnfα 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 SDF1a, 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. 1a). 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-u


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The effects of ITK on HIV replication were observed over longer periods of time. The activity of this compound was confirmed in an ITK in vitro 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. 1b). 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. 1b). 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. 2a). 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. 2b 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. 2b).

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. 2c). To determine whether ITK affects HIV-induced cytoskeletal changes, Jurkat cells were treated with siITK or siGFP and stimulated with 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 (data not shown). Jurkat cells treated with SDF1α or gp120-coated beads, siITK-treated cells showed impaired actin polarization to these stimuli (Fig. 2d 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. BMS509744 was isolated for f t e rinfection 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-
transfected Jurkat cells (Fig. 3a) or BMS vs. control-treated primary CD4 cells (Fig. S5a). 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. 3b, Fig. S5b). In contrast, when the virus entered by a receptor specific route, we observed a decrease in reverse-transcribed proviral DNA (Fig. 3c) and integration (Fig. S5c) in siITK-treated cells, consistent with their partial defect in viral entry. Similar results were obtained in activated primary human CD4+ T cells (Fig. S5d). 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. 4a). 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. 4b). 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. 4c).

**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 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-PLAP nef+ (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 66% compared with S-control-treated cells 24 h after siRNA treatment (Fig. 6a). Furthermore, reduction of ITK led to fewer infected cells (48%...
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.

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 leukemia 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, CXC4R, 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 Ca2++, 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 Th1 responses, leading to interest in ITK as a therapeutic target for treatment of Th12-mediated diseases including asthma and hypersensitivity (22). It is, therefore, of interest that some report that Th12 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-Th12 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 chemokinetic 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 antiretroviral therapy.
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 Heps. 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-Hypaque 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), or 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 (78:7138). CD4+ T cells (Beckman Coulter) were stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 2 µg/ml phytohemagglutinin (Sigma), or with or without 20 units/ml of IL-2 (Preprotech).

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For virus entry, cells were incubated for 5 min with trypsin and then washed with 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 Alexa488-phalloidin (Molecular Probes) and analyzed on an inverted Zeiss Axiohot 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 HIV- and viral-specific primers (34, 36). GAPDH was used to normalize DNA concentration.

Antibodies/Immunoblots. Cells were lysed in lysis buffer [1% Triton X 100, 20 mM Heps, 50 mM B-glycerophosphate, 2 mM EGTA, 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 plasmid 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 pM12 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 g for 1 h at 4°C, pelleted VLPs resuspended in 1 ml of PBS, and p24 levels measured by ELISA.

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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 for 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 102 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 Alexa488-phalloidin (Molecular Probes) and analyzed on an inverted Zeiss Axiohot 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).

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.

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