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Interleukin 2-inducible T cell kinase (ITK) facilitates efficient egress of HIV-1 by coordinating Gag distribution and actin organization

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ABSTRACT

Interleukin 2-inducible T cell kinase (ITK) influences T cell signaling by coordinating actin polymerization and polarization as well as recruitment of kinases and adapter proteins. ITK regulates multiple steps of HIV-1 replication, including virion assembly and release. Fluorescent microscopy was used to examine the functional interactions between ITK and HIV-1 Gag during viral particle release. ITK and Gag colocalized at the plasma membrane and were concentrated at sites of F-actin accumulation and membrane lipid rafts in HIV-1 infected T cells. There was polarized staining of ITK, Gag, and actin towards sites of T cell conjugates. Small molecule inhibitors of ITK disrupted F-actin capping, perturbed Gag-ITK colocalization, inhibited virus like particle release, and reduced HIV replication in primary human CD4+ T cells. These data provide insight as to how ITK influences HIV-1 replication and suggest that targeting host factors that regulate HIV-1 egress provides an innovative strategy for controlling HIV infection.

Introduction

The HIV-1 group-specific antigen (Gag) protein is necessary and sufficient for the formation of virus like particles (VLPs) (Gheysen et al., 1989). Virus assembly is a multistep process which includes Gag transport to the site of assembly, binding to the lipid bilayer, multimerization, budding and pinching off of the viral particle from the host membrane. In infected T cells Gag polyproteins (Pr55Gag) are targeted to the plasma membrane (Freed, 1998; Garnier et al., 1998; Tritel and Resh, 2000) where they preferentially localize at lipid rafts and assemble into immature viral particles (Garoff et al., 1998). The HIV-1 Gag matrix (MA) domain is required for Gag targeting to the plasma membrane (Spearman et al., 1994). Critical regions within the MA domain that mediate membrane targeting include N-terminus myristylation signals and the highly basic domain, which directs Gag to rafts enriched with phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P(2)) (Saad et al., 2008, 2007, 2006; Zhou et al., 1994). Deleting or mutating the highly basic domain alters the cellular distributions of Gag away from the plasma membrane and into intracellular compartments such as the endoplasmic reticulum, late endosomes or MVBs (Facke et al., 1993; Ono et al., 2004; Ono and Freed, 2004; Zhou and Resh, 1996). Identifying cellular factors that coordinate and regulate Gag trafficking and intracellular localization would provide insights into mechanisms for this critical step in HIV-1 replication as well as suggest potential strategies to block late stages of HIV egress.

Productive HIV-1 infection in T cells requires T cell activation (Oswald-Richter et al., 2004; Stevenson et al., 1990) and reorganization of the cytoskeleton (Fackler and Krausslich, 2006; Gladnikoff et al., 2009; Jolly et al., 2004; Vorster et al.). Interleukin-2 inducible T cell kinase (ITK) modulates T cell signaling (Andreotti et al.) by coordinating changes in cellular organization during T cell activation (Gomez-Rodriguez et al., 2007), including accumulation of F-actin at sites of receptor engagement or within immunological synapses. Studies performed with T cells lacking ITK show defects in actin responses including extending unstable lamellipodia upon T cell receptor (TCR) stimulation (Berge et al., 2010; Carrizosa et al., 2009), an inability to properly polarize upon contact with antigen presenting cells (Labno et al., 2003), and reduced accumulation of activated

Abbreviations: ITK, Interleukin 2-inducible T cell kinase; Gag, HIV-1 group-specific antigen; MVB, multivesiclar bodies; VSV-G, vesicular stomatitis virus glycoprotein; PLAP, placental alkaline phosphatase; VLP, virus like particles

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cdc42 and Vav1 at site of receptor engagement (Dombroski et al., 2005). ITK is activated and targeted to the plasma membrane downstream of TCR signaling and chemokine receptor CXCR4 signaling induced by SDF1α (Fischer et al., 2004). Since these receptors are utilized by HIV-1 to gain access into target cells and T cell activation is necessary for efficient HIV-1 replication, we posit that ITK impacts HIV-1 replication.

We recently demonstrated that ITK facilitates HIV-1 replication and is necessary for efficient viral entry, proviral transcription, and viral particle assembly and release (Readinger et al., 2008). In this study, we utilize wide field fluorescence deconvolution microscopy to gain a better understanding of mechanisms by which ITK enhances viral particle assembly and release. Our findings show that ITK and Gag colocalize at sites of actin polymerization and membrane lipid rafts. Chemical inhibition of ITK disrupts actin capping, perturbs Gag-ITK colocalization and correlates with decreased virus like particle release. Our findings demonstrate that ITK is a potential novel drug target for inhibiting HIV assembly and release in infected T cells.

Results

ITK and Gag colocalize at the plasma membrane in distinct lipid raft microdomains

We have shown that ITK facilitates VLP production and that the PH and SH2 domains of ITK are critical for this function (Readinger et al., 2008). To begin to investigate how ITK regulates VLP assembly and whether it influences Gag trafficking, we utilized a Gag mutant, $\Delta MA^{(16-99)}$, which has a deletion in the highly basic region of MA domain. Although this mutation does not prevent VLP release (Supplemental Fig. 1), ΔMA Gag does not bind PIP2 and is diverted from the plasma membrane, preferentially targeting intracellular compartments, such as ER and endosomal vesicles (Facke et al., 1993; Hermida-Matsumoto and Resh, 2000; Ono and Freed, 2004; Ono et al., 2000; Yuan et al., 1993). To determine if ITK was able to enhance ΔMA Gag VLP production, we transfected HEK293T cells with plasmids expressing ITK-GFP fusion protein and Gag-Cherry or ΔMA Gag-Cherry fusion proteins. The fluorescent tags were used to assess the expression of transfected proteins and determine their intracellular localization by immunofluorescence microscopy. VLP release was determined by measuring $p24^{gag}$ in transfected cell supernatants by ELISA, while immunoblots of whole cell lysates confirmed Gag, ΔMA Gag, and ITK expression. ITK enhanced VLP release by greater than 7 fold when cotransfected with Gag-Cherry (Fig. 1a) consistent with our previous findings (Readinger et al., 2008), whereas ITK did not facilitate release of ΔMA Gag VLPs. Western blot analysis confirmed that comparable levels of Gag or ΔMA Gag were expressed in the absence or presence of ITK (Fig. 1b). These data suggest that ITK does not influence Gag trafficking but enhances VLP release after Gag has been delivered to the plasma membrane.

We used deconvolution microscopy to visualize the location of ITK and Gag and determine if they colocalized at the plasma membrane. ITK was observed dispersed throughout the plasma membrane when ITK-GFP was overexpressed in HEK293T cells (Fig. 2a). In contrast, mPH-ITK, which harbors a point mutation within the Pleckstrin Homology (PH) domain that disrupts the ability of ITK to bind PIP3, is redirected from the plasma membrane to intracellular compartments. Similarly, Gag-Cherry was expressed throughout the plasma membrane, whereas, ΔMA Gag-Cherry was redirected from the plasma membrane and into intracellular compartments consistent with previously described patterns for HIV-1 Gag and ΔMA Gag (Hermida-Matsumoto and Resh, 2000; Ono and Freed, 2004; Ono et al., 2000; Yuan et al., 1993). When ITK and Gag were co-expressed we observed that in 100% of the cells ITK and Gag colocalized within the plasma membrane with an average of Pearson's coefficient $r=0.87$, often in distinct domains, which were observed in approximately 45% of the cells (Fig. 2b), whereas, capping or clustering of Gag-cherry was not observed in cells expressing only Gag-cherry (Fig. 2a). Coexpressing mPH-ITK with Gag did not result in capping of Gag (Fig. 2b) indicating that the ITK PH domain and membrane targeting are required for the ability of ITK to influence Gag distribution. In addition, ITK was not able to redirect ΔMA Gag from intracellular compartments to the plasma membrane (Fig. 2c). Coexpressing mPH-ITK and ΔMA Gag resulted in both molecules targeting distinct intracellular compartments. These data suggest that although Gag and ITK can independently traffic to the plasma membrane, once at the membrane they functionally interact to form distinct domains where they colocalize.

To confirm ITK and Gag colocalize in the context of infected T cells, we visualized the location of endogenous ITK and Gag in

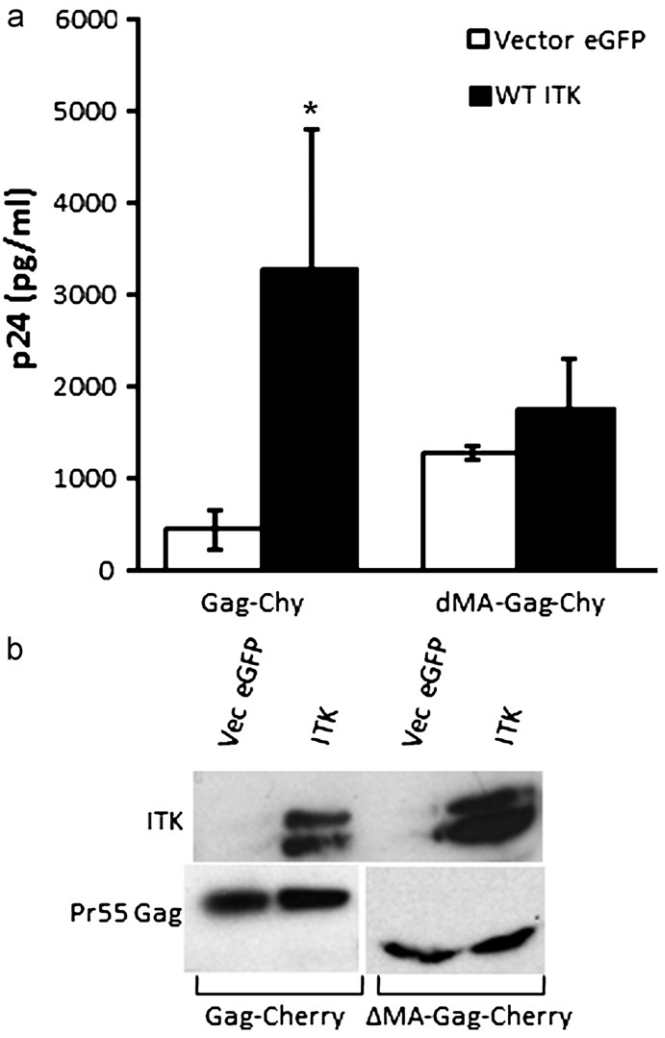

Fig. 1. ITK-mediated enhancement of VLP release requires Gag targeting to the plasma membrane. (a) HEK293T cells were transiently transfected with Gag-Cherry, or ΔMA Gag-Cherry DNA plus vector-GFP (control) or ITK-GFP DNA constructs. Supernatants were spun on 20% sucrose cushion to purify extracellular virus like particles and assayed by p24 ELISA. *p*24 values were normalized to Gag associated with whole cell extracts as determined by immunoblots and densitrometry (b). These data are from a single transfection experiment performed in triplicate and represent greater than three independent experiments. Error bars show the standard deviation between the triplicate transfections for each condition. * indicates a *P* value <0.05 as determined by a Student's *t*-test.

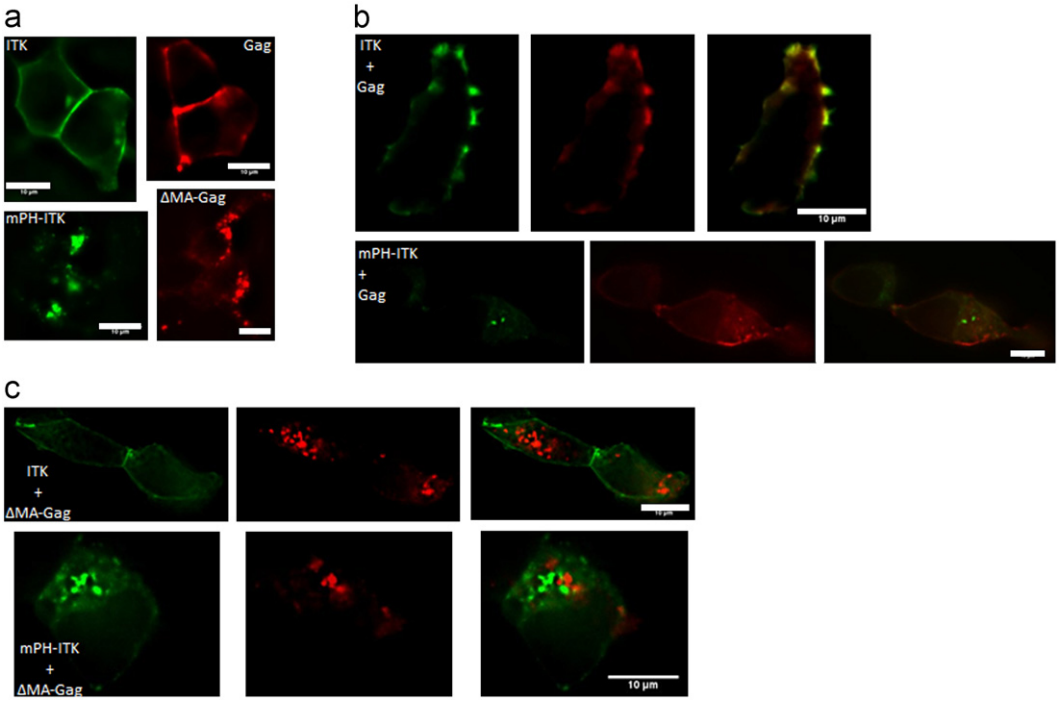

Fig. 2. ITK colocalizes with Gag at the plasma membrane in transfected HEK293T cells. (a) HEK293T cells were cotransfected with (a)–(c) Gag-Cherry, ΔMA Gag-Cherry, ITK-GFP, or mPH-ITK-GFP (which lacks a functional pleckstrin homology domain). Cells were fixed and viewed using a Nikon Fluorescence microscope at 60× oil immersion, Image J software was used for deconvolution and image analysis.

HIV-1 infected Jurkat T cells. Gag expression was predominantly detected at the plasma membrane of HIV-1 infected T cells (Fig. 3a), often in discrete patches or caps. Consistent with the above findings, ITK staining overlapped with Gag staining suggesting that Gag and ITK are found in similar plasma membrane lipid domains (average Pearson's coefficient measures $r=0.93$). This was further explored by determining if ITK and Gag were targeting cholesterol rich lipid raft regions. Infected Jurkat T cells were stained with FITC-conjugated cholera toxin B which binds GM1 a component of lipid rafts, as well as, anti-Gag and anti-ITK antibodies. As shown in Fig. 3b, Gag and ITK were present at regions that stained with cholera toxin B, indicating that in HIV infected cells Gag and ITK colocalize in lipid raft microdomains (for Gag and CT-B staining average Pearson's coefficient measures $r=0.813$, for ITK and CT-B staining average Pearson's coefficient measures $r=0.736$).

Gag, ITK, and F-actin accumulate at sites of T cell contact

HIV particle transfer via cell-to-cell contact is more efficient than infection by cell-free virions (Carr et al., 1999; Dimitrov et al., 1993; Phillips, 1994). This is in part due to a redistribution of Gag and a directional release of HIV-1 towards the uninfected target cell (Johnson and Huber, 2002). This capping of Gag is associated with localized changes to the cytoskeleton including actin polymerization (Jolly et al., 2004). Therefore, we examined the distribution of ITK, Gag and actin during HIV infection especially in juxtaposed T cells. As expected, we observed Gag capping in HIV-1 infected cells directed towards the neighboring cell (Fig. 3c). ITK is also polarized, colocalizing with Gag at regions where T cells are in close proximity (average Pearson's coefficient measures $r=0.67$). Similarly, F-actin, as detected by staining cells with Phalloidin, accumulated at sites where T cells were in close proximity or in contact and colocalized with ITK (Fig. 4; average Pearson's coefficient measures $r=0.89$). These data indicate that ITK, Gag and F-actin are located in overlapping and discrete regions in T cell-T cell conjugates.

Compounds that inhibit ITK diminish VLP release, alter Gag-ITK colocalization at the plasma membrane and disrupt actin polymerization

We were interested in determining whether compounds that targeted ITK activity could be used to block HIV-1 assembly and release. We performed VLP assays by cotransfecting Gag and ITK in the presence or absence of ITK inhibitors BMS509744 or CNX-225. BMS509744 is a chemical inhibitor which specifically inhibits ITK activation (Lin et al., 2004) and CNX-225 is a selective small molecule kinase inhibitor that covalently binds Tec kinases. CNX-225 structure and profile for inhibition of Tec kinase family members are shown in supplemental Fig. S2. ITK enhanced VLP production by greater than 5 fold (Fig. 5a) in DMSO treated control samples, consistent with the above data (Fig. 1a) and our previous report (Readinger et al., 2008). Induction of VLPs was not observed in cells treated with either inhibitor suggesting that specific chemical inhibitors reduce the ability of ITK to facilitate HIV assembly and release. In order to determine whether the ITK inhibitor CNX-225 inhibited HIV replication in human primary CD4+ T cells, HIV infected cells were treated with the inhibitor 24 h post HIV infection. CNX-225 reduced HIV replication by approximately 70% compared to the DMSO vehicle control treatment (Fig. 5b). These results are similar to our previous findings which showed that BMS509744 inhibited HIV replication in primary human CD4+ T cells (Readinger et al., 2008).

Since ITK influenced Gag membrane location and cytoskeleton organization, we were interested in determining if the ITK inhibitors altered Gag and actin distribution. Infected Jurkat T cells were treated with BMS509744 or CNX-225. Deconvolution microscopy was employed to visualize how these compounds influenced intracellular distribution of ITK, Gag and F-actin. As expected in DMSO treated controls ITK and Gag colocalized at the

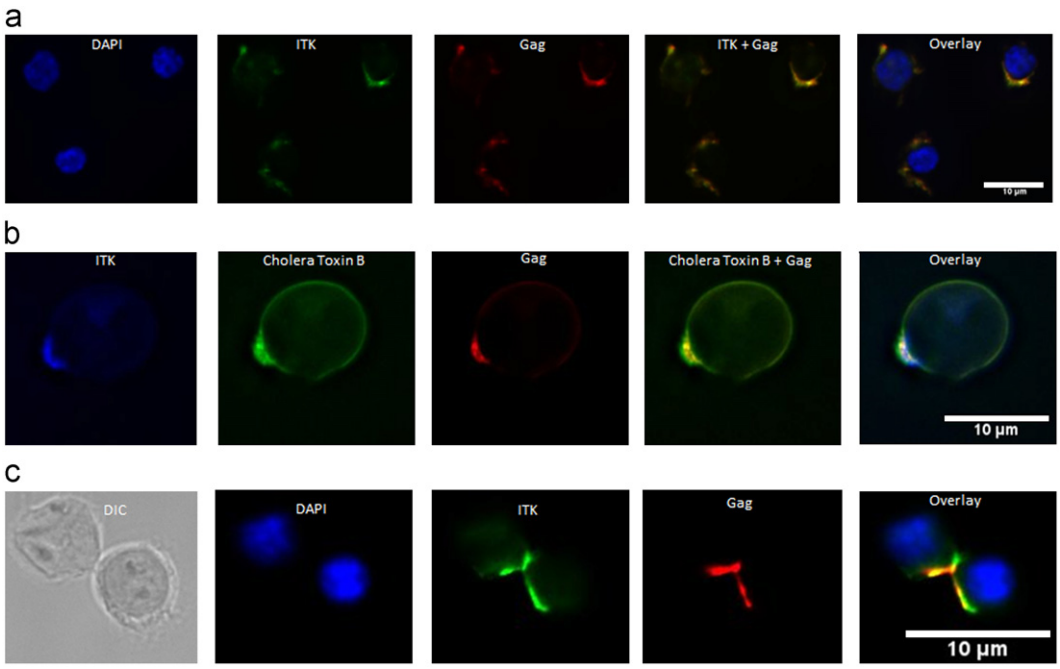

Fig. 3. ITK and Gag colocalize in the plasma membrane in lipid rafts and at sites of T cell-T cell contact in HIV infected T cells. (a) Jurkat cells were infected with VSVG-HXB-PLAP-nef+ virus, and enriched for HIV infected cells using magnetic beads coated with anti-PLAP antibody. Infected cells were treated with DMSO for 30 min, then fixed, permeabilized and intracellularly labeled for ITK (green) and Gag (red) expression with specific antibodies and counterstained with DAPI to detect nuclei (blue). (b) 72 h post-infection infected Jurkat cells were incubated with cholera toxin B conjugated-FITC, washed and fixed. Cells were permeabilized and intracellularly labeled for ITK expression (blue) and Gag expression (red). (c) Jurkat cells were infected with VSV-G pseudotyped HXB-PLAP-nef+ virus, treated with DMSO and 72 h post infection cells were fixed, permeabilized and stained for intracellular ITK (green), Gag (red) and counterstained for DAPI (blue). Cells were imaged using Nikon Fluorescence microscope at 60× oil immersion, Image J software was used for deconvolution and image analysis.

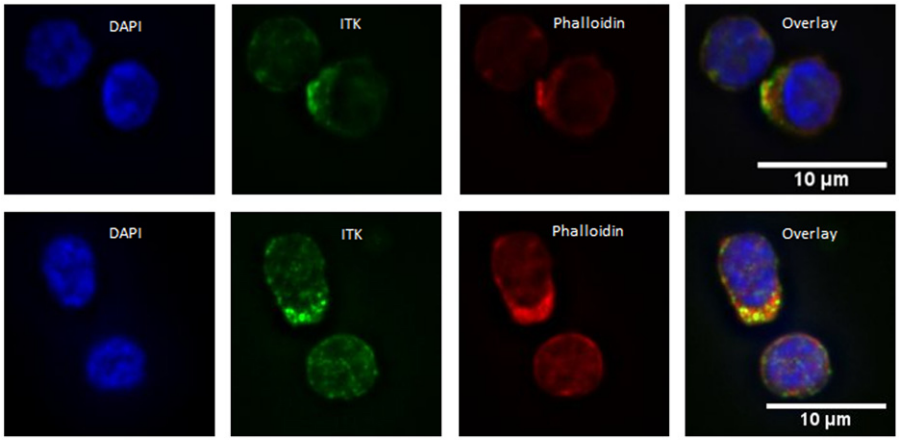

Fig. 4. ITK colocalizes with actin polymerization in infected T cell conjugates. Jurkat cells were infected with a VSV-G pseudotyped HXB-PLAP-nef+, and 72 h post infection cells were sorted for HIV positive cells, fixed, permeabilized and intracellularly labeled for ITK expression (green), Phalloidin (red), and DAPI to detect nuclei (blue). All images were taken using a Nikon fluorescence microscope at 60× oil immersion, Image J software was used for deconvolution and image analysis.

plasma membrane (average Pearson's Coefficient measures $r=0.71$). In cells treated with either BMS509744 or CNX-225 ITK inhibitors, ITK was more evenly dispersed throughout the cell and no longer colocalized with Gag in discrete domains as indicated in the loss of Pearson's positive correlative staining (Fig. 5c and d). ITK and Phalloidin colocalized at sites of cell–cell contact in vehicle control treated cells (average Pearson's coefficent measures $r=0.89$). However, treating cells with BMS509744 or CNX-225 decreased F-actin polymerization and capping towards sites of T cell–T cell contact (Fig. 5e). These data indicate Tec kinase chemical inhibitors diminish the recruitment of ITK to the plasma membrane perturbing Gag-ITK colocalization, disrupting actin polymerization, and inhibiting HIV release and replication. Collectively our data demonstrates that targeting ITK is an effective strategy to inhibit HIV assembly and release.

Discussion

In this study, we demonstrate that the ability of ITK to regulate HIV assembly and release correlates with its function to orchestrate actin cytoskeleton rearrangements. ITK and Gag colocalize within cholesterol rich lipid domains in the plasma membrane of infected T cells; however, ITK does not enhance VLP release of Gag

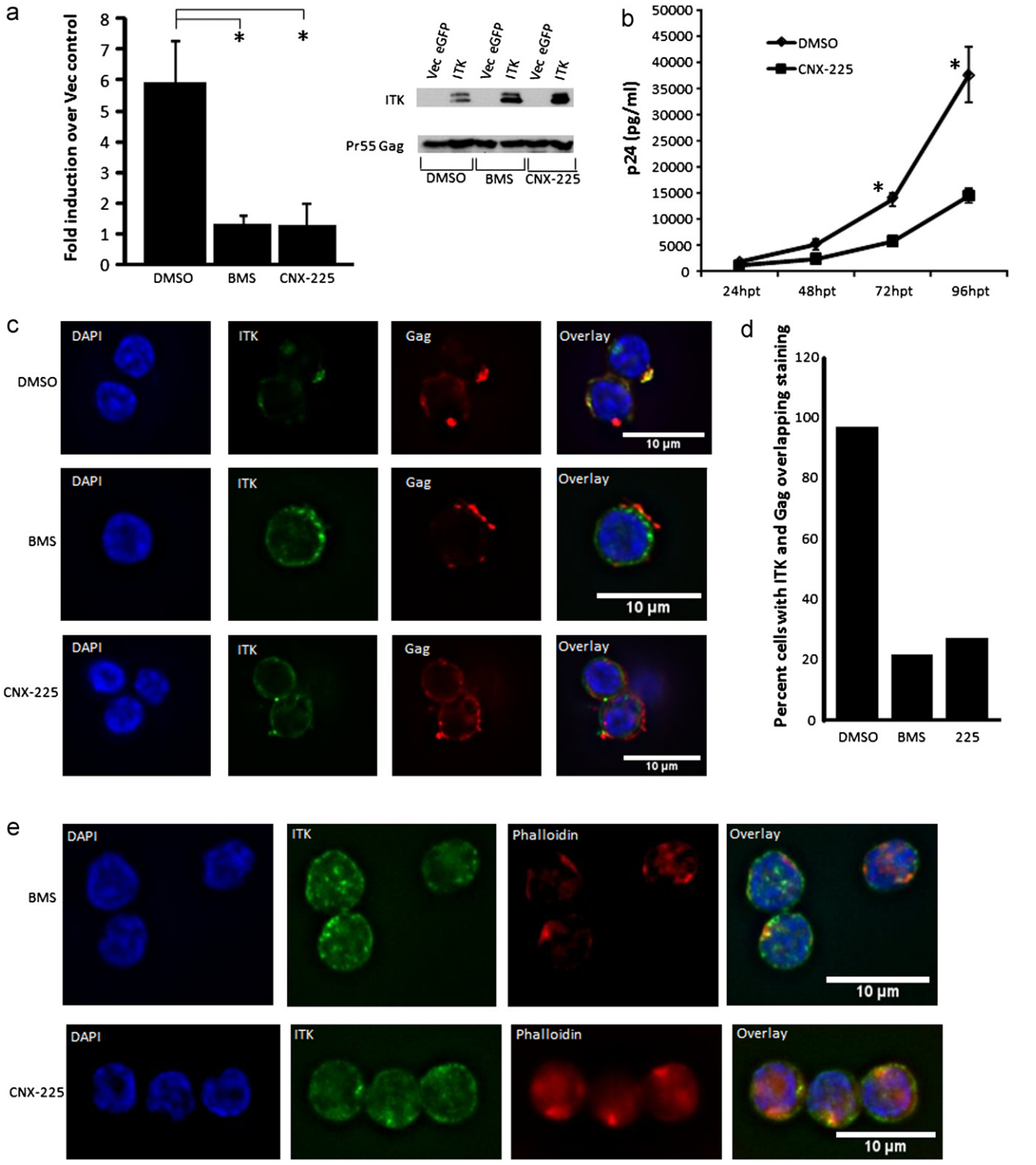

Fig. 5. Chemical inhibition of ITK disrupts Gag colocalization, alters actin organization, decreases VLP release and inhibits HIV replication. (a) HEK293T cells were co-transfected with Gag-opt and Vector- eGFP or ITK-eGFP, treated with DMSO, BMS or CNX-225 for 72 h. Supernatants were collected and spun on a 20% sucrose cushion, and assayed by p24 ELISA. *p*24 values were normalized to Gag associated with whole cell extracts as determined by immunoblots and denistrometry. Fold induction of VLPs over vector control is shown. This is a single experiment performed in triplicate and represents at least three independent treatments. Error bars calculated as standard deviation and * indicates *P* value <0.05 as determined by a Student's *t*-test. Immunoblots were also performed to confirm ITK expression. (b) Human primary CD4+ T cells were infected with VSV-G pseudotyped HXB-PLAP-nef+ virus, 24 h post infection cells were treated with CNX-225 inhibitor (1 μM) or equivalent DMSO and cultured in the presence of treatment for 4 days. Supernatants were sampled at indicated times and HIV replication was assessed by p24 ELISA. These data are triplicate infections from a single donor and are representative of results obtained from three different donors. The error bars are standard deviation between the triplicate samples and * indicates *P* value <0.05 as determined by a Student's *t*-test. (c)–(e) Jurkat cells were infected with a VSV-G pseudotyped HXB-PLAP-nef+, 72 h post infection cells were sorted for HIV positive cells. HIV positive cells were treated with BMS509744 (10 μM) or CNX-225 inhibitor (1 μM) or equivalent DMSO control for 30 min. Cells were washed, fixed, permeabilized, and intracellularly labeled for ITK expression (green) and Gag expression (red). (d) Percent Jurkat cells following treatment in which ITK and Gag staining overlapped. At least 60 cells were counted for each treatment. (e) HIV infected Jurkat cells were intracellularly labeled for ITK expression (green) and F-actin using Phalloidin (red). The cells were also stained with DAPI to detect nuclei (blue). Comparable DMSO treated cells are shown in Fig. 4. All images were taken using a Nikon fluorescence microscope at 60× oil immersion, Image J software was used for deconvolution and image analysis.

harboring mutations that redirect it away from the plasma membrane to intracellular compartments. These data indicate that physical proximity to the site of viral particle release is critical for ITK induced enhancement of VLP release.

ITK and Gag colocalize in discrete membrane domains suggesting that ITK and Gag influence each other's distribution in the plasma membrane. Furthermore, both ITK and Gag are associated with cholesterol rich rafts which have been described as preferred sites of virus assembly and release. It is possible that ITK is physically interacting with Gag at the plasma membrane; ITK has been shown to interact with many proteins through its SH2-SH3 domains; however, we are unable to coimmunoprecipitate ITK and Gag (data not shown), suggesting that ITK and Gag indirectly interact. The functional interaction between ITK and Gag may be restricted to the plasma membrane since ITK cannot rescue ΔMA-Gag localization to plasma membrane or enhance ΔMA-Gag VLP release.

Our data suggest that ITK facilitates HIV release by coordinating actin reorganization. In addition, we observe a high density of Gag and ITK at sites of dynamic actin polymerization such as membrane protrusions or extensions (Fig. 2b and data not shown). For HIV entry and egress the virus must traverse the barrier presented by cytoskeleton. Inhibitors that block actin and tubulin assembly or disassembly decrease virus release and infectivity (Jolly et al., 2004, 2007) highlighting the importance of the cytoskeleton in HIV replication. Furthermore, cell host machinery is co-opted to help mediate reorganization of the cytoskeleton to assure efficient replication. For example, HIV-1 entry is regulated in resting $CD4^+$ T cells by cofillin, which depolymerizes the actin network to overcome this physical barrier to viral entry (Yoder et al., 2008). Similarly, the actin binding proteins ERM (Barrero-Villar et al., 2009) and filamin A are required for efficient HIV infection (Jimenez-Baranda et al., 2007). Filamin A has recently been shown to also have a novel role in Gag intracellular trafficking and distribution at the plasma membrane (Cooper et al., 2011). In addition, live fluorescent microscopy showed that viral particles utilize cytoplasmic dynein and the microtubule network to migrate towards the nucleus during early HIV infection (McDonald et al., 2002). ITK has been demonstrated to coordinate spatiotemporal organization of signaling molecules in response to T cell activation and mediate events such as actin polymerization and capping upon T cell receptor engagement and CXCR4 chemokine receptor signaling (Dombroski et al., 2005; Readinger et al., 2008; Singleton et al., 2011). We posit that it is the ability of ITK to coordinate cytoskeleton organization which mediates HIV assembly and release. It is probable that ITK may be targeting and influencing activation of specific factors that are upstream regulators of the actin binding proteins ERM and filamin A such as Vav, RhoA and cdc42, thus influencing the clustering of Gag.

An additional mechanism by which ITK may facilitate HIV release and infection is by coordinating the organization of protein and lipid domains within the plasma membrane. Proper formation of the immunological synapse, a structure between antigen presenting cells and T effector cells which is necessary for efficient T cell signaling and function, requires ITK (Grasis et al., 2003; Schaeffer et al., 1999). T cells that lack or express mutant versions of ITK show impaired TCR induced actin polymerization, cell polarization and regulation of the signaling events involved in the cytoskeletal reorganization (Grasis et al., 2003; Schaeffer et al., 1999). A structure that is similar to the immunological synapse, the virological synapse, forms between an HIV infected cell and an uninfected target cell. Virological synapse formation requires the dynamic redistribution of extracellular receptors on the surface of the target cell, intracellular proteins and membrane lipid distribution on the surface of the infected cell, all of which enables efficient viral particle release. We speculate based on the changes we observe in Gag distribution and actin polymerization and capping in juxtaposed infected cells upon inhibition of ITK activity that ITK may coordinate the generation of functional virological synapses.

In addition to ITK, other kinases have been implicated as regulators of HIV replication including Lck (Cheng et al., 1999; Phipps et al., 1996) and Zap70 (Sol-Foulon et al., 2007). Lck targets Zap70 and ITK upon TCR engagement. Zap70 and ITK phosphorylation recruits additional T cell kinases which lead to the formation of a signalasome anchored by LAT and SLP76 assuring efficient T cell signaling. Furthermore, unlike ITK, Lck has been shown to directly interact with Gag and inhibiting its activity in T cells leads to accumulation of intracellular viral particles. (Strasner et al., 2008). CNX-225 did modestly inhibit Src kinase Lyn, however, Lyn is not expressed in CD4+ T cells (Corey and Anderson, 1999; Denny et al., 2000; Olszowy et al., 1995; Yamanashi et al., 1989) and knocking down the related T cell restricted tyrosine kinase Fyn had no significant impact on HIV release (data not shown) suggesting that these kinases have a minimal role in HIV egress. Since Lck can phosphorylate Itk, we propose that Lck and ITK cooperate to assure that Gag properly targets and is retained in discrete plasma membrane domains as well as coordinating the cytoskeletal changes and signalasome formation which assures efficient HIV egress. It should be noted that the multifunctional viral protein Nef has been reported to interact with a number of kinases and signal transduction adapter proteins; however, overexpression of Nef does not appear to influence ITK effects on HIV in our experimental systems, though a more complete analysis of physical and functional interactions between Nef and ITK is necessary. Furthermore, other viruses may co-opt ITK to assure efficient replication in T cells. For example, it was recently reported that ITK is activated by influenza A infection of T cells and enhances virus replication (Fan et al., 2012).

We propose a model in which ITK facilitates HIV release by coordinating signals that generate a scaffold required for optimal Gag targeting into proper lipid domains facilitating viral particle assembly and release. Discovering new therapeutic approaches which can be used in combination with current retrovirals to combat HIV replication targeted at the late stages of the viral life cycle is of high priority. Currently there are a limited number of compounds that have been identified as targeting the late stages of HIV replication, none of which have been approved for treatment. Our findings would suggest that ITK inhibitors may offer a potential strategy for reducing viral particle release from infected T cells. In addition, current treatments for HIV infection target viral proteins which are highly prone to mutations and selection for drug-resistant viruses. Targeting cellular proteins required for HIV replication would be predicted to be less susceptible to mutations, thus reducing the probability of selection for drug-resistant viruses. Finally, we have previously shown that inhibition of ITK blocks HIV infection and replication (Readinger et al., 2008), coupled with these new findings that ITK facilitates VLP formation, targeting ITK could provide a multi-faceted anti-HIV therapeutic strategy.

Materials and methods

Cells, plasmids and transfections

Jurkat E6.1 cells (American Type Culture Collection) were cultured in RPMI medium 1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M L-glutamine. Primary human CD4+ T cells were isolated from peripheral blood Leukopacks and positively sorted for CD4

expression (Dynabeads CD4+ isolation kit, Invitrogen) and maintained in culture in RPMI medium 1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M L-glutamine. HEK293T human embryonic kidney cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M L-glutamine.

Plasmids used in these experiments were Gag-cherry, p96ZM651 Gag-opt (AIDS Research and Reference Reagent Program, NIAID, Drs. Yingying Li, Feng Gao, and Beatrice H. Hahn) along with 2 μg of eGFP vector, ITK-eGFP fusion protein (generously provided by Avery August, Cornell University) or mPH ITK-eGFP (generously provided by Dr. Pamela Schwartzberg, NIH; (Berg et al., 2005) and $\Delta MA^{(16-99)}$ Gag-cherry. The expression construct, encoding the codon-optimized Gag-eGFP fusion protein, was obtained from the NIAID AIDS Reference and Reagent Program (contributed by Dr. George Pavlakis). HIV-1 Gag-mCherry expression plasmid that expresses a red fluorescent Gag-mCherry fusion protein has been described previously (Izquierdo-Useros et al., 2009), and was constructed by swapping the mCherry sequence for eGFP using BamHI and NotI restricition enzymes. The MA deficient ΔMA16-99-Gag-mCherry plasmid is isogenic to HIV-1 Gag-mCherry, except for the presence of the indicated in-frame deletion (amino acids 16–99) in the MA coding region. Briefly, HIV gag sequence was digested with ClaI and HindIII restriction enzymes to remove the intervening sequence. The 5′ ends were filled in with the large fragment of Klenow DNA polymerase to create blunt ends and that were then self-ligated to create an in-frame deletion of 84 amino acids, and the mutation (in-frame deletion) verified by sequencing.

Cells were cotransfected with the indicated plasmids using calcium phosphate. Media was replaced 3 h post-transfection, and cells were cultured for 16 h before collecting supernatants or harvesting cells for assays. Transfections were typically performed in triplicates and the data shown are representative of at least three independent experiments.

For some experiments cells were cultured for 72 h with ITK inhibitors BMS509744 (10 μM) (Readinger et al., 2008) or CNX-225 (1 μM) a covalent small molecule specific Tec family kinase inhibitor (Avila Therapeutics) or DMSO vehicle. The structure of the CNX-225 inhibitor and a summary of its specific activity are shown in the supplementary Fig. S1. The ability of CNX-225 to inhibit Tec kinases was determined using HotSpot^{SM} technology and utilized radioisotope-based P81 filtration (Reaction Biology Corporation, Malvern, PA). CNX-225 was dissolved in pure DMSO to the final 1 μM test concentration. Substrates were prepared and were then added to the substrate solution followed by kinase addition pre-incubation for 30 min at room temperature. ^{33}P-ATP (10 μM) was delivered into the reaction mixture to initiate the reaction and continued for 2 h at room temperature. The reaction was terminated and any unreacted phosphate was washed away using 0.1% phosphoric acid prior to detection utilizing a proprietary technology (Reaction Biology Corp.; Malvern, PA, USA). The study was performed in duplicate and 10 μM staurosporine, a non-selective, ATP-competitive kinase inhibitor, was used as the positive control.

HIV infections

Replication competent HXB-PLAP-Nef+ (obtained from the National Institutes of Health AIDS Research and Reference Reagent program (Chen et al., 1996)) virus stocks were generated by cotransfecting expression plasmids for HIV-PLAP provirus with VSG-G envelope as described previously (Readinger et al., 2008). Jurkat E6.1 cells were infected with VSV-G pseudotyped HXB-PLAP-Nef+ virus for 4 h and then cultured for 72 h. HIV-1 infected PLAP positive cells were selected for using Dynal magnetic beads coated with anti-PLAP antibody (Sigma). The PLAP positive cells were fixed in 4% paraformaldehyde or treated with BMS509744 (10 μM), CNX-225 (1 μM) or DMSO for 30 min before fixing and visualizing. All data shown represent a minimum of at least three independent infections. Primary human CD4+_T cells were stimulated with PMA (10 ng/ml) and PHA (2 μg/ml) for 16 h prior to HIV infection. The primary human CD4+ T cells were infected via spinocculation using VSV-G psuedotyped HXB-PLAP-nef+ provirus and polybreene (8 μg/ml) at 2700RPM for 90 min. After spinocculation the CD4+ T cells were cultured in fresh media for 24 h. The cells were washed with PBS 3× prior to CNX-225 (1 μM) or DMSO treatment, cells were left to culture in the presence of the ITK inhibitor and samples were taken at 24–96 h post inhibitor treatment for HIV replication analysis. The data shown is a representative experiment of three infections from three different donors.

Virus like particle assays

HEK293T cells were cotransfected with expression vectors for Gag and ITK. Following transfection whole cell lysates were prepared with a lysis buffer (10 mM Tris–HCl, ph 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 2 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% NP-40 1 mM PMSF and 100 μl of cocktail inhibitor set III), and Gag and ITK expression was monitored by immunoblots or fluorescent microscopy using anti-HIV p24 (anti-HIV p24, 183-H12-5C from the National Institutes of Health AIDS Research and Reference Reagent program) and/or anti-ITK antibodies (2F12, Upstate). Cell supernatants were collected and spun over 20% sucrose cushions at 100,000 × *g* for 1 h at 4 °C to pellet virus like particles (VLPs), which were resuspended in 1 ml of PBS. Extracellular p24 levels were measured by ELISA (26).

Immunofluorescence microscopy

For imaging transfected HEK293T cells, cells were plated 24 h prior to transfection on glass and following transfection cells were fixed with a final concentration of 2% paraformaldehyde, and mounted on glass slides for microscopy analysis.

To image intracellular HIV-1, paraformaldehyde fixed HIV-1 infected Jurkat cells were washed in PBS before permeabilizing for 15 min in 0.1% Triton X-100. Cells were then blocked/enhanced with Image-IT signal enhancer (Molecular Probes) for 30 min at room temperature. The cells were washed with ice cold PBS and stained with primary antibodies against Gag (anti-HIV p24, 183-H12-5C from the National Institutes of Health AIDS Research and Reference Reagent program), and ITK (anti-ITK [Y401], AB32039 Abcam) for 1 h at room temperature. Cells were once again washed extensively with ice cold PBS before staining with the secondary antibodies. Secondary antibodies used were Alexa Fluor 594 goat anti-mouse, Alexa Fluor 488 Goat anti-Rabbit (obtained from Molecular Probes) at 8 μg/ml concentration for 1 h at room temperature. Phalloidin staining was performed with Alexa Fluor 635 Phalloidin (Molecular Probes) for 30 min in the dark at room temperature. Cholera Toxin-B staining was performed to identify lipid rafts in living cells for 30 min in the dark at 4 °C with CT-B FITC (Molecular Probes), washed with PBS then fixed. DAPI (4′,6diamidino2phenylindole from Molecular Probes) was used as a nuclear stain at a concentration of 300 ng/ml for 10 min in the dark at room temperature. Samples were washed with ice cold PBS then wet mounted on glass slides for microscopy analysis.

All microscopy was performed using a Nikon deconvolution wide-field Epifluorescence system at Boston University Cellular Imaging Core. ImageJ software was used to perform image analysis including, parallel Iterative Deconvolution 3D analysis, background subtraction and colocalization analysis via JACoP. Pearson's coefficent of colocalization values are indicated as *r* values, where perfect colocalization of both signals equals 1. All images shown are representatives a minimum of three independent transfections or HIV-1 infections. Cell quantification was performed by counting at least 60 cells for each set of infections and over 125 cells for transfection experiments.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.11.015.

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