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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

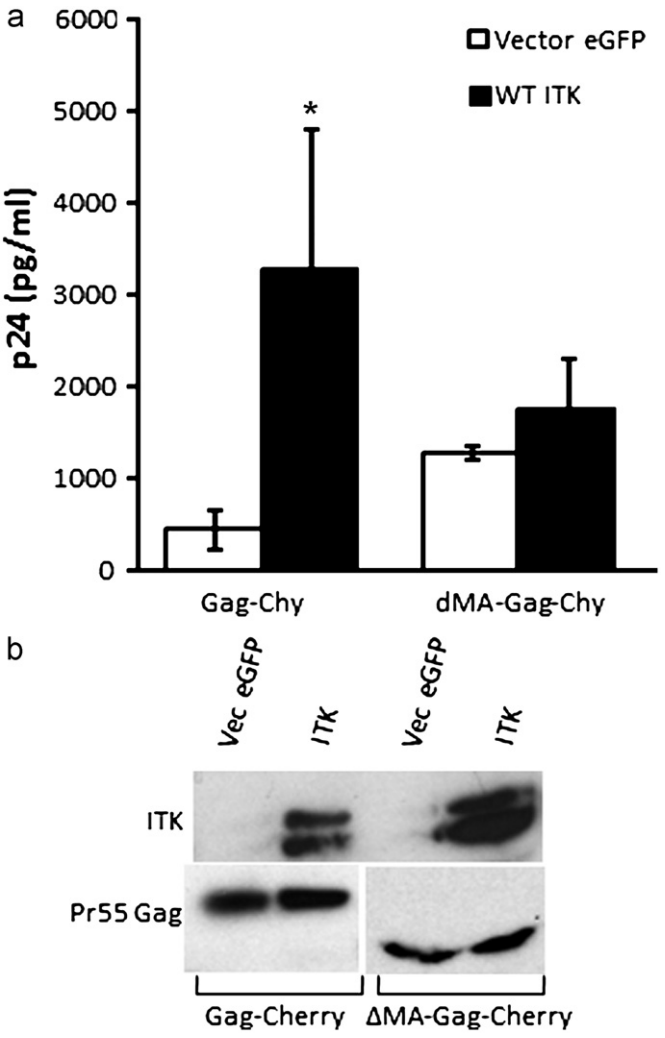

Fig. 1. ITK-mediated enhancement of VLP release requires Gag targeting to the plasma membrane. (a) HEK293T cells were transiently transfected with Gag-Cherry, or ΔMA Gag-Cherry DNA plus vector-GFP (control) or ITK-GFP DNA constructs. Supernatants were spun on 20% sucrose cushion to purify extracellular 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.