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The Src Kinase Lck Facilitates Assembly of HIV-1 at the Plasma Membrane

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HIV type 1 (HIV-1) assembly and egress are driven by the viral protein Gag and occur at the plasma membrane in T cells. Recent evidence indicates that secretory vesicles and machinery are essential components of virus packaging in both T cells and macrophages. However, the pathways and cellular mediators of Gag targeting to the plasma membrane are not well characterized. Lck, a lymphoid specific Src kinase critical for T cell activation, is found in the plasma membrane as well as various intracellular compartments and it has been suggested to influence HIV-1 replication. To investigate Lck as a potential regulator of Gag targeting, we assessed HIV-1 replication and Gag-induced virus-like particle release in the presence and absence of Lck. Release of HIV-1 and virus-like particles was reduced in the absence of Lck. This decrease in replication was not due to altered HIV-1 infection, transcription or protein translation. However, in T cells lacking Lck, HIV-1 accumulated intracellularly. In addition, expressing Lck in HeLa cells promoted HIV-1 Gag plasma membrane localization. Palmitoylation of the Lck unique domain, which is essential for directing Lck to the plasma membrane, was critical for its effect on HIV-1 replication. Furthermore, HIV-1 Gag directly interacted with the Lck unique domain in the context of infected cells. These results indicate that Lck plays a key role in targeting HIV-1 Gag to the plasma membrane in T cells.

The assembly and release of HIV type 1 (HIV-1)* are driven by the viral protein Gag. Expression of Gag in the absence of any other viral factors is sufficient for the formation and release of virus-like particles (VLP) (1). HIV-1 Gag interacts with the Golgi membrane in fibroblasts (2) and traffics through the late endosomal compartment on the way to the plasma membrane, the primary site of viral assembly and release in T cells (3). In contrast, HIV-1 is assembled at and buds into multivesicular bodies in macrophages (4, 5), although this model has been recently challenged (6). The observation that HIV-1 assembles at different sites in T cells and macrophages suggests that cell specific factors are operative in Gag targeting. Although several cellular proteins have been determined to be critical for HIV-1 budding (7–9), the pathways and cellular factors involved in regulating Gag trafficking during virus assembly have only recently begun to be identified (10–13).


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Materials and Methods

Cells and plasmids

Human acute T cell leukemia cell line Jurkat E6-1, obtained from American Type Culture Collection (ATCC) and JCaM1.6 (JCaM, described in Ref. 27), a Lck-deficient line derived from Jurkat, were maintained in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2 M l-glutamine, and 2 mg/ml hygromycin B (C35A only; Invitrogen). JCaM-Lck cells were generated by limiting dilution and G418 (Sigma-Aldrich) selection of JCaM cells transfected with 15 μg of pME6-Lck by electroporation with a BTX Electro Square Porator T820 (215 V, 65 ms, low voltage, 1 pulse). Pooled cells and several JCaM-Lck clones were used for analysis. Human embryonic kidney cells 293T (ATCC), and HeLa cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.2 M l-glutamine. The HIV-1 infectious cDNA clone with plasmatic alkaline phosphatase (PLAP) insert pHXBnPLAP-IRES-Nef+ (HIV-PLAP) was obtained from...
the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Germantown, MD) and has been described by Chen et al. (28). This construct was generated by inserting PLAP in place of the nef gene and Nef expression restored through its reinsertion with an upstream IRES element. Clone pBS-HXB2 was obtained from Dr. N. Landau (The Scripps Research Institute, La Jolla, CA). The LC1, LC2, LC1/2, unique domain (UD)-GFP, Lck/Src, and Src/Lck mutant constructs were provided by Dr. M. Davis (University College London, London, U.K.) (29, 30).

With the exception of UD-GFP, these constructs were subcloned into a pC I vector using convenient restriction sites. The p90ALcK GFP mutant was from Dr. M. Harrison (Purdue University, West Lafayette, IN). The pcDNA3.1 Lck, K154, F505, and R273 mutant Lck constructs were provided by Dr. J. Won (Mogam BioTechnology Research Institute, Gyeonggi-do, Korea) (31). The mouse pCino-c-Src construct was from Dr. J. Lavoie (Université Laval, Québec, Montreal, Canada) (32). The Fyn and Fyn C3.65 constructs were obtained from Dr. M. Resh (Memorial Sloan-Kettering Cancer Center, New York, NY) (33, 34).

**Generation of infectious viruses and infections**

The generation, collection, and infection with conditioned medium containing vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 by transient transfection of 293T cells using the calcium phosphate method has been previously described (35). Conditioned medium was filtered with 0.45-µm syringe filter (Whatman) before infection. For HIV-1, PLAP virus was infected by flow cytometry using murine anti-PLAP Ab (Sigma-Aldrich) and FITC-conjugated anti-mouse Ab (BD Pharmingen). HIV replication was measured by p24 ELISA (PerkinElmer) per the manufacturer’s instructions.

**Immunofluorescence microscopy**

Infected Jurkat and JCaM cells (5 days postinfection) and transfected HeLa cells (48 h posttransfection) were harvested at 5 days postinfection, washed in cold PBS, and fixed with 2% paraformaldehyde in PBS for 30 min at 22°C. Fixed cells were washed twice in staining medium (ice-cold 1% FCS/PBS) and permeabilized with 0.1% Triton X-100 for 30 min at 22°C. Permeabilized cells were blocked with 1% or 3% BSA/PBS for 30 min at 22°C and/or 0.01% saponin for 30 min at 22°C before incubation for 1 h on ice with mouse anti-HIV-1 capsid protein p17 (13-103-100; Advanced Biotecnologies) in 1% BSA/PBS with occasional mixing. Two washes in staining medium removed unbound primary Ab before the addition of Alexa Fluor 660 goat anti-mouse IgG (H+L) (Molecular Probes) in 1% BSA/PBS for 30 min on ice in the dark. Excess Ab was removed by two washes with staining medium and stained with mouse anti-CD63 (sc-5275 FITC; Santa Cruz Biotechnology) or rabbit anti-Lck (Santa Cruz Biotechnology) followed by the secondary Ab conjugated to Alexa Fluor 647 (Invitrogen). At least 99 HIV-infected cells were counted to determine whether Gag staining was intracellular or at the plasma membrane.

**Electron microscopy**

Cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4; Electron Microscopy Sciences) for 2 h on ice. Three 10-min washes with cold 0.1 M sodium cacodylate buffer (pH 7.4) were performed before fixing cell pellets for 2 h at 22°C with 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer. After three washes, cells were dehydrated in a graded ethanol series, 25%, 50%, 70%, 90%, 100% for 15 min each. Infiltration of the cell pellets was accomplished with a 1:1 mixture of EMbed-812 resin, medium formulation (Electron Microscopy Sciences), and propylene oxide, overnight. After two changes of undiluted resin for 4 h per exchange, pellets were placed into a 70°C oven overnight to cure. Ultra-thin sections were cut to 60-nm thickness using a Microtome diamond knife and placed on an RMC MT-7000 ultra microtome. Sections were collected on 300-mesh copper grids and stained with 5% uranyl acetate in methanol and Reynolds lead citrate for 15 and 15 min, respectively. Sections were observed and photographed using a Philips EM-400 electron microscope operated at 80 kV.
Results

Lck is required for efficient HIV-1 replication

To examine whether Lck regulates HIV-1 replication, Jurkat T cells and JCaM cells, a Jurkat-derived cell line that lacks a functional Lck protein (27, 37), were infected with HIV-PLAP pseudotyped with a VSV-G envelope. At 5 days postinfection, p24 ELISA was performed to assay viral replication. Lck protein expression was confirmed by immunoblot. Results shown are from a single experiment that includes at least three independent infections. Error bar shows the SD between infections. Data are representative of five independent experiments. B, Jurkat and JCaM cells were infected with HIV-PLAP, and p24 was monitored over the course of 7 days. Results shown are from a single experiment with each data point representing three independent infections. These data are representative of three experiments. C, Positively selected CD4+ cells were activated and transfected with a nontarget control or Lck in a Mission short hairpin RNA (shControl or shLck) vector by electroporation. After 72 h, cells were infected with HIV-PLAP, and p24 was monitored for 5 days at which time viral replication was quantified using p24 ELISA. Lck protein expression was evaluated by immunoblot. These data represent four independent transfections. Values were normalized to control cells, which were set at 100%. Error bar represents SD. p = 0.003, using a paired t test with two tails.

Whether Lck activity was required for efficient HIV-1 replication in primary T cells was examined by cotransfecting primary CD4+ T cells with HIV-1 HXB cDNA plus control or Lck short hairpin RNA and measuring virus production by p24 ELISA. HIV-1 replication was ∼30% lower in primary CD4+ T cells in which Lck expression was reduced using Lck short hairpin RNA as compared with controls (Fig. 1C). Therefore, these findings with primary CD4+ T cells, together with the results from our Lck-deficient cell lines, indicate that Lck promotes efficient HIV-1 replication in T cells.

Lck targets HIV-1 Gag to the plasma membrane

To identify Lck-dependent processes critical for HIV-1 replication, we examined HIV-1 infection and protein levels in HIV-PLAP-infected Jurkat and JCaM cells. We initially analyzed proviral integration in these cells using a semiquantitative nested PCR technique (36). Comparable amounts of integrated provirus were observed in both infected Jurkat and JCaM cells (Fig. 2, A and B), indicating that Lck is regulating a step after proviral integration. In addition, protein expression of HIV-1 Gag p55 and p24 were similar or greater in infected JCaM as compared with Jurkat cells as determined by immunoblotting (Fig. 2C and data not shown). These observations verify that Jurkat and JCaM cells are equally susceptible to HIV-1 infection and suggest that Lck is participating in late events of the virus life cycle such as virus assembly or release. To confirm that Lck is involved in HIV-1 packaging or egress, we cotransfected Lck and HIV-1 Gag into 293T cells and measured VLP production by p24 ELISA. VLP production in the presence of Lck was increased by ∼4-fold compared with control cells without Lck (Fig. 2D), signifying that Lck enhances HIV-1 Gag assembly and release. Fyn, a src family member also found in T cells similarly enhanced VLP release by ∼3-fold, whereas c-Src did not increase VLP release (Fig. 2, E and F).

Virus assembly and release are driven by Gag and preferentially occur at the plasma membrane in T cells. Because our experiments implicated Lck in these processes, we examined Gagp17 localization in HIV-1-infected Jurkat, JCaM, and JCaM-Lck cells using confocal microscopy. As expected, Gagp17 was detected primarily at the plasma membrane in Jurkat (Fig. 3B) and JCaM-Lck cells (Fig. 3F). In contrast, Gagp17 was detected at the plasma membrane as well as intracellularly in the HIV-infected JCaM cells (Fig. 3D). Intracellular Gagp17 staining was observed in ∼11% of the infected Jurkat cells, whereas 56% of the JCaM cells were positive for intracellular Gagp17 staining as determined by immunofluorescence and confocal microscopy. To identify the intracellular compartment containing Gagp17 in JCaM, HIV-infected Jurkat and JCaM cells were stained for both HIV-1 Gagp17 and CD63, a late endosomal marker. Colocalization of HIV-1 Gagp17 percentage of JCaM cells expressing HIV-1 at both 3 and 5 days postinfection was equal or greater than that observed in Jurkat cells, precluding differential infection and expression as explanations for the reduction in virus replication (data not shown). Furthermore, diminished HIV-1 replication in JCaM was not due to a general decrease in cellular metabolism or inability to signal because no decrease in virus replication was observed in cells lacking Zap70, a protein tyrosine kinase immediately downstream of Lck (data not shown). To confirm that the decrease in HIV-1 production was due to the absence of Lck in JCaM cells, several clonal and pooled Lck-expressing JCaM cell lines (JCaM-Lck) were generated and tested for their ability to support HIV-1 replication. Re-introducing functional Lck into JCaM cells restored HIV-1 replication to levels observed in infected Jurkat cells (Fig. 1A and data not shown).

FIGURE 1. HIV-1 replication is reduced in the absence of Lck. A, Jurkat, JCaM, and JCaM that stably express Lck (JCaM + Lck) cells were infected with HIV-PLAP pseudotyped with a VSV-G envelope. At 5 days postinfection, p24 ELISA was performed to assess viral replication. Lck protein expression was confirmed by immunoblot. Results shown are from a single experiment that includes at least three independent infections. Error bar shows the SD between infections. Data are representative of five independent experiments. B, Jurkat and JCaM cells were infected with HIV-PLAP, and p24 was monitored over the course of 7 days. Results shown are from a single experiment with each data point representing three independent infections. These data are representative of three experiments. C, Positively selected CD4+ cells were activated and transfected with a nontarget control or Lck in a Mission short hairpin RNA (shControl or shLck) vector by electroporation. After 72 h, cells were infected with HIV-PLAP, and p24 was monitored for 5 days at which time viral replication was quantified using p24 ELISA. Lck protein expression was evaluated by immunoblot. These data represent four independent transfections. Values were normalized to control cells, which were set at 100%. Error bar represents SD. p = 0.003, using a paired t test with two tails.
and intracellular CD63 was observed in the majority of JCaM cells, whereas CD63 colocalized with HIV-1 Gagp17 in infected Jurkat cells only at the plasma membrane (Fig. 3, G and H). Electron microscopy corroborated these results showing that viral particles were exclusively associated with the plasma membrane in Jurkat cells but were detected both at the plasma membrane and in membrane-bound intracellular vesicles in JCaM cells (Fig. 4).

FIGURE 3. Intracellular accumulation of Gag in the absence of Lck. Jurkat (A and B), JCaM (C and D), and JCaM-Lck (E and F) cells were infected with HIV-PLAP. Five days after infection, cells were stained for HIV-1 Gagp17 (red) (B, D, and F). Phase contrast images of the cells are shown in A, C, and E. Jurkat (G) and JCaM (H) cells were infected and stained with both Gagp17 (red) and CD63 (green) as shown. CD63/Gag image shows an overlay of these two stains. Cells were visualized using an Olympus IX70 confocal laser-scanning microscope with a ×60 (NA1.4) oil objective.

FIGURE 4. HIV is present in intracellular vesicles in the absence of Lck. Electron micrographs of HIV-PLAP-infected Jurkat T cells (A and B) and JCaM cells (C and D). Magnification shown in A and C is ×6000, whereas magnification in B and D are ×13,000. Areas boxed in A and C represent enlarged view in B and D. Arrows highlight location of virus particles. Scale bar represents 1 micron.
These data suggest that Lck regulates Gag trafficking from the intracellular compartments to the plasma membrane during HIV-1 assembly in T cells.

HIV-1 packaging and release has been reported to occur in intracellular vesicles in some cells such as macrophages (3) and HeLa cells (38). To determine whether Lck could redirect the site of virus assembly to the plasma membrane, HeLa cells were cotransfected with Lck and HIV-1 Gag and Gag localization was determined by fluorescent microscopy. The majority of HeLa cells transfected with control vector had intracellular Gag with only ~38% of the cells having Gag localized to the plasma membrane (Fig. 5). When Lck was overexpressed in HeLa cells, the percentage of cells that had Gag associated with the plasma membrane increased to ~80% (Fig. 5). It should be noted that overexpression of Lck did not enhance the release of VLPs in HeLa cells (data not shown), although redirecting Gag to the plasma membrane from intracellular sites of assembly does not necessarily enhance VLP release (3). These data suggest that Lck expression alters the site of assembly in the context of HeLa cells.

**Lck palmitoylation is required for efficient virus production**

To gain insight into how Lck influences HIV-1 assembly, we cotransfected HIV-1 Gag with Lck expression constructs that harbored mutations in various functional domains. Both the Src homology (SH)2 (K154) and SH3 (W97A LckGFP) Lck mutants produced VLP at equal or higher levels than that of wild-type Lck (Fig. 6B), implying that neither of these domains participate in, and may even inhibit VLP production. A kinase dead Lck (R273) supported similar or elevated levels of VLP whereas a constitutively active Lck (F505) did not enhance VLP release compared with wild-type Lck (Fig. 6B), suggesting that kinase activity is dispensable for or possibly suppresses HIV-1 packaging and replication.

Palmitoylation of Lck is essential for its localization in plasma membrane lipid rafts and HIV-1 is packaged and released from these lipid rafts in T cells. Thus, we were interested in assessing the importance of Lck palmitoylation in HIV-1 replication. Lck expression constructs that included mutations in individual (LC1 and LC2) as well as both critical residues required for palmitoylation (LC1/2) (29) were reduced by greater than 80% compared with wild-type Lck in their ability to mediate VLP release from both 293T and JCaM cells (Fig. 6). It should be noted that previous studies have reported the kinase activities of these mutations to be equivalent to their palmitoylated counterparts in unstimulated cells.
mined by fluorescence microscopy. GFP expression was evaluated by immunoblot, and GFP expression was determined by fluorescence microscopy. A, HIV Gagp55 and pCl, UD-Src/Lck, or UD-Lck/Src were cotransfected into 293T cells, and VLP release was assessed by p24 ELISA. HIV Gagp55, Lck, and Src protein expression were measured by immunoblot.

These data indicate that the palmitoylation sites, but not SH2, SH3, or the kinase activity of Lck are critical for its effect on HIV-1 Gag. Similarly, a Fyn palmitoylation mutant, Fyn C3,6S, did not increase VLP production in 293T cells (data not shown), reinforcing the importance of palmitoylation for this activity of Src kinases.

Lck palmitoylation occurs at cysteines 3 and 5 in the UD. To confirm the significance of Lck palmitoylation and the UD in HIV-1 assembly, VLP release was assessed following cotransfection with several components of the cellular protein sorting pathway, including Lck and components of the vacuolar protein sorting pathway. These data have shown that Lck palmitoylation is required for efficient VLP release. In the absence of Lck, HIV-1 Gagp17 accumulated intracellularly as well as at the plasma membrane. In addition, overexpression of Lck in HeLa cells promoted Gag localization to the plasma membrane. Together, these data imply that Lck facilitates the targeting of HIV-1 Gag to the plasma membrane.

FIGURE 7. The Lck UD is sufficient for enhancing VLP release. A, 293T cells were cotransfected with HIV-1 Gag,55 and pEGFP or UD-GFP, and VLP release was quantified using p24 ELISA. Gagp55 protein expression was evaluated by immunoblot, and GFP expression was determined by fluorescence microscopy. B, HIV Gagp55 and pCl, UD-Src/Lck, or UD-Lck/Src were cotransfected into 293T cells, and VLP release was assessed by p24 ELISA. HIV Gagp55, Lck, and Src protein expression were measured by immunoblot.

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HIV-1 assembly and budding occur at the plasma membrane in T cells (42–44). In contrast, HIV-1 is both packaged and released into the multivesicular bodies in macrophages, although this model has been recently challenged (6). It has been shown that ubiquitin and components of the vacuolar protein sorting pathway are required for HIV-1 assembly and budding in both cell types (9, 13). However, the pathways and mechanisms by which HIV-1 Gag couples to this machinery and is targeted to the site of virus assembly are not well defined. The distinct locations for HIV-1 packaging and egress in different cell types suggest that cell-specific factors partially determine the site of virus assembly. Lck, a T cell-specific Src kinase, is located at both the plasma membrane (45) and in microvesicles (15), and binds the ubiquitin binding proteins p62 (46) and c-Cbl (47). In fact, a recent report demonstrated an accumulation of Lck in the endosomal compartment of HIV-1-infected cells as compared with uninfected cells (48). It is possible that Lck and c-Cbl play a role in targeting proteins into these intracellular vesicles. Furthermore, Lck indirectly interacts with several components of the cellular protein sorting pathway, including Lck and components of the vacuolar protein sorting pathway. These data indicate that the Lck UD is sufficient for its function in HIV-1 assembly.

Lck physically interacts with HIV-1 Gag

It is possible that Lck in part promotes VLP release by binding to HIV-1 Gag; therefore, we performed coimmunoprecipitation assays to determine whether HIV-1 Gag and Lck physically interact. As shown in Fig. 8A, an Lck Ab effectively pulled down a complex comprised of HIV-1 Gag and Lck from extracts prepared from 293T cells cotransfected with both of these proteins. This complex was not obtained from control cells that were transfected with either Lck or HIV-1 Gag individually. Furthermore, this interaction was mediated through the UD because HIV-1 Gag coimmunoprecipitated with UD-GFP (Fig. 8B). Importantly, HIV-1 Gag and Lck were coimmunoprecipitated from HIV-1 infected Jurkat cells, whereas, Gag-Lck complexes were not detected in infected JCaM cells that lack Lck (Fig. 8C). These results confirm that Gag and Lck physically interact in the context of HIV-1 infected cells. Based on these findings, we propose that the UD of Lck binds HIV-1 Gag during virus assembly and facilitates efficient virus release.

Discussion

Previous studies have suggested that Lck plays a role in HIV-1 transcription, replication, and pathogenesis (22, 23, 39–41). In this study, we have identified a novel function for Lck in the later stages of the HIV-1 life cycle, specifically viral packaging. The ability of Lck to directly influence the targeting of HIV-1 Gag in 293T cells implies that this activity of Lck is CD4-independent, and distinct from its role in T cell signaling. The observation that HIV-1 replication occurs in the absence of Lck indicates that Lck is not necessary for but does increase the efficiency of HIV-1 Gag assembly. In the absence of Lck, HIV-1 Gag17 accumulated intracellularly as well as at the plasma membrane. In addition, overexpression of Lck in HeLa cells promoted Gag localization to the plasma membrane. Together, these data imply that Lck facilitates the targeting of HIV-1 Gag to the plasma membrane.

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including the adapter complexes AP-1 and AP-3 (11, 49), TGN38/41 (50, 51), Rab6 (51), and atypical protein kinase C (52). Thus, Lck may regulate HIV-1 assembly by acting as an adapter protein for these or other cellular and viral proteins, such as HIV-1 Gag. In addition, various ESCRT proteins have been reported to be involved in virus assembly and release (9) and it is possible that Lck interacts with components of this complex to influence HIV-1 assembly at the plasma membrane. Alternatively, Lck may influence the kinetics of endocytosis of newly formed virus particles at the plasma membrane (6), as it does with CD4 (53, 54). This possibility was not directly tested in the current study and warrants further investigation.

The primary function of Lck is as a tyrosine kinase, and it is possible that Lck enhances HIV-1 assembly through phosphorylation of adapter complex proteins. Our data indicate that the Lck kinase domain is dispensable for its effect on HIV-1 Gag assembly. Interestingly, Yousefi and colleagues (41) reported an inverse relationship between Lck kinase activity and HIV-1 replication in T cell lines. Thus, it is possible that Lck adapter activity is mediated through direct binding rather than phosphorylation of proteins. Furthermore, neither the SH2 nor SH3 domains are required for the ability of Lck to promote HIV-1 VLP release.

We show that constructs in which Lck palmitoylation was compromised were unable to rescue efficient HIV-1 packaging and that the Lck UD was sufficient to influence VLP release indicating that palmitoylation and plasma membrane localization of the Lck UD are critical for the ability of Lck to impact HIV-1 assembly. Lck is palmitoylated on the intracellular membranes of the early exocytic pathway, which allows for its subsequent transport to the plasma membrane (45). Thus, HIV-1 Gag may be usurping this property of Lck for HIV-1 assembly. Our demonstration of a UD-mediated interaction between HIV-1 Gag and Lck substantiates this hypothesis. Interestingly, the Lck SH3 mutant (W97ALckGFP), which has been shown to have a higher repalmitoylation rate and increased presence in lipid rafts (55), enhanced VLP production over that of wild-type Lck. Fyn, another Src family kinase found in T cells, is also palmitoylated and had a comparable function in HIV-1 packaging as suggested by a previous observation that a chimeric construct consisting of the N-terminal sequence of Fyn fused to the remainder of Gag exhibited a heightened affinity for plasma membrane “barriers” and an enhancement of VLP release (56). We have confirmed these results by demonstrating that overexpressing Fyn promotes VLP production and that palmitoylation is critical for this activity. Although Lck and Fyn appear to have redundant activities for VLP formation, reduction of Lck is sufficient to induce intracellular accumulation of virus and decrease HIV replication in CD4+ T and Jurkat cells. However, the fact that Gag is still observed at the plasma membrane and virus replication is not completely inhibited when Lck expression is diminished in T cells suggests that Fyn may have a role in these processes. Other palmitoylated Src kinases may influence HIV-1 assembly in non-T cells. In contrast, c-Src, a nonpalmitoylated Src kinase, had no effect on VLP production in 293T cells. The Src/Lck chimeric construct did show modest enhancement in VLP release in 293T cells (Fig. 7B) suggesting that either a deleted Src domain inhibits VLP production or that there are additional domains in Lck that influence VLP release; however, the increase in VLP production in the presence of Src/Lck was significantly less than that in cells overexpressing Lck/Src.

Another potential mediator of the effect of Lck on HIV-1 assembly and release is the viral protein Nef. Lck physically interacts with Nef (19), and Nef has been shown to induce functional and structural changes in the endosomal compartment (20). However, although Nef may be influencing Lck activity, it is not likely to be critical for the Lck-induced enhancement of HIV-1 assembly for several reasons. First, it was not present in the cotransfection experiments in which this effect was observed. In addition, Nef binds the SH2 and SH3 domains of Lck, which are dispensable for mediating VLP release.

It is interesting to note that Src family kinases have been implicated in influencing the replication of a number of viruses, including hepatitis B virus (57, 58), vaccinia virus (59), and mouse polyoma virus (60). Furthermore, herpesvirus saimiri encodes a protein, Tip, which recruits Lck into endosomal vesicles (61). In addition, HIV-1 Vif has been shown to interact with the Src kinase Hck in macrophages (62). Finally, it has been suggested that c-Yes contributes to RSV and dengue budding and release (63). Similar to our findings for Lck and HIV-1, c-Yes plays a role in the transit of the assembled West Nile virion from the endoplasmic reticulum through the cellular secretory pathway (64) and inhibition of c-Src activity interferes with dengue virus assembly in the endoplasmic reticulum (65). These data suggest that Lck or other Src kinases have more general roles in virus replication, including virus assembly and release. Finally, other kinases that regulate T cell activation, such as the Tec kinases, may impact the late stages of HIV replication (66).

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Disclosures

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References


45. Mingozzi, M. J., and M. Marsh. 1999. Trafficking of an acylated cytosolic protein: newly synthesized p56 travels to the plasma membrane via the exo-