The Journal of Immunology

RESEARCH ARTICLE | SEPTEMBER 01 2008

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

Amy B. Strasner; ... et. al

J Immunol (2008) 181 (5): 3706–3713.
https://doi.org/10.4049/jimmunol.181.5.3706

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023

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

Amy B. Strasner,* Malini Natarajan,* Tom Doman,† Douglas Key,† Avery August,*†‡ and Andrew J. Henderson[2§]

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 Journal of Immunology,* **2008, 181: 3706–3713.**

The assembly and release of HIV type 1 (HIV-1)[3] 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).

Lck, a lymphoid specific Src kinase found predominantly in T cells, plays a critical role in T cell activation. The majority of Lck is associated with CD4 and plasma membrane lipid rafts; however, it is also found associated with the Golgi network (14) and in the endosomal compartment (15, 16). Lck interacts with several ubiquitin-binding proteins that have critical functions for protein trafficking (17, 18), although a role for Lck in the secretory pathway has not been established. In the context of HIV-1, Lck binds the viral protein Nef, which has been implicated in altering the structure and function of the endosomal compartment (19, 20). Lck is also activated following HIV-1 infection (21) and mediates syncytium formation (22, 23). Furthermore, Lck protein levels were reported to be altered in some HIV-1 patients (24), and it has been proposed that increased Lck activity following T cell stimulation leads to reactivation of latent HIV-1 (25). Finally, Lck is present in the HIV-1 virion (26), implying an important role in the late stages of the viral life cycle. Based on these observations and its presence at critical sites of HIV-1 assembly and release, we hypothesized that Lck is a cellular regulator of HIV-1 Gag targeting. We report in this study that Lck assists in directing Gag to the plasma membrane and participates in efficient production of HIV-1 from T cells.

Materials and Methods

Cells and plasmids

Human acute T cell leukemia cell line Jurkat E6-1, obtained from American Type Culture Collection (ATCC) and J.CaM1.6 (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 pMEX-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 placental alkaline phosphatase (PLAP) insert pHXBnPLAP-IRES-Nef+ (HIV-PLAP) was obtained from

*Intergrated Bioscience Program in Immunobiology, Huck Institute of the Life Sciences, †Department of Veterinary and Biomedical Sciences, and ‡Center of Molecular Immunology and Infectious Diseases, The Pennsylvania State University, University Park, PA 16802; and §Department of Medicine, Section of Infectious Diseases, Center for HIV/AIDS Care and Research, Boston University School of Medicine, Boston, MA 02118

Received for publication July 2, 2007. Accepted for publication June 29, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work is supported by Penn State Tobacco Formula Funds and by Grants AI46261 and AI62467 from the National Institutes of Health (to A.J.H.).

[2] Address correspondence and reprint requests to Dr. Andrew J. Henderson, Department of Medicine, Section of Infectious Diseases, Center for HIV/AIDS Care and Research, Evans Biomedical Research Center, Boston University School of Medicine, 650 Albany Street, Boston, MA 02118-2393. E-mail address: andrew.henderson@bmc.org

[3] Abbreviations used in this paper: HIV-1, HIV type 1; VLP, virus-like particle; VSV-G, vesicular stomatitis virus glycoprotein; PLAP, placental alkaline phosphatase; UD, unique domain; SH, Src homology.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023

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.-J. Bijlmakers (University College London, London, U.K.) (29, 30). With the exception of UD-GFP, these constructs were subcloned into a pCI vector using convenient restriction sites. The W97ALck 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, Gyunggido, Korea) (31). The mouse pCIneo-c-Src construct was from Dr. J. Lavoie (Université Laval, Québec, Montreal, Canada) (32). The Fyn and Fyn C3,6S constructs were obtained from Dr. M. Resh (Memorial Sloan-Kettering Cancer Center, New York, NY) (33, 34).

Generation of infectious virus 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-PLAP viruses, infection was assessed 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.

Integrated HIV-1 provirus was detected using a previously described semiquantitative Alu-based nested PCR technique (36). Briefly, 250 ng of genomic DNA isolated from infected cells was amplified with primers specific for the HIV long terminal repeat and chromosomal Alu repeats Alu3 5′ (AGGCAAGCTTTATTGAGGCTTAAGC-3′) and Alu5 5′ (TC CCAGCTACTCGGGAGGCTGA GG-3′). Following an initial incubation at 94°C for 3 min, 22 cycles of amplification were conducted using the following conditions: 94°C for 30 s, 70°C for 30 s, and 70°C for 5 min. A final incubation at 72°C for 10 min was performed, the resulting product diluted 10-, 50-, or 250-fold as indicated and subjected to a second round of amplification using a primer set internal to the HIV long terminal repeat for NI-3 (5′-GCCACTCCCCIGTCCCGCCC-3′) and NI-5 (5′-CACACA CAAGGCTACTTCCCT-3′). PCR conditions were as follows: 94°C for 12 min followed by 29 cycles of amplification at 95°C for 30 s, 69°C for 30 s, and 72°C for 1 min and an additional incubation at 72°C for 10 min. The final product was visualized on a 1% agarose gel. β-actin primer set 5′ (CCTAAGGCCAACCGTGAAAAG-3′) and 3′ (TCTTCATGGTG CTAGGAGCCA) was also amplified to serve as a loading control.

Coimmunoprecipitations and immunoblots

Whole cell extracts were prepared by suspending cells in lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0 mM EDTA (pH 8.0), 2.0 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Nonidet P-40, 1.0 mM PMSF, 1.0 mM pepstatin) at 4°C for 30 min. Whole cell lysates were precleared with 10 μl of protein agarose A/G beads (Santa Cruz Biotechnology) and immunoprecipitated with prewashed mouse anti-Lck monoclonal (1 μg, Lck 3A5; Santa Cruz Biotechnology) or mouse monoclonal anti-GFP (2 μg, ab1218; Abcam) Ab-coated protein agarose A/G beads. Complexes were washed three times with lysis buffer. Samples were mixed with 2X SDS loading buffer containing DTT and heated at 100°C for 3 min before resolving by SDS-PAGE with 10% polyacrylamide unless otherwise specified. Proteins were transferred to polyvinylidene difluoride membrane (Millipore), blocked with 5% nonfat dry milk in PBS with 0.02% v/v Tween 20, and detected with primary Abs against human Lck (Lck 3A5; Santa Cruz Biotechnology), c-Src (H12; Santa Cruz Biotechnology), HIV-1 p24 (183-H12-5C; AIDS Research and Reference Reagent Program, National Institutes of Health, Germantown, MD), GFP (ab1218; Abcam) or mouse β-actin (clone AC-15; Sigma-Aldrich), and mouse Trueblot (for coimmunoprecipitations only; eBioscience) or an HRP-conjugated secondary Ab against mouse IgG (Sigma-Aldrich). Chimeric constructs were detected using primary Abs against Lck (ab18896; Abcam) or c-*src* (sc-18; Santa Cruz Biotechnology). Blots were developed using an ECL-plus kit (Amersham Biosciences). For reprobing, blots were stripped with 100 mM 2-ME, 62.5 mM Tris-HCl (pH 6.7), 2% w/v SDS for 45 min at 65°C with intermittent shaking, and reblocked for 1 h before reprobing.

Transfection and assessment of VLPs

HeLa cells (2.5×10^5 cells) were plated on glass cover slips and incubated at 37°C overnight before transfection. The 293T cells were cotransfected with 10 μg of p96ZM651gag-opt (AIDS Research and Reference Reagent Program, National Institutes of Health, Germantown, MD) contributed by Drs. F. Gao, Y. Li, and B. H. Hahn, Duke University Medical Center, Durham, NC (11) and 10 μg of pCI (Promega), pCI Lck, pcDNA3.1, pcDNA3.1 Lck, pMEX, pMEX Lck, K154, F505, or R273,LC1, LC2, LC1/2, or pCineo-c-Src, Fyn, Fyn C3,6S using the calcium phosphate method (35). In preparation for transfection, JCaM cells were washed once and subsequently resuspended with 250 μl of 20 mM HEPES/RPMI 1640. The 7.5 μg of p96ZM651gag-opt and 7.5 μg of pCI, Lck, or LC1/2 was transfected into JCaM cells by electroporation with a BTX Electro Square Porator T820 (215V, 65 ms, low voltage, 1 pulse). The 10×10^6 $CD4^+$ T cells were stimulated daily with 10 ng/ml PMA and 2 μg/ml PHA for 2 days before transfection. Activated cells were washed once with and resuspended in 400 μl of RPMI 1640 supplemented with 5% FCS, to which 40 μg of either a nontarget control or Lck (sequence no. 55434 from Sigma-Aldrich) in a Mission short hairpin RNA vector (Sigma-Aldrich) was added. The cell-DNA mixture was incubated on ice for 10 min, transfected by electroporation using a BTX Electro Porator (250 V, 40 ms, low voltage, 1 pulse), and returned to ice for an additional 5 min. Transfected cells were then recovered in RPMI 1640 supplemented with 10% FCS at 37°C. VLP production was quantified from cell supernatants 48 h posttransfection by p24 ELISA. In addition, these results were confirmed by purifying VLPs. To do this, cell supernatants were layered over a 20% sucrose gradient in Tris EDTA buffer (1 M NaCl, 0.10 M Trizma base, 0.01 M EDTA) and pelleted by ultracentrifugation at $100{,}000 \times g$ for 1 h before assessing with p24 ELISA. Comparable results were obtained using cell supernatants and virus-like particles. Lck, c-Src, and Gag protein expression was confirmed by immunoblot.

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 3 min at 22°C. Permeabilized cells were blocked with 1% or 3% BSA/PBS for 30 min at 22°C before incubation for 1 h on ice with mouse anti-HIV-1 capsid protein p17 *gag* (13-103-100; Advanced Biotechnologies) 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 (Lamp-3) FITC (sc-5275 FITC; Santa Cruz Biotechnology) or rabbit anti-Lck (Santa Cruz Biotechnology). Following two washes, cells were resuspended in staining medium and visualized using an Olympus IX70 laser-scanning confocal microscope with a ×60 (NA1.4) oil objective. 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%, 95%, 2 × 100% for 15 min each. Infiltration of the cell pellets was accomplished with a 1:1 mixture of EMbed-Araldite 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 a thickness of 80 nm using a MicroStar diamond knife and 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 10 min, respectively. Sections were observed and photographed using a Philips EM-400 electron microscope operated at 80 kV.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023

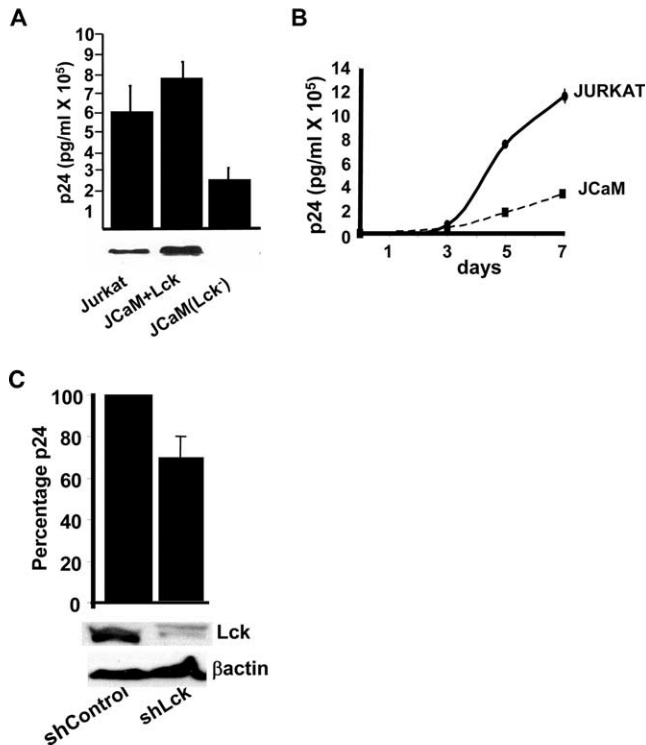

FIGURE 1. HIV-1 replication is reduced in the absence of Lck. *A*, Jurkat, JCaM, and JCaM that stably express Lck (JCaM + Lck) cells were infected with HIV-PLAP pseudotyped with a VSV-G envelope. At 5 days postinfection, p24 ELISA was performed to assess viral replication. Lck protein expression was 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 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.

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. HIV-PLAP expresses PLAP on the surface of infected cells upon viral transcription (28), thus providing a marker for infected cells that can be detected by flow cytometry. Because JCaM cells have reduced CD4 expression (data not shown), HIV-PLAP was pseudotyped with a VSV-G envelope allowing it to bypass the CD4 receptor and enter the cell via endocytosis. At various times postinfection, HIV-1 replication as assessed by p24 ELISA was found to be reduced 50–86% in T cells lacking Lck as compared with those with Lck (Fig. 1, *A* and *B*). We determined the time point with the largest p24 difference and the least amount of syncytium formation or cell death to be at 5 days postinfection, thus all subsequent experiments were performed at this time. Flow cytometric analysis for the PLAP surface marker showed that the 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. 1*A* and data not shown).

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. 1*C*). 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. 2*C* 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. 2*D*), 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. 3*B*) and JCaM-Lck cells (Fig. 3*F*). In contrast, Gagp17 was detected at the plasma membrane as well as intracellularly in the HIV-infected JCaM cells (Fig. 3*D*). 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

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023

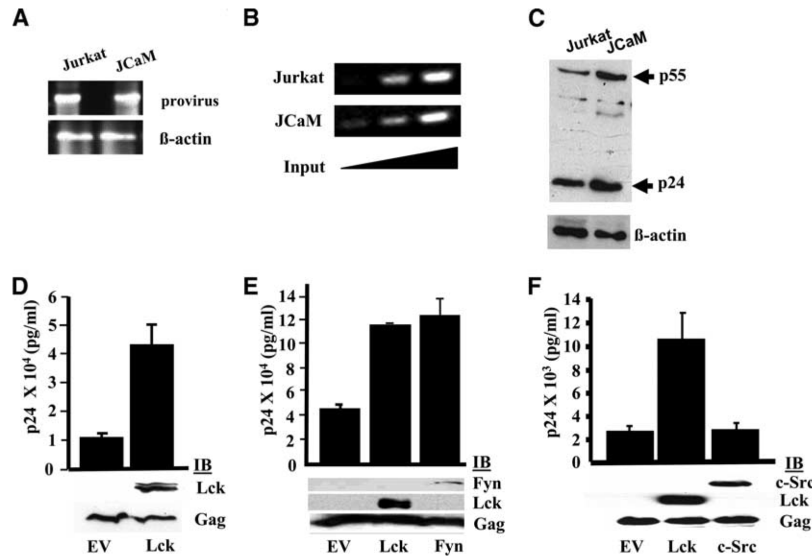

FIGURE 2. Lck is required for HIV-1 Gag assembly. *A*, Jurkat and JCaM cells were infected with HIV-PLAP for 5 days. Genomic DNA was isolated to measure proviral integration using a nested PCR as described in *Materials and Methods*. *B*, A repeat of the PCR shown in *A* is shown, except that the first-round product was diluted by 250-, 50-, or 10-fold before the second round of PCR to demonstrate that PCR was linear. PCR products were visualized on an ethidium bromide stained agarose gel. *C*, Jurkat and JCaM cells were infected with HIV-PLAP and at 5 days postinfection, cells were lysed and Gag protein expression was determined by immunoblotting. *D*, HIV-1 Gagp55 and a Lck expression vector or empty vector (EV) were cotransfected in 293T cells. VLPs were purified from cell supernatant with a sucrose gradient and release assessed by p24 ELISA. These transfections were performed in triplicate, and error bar represents SD between samples. Immunoblots below p24 data show protein expression in cells used in this experiment. In some experiments, a slower migrating anti-Lck cross-reactive band was detected. Results shown are from a single experiment that is representative of five experiments. *E*, HIV-1 Gagp55, Lck, and Fyn or empty expression vector were cotransfected into 293T cells, and VLP release was assessed by p24 ELISA. These transfections were performed in triplicate and error bar represents SD between samples. Immunoblots below p24 data show protein expression in cells used in this experiment. Results shown are from a single experiment that is representative of three experiments. *F*, HIV-Gagp55 and a c-Src expression vector or empty vector were cotransfected in 293T cells and VLP release measured by p24 ELISA. These transfections were performed in triplicate and error bar represents SD between samples. Immunoblots below p24 data show protein expression in cells used in this experiment. Results shown are from a single experiment representative of three experiments.

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).

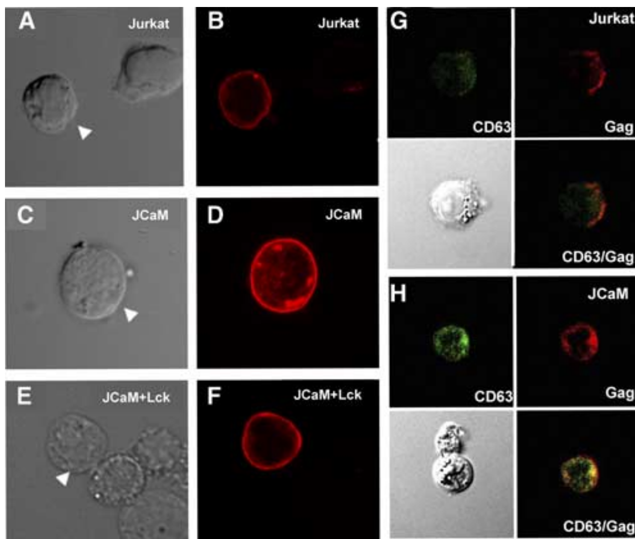

FIGURE 3. Intracellular accumulation of Gag in the absence of Lck. Jurkat (*A* and *B*), JCaM (*C* and *D*), and JCaM-Lck (*E* and *F*) cells were infected with HIV-PLAP. Five days after infection, cells were stained for HIV-1 Gagp17 (red) (*B*, *D*, and *F*). Phase contrast images of the cells are shown 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.

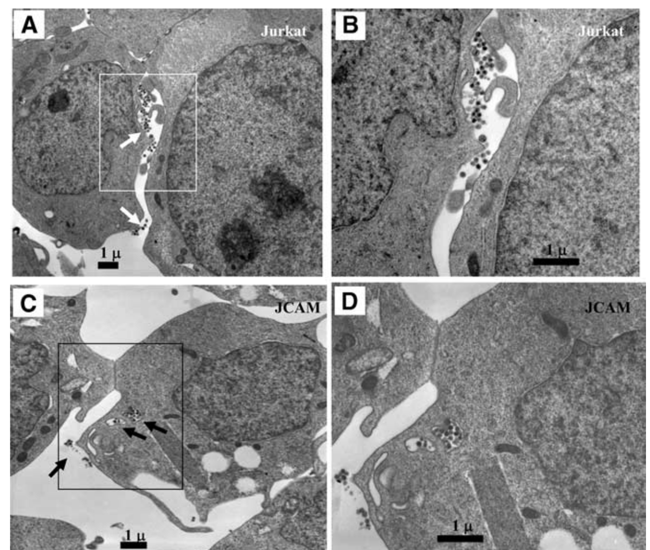

FIGURE 4. HIV is present in intracellular vesicles in the absence of Lck. Electron micrographs of HIV-PLAP-infected Jurkat T cells (*A* and *B*) and JCaM cells (*C* and *D*). Magnification shown in *A* and *C* is ×6000, whereas magnification in *B* and *D* are ×13,000. Areas boxed in *A* and *C* represent enlarged view in *B* and *D*. Arrows highlight location of virus particles. Scale bar represents 1 micron.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023

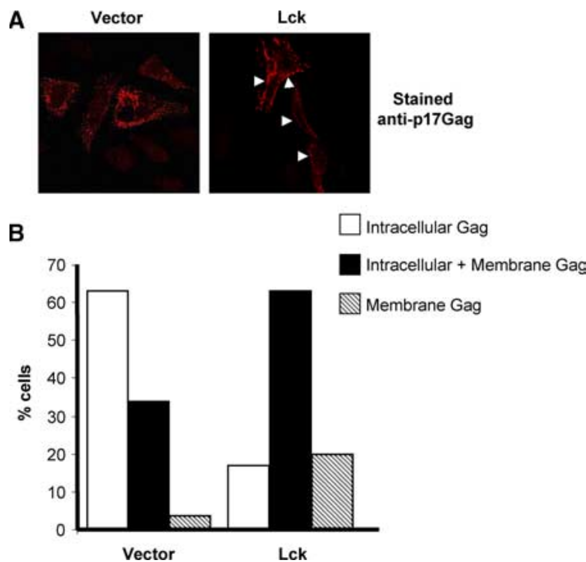

FIGURE 5. Lck directs HIV-1 Gag to the plasma membrane in HeLa cells. *A*, HeLa cells were plated on glass coverslips and cotransfected with HIV-1 Gag plus empty vector or Lck. *A*, At 48 h posttransfection, cells were fixed, permeabilized, and stained for HIV-1 Gagp17 (red). Cells were visualized using an Olympus FV1000 with an Olympus IX81 inverted microscope with a ×60 (NA1.4) oil objective. Gag at the plasma membrane is highlighted on *right* (arrowheads). *B*, Percentage of HeLa cells with intracellular, membrane, or intracellular plus membrane Gag following transfections. A minimum of 99 cells were counted.

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 (W97ALckGFP) Lck mutants produced VLP at equal or higher levels than that of wild-type Lck (Fig. 6*B*), 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. 6*B*), 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

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023

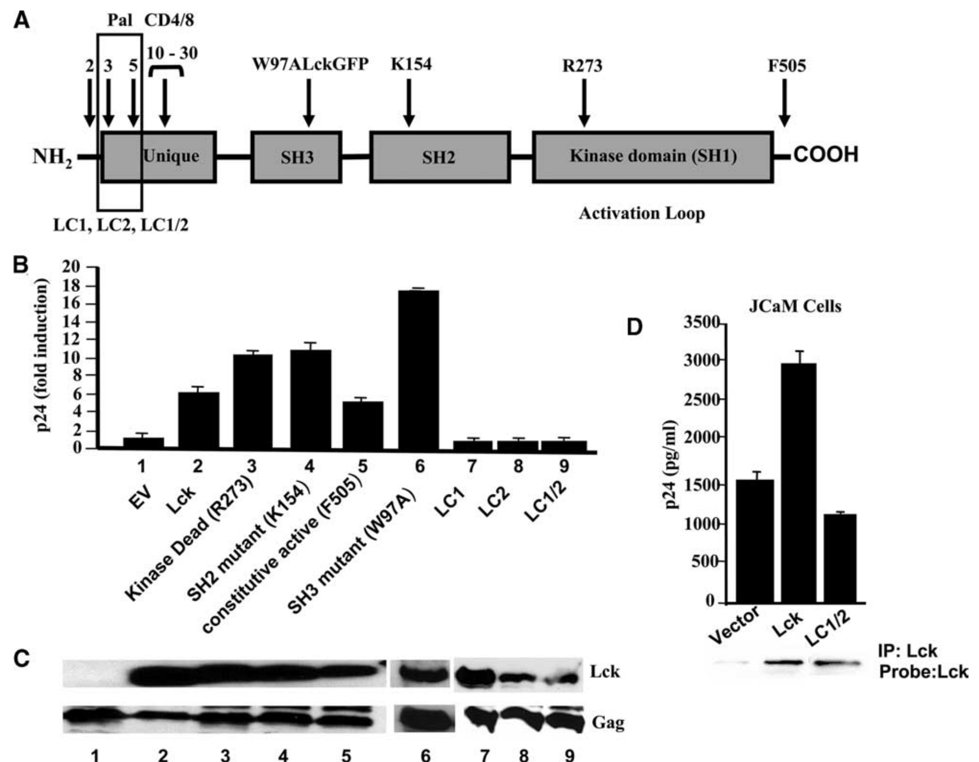

FIGURE 6. Lck palmitoylation is required for efficient HIV-1 replication. *A*, Schematic of the structure of Lck. Locations of point mutations used in this experiment are indicated by arrows. *B*, 293T cells were cotransfected with HIV-1 Gagp55 and pCI, pCI Lck, Lck R273, Lck F505, Lck K154, W97A Lck GFP, LC1, LC2, or LC1/2, and VLP release was measured by p24 ELISA. These transfections were performed in triplicate and error bar represents SD between samples. C, Immunoblots correspond with p24 data (in *B*) to show protein expression in cells used in this experiment. Results shown represent three independent transfection experiments in which values were normalized to empty vector controls, which were set at 1.0. *D*, JCaM cells were cotransfected with HIV Gagp55 and pCI, Lck, or LC1/2 by electroporation. VLP release was assessed by p24 ELISA. Lck was immunoprecipitated from whole cell lysates and protein expression determined by immunoblots.

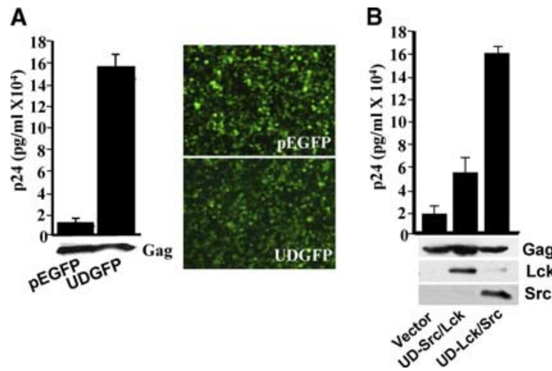

FIGURE 7. The Lck UD is sufficient for enhancing VLP release. *A*, 293T cells were cotransfected with HIV-1 Gagp55 and pEGFP or UD-GFP, and VLP release was quantified using p24 ELISA. Gagp55 protein expression was evaluated by immunoblot, and GFP expression was determined by fluorescence microscopy.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

(37). 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, (33, 34), 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 of HIV-1 Gag and Lck chimeric constructs with or without the UD. Chimeric constructs included a fusion protein in which the Lck UD replaced the Src UD and an Lck UD-GFP construct. As a control, a vector in which the Src UD replaced the Lck UD was also included. Overexpression of proteins with the Lck UD produced 8- to 15-fold increases in VLP release, comparable to or greater than those observed with wild-type Lck (Fig. 7), whereas the Src UD exhibited only a 3-fold enhancement of VLP release. These data indicate that the Lck UD is sufficient for its function in HIV-1 assembly.

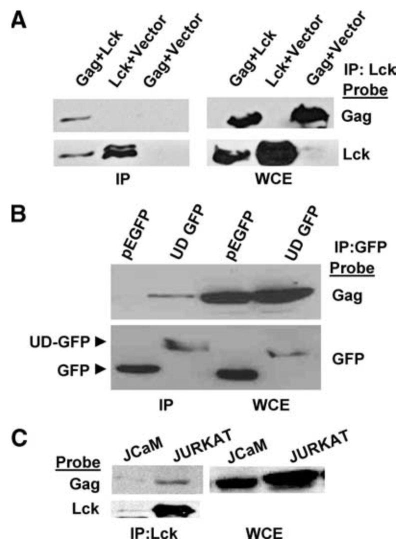

FIGURE 8. HIV-Gag and Lck physically interact. *A*, Lck was immunoprecipitated from whole cell lysates of 293T cells cotransfected with empty vector (EV), HIV-1 Gagp55, or Lck expression constructs as indicated and probed with either anti-HIV-1 Gag p24 Ab or anti-Lck Ab. Lck and HIV-1 Gagp55 expression were confirmed by immunoblotting whole cell extracts. In some experiments, a slower migrating anti-Lck cross-reactive band was detected. *B*, GFP was immunoprecipitated from whole cell lysates of 293T cells cotransfected with HIV-Gagp55 and either pEGFP or UD-GFP and probed with either anti-HIV-Gag p24 or anti-GFP Ab. GFP and HIV-Gagp55 expression was confirmed by immunoblot of whole cell extracts. *C*, Lck was immunoprecipitated from whole cell lysates of infected Jurkat and JCaM cells (5 days postinfection) and probed with either anti-HIV-1 Gag p24 Ab or anti-Lck Ab. HIV-1 Gag expression was confirmed by immunoblots of whole cell extracts.

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. 8*A*, 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. 8*B*). 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. 8*C*). 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 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.

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,

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023

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 maybe influencing 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 "barges" 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. 7*B*) 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).

Acknowledgments

We are grateful to Elaine Kunze, Susan Margagee and Nicole Bem, the technicians in the Center for Quantitative Cell Analysis at Pennsylvania State University for assistance. In addition, Kenny Gerszberg provided technical support for several experiments. We also appreciate the critical feedback from Drs. John Wills, Biao He, and Anthony Schmitt.

Disclosures

The authors have no financial conflict of interest.

References

1. Freed, E. O. 1998. HIV-1 Gag proteins: diverse functions in the virus life cycle. *Virology* 25: 1–15.
2. Ono, A., J. M. Orenstein, and E. O. Freed. 2000. Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. *J. Virol.* 74: 2855–2866.
3. Ono, A., and E. O. Freed. 2004. Cell-type dependent targeting of human immunodeficiency virus type 1 assembly to the plasma membrane and the multivesicular body. *J. Virol.* 78: 1552–1562.
4. Pelchan-Matthews, A., B. Kramer, and M. Marsh. 2003. Infectious HIV-1 assembles in late endosomes in primary macrophages. *J. Cell Biol.* 162: 443–455.
5. Raposo, G., M. Moore, D. Innes, R. Leijendekker, A. Leigh-Brown, P. Benaroch, and H. Geuze. 2002. Human macrophages accumulate HIV-1 particles in MHCII compartments. *Traffic* 3: 718–729.
6. Jouvenet, N., S. J. D. Neil, C. Bess, M. C. Johnson, C. A. Virgen, S. M. Simon, and P. D. Bieniasz. 2006. Plasma membrane is the site of productive HIV-1 particle assembly. *PLoS* 4: 2296–2310.
7. Garrus, J. E., U. K. von Schwedler, O. W. Pornillos, S. G. Morham, K. H. Zavitz, H. E. Wang, D. A. Wettstein, K. M. Stray, M. Cote, R. L. Rich, et al. 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 107: 55–65.
8. Strack, B., A. Calistri, S. Craig, E. Popova, and H. G. Gottlinger. 2003. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. *Cell* 114: 689–699.
9. von Schwedler, U. K., M. Stuchell, B. Muller, D. M. Ward, H. Y. Chung, E. Morita, H. E. Wang, T. Davis, G. P. He, D. M. Cimbora, et al. 2003. The protein network of HIV budding. *Cell* 114: 701–713.
10. Alroy, I., S. Tuvia, T. Greener, D. Gordon, H. M. Barr, D. Taglicht, R. Mandil-Levin, D. Ben-Avraham, D. Konforty, A. Nir, et al. 2005. The *trans*-Golgi network-associated human ubiquitin-protein ligase POSH is essential for HIV type 1 production. *Proc. Natl. Acad. Sci. USA* 102: 1478–1483.
11. Gao, F., Y. Li, J. M. Decker, F. W. Peyerl, F. Bibollet-Ruche, C. M. Rodenburg, Y. Chen, D. R. Shaw, S. Allen, R. Musonda, et al. 2003. Codon usage optimization of HIV-1 subtype C gag, pol, env, and nef genes: in vitro expression and immune responses in DNA-vaccinated mice. *AIDS Res. Hum. Retroviruses* 19: 817–823.
12. Ono, A., S. D. Ablan, S. J. Lockett, K. Nagashima, and E. O. Freed. 2004. Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 gag targeting to the plasma membrane. *Proc. Natl. Acad. Sci. USA* 101: 14889–14894.
13. Nydegger, S., M. Foti, P. Spearman, and M. Thali. 2003. HIV-1 egress is gated through late endosomal membranes. *Traffic* 4: 902–910.
14. Hu, H., C. E. Rudd, and H. Schneider. 2001. Src kinases Fyn and Lck facilitate the accumulation of phosphorylated CTLA-4 and its association with PI-3 kinase

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023

in intracellular compartments of T-cells. *Biochem. Biophys. Res. Commun*. 288: 573–578.
15. Blanchard, N., D. Lankar, F. Faure, A. Regnault, C. Dumont, G. Raposo, and C. Hivroz. 2002. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/ζ complex. *J. Immunol.* 168: 3235–3241.
16. Marie-Cardine, A., S. Fischer, J. P. Gorvel, and I. Maridonneau-Parini. 1996. Recruitment of activated $p56^{lck}$ on endosomes of CD2-triggered T cells, colocalization with Zap-70. *J. Biol. Chem*. 271: 20734–20739.
17. Scott, P. M., P. S. Bilodeau, O. Zhdankina, S. C. Winistorfer, M. J. Hauglund, M. M. Allaman, W. R. Kearney, A. D. Robertson, A. L. Boman, and R. C. Piper. 2004. GGA proteins bind ubiquitin to facilitate sorting at the *trans*-Golgi network. *Nat. Cell Biol*. 3: 252–259.
18. Umebayashi, K., and A. Nakano. 2003. Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. *J. Cell Biol.* 161: 1117–1131.
19. Cheng, H., J. P. Hoxie, and W. P. Parks. 1999. The conserved core of human immunodeficiency virus type 1 Nef is essential for association with Lck and for enhanced viral replication in T-lymphocytes. *Virology* 264: 5–15.
20. Stumptner-Cuvelette, P., M. Jouve, J. Helft, M. Dugast, A. S. Glouzman, K. Jooss, G. Raposo, and P. Benaroch. 2003. Human immunodeficiency virus-1 Nef expression induces intracellular accumulation of multivesicular bodies and major histocompatibility complex class II complexes: potential role of phosphatidylinositol 3-kinase. *Mol. Biol. Cell* 14: 4857–4870.
21. Phipps, D. J., S. E. Read, J. P. Piovesan, G. B. Mills, and D. R. Branch. 1996. HIV infection *in vitro* enhances the activity of *src*-family protein tyrosine kinases. *AIDS* 10: 1191–1198.
22. Briand, G., B. Barbeau, J. Corbeil, and M. Tremblay. 1997. Enhancement of HIV-1-induced syncytium formation in T cells by the tyrosyl kinase $p56^{lck}$. *Virology* 231: 10–19.
23. Yoshida, H., Y. Koga, Y. Moroi, G. Kimura, and K. Nomoto. 1992. The effect of $p56^{lck}$, a lymphocyte specific protein tyrosine kinase, on the syncytium formation induced by human immunodeficiency virus envelope glycoprotein. *Int. Immunol*. 4: 233–242.
24. Cayota, A., F. Vuillier, J. Siciliano, and G. Dighiero. 1994. Defective protein tyrosine phosphorylation and altered levels of $p59^{fyn}$ and $p56^{lck}$ in CD4 T cells from HIV-1 infected patients. *Int. Immunol*. 6: 611–621.
25. Brooks, D. G., P. A. Arlen, L. Gao, C. M. R. Kitchen, and J. A. Zack. 2003. Identification of T cell-signaling pathways that stimulate latent HIV in primary cells. *Proc. Natl. Acad. Sci. USA* 100: 12955–12960.
26. Ott, D. E., L. V. Coren, D. G. Johnson, B. P. Kane, R. C. Sowder II, Y. D. Kim, R. J. Fisher, X. Z. Zhou, K. P. Lu, and L. E. Henderson. 2000. Actin-binding cellular proteins inside human immunodeficiency virus type 1. *Virology* 266: 42–51.
27. Straus, D. B., and A. Weiss. 1992. Genetic evidence for the involvement of the *lck* tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell* 70: 585–593.
28. Chen, B. K., R. T. Gandhi, and D. Baltimore. 1996. CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of *vpu*, *env*, and *nef*. *J. Virol.* 70: 6044–6053.
29. Bijlmakers, M. J., M. Isobe-Nakamura, L. J. Ruddock, and M. Marsh. 1997. Intrinsic signals in the unique domain target $p56^{lck}$ to the plasma membrane independently of CD4. *J. Cell Biol.* 137: 1029–1040.
30. Bijlmakers, M. J., and M. Marsh. 2000. Hsp90 is essential for the synthesis and subsequent membrane association, but not the maintenance, of the Src-kinase $p56^{lck}$. *Mol. Biol. Cell* 11: 1585–1595.
31. Hur, Y. G., Y. Yun, and J. Won. 2004. Rosmarinic acid induces $p56^{lck}$-dependent apoptosis in Jurkat and peripheral T cells via mitochondrial pathway independent from Fas/Fas ligand interaction. *J. Immunol.* 172: 79–87.
32. Lavoie, J. N., C. Champagne, M. C. Gingras, and A. Robert. 2000. Adenovirus E4 open reading frame 4-induced apoptosis involves dysregulation of Src family kinases. *J. Cell Biol.* 150: 1037–1056.
33. Alland, L., S. M. Peseckis, R. E. Atherton, L. Berthiaume, and M. D. Resh. 1994. Dual myristylation and palmitoylation of Src family member $p59^{fyn}$ affects subcellular localization. *J. Biol. Chem.* 269: 16701–16705.
34. van't Hof, W., and M. D. Resh. 1997. Rapid plasma membrane anchoring of newly synthesized $p59^{fyn}$: selective requirement for NH_2-terminal myristoylation and palmitoylation at cysteine-3. *J. Cell Biol.* 136: 1023–1035.
35. Cook, J. A., L. Albacker, A. August, and A. J. Henderson. 2003. CD28-dependent HIV-1 transcription is associated with with *vav*, *rac*, and NF-κB activation. *J. Biol. Chem.* 278: 35812–35818.
36. Butler, S. L., M. S. T. Hansen, and F. D. Bushman. 2001. A quantitative assay for HIV DNA integration *in vivo*. *Nat. Med.* 7: 631–634.
37. Goldsmith, M. A., and A. Weiss. 1987. Isolation and characterization of a T-lymphocyte somatic mutant with altered signal transduction by the antigen receptor. *Proc. Natl. Acad. Sci. USA* 84: 6879–6883.
38. Sherer, N. M., M. J. Lehmann, L. F. Jimenez-Soto, A. Ingmundson, S. M. Horner, G. Cicchetti, P. G. Allen, M. Pypaert, J. M. Cunningham, and W. Mothes. 2003. Visualization of retroviral replication in living cells reveals budding into multivesicular bodies. *Traffic* 4: 785–801.
39. Coudronniere, N., J. Corbeil, V. Robert-Hebmann, J. M. Mesnard, and C. Devaux. 1998. The lck protein tyrosine kinase is not involved in antibody-mediated CD4 (CDR3-loop) signal transduction that inhibits HIV-1 transcription. *Eur. J. Immunol*. 28: 1445–1457.
40. Tremblay, M., S. Meloche, S. Gratton, M. A. Wainberg, and R.-P. Sekaly. 1994. Association of $p56^{lck}$ with the cytoplasmic domain of CD4 modulates HIV-1 expression. *EMBO J.* 13: 774–783.
41. Yousefi, S., X.-Z. Ma, R. Singla, Y.-C. Zhou, D. Sakac, M. Bali, Y. Liu, B. M. Sahai, and D. R. Branch. 2003. HIV-1 infection is facilitated in T cells by decreasing $p56^{lck}$ protein tyrosine kinase activity. *Clin. Exp. Immunol.* 133: 78–90.
42. Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, et al. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for aquired immunodeficiency syndrome (AIDS). *Science* 220: 868–871.
43. Gelderblom, H. R., E. H. Hausmann, M. Ozel, G. Pauli, and M. A. Koch. 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. *Virology* 156: 171–176.
44. Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* 225: 840–842.
45. Bijlmakers, M. J., and M. Marsh. 1999. Trafficking of an acylated cytosolic protein: newly synthesized $p56^{lck}$ travels to the plasma membrane via the exocytic pathway. *J. Cell Biol.* 145: 457–468.
46. Vadlamudi, R. K., I. Joung, J. L. Strominger, and J. Shin. 1996. p62, a phosphotyrosine-independent ligand of the SH2 domain of $p56^{lck}$, belongs to a new class of ubiquitin-binding proteins. *J. Biol. Chem*. 271: 20235–20237.
47. Rao, N., S. Miyake, A. L. Reddi, P. Douillard, A. K. Ghosh, I. L. Dodge, P. Zhou, N. D. Fernandes, and H. Band. 2002. Negative regulation of Lck by Cbl ubiquitin ligase. *Proc. Natl. Acad. Sci. USA* 99: 3794–3799.
48. Thoulouze, M. I., N. Sol-Foulon, F. Blanchet, A. Dautry-Varsat, O. Schwartz, and A. Alcover. 2006. Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse. *Immunity* 24: 547–561.
49. Coleman, S. H., D. Hitchin, C. M. Noviello, and J. C. Guatelli. 2006. HIV-1 Nef stabilizes AP-1 on membranes without inducing ARF-1 independent de novo attachment. *Virology* 345: 148–155.
50. Stephens, D. J., and G. Banting. 1997. Insulin dependent tyrosine phosphorylation of the tyrosine internalisation motif of TGN38 creates a specific SH2 domain binding site. *FEBS Lett*. 416: 27–29.
51. Jones, S. M., J. R. Crosby, J. Salamero, and K. E. Howell. 1993. A cytosolic complex of p62 and rab6 associates with TGN38/41 and is involved in budding of exocytic vesicles from the *trans*-Golgi network. *J. Cell Biol.* 122: 775–788.
52. Sanchez, P., G. D. Carcer, I. V. Sandoval, J. Moscat, and M. T. Diaz-Meco. 1998. Localization of atypical protein kinase C isoforms into lysosome targeted endosomes through interaction with p62. *Mol. Cell Biol.* 18: 3069–3080.
53. Kim, Y. H., S. H. Chang, J. H. Kwon, and S. S. Rhee. 1999. HIV-1 Nef plays an essential role in two independent processes in CD4 down-regulation: dissociation of the CD4–$p56^{lck}$ complex and targeting of CD4 to lysosomes. *Virology* 257: 208–219.
54. Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagard, and M. Marsh. 1992. The protein tyrosine kinase $p56^{lck}$ inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. *J. Cell Biol.* 117: 279–290.
55. Hawash, I. Y., K. P. Kesavan, A. I. Magee, R. L. Geahlen, and M. L. Harrison. 2002. The Lck SH3 domain negatively regulates localization to lipid rafts through an interaction with c-Cbl. *J. Biol. Chem.* 277: 5683–5691.
56. Lindwasser, O. W., and M. D. Resh. 2001. Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane domains. *J. Virol.* 75: 7913–7924.
57. Klein, N. P., M. J. Bouchard, L.-H. Wang, C. Kobarg, and R. J. Schneider. 1999. Src kinases involved in hepatitis B virus replication. *EMBO J.* 18: 5019–5027.
58. Klein, N. P., and R. J. Schneider. 1997. Activation of Src family kinases by hepatitis B virus HBx protein and coupled signaling to Ras. *Mol. Biol. Cell* 17: 6427–6436.
59. Newsome, T. P., N. Scaplehorn, and M. Way. 2004. Src mediates a switch from microtubule-to actin-based motility of vaccinia virus. *Science* 306: 124–128.
60. Messerschmitt, A. S., N. Dunant, and K. Ballmer-Hofer. 1997. DNA Tumor viruses and Src family tyrosine kinases, an intimate relationship. *Virology* 227: 271–280.
61. Park, J., B.-S. Lee, J.-K. Choi, R. E. Means, J. Choe, and J. U. Jung. 2002. Herpesviral protein targets a cellular WD repeat endosomal protein to downregulate T lymphocyte receptor expression. *Immunity* 17: 221–233.
62. Hassaine, G., M. Courcoul, G. Bessou, Y. Barthalay, C. Picard, D. Olive, Y. Collette, R. Vigne, and E. Decroly. 2001. The tyrosine kinase Hck is an inhibitor of HIV-1 replication counteracted by the viral Vif protein. *J. Biol. Chem.* 276: 16885–16893.
63. Garnier, L., J. W. Wills, M. F. Verderame, and M. Sudol. 1996. WW domains and retrovirus budding. *Nature* 381: 744–745.
64. Hirsch, A. J., G. R. Medigeshi, H. L. Meyers, V. DeFilippis, K. Fruh, T. Briese, W. I. Lipkin, and J. A. Nelson. 2005. The Src family kinase c-Yes is required for maturation of West Nile virus particles. *J. Virol.* 79: 11943–11951.
65. Chu, J. J., and P. L. Yang. 2007. c-Src protein kinase inhibitors block assembly and maturation of dengue virus. *Proc. Natl. Acad. Sci. USA* 104: 3520–3525.
66. Readinger, J. A., G. M. Schiralli, J. K. Jiang, C. J. Thomas, A. August, A. J. Henderson, and P. L. Schwartzberg. 2008. Selective targeting of ITK blocks multiple steps of HIV replication. *Proc. Natl. Acad. Sci. USA* 105: 6684–6689.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/5/3706/1267607/zim01708003706.pdf by guest on 27 January 2023