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ABSTRACT

The assembly and release of retroviruses from the host cells require dynamic interactions between viral structural proteins and a variety of cellular factors. It has been long speculated that the actin cytoskeleton is involved in retrovirus production, and actin and actin-related proteins are enriched in HIV-1 virions. However, the specific role of actin in retrovirus assembly and release remains unknown. Here we identified LIM kinase 1 (LIMK1) as a cellular factor regulating HIV-1 and Mason-Pfizer monkey virus (M-PMV) particle release. Depletion of LIMK1 reduced not only particle output but also virus cell-cell transmission and was rescued by LIMK1 replenishment. Depletion of the upstream LIMK1 regulator ROCK1 inhibited particle release, as did a competitive peptide inhibitor of LIMK1 activity that prevented cofilin phosphorylation. Disruption of either ROCK1 or LIMK1 led to enhanced particle accumulation on the plasma membrane as revealed by total internal reflection fluorescence microscopy (TIRFM). Electron microscopy demonstrated a block to particle release, with clusters of fully mature particles on the surface of the cells. Our studies support a model in which ROCK1- and LIMK1-regulated phosphorylation of cofilin and subsequent local disruption of dynamic actin turnover play a role in retrovirus release from host cells and in cell-cell transmission events.

IMPORTANCE

Viruses often interact with the cellular cytoskeletal machinery in order to deliver their components to the site of assembly and budding. This study indicates that a key regulator of actin dynamics at the plasma membrane, LIM kinase, is important for the release of viral particles for HIV as well as for particle release by a distantly related retrovirus, Mason-Pfizer monkey virus. Moreover, disruption of LIM kinase greatly diminished the spread of HIV from cell to cell. These findings suggest that LIM kinase and its dynamic modulation of the actin cytoskeleton in the cell may be an important host factor for the production, release, and transmission of retroviruses.

The cellular actin network is critical for cell morphogenesis, cell migration, distribution of organelles, and other fundamental cellular functions (1, 2). Actin exists in the cell in two different forms: actin monomers (G actin) and actin filaments (F actin). Actin filaments undergo dynamic polymerization and depolymerization that produce organized protrusions such as filopodia, lamellipodia, microvilli, podosomes, and membrane ruffles (3). These structures are modified by a number of actin binding proteins and by members of the Rho protein family of small GTPases. Many pathogens have developed strategies to regulate the actin cytoskeleton in order to harness the mechanical forces generated by polymerizing/depolymerizing actin filaments to exit the infected cell and spread from one cell to another. Listeria monocytogenes and Shigella flexneri induce actin polymerization through specific F-actin polymerizing proteins, leading to the formation of actin comet tails that propel the bacteria through the host cytoplasm and into target cells (4). Vaccinia virus (VV) exits the host cell and also induces formation of dense actin comet tails underneath virions, which drive the virions away from the cell and are important for cell-cell dissemination (5). Measles virions budding off the plasma membrane are often associated with cortical actin filaments. These actin filaments exclusively protrude into virus particles and interact with viral nucleocapsids (6). Moloney murine leukemia virus (MLV) has been shown to transit along filopodial bridges from an infected cell toward noninfected cells (7, 8).

There is considerable circumstantial evidence for the involvement of actin in HIV particle assembly or release. Actin and the actin-related proteins ezrin and cofilin are highly represented in HIV-1 virions (9). Cryo-electron tomographic analysis of HIV-1 assembly sites revealed that one-half of the HIV budding sites were present on actin-filled filopodia, where actin filaments were aligned toward the budding sites (10). Studies employing inhibitors of actin polymerization such as cytochalasin D and latrunculin B generally showed only a modest decrease in virus release (11, 12), while cell-cell transmission has been shown to be more significantly disrupted by these inhibitors (12).

LIM kinase 1 (LIMK1) is a serine protein kinase involved in the regulation of actin polymerization. Once activated, LIM kinase phosphorylates and inactivates the actin depolymerizing factor cofilin, which results in an increase in filamentous actin (13, 14). Here we identified LIMK1 and its upstream activator ROCK1 as...
cellular factors regulating HIV-1 and Mason-Pfizer monkey virus (M-PMV) release. Both particle release and cell-cell viral transmis-

sion were greatly reduced following LIMK1 deletion. Re-

deply, depletion of LIMK1 or its upstream regulator ROCK1 caused an accumulation of mature HIV-1 virions at the plasma

membrane.

MATERIALS AND METHODS

Cells. HeLa and Cos-1 cells were maintained in Dulbecco's modified Ea-

gle's medium (DMEM) containing 10% fetal bovine serum (FBS) and
antibiotics at 37°C with 5% CO₂. Jurkat T cells were cultured in RPMI
medium supplemented with 10% FBS, 2 mM glutamine, and peni-
cillin-streptomycin antibiotics.

Plasmids and peptides. pSARM-X is an M-PMV proviral expression

vector that expresses the M-PMV genome under the control of the vir-

al long terminal repeat (LTR) promoter. pSARM-GagGFP-M100A is an M-

PMV proviral plasmid in which Gag-green fluorescent protein (GFP)
coding sequences were inserted in place of the Gag gene; M100A signifies
a methionine-to-alanine substitution in Gag that eliminates an internal

long terminal repeat (LTR) promoter. pSARM-GagGFP-M100A is an M-

PMV vector. The vector was constructed by amplifying LIMK1 coding se-
quences with the following oligonucleotides: LIMK1Start 5'-ACGGTTGACGCTACTTTGT-3' and LIMK1(gly)3FLAG 3'-

TAGATCACTTATCGTCGTCATCCTTGTAATCTCCTCCTCCGTCG

AGGAGGTCGATCTCGTCCAGGGGCACATTTCGG-3'.

The primers used were LIMK1 resistant cDNA-F1 5'-GGCTGGTTTCCTGGATGAGGAGATCGATTTC

TGGATGAAATCGATCTCCTCATCCAGGAAACCAGCC-3', LIMK1 resistant cDNA-R1 3'-GGCTGTGGTTTTCCTGGATGAGGAGATCGATTTC

ATCAGCTGAGGTGCTGTCATGTGGTAACTCTCCTCCTCGTCG

GGGACCTCAGGGTGTCGACAGGAGAGATCGATTTC

CC-3', and LIMK1 resistant cDNA-R2 3'-GGCTGTGGTTTTCCTGGATGAGGAGATCGATTTC

AGGAGGTCGATCTCGTCCAGGGGCACATTTCGG-3'.

Rev S3 and S3 peptide were synthesized by GenScript (Piscataway, NJ, USA). The amino acid sequences are as follows: Rev S3 peptide, NFVKIVGDSWAVGASMQRQIKWFKQNRMRKKWK; and S3 peptide, MASVGAVSDGVKVFVRNFQNRMRKWK.

Pak18 peptide and Pak18–R192A were purchased from EMD Millipore (Billerica, MA, USA). The amino acid sequences are as follows: Pak18, RRKRQRQR-2PPV1ARPHEKSVYTR, and Pak18–R192A, RRKRQRQR-2PPV1ARPHEKSVYTR.

Virus stocks and infections. VSV-G-pseudotyped or wild-type HIV-

1NL4-3 was generated by transfecting 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). On day 2 posttransfection, viruses were harvested from the supernatant, filter sterilized, and subjected to Western blotting.

Subtilisin digestion. HeLa cells were transfected with NL4-3 proviral vector. Constitutively released particles were harvested from culture supernatant by centrifugation through 20% sucrose cushion prior to SDS-PAGE and Western blot analysis.

Linear sucrose density gradient analysis of virions. HeLa cells were transfected with HIV NL4-3 proviral vector and LIMK1–FLAG expression vector. Virions were purified by centrifugation through 20% sucrose as described above and then resuspended in buffer (Tris-Cl [pH 8.0], 150 mM NaCl, 5 mM CaCl₂). Thereafter, the virions were treated with different concentrations of the protease subtilisin (Sigma) for 15 min at 37°C. The reaction was stopped by addition of 1 mM phenylmethylsulfonyl fluoride plus protease inhibitors, and the virions were repurified by centrifugation through 20% sucrose cushion prior to SDS-PAGE and Western blot analysis.

Immunofluorescence microscopy. HeLa cells or Cos-1 cells expressing HIV-1 Gag-mCherry or pSARM-GagGFP-M100A were analyzed in 35-mm² glass bottom dishes coated with poly-1-lysine (MatTek, Ashland,
Wen et al.

A

B

C

D

E

HeLa

COS1

LIMK1 shRNA:

Scr shRNA:

LIMK1 cDNA:

LIMK1 cDNA*:

HIV-1 Infectivity

% Particle Release

M-PMV Infectivity
MA). Live cells were examined by total internal reflection fluorescence microscopy (TIRFM) as described below. HeLa or Cos-1 cells expressing NL4-3 or pSARM-X were also grown in MatTek dishes. For staining, these cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were then permeabilized for 5 min with 0.2% Triton X-100 and blocked in Dako blocking buffer for 30 min. After blocking, the cells were incubated in primary antibody for 1 h and then secondary antibody for 1 h prior to examination by TIRFM. Primary and secondary antibodies were diluted in Dako antibody diluent.

**TIRFM image acquisition and analysis.** The imaging station was a Deltavision imaging system developed by Applied Precision (Issaquah, WA [part of GE Healthcare]). Through-the-objective TIRFM was performed by using an inverted Olympus IX-70 microscope with a 60×, 1.45-numerical-aperture (NA) TIRF objective and 486-nm or 561-nm lasers. Imaging processing and deconvolution were performed using softWoRx software. The number of HIV Gag-iGFP puncta and M-PMV Gag-GFP puncta at the plasma membrane was determined using the measurements module of Volocity 6.2.1. The colocalization pixels between Gag and LIMK1-FLAG were determined with the colocalization module of Volocity 6.2.1.

**Electron microscopy.** HeLa cells stably expressing control shRNA or LIMK1 shRNA were transfected with NL4-3 provirus and cultured in 6-well plates. Forty-eight hours following transfection, cells were fixed in paraformaldehyde-glutaraldehyde, 2.5% each in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA) overnight at 4°C and embedded in Epon. Ultrathin sections were cut and images obtained using a Hitachi H-7500 transmission electron microscope at 75 kV.

**Cell-cell transmission studies.** For the analysis of HIV cell-cell transmission, donor HeLa cells were transfected with HIV NL4-3. On day 1 posttransfection, Jurkat cells were added to the donor cells and were either centrifuged at 300 × g for 10 min to ensure cell-cell contact of Jurkat cells with HeLa cells or gently shaken at 50 rpm to inhibit intercellular contacts. After 2 h of coculture, the Jurkat cells were harvested, washed, and incubated at 37°C. After two additional days of culture, Jurkat cells were stained for CD3 and intracellular p24, followed by flow cytometry analysis. For the analysis of M-PMV cell-cell transmission, donor Cos-1 cells were transfected with M-PMV pSARMX. Target Jurkat cells were prelabeled with CellTracker Red CMTPX (Invitrogen) for 20 min at 37°C. On day 1 posttransfection, target cells were added to the donor cells and were either centrifuged at 300 × g for 10 min to ensure cell-cell contact of Jurkat cells with Cos-1 cells or gently shaken at 50 rpm to inhibit intercellular contacts. After 2 days of coculture, the cells were harvested and stained for M-PMV p27 antigen. The percentage of infected target cells was analyzed by flow cytometry. Cell-cell transmission events were derived by subtracting the cell-free transmission events (rocking plates) from the total transmission events (static plates).

**Statistical analysis.** Statistical comparison between the groups was performed using the unpaired t test in GraphPad Prism5. Differences were considered statistically significant at P values of ≤0.05.

**RESULTS**

**LIMK1 plays a role in both HIV-1 and M-PMV particle output.** We performed an siRNA screen using a commercially available siRNA library targeting 140 cellular membrane-trafficking genes in order to identify the cellular factors involved in both HIV-1 and M-PMV virion assembly or release. The readout of this screen was HIV-1 and M-PMV particle release as measured by supernatant reverse transcriptase activity. From the screen results, we further evaluated 14 genes that consistently led to diminished particle output for both viruses. A complete description of the screen validation and results is presented in a separate report (X. Wen, L. Ding, E. Hunter, and P. Spearman, submitted for publication). This screening strategy identified LIMK1 and its upstream activator ROCK1 as gene products whose depletion reduced both HIV-1 and M-PMV particle output. Therefore, we hypothesized that the cellular LIM kinase pathway and its regulation of dynamic actin polymerization may play a role in retrovirus particle release.

In order to test this hypothesis, we first depleted endogenous LIMK1 using siRNA and examined the effects on both HIV-1 and M-PMV particle output by Western blotting as shown in **Fig. 1A** and B. TSG101 is required for both HIV-1 and M-PMV late budding events and was included as a positive control (21, 22). LIMK1 levels in LIMK1-siRNA-treated cells were significantly reduced in HeLa (95%) and in Cos-1 (70% to 80%) cells compared with control siRNA-treated cells. Consistent with previous results from the screen, depletion of LIMK1 significantly reduced both HIV-1 (Fig. 1A) and M-PMV (Fig. 1B) particle output and also reduced viral infectivity compared with control cells. P24 antigen enzyme-linked immunosorbent assay (ELISA) analyses of cell lysates and supernatants in the same experiment revealed that HIV particle release from LIMK1-depleted HeLa cells was decreased more than 4.5-fold compared with control cells (Fig. 1A). To further evaluate the role of LIMK1 in HIV-1 and M-PMV particle output, we generated stable HeLa and Cos-1 cell lines expressing control shRNA or LIMK1 shRNA and then examined the effects on particle output. The LIMK1 levels were significantly reduced in LIMK1 stable knockdown HeLa cells (approximately 95%) and in LIMK1 stable knockdown Cos-1 cells (90%) as shown in the top panel of **Fig. 1C**. HIV-1 and M-PMV particle output were each significantly decreased by LIMK1 depletion (Fig. 1C). However, in order to rule out off-target effects, we next restored cellular levels of LIMK1 using an shRNA-resistant FLAG-tagged LIMK1 cDNA. Figure 1C demonstrates that LIMK1 cDNA with silent mutations rendering it resistant to shRNA-mediated depletion was able to fully restore both HIV-1 and M-PMV output (Fig. 1C, LIMK1 cDNA* lanes). In contrast, LIMK1 levels were not restored with the unaltered LIMK1 cDNA. Accompanying the restoration of LIMK1 function and particle output, the infectivity of the released particles was almost fully restored (Fig. 1D and E). These data support a specific role for LIMK1 in particle output for both HIV-1 and M-PMV.
Role of cofilin in both HIV-1 and M-PMV particle output. LIMK1 is a serine protein kinase involved in the regulation of actin polymerization (23). To date, the only known target of LIMK1 is cofilin, whose only known function is the regulation of actin dynamics. Once activated, LIMK1 in turn phosphorylates and inactivates the actin depolymerizing factor cofilin, which results in net actin polymerization (13, 14). We hypothesized that the balance between active and inactive cofilin at the particle assembly site may regulate retroviral particle budding or release. Therefore, we examined the role of cofilin in HIV-1 and M-PMV assembly and release. HeLa or Cos-1 cells were transfected with siRNAs against cofilin-1 and LIMK1, either individually or together, followed by a second transfection with siRNA and NL4-3 proviral DNA. Western blot analysis demonstrated a nearly 75% knockdown of cofilin-1 protein in HeLa cells transfected with cofilin-specific siRNA and a 60% knockdown of cofilin-1 protein in Cos-1 cells, compared with cells transfected with control siRNA (Fig. 2). Both LIMK1 depletion and cofilin-1 depletion caused a significant reduction in HIV-1 and M-PMV particle output. We failed to observe an additive effect of depleting both LIMK1 and cofilin together (Fig. 2A and B, rightmost lanes). The number of infectious particles released into the cellular supernatants was reduced following cofilin depletion, reflecting the diminished overall particle release (Fig. 2C). The finding that depletion of both the upstream kinase that inactivates cofilin’s actin-severing activity and cofilin itself reduced particle output suggested to us that a balance between actin polymerization and severing must be maintained during retrovirus assembly or release.

ROCK1 is the upstream activator of LIMK1 for HIV-1 and M-PMV particle output. LIMK1 is normally activated through phosphorylation by ROCK1 and members of the Pak family, including Pak1, Pak2, and Pak4 (24–26). We examined the role of each of these upstream kinases in our experimental systems for HIV-1 and M-PMV particle output. HeLa or Cos-1 cells were transfected with siRNAs against Pak1, Pak2, and Pak4, either individually or together, or with siRNA against ROCK1, followed by a second transfection with siRNA as well as the proviral expression vector. Knockdown efficiency was assessed by Western blotting and varied from 90 to 95% as shown in Fig. 3A and B. Depletion of ROCK1 led to markedly reduced HIV-1 and M-PMV particle output, while little effect was seen with depletion of Pak1, Pak2, or Pak4, either individually or together. Similarly, the infectivity of the released particles was significantly reduced upon ROCK1 depletion but not upon depletion of Pak kinases (Fig. 3C and D). Taken together, these experiments demonstrate that the ROCK1-LIMK1-cofilin-1 pathway plays an active role in both HIV-1 and M-PMV particle output.

Inhibition of LIMK1 kinase activity by S3 peptide decreases both HIV-1 and M-PMV particle output. As an alternative approach to examine the role of the ROCK1-LIMK1-cofilin pathway in HIV-1 and M-PMV particle output, we employed a synthetic peptide that acts as a competitive inhibitor for binding to LIMK1, thus inhibiting cofilin phosphorylation. Inhibition of cofilin phosphorylation by S3 peptide was previously utilized by Yoder et al. to examine the effects on HIV-1 infection of resting T cells (27). The S3 peptide includes the N-terminal 16 residues of human cofilin as well as a membrane-permeable peptide segment (28) (Fig. 4A). The reverse sequence (Rev S3) peptide served as a negative control in these experiments. We first established that S3 inhibits LIMK1-mediated cofilin phosphorylation in both HeLa and Cos-1 cells. HeLa and Cos-1 cells were transfected with empty vector or LIMK1 expression vector, followed by treatment with S3 peptide or Rev S3 peptide. The levels of phospho-cofilin and total cofilin were determined by Western blotting 24 h after transfection as shown in Fig. 4B. Overexpression of LIMK1 induced a marked enhancement in the levels of phospho-cofilin without affecting the levels of total cofilin in both HeLa and Cos-1 cells. This enhancement was efficiently inhibited by S3 but not by Rev S3. We then turned to an examination of the effect of S3 on HIV-1 and M-PMV viral particle output. We observed that both HIV-1 and M-PMV particle outputs were significantly diminished by S3 treatment (Fig. 4C). In contrast, Rev S3 treatment had little effect on the particle output of HIV-1 and M-PMV. Taken together, these results suggest that inhibiting LIMK1 kinase activity by S3

FIG 2 Effect of cofilin depletion on HIV-1 and M-PMV virus output. (A) HeLa cells were transfected in a manner similar to that described in Fig. 1A with the indicated siRNAs. Virions and cell lysates were harvested and subjected to immunoblotting with anti-p24 antibody. (B) Cos-1 cells were transfected in a manner similar to that described in Fig. 1B with the indicated siRNAs. Virions and cell lysates were harvested and subjected to immunoblotting with M-PMV anti-p27 antibody. (C) Infectivity of the released HIV-1 particles from the experiment shown in panel A was determined on TZM-bl indicator cells, and HIV-1 particle release was assessed using a p24 antigen ELISA. The infectivity of released M-PMV particles (right graph) from the experiment shown in panel B was determined by intracellular staining and flow cytometry.
impairs both HIV-1 and M-PMV particle release, consistent with the depletion studies already presented.

In order to further examine the potential role of PAKs in HIV-1 and M-PMV particle output, we inhibited the activity of PAKs by using a PAK-inhibitory peptide, PAK18. PAK18 selectively blocks the PIX-PAK interaction, which is essential for complete activation of PAK (29). The inhibitory capability of PAK18 is rendered inactive with a single amino acid mutation, R192A, which serves as an inactive control peptide. As a positive control, we examined the effect of this peptide on the P-Raf1 (Ser 338) level, a direct PAK substrate (30). As shown in Fig. 4D, PAK activity was reduced in PAK18-treated cells as indicated by the levels of phospho-Raf1, while the inactive control peptide PAK18–R192A (C-PAK) had no impact. We next examined the effect of PAK18 on HIV-1 and M-PMV viral particle output. We observed that treatment with either PAK18 or the inactive control peptide had minimal effect on HIV-1 and M-PMV particle output, while S3 peptide treatment led to markedly reduced HIV-1 and M-PMV particle output (HIV-1, Fig. 4E; M-PMV, Fig. 4F). These results further confirmed that the Pak kinase family is not involved in modulating HIV-1 or M-PMV particle output and lend support for the role of ROCK1 as the upstream kinase involved in LIMK1 activation relevant to particle output.

Disruption of the cellular ROCK1-LIMK1-cofilin pathway caused an accumulation of Gag puncta at the plasma membrane. Our finding that the HIV-1 particle output is diminished
by the disruption of the cellular ROCK1-LIMK1-cofilin pathway suggested that the HIV-1 life cycle is arrested at the level of either assembly, budding, or release. In order to identify which step of the HIV-1 life cycle is arrested upon disruption of the ROCK1-LIMK1-cofilin pathway, we examined HIV-1 virion assembly sites at the plasma membrane by total internal reflection fluorescence microscopy (TIRFM). HeLa cells were transfected with control siRNA or siRNA against LIMK1 or ROCK1, followed by a second transfection with siRNA as well as HIV Gag-iGFP and wild-type untagged Gag expression vectors. Fourteen hours after the second transfection, images of HIV-1 assembly sites at the plasma membrane were taken. We observed that LIMK1 depletion caused a prominent accumulation of Gag-iGFP puncta at the plasma membrane compared with control siRNA-transfected cells (Fig. 5A, B, C, D, E, F).
FIG 5 Analysis of particle puncta by TIRFM. (A) (Top row) HeLa cells were first transfected with siRNA against ROCK1 or LIMK1, followed by cotransfection with siRNA and expression vectors of HIV Gag-iGFP and HIV Gag at a 1:3 ratio. Fourteen hours after the second transfection, Gag-iGFP puncta at the plasma membrane were imaged by TIRFM. For clarity, we have drawn the cell border as a solid white line on the image panels. (Middle row) Cos-1 cells were first transfected with siRNA against ROCK1 or LIMK, followed by the cotransfection of siRNA and expression vectors of pSARMX Gag-GFP and pSARMX. Fourteen hours after the second transfection, particle puncta on the plasma membrane were imaged by TIRFM as above. (Bottom row) HeLa cells were transfected with HIV iGFP and HIV Gag expression vectors. Sixteen hours posttransfection, the cells were treated with Rev S3 or S3 peptide at a final concentration 30 μM for 2 h, and particle puncta were imaged by TIRFM. (B) Quantitation of the HIV or M-PMV puncta at the plasma membrane from the experiments depicted above was performed using Volocity 6.2.1 measurements module. Error bars represent standard deviations (SD).
In addition, ROCK1 depletion also caused a prominent accumulation of Gag-iGFP puncta at the plasma membrane compared with control siRNA transfected cells (Fig. 5A, top row). We next examined M-PMV virion assembly sites at the plasma membrane following the depletion of ROCK1 or LIMK1. To do this, we employed a unique M-PMV Gag-GFP virus developed in the Hunter laboratory (pSARM-GagGFP-M100A) (15). As shown in Fig. 5A, middle row, both ROCK1 depletion and LIMK1 depletion caused a prominent accumulation of M-PMV Gag-GFP puncta at the plasma membrane compared with control siRNA-transfected cells. We then examined the effect of S3 peptide on HIV-1 virion assembly sites at the plasma membrane. Consistent with the results of LIMK1 depletion and ROCK1 depletion, inhibiting LIMK1 kinase activity by S3 peptide similarly led to an accumulation of Gag-iGFP puncta at the plasma membrane (Fig. 5A, bottom row). Quantitation of these results demonstrated statistical significance (Fig. 5B). These results suggested to us that the defect in retrovirus production caused by disruption of the ROCK1-LIMK1-cofilin pathway did not prevent particle formation but was likely at the level of either budding or release.

LIMK1 depletion causes HIV-1 virion retention on cell surfaces. In order to identify the stage of the late defect caused by disruption of the ROCK1-LIMK1-cofilin pathway, we examined LIMK1-depleted cells by transmission electron microscopy. In

FIG 6 Transmission electron microscopic analysis of HIV particles following LIMK1 depletion. (A) Electron micrograph showing plasma membrane of the control shRNA stably expressing HeLa cells after transfection with HIV-1 NL4-3. The image is representative of more than 50 cells examined. Rare particles were seen without obvious clusters on the plasma membrane (arrows). (B to D) Electron micrographs of the LIMK1 shRNA stably expressing HeLa cells following transfection of HIV-1 NL4-3 expression vector. (B) Clusters of mature HIV particles adjacent to the plasma membrane are shown. The boxed area in panel B is shown at higher magnification in panel C. (D) Focal cluster of retained particles from a different cell than that shown in panel B or C. The panels with clustered virions shown are representative of more than 50 cells examined in three separate experiments. Size bars are 1 μm for panels A and B and 0.2 μm for panels B and C.
control shRNA-expressing HeLa cells, we observed occasional budding structures and mature virions associated with the plasma membrane (Fig. 6A). In contrast, in LIMK1-shRNA stably expressing cells, we observed clustered accumulations of mature virion particles on the cell surfaces (Fig. 6B to D). The fact that the majority of the particles were mature fit well with the findings from Western blot analysis, as a Gag cleavage defect was not observed in LIMK1-depleted cells (Fig. 1). Similarly, the accumulation of mature particles attached to the plasma membrane was consistent with the findings by TIRFM showing an accumulation of fluorescent puncta on the plasma membrane. The appearance of focal clusters of particles was similar to those seen with Vpu-deficient viruses captured by tetherin (addressed later in this work) (31, 32). Taken together, our results suggest that disruption of the ROCK1-LIMK1-cofilin pathway causes a defect in HIV-1 particle release and not in earlier events of virion assembly or budding.

The cellular LIMK1 pathway plays an important role in HIV-1 cell-cell transmission. The results presented here indicate that the cellular LIMK1 pathway plays a role in both HIV-1 and M-PMV particle release. However, the effect of knockdown or peptide-mediated disruption of LIMK1 function on particle release into the supernatant was only partial (4- to 5-fold for HIV in Fig. 1). To address the potential significance of this pathway to HIV or M-PMV spread, we next asked whether LIMK1 is required for HIV-1 and M-PMV cell-cell transmission. For HIV-1 cell-cell transmission experiments, HeLa cells stably expressing control shRNA and LIMK1-shRNA were transfected with HIV NL4-3 provirus, along with empty vector or an shRNA-resistant FLAG-tagged LIMK1 vector. Jurkat cells were then cocultured for 2 h with the virus producer cells either in direct contact with the adherent cells in a dish, or with gentle shaking to prevent stable cell-cell contacts from forming. The infected Jurkat target cells were then quantified by intracellular p24 staining using flow cytometry. Infected Jurkat cells detected from the unshaken coculture represented total transmission events (cell-cell and cell-free events). Cell-cell transmission events were derived by subtracting the cell-free transmission events from the total transmission events. We found that HIV-1 cell-cell transmission was significantly diminished in HeLa cells stably expressing LIMK1-shRNA compared to HeLa cells stably expressing control shRNA as shown in Fig. 7B. The reduced HIV-1 cell-cell transmission in LIMK1-shRNA-expressing HeLa cells was restored to normal levels when LIMK1-FLAG* protein was reintroduced, demonstrating that LIMK1 is required for the efficient HIV-1 cell-cell transmission and providing a specificity and toxicity control (Fig. 7B, rightmost bar). Because the cellular manipulation of LIMK1 was performed only in producer cells, these experiments should not be confounded by the role of LIMK1 on HIV entry events (34). Next, in order to quantify M-PMV cell-cell transmission, we performed similar experiments in Cos-1 cells stably expressing control shRNA and LIMK1-shRNA. Jurkat cells were again used as targets in this assay, as simian type D retroviruses have a broad host range (35, 36), and this allowed us to be internally consistent with the cell-cell transmission studies of HIV-1. As with HIV-1, we observed a significant reduction in M-PMV cell-cell transmission from LIMK1-depleted Cos-1 cells, and this defect was completely rescued when LIMK1-FLAG* protein was reintroduced (Fig. 7C). Therefore, despite demonstrating only a partial block to particle release into the supernatant, cell-cell transmission was significantly diminished for both viruses when LIMK1 was disrupted. This suggests that LIMK-mediated effects on actin dynamics may have important consequences for viral spread.

LIMK1 colocalizes with HIV-1 particle assembly sites and is incorporated into HIV-1 virions. Since our data indicated that
LIMK1 activity is involved in HIV-1 particle release, so we reasoned that LIMK1 might localize at particle assembly sites on the plasma membrane. Therefore, we examined the localization of the FLAG-tagged LIMK1 protein in the presence of Gag-mCherry (37) by TIRFM. Indeed, LIMK1-FLAG puncta colocalized significantly with Gag-mCherry at the plasma membrane (Fig. 8A to D). We next repeated this experiment using untagged NL4-3 provirus and immunostaining for Gag, followed by colocalization experiments using TIRFM. We observed significant colocalization of LIMK1-FLAG and HIV-1 particle assembly sites using the untagged proviral vector (Fig. 8E to H). Quantitation of colocalization from 20 cells examined by immunostaining and TIRFM revealed a colocalization coefficient of 0.76 (±0.16) for Gag pixels colocalizing with LIMK1-FLAG pixels. Notably, LIMK1 was expressed in a very punctate manner at the plasma membrane even in the absence of Gag (most evident in Fig. 8K [green puncta]), raising the possibility that it shares a common membrane microdomain, rather than being itself recruited by HIV-1 particle components to the budding site. TIRFM examination of peripheral F-actin bundles revealed a partial colocalization of LIMK1 puncta with actin the budding site. TIRFM examination of peripheral F-actin bundles revealed a partial colocalization of LIMK1 puncta with actin the budding site. Moreover, we showed that upon addition of aggregating factor sheets, both virion-associated LIMK1 and the virion core protein gp120 were resistant to detergent extraction, indicating that LIMK1 is incorporated into the HIV-1 particles. HeLa cells were transfected with empty vector or HIV-1 NL4-3 proviral vector. The supernatants were harvested, clarified by low-speed centrifugation, and subsequently concentrated through a 20% sucrose cushion. We observed that endogenous LIMK1 cosedimented with released HIV-1 particles (Fig. 8M). We further reasoned that if LIMK1 is incorporated within HIV-1 particles, virion-associated LIMK1 should be resistant to protease digestion in the absence of detergents. Therefore, we examined the resistance of virion-associated LIMK1 to exogenously added protease subtilisin. As shown in Fig. 8N, purified virions were either mock treated or treated with 5 or 50 ng/ml of subtilisin. HIV-1 envelope protein gp120 was significantly degraded by subtilisin, consistent with the location of this protein on the outside of the viral particle membrane. In contrast, both virion-associated LIMK1 and the virion core protein p24 were largely resistant to subtilisin digestion, indicating that endogenous LIMK1 is incorporated within HIV-1 particles. We next purified HIV-1 particles from HeLa cells transfected with HIV-1 NL4-3 and LIMK1-FLAG expression vectors and examined LIMK1 cosedimentation with Gag on a linear 20 to 60% sucrose gradient. As expected, LIMK1 and p24 were found in the same fractions with a peak density of 1.15 to 1.16 g/ml (Fig. 8O). Taken together, these results strongly suggest that LIMK1 is concentrated in HIV-1 particle assembly sites and incorporated into particles. The basis for recruitment of LIMK1 to the particle assembly site is currently unknown, as is the potential function of LIMK1 in the released particles.

LIMK1-mediated effects on HIV-1 virus output are not mediated by tetherin. In early 2008, tetherin was identified as the cellular restriction factor for HIV-1 virus release that is counteracted by Vpu (32, 38). Tetherin is a broadly specific inhibitor of enveloped particle release (39). We describe here a late defect that resulted in the accumulation of mature particles on the plasma membrane that appears somewhat similar to the accumulation seen with Vpu-deficient virus in the presence of tetherin. Therefore, we asked whether the effect of LIMK1 depletion on HIV-1 release was tetherin dependent or tetherin independent. We first examined whether LIMK1 depletion affected the cell surface levels of tetherin in the absence or presence of Vpu. Both control shRNA and LIMK1 shRNA-expressing HeLa cells were transfected with either empty vector or GFP-Vpu expression vector, and cell surface tetherin levels were measured by flow cytometry. Our results showed that tetherin cell surface levels in LIMK1-depleted cells were comparable to the level in control cells in the absence of Vpu. Furthermore, Vpu was able to efficiently decrease cell surface levels of tetherin about 2-fold in both control cells and LIMK1-depleted cells, indicating that LIMK1 depletion did not interfere with Vpu function (Fig. 9A). We then asked if expression of Vpu is able to restore HIV-1 release following LIMK1 depletion. Both control shRNA and LIMK1 shRNA stably expressing HeLa cells were transfected with either NL4-3 or vpu-deficient NL-Udel expression vector, together with empty vector or Vpu expression vector as indicated in Fig. 9B. Western blot analysis was performed 48 h posttransfection to evaluate virus particle release. Expression of Vpu efficiently restored NL-Udel particle release in control shRNA-treated cells (Fig. 9B, compare lane 4 to lane 3). However, Vpu expression failed to restore the LIMK1 depletion-mediated defect in particle release (Fig. 9B, compare lane 8 to lane 4). Results from this experiment were perhaps even more revealing when assessed by p24 ELISA and calculation of the particle release ratio [total supernatant p24/(total cellular p24 + total supernatant p24)] (Fig. 9B, bar graph below lanes, compare lane 4 with lane 8). Thus, while the tetherin-mediated restriction of NL-Udel created a more profound defect in particle release, Vpu could rescue NL-Udel particle release in the face of LIMK1 depletion only to levels previously seen upon LIMK1 depletion, not to levels seen in control cells. Despite finding no evidence for tetherin in-

![Fig 8](https://jvi.asm.org/6917)
volvement in the particle release defect induced by LIMK1 depletion, mild protease treatment (subtilisin) of cells was able to enhance the release of particles retained on the plasma membrane following LIMK1 depletion (Fig. 9C). This suggests that the mechanism of particle retention is protein dependent, yet distinct from tetherin.

DISCUSSION

In this study, we examined the effects of actin modulation by ROCK1 and LIMK1 on retrovirus particle release. We initially identified this pathway as a regulator of HIV-1 and Mason-Pfizer monkey virus (M-PMV) release through an siRNA screen and then verified the inhibitory effects of LIMK1 knockdown as well as disruption of its upstream activator ROCK1 and downstream target cofilin. By utilizing two distantly related retroviruses in our screen and in our subsequent experiments, we hoped to generate findings that would be generalizable to the late events in retrovirus particle budding or release. Our results suggest an important role for modulation of actin dynamics by the ROCK1-LIMK1-cofilin pathway in retrovirus particle release.

Actin is incorporated into HIV particles, together with actin-binding proteins cofilin, ezrin, and moesin (9, 40). Actin has been shown to interact with the nucleocapsid (NC) domain of Gag (41, 42). Filamin A interacts with Gag, and disruption of this interaction led to diminished particle release (43). Some investigators have found that disruption of actin polymerization through the use of cytochalasin D, latrunculin, or other actin polymerization inhibitors diminishes particle release (11, 12, 44). Inhibitors of actin polymerization also disrupt cell-cell transmission of HIV (12). Thus, a number of suggestive pieces of evidence point to a role for actin and actin-related functions in HIV assembly or release, while a unifying model for the role of actin at this stage of the viral life cycle has not yet been derived.

LIMK1 and LIMK2 are serine kinases that regulate actin polymerization through phosphorylation of the actin depolymerizing factor ADF/cofilin. Cofilin acts as an F-actin-severing protein.
LIMK activity in turn activated by upstream kinases such as ROCK1 and is in this way responsive to the activation of Rho family GTPases. LIMK1 has previously been implicated in HIV entry (34). In this study, HIV-1 gp120 transiently activated LIMK1 and stimulated actin polymerization, contributing to CXCR4/CD4 clustering and enhancing HIV entry. Depletion of LIMK1 in T cell lines has also been noted to diminish polarized, actin-rich caps (45), and polarized regions of actin polymerization have been associated with a high density of particle budding sites in other studies (12, 46–48). Our study is the first to directly implicate ROCK1-LIMK1-cofilin in retroviral release and cell-cell transmission events.

There were several unexpected findings in this study. We initially suspected that the block to particle production might be at the level of budding, with the idea that actin polymerization may play a role in membrane deformation or in generating a localized outward force on the Gag lattice. Another potential mechanism that could arrest release would be interference with a localized outward force on the Gag lattice. Another potential mechanism that could arrest release would be interference with the final scission event, as actin dynamics are implicated in scission of endosomes during clathrin-independent endocytosis (49). However, we did not observe defects in budding, and our electron microscopic analysis demonstrated a defect in release of clusters of mature virions without any significant membrane connection rather than revealing incomplete scission. We were also surprised to find that LIMK1 is recruited to and concentrated in HIV-1 assembly sites at the plasma membrane and is incorporated into HIV-1 particles. It is possible that HIV-1 hijacks cellular LIMK1 to regulate the dynamic turnover of actin filaments within a localized microdomain as particles are budding, facilitating the ultimate release of newly formed HIV-1 particles. A close parallel to the role of very localized regulation of actin dynamics as proposed here for retrovirus release may be found in studies of localized cofilin within dynamic filopodia. Cofilin is concentrated within the tips of filopodia during the retraction phase of filopodial protrusion and retraction (50), where it serves to disassemble actin bundles. It is conceivable that LIMK1 concentrated in particle assembly sites serves as a localized inhibitor of cofilin to transiently enhance actin filament generation and contribute to particle budding and release.

The block to particle release observed upon depletion of LIMK1 or ROCK1 in the present study remains incompletely explained. We observed mature particles that appeared to have completed the budding process but remained in proximity to the plasma membrane, sometimes in large focal clusters. This tetherin-like phenotype was shown clearly not to be due to tetherin function, but it was relieved by protease digestion. Although we do not yet know the mechanism of particle retention, we postulate that another protein present at the plasma membrane must retain mature particles upon disruption of normal actin dynamics at the particle budding site. Because both HIV-1 and M-PMV particles were retained in clusters on the plasma membrane, it is unlikely that the mechanism of particle retention involves direct interaction with specific viral proteins. This aspect of our study remains puzzling and will require further evaluation. One indication that this block may have significance comes from our studies of cell-cell transmission. We found that both HIV-1 and M-PMV were very inefficiently spread from cell to cell upon depletion of LIMK1. This may have resulted from the inability of the retained particle clusters to transmit to target cells, as well as from the effect of LIMK1 depletion on the formation of polarized cell-cell contacts. The RhoA/ROCK1/LIMK1/cofilin axis is required for formation of the immunologic synapse (51, 52) and is likely to play a role in the virologic synapse as well.

M-PMV is a betaretrovirus that assembles viral capsids in a perinuclear location in close proximity to the microtubule-organizing center. M-PMV capsids then are directed to the plasma membrane, where budding and release occur (53). This mode of assembly and release is quite distinct from that of HIV, which assembles immature capsids on the plasma membrane during the budding process. The finding that disruption of the ROCK1-LIMK1-cofilin pathway affected release and cell-cell transmission of both M-PMV and HIV in a similar manner suggests that actin dynamics at the particle budding site play a role in particle release that is generalizable and may extend to additional enveloped viruses that bud from the plasma membrane.

In conclusion, we have identified for the first time that modulation of actin dynamics through ROCK1-LIMK1-cofilin affects retrovirus release. Several areas remain to be explored, including the significance of particle-incorporated LIMK1 and the identity of the protein(s) retaining particles following budding when LIMK1 activity is disrupted. This study adds to growing evidence for a dynamic role of actin in the late events of the retrovirus life cycle.

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