Molecular Characterization of Feline Immunodeficiency Virus Budding[▽]

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Infection of domestic cats with feline immunodeficiency virus (FIV) is an important model system for studying human immunodeficiency virus type 1 (HIV-1) infection due to numerous similarities in pathogenesis induced by these two lentiviruses. However, many molecular aspects of FIV replication remain poorly understood. It is well established that retroviruses use short peptide motifs in Gag, known as late domains, to usurp cellular endosomal sorting machinery and promote virus release from infected cells. For example, the Pro-Thr/Ser-Ala-Pro [P(T/S)AP] motif of HIV-1 Gag interacts directly with Tsg101, a component of the endosomal sorting complex required for transport I (ESCRT-I). A Tyr-Pro-Asp-Leu (YPDL) motif in equine infectious anemia virus (EIAV), and a related sequence in HIV-1, bind the endosomal sorting factor Alix. In this study we sought to identify and characterize FIV late domain(s) and elucidate cellular machinery involved in FIV release. We determined that mutagenesis of a PSAP motif in FIV Gag, small interfering RNA-mediated knockdown of Tsg101 expression, and overexpression of a P(T/S)AP-binding fragment of Tsg101 (TSG-5') each inhibited FIV release. We also observed direct binding of FIV Gag peptides to Tsg101. In contrast, mutagenesis of a potential Alix-binding motif in FIV Gag did not affect FIV release. Similarly, expression of the HIV-1/EIAV Gag-binding domain of Alix (Alix-V) did not disrupt FIV budding, and FIV Gag peptides showed no affinity for Alix-V. Our data demonstrate that FIV relies predominantly on a Tsg101-binding PSAP motif in the C terminus of Gag to promote virus release in HeLa cells, and this budding mechanism is highly conserved in feline cells.

Feline immunodeficiency virus (FIV) is a nonprimate lentivirus that is found ubiquitously in feral cats and causes AIDS in domestic cats (*Felis catus*) (50, 57, 73). Approximately 10% of all domestic cats worldwide are infected with FIV (12), and its pathogenesis is similar to that observed for human immunodeficiency virus type 1 (HIV-1) in humans (10, 29, 46, 76). The potential use of cats as a model host for infectious diseases, such as feline leukemia virus and FIV, stimulated a complete genome sequencing project for *Felis catus*; the results were recently submitted to GenBank (National Center for Biotechnology Information). By radiation hybrid mapping, 96% of all feline microsatellite markers were found to have identifiable orthologs in both canine and human genomes (45). Thus, FIV in domestic cats represents an important small-animal model for HIV vaccine research, for testing potential antiretroviral agents, and for understanding the cell biology of lentiviral replication (10, 29, 32). FIV has also been developed as an ideal lentiviral vector system for the delivery of genes and

small interfering RNAs (siRNAs), since it has the capacity to transduce nondividing cells for long-term gene expression but is not infectious or pathogenic in humans (24, 28, 36, 52, 58, 60). Despite the importance of the FIV system, little is known, relative to the primate lentivirus systems, about the molecular mechanisms of FIV Gag protein trafficking, virus assembly, release, and replication (29).

The Gag precursor protein is the primary structural component of retrovirus particles (19). Expression of Gag alone in appropriate host cells is sufficient to drive the assembly and release of noninfectious, immature viruslike particles (VLPs). Unspliced genomic mRNA transcripts are translated into a Gag polyprotein precursor that traffics within the cytoplasm to specific membrane-associated sites, typically at the plasma membrane, where virus assembly takes place. Specific targeting of retroviral Gag proteins to the site of virus assembly is largely regulated by the matrix (MA) domain (19), and this appears to be the case for FIV as well (37). At the site of assembly, Gag molecules associate with the lipid bilayer and multimerize, thereby inducing membrane curvature away from the cytoplasm, resulting in the formation of spherical immature particles. The release of virions from the plasma membrane requires specific host cell factors (6, 13, 43). Virion maturation, which is virtually concomitant with particle release, is triggered by the activation of the *pol*-encoded viral protease (PR), which cleaves Gag precursors into the mature Gag proteins: MA, capsid (CA), nucleocapsid (NC), and C-terminal peptide (p6 in

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HIV-1). Cleavage of the less abundant Gag-Pol precursor proteins also occurs at this time, resulting in the generation of several enzymes, including the mature PR, reverse transcriptase (RT), integrase (IN), and a dUTP-ase in nonprimate lentiviruses such as FIV and equine infectious anemia virus (EIAV) (49). When visualized by thin-section transmission electron microscopy (EM), released immature particles appear as hollow spheres, whereas mature lentiviral particles contain electron-dense conical cores.

We and others have shown that short peptide motifs in retroviral Gag precursor proteins are essential for proper release of assembled virions from infected cells in culture; these motifs are therefore referred to as “late” (L) domains (6, 13, 15, 23, 26, 43, 47, 55, 67, 72). Retroviral late domains apparently mimic cellular proteins that interact with the highly conserved endosomal sorting complexes required for transport (ESCRT-I, -II, and -III) first identified by studying yeast vacuolar protein sorting (Vps) mutants (2, 3, 5, 30, 42, 56). To date, three retroviral late domains motifs have been described: Pro-Thr/Ser-Ala-Pro [P(T/S)AP], Tyr-Pro-Asp-Leu (YPDL or the related sequence LYP_xLxxL, where “x” is any amino acid), and Pro-Pro-Pro-Tyr (PPPY). The P(T/S)AP motif is conserved among a number of lentiviruses (Fig. 1A) and functions by interacting directly with the ESCRT-I component Tsg101 (14, 21, 41, 53, 54, 66). EIAV utilizes a YPDL motif (Fig. 1A) to interact with the ESCRT-associated protein Alix (apoptosis-linked-gene-2 [ALG2]-interacting protein X; formerly referred to as AIP1) for virus release (11, 40, 63, 67). HIV also contains a degenerate form of this motif, LYP_xLxxL, which binds Alix (11, 18, 33, 40, 44, 63) and can facilitate virus release under specific conditions (63). The third type of retroviral late domain, PPPY, is found in a number of retroviruses, including murine leukemia virus (75), Rous sarcoma virus (72), human T-cell leukemia virus (25, 31, 68), and Mason-Pfizer monkey virus (74). PPPY motifs promote retrovirus release by interacting with Nedd4-like ubiquitin ligases that may be peripherally associated with endosomal sorting machinery (39). FIV contains a potential Tsg101-binding (PSAP) motif near the C terminus of its Gag precursor protein and also bears an LxxL motif that could possibly serve as a binding site for Alix (Fig. 1A). We hypothesized that the PSAP motif of FIV Gag was likely to interact with Tsg101 in human cells in the same manner as HIV-1 Gag, based on its high degree of conservation, and would thus be required for virus release in human cells. Similarly, a feline homolog of Tsg101 could potentially interact with FIV Gag via the PSAP motif and promote virus particle budding in feline cells. In addition, we speculated that the LLDL motif of FIV Gag might serve an auxiliary role in virus budding by interacting with Alix in either human or feline cells.

HIV-1 release is strongly inhibited by the depletion of endogenous Tsg101 (21) and by overexpression of the N-terminal, Gag-binding domain of Tsg101 (TSG-5') (14). The release of other retroviruses whose budding is stimulated by a P(T/S)AP late domain is also disrupted by TSG-5' (62) and by overexpression of full-length Tsg101 or other dominant-negative Tsg101 fragments (22). All of the retroviruses tested are sensitive to expression of an ATPase-deficient mutant of the AAA ATPase Vps4A (13), a critical enzyme required for the membrane dissociation and recycling of ESCRT components

at the completion of each vesicular sorting cycle (4). Finally, the release of viruses such as HIV-1 and EIAV that bear a binding site for Alix is blocked by the Gag-binding fragment (V domain) of Alix (11, 33, 44). The inhibitory activity of these dominant-negative budding inhibitors is late domain dependent (62), making them useful tools in identifying and characterizing viral late domains and in elucidating budding machinery.

As a first step toward gaining a better understanding of FIV assembly and release, we undertook a study aimed at characterizing both the viral and the cellular determinants of FIV budding. We observed that mutation of the FIV PSAP motif severely restricted virus release, as did Tsg101 depletion and dominant-negative Tsg101-based budding inhibitors. We also demonstrated direct binding of FIV-derived PSAP-containing peptides to Tsg101. However, in contrast to a previous report (38), mutation of the LxxL motif had no significant effect on virus particle production. Furthermore, FIV release was unaffected by Alix depletion or by Alix V domain overexpression, and binding between a C-terminal FIV Gag peptide and Alix was not detected. These results demonstrate that the PSAP motif of FIV Gag is the dominant late domain for this nonprimate lentivirus.

MATERIALS AND METHODS

Plasmids, mutagenesis, and siRNA. pFIV-34TF10 is an infectious molecular clone of the Petaluma isolate (51) obtained from J. Elder (Scripps Research Institute, La Jolla, CA) through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. pFIV-Orf2rep, a derivative of pFIV-34TF10 with a repaired Orf2 gene that increases infectivity in feline lymphocytes (69), was also donated by J. Elder. pFP93, which is an FIV gag-pol expression vector deleted for Env, Vif, LTRs, and the FIV RNA packaging signal, expresses noninfectious FIV-Petaluma virus-like particles in human cells using a cytomegalovirus (CMV) promoter (58) and was a gift from E. Poeschla (Mayo Clinic, Rochester, MN). pHCMV-G, which expresses the G glycoprotein of vesicular stomatitis virus (VSV), was a gift from J. Burns (University of California, San Diego). Site-directed mutagenesis of FIV vectors was performed with synthetic complementary oligonucleotides (Sigma Genosys) using a QuikChange II XL mutagenesis kit (Stratagene). FIV DNA encoding potential late domain motifs (PSAP, LLDL) in the p2 domain of Gag was mutated to GCA TCT GCA GCT, resulting in a P6A/P9A mutation (PSAP⁻), or GCA TTG GAT TCG, resulting in a L15A/L18S mutation (LLDL⁻). Changes from wild-type (WT) DNA sequence are underlined. This region encoding Gag p2 overlaps with the *pol* open reading frame, and all changes in Gag were designed to make either silent or conservative mutations in *pol*, to minimize disruption. Vectors for expression of EIAV (pPRE/GagEIAV) and HIV-1 (pNL4-3) have been described (1, 48). Hemagglutinin (HA)-tagged derivatives of Tsg101—TSG-5' and TSG-3' in the pcGNM2 expression vector—were gifts of Z. Sun (Stanford University), and the construction of the full-length Tsg101 expression vector (pcGNM2/TSG-F) has been described (22). pcGNM2/TSG-5'(zeo) was constructed by cloning a PCR product containing the zeocin resistance gene from pcDNA3.1(–)/zeo (Invitrogen) downstream of the open reading frame for TSG-5' in pcGNM2/TSG-5'. pEGFP-C2:VPS4A(E228Q), expressing an ATPase-deficient mutant of VPS4A fused to eGFP (8), was a gift from P. Woodman (University of Manchester, Manchester, United Kingdom). Expression vectors for the V domain of hAlix [pcGNM2/hAlix(364-716) and pGST(Parallel2)-hAlix(364-716)] have been described (44). Duplex siRNA was synthesized (Dharmacon) based on sequences specific for human Tsg101 that have been described (7, 16). Negative control nontargeting duplex siRNA, which has the same GC content as Tsg101 siRNA, was (sense) 5'-AAG CTT CCC GAT GAC ACT ACC-3'.

Cell culture and transmission EM. Crandall feline kidney (CrFK) cells (a gift from S. Le Grice, HIV Drug Resistance Program, National Cancer Institute, Frederick, MD) and HeLa cells were maintained in Eagle minimal essential medium (American Type Culture Collection) supplemented with 10% fetal bovine serum (FBS; HyClone), penicillin, streptomycin, and glutamine (Gibco). For visualization of virions and VLPs by transmission EM, cells were either infected with cell-free preparations of FIV produced in CrFK cells or were

FIG. 1. A highly conserved P(T/S)AP motif in FIV Gag is required for FIV release and replication. (A) An alignment of C-terminal sequences from lentiviral Gag proteins is shown, with the highly conserved P(T/S)APP motif highlighted (GenBank; National Center for Biotechnology Information; Entrez Genome). Abbreviations: SIVagm, SIV from African green monkey; Visna, visna/maedi lentivirus; BIV, bovine immunodeficiency virus. The host species of each virus is indicated. Sites of protease cleavage in the C-terminal domain of Gag liberating HIV-1 p6 and FIV p2 are marked with an arrow, resulting in the proposed numbering of amino acids in FIV p2 (17, 34, 65). Domains of FIV Gag and residues altered by site-directed mutagenesis in the present study are shown. (B) The PSAP motif in FIV Gag (p2) is required for efficient virus release. HeLa cells were transfected with an FIV-expression vector (FP93) and metabolically radiolabeled with [³⁵S]Met/Cys. FIV proteins were detected in cell or virus fractions by immunoprecipitation with anti-FIVp24gag and resolved by SDS-PAGE. p50, full-length Gag precursor protein (MA-CA-p1-NC-p2); Gag processing intermediates p47, p40, and p33; p24, capsid (CA). The ~45-kDa band detected in mock-transfected cells is a cross-reactive protein. Gag protein levels were quantified by phosphorimager analysis, and the relative virus release efficiency was calculated as the ratio of virion-associated Gag to total Gag (cells plus virus), normalized to the WT FIV release, and the results were averaged from at least three independent experiments. Error bars indicate the standard deviations (SD). (C) CrFK cells were infected with RT-normalized WT or mutant FIV(Orf2rep) pseudotyped with VSV-G. The RT levels in culture supernatants were measured prior to each cell passage.

transfected with FIV expression vectors. Transfections were carried out in six-well tissue culture dishes (Costar). Each well was seeded with 0.3×10^6 to 0.6×10^6 cells, followed by incubation for 12 to 24 h. Transfection medium contained 5 μ l of Lipofectamine 2000 (Invitrogen) and 3 μ g of FIV DNA

diluted in 0.5 ml of Opti-MEM (Gibco). Cells were fixed in a 2% glutaraldehyde–100 mM sodium cacodylate solution and stored at 4°C. The methods for sample preparation and visualization of fixed cells by transmission EM were as described previously (20).

Virus release assays. FIV, EIAV, or HIV-1 proteins were detected by radio-immunoprecipitation assay (RIPA) based on methods previously described (20, 59, 70) with several modifications. Cultures of HeLa or CrFK cells were seeded at a density of 6×10^4 cells/cm² 24 h prior to transfection. Cells were transfected by using Lipofectamine 2000 according to the manufacturer's suggested protocol. Vectors expressing FIV, HIV-1, or EIAV were cotransfected with vectors over-expressing ESCRT-related proteins [TSG-F, TSG-5', TSG-3', Vps4A(E228Q), and Alix-V] or an empty vector [pBluescript SK(+); Stratagene]. Cells were washed briefly with DPBS and then metabolically labeled at 37°C with [³⁵S]Met/Cys (Express protein labeling mix; Perkin-Elmer) in labeling medium (RPMI 1640 with 25 mM HEPES Cys⁻/Met⁻ [Specialty Media] with 5% FBS). Released virions or VLPs were collected by filtration (0.4 μ m, Millex-HA; Millipore) and ultracentrifugation at 100,000 $\times$ g for 45 min. Cell and virion samples were lysed in cell lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris [pH 7.5], and protease inhibitors [Complete; Roche]). Insoluble material from cell lysates was concentrated by microcentrifugation, and the supernatant was precleared by adsorption with protein G-agarose (Invitrogen) suspended in RIPA buffer (0.1% Triton X-100, 300 mM NaCl, 50 mM Tris [pH 7.5]) and 0.1% bovine serum albumin (BSA). Virion and precleared cell lysates were immunoprecipitated with either mouse anti-FIV p24gag (clone PAK3-2C1), horse anti-EIAV ("Lady" serum; kindly provided by R. Montelaro, University of Pittsburgh, Pittsburgh, PA), or human anti-HIV-IG (obtained from the NIH AIDS Reference and Reagent Program) bound to protein G-agarose at 4°C. Immunoprecipitated cell lysates were washed three times in RIPA buffer and once with SDS-DIC wash (0.1% sodium dodecyl sulfate, 300 mM NaCl, 50 mM Tris [pH 7.5], 2.5 mM deoxycholic acid). Immunoprecipitated virus lysates were washed once with RIPA buffer. Immunoprecipitated proteins were eluted by boiling in Laemmli sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE) in 12% acrylamide with 0.4% AcrylAide cross-linker (Lonza), fixed in 40% methanol-10% acetic acid-7.5% glycerol, and dehydrated. Labeled proteins were detected by autoradiography on phosphorimaging plates (Fujifilm) and quantitated by using QuantityOne software (Bio-Rad). Virus release efficiency was calculated as the ratio of released Gag over total Gag protein, normalized to the positive control (uninhibited WT Gag).

Immunofluorescence assays. Transfected cells were suspended by trypsinization, seeded onto eight-well chamber slides (Lab-Tek II; Nalge Nunc International) in normal growth medium (Eagle minimal essential medium with 10% FBS) at a density of 1×10^4 to 5×10^4 cells per well, and incubated for 18 to 24 h at 37°C. The adherence of cells was verified by phase-contrast microscopy, and then the cells were washed briefly with Dulbecco phosphate-buffered saline with Ca²⁺ and Mg²⁺ (DPBS+CaMg; Cambrex), fixed with 3.7% formaldehyde (Sigma) for 15 min, washed with 0.1 M glycine for 5 min, washed twice briefly with DPBS+CaMg, permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 3% BSA (Sigma) for 5 min. Tsg101 derivatives were detected with rabbit anti-HA polyclonal antibody (Y-11; Santa Cruz) diluted 1:100 in 3% BSA. Primary antibodies were detected with Alexa 594-conjugated secondary antibodies (Molecular Probes) at a 1:100 dilution in 3% BSA. All solutions were prepared in DPBS+CaMg. Slides were mounted in Fluoromount-G (Electron Microscopy Sciences) and visualized with a Leica DM IRE2 inverted microscope equipped with a halogen lamp and a 63 $\times$ APO oil-immersion objective lens. Images obtained from a Retiga Exi charge-coupled device camera (Qimaging Corp.) were processed by using OpenLab software (Improvision).

Construction of the stable CrFK/TSG-5' cell line. CrFK cells were transfected with pcGNM2/TSG-5'(zeo) or pcDNA3.1/zeo(-) as a negative control and then selected for resistance to phleomycin (InvivoGen) at 50 μ g/ml. Stable HA-tagged TSG-5' expression was verified at 2 months posttransfection by Western blotting and was evaluated over the course of 1 year in culture by immunofluorescence assay.

Preparation of FIV or FIV/VSV-G and FIV RT assays. FIV-Petaluma was obtained from CrFK cells transfected with pFIV-34TF10. Cell-free virus stocks were prepared by 0.4- μ m-pore-size filtration of cultured supernatants, which were then quantitated by RT assay using methods described for HIV-1 (71). For FIV/VSV-G, FIV(Orf2rep) clones were pseudotyped with VSV-G by transfection of HeLa cells with three plasmids (pFIV-Orf2rep, pFP93, and pHCMV-G) mixed with Lipofectamine 2000. (For more information on these vectors, see above.) Levels of VSV-G/FIV released into the culture medium were quantitated by RT assay. Supernatants were collected at 48 h posttransfection, when highest levels of RT activity were consistently observed. FIV titers achieved during replication experiments were also quantified from cultured cell supernatants by RT assay.

Western blot. Lysates prepared from cells treated with cell lysis buffer (described above) were denatured by boiling with Laemmli sample buffer containing 0.1 M dithiothreitol, resolved by SDS-PAGE, transferred by using a semidry

blotting apparatus to polyvinylidene difluoride membranes (Immobilon-P; Millipore), and blocked with 5% milk in Tris-buffered saline (pH 7.4) with 0.1% Tween 20. Membranes were probed with mouse anti-HA (clone HA-7; Sigma) at a 1:10,000 dilution in blocking solution to detect HA-tagged Tsg101 protein. Primary antibody was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (Amersham) at a 1:25,000 dilution in blocking solution, and HRP was detected by using Western Lightning chemiluminescent reagent Plus (Perkin-Elmer). Alix protein was detected with rabbit anti-Alix (kindly provided by W. Sundquist, University of Utah) and HRP-conjugated goat anti-rabbit immunoglobulin G. Blots were exposed to BioMax XAR film (Kodak) and developed with an X-ray film processor (Kodak). For quantitation of specific protein levels, blots were photographed with a digital camera enhanced for chemiluminescent detection (AlphaInnotech) and analyzed with QuantityOne (Bio-Rad) software.

Fluorescence anisotropy. Custom peptides were synthesized and N terminally labeled with fluorescein isothiocyanate (FITC; EZBioLabs). FITC-tagged peptides at 50 nM were incubated with 1 to 100 μ M concentrations of either purified Tsg101 UEV domain or Alix V domain protein. Methods for detection of protein-peptide interactions by fluorescence anisotropy have been described previously (35).

Cloning and protein purification of Tsg101-UEV. The cloning and purification of the Tsg101 UEV domain has been described (35), and the resulting expression plasmid was a gift from M. Javad Aman (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD). Briefly, the human Tsg101 UEV domain was cloned into pET21b (Novagen) to encode a C-terminal His-tagged fusion protein. Protein was expressed in *Escherichia coli* BL21(DE3)pLysS (Novagen), upon induction with IPTG (isopropyl- β -D-thiogalactopyranoside) and purified from solubilized cell pellets by using the HisTrap HP kit (Amersham Biosciences). Buffer exchange into PBS and protein concentration determinations were performed by using Vivaspin 20 columns rated with a molecular weight cutoff of 5,000 (Vivascience). The purified protein was verified by SDS-PAGE, and the protein concentration estimated with a Quick-Start Bradford protein assay (Bio-Rad).

Cloning and purification of Alix-V. The cloning and methods for purification of GST-Alix-V have been described (44, 61). Briefly, cDNA encoding residues 364 to 716 of human Alix was cloned into the pGST-parallel2 vector for expression in *E. coli* Rosetta2(DE3) cells (Novagen). Cells were cultured in LB containing ampicillin (100 μ g/ml) and chloramphenicol (34 μ g/ml) at 37°C to an optical density at 600 nm of 1.0. Overexpression of GST-tagged protein was induced with 0.5 mM IPTG, and cultures were incubated at 25°C for 24 h. Cell pellets were solubilized in extraction buffer (20 mM HEPES [pH 7.3], 100 mM NaCl, 5 mM MgCl₂, 5% glycerol, protease inhibitors [Roche]), digested with lysozyme (0.5 mg/ml), and treated with benzonase (10 U/ml). GST-tagged protein was purified by using a GSTrap column (GE Healthcare). The GST tag was cleaved by using His₆-TEV protease, and both GST and TEV protease were removed sequentially with GSTrap and HisTrap columns (GE Healthcare) and then dialyzed into PBS (pH 7.4). Each stage of protein purification was monitored by SDS-PAGE, and the final protein concentration was calculated by Bradford assay (Bio-Rad). Further concentration of protein was achieved by using Vivaspin 20 columns with a 10,000-molecular-weight cutoff (Vivascience).

RESULTS

FIV release and replication require the PSAP but not the LLDL motif. To evaluate the function of either of the two putative late domain motifs in FIV Gag, we introduced a P6A/P9A (PSAP⁻) mutation or an L15A/L18S (LLDL⁻) mutation into FIV Gag p2 (Fig. 1A) in two distinct FIV expression vectors: pFP93, for expression of FIV Gag in human cells via a CMV promoter, and pFIV-Orf2rep, an infectious FIV molecular clone. We then determined the effect of these mutations on virus release efficiency (Fig. 1B). We observed that the LLDL⁻ mutation had no significant effect on virus particle production, whereas the PSAP⁻ mutation induced a severe defect. Similar results were obtained in CrFK cells (data not shown). We observed delayed replication of the PSAP⁻ mutant in CrFK cells in one experiment (Fig. 1C, left panel), and RT activity in the medium eventually reached levels comparable to those of the WT. Further analysis indicated that this was

FIG. 2. Effect of PSAP⁻ and LLDL⁻ mutations on the morphogenesis of FIV budding. HeLa (left panels) or CrFK (right panels) cells were transfected with plasmids expressing WT or mutant FIV Gag and then fixed 2 days posttransfection. Typical images of both normal and defective virions and budding intermediates found associated with the plasma membrane are shown. All scale bars represent 100 nm unless indicated otherwise.

the result of reversion of the mutant back to WT. Repetition of the experiment (Fig. 1C, right panel) better demonstrated a complete lack of FIV PSAP⁻ mutant replication in CrFK cells over a similar period of time. Replication of the LLDL⁻ mutant appeared to be similar to the WT in both experiments. Thus, in agreement with our biochemical data, replication of FIV-Orf2rep in CrFK cells was also inhibited by the PSAP⁻ mutation, whereas the LLDL⁻ mutation had no effect (Fig. 1C).

When transfected cells were examined by transmission EM, the budding of PSAP⁻ mutant VLPs from the plasma membrane of CrFK and HeLa cells consistently appeared defective relative to WT FIV, whereas the LLDL⁻ mutant VLPs appeared normal (Fig. 2). Mature WT particles produced in HeLa or CrFK cells (Fig. 2) were ca. 125 to 150 nm in diameter with condensed conical cores. Production of fully released WT immature particles, which were spherical and lacking an elec-

tron-dense core, was common in HeLa cells, approximately equal to the number of mature particles. In CrFK cells, fully released WT immature particles were rare, but some budding immature particles with nearly completed virion closure appeared tethered to the plasma membrane by short membrane extensions of ~100 nm in length. In contrast, budding tubular PSAP⁻ particles produced in HeLa cells appeared deficient in virion closure and were often found tethered to the plasma membrane by long stalks, extending 500 nm or more. No mature released PSAP⁻ particles were detected in either cell type. In CrFK cells, budding and released immature PSAP⁻ particles were also highly aberrant and included a mixture of comet-shaped and tubular structures, containing either singlet or doublet heads and highly extended tails, or irregularly shaped doublets and triplets with multiple crescent-shaped zones of electron density. These crescent-shaped zones appeared to be a result of multiple incomplete virion closures within a larger,

FIG. 3. FIV release is sensitive to dominant-negative disruption of Tsg101 and Vps4A. (A) HeLa cells were mock transfected (lane “–”) or were cotransfected with FIV expression vector (FP93) and pBS empty vector (lane “+”), or expression vectors encoding TSG-F (lane F), TSG-5’ (lane 5’), TSG-3’ (lane 3’) or Vps4A(E228Q)-eGFP (lane V). Transfected cells were metabolically radiolabeled with [³⁵S]Met/Cys; FIV proteins were detected in cell or virus fractions by immunoprecipitation with anti-FIVp24gag and resolved by SDS-PAGE. FIV Gag products p50, p40, p33, and p24 are described in the Fig. 1 legend. Relative virus release was determined as indicated in the Fig. 1 legend based on data obtained from five independent experiments ± SD. FIV release was found to be significantly different from the negative control (pBS) in all experimental samples by a two-tailed one-sample *t* test (*P* < 0.05). (B) HeLa or CrFK cells were transfected with the same vectors used in virus release assays described for panel A. Exogenous Tsg101 was visualized by immunofluorescence using a rabbit anti-HA antibody (red); Vps4EQ was visualized directly via its green fluorescent protein tag (green).

contiguous particle that had been released from the cell. In contrast to the PSAP[−] mutant, budding and maturation of LLDL[−] mutant FIV particles from either cell type did not appear to be inhibited and was not significantly different from

the WT in morphology and size. Release of immature, spherical ~100-nm particles from HeLa cells, which was typical of WT FIV, was also observed for the LLDL[−] mutant (data not shown). These data suggest that FIV depends predominantly

FIG. 4. Full-length and truncated forms of Tsg101 disrupt FIV budding. HeLa and CrFK cells were transfected with FIV expression vector alone (FIV only) or were cotransfected with expression vectors encoding TSG-F, TSG-5', or TSG-3'. Cells were fixed for EM 1 day posttransfection. Typical images of both normal and defective virions and budding intermediates associated with the plasma membrane are shown. Scale bars represent 100 nm, unless indicated otherwise.

on the P(T/S)AP domain for virus release in HeLa and CrFK cells. Consistent with our biochemical data, the LLDL⁻ mutation had no apparent effect on the production of mature virions.

FIV release depends on ESCRT machinery and Tsg101 function. We previously reported that overexpression of full-length Tsg101 (TSG-F), N- or C-terminal fragments of Tsg101 (TSG-5' and TSG-3', respectively), or an ATPase-deficient mutant of Vps4A (E228Q) each inhibits the release of a unique subset of ESCRT-utilizing retroviruses depending on late domain differences (14, 22, 62). To examine the sensitivity of FIV release to overexpression of these dominant-negative budding inhibitors, we cotransfected cells with FIV vectors and plasmids expressing TSG-F, TSG-5', TSG-3', or Vps4A(E228Q). The effect on virus particle production was determined by RIPA (Fig. 3A). We observed that FIV behaves much like HIV-1 in HeLa cells in terms of its sensitivity to TSG-5' and TSG-3', and expression of Vps4A(E228Q) profoundly inhibited virus release in both cell types. Inhibition of FIV release by TSG-5' and TSG-3' in CrFK cells was less pronounced than

in HeLa cells but was still statistically significant when measured by radioimmunoprecipitation analysis (data not shown). We also examined the expression and localization of TSG-F, TSG-5', TSG-3', and Vps4A(E228Q) in HeLa and CrFK cells by immunofluorescence assay (Fig. 3B). We observed that the subcellular localization of each expressed protein was comparable in HeLa and CrFK cells.

To further demonstrate the defect in FIV release induced by TSG-F, TSG-5', and TSG-3' expression, we examined FIV-transfected HeLa and CrFK cells by EM (Fig. 4). Several types of aberrant FIV budding structures were induced in both cell types upon cotransfection with TSG-F, TSG-5', or TSG-3' that were not typically observed in negative controls (FIV only). For example, cotransfection with TSG-F produced large electron-dense protrusions from the plasma membrane, which were often hydra-shaped with many branches of apparently stalled budding events. In HeLa cells, cotransfection with TSG-5' induced a series of highly extended virion budding structures (>0.5 μm in length) along the plasma membrane, and released particles were either comet-shaped with tubular

tails and several electron-dense crescents at the heads or doublets tethered to each other by long extensions of membrane. These data suggest that the mechanisms of FIV release in both CrFK and HeLa cells are similar to those of HIV-1, in terms of sensitivity to manipulation of the ESCRT machinery that is required for ESCRT turnover (Vps4A) (Fig. 3), and that perturbation of Tsg101 function (Fig. 3 and 4) is sufficient to inhibit virus release.

FIV replication in CrFK cells is sensitive to stable TSG-5' expression. To determine whether inhibition of FIV release could be induced by nontoxic, stable expression of TSG-5' protein, and, if so, whether this would disrupt FIV replication in feline cells, we engineered a stable TSG-5'-expressing CrFK cell line, CrFK/TSG-5'(zeo). Stable TSG-5' expression, which was maintained by selection in phleomycin (zeo), was verified in long-term cultures by anti-HA Western blot (Fig. 5A, inset) and did not affect cell viability, as measured by the rate of cellular replication in culture (data not shown). As a control, we generated a parallel cell line, CrFK(zeo), expressing only the phleomycin-resistance gene (zeo). CrFK/TSG-5'(zeo) and CrFK(zeo) cells were infected with a 10-fold serial dilution of FIV, and virus replication was monitored based on RT activity. FIV replication was readily detectable in control CrFK(zeo) cells within 4 weeks, regardless of the viral input (Fig. 5A). In contrast, FIV replication in CrFK/TSG-5'(zeo) cells was only detectable when the highest input (10^7 cpm RT activity) was used and occurred with a significant delay (1 month) relative to that observed in control CrFK(zeo) cells. To determine whether FIV replication in CrFK/TSG-5'(zeo) cells detected in delayed chronic infections was due to viral escape from TSG-5'-mediated inhibition, cell-free virus was harvested from both CrFK/TSG-5'(zeo) and CrFK(zeo) cells and used to infect FIV-naïve cells. Virus harvested from either CrFK/TSG-5'(zeo) or CrFK(zeo) cells consistently showed a similarly delayed replication in CrFK/TSG-5'(zeo) cells relative to CrFK(zeo) cells upon repeated passage (data not shown), arguing against emergence of TSG-5'-resistant virus in CrFK/TSG-5'(zeo) cells.

To determine whether the inhibition of FIV replication in CrFK/TSG-5'(zeo) cells occurs at the level of virus release, we transfected either control or TSG-5'-expressing cells with pFIV-34TF10 and examined fixed cultures by EM at 2 days posttransfection (Fig. 5B). Mature virions were easily detected at or near the plasma membrane in FIV-transfected CrFK(zeo) cells. In contrast, in FIV-transfected CrFK/TSG-5'(zeo) cells mostly defective doublet particles were released, or apparent accumulations of FIV Gag at the plasma membrane were detected, and no mature virions were observed. To confirm these results biochemically, we measured the efficiency of FIV release in CrFK/TSG-5'(zeo) versus CrFK(zeo) cells. Consistent with FIV release assays in TSG-5'-transfected cells (data not shown), FIV release was inhibited by twofold in CrFK/TSG-5'(zeo) cells relative to CrFK(zeo) cells (Fig. 5C). Processing of cellular Gag p50 to p24 was also inhibited by twofold, which again suggests decreased activation of the viral protease as a result of virus release inhibition. This relative decrease in cellular p24 in the presence of TSG-5' was consistently more apparent in CrFK cells than in HeLa cells. These data indicate that FIV replication in chronically infected cells can be inhibited at the level of virus release by the nontoxic expression of

FIG. 5. FIV release and replication are inhibited in CrFK cells by stable TSG-5' expression. (A, inset) Cell lysates from control CrFK(zeo) or TSG-5'-expressing cells [CrFK/TSG-5'(zeo)] at 4 months posttransfection were subjected to SDS-PAGE and immunoblotted with rabbit anti-HA antibody to detect stably expressed TSG-5' protein. (A) Control [CrFK(zeo)] or TSG-5'-expressing cells were infected with 10-fold serial dilutions (10^5 to 10^7 RT cpm) of cell-free WT FIV (34TF10). RT activity in cultured supernatants was determined prior to each cell passage. (B) Control CrFK(zeo) or TSG-5'(zeo)-expressing cells were transfected with pFIV-34TF10 and fixed for EM at 2 days posttransfection. Typical lentiviral particles associated with the plasma membrane are shown. Scale bars, 100 nm. (C) FIV release assays in control [CrFK(zeo)] or TSG-5'(zeo)-expressing cells. The results were averaged from five independent experiments.

FIG. 6. Tsg101 depletion inhibits FIV but not EIAV release. (A) HeLa cells were serially transfected with siRNA at both 0 and 24 h and with the TSG-F expression vector at 24 h. Cell lysates, prepared at 48 h, were subjected to SDS-PAGE and immunoblotted with anti-HA antiserum. Exogenous Tsg101 expression was undetectable after cotransfection with as little as 2.5 nM Tsg101-specific siRNA but not with negative control siRNAs. In contrast, levels of endogenous Alix were not affected by Tsg101 siRNA. (B) HeLa cells were transfected with FIV or EIAV expression vectors in the absence of siRNA (—) or in the presence of 5 nM control siRNA (neg.) or Tsg101-specific siRNA. Transfected cells were metabolically radiolabeled with [35 S]Met/Cys. FIV and EIAV proteins were detected in cell and virus fractions by immunoprecipitation with anti-FIVp24gag (FIV) or anti-EIAV horse antiserum (EIAV) and resolved by SDS-PAGE. FIV Gag products p50, p40, p33, and p24 are described in the Fig. 1 legend. In EIAV immunoprecipitations, p55 is the 55-kDa EIAV Gag precursor. Relative virus release efficiency was determined as indicated in the Fig. 1 legend, based on data obtained from three independent experiments, $\pm$ the SD.

a dominant-negative PTAP-specific inhibitor, TSG-5', and that resistance to this inhibitor does not develop readily.

FIV release is inhibited by siRNA-mediated knockdown of Tsg101 expression. To determine whether FIV requires human Tsg101 for efficient virus release in HeLa cells, we depleted endogenous levels of Tsg101 by siRNA transfection. First, to confirm gene-specific siRNA-mediated knockdown of Tsg101 protein expression, cells were transfected with siRNAs targeted to Tsg101 versus negative-control (nontargeting) siRNA without exogenous DNA. At 24 h posttransfection, siRNA transfection was repeated along with DNA expressing HA-tagged Tsg101 (TSG-F). Cells were lysed at 48 h, and the specific knockdown of Tsg101 expression was confirmed by immunoblotting with rabbit anti-HA (Fig. 6A). Neither Tsg101 nor control siRNA had any detectable effect on the endogenous protein expression levels of a nontargeted ESCRT-associated protein, Alix, even at the highest concentration of siRNA. By using serial siRNA (5 nM) transfections, parallel virus release assays were performed in HeLa cells with vectors expressing FIV or EIAV Gag (Fig. 6B). FIV release was inhibited by knockdown of Tsg101, whereas EIAV release was not. Similar to previous results with HIV-1 (21, 64), these data demonstrate that efficient FIV release in HeLa cells depends on Tsg101 protein expression.

FIV release is not inhibited by a dominant-negative Gag-binding fragment of Alix. To determine whether FIV release could be facilitated by interactions of FIV Gag with cellular Alix protein that are not blocked by the Gag LLDL⁻ mutation, we coexpressed FIV Gag with the V domain of Alix (Fig. 7) that is known to bind both EIAV and HIV-1 Gag and act as a dominant-negative inhibitor of virus release (18, 33, 44). Assays for FIV and HIV-1 release in HeLa cells, either in the presence or absence of Alix V domain expression, were performed in parallel (Fig. 7). As a positive control, inhibition of EIAV particle release by Alix V domain expression was also tested. As we reported previously (44), Alix V domain expression greatly inhibited both EIAV and HIV-1 release. In contrast, FIV release was not significantly inhibited under these conditions (Fig. 7). These data suggest that, unlike HIV-1 and EIAV Gag, either FIV Gag does not interact with the Alix protein or that any interaction between the two proteins possibly mediated by the Alix V domain has no effect on FIV release in HeLa cells.

FIV C-terminal Gag peptides do not bind the V domain of Alix. To determine whether FIV Gag is capable of binding to the Alix-V domain (Fig. 7), we measured the binding affinity of purified Alix V domain (Alix-V) for synthetic Gag fragments in vitro by fluorescence anisotropy (Fig. 8A). Fluorescently labeled peptides were synthesized based on the amino acid sequence found at or near the C terminus of FIV, HIV-1, and EIAV Gag, which contain known and putative late domains. To detect binding and measure binding affinity, a fixed amount of Gag peptide in solution was incubated with various amounts of Alix-V. As reported previously (44), the EIAV Gag peptide, containing the canonical LYPDL Alix-binding motif, showed a strong affinity for Alix-V. The WT HIV-1 Gag peptide, containing the related LYPL_nLxxL motif, also bound Alix-V with similar affinity. Importantly, neither the WT nor the LLDL⁻ mutant FIV Gag peptide bound to Alix-V.

FIG. 7. Dominant-negative Alix V domain fragment inhibits the release of HIV-1 and EIAV, but not that of FIV. The domain structure of Alix protein is shown (top), with the Bro1, "V" (amino acids 364 to 716), and C-terminal proline-rich domains indicated. HeLa cells were cotransfected with expression vectors for EIAV, HIV-1, or FIV and empty vector (lanes "-") or plasmid expressing the Alix V domain (lanes "+"). Transfected cells were metabolically radiolabeled with [³⁵S]Met/Cys. EIAV, HIV-1, and FIV proteins were detected in cell and virus fractions by immunoprecipitation with anti-EIAV horse antiserum (EIAV), HIV-Ig (HIV-1), or anti-FIVp24gag (FIV) and were resolved by SDS-PAGE. FIV Gag products p50, p40, p33, and p24 are described in the Fig. 1 legend. In EIAV samples, p55 denotes the 55-kDa EIAV Gag precursor. In HIV-1 samples, the Gag precursor protein Pr55^{Gag} (p55) and the mature CA protein (p24) are indicated. Averages of relative virus release efficiency, determined as indicated in the Fig. 1 legend, were based on data obtained from three independent experiments (for FIV and HIV-1) ± the SD and one representative experiment for EIAV.

The C terminus of FIV Gag binds Tsg101. To determine whether FIV Gag has any binding affinity for the PTAP-binding ubiquitin E2 variant (UEV) domain of Tsg101, we again measured the interaction between purified UEV domain (Tsg101-UEV) and fluorescent peptides derived from the C terminus of FIV Gag (containing the PSAP motif) by fluorescence anisotropy. Peptides based on the C termini of HIV-1 Gag (containing the PTAP motif) and EIAV Gag (containing the LYPDL motif) served as positive and negative controls, respectively. A fixed amount of Gag-derived peptide in solution was incubated with various amounts of Tsg101-UEV (Fig.

FIG. 8. Analysis of Alix-V and Tsg101-UEV binding to peptides derived from FIV, HIV-1, and EIAV Gag by fluorescence anisotropy. (A) Alix-V protein binds to peptides derived from HIV-1 and EIAV Gag, but not FIV Gag. (B) Tsg101-UEV protein binds to FIV and HIV-1 Gag, but not EIAV Gag. Protein-peptide interactions were detected by fluorescence anisotropy upon the addition of increasing amounts of either purified Alix-V protein (A) or purified Tsg101-UEV protein (B) to a fixed concentration (50 nM) of FITC-labeled peptides, based on FIV, EIAV, or HIV-1 Gag C-terminal sequences. Calculated dissociation constants (K_d) are indicated.

8B). As expected, WT PTAP-containing HIV-1 peptide bound to Tsg101-UEV, whereas WT EIAV Gag and FIV PSAP⁻ mutant peptides did not. Both WT and LLDL⁻ mutant FIV peptides demonstrated ~6-fold-greater affinity than HIV-1

Gag peptides for binding to Tsg101-UEV. These data demonstrate a direct binding between the PSAP motif of FIV Gag and the UEV domain of Tsg101 protein and are consistent with a requirement for both Tsg101 and the PSAP motif of FIV Gag in FIV release.

DISCUSSION

In the present study, we have defined the late domain of FIV and examined the interactions between late domains within Gag and proteins associated with ESCRT machinery that facilitate virus release at the plasma membrane. As has been observed for HIV-1 and other retroviruses, we found that maintaining ESCRT function in host cells is required for efficient FIV release, and virus release can be manipulated by the expression of either full-length or dominant-negative forms of ESCRT-related components in transfected human (HeLa) cells (14, 22, 62).

We demonstrate that FIV utilizes a PSAP late domain motif near the C terminus of Gag to interact with the UEV domain of human Tsg101 in HeLa cells to enhance virus release. This is partly based on sensitivity of FIV release to site-directed mutagenesis of proline residues in the PSAP motif of Gag (Fig. 1). This interaction appears to be conserved in feline cells, since release and replication of our FIV PSAP⁻ mutant are severely inhibited in CrFK cells. In previous studies (15, 26) we have analyzed the virus release efficiency and replication kinetics of analogous HIV-1 mutants in a wide range of cell types, including epithelial cell lines, T-cell lines, and primary cells. We observed that the phenotype of HIV-1 PTAP mutations is strongly cell type dependent, with a complete block in some cell systems and a much more modest phenotype in others. Our results regarding the PSAP late domain of FIV Gag are consistent with those obtained with HIV-1. Furthermore, mutation of the PSAP motif also abolishes the normally high affinity of FIV Gag C-terminal peptides for binding to purified Tsg101 UEV domain *in vitro* (Fig. 8). Our measurement of the affinity of HIV-1 Gag C-terminal peptide (containing the PTAP late domain) for the UEV domain of Tsg101 is consistent with previous reports (35, 54). Surprisingly, peptides derived from FIV Gag (PSAP) bound Tsg101-UEV with consistently higher affinity than HIV-1 Gag (PTAP) peptide in our fluorescence anisotropy assay. Since mutagenesis of the LLDL domain at the C terminus of the FIV Gag peptide had no effect on Tsg101-UEV binding *in vitro*, the residues immediately flanking the P(T/S)AP motif seem to be important for this phenomenon. The potential ability of peptides derived from the C-terminal domain of FIV Gag to competitively inhibit the interaction of HIV-1 Gag with cellular Tsg101 in infected cells is currently under investigation.

FIV release requires Tsg101, based on siRNA-mediated depletion of Tsg101 expression in HeLa cells (Fig. 6). Although our detection of endogenous Tsg101 levels was inefficient, expression from HA-tagged Tsg101 cDNA in transfected cells was highly suppressed using a Tsg101-specific siRNA that has been previously reported (7, 16). Our data demonstrate that a direct interaction between FIV Gag and endogenous Tsg101 in HeLa cells makes FIV release sensitive to dominant-negative inhibition by TSG-5', which encodes the UEV domain. Interestingly, we also observed inhibition of virus release and rep-

lication upon TSG-5' expression in feline kidney cells (CrFK), which was accompanied by a decrease in the processing of Gag p50 (Fig. 5). These observations suggest that feline Tsg101 and its interaction with the PSAP motif in FIV Gag is highly conserved in feline cells. Our biochemical assays likely underestimate the severity of the defect imposed by Tsg101 disruption, since much of the Gag that is released from cells in the presence of dominant-negative Tsg101-based inhibitors is in the form of highly defective structures that have failed to complete membrane scission (Fig. 4). This dual effect on particle release and proper virion morphogenesis and maturation likely explains the severe inhibition of virus replication imposed by stable TSG-5' expression in feline kidney cells (Fig. 5). TSG-5' expression does not appear to be toxic and can be sustained indefinitely in stably transfected CrFK cell lines. We are currently investigating whether FIV can develop resistance to TSG-5' expression in these cells. To our knowledge, this is the first report of a dominant-negative ESCRT component, or any other inhibitor of retroviral late domain function, being stably expressed in a cell line that supports retroviral replication.

In our virus release assays, we often observed an accumulation of FIV Gag processing intermediates or a decrease in the cellular p24 to p50 ratio relative to uninhibited virus controls in the presence of virus release inhibitors (Fig. 3), especially Vps4A(E228Q), and the effect was more pronounced in CrFK cells (Fig. 5). Inhibition of HIV-1 release in the presence of Alix-V also led to a decrease in the ratio of cellular Gag p24 to p55 (Fig. 7), which is consistent with our previous report (44). Similar effects on Gag processing through manipulation of endogenous ESCRT machinery have been found in other studies (9, 14, 21, 22, 42). These observations are also consistent with an inhibition of virus release, because the maturation of particles through the activation of protease and cleavage of the Gag precursor protein occur during or shortly after particle release.

In our virus release assays, FIV release efficiency appeared to be less inhibited in CrFK cells than in HeLa cells when either TSG-5' or TSG-3' was expressed, relative to controls. The reason for this is not entirely clear, because our EM data suggest a defect in virus release under the same conditions in CrFK cells. A higher percentage of total FIV Gag was found in the supernatant of transfected CrFK cells compared to HeLa cells, suggesting a higher baseline of virus release efficiency that may be more difficult to inhibit. Similarly, release of HIV-1 from 293T cells and most T-cell lines is also more efficient than transfected HeLa cells, as suggested by less accumulation of cellular p24 and a higher proportion of released Gag, and is less sensitive to mutation of the PTAP late domain (15). Furthermore, the sensitivity of infectious HIV-1 release to TSG-5' or TSG-3' expression in these cell lines has not yet been reported. Expression of either construct in CrFK, and their expected cellular localization compared to transfected HeLa cells, appeared to be similar as determined by immunofluorescence microscopy, including the formation of TSG-3'-induced cellular structures (22, 27). Our EM images did suggest that vesicles or extracellular membrane-associated debris containing incompletely assembled or defective virus particles are possibly being released from CrFK cells into the medium. Any extracellular membrane containing Gag that is not removed by filtration can potentially be concentrated by ultra-

centrifugation and detected by anti-FIVp24 immunoprecipitation as virion-associated Gag. We are currently developing FIV infectivity assays to address this issue.

We and others have recently reported that both EIAV and HIV-1 are highly sensitive to the expression of a dominant-negative fragment of Alix (Alix-V) containing only the Gag-binding V domain (18, 33, 44). In contrast, the C terminus of FIV Gag did not interact with Alix-V *in vitro*, and FIV release was completely insensitive to Alix-V expression. We also found that RNA interference-mediated knockdown of Alix had no effect on FIV release in HeLa cells (unpublished results). Site-directed mutagenesis of the LLDL motif at the C terminus of FIV Gag to ALDS had no effect on virus release, in contrast to a previous report using the same mutation in another molecular clone (FIV-14), which encodes the same Petaluma isolate of FIV used in our study (38). We confirmed our result in several independent experiments using two different FIV expression vectors in both HeLa and CrFK cells. Mutation of the LLDL domain in Gag to QSGS also had no effect on virus release in feline cells but did inhibit virus replication (our unpublished results). Thus, it seems unlikely that the LLDL motif in FIV Gag is a late domain. Rather, we hypothesize that this sequence may serve another function; for example, based on sequence similarities with known clathrin-binding cellular proteins, it may be a clathrin-binding motif. This possibility is currently under investigation.

Despite the low level of sequence conservation between FIV and HIV-1, there have been numerous observed similarities between FIV and HIV-1 in terms of pathogenesis and virus biology in both feline and human cells. Identification of cellular mechanisms that are either shared or highly divergent between FIV and HIV-1 has profound implications for the relevance of domestic cats as a nonprimate model for AIDS and for the molecular requirements for efficient long-term gene expression from nonpathogenic FIV gene therapy vectors in human cells. In terms of the relationship between cellular ESCRT proteins and retroviral late domains, the data presented here illustrate a high degree of conservation between FIV and HIV-1 in both human and feline cells in their requirement for an interaction with Tsg101. In contrast to HIV-1, our data suggest that Alix is not likely to be involved in FIV release in human cells. A feline homolog of Alix has been identified in the domestic cat genome, although the cDNA sequence of the homologous Gag-binding V domain has not yet been determined. It remains formally possible that feline Alix may interact with FIV Gag by using an unidentified late domain. Nevertheless, such an interaction may only be required for FIV release and replication in specific cell types, such as feline lymphocytes, in conjunction with PSAP late domain function and Tsg101.

This study represents the first step in defining host cell machinery required for FIV assembly and release. Further studies will expand on the cell biology of FIV replication.

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