VIRAL ENVELOPE PROTEINS AND THE HIV-1 ACCESSORY GENE VPU MEDIATE SELECTIVITY OF VIRAL AND HOST PROTEINS IN RETROVIRAL ASSEMBLY

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DEDICATION

I believe there exists, & I feel within me, an instinct for the truth, or knowledge or discovery, of something of the same nature as the instinct of virtue, & that our having such an instinct is reason enough for scientific researches without any practical results ever ensuing from them.

— Charles Darwin

This work is dedicated to those who have fostered my love of science by sharing their own love, insight and strength so generously through the many years: my parents Linda Post-Lucas and Rick Lucas, my first scientific mentor Dr. Laura Fielden, and my dearest friends Chris, Jen, Terri, Maggie, Karen and my companion, Elfin.
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ABSTRACT

Retroviruses are enveloped RNA viruses that assembly primarily at the plasma membrane of the host cell. During budding from the membrane, they acquire their own glycoproteins as well as a lipid bilayer derived from the cell. The assembly process is complex and involves protein:protein, protein:RNA, and, likely, protein:lipid interactions between viral components and the cell. These interactions appear to be specific and retroviruses are selective in acquisition of proteins. Compatibility during retrovirus assembly appears to be mediated by multiple factors: physical compatibility between glycoproteins and viral structural proteins, trafficking of proteins to appropriate locations, lipid interactions between Gag, Env and the plasma membrane, and microdomain association. In addition to mediating coalescence of appropriate factors, retroviruses appear equally equipped at excluding select host cell proteins and have evolved a number of genes to do so.

Surprisingly, retrovirus assembly has remained enigmatic and there are no anti-retroviral drugs that target the assembly stage of HIV replication. Much of the knowledge we have garnered on retrovirus assembly has been through a process known as pseudotyping, where the core structural proteins will accept the glycoproteins of an unrelated virus. Here we present work outlining contributions of the envelope protein from murine leukemia virus to assembly with the lentiviral vector human immunodeficiency virus-1 (HIV-1). We subsequently observed an interesting phenotype, where an HIV-1 accessory gene known as Vpu restricts the envelope protein
from gibbon ape leukemia virus (GaLV Env) from assembling with HIV-1. Further studies from our lab demonstrated that Vpu recognizes GaLV Env in a manner almost identical to CD4, the natural cellular target of Vpu, and that GaLV Env is essentially a CD4 analogue. Interestingly, we have found that Vpu restricts both target proteins in a manner that does not fit with the previously described Vpu-restriction model for CD4. Collectively, the GaLV Env model offers a new tool for more carefully investigating how the HIV-1 accessory gene Vpu downmodulates the host cell receptor CD4.
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I. Introduction

Retroviridae.

Retroviruses are enveloped, RNA viruses that represent a unique phylogenetic branch because of their ability to reverse transcribe their genomic RNA into DNA and integrate into the host genome. Retroviruses encode three major proteins: Pol, Gag, and Env (Figure 1-1a). Enzymatic proteins, such as reverse transcriptase and integrase, are encoded in Pol. The core structural protein, Gag, comprises the “body” of the virus and packages the genome (Figure 1-1b). The envelope (Env) glycoprotein binds the virus to target receptors, mediating fusion between the virus and the host cell.

The origin of retroviruses is unknown, however because of their unique reverse transcription and integration process, the family is thought to be monophyletic (Figure 1-2). Retroviruses are classified as either “simple” or “complex”. Murine leukemia virus (MLV) is considered a “simple” retrovirus, in that its genome encodes only Gag, Pol and Env. Other complex retroviruses, such as the lentiviruses, encode accessory genes which modulate host immune response and promote viral fitness. This is not to imply that “simple retroviruses” are less complex; indeed, many have encoded multiple accessory-type functions within single gene product like Env or Gag. For example, Gag of Moloney murine leukemia virus (MoMLV) has an upstream initiation start site that
encodes glycogag, which functions in a manner similar to Nef by enhancing infectivity (178).

The retroviral lifecycle begins when the virus binds to a host cell through interactions between Env and a target receptor with potential coreceptors (Figure 1-3). Binding initiates type-I fusion, blending the lipid envelope coating the viral core and the host plasma membrane and the core is released into the cytoplasm. The core is composed of mature subunits of Gag. How or when the viral core uncoats is unknown, but both rapid and delayed uncoating is detrimental to the virus. Reverse transcription is enzymatically mediated by viral reverse transcriptase (RT), which is packaged within each virion. RT accesses at least one strand of the dimeric RNA to reverse transcribe the viral RNA into double stranded DNA. The viral genome is flanked by two LTRs (long-terminal repeats) which allow RT to jump from head to tail during transcription. The nascent double stranded DNA is either imported into the nucleus (as is the case for lentiviruses) or accesses the host chromosomal DNA during cell division when the nuclear membrane is broken down. The viral DNA now begins the integration process through a series of biochemical interactions mediated by the viral enzyme integrase (IN). The raw ends of the viral DNA go through a nucleophilic attack, exposing ends which are then joined by IN with the host cell genome. Emerging evidence now suggests a role for host proteins in promoting integration. Following integration, the host cell begins to synthesize viral proteins and the new viral RNA. Gag is produced in
the cytoplasm and Env is synthesized in the rough endoplasmic reticulum (ER), and eventually coalesce at the plasma membrane of the cell to produce the nascent virus.
Figure 1-1. HIV-1 genomic and virus schematics.

(A) HIV-1 genome is comprised of 8 genes flanked by long terminal repeats (LTR). Of these genes, *gag*, *pol*, and *env* are found in all retroviruses. HIV-1 and other lentiviruses contain additional accessory genes. HIV-1 encodes *vif*, *vpu*, *nef*, *vpr*, *rev*, and *tat*. *Vpu* is unique to HIV-1 and a few closely related strains of SIV, but in not found in HIV-2. Figure adapted from (72). (B) HIV-1 viral particle is comprised of a dimeric RNA genome housed within core structural proteins (nucleocapsid, capsid [CA], and matrix [MA]), which are produced from protease cleavage of the polyprotein Gag. The viral core is coated by the host-lipid bilayer and the viral envelope (Env) protein, a heterodimer of gp120 and gp41 in HIV. Image NIH, open public access.
Figure 1-1. HIV-1 genomic and virus schematics.

A.

- **LTR**
  - Long terminal repeat
  - Contains control regions that bind host transcription factors

- **vif**
  - Viral infectivity factor (p23)

- **vpu**
  - Viral protein U
  - Promotes CD4 degradation and influences virion release

- **env**
  - gp160 envelope protein
  - Cleaved in endoplasmic reticulum to gp120 (SU) and gp41 (TM)
  - gp120 mediates CD4 and chemokine receptor binding, while gp41 mediates fusion

- **nef**
  - Negative effector (p24)
  - Promotes downregulation of surface CD4 and MHC I expression
  - Blocks apoptosis

- **gag**
  - Pr55
  - Polyprotein processed by PR

- **pol**
  - Polymerase
  - Encodes a variety of viral enzymes

- **vpr**
  - Viral protein R (p15)
  - Promotes G2 cell-cycle arrest

- **rev**
  - Regulator of viral gene expression (p19)

- **tat**
  - Transcriptional activator (p14)
  - Binds TAR

B.

- **gp120**
  - Docking Glycoprotein

- **gp41**
  - Transmembrane Glycoprotein

- **Lipid Membrane**

- **Capsid**

- **Matrix**

- **RNA**

- **Reverse Transcriptase**
Figure 1-2. Phylogenetic tree of the family Retroviridae.

An unrooted phylogenetic tree of retroviruses. Simple retroviruses contain the genes Gag, Pol, and Env, while complex retroviruses contain additional genes known as accessory genes. Figure adapted from (243).
Figure 1-2. Phylogenetic tree of the family *Retroviridae.*
Figure 1-3. The HIV-1 life cycle.

The virus binds to the host cell through interactions between Env and the host cell receptor CD4 and coreceptors CXCR4 and CCR5. Following fusion events, reverse transcription, uncoating and trafficking of the viral DNA to the host nucleus occur, although the exact order of these steps are unknown. Viral DNA integrates into the host cell genome with help from the viral encoded integrase (IN). The host cell then synthesizes new viral RNA genomes, core structural proteins and Env. The genome and viral proteins assemble at the plasma membrane. The new viral particle buds and viral protease cleaves the Gag precursor protein into the structural subunits to give the virus its dense cone-type core. Image reproduced with permission (244).
Figure 1-3. The HIV-1 life cycle.
Glycoproteins.

The retroviral Env protein mediates binding of the viral particle to the host receptor and entry of the virus into target cells. In its mature form, each Env is a heterodimeric glycosylated protein that ultimately trimerizes to form the fusogenic complexes. All Envs are type-I transmembrane proteins that contain an extracellular surface subunit (SU) and a transmembrane subunit (TM), which contains extracellular, transmembrane and intracellular regions (Figure 1-4). SU and TM work in a coordinated fashion to regulate fusogenic potential and activation of the trimer upon receptor binding.

Env, itself, goes through a complex series of steps in its synthesis, maturation and posttranslational modification (Figure 1-5). Most retroviral Envs are expressed on their own mRNA, although HIV-1 Env is located on a bicistronic mRNA with the viral accessory gene Vpu (205). Env is synthesized in the rough ER as a precursor polyprotein (Pr85 and gp160 in the cases of MLV and HIV-1, respectively). Following translation, the signal peptide is cleaved and the precursor Env is thought to trimerize prior to exportation from the ER to the Golgi.

Env goes through up to three critical changes in the Golgi: modification of glycosylation, cleavage into SU-TM, and the addition of fatty acids. A crucial step in the maturation of retroviral Env proteins is cleavage of the precursor Env by cellular furin or furin-like proteases to produce mature Env. HIV-1 Env maturation likely occurs with furin-7, but will occur in the presence of other furin family members, albeit less
Furin recognizes highly conserved cleavage sites: HIV-1 requires conservation of a cleavage site Lys/Arg-X-Lys/Arg-Arg, where X is any amino acid (61), while Rous sarcoma virus (RSV) requires a Arg-Arg-Ly-Arg (176). Cleavage of SU-TM happens rapidly, about 30- to 90-min post-synthesis (213). The SU and TM subunits remain linked to one another through disulfide bonds. This important structural modification potentiates the CXXC-thiol disulfide bond bridging the nascent heterodimer while holding Env in a meta-stable state prior to fusion with a target receptor (121, 213).

Furin cleavage of Env is required. In the case of MLV Env, uncleaved Env is not incorporated into viral particles and exhibits a reduction in cell surface expression, presumably because of misfolding (4, 9, 70, 230). Similar studies conducted in HIV, mouse mammary tumor virus (MMTV) and RSV resulted in more appreciable surface expression but particles, again, were highly reduced in infectivity (48, 52, 69, 76). While the MLV precursor Env can be incorporated into the virus, the mature Env appears to be preferentially incorporated (257). Interestingly, when cleavage is blocked, Env includes a higher weight molecular species suspected to be an uncleaved Env precursor with complex oligosaccharide side chains (61, 62, 144). This suggests that an undefined intermediate species of Env exists during maturation.

Some Env proteins, specifically members of the gammaretroviruses, undergo an additional cleavage event upon the virus budding from the cell. This final step in maturation removes a small ~16 amino acid, C-terminal motif, known as the R peptide
and potentiates fusogenic activity of the SU subunit (110, 128, 179, 180, 224, 253).

Cleavage of the R peptide is mediated by the viral protease and is thought to occur at the time or immediately following release of the nascent particle. The R peptide suppresses premature activation of Env fusogenicity until the peptide is cleaved at a conserved leucine-valine dipeptide site (1, 13, 109). In constructs with R peptide deletions, Env proteins are prematurely active, causing fusion between cells expressing both Env and the receptor.

After leaving the Golgi, Env is trafficked to the cell surface where it may interact with Gag directly or through cellular factors mediating indirect interactions. Multiple features in retroviral Envs appear to modulate the assembly success with Env and core structure proteins. Compatibility appears to be controlled by multiple features including: 1) location of membrane assembly, 2) direct interactions between Gag and Env, and 3) cellular sorting of viral proteins and their ability to coalesce.
Figure 1-4. Envelope is a heterodimer that forms a functional trimeric complex.

Retroviral Env proteins consist of two subunits that are produced from the precursor Env. The surface subunit (SU) is extracellular and mediates binding between the virus and a target host cell receptor. The transmembrane subunit (TM) regulates activity of SU and contains extracellular, transmembrane and cytoplasmic regions. SU is connected to TM through a disulfide bridge, which goes through conformational changes between the pre-fusion and post-fusion states.
Figure 1-4. Envelope is a heterodimer that forms a functional trimeric complex.
Figure 1-5. Envelope is a transmembrane protein that undergoes processing and maturation.

Retroviral Env proteins are synthesized in the ER, where they undergo cleavage of the signal peptide, trimerize and are exported to the Golgi for modification of glycosylation and furin cleavage. All retroviral Env proteins are synthesized as precursor polyproteins, which are subsequently cleaved into the SU and TM subunits. Env is ultimately targeted to the plasma membrane for incorporation into viral particles, although many Env proteins undergo significant endosomal recycling. Image from (32).
Figure 1-5. Envelope is a transmembrane protein that undergoes processing and maturation.

Processing of gp160 and oligosaccharide modification

Cotranslational glycosylation of gp160, signal peptide cleavage, and oligomerization
**Retrovirus assembly.**

The assembly of a nascent virus ultimately requires the union of a dimeric RNA genome housed within core structural proteins decorated with Env in a lipid bilayer. In its simplest, assembly occurs in three steps: (1) Gag is trafficked to the cell surface where Env is embedded, (2) Env coalesces at sites of Gag multimerization, and (3) the virus buds from the cell resulting in the final maturation of structural proteins (Figure 1-6). This is, of course, an oversimplification; retrovirus assembly likely occurs in multiple overlapping and interacting steps within both the cytoplasm and at the plasma membrane.

The study of virus assembly has been greatly advanced using a process known as pseudotyping, in which core structural proteins of one virus will accept the glycoproteins of an unrelated virus. For example, MLV Env is readily accepted by unrelated HIV-1 Gag. This natural biological library allows the rapid investigation of similarities and differences in assembly pathways used by different viruses. Pseudotyping to generate gene therapy vectors has been of intense interest, as the glycoprotein employed mediates specificity of the cell target. Additionally, lentiviral vectors have the unique ability to transduce non-dividing cells and integrate a gene of interest into the host cell chromosome. Pseudotyping does occur in nature. For example, many endogenous retroviruses no longer encode a functional Env and thus cannot produce infectious particles. However, in the presence of an actively replicating retrovirus, some endogenous retroviruses can steal Env to produce infectious particles.
in a process known as Env trans-complementation (103). Through pseudotyping studies it has become clear that specificity occurs between Gag and Env and this relationship is largely enforced by the properties of both proteins.

Gag is synthesized in the cytoplasm as the unprocessed core structural polyprotein (Pr55 for HIV-1), which, upon maturation, is cleaved by viral protease into three regions: matrix (MA), capsid (CA) and nucleocapsid (NC). Each region of Gag contributes to different steps in assembly and Gag alone is sufficient to mediate the release of virus like particles. Gag is targeted to the plasma membrane for assembly through interactions between MA and the inner membrane leaflet. This interface appears to be mediated by electrostatic interactions between conserved, highly basic residues (HBR) found in the N-terminus of MA in all retroviruses (131, 158). For example, RSV and HIV-1 both use strong negative charges in the headgroup to facilitate assembly (43, 44). HIV-1 MA contains fewer hydrophobic charges but has a fatty-acid modification required for assembly, a myristle group, added to the N-terminus of MA that is required for assembly of HIV (24). The exact role of the myristate is unknown but it appears to promote interactions between Gag and the inner plasma membrane through charged interfaces. More specifically, the HBR in HIV MA is affiliated with the lipid phosphatidylinositol-(4,5)-biphosphate [PI(4,5)P₂], which is enriched in “lipid rafts” at the basolateral plasma membrane (40, 169). Depletion of PI(4,5)P₂ results in a reduction in infectivity and mislocalization of Gag in the cytoplasm and perinuclear regions (40, 152). Other data supports a co-association between HIV-1 MA and
PI(4,5)P₂ enrichment. HIV-1 and MLV exhibit a two-fold enrichment in PI(4,5)P₂ relative to cellular membranes, and deletion of HIV-1 MA abolishes this fortification (31). Collectively, these finding suggest that the MA domain either targets Gag to PI(4,5)P₂ enriched budding sites or, alternatively, Gag facilitates PI(4,5)P₂ enrichment at budding sites.

With few possible exceptions, retroviruses are thought to bud from the plasma membrane. In polarized Madin-Darby canine kidney cells (MDCK) assembly occurs at the basolateral membrane (47, 124, 125), possibly due to the enrichment of negatively charged lipids at this interface. Polarization of cells creates different membrane potentials by selectively targeting ceramides to the apical membrane and PI(4,5)P₂ to the basolateral membranes (reviewed in 41, 123, 211, 212). Some plasma membrane budding viruses like influenza and retroviruses are thought to bud from “lipid rafts” because they exhibit enrichment in some lipid raft species and are sensitive to disruption of rafts. Historically, lipid rafts are considered liquid ordered (Lₒ) domains with tight packing of cholesterols, lipids and select cellular proteins like CD4 and GPI anchored proteins. In practice, lipid rafts have been defined as detergent insoluble at 4°C and are disrupted by cholesterol depletion with methyl-β-cyclodextrin. Depletion of cholesterol limits production of viral particles (170, 177) and disrupts the ability of HIV-1 Gag to both bind the plasma membrane and multimerize (172). Work by Chan et al. demonstrates differences in the envelope lipid composition between HIV-1 and MLV, suggesting variability in budding locations (31). Based on electrospray ionization mass
spectrometry (ESI-MS) HIV-1 and MLV are enriched in cholesterol, including raft associated phosphinositides and sphingolipids (PS, PIP₂, SM and dhSM), and reduced in select non-raft species relative to the cellular PM or total cell membranes (31). These enrichments all suggest that viral envelopes contain a “lipid-raft” type profile.

Increasing evidence supports that microdomains are far more biologically complex than static liquid ordered (lipid raft) and disordered domains. It has been established for some time that HIV incorporates tetraspanins presumably through budding in select tetraspanin-enriched microdomains (TEMs) (233). Tetraspanins are a unique family of proteins, having a four membrane pass, a cytoplasmic tail of varying length and are typically palmitoylated. Their exact role in cells is unclear, but they appear to function as organizers and regulate fusion and signaling activity (reviewed in 80). HIV is enriched in CD9, CD63, CD81, and CD82 when produced in multiple cell types, including macrophages, dendritic cells and T-cells (reviewed in 227). Tetraspanins appear to mediate some benefit to the virus by regulating activity of HIV-1 Env. In the absence of tetraspanins, infected cells form syncytia with neighboring cells, while in the presence of tetraspanins, Env activity is reduced (108, 245). Tetraspanin enrichment does not appear to promote the actual budding process (227), however, they colocalize with TSG101 and VPS28, components of the endosomal sorting complex required for transport 1 (ESCRT1) that are necessary in HIV budding (163).

TEM studies provide some of the most interesting information describing where retroviruses and other membrane budding viruses may actually assemble and how
microdomains function in the cell. Previously, it was thought that viral components were targeted to preexisting, large raft structures. However, recent research in virology demonstrates the highly plastic nature of TEMs. Small, transient TEMs likely exist on the plasma membrane, but HIV Gag induces the coalescence of small TEMs into larger platforms from which the virus buds (107). Multimerization of Gag is required, and in its absence, redistribution of tetraspanins is not observed (107). Virus induced TEMs are able to trap some tetraspanins like CD9, while other tetraspanins and GPI (a lipid raft marker) are able to freely diffuse in and out of the TEM. Perhaps not surprisingly, the ability of Gag to sequester TEMs is highly dependent on cholesterol. Gag induction of TEMs may result in “coclustering” between tetraspanins and lipid raft markers (85). It is likely that cholesterol helps to stabilize microenvironment scaffolding which in turn promotes membrane charge and thickness, factors which retain or exclude specific proteins and lipids. TEMs appear to be enriched in other viruses as well. Although initial studies suggested influenza buds from a unique TEM site (100), subsequent work demonstrates that influenza is also enriched in CD9 and CD81 (209) and that they enhance virus release (unpublished results, reviewed in 228).

Microdomains are important in retroviruses assembly, although it has yet to be determined how viruses induce microdomains and use enriched tetraspanins and/or lipid species to enhance their fitness. These interactions likely promote both coalescence of and interactions between Gag and Env proteins to help facilitate assembly.
Figure 1-6. Retrovirus assembly occurs at the plasma membrane.

(A) HIV-1 Gag contains multiple domains that regulate assembly. The matrix (MA) domain facilitates interactions between Gag and the plasma membrane through interactions in the highly basic region (HBR). Multimerization of Gag through CA and NC likely enhances Gag-plasma membrane interactions. (B) Gag, the viral structural protein, begins to multimerize in the cytoplasm while Env coalesces at the budding locations. Virus budding is not dependent on Env and Gag is sufficient to alone induce the formation of virus like particles. Image reproduced with permission from (168).
Figure 1-6. Retrovirus assembly occurs at the plasma membrane.
Direct interactions with Gag.

Some retroviral species’ Env proteins show incompatibility with non-native Gags and this largely is the case with lentiviruses. This mismatch appears to be enforced by the lentiviruses’ remarkably long cytoplasmic tails (CT) relative to other retroviruses. Gammaretroviruses like MLV typically have ~20 amino acid long tails, while lentiviruses range from FIV (53 amino acids) to EIAV (more than 200 amino acids) (Figure 1-7) (32). The specific purpose of such extensive tails is unknown, although in the case of SIV it appears that the full length tail is important in both transduction and assembly, and greatly enhances pathogenicity in the simian cell types (238). Similar studies on HIV-1 has shown that the CT is required in some cell lines, specifically T cells (157). Partial truncation of the FIV CT negatively affects fusogenic behavior and/or surface expression, while dramatic truncation enhances incorporation (30). Pseudotyping studies have demonstrated that the CT significantly impacts compatibility between Env and some Gags.

Lentiviruses may owe their extreme selectivity due to physical limitations (steric hindrance) imposed by their long CT. Specific interactions may occur between lentiviruses and their Env proteins to accommodate Env in the tightly packed virus. Evidence supports the Gag-Env steric hindrance hypothesis from the perspective of both the CT and Gag. Truncation of the majority of the CT alleviates incompatibilities between HIV-1 Env and MLV Gag, and RSV Env and HIV-1 Gag (119, 140). This same enhanced Env activation and incorporation has been shown in HIV-1 and SIV CT
truncation mutants (97, 216, 237, 259). Collectively, multiple studies demonstrate that cytoplasmic tails contribute to Gag incompatibility.

From the perspective of Gag, the MA domain regulates acceptability of the CT. Mutations in HIV-1 MA prohibit incorporation of HIV-1 Env, but allow amphotropic MLV (A-MLV) Env (139). However, this HIV-1 MA mediated restriction can be alleviated by the removal of HIV-1 Env CT (139). These results suggest that MA enforces a steric hindrance-type incompatibility between Env and Gag (140, 197) and that removal of most of the cytoplasmic tail of HIV and SIV Env enhances compatibility between lentiviral Envs and unrelated Gags (139).

**Cellular factors mediating coalescence.**

Cellular factors may help Env and Gag to coalesce by either directly facilitating interactions or by targeting either viral protein to assembly sites. Although the concept of a direct cellular intermediate linking Gag to Env is attractive, to date, findings have been limited. One candidate protein is known as tail-interacting protein (TIP47) (126). The cellular function of TIP47 is unknown, but it may function in trafficking of lipids (25, 250) and Rab9 (2, 78). Knockdown of TIP47 has been reported to prevent HIV Env but not vesicular stomatitis virus G (VSV-G) incorporation into viral particles (6, 126).

Alternatively, cellular proteins may target Env and Gag to the same location. Retrovirus Env proteins typically contain one or more trafficking motifs in the CT that may play a role in such a scenario. Tyrosine and dileucine trafficking motifs are thought
to link Env proteins with adapter complexes (AP), which are also used by retroviral Gags (5, 49). Therefore, the AP trafficking pathways may logically offer the opportunity for colocalization of viral proteins.

The most common of these motifs is a tyrosine trafficking motif Yxxφ, where x is any amino acid and φ is a bulky amino acid, typically lysine although isoleucine and methionine are also observed (166). The tyrosine motif promotes endocytosis and retrograde transport from the plasma membrane to the TGN through interactions with the clathrin adapter protein complexes (AP) 1, 2, 3 and 4 (reviewed in 22, 29, 206). Disruptions in the CT tyrosine-motif in MLV and Mason-Pfizer monkey virus (MPMV) cause a relocalization of chimeric Envs from the TGN to endosomes and late endosomes (12). A similar phenotype has been observed for RSV, however the tyrosine-motif’s activity appears to also be regulated by a palmitic fatty acid modification in the membrane spanning domain, which will be discussed later (166).

Beyond its ability to traffic Env proteins, the Env tyrosine-sorting motif appears to play an important role in virus assembly and pathogenesis. In the Env proteins of HIV-1 and oncogenic retroviruses MLV and MPMV, the tyrosine motif mediates basolateral targeting of virus budding in polarized MDCK cells, as detected by ELISA (124, 125). The tyrosine motif enhances cell-to-cell transmission, but appears to have little effect on cell free transmission (47). Interestingly, mice infected with a tyrosine mutant MLV produced dramatic pathogenesis, atypical of wildtype infection (45). Most retroviruses chronically infect the host and are slow to elicit symptoms, possibly to
avoid immune detection or severe detriment to the host. Although the exact role of the tyrosine motif is unknown, its conservation and ability to alter the distribution of viral proteins and restrict host pathogenesis all support an important function.

The second commonly observed trafficking motifs in the Env proteins is the dileucine motif. The dileucine motif is typically defined as [D/E]XXL[L/I], although in the case of retroviral Envs the motif is more loosely defined as L[L/I]. The motif is found in the cytoplasmic tail of proteins and interacts with clathrin adaptor protein 1 (AP-1) (reviewed in 29, 206). However, reports with HIV-1 Env are mixed as to which AP interacts with the dileucines, with either AP-1 (252) or AP-2 (27). Nonetheless, they appear to be important in Env distribution. Disruption of the dileucine motif in MLV or MPMV results in mislocalization of Env from the TGN to endosomes (12).

Another sorting motif appears to be unique to RD114, an endogenous feline retrovirus Env. RD114 Env fails to assemble with SIV Gag (189, 190). Interestingly, this incompatibility is enforced by an acid sorting motif and this motif can be transferred to MLV Env to create incompatibility with SIV Gag (15). The cellular sorting protein PACS-1 recognizes the acid motif, and distributes RD114 Env to the late endosomes (15, 190). However, PACS-1 was not required for the redistribution of RD114 Env when SIV Gag was present, suggesting a role of both a cellular sorting protein and the viral core structural proteins in the distribution of Env (15).

Many viral glycoproteins are palmitoylated prior to incorporation, including influenza, Sindbis, RSV, MLV, simian immunodeficiency virus (SIV) and HIV (82, 89, 165,
Palmitate, a fatty acid, is added to one or more cysteines, which are often located in the transmembrane or are in the membrane proximal CT of the glycoprotein. Palmitoylation is thought to help promote interactions with lipids, and in the case of Env proteins, enhance interactions with lipid rafts at the plasma membrane. HIV-1 has two palmitoylation sites in the CT, although 5% of HIV Envs lack both cysteines, suggesting that the modification is beneficial but not required (10). Mutating both cysteines in HIV Env decreases lipid raft association, incorporation into viral particles and infectivity (10, 185). Similar effects have been observed in MLV Env, notably a decrease in surface expression but not fusogenicity (122). In RSV Env, which is palmitoylated twice in the transmembrane region, palmitoylation enhanced stability of Env and prevented rapid endocytosis observed in mutants (165). Although palmitoylation is not absolutely required, it does appear to offer benefit to Env by promoting interactions with lipids rafts or some other microdomain and/or preventing endocytosis.
Figure 1-7. The cytoplasmic tail varies in length among retroviruses.

Lentiviruses contain remarkably long cytoplasmic tails relative to other retroviruses. The length of the cytoplasmic tail appears to play a role in restricting some pseudotyping combinations. Image from (32).
Figure 1-7. The cytoplasmic tail varies in length among retroviruses.
Gibbon ape leukemia virus envelope.

Gibbon ape leukemia virus (GaLV) is a gammaretrovirus most closely related to murine leukemias. The virus is, most likely, an MLV recently introduced into captive gibbon ape population by rodents. Pathologically, the virus induces leukemias in apes. GaLV is thought to share recent common ancestry with koala endogenous retrovirus (KoRV). In a remarkable case of modern retroviral endogenization, KoRV has been in the koala population 100 years or less as an endogenous retrovirus (222, 223). The virus causes leukemia, lymphoma and immunosuppression leaving koalas susceptible to opportunistic infections.

GaLV Env is able to target a common receptor Pit-1 (57), is low in cytotoxicity relative to many viral glycoproteins, a good candidate for gene therapy, and has been of interest in virus assembly. GaLV Env has a well-known incompatibility with lentiviral Gags, which is mediated by features in the cytoplasmic tail (13, 38, 147, 217, 218). Modifications in the GaLV Env CT alleviate this restriction and permit pseudotyping with lentiviral vectors. Successful GaLV Env vectors with alterations in the CT have been used to successfully transduce human cord blood CD34+ progenitor cells with 90% efficiency and transplant the cells into NOD/SCID mice (181). From the perspective of pseudotyping, GaLV Env and RD114 Env are two of the few short tailed Env proteins exhibiting cytoplasmic tail incompatibilities with lentiviruses.

Vpu.
Lentiviruses are complex retroviruses; in addition to Gag, Pol and Env, they contain a suite of accessory genes that promote replication of the virus through enhancement of infectivity, assembly and counteraction of host defenses. HIV-1 contains 6 accessory genes: vif, vpr, rev, tat, nef, and vpu. Viral protein U (Vpu) is an accessory gene found exclusively in HIV-1 and a few closely related strains of SIV. Vpu functions to restrict host cellular proteins detrimental to viral replication post-infection including CD4, tetherin (CD317 or BST-2), NTB-A, IκB, CD71 and MHC II (17, 34, 88, 159, 207, 249). Two main functions have been attributed to Vpu: degradation of the host cell receptor CD4 and enhanced viral release (EVR) through antagonism of the antiviral protein tetherin.

Vpu is an 81-86 amino acid, type-1 transmembrane protein containing a transmembrane domain (TMD) and cytoplasmic tail (CT) (Figure 1-8) (53). HIV-1 encodes Vpu on a bicistronic mRNA downstream of the Env open reading frame (205). Vpu is predicted to have three α-helices, with one in the TMD and two in the CT. The transmembrane is sufficient to promote the assembly of Vpu pentamers in vitro (138), however the significance, if any, of Vpu multimerization is unknown and appears unimportant in tetherin downmodulation (235). Work done by FRET suggests that Vpu forms multimers in the Golgi and within vesicles, but not within the ER, the primary site for CD4 targeting (87). The TMD has been shown to mediate direct binding to and antagonism of tetherin (55, 132, 201, 234), but results are conflicted on the role of the TMD on CD4 targeting (135, 201, 231).
The Vpu CT is thought to interact with cellular factors to promote modulation of target proteins. Between the two CT α-helices is a highly conserved hinge region required for Vpu-mediated degradation of target proteins (202). The putative EDSGNESE motif is highly conserved among Vpu groups and subtypes and always contains constitutively phosphorylated serines. Vpu itself is phosphorylated at S52, S56 by casein kinase II (CK-II) (202). Phosphorylation results in the recruitment of β-TrCP (Beta-transducin repeats-containing protein), a component of the Skp-Cullin-F Box E3 ubiquitin ligase complex (SCF). This motif resembles the common DSG(X)$_{2+n}$S destruction motif, which is specifically recognized by β-TrCP (reviewed in 63). β-TrCP contains a series of N-terminal WD40 repeats and a C-terminal F-box region that specifically binds Vpu (142). Interestingly, Vpu appears to avoid its own degradation while facilitating this interaction between β-TrCP and target proteins. Some studies suggest that β-TrCP binding is not required for restriction of Vpu targets. Disruption of S52 alone is sufficient to negate antagonism of either CD4 or tetherin, likely through prevention of Vpu phosphorylation (195). Interestingly, the S52A mutant demonstrates low levels of tetherin inhibition, possibly through its ability to bind to but not redistribute or degrade tetherin (195). Outside of the hinge region, Vpu contains two highly conserved, overlapping trafficking motifs located in the second α-helix: a tyrosine sorting motif (YXXφ) and a dileucine sorting motif ([D/E]XXX[L/I]). These motifs are important in cellular localization of Vpu and disruption of the overlapping motifs increases CD4 surface expression (188).
**Figure 1-8.** The HIV-1 accessory gene Vpu is an 81-86 amino acid, type-1 transmembrane protein.

(A) Vpu contains a transmembrane region (TMD) with one α-helix and a cytoplasmic tail (CT) with two α-helices. The constitutively phosphorylated serines are located in the hinge region. (B) The sequence of Vpu is highly conserved among different strains of HIV-1. Proposed trafficking motifs are highlighted in blue and the critical hinge region is boxed in red. Image reproduced with permission from (53).
Figure 1-8. The HIV-1 accessory gene Vpu is an 81-86 amino acid, type-1 transmembrane protein.
Effects on CD4.

HIV Env recognizes and binds to the host cell receptor CD4 and the coreceptors CXCR4 and CCR5 to mediate entry into the cell. CD4 is a 55-kDa, type-I transmembrane protein with four extracellular IgG-like domains known as D1-D4. Upon infection, many retroviruses downmodulate target host cell receptors. In the case of HIV, downmodulation of CD4 provides fitness benefits for the virus, likely through two methods: 1) it prevents “super-infection” of cells that are already infected (246) and 2) CD4 interacts with Env in the ER, and thus Env must be liberated from CD4-retention or alternatively Vpu prevents the formation of non-functional gp120-CD4 complexes (118).

Indeed, the downmodulation of CD4 by HIVs is so important that three genes encode proteins to aid in restriction: Nef, Vpu, and Env (33). Nef is a cytoplasmic protein that primarily targets CD4 at the plasma membrane, directing clathrin-mediated endocytosis and lysosomal degradation at the plasma membrane or alternatively retention and degradation of newly synthesized CD4 through interactions with the CD4 cytoplasmic (46).

HIV-1 Env, Vpu and CD4 are transmembrane proteins synthesized within the ER. Vpu prevents interactions between Env and CD4, and Env is liberated and allowed to be incorporated into viral particles. In the absence of Vpu, CD4 appears to have multiple negative consequences on virus fitness. HIV-1 Env complexes with CD4, which results in the retention of Env in the ER (16, 42, 91) and prevents precursor Env cleavage and maturation (16). Viral particles that incorporate CD4 are less efficient at binding to
target cells (220). Additionally, without Vpu and Nef, CD4 sequesters Env in the ER, thus prevents viral particles from incorporating Env (112).

Vpu interaction with CD4 occurs in the rough ER between the cytoplasmic tails of the two proteins, specifically the hinge region of Vpu and the LSEKKT stretch in CD4 (19). At this time, it is unclear whether TMD interactions are required between Vpu and CD4. A commonly used Vpu containing a “scrambled” transmembrane region (VpuRD) is fully functional in CD4 restriction (202), however two studies found that disruption of a conserved tryptophan located in the C-terminal region of the Vpu TMD greatly reduced anti-CD4 activity (135, 231). From the perspective of the CD4 transmembrane it is unknown what contribution the CD4 TMD makes. Work done with chimeric CD4/CD8 proteins, where extracellular, TMD and the CT were exchanged between the two proteins indicates that only the CT is necessary for Vpu targeting (248). However, chimera between vesicular stomatitis protein G (VSV-G) and CD4 suggests a role of the TMD in Vpu sensitivity (26).

Vpu targeting of CD4 is thought to occur in two steps: retention and degradation (Figure 1-9) (136). In the first step, Vpu recognizes and binds to the LSEKKT motif in the CD4 cytoplasmic tail and retains CD4 in the ER, preventing it from reaching the PM (19, 248). Vpu is then phosphorylated at serines 52, 56 by CK-II, which subsequently recruits the Skp1-Cullin-F-box$^B$-TrCP E3 ubiquitin ligase machinery (52, 56). Recruitment of the complex is thought to facilitate K48 and/or some other polyubiquitination of CD4 at any combination of serine, threonine or lysine residues (11, 136, 142, 200). CD4
ubiquitination promotes dislocation of the protein from the ER via some variant of the ERAD (ER-associated degradation) dislocation complex and ultimately targets CD4 for proteasomal degradation (11). The entire process appears to happen rapidly, with Vpu reducing the half-life of CD4 from 6h to 12min (249). Interestingly, when steps are abrogated, restriction can partially occur. Mutation of serines 52, 56 prevents β-TrCP recruitment but still allows Vpu to retain CD4 in the ER. However, in the presence of proteasome inhibition, ERAD dislocation does not occur (200). Altogether, mutagenic studies demonstrate an absolute requirement for interactions between the CT of Vpu and CD4, and a potential role for TMD interactions.
Figure 1-9. Vpu targets CD4 in the rough endoplasmic reticulum and directs it for degradation.

Vpu binds CD4 through interactions in the cytoplasmic tail. The critical serines in the Vpu hinge region are constitutively phosphorylated by casein kinase-II (CK-II), which subsequently recruits SCFβ-TrCP E3 ubiquitin ligase machinery. CD4 is polyubiquitinated, dislocated from the ER and targeted to proteasomal degradation. Image reproduced with permission from (53).
Figure 1-9. Vpu targets CD4 in the rough endoplasmic reticulum and directs it for degradation.
**Antagonism of tetherin.**

Tetherin (BST-2 or CD317) is an innate, antiviral protein whose expression is induced by IFN-α and is found in many warm blooded-vertebrates. As suggested by the name, tetherin physically “tethers” many species of budding viruses to the PM, creating a web-like mass of mature viral particles bound to the cell. HIV-1, HIV-2, SIV, Ebola, Kaposi sarcoma-associated virus (KSRV) and influenza appear to have independently evolved mechanisms to overcome tetherin (18, 21, 160, 173, 242). For example, HIV-2 Env has an analogous function to Vpu. Mechanistically, HIV-2 Env utilizes an endocytic motif within the cytoplasmic tail to redirect but not degrade tetherin (18, 21, 114). In the case of HIV-1, the accessory gene Vpu counteracts human tetherin’s antiviral activities.

Tetherin is a protein with unusual topology. Structurally, tetherin is a type-2 protein with an N-terminal transmembrane domain anchor, an extra cellular coiled-coil domain, and C-terminal glycophaspatidylinositol (GPI) anchor (111, 198, 219). Initially, it was assumed that the GPI-anchor was embedded within a “lipid raft” and that the C-terminal anchor bound to the virus during budding. However, recent work demonstrates the N-terminus embeds within the virus, while the GPI-anchor remains within the cell (115). This work was particularly interesting in that it suggested that the GPI-anchor localizes proximal to viral assembly patches on the cell surface and not lipid rafts. Direct interaction occurs between the TM regions of Vpu and tetherin, and disruption in either protein negates binding. Tetherin functions as dimers or possibly

The mechanism by which Vpu antagonizes tetherin is still unclear. Initially, Vpu was thought to remove tetherin from the PM and target it for proteasomal or lysosomal degradation, ultimately leading to a reduction in surface expression and an enhancement of viral release (79, 150). Similar to CD4-modulation, β-TrCP was required for degradation of tetherin (141). However, Vpu antagonism is more complex than initially thought and involves a restriction model distinct from that of Vpu-CD4 (201). It is currently unclear whether Vpu ultimately targets tetherin for degradation (66, 141, 142, 150).

Subsequent work demonstrated tetherin antagonism does not require surface down-modulation or intracellular depletion or degradation (151). Most groups now agree that neither total intracellular depletion, surface down-modulation, nor β-TrCP activity towards tetherin is required for antagonism (68, 151, 196, 225). Like down-modulation of CD4, Vpu requires β-TrCP for degradation of tetherin although β-TrCP is not required for restriction of tetherin (51, 55, 150, 196, 225). The reduction in tetherin from the surface is not required for promoting antagonism of tetherin (68). Increasing evidence supports the primary role of Vpu is to alter the trafficking and the subsequent density of tetherin at viral budding sites through an unknown mechanism. Interaction between Vpu and tetherin seems to mainly occur in the Golgi and disruptions in Golgi colocalization interrupt antagonism (235). To modulate tetherin, Vpu may employ one
or more of the following restrictions to trafficking: (1) slow the recycling rate of internalized tetherin to the PM or (2) down-modulate the supply of nascent tetherin (54, 55, 113, 196). Work conducted by Schmidt et al. demonstrates that Vpu alters the rate of tetherin recycling, the anterograde transport of newly synthesized tetherin, but not the rate of endocytosis from the PM (196). Nonetheless, considerable controversy exists in understanding the exact mechanism by which Vpu restricts tetherin.

In order to understand how Vpu may exploit microdomains and cellular proteins to modulate tetherin, a few studies have investigated the role of sorting factors. Vpu may require lipid rafts for function, as disruption of cholesterol prohibits anti-tetherin activities (239). Depletion of Rab7A, a RAS-related GTP-binding protein, limited the ability of Vpu to reduce tetherin expression at the PM (28). Rab GTPases regulate endo- and exocytosis, and membrane-related trafficking events. Rab7A also has a role in the maturation of HIV-1 Env proteins from the immature gp160 to the SU (gp120) and TM (gp41) subunits (28). Whether these two Rab7A associated functions are related or coincidental has yet to be determined. HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), a subunit within ESCRT-0 (Endosomal Sorting Complexes Required for Transport), appears to be required for tetherin restriction by Vpu (94). The role of an ESCRT complex is interesting for two reasons. First, ESCRT complexes are exploited by HIV and other retroviruses during the assembly process and are thought to function as assembly platforms (14). Second, Hrs interacts with and sorts ubiquitinated proteins.
Ubiquitin is associated with protein modification through a process known as ubiquitination or ubiquitylation. Ubiquitination may occur as monoubiquitination or polyubiquitination, the addition of a chain of ubiquitins added onto lysine acceptors. Polyubiquitination of protein may result in its subsequent degradation but may also alter trafficking of ubiquitinated proteins. After the identification of a non-canonical ubiquitination motif (serine/threonine) in both CD4 and the KSRV-targeted tetherin, the Guatelli lab identified a Vpu-mediated ubiquitinated sequence in tetherin, serine-threonine-serine (3S,4T,5S) that is ubiquitinated in the presence of Vpu (136, 232, 241). In addition, the two cysteines and two lysines also appear to be able to accept ubiquitination, and mutation of any one acceptor motif did not abrogate ubiquitination, suggesting that Vpu mediated ubiquitination of tetherin is somewhat plastic (232). This motif had previously been overlooked because it fails to fit the traditional lysine-ubiquitin acceptor site.

These findings collectively support multiple steps in Vpu’s antagonism of tetherin. Currently, studies suggest that Vpu ultimately targets tetherin for degradation through and CK-II mediated phosphorylation of Vpu and the subsequent recruitment of β-TrCP (195). Multiple steps in restriction pathways are not uncommon in the interplay between host and virus. In conclusion, our scientific understanding of Vpu-tetherin targeting model is still not understood. Variations in these experiments can be dependent on multiple factors, including the types of cells used and the tetherin.
expression method (exogenous or endogenous) (151). Further studies will be needed to
determine how Vpu antagonizes tetherin.
II. Two distinct mechanisms regulate recruitment of murine leukemia virus envelope protein to retroviral assembly sites

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**ABSTRACT**

The cytoplasmic tail domain (CTD) of retroviral envelope (Env) proteins has been implicated in modulating Env incorporation into viral particles. We generated a panel of murine leukemia virus (MLV) Env mutants and analyzed their ability to be recruited to human immunodeficiency virus-1 (HIV-1) assembly sites. Surprisingly, the entire CTD was dispensable for recruitment to assembly sites, but a mutation that disrupted the furin cleavage site in Env abolished recruitment. To determine if MLV Env can show selectivity for homologous assembly sites, cells were co-transfected with both HIV-1 and MLV assembly components along with each MLV Env construct and assayed for infectious particle production. MLV Env selectively formed infectious particles with the MLV components at the expense of infectious HIV-1 infectious particle production, but truncation of the CTD progressively reduced this selectivity. Collectively these data
suggest that there are two separable mechanisms that govern MLV Env recruitment to viral assembly sites.
INTRODUCTION

Fundamentally, the formation of an infectious retrovirus is the result of the coordinated assembly of at least three viral components: the genome, the core structural proteins (Gag and GagPol) and an envelope glycoprotein (Env). Although Env is expendable for nascent particle assembly and budding, it is required for selectively binding to specific receptors and mediating entry into the host cell. MLV Env (gp85) forms a heterotrimeric, type-1 transmembrane protein and is produced as a single protein that is cleaved into a transmembrane (TM or p15E) subunit and an extracellular surface (SU or gp70) subunit. The SU subunit of ecotropic MLV Env mediates binding to the host cell surface receptor, murine cationic amino acid transporter-1 (mCAT-1), and the TM subunit of Env contributes to membrane fusion following receptor binding. The MLV Env TM subunit contains an N-terminal ectodomain followed by a membrane-spanning domain and a 35 amino acid C-terminal cytoplasmic tail domain (CTD).

In a process known as pseudotyping, some enveloped viruses are capable of incorporating heterologous viral glycoproteins during assembly. The mechanism by which particular viral glycoproteins are sorted to assembly sites of unrelated viruses remains unknown. Using a scanning electron microscopy technique (SEM), we recently demonstrated that MLV Env is specifically recruited to HIV-1 budding sites during assembly (98). It has been shown for several Env proteins, including those of HIV-1, RD114, GaLV and others, that the CTD of Env dictates pseudotyping restrictions (37,
189-191, 197, 217). These studies suggest that amino acid sequences in the CTD mediate Env inclusion into viral particles, possibly through cytoplasmic interactions with Gag, GagPol or cellular cytoplasmic proteins.

Within the MLV Env CTD, several elements regulate Env incorporation and fusogenicity. The C-terminus of the MLV Env CTD is known as the R peptide, a 16 amino acid sequence that is cleaved off by the viral protease during virus maturation (13, 71, 81). Among gammaretroviruses, the R peptide includes a conserved leucine-valine dipeptide cleavage site and a tyrosine (YXXL) motif that has been implicated in promoting endocytosis of the Env protein (1, 12, 13, 110, 124, 128). Mutation of the tyrosine to an alanine does not disrupt fusogenicity, but does increase Env expression on the cell surface presumably through disruption of the R peptide endocytosis motif (1). Additionally, some mutations at the tyrosine position have been shown to reduce Env incorporation into viral particles, a possible result of a defect in Env trafficking (224). Although R-minus Env is highly fusogenic, large defects in the context of MLV particles have been reported. MLV produced with R-minus Env resulted in a decrease in cellular transduction relative to wildtype MLV Env particles (1, 93, 109, 180, 186). In cell culture, the R peptide is required as demonstrated by the rapid reversion of MLV R-minus Env mutants to an Env with a functional R peptide (229). Fractionation studies have shown R peptide association with immature particles, suggesting a role for R peptide-mediated assembly between Env and budding particles (3).
Prior to its cleavage, the R peptide functionally suppresses premature Env fusogenicity. MLV Env proteins become highly fusogenic in mutants that lack the R peptide (110, 128, 179, 180, 224, 253). When R peptide cleavage is inhibited, MLV Env proteins are still incorporated into viral particles but have greatly diminished fusogenic activity (128, 180). A conserved leucine, located in the second position of the R peptide N-terminus, is critical in the suppression of premature Env activation. Mutation of this amino acid results in enhanced Env fusogenicity in the absence of R peptide cleavage (109, 110, 224, 253). Some mutations in the R peptide can also alter the conformation of the Env ectodomain (1). The mechanistic action of R peptide removal on SU subunit activation has recently been elucidated further. Cleavage of the R peptide facilitates the isomerization of the disulfide bond bridging the SU and TM subunits by reducing the ability of the R peptide to maintain stability within the CTD alpha-helical coil and interfere with the activity of CXXC-thiol in the SU subunit (128).

Although the R peptide is a prominent factor in the activation of Env, other portions of the CTD contribute to incorporation and fusion. Truncations of the MLV CTD demonstrate a correlation between reduced tail length and a reduction in infectivity and incorporation, despite similar levels of Env expression at the cell surface (93, 186). Truncations in the CTD greater than the last 31 C-terminal amino acids result in a dramatic decrease in incorporation and infectivity (186).

Like all retroviral Env proteins, the precursor MLV Env is assembled into a trimeric structure in the endoplasmic reticulum and is subsequently cleaved into SU and
TM subunits by a cellular furin or furin-like protease. The cleavage of Pr85 into subunits is required and considered to be the first step towards MLV Env activation (62, 144, 213, 257). The SU and TM subunits remain associated with each other through a disulfide bond bridge by a CXXC-thiol located in the SU subunit. Interestingly, reports vary on whether the cleavage of retroviral Env proteins is required for trafficking to the plasma membrane or incorporation into viral particles (48, 52, 62, 69, 76, 134, 144, 154, 257).

We investigated a series of MLV Env mutants with truncations of the CTD, a furin-cleavage defective construct, as well as an MLV Env chimera containing the CTD of the gammaretrovirus RD114. We demonstrate that all MLV Env truncations are robustly recruited to viral budding sites of HIV-1. Surprisingly, we found that the CTD is not required for recruitment of MLV Env to viral budding sites, but that the CTD does mediate Env discrimination between MLV and HIV-1 sites of assembly. Of the Env mutants investigated, only the furin-cleavage defective mutant failed to associate with viral budding sites or produce infectious particles. Our findings suggest that the minimal factors facilitating generic recruitment and incorporation of Env lie outside of the CTD while elements regulating viral specificity are located within the CTD.

RESULTS

Properties of MLV Env mutants
Previous studies have demonstrated that the Env CTD has a critical role in promoting Env protein incorporation into viral particles and modulating fusogenicity. We created a library of expression constructs containing sequential C-terminal truncations of the CTD as well as a processing defective MLV Env (R479G) that lacks the pr85 cleavage site required to produce the SU and TM subunits (Figure 2-1). In addition, we engineered an MLV chimeric construct that contains the CTD of the feline gammaretrovirus RD114 (Figure 2-1). An equivalent glycoprotein construct has been shown to be incompatible with the lentivirus simian immunodeficiency virus (SIVmac) (15, 189-191).

The six constructs were evaluated to characterize their relative infectivity, fusogenicity and surface expression (Table 1). These assays were performed primarily to confirm that our MLV Env constructs behave similarly to previously described MLV Env mutants. First, we determined whether each MLV Env construct was capable of forming infectious particles in the context of either lentiviral (HIV-1) core particles or homologous gammaretrovirus (MLV) core particles. Both gammaretroviral or lentiviral particles were able to pseudotype with all Env chimeras up to deletion of the C-terminal 31 amino acids. The furin-cleavage mutant, R479G, was unable to generate a significant viral titer. These findings are generally consistent with previous reports (37, 62, 93, 186). Surprisingly, we did not observe a decrease in infectivity with HIV-1 upon substitution of the MLV CTD with the RD114 Env CTD. Although a similar MLV Env construct has been
reported to be incompatible with SIVmac (191), its compatibility with HIV-1 has not been reported.

Syncytial assays were performed to determine the fusogenic properties of the MLV Env constructs. As expected, the removal of the R peptide (Δ16) promoted the formation of syncytia. Some syncytia were also observed with the other truncation mutants that lacked the R peptide, but essentially no syncytia were observed with wildtype MLV Env, R479G, or the MLV/RD114 chimera. These findings are consistent with previous reports (93, 109, 180, 186, 224, 253).

To exclude the possibility that reductions in infectivity could be explained by a decrease in MLV Env present at the cellular plasma membrane, the surface abundance of the MLV Env proteins was quantified via flow cytometry. Each of the six proteins displayed significant surface expression, although there was some reduction with select truncations and with the R479G mutant. These data are consistent with reports by others using similar MLV Env modifications demonstrating that Env truncations are capable of plasma membrane expression (93, 186).

The MLV Env cytoplasmic tail is dispensable for recruitment to HIV-1 assembly sites

Because all MLV Env truncations, with the exception of Δ33, readily produced infectious particles with HIV-1, we next sought to determine if infectivity of pseudotyped particles was the result of specific or non-specific recruitment of each Env to HIV-1 assembly sites. It has been estimated that a single infectious retroviral particle
requires inclusion of less than ten Env trimers (104, 137, 255) and it is plausible that sufficient levels of Env could be passively incorporated into particles as they bud from the cellular plasma membrane. However, we have previously demonstrated, using an SEM imaging technique, that MLV Env incorporation into HIV-1 particles is not random, but MLV Env is in fact robustly recruited to HIV-1 assembly sites (98). To determine if the CTD of MLV Env modulates this recruitment, we imaged the distribution of each of the MLV Env constructs relative to HIV-1 assembly sites by SEM. Surprisingly, we found that all truncation mutants, including the mutant lacking its entire CTD, were recruited to budding HIV-1 particles (Figure 2-2). The MLV/RD114 chimera was also robustly recruited to HIV-1 assembly sites. The only Env mutant that was not recruited to HIV-1 assembly sites was R479G. This protein appeared somewhat clustered in distribution on the surface of cells, but these clusters did not coincide with HIV-1 assembly sites. Although this result is consistent with some previous studies that have suggested that furin-cleavage defective Env proteins are excluded from viral particles despite cell surface expression (52, 154), it was nonetheless remarkable to see the dramatic shift of protein away from HIV-1 assembly sites due to an alteration in the ectodomain of the protein. These images demonstrate that the CTD of Env is not required for recruitment of MLV Env to HIV-1 budding sites.

**MLV Env displays selectivity for MLV particles**
Because MLV Env is known to be a promiscuous viral glycoprotein that is robustly recruited to HIV-1 assembly sites (Figure 2-2) and was able to efficiently form infectious particles when combined with HIV-1 (Table 2-1), we wished to determine if MLV Env demonstrates intrinsic selectivity for MLV cores. Because the SEM assay relies on identifying assembly sites based on their surface topology, it was not feasible to compare recruitment of Env to two different types of viral assembly that would be morphologically similar. Instead, we employed an infectivity-based competition assay to determine whether MLV Env displays viral selectivity. To produce MLV and HIV-1 particles, we utilized an MLV reporter construct that expresses the red fluorescent protein TdTomato in infected cells and an HIV-1 reporter that expresses green fluorescent proteins (GFP) in infected cells. Along with their respective fluorescent reporters, plasmids encoding the MLV and HIV-1 structural components were transfected into cells and allowed to pseudotype with a single viral glycoprotein. Viral medium was transferred to 293T mCAT-1 cells and the relative viral infectivity output was determined by flow cytometry. As a control, the promiscuous rhabdoviral glycoprotein VSV-G was utilized instead of MLV Env. Infected cells were gated as either green or red fluorescent populations and cells co-infected with HIV-1 and MLV were excluded from infectivity analyses for consistency.

Individually, both HIV-1 and MLV cores were able to efficiently form infectious particles with VSV-G, and when the two viral cores were simultaneously introduced, the output of infectious particles was equivalent for the two types of virus (Figure 2-3, top).
When these transfections were conducted with MLV Env, both individually expressed MLV and HIV-1 particles were able to form infectious particles. However, when viral particles were produced in the presence of both cores there was a dramatic shift towards the production of infectious MLV particles at the expense of infectious HIV-1 particle production (Figure 2-3, bottom). The ratio of infectious MLV to HIV-1 was approximately 100-fold higher with MLV Env than with VSV-G. These data demonstrate that, although MLV Env is attracted to HIV-1 assembly sites, it displays a distinct preference for MLV assembly sites.

The CTD of MLV Env dictates viral selectivity

Although the MLV Env CTD clearly is not required for recruitment to HIV-1 budding sites, we questioned if the CTD contributes to the protein’s selectivity for MLV viral particles. To address this query, each of the MLV Env mutants was subjected to the competition assay described in the previous section. The results confirmed that MLV and HIV-1 cores formed infectious particles with each of the MLV Env constructs, except R479G (Figure 2-4). In general, progressive truncation reduced the infectivity of MLV Env with both particle types (Figure 2-4A and B). When the two types of particles were produced simultaneously in cells, MLV Env again showed a strong preference for forming infectious MLV particles. However, truncation of the MLV Env CTD progressively reduced this selectivity (Figure 2-4C and D). In the complete absence of the CTD, MLV Env displayed little preference for MLV over HIV-1 cores. Collectively,
these data suggest that determinants that contribute to MLV Env selectivity reside within the CTD.

**DISCUSSION**

Through the use of two novel assays, we have analyzed several MLV Env mutants that are similar or identical to constructs previously described. We utilized an SEM assay to determine whether the Env proteins were actively recruited to HIV-1 budding sites and an infectivity-based competition assay to determine whether selectivity between HIV-1 and MLV occurred. Using the SEM assay we observed that MLV Env is recruited to HIV-1 budding sites in a manner that is independent of the CTD. Using the competition assay we observed that MLV Env preferentially forms infectious particles with MLV cores and this selectivity is dependent on the CTD. Collectively these data suggest that at least two distinct and separable mechanisms modulate recruitment of MLV Env to retroviral budding sites. We will refer to these two mechanisms as *generic recruitment* (CTD independent) and *specific recruitment* (CTD dependent).

**Generic recruitment**

MLV Env is a promiscuous glycoprotein that is recruited to sites of retroviral assembly of viruses as divergent as HIV-1 and Rous sarcoma virus (RSV) (98). Because
the CTD of Env is not required for recruitment, the mechanism is likely not based on a direct protein-protein interaction between Env and GagPol. Although unlikely, we cannot exclude the possibility that Gag binds to a very short portion of the CTD that remains partially exposed in the cytoplasm. Instead, we favor the hypothesis that recruitment occurs indirectly. Several possible mechanisms could support indirect interaction between viral proteins, including the following: 1) a direct protein intermediate links viral proteins, 2) cellular trafficking factors independently target Gag and Env to the same cellular microenvironment, or 3) viral proteins independently co-associate with a non-protein intermediate.

The feasibility of a cellular protein intermediate that directly links Gag and Env is easy to propose and has some precedent. One such direct protein intermediate is TIP47, which has been proposed to function as a protein connector between HIV-1 Gag and the HIV-1 Env cytoplasmic tail (127). However, because the CTD of MLV Env is not required for recruitment to budding HIV-1 particles, the relevant interaction domain of MLV Env would be restricted to the membrane spanning domain and/or the ectodomain. A direct protein intermediate would likely be complex in structure and/or function in order to mediate signaling between at least two distinct cellular domains in which viral Env and Gags reside. If this proposed intermediate were required to maintain the interaction between Gag and Env, it would be expected to be abundant in budded particles. However, extensive HIV-1 proteomic studies (35, 192, 258) have identified no obvious cellular protein intermediate.
In an alternative model, a cellular factor separately targets MLV Env and HIV-1 Gag to the same cellular region, such as a specific site on the plasma membrane or a specific endocytic compartment, where Gag and Env co-assemble into particles. According to this model the cellular trafficking machinery would function transiently as an intermediate facilitator and thus might not be present during the budding process. This mechanism fails to readily explain why some viral glycoproteins can form pseudotypes with a wide variety of viruses, but others can only form pseudotypes with a subset of viruses.

Finally, in a third model, a non-protein intermediate fosters an environment favorable for pseudotyping. Multiple studies have suggested that retroviral assembly occurs at lipid rafts on the plasma membrane and that Env proteins are independently attracted to these sites (23, 117, 177, 240). Based on this hypothesis, a lipid microenvironment essentially serves as an intermediate during viral assembly. However, our previous and current data do not support a simple raft model. The raft hypothesis proposes that during assembly Gag targets Env proteins that are stably present in pre-existing clusters at the plasma membrane. However, we have previously shown that wildtype MLV Env protein is essentially random in its plasma membrane distribution in the absence of Gag (98). In addition, the viral glycoprotein VSV-G is considered to be a non-raft protein (8, 194) and yet it efficiently pseudotypes with most viruses and is recruited to HIV-1 budding sites (98).
Our data are consistent with a model where Gag assembly creates a unique lipid environment at the assembly site and that this environment is favored by certain viral glycoproteins. This unique lipid region is perhaps similar to what is defined as a lipid raft or a liquid ordered domain. If some glycoproteins, such as VSV-G or MLV Env, have a higher affinity for a specific microenvironment than other viral glycoproteins, it could explain why promiscuous glycoproteins pseudotype efficiently with many different types of virus.

This model does not exclude the possibility that additional factors either enforce (see next section) or inhibit Env incorporation. For instance, non-lentiviral retroviruses can be pseudotyped only with HIV-1 Env that lacks the cytoplasmic tail (59, 139, 247). This restriction could be explained if the HIV-1 Env is recruited to budding sites in a manner similar to that for MLV Env, but is excluded from incorporation because of steric hindrance from its long CTD. In addition, some cellular proteins may have an affinity for retroviral assembly sites but fail to be incorporated into particles due to interactions with other cellular proteins. For instance, this may explain why the protein human CD4 is efficiently incorporated into RSV particles when expressed in quail cells (256).

Specific recruitment.

In the presence of both MLV and HIV-1 cores, competition studies demonstrated a preferential production of MLV cores with wildtype MLV Env that supersedes the ability of HIV-1 to productively pseudotype with wildtype MLV Env. Although not
known, we speculate that MLV cores may sequester available MLV Env from HIV-1 cores. However, in the absence of the MLV Env CTD, infectious particles of MLV and HIV-1 were produced in equal proportion to one another. As a result, we propose that the CTD of MLV Env facilitates selective incorporation into MLV particles.

A reasonable model for specific CTD-dependent recruitment would support a direct interaction between the MLV structural protein Gag and the CTD of Env. Substantial evidence from other retroviruses suggests that the MA domain of Gag directly interacts with the cytoplasmic tail of native Env glycoproteins. In one such example, mutations in HIV-1 Env that block incorporation into HIV-1 particles can be circumvented by compensatory mutations in HIV-1 MA (156). The R peptide of MLV Env has been shown to predominantly associate with immature particles, suggesting an R peptide-facilitated interaction between Env and the assembling viral particle (3). Furthermore, wildtype HIV-1 Env becomes fully fusogenic only after maturation of HIV-1 Gag, suggesting a direct interaction between HIV-1 Gag and Env (95, 251). Although a direct interaction between MA and Env is the most logical explanation for specific recruitment, other contributing mechanisms cannot be excluded.

**MATERIALS AND METHODS**

**Plasmid constructs.**
The ecotropic Friend MLV Env, yellow fluorescent protein (YFP)-tagged MLV Env, and MLV GagPol constructs were kindly provided by Walther Mothes (Yale University) (210). The codon-optimized late domain deficient HIV-1 Gag construct used in SEM images has been described previously (98). Truncations of MLV Env were generated by oligonucleotide linker insertion between the ClaI site (33 amino acids upstream of the stop codon) and the EcoRI site (located just downstream of the stop codon). The R479G mutation was introduced by two-step PCR. The MLV reporter construct pQCXIP-TdTomato was described previously (129). The HIV-1 packaging construct CMV ΔR8.2 was obtained from Addgene (plasmid #12263) (260). The reporter construct pSIN18.cPPT.hEF1a.EGFP.WPRE was designed by Michal Gropp and Benjamin Reubinoff (74).

Cells.

The 293FT cell line was obtained from Invitrogen. The 293T mCAT-1 cell line stably expresses the target cellular receptor for ecotropic MLV Env, murine cationic transporter-1, and was obtained, kindly, from Walther Mothes. Cell lines were maintained in DMEM supplemented with 10\% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM non-essential amino acids, and 0.5 mg/ml G418.

Infectivity assay.
Infectivity competition studies were performed by transfecting 293FT cells in 6-well plates with FuGene 6 (Roche). Each MLV Env construct (200 ng) was co-transfected with HIV CMV ΔR8.2 (150 ng), HIV reporter construct pSIN18.cPPT.hEF1a.EGFP.WPRE (150ng), MLV GagPol expression construct (250ng) and the MLV reporter construct pQCXIP-TdTomato (250ng). In experiments where the viruses were expressed individually, the DNA for the second expression construct and reporter was replaced with filler DNA. The media was replaced 24 hours post-transfection to remove residual transfection reagent and viral media was collected ca. 48 h posttransfection, frozen at -80°C for at least 4 h, thawed, and 1 ml of media was transferred onto 293T mCAT-1 cells with 10 ug/ml polybrene (Invitrogen). Cells were collected 48 hours later, fixed with 4% paraformaldehyde and infectivity was measured as infectious units per ml as determined by flow cytometry with an Accuri flow cytometer.

**Surface labeling.**

For each construct, 293FT cells were transfected with 1000 ng of each YFP-tagged Env. Media was changed 24 h posttransfection to remove residual transfection reagent. At 48 hours posttransfection, cells were collected, labeled with an anti-GFP Alexa Fluor-647 antibody with 1% goat serum for 1 h at 4°C (1:1000, Invitrogen), fixed with 4% paraformaldehyde and analyzed by FACS. Surface expression was calculated as mean channel FL-2 fluorescence to mean channel GFP fluorescence for each construct relative to wildtype MLV Env expression levels.
**Syncytia assay.**

In order to determine fusogenic activity of the MLV Env constructs, 293T mCAT-1 cells were co-transfected with each of the 1000 ng MLV Env constructs along with 20 ng of plasmid expressing mCherry. The mCherry allowed the transfection to be detected by fluorescence microscopy. Cells were fixed ca. 24 hours posttransfection and were treated with Hoechst stain to identify cell nuclei. Cells were imaged via fluorescent microscopy and the number of cells and the number of nuclei were counted per view-field. Syncytia were quantified with the cell fusion index formula as defined by \[1 - \frac{\text{number of cells}}{\text{number of nuclei}}\].

**SEM.**

The distribution of MLV Env and virions on the cell surface was imaged via SEM, as previously described (98). Briefly, cells were plated onto coverslips coated with a patterned gold grid and were transfected with a late domain defective HIV-1 Gag expression vector (500 ng) and a YFP-tagged Env expression vector (500 ng) with FuGene 6 according to manufacturer’s protocol (Roche). At 20 hours posttransfection, plated cells were fixed with 4% paraformaldehyde and the grid locations of individual transfected cells were recorded. Cells were labeled with primary mouse anti-GFP (1:25, Sigma) and 10- to 12-nm gold conjugated anti-mouse secondary antibody (1:20, Jackson ImmunoResearch). Subsequently, cells were fixed with 2.5% glutaraldehyde,
dehydrated in ethanol, critical point dried, coated with carbon, and imaged with a Hitach S-4700 FE-SEM.

ACKNOWLEDGEMENTS

We thank Walther Mothes and Vineet KewalRemani for reagents. SEM studies were performed at the University of Missouri Electron Microscopy Core facility. This research was supported by U.S. Public Health Service grant AI73098 and the Arnold and Mabel Beckman Foundation Young Investigator Program.
Figure 2-1. Schematic of MLV Env protein constructs.

Sequences are the C-terminal cytoplasmic tails of ecotropic MLV Env, an MLV/RD114 chimera and MLV Env mutants. Specific mutations are shown below MLV wildtype Env. The cytoplasmic tail domain is shown in bold, the membrane proximal domain is underlined, an arrow indicates the point mutation made at the furin cleavage site. The YFP insertion site is shown by the triangle for mutants used in fluorescent cell surface expression, syncytial and SEM assays. SU=surface domain, TM=transmembrane domain, black column= transmembrane spanning helix.
Figure 2-1. Schematic of MLV Env protein constructs.

MLV: GPC\underline{ILNRLVQFVKDRISVVQAL} VLTQQYHQLKFIEYEP
MLV/RD114: GPC\underline{ILNRLMAFINDRLNVVHAM} VLAQYQALKAEAEAEQD
MLVΔ16: GPC\underline{ILNRLVQFVKDRISVVQAL}
MLVΔ25: GPC\underline{ILNRLVQFVK}
MLVΔ31: GPC\underline{ILNR}
MLVΔ33: GPC\underline{IL}
Table 2-1. Properties of MLV Envelope mutations.

a. Infectivity values for each construct are shown as infectious units (IU) per ml relative to wildtype MLV Env (+SD, n=3). b. The relative cell fusion index (CFI) was calculated per field by first determining the CFI relative to the CFI of each treatment group as described in materials and methods. c. Truncations in the Env cytoplasmic tail fail to alter cell surface expression. Calculated as mean channel FL-2 fluorescence to mean channel GFP fluorescence for each construct relative to wildtype expression levels. Values shown at the average of two independent experimental replicates.
Table 2-1. Properties of MLV Envelope mutations.

<table>
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<tr>
<th>Env construct</th>
<th>Infectivity *</th>
<th>Syncytia b</th>
<th>Cell surface expression (FACS) c</th>
</tr>
</thead>
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<td>MLV Core</td>
<td></td>
</tr>
<tr>
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<td>100</td>
<td>0±0</td>
</tr>
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<td>MLV/RD114</td>
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<tr>
<td>MLV R479G</td>
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</table>
Figure 2-2. Cell surface distribution of MLV Env mutants relative to HIV-1 assembly sites.

293mCAT-1 cells were transfected and labeled for Env. Left, secondary electron images of HIV-1 assembly sites. Right, backscatter electron images of gold-labeled Env. Scale bars = 200-nm.
Figure 2-2. Cell surface distribution of MLV Env mutants relative to HIV-1 assembly sites.
Figure 2-3. MLV cores outcompete HIV-1 cores for MLV Env proteins.

Infectious particle output was determined by flow cytometry. The input viral cores, HIV-1 or MLV, are shown top and the input viral glycoprotein, VSV-G or MLV Env mutant, is shown left. Numbers represent the percentage of the total cells in the defined gates. 293FT cells were transfected with either MLV Env or VSV-G along with MLV proviral components expressing TdTomato and/or HIV proviral components expressing GFP. Viral media from transfected cells were transferred onto 293T mCAT-1 cells.
Figure 2-3. MLV cores outcompete HIV-1 cores for MLV Env proteins.
Figure 2-4. Env cytoplasmic tail length affects MLV specificity and infectivity in pseudotyped particles.

Infectivity assays were performed by transfecting 293FT cells and 48 h later transferring viral media onto 293T mCAT-1 cells. (A) MLV GagPol with a fluorescent MLV genome reporter or (B) an HIV-1 provirus with a GFP reporter was co-transfected with each Env construct into 293FT cells. (C) Competition assays were performed as described above, but both proviral components were co-transfected with each Env construct into each well. (D) Relative infectivity was calculated for each Env treatment by first calculating the ratio MLV IU (infectious units) per ml to HIV-1 IU per ml and then each value was normalized by the ratio of VSV Env with MLV core to HIV-1 core. A representative experimental result from one of three replicates is shown for parts A and B. Means and +SD from all three experiments are reported in Table 1.
Figure 2-4. Env cytoplasmic tail length affects MLV specificity and infectivity in pseudotyped particles.
III. The pseudotyping incompatibility between HIV-1 and GaLV Env is modulated by Vpu.

Note: This work was published in Journal of Virology (129).

ABSTRACT

The Env protein from gibbon ape leukemia virus (GaLV) has been shown to be incompatible with human immunodeficiency virus 1 (HIV-1) in the production of infectious pseudotyped particles. This incompatibility has been mapped to the C-terminal cytoplasmic tail of GaLV Env. Surprisingly, we found that the HIV-1 accessory protein Vpu modulates this incompatibility. The infectivity of HIV-1 pseudotyped with MLV Env was not affected by Vpu. However, the infectivity of HIV-1 pseudotyped with an MLV Env with the cytoplasmic tail from GaLV Env (MLV/GaLV Env) was restricted 50- to 100-fold by Vpu. A Vpu mutant containing a scrambled membrane-spanning domain, VpuRD, was still able to restrict MLV/GaLV Env, but mutation of the serines residues at positions 52 and 56 completely alleviated the restriction. Loss of infectivity appeared to be caused by reduced MLV/GaLV Env incorporation into viral particles. The mechanism of this downmodulation appears to be distinct from Vpu mediated CD4 downmodulation because Vpu expressing cells that failed to produce infectious HIV-1
particles nonetheless continued to display robust surface MLV/GaLV Env expression. In addition, if MLV and HIV-1 were simultaneously introduced into the same cells, only the HIV-1 particle infectivity was restricted by Vpu. Collectively these data suggest that Vpu modulates the cellular distribution of MLV/GaLV Env preventing its recruitment to HIV-1 budding sites.
INTRODUCTION

The gammaretrovirus GaLV has been widely used for gene therapy because of its wide host cell tropism and non-pathogenicity (7, 65, 86, 101, 102, 148). The host cell receptor for GaLV Env has been cloned and identified as a sodium-dependent phosphate transporter protein (164, 167). Like other retroviruses, GaLV encodes a single transmembrane surface glycoprotein (GaLV Env), which is cleaved into surface (SU) and transmembrane (TM) subunits (Fig 1). The TM domain of GaLV Env contains a short 30 amino acid C-terminal cytoplasmic tail. Although GaLV Env functions well when coupled (pseudotyped) with MLV based retroviral vectors, it has been shown to be completely incompatible with HIV-1 (37, 217). When GaLV Env is expressed with HIV-1, essentially no infectious HIV-1 particles are produced (37, 217). The mechanism for this infectivity downmodulation is unknown, but the component of GaLV Env responsible for the restriction has been mapped to the cytoplasmic tail. Replacing the cytoplasmic tail of GaLV Env with the equivalent sequence from MLV Env ameliorates the restriction. Likewise, replacing the cytoplasmic tail of MLV Env with that from GaLV Env confers the restriction (37).

Vpu is an 81 amino acid HIV-1 accessory protein produced from the same mRNA as the HIV-1 Env gene. The N-terminus of Vpu contains a membrane-spanning domain followed by a 50 amino acid cytoplasmic domain. Vpu is unique to HIV-1 and a few closely related SIV strains. The best characterized roles for Vpu in the HIV-1 lifecycle are
modulation of host proteins CD4 and tetherin (also known as BST-2, CD317, and HM1.24) (159, 234, 249). Vpu promotes the degradation of CD4 in the endoplasmic reticulum through a proteasome-dependent mechanism (199). The cytoplasmic tail of Vpu physically interacts with the cytoplasmic tail of CD4 and recruits the human beta-transducing repeat-containing protein (β-TrCP) and E3 ubiquitin ligase components to polyubiquitinate and ultimately trigger the degradation of CD4 (142). Two serine residues at positions 52 and 56 of Vpu are phosphorylated by casein kinase-2 and are required for CD4 degradation (202, 204). The membrane-spanning domain of Vpu is not specifically required for CD4 degradation. A mutant protein containing a scrambled membrane-spanning sequence, VpuRD, is still able to trigger the degradation of CD4 (204). The region of CD4 that is targeted by Vpu is approximately 17-13 amino acids from the C-terminus in the cytoplasmic tail (Figure 1-1) (34, 116).

In addition to degrading CD4, Vpu has also long been known to enhance viral release (EVR) in certain cell lines (105, 226). Recently, the type-I interferon-induced host protein tetherin was identified as being responsible for this Vpu-modulated restriction (159, 234). In the absence of Vpu, tetherin causes particles to remain tethered (hence the name) to the host cell post-fission. Although Vpu counteracts the function of tetherin, the exact mechanism has not been fully elucidated. However, the mechanism for tetherin antagonism appears to be distinct from that for modulating CD4. Mutation of the serines 52 and 56 of Vpu abolish CD4 degradation, but only reduce EVR activity (50, 141, 149, 204). Some EVR activity remains even when much of
the Vpu cytoplasmic tail is deleted (201). In addition, many mutations in the membrane-spanning domain, such as Vpu<sub>RD</sub>, do not affect CD4 degradation yet completely abolish EVR activity (174, 200, 231). The critical residues in tetherin for recognition by Vpu appear to be in the membrane-spanning domain and not the cytoplasmic tail (77, 145, 183). Although β-TrCP is required for complete EVR activity, there is no consensus whether the degradation of tetherin is proteosomal or lysosomal mediated (50, 67, 149), or whether degradation is required at all. In some cases there can be some EVR activity in the absence of tetherin degradation (141, 151).

Here we demonstrate that Vpu is responsible for the incompatibility between HIV-1 and GaLV Env. Glycoproteins containing the cytoplasmic tail from GaLV Env are prevented from being incorporated into HIV-1 particles by Vpu, effectively reducing infectious particle production by 50- to 100-fold. The serines at positions 52 and 56 are required for this restriction, but the membrane-spanning domain is not. Although the mechanism for this restriction appears similar to CD4 degradation, there are apparent differences. Vpu does not prevent surface expression, and it does not prevent its incorporation into MLV particles. Therefore, the mechanism of restriction appears to involve a system that does not rely directly on protein degradation.

**MATERIALS AND METHODS**
Plasmid constructs.

The ecotropic Friend MLV Env and YFP-tagged MLV Env constructs were kindly provided by Walther Mothes (Yale University) (210). The codon-optimized late domain deficient HIV-1 Gag construct used in scanning electron microscopy images has been described previously (98). The MLV/GaLV Env expression vectors were constructed using oligonucleotide generated linkers to replace the ClaI to EcoRI (located 100 nt upstream and just downstream of the stop codon, respectively) fragment of the parent MLV Env expression constructs. The NL4-3 derived HIV-CMV-GFP was kindly provided by Vineet KewalRamani at NCI-Frederick (260). The HIV-1 packaging construct CMV ΔR8.2 containing all HIV-1 accessory genes was obtained from Addgene (plasmid #12263) (260). The HIV-1 construct CMV ΔR8.91 lacking Vpr, Vif, Vpu, and Nef, was previously described (260). The HIV-1 packaging construct lacking Vif and Vpr was engineered by replacing the Sall-Xbal fragment of CMV ΔR8.91 with the equivalent sequence from CMV ΔR8.2. The construct lacking Vpu was engineered by replacing the Sall-BamHI fragment of CMV ΔR8.2 with the equivalent sequence from CMV ΔR8.91. The construct lacking Nef was engineered by replacing the BamHI-Xbal fragment of CMV ΔR8.2 with the equivalent sequence from CMV ΔR8.91. The construct lacking Vpr and Nef was engineered by replacing the Sall-Xbal fragment of CMV ΔR8.2 with the equivalent sequence from CMV ΔR8.91. The construct lacking Vif, Vpr, and Nef was engineered by replacing the Sall-BamHI fragment of CMV ΔR8.91 with the equivalent sequence from CMV ΔR8.2. The construct lacking Vif, Vpr, and Vpu was engineered by
replacing the BamHI-XbaI fragment of CMV ΔR8.91 with the equivalent sequence from CMV ΔR8.2. The reporter construct pSIN18.cPPT.hEF1a.EGFP.WPRE was designed by Michal Gropp and Benjamin Reubinoff (74). Vpu was added to the HIV-CMV-GFP provirus by replacing the BamHI-Sall fragment of with the equivalent sequence from CMV ΔR8.2 to create HIV-CMV-GFP+Vpu. The HIV-CMV-GFP+VpuRD and CMV-GFP+VpuS2/S6 were engineered by PCR mutagenesis using HIV-CMV-GFP+Vpu as a parent. The mutations introduced have been previously described (202). The GFP-tagged MLV Env and MLV/GaLV Env were generated by replacing the YFP sequence with eGFP sequence from pEGFP-N1 (Clontech). The MLV packaging construct expressing GagPol was kindly provided by Walther Mothes. The MLV reporter construct expressing TdTomato was constructed by introducing the TdTomato sequence from pRSET-B TdTomato (208), kindly provided by Roger Tsien, into the MLV reporter vector pQCXIP (Clontech).

**Cells.**

The 293FT cell line was obtained from Invitrogen. The cell line expressing the ecotropic MLV Env receptor, 293T mCAT-1, was kindly provided by Walther Mothes. Both cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM non-essential amino acids, and 0.5 mg/ml G418.
**Scanning electron microscopy.**

The method for imaging the distribution of MLV Env on the cell surface has been described previously (98). Briefly, cells were plated onto coverslips coated with patterned gold and transfected with a late domain defective HIV-1 Gag expression vector and an YFP-tagged Env expression vector using FuGene 6 (Roche). Transfected cells were fixed with 4% paraformaldehyde approximately 20 hours post-transfection and the locations of individual transfected cells on the finder grids were recorded. Cells were labeled with primary mouse anti-GFP (Sigma) and 12nm gold conjugated anti-mouse secondary antibody (Jackson ImmunoResearch). Cells were then fixed with 2.5% glutaraldehyde, dehydrated in ethanol, critical point dried, coated with carbon, and imaged with a Hitach S-4700 FE-SEM.

**Infectivity assays.**

Infectivity assays using the CMV ΔR8.2 and its derivatives were performed by transfecting a 35mm dish of 293FT cells with 400 ng packaging construct, 400 ng pSIN18.cPPT.hEF1a.EGFP.WPRE, and 200 ng MLV Env or MLV/GaLV Env using FuGene 6. The media was replaced 24 hours post-transfection to remove residual transfection reagent. Supernatant was collected 48 hours post-transfection, filtered through 0.45 micron filters, and 1 ml of media was added to fresh 293T mCAT-1 cells in the presence of 10 µg/ml of polybrene. Cells were collected 48 hours later, fixed with 4% paraformaldehyde, and analyzed to determine the percent infected by flow cytometry.
at the University of Missouri Cell and Immunobiology Core facility. Infectivity assays using HIV-CMV-GFP and its derivatives were performed as above, except transfections were carried out with 500 ng of the proviral DNA and 500 ng of the Env expression construct. Infectivity assays that combined both HIV-1 and MLV proviruses were performed with 800 ng of the HIV-CMV-GFP or HIV-CMV-GFP+GFP, 40 ng of the MLV GagPol expression construct, 40 ng of pQCXIP-TdTomato, and 120 ng of MLV Env or MLV/GaLV Env.

**Western Blots.**

Transfections for westerns were performed as described for infectivity assays. Viral supernatants were filtered with a 0.45 micron filter, and pelleted through a 20% sucrose solution. Viral pellets were resuspended in 1X SDS-PAGE loading buffer and the equivalent of 0.5 ml of supernatant was analyzed by 10% discontinuous SDS-PAGE. Cells were washed, pelleted and resuspended in 1% SDS-PAGE loading buffer and approximately 4% of the cell suspension was analyzed in parallel with supernatant. Proteins were transferred using the iBlot™ dry blotting system (Invitrogen). Membranes were blocked with 5% non-fat dry milk, and probed with goat anti-MLV Env (kindly provided by Alan Rein, NCI-Frederick) diluted 1:10,000 or anti-HIV CA hybridoma media diluted 1:1000 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 p24 hybridoma (183-H12-5C) from Bruce Chesebro.
Blots were then probed with horseradish peroxidase conjugated anti-goat diluted 1:20,000 or anti-mouse diluted 1:10,000 (both from Sigma).

**Surface labeling.**

Transfections for protein surface labeling were performed as for infectivity assays except GFP-tagged Env expression vectors, which are more appropriate for flow cytometry, were used. The media was changed 24 hours post-transfection to remove residual transfection reagent. Media and cells were collected 48 hours post-transfection. Infectivity from the supernatant was assayed as described for infectivity assays. Cells were resuspended with PBS containing 1 mM EDTA, chilled to 4°C, blocked with 5% goat serum, and labeled with Alexa Fluor® 647 conjugated anti-GFP (Invitrogen) diluted 1:1000 in PBS containing 1% goat serum for 1 hr at 4°C. After washing, cells were fixed with 4% paraformaldehyde and analyzed by flow cytometry.

**Statistical analysis.**

Student’s t-test, ANOVA and Tukey-Kramer HSD analyses were performed with the statistical software JMP (SAS Institute Inc., Cary, NC)

**RESULTS**

The GaLV Env tail does not exclude glycoproteins from HIV-1 assembly sites.
Our lab developed a novel scanning electron microscopy (SEM) technique for visualizing recruitment or exclusion of surface proteins to HIV-1 assembly/budding sites. With this technique, we have demonstrated that certain foreign viral glycoproteins, including MLV Env, are efficiently recruited to HIV-1 assembly sites (Figure 3-2A,(98)). Because MLV Env glycoproteins containing the cytoplasmic tail from GaLV Env have been shown to be incompatible with HIV-1 in infectivity assays, we predicted that an Env protein with a GaLV tail would be excluded from HIV-1 assembly sites. To test this hypothesis, we replaced the last 30 amino acids of a YFP-tagged ecotropic MLV Env (210) with the equivalent sequence from GaLV Env (henceforth referred to as MLV/GaLV Env) and observed its distribution on the cell surface relative to HIV-1 assembly sites. Surprisingly, MLV/GaLV Env was not excluded from HIV-1 assembly sites and in some instances appeared recruited to them (Figure 3-2B).

**The GaLV Env tail is not inherently incompatible with HIV-1.**

In previous studies equivalent MLV/GaLV Env chimeras were found to be over 1000-times less infectious than the parent MLV Env when coupled with HIV-1 (37, 217). To determine if our MLV/GaLV Env chimera could form infectious particles with HIV-1, we performed a single-round infectivity assay combining MLV/GaLV Env with a minimal HIV-1 provirus lacking all non-essential accessory proteins, HIV-CMV-GFP (155). This provirus contains a CMV-driven green fluorescent protein (GFP), which serves as a reporter, in place of the Nef gene. This provirus was co-transfected with either wildtype
ecotropic MLV Env or the MLV/GaLV Env chimera into 293FT cells. Although the YFP-tagged Env is capable of forming infectious particles, a non-YFP tagged Env was used for this infectivity experiment. Infectious particles were collected two days post transfection, filtered, and used to transduce 293T mCAT-1 cells (a stable cell line expressing the mCAT-1 receptor). Infectivity was determined by counting the number of fluorescent cells by flow cytometry. The infectivity of MLV/GaLV Env with HIV-1 was reduced compared to MLV Env, but was considerably higher than had been previously observed (37, 217).

**HIV-1 incompatibility with GaLV is modulated by Vpu.**

Because these findings with MLV/GaLV Env conflict with the results of previous studies, we tested our MLV/GaLV chimera with the HIV-1 construct that was found to be incompatible with MLV/GaLV Env previously, ΔR8.2 (Fig. 3A) (217, 260). Two obvious differences exist between our minimal proviral construct and ΔR8.2: (1) ΔR8.2 contains a CMV promoter in place of the 5’ LTR and packaging sequence; and (2) ΔR8.2 contains the accessory genes Vif, Vpr, Vpu, and Nef. Because ΔR8.2 does not produce a packageable genomic RNA, it was co-transfected with a reporter plasmid that contained the viral packaging sequence and a GFP reporter gene, pSIN18.cPPT.hEF1a.EGFP.WPRE (74). To determine if the accessory genes influenced the incompatibility between HIV-1 and MLV/GaLV Env, we also tested a derivative of ΔR8.2 that lacks the four accessory genes, ΔR8.91 (260). When coupled with wildtype MLV Env, both constructs produced
high viral titers. However, when coupled with MLV/GaLV Env, only the construct lacking accessory genes was able to produce a significant viral titer (Figure 3B-C). This observation indicated one or more of the accessory genes in ΔR8.2 prevent HIV-1 from forming infectious particles with MLV/GaLV Env. To determine which of the accessory genes contributed to the restriction, we made a series of constructs lacking one or more accessory genes and tested each of these derivatives for infectivity (Figure 3B-C). All of the ΔR8.2 derivatives were able to efficiently produce infectious particles when combined with MLV Env. However, the infectivity of all four constructs that contained Vpu dropped to background levels when combined with MLV/GaLV Env. The four constructs that contained Nef also displayed a reduction in infectivity when coupled with MLV/GaLV Env, although the restriction was not as striking as with Vpu. Vif and Vpr did not appear to contribute to the MLV/GaLV Env restriction. Because Vpu appeared to be the major contributor to the incompatibility between HIV-1 and MLV/GaLV Env, this protein was selected for further study.

To confirm that Vpu mediated the restriction, we re-introduced Vpu back into the HIV-CMV-GFP provirus background (Figure 3-4). The proviruses, containing or lacking Vpu, were co-transfected with MLV Env or MLV/GaLV Env into 293FT cells. The supernatant was collected after 48 hours, filtered, transferred to 293T mCAT-1 cells, and infectivity was measured by flow cytometry (Figure 3-4). Because 293FT cells do not constitutively express tetherin, Vpu should not enhance viral release in these cells through the antagonism of tetherin. The addition of Vpu slightly increased the
infectivity of HIV-1 with MLV Env, but the difference was not significant (two-tailed Student’s t-test, n=7, p=0.76). The addition of Vpu significantly diminished the infectivity of HIV-1 with MLV/GaLV Env (two-tailed Student’s t-test, n=7, p=0.02) with an average drop in infectivity of more than 60-fold.

**Vpu prevents MLV/GaLV Env from being incorporated into HIV-1 particles.**

To analyze the nature of the Vpu mediated restriction of MLV/GaLV Env we introduced two Vpu mutants into HIV-CMV-GFP (Figure 3-5). The first, Vpu_{52/56}, contains two alanine substitutions at positions 52 and 56 of the cytoplasmic domain. The second mutant, Vpu_{RD}, contains a scrambled Vpu membrane-spanning domain. Each provirus was transfected along with MLV Env or MLV/GaLV Env and the expression, incorporation, and infectivity was determined for each. HIV-1 protein production and particle release were not affected by the expression of Vpu or MLV/GaLV Env in this context (Figure 3-5A). In the presence of wildtype Vpu, a moderate reduction in the amount of MLV/GaLV Env in expressing cells was observed (Figure 3-5A, cells, compare lanes 9 and 10). This reduction varied between experiments where on two occasions there was no reduction, but on four occasions there was a 2-4 fold reduction (as shown in Figure 3-5A). In contrast, the Vpu mediated reduction of MLV/GaLV Env in virions was consistent and drastic. In six out of six experiments, Vpu caused an acute reduction in the amount of MLV/GaLV Env in virions (Figure 3-5A, virions, compare lanes 9 and 10) and in half of these experiments the MLV/GaLV Env in virions was undetectable. This
lack of MLV/GaLV Env in virions correlated with the loss in infectivity (Figure 3-5A, bottom). The HIV-CMV-GFP provirus containing VpuRD incorporated MLV Env but not MLV/GaLV Env into virions while the provirus containing Vpu52/56 incorporated both MLV and MLV/GaLV Env. The infectivity experiment was repeated six times and the average infectivity and standard deviations are reported (Figure 3-5B). As was suggested by the immunoblot analysis, Vpu type had a significant effect on the ability of MLV/GaLV Env to form infectious particles with HIV-1 (ANOVA, F3,20=19.88, p<0.0001). Wildtype Vpu or VpuRD significantly reduced the infectivity of MLV/GaLV Env pseudotyped particles compared to no Vpu or Vpu52/56 (Figure 3-5B). There was also a variation in infectivity among Vpu constructs pseudotyped with MLV Env but the difference was very slight (ANOVA, F3,20=5.04, p=0.0092). Because Vpu52/56 fails to recruit the E3 ubiquitin ligase complex during CD4 downmodulation (142), these data are consistent with Vpu targeting MLV/GaLV Env for degradation in a manner synonymous with CD4 degradation. However, the reduction in MLV/GaLV Env expression levels appeared modest compared to the loss in incorporation and infectivity.

**Vpu does not prevent the surface expression of MLV/GaLV Env.**

Although we did not observe a severe decrease in MLV/GaLV Env expression levels, it is plausible that Vpu prevents MLV/GaLV Env from reaching the plasma membrane by triggering its degradation at a post-translational stage or by interfering with its trafficking. To test whether Vpu prevents MLV/GaLV Env from reaching the
plasma membrane, we transfected HIV-CMV-GFP (with or without Vpu) along with MLV Env or MLV/GaLV Env tagged with GFP. The transfected cells were stained live with an Alexa-Fluor 647-conjugated antibody against GFP at 4°C and analyzed by flow cytometry to assay for Env expression on the cell surface (Figure 3-6A and B). Scatter plots denote total Env surface staining (Y-axis) to total transfection level (X-axis). Although HIV-CMV-GFP and Env-GFP both contribute to the overall mean cellular GFP fluorescent intensity (X-axis), the signal from Env-GFP was negligible compared to the signal from HIV-CMV-GFP. To ensure only surface GFP was stained, we also analyzed cells transfected with HIV-CMV-GFP alone (first two columns). A portion of cells exhibited non-specific staining, but the staining occurred equally in untransfected cells (not shown) and did not correlate with increased cytoplasmic GFP expression. Both MLV and MLV/GaLV Env displayed robust surface expression (Y-axis), which directly correlated with overall cellular expression levels (X-axis). The presence of Vpu did not considerably alter the surface expression of MLV or MLV/GaLV Env. The supernatant from the same transfection was transferred onto fresh 293T mCAT-1 cells to assay for infectivity. The supernatant from the cells expressing Vpu and MLV/GaLV Env contained approximately 95-fold fewer infectious particles than cells lacking Vpu. Although in some instances we have witnessed a minor (2-4 fold) reduction in relative MLV/GaLV Env surface staining in the presence of Vpu, we chose to show this experiment because it demonstrates that the restriction in infectivity is not dependent on significant MLV/GaLV Env surface downmodulation.
**HIV-1 Vpu does not prevent MLV/GaLV Env from being incorporated into MLV particles.**

It was shown previously that the co-transfection of ΔR8.2 (containing the HIV-1 accessory genes) and an MLV viral construct along with GaLV Env resulted in negligible infectious HIV-1 production, but normal levels of infectious MLV particle production (37). These data suggested that the incompatibility between HIV-1 and MLV/GaLV Env was not the result of global downregulation of the GaLV Env protein. To confirm this finding we configured a fluorescence based dual infectivity assay using HIV-CMV-GFP, which produces fluorescent green infected cells, and an MLV packaging construct and genome containing TdTomato, which produces fluorescent red infected cells. The plasmids for producing these single round infectious particles were co-transfected in 293FT cells with MLV or MLV/GaLV Env and in the presence or absence of Vpu (Fig. 7). Because the MLV structural components are more efficient at producing infectious particles with MLV/GaLV Env than HIV-1, the ratio of HIV-1 components to MLV components was adjusted such that a roughly equivalent amount of HIV-1 and MLV infections were produced in the absence of Vpu (Figure 3-7B, top left panel). When the parallel transfection was performed with a Vpu-containing HIV-1 provirus, the output of infectious HIV-1 particles was dramatically reduced but the output of infectious MLV particles was essentially unchanged (Figure 3-7, top right). As a control, the same ratio of packaging components was transfected into cells along with MLV Env. HIV-1 was
much more efficient at forming infectious particles with MLV Env than with MLV/GaLV Env, resulting in a much higher ratio of HIV-1 to MLV infections. However, the inclusion of Vpu did not alter the output of infectious HIV-1 or MLV particles with MLV Env.

**DISCUSSION**

There have been many examples of virus/glycoprotein pairs that do not efficiently produce infectious pseudotyped virus together. For example, HIV-1 does not pseudotype efficiently with RSV Env, influenza hemagglutinin, or lymphocytic choriomeningitis glycoprotein (37, 120). However, most of these virus/glycoprotein pairs do yield some infectious pseudotyped particles, albeit at an inefficient level. The incompatibility between HIV-1 and GaLV Env (specifically Env proteins containing the GaLV cytoplasmic domain) is unique because it is so absolute, yielding essentially no infectious particles. It is now clear that this extreme incompatibility is the result of at least two factors. First, HIV-1 shows a general incompatibility with the GaLV Env cytoplasmic tail that is not related to Vpu (Figure 3-4). This incompatibility has not been addressed here but is likely the result of poor fusogenicity. Second, MLV/GaLV Env incorporation into HIV-1 particles is prevented by the HIV-1 accessory protein Vpu (and possibly Nef) resulting in a further 50- to 100-fold decrease in infectivity.
The mechanism for how Vpu modulates MLV/GaLV Env is only partially elucidated here. In some ways the mechanism seems similar to the downmodulation of CD4 by Vpu. Like with CD4, the sequence in Env responsible for the Vpu modulation exists in the C-terminal cytoplasmic tail. Also like CD4, the conserved 52/56 serine residues in the cytoplasmic tail of Vpu are required for the modulation, but the precise membrane spanning sequence is dispensable. However, while CD4 is prevented from accumulating on the cell surface and is degraded by Vpu, neither appears to occur to a significant extent with MLV/GaLV Env. The reduction in MLV/GaLV Env expression level is more modest than previously reported (37), but it should be noted that the current studies were performed with a different Env background, cells were not treated with cyclohexamid to prevent new protein synthesis, and Nef, which likely targets MLV/GaLV Env independently, was not included in the majority of our experiments.

Although the ultimate downmodulation of infectious particle production by Vpu appears to be due to lack of Env incorporation into viral particles, it is not clear how this is accomplished. It is particularly intriguing that Vpu does not prevent MLV/GaLV Env from producing infectious particles with MLV packaging proteins. There are two explanations for this observation. Either Vpu alters MLV/GaLV Env trafficking in a way that affects the two types of retroviruses differently, or Vpu alters MLV/GaLV Env in a way that would affect both viruses, but MLV structural components are able to overcome this restriction. For example, it is plausible that Vpu binds the cytoplasmic tail
of MLV/GaLV Env and sequesters it away from assembly sites, but MLV Gag binds with
greater affinity to the cytoplasmic tail to subvert this sequestration.
The observation that HIV-1 Vpu causes an incompatibility with an unrelated viral
glycoprotein is surprising. This incompatibility seems to be specific and robust, but it is
not obvious how or why HIV-1 would acquire the ability to target a specific foreign viral
glycoprotein. HIV-1 and GaLV do not infect the same host so it is not likely that
downmodulation of GaLV Env provides a selective advantage for HIV-1. The targeting of
GaLV Env could be the result of a coincidental similarity between the GaLV Env
cytoplasmic tail and a natural target of Vpu. GaLV Env has certain similarities with CD4;
both are type-1 surface glycoproteins that are targeted by Vpu through elements in
their cytoplasmic tails. However, the cytoplasmic tails of CD4 and GaLV Env display no
obvious sequence similarity (Figure 3-1), though it is plausible that they contain a
conserved structural element not recognized in the primary sequence. One could
postulate that GaLV Env mimics some host cell protein that is a natural target of Vpu,
but there is currently no experimental evidence to support this speculation.

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the University of Missouri Electron Microscopy Core facility. This research was supported by U.S. Public Health Service grant AI73098 and the Arnold and Mabel Beckman Foundation Young Investigator Program.

**Figure 3-1. Schematic of MLV Env protein.**

Sequences are the C-terminal cytoplasmic tails of MLV Env, GaLV Env, and human CD4. GaLV sequences in boldface are residues that have been shown to modulate the HIV-1 incompatibility (31). Underlined sequences in CD4 are amino acids required for Vpu-mediated downmodulation (34, 116). Arrows denote the location of MLV/GaLV tail substitution. SU, surface domain; TM, transmembrane domain.
Figure 3-1. Schematic of MLV Env protein.
Figure 3-2. Distribution of MLV Env relative to HIV-1 assembly sites.

293T mCAT-1 cells were cotransfected with a plasmid expressing late domain-defective HIV-1 Gag and a plasmid expressing YFP-tagged MLV Env (A) or YFP-tagged MLV/GaLV Env (B). Env was labeled with 12-nm gold and imaged by SEM. Left, secondary electron images of HIV-1 assembly sites. Right, backscatter electron images of gold-labeled Env. Scale bars, 200 nm.
Figure 3-2. Distribution of MLV Env relative to HIV-1 assembly sites.
Figure 3-3. HIV-1 accessory genes modulate MLV/GaLV Env restriction.

(A) Schematic of HIV-1 packaging constructs ΔR8.2. (B) ΔR8.2 or its derivatives containing the accessory genes shown were cotransfected with reporter vector pSIN18.cPPT.hEF1a.EGFP.WPRE and either MLV Env or MLV/GaLV Env. Viral supernatant was transferred from transfected 293FT cells to 293T mCAT-1 cells, and infectivity was determined by flow cytometry. (C) Output of the same experiment with the infectivity of each construct expressed as the ratio of infections with MLV/GaLV Env to infections with MLV Env.
Figure 3-3. HIV-1 accessory genes modulate MLV/GaLV Env restriction.

A.

ΔR8.2

ΔEnv

B.

- Infectious Units/ml

Vif, Vpr

Vpu

Nef

C.

- Infectivity ratio of MLV/GaLV Env to MLV Env

Vif, Vpr

Vpu

Nef
**Figure 3-4. Vpu modulates HIV-1 infectivity with MLV/GaLV Env.**

Infectivity of MLV Env, MLV/GaLV Env, or an empty DNA vector (control) pseudotyped with HIV-1 was determined in the absence (▪) or presence (□) of Vpu. Infectivity was measured as infectious units (I.U.) per ml of viral supernatant transferred from transfected 293FT cells to 293T mCAT-1 cells 48 h posttransfection. The averages and standard deviations of infectious units per milliliter of seven independent experiments with the same combinations are shown. Significant differences (*, P < 0.05) and nonsignificant differences (NS) were determined by two-tailed Student's t test for each envelope type in the presence or absence of Vpu.
Figure 3-4. Vpu modulates HIV-1 infectivity with MLV/GaLV Env.
Figure 3-5. Vpu prevents MLV/GaLV Env from being incorporated into HIV-1 particles.

(A) 293FT cells were transfected with HIV-CMV-GFP containing no Vpu (Vpu−), Vpu wt, VpuRD, or VpuS2/S6, and MLV Env or MLV/GaLV Env. For the upper panel, Western blot analysis performed on the transfected cells and pelleted viral supernatants. The lower panel shows the infectivity output from the same experiment. Infectious units per ml were normalized to p24 levels. (B) The averages and standard deviations of infectious units per milliliter of six independent experiments with the same combinations are shown. Significantly different means for each Env treatment are indicated by unique letters (Tukey-Kramer HSD, $P < 0.05$).
Figure 3-5. Vpu prevents MLV/GaLV Env from being incorporated into HIV-1 particles.

A. 

<table>
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<th>p55 Gag</th>
<th>Relative Env/p55 in cells</th>
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<td>Vpu wt</td>
<td>Vpu wt</td>
</tr>
<tr>
<td>HIV+MLV Env</td>
<td>Vpu</td>
<td>Vpu wt</td>
<td>Vpu wt</td>
</tr>
<tr>
<td>HIV+MLV/GaLV Env</td>
<td>Vpu</td>
<td>Vpu wt</td>
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</table>

B. 

<table>
<thead>
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<th>Env</th>
<th>p24 CA</th>
<th>I.U. per ml</th>
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<tr>
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<td>Vpu wt</td>
<td>Vpu wt</td>
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<tr>
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<td>Vpu</td>
<td>Vpu wt</td>
<td>Vpu wt</td>
</tr>
<tr>
<td>HIV+MLV/GaLV Env</td>
<td>Vpu</td>
<td>Vpu wt</td>
<td>Vpu wt</td>
</tr>
</tbody>
</table>
Figure 3-6. Vpu does not prevent MLV/GaLV Env surface expression.

(A) Schematic of surface labeling experiment. 293FT cells were transfected HIV-CMV-GFP (+/- Vpu) in the presence or absence of MLV Env-GFP or MLV/GaLV Env-GFP. At 48 h posttransfection, cells were collected and stained live for surface GFP expression with Alexa Fluor 647-conjugated anti-GFP antibody, and the supernatant was transferred to 293T mCAT-1 cells and assayed for infectivity. (B) Surface expression and infectivity of Env proteins. The average surface GFP intensity/total GFP intensity of transfected cells is reported in the upper right-hand corner of surface expression scatter plots. Infectivity scatter plots show infections on the x axis; the y axis (FL2) is not relevant for this experiment, but is used to maintain visual consistency. Infectivity is shown in each plot as the percentage of the 293T mCAT-1 cells infected with HIV-CMV-GFP.
Figure 3-6. Vpu does not prevent MLV/GaLV Env surface expression.

A. HIV-CMV-GFP (+/− Vpu) + MLV Env-GFP (wt or GaLV tail) supernatant

B. 

<table>
<thead>
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<th>Surface Expression</th>
<th>Infectivity</th>
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<td>0.03</td>
</tr>
<tr>
<td>HIV-CMV-GFP+Vpu</td>
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Figure 3-7. Vpu does not prevent the production of infectious MLV particles.

(A) Schematic of dual infection assay. 293FT cells were transfected with HIV-1 and MLV assembly components, along with MLV or MLV/GaLV Env. At 48 h posttransfection the supernatant was transferred to 293T mCAT-1 cells. The ratio of HIV-1 to MLV was adjusted so that similar HIV-1 and MLV infectious particles were produced with MLV/GaLV Env and so that same ratio was used in each of the four transfections. (B) Flow cytometry output of 293T mCAT-1 infections. MLV infections display red fluorescence (y axis), and HIV-1 infections display green fluorescence (x axis). Infectivity is shown in each plot as percentage of the 293T mCAT-1 cells infected, excluding double-positive cells.
Figure 3-7. Vpu does not prevent the production of infectious MLV particles.
IV. Vpu appropriates overlapping features for downmodulation of distinct targets, tetherin and gibbon ape leukemia virus envelope

Note: The research presented in chapter IV was conducted equally between T.M. Lucas and S.K. Janaka. The manuscript and figures were written and prepared by T.M. Lucas and revised by all authors.

ABSTRACT

The HIV-1 accessory gene Vpu modulates many host proteins such as CD4 and tetherin during the course of infection, promoting viral fitness. Here, we provide the findings for a systematic and comprehensive mutagenic scan of Vpu on two distinct targets, tetherin and a CD4 analogue, gibbon ape leukemia virus envelope (GaLV Env). Using parallel assays, we observed considerable overlap in the Vpu sequences required to modulate these two disparate proteins.

INTRODUCTION

Vpu is an 81-86 amino acid, type-1 transmembrane protein found in HIV-1 and a few closely related strains of SIV. Vpu exhibits a wide range of functions, including
modulation of host proteins CD4, tetherin, IkB, MHC-II, NTB-A, and the gammaretroviral gibbon ape leukemia virus (GaLV) envelope (Env) (17, 19, 39, 88, 92, 129, 207, 249). Of these functions, Vpu’s ability to degrade cellular CD4 and tetherin (BST-2, CD137) have been the best described (20, 75, 162, 187). CD4 is the primary receptor for HIV-1. Vpu targets newly synthesized CD4 in the rough endoplasmic reticulum (RER) through interactions between the cytoplasmic tails of Vpu and CD4, recruiting the Skp1-Cullin-β- TrCP E3-ubiquitin ligase complex, resulting in the subsequent proteasomal degradation of CD4 (19, 34, 64, 136, 142, 200, 248). The cytoplasmic tail (CT) of Vpu is unambiguously required for CD4 modulation, but it is disputed whether the membrane spanning domain (MSD) also plays a specific role (84, 135, 188, 201, 231).

Tetherin is an interferon inducible, type-II transmembrane anti-viral protein with a C-terminal GPI-anchor. Tetherin, as its name suggests, “tethers” many budding, enveloped viruses or viral like particles to the plasma membrane (PM), including retroviruses, Ebola, Kaposi sarcoma-associated herpes virus (KSHV) and influenza virus like particles (160, 161, 173, 242). Vpu-mediated antagonism of tetherin requires an interaction between the MSDs of Vpu and tetherin, but as of yet, there is no consensus on the precise mechanism by which Vpu modulates tetherin activity. Vpu has been reported to reduce tetherin surface expression by altering the rate of recycled and/or restricting newly synthesized tetherin from reaching the PM (54, 55, 79, 150, 196, 214). However, it has been reported that Vpu can modulate tetherin activity in the absence of surface downmodulation and intracellular depletion (151). Some studies suggest that
tetherin can be degraded through β-TrCP mediated targeting to lysosomes or the proteasome (51, 141, 150).

Although the mechanisms for CD4 and tetherin antagonism are believed to be distinct, evidence suggests that Vpu contains some shared features required for modulation of both proteins. For instance, complete proscription of either target requires two critical serines housed in the Vpu cytoplasmic tail, which is also required for interaction with β-TrCP and degradation (141, 142, 202). Vpu mutants lacking these serine residues retain some activity against tetherin but not CD4 (11, 55). Direct parallels between Vpu modulation of tetherin and CD4 are difficult to draw due to differences in the assays employed. Studies investigating tetherin antagonism have relied heavily on detection of viral particle release, through protein release or infectious virus production, although some studies have also measured tetherin modulation directly. Reports on CD4 down-modulation typically rely on biochemical assays measuring total protein or surface expression. Additionally, Vpu studies have used different cell types, multiple methods of introducing CD4 or tetherin targets (endogenous or exogenous), and different methods of producing Vpu (native or codon-optimized, contained in the provirus or introduced in trans, etc). These distinct methods of analysis have likely contributed to apparent differences observed by different groups studying Vpu.

We and others found that Vpu prevents GaLV Env incorporation into HIV-1 particles, likely through a shared structural recognition motif in the Env cytoplasmic tail
that mimics the critical motif found in the cytoplasmic tail of CD4 (39, 92, 129). Based on these findings, we currently believe Vpu recognizes GaLV Env as a CD4 analogue. Modulation of GaLV Env by Vpu is sensitive and well suited for a comparative study with the modulation of tetherin by Vpu. Modulation of both targets can be studied in the same cell type using Vpu encoded in the provirus with infectivity as the output for both.

**METHODOLOGY AND RESULTS**

To determine specific regions of Vpu responsible for antagonism of tetherin and GaLV Env, we generated a library of Vpu mutants and introduced them into a reduced HIV-1 M group (HXB 2) proviral construct containing an E2Crimson reporter gene (Figure 4-1A). For tetherin modulation assays, each provirus was transfected with a VSV-G expression plasmid alone, or in combination with an HA-tagged tetherin expression construct, kindly provided by P. Bieniasz (175) (Figure 4-1B, left). In this case, Vpu activity was measured by comparing the infectivity in the presence and absence of tetherin. For assaying GaLV Env modulation, an internally controlled system was used where each mutant was transfected with a mixture of plasmids expressing the previously described Vpu-sensitive chimeric MLV Env containing the GaLV Env cytoplasmic tail, herein referred to simply as GaLV Env, and a Vpu-insensitive Rous sarcoma virus Env lacking the cytoplasmic tail (RSVΔCT) (Figure 4-1B, right). Virus was
collected and used to infect 293T mCAT-1, expressing the MLV Env receptor (293mCAT-1) and 293T TVA, which expresses the RSV receptor (293TVA). Vpu activity was measured by comparing the ratio of RSV Env pseudotyped infectious virus to MLV Env pseudotyped infectious virus. For both assays, infections were quantified by flow cytometry, and activity was expressed by normalizing to a provirus with wildtype Vpu (Vpu wt) (100% activity) and a Vpu-deficient provirus (ΔVpu) (0%). It should be noted that the raw output is inverted for the two assays: with tetherin, Vpu enhances infectivity, but with GaLV Env, Vpu inhibits infectivity.

Previous studies have demonstrated that Vpu’s transmembrane domain (TMD) and cytoplasmic tail (CT) promote tetherin antagonism while only the Vpu cytoplasmic tail CT has been identified for GaLV Env restriction (39, 55, 129, 201, 234). VpuRD, a transmembrane “scrambled” mutant, is known to fully restrict CD4, but is ineffectual against tetherin (201). However, there have been conflicting reports about the importance of the TMD in CD4 restriction, with some studies suggesting a role of a conserved tryptophan (W22) in the C-terminal region (135, 231). We therefore sought to further investigate the role of Vpu’s TMD by employing two previously described TMD mutants: VpuRD and W22L (135, 200, 201). We introduced both of these mutants into our proviral system and tested their activity against tetherin and GaLV Env (Figure 4-2). As previously reported, both VpuRD and W22 mutants had decreased activity against tetherin (215, 234, 235). However, both mutants exhibited wildtype activity against GaLV Env. In addition, we also included serine to alanine mutations at positions
53, 57. These serines are highly conserved and have been previously reported to be essential in tetherin and CD4 downmodulation (196). As expected, the serines are important in downmodulation of both targets.

The subcellular location where CD4 and tetherin are targeted appears to be distinct. While action against CD4 has been reported to be exclusively in the RER, action against tetherin is generally believed to occur in a post-ER compartment (56, 136, 188, 236). Previous studies demonstrated that Vpu retention in the RER by a putative retrieval motif prevents downmodulation of tetherin at the PM (235, 236). We found that placement of the KKDQ ER-retention motif on the C-terminus of Vpu, exactly as previously described (236), reduced its ability to restrict either target, though the effect on tetherin restriction was more severe. These data are consistent with direct or indirect interactions between Vpu and both target proteins in a post-ER region.

Next, we generated truncation mutations in Vpu to determine the minimal sequence required for modulation of the two targets. For both tetherin and GaLV Env, truncation beyond 13 C-terminal amino acids (Δ13) resulted in a decrease in Vpu function, although for tetherin this decrease was progressive (Figure 4-2). To identify critical regions upstream of Δ13, we mutated two residues at a time to alanine and assayed for activity. For both targets, Vpu was most sensitive to mutations within the conserved hinge region while upstream regions were less sensitive (Figure 4-3A). Unlike reported findings for HxBH10 Vpu R30A,K31A (56), located within the YRKIL trafficking motif, we did not observe a decrease in infectivity for tetherin in our HXB2 Vpu system.
Although both are subtype B, variances in amino acid sequence between both Vpus may explain differences. We then sought to identify specific amino acids required in the CT by scanning individual point mutants through substitution of alanine for individual amino acids, with the exception of alanine which was substituted with serine. Interestingly, almost all amino acids within the Vpu-hinge region, not solely the serines 53, 57, were sensitive to disruption (Figure 4-3B). These results demonstrate Vpu’s requirement for conservation of the hinge region for antagonism of two distinct protein targets. Because alanine substitution should not affect physical accessibility of the hinge region by proteins such as β-TrCP, we speculate that modification of the conserved features, such as the acidic amino acids, disrupts recognition of Vpu by cellular factors or Vpu’s ability to interact with targets.

DISCUSSION

Here we have identified shared critical features in Vpu required for restriction of two distinct proteins, tetherin and the glycoprotein GaLV Env. With the exception of the TMD region, Vpu requires similar features to counteract both targets. Our Vpu screen raises the question: why are similar features in Vpu required for modulation of two disparate target proteins? We propose that Vpu utilizes multiple regions perhaps for three somewhat overlapping steps in both restriction pathways: i) retention through
interaction, ii) modification and redirection, and iii) degradation. In the case of tetherin, interaction occurs between the TMDs and for CD4 interaction occurs in the CT and is absolutely required for antagonism (54, 90, 184, 201, 235). The importance of TMD interactions is highly evident in the evolution of species and subtype specificity of Vpu antagonism of tetherin (106, 193, 236). In the second step, we postulate that Vpu’s CT-hinge region is required for both tetherin and GaLV Env modification and redirection. The hinge region likely represents a collective β-TrCP recognition motif, with serines housed within a conserved acidic stretch of amino acids. How Vpu modifies and subsequently redirects targets is not yet fully understood, although emerging data suggests a role of ubiquitination of both tetherin and CD4. Tetherin appears to be monoubiquitinated while CD4 is polyubiquitinated, hallmarks of redirection for lysosomal and proteasomal degradation, respectively (136, 173, 232). In the final step of restriction, degradation of targets may occur. CD4 is directed for degradation through ERAD-proteasomal targeting (19, 34, 142, 200, 248, 249). However, the role of degradation for tetherin is unclear, with some data suggesting lysosomal (51, 150) or proteasomal degradation (141). Interestingly, although tetherin restriction can occur independently of the degradation, possibly through retention-based interactions, recent work demonstrates a significant role for lysosomal degradation of newly synthesized tetherin (54). We suspect that degradation may represent a late stage in restriction and may not be required until available Vpu becomes saturated.
Through our systematic alanine mutagenic library of the Vpu cytoplasmic tail, we identified specific amino acids contributing to the antagonism of two distinct targets, tetherin and a CD4 analogue, GaLV Env. Interestingly, we demonstrated a role for multiple amino acids within the CT hinge region and the importance of Vpu localization in restriction. Altogether our findings, along with other mutagenic Vpu studies, suggest that Vpu has unique regions mediating interaction with targets, while it uses conserved features within the CT to ultimately redirect and potentially degrade target proteins.

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Figure 4-1. Schematics of HIV-1 proviral construct and experimental assay.

(A) HIV-1 HXB2 proviral construct with E2Crimson reporter showing enlargement of Vpu schematic outlining critical features in Vpu. Dotted outline predicted α-helices (84), bold script indicates the hinge region and underlined script highlights phosphorylated serines at positions 53,57. (B) For tetherin assays, 293FT cells (Invitrogen) were transfected with the following expression constructs: provirus (425 ng) and VSV-G (25 ng) with or without 12.5 ng of tetherin (kindly provided by P. Bieniasz) in a total of 500 ng. For GaLV Env assays, cells received 500 ng provirus, 25 ng of RSV Env ΔCT, and 475 ng MLV/GaLV Env (GaLV Env) (92, 129). At 48hr post-transfection, media was frozen overnight and used to infect target cells. Infected cells were fixed and analyzed by FACS on an Accuri flow cytometer. Flow plots illustrate typical data output for positive controls.
Figure 4-1. Schematics of HIV-1 proviral construct and experimental assay.

A. HIV-1 HXB2:

![Diagram of HIV-1 proviral construct]

Vpu:

- α-helix 1
- α-helix 2
- Hinge
- α-helix 3

Transmembrane region (TMD)  Cytoplasmic tail domain (CT)

B.

1. Transfect
   - Provirus
   - HIV-1 E2Crimson reporter (Vpu+ΔVpu)
   - Glycoprotein: VSV-G
   - + No tetherin
   - + Tetherin

2. Collect virus

3. Infect target cells
   - 293FT
   - 293CAT-1

4. Assay infectivity
   - 40%
   - 33%
   - 0.2%
   - 27%
   - 38%
   - 0.9%
   - 4.8%
   - 25%

Infectivity: E2Crimson
Figure 4-2. Features required for Vpu-mediated antagonism of targets.

Tetherin (dark bars) and GaLV Env (light bars). (Top) Location of VpuRD, W22L (bold), critical serines 53,57 (underline) and truncations (arrows) are noted in the Vpu schematic. (Bottom) Relative Vpu activity is shown as mean averages (n=3-4, ±SE) calculated by normalizing infectious units per ml for each mutant Vpu relative to Vpu wildtype (Vpu wt) (100%) and no Vpu (ΔVpu) (0%).
Figure 4-2. Features required for Vpu-mediated antagonism of targets.
Figure 4-3. Alanine mutagenic scan of Vpu reveals antagonistic regions for downmodulation of tetherin and GaLV Env.

Tetherin (dark bars) and GaLV Env (light bars). Amino acids were mutated to alanine, with the exception of alanine which was mutated to serine (A) A double alanine-mutagenic scan was performed on the cytoplasmic tail region of Vpu (double mutations, underlined). (B) An individual amino acid alanine scan was analyzed for amino acids identified in the double-alanine scan (bold, underlined) and relative Vpu activity was measured. Relative Vpu activity is shown are mean averages (n=3-4, ±SE) calculated by normalizing infectious units per ml for each mutant Vpu relative to Vpu wildtype (Vpu wt) (100%) and no Vpu (ΔVpu) (0%).
Figure 4-3. Alanine mutagenic scan of Vpu reveals antagonistic regions for downmodulation of tetherin and GaLV Env.

A. Vpu: cytoplasmic tail

B. Vpu: cytoplasmic tail
V. Vpu restricts GaLV Env and CD4 incorporation into viral particles through disruption of colocalization at the cellular surface

ABSTRACT

HIV-1 relies on a suite of viral accessory genes to help promote virus assembly and to restrict host defenses. One of these genes, Vpu counteracts the innate immune factor tetherin (CD317 or BST-2) and downmodulates the T-cell signaling receptor CD4. Vpu is thought to restrict CD4 from incorporation into viral particles through direct interactions in the endoplasmic reticulum that redirect CD4 for degradation through ubiquitination, dislocation, and targeting to the proteasome.

We recently described a new behavior for Vpu, an ability to recognize and restrict a viral foreign glycoprotein, gibbon ape leukemia virus (GaLV) envelope (Env), from incorporation into the assembling virus. Further investigation by our lab suggests that Vpu mistakenly recognizes GaLV Env as a CD4 analogue. Because of the similarities between GaLV Env and CD4, we extended our GaLV Env model to carefully investigate Vpu-mediated restriction of CD4 through a series of unique and highly sensitive assays that could not historically be applied to CD4 biochemical studies. Our findings shed new light on how Vpu prohibits CD4, suggesting that degradation is not required, and that Vpu causes mislocalization of target proteins. We postulate that Vpu targets immature
forms of Env and CD4 and prevents their maturation, a step required for appropriate targeting of either protein to viral assembly sites.

INTRODUCTION

HIV-1 encodes a series of accessory genes that enhance viral fitness by promoting assembly and counteracting anti-viral host factors. Of these genes, Vpu produces a 16-kDa, type-1 transmembrane protein that targets multiple cellular proteins, including the HIV receptor CD4 and the innate anti-viral protein tetherin (CD317 or BST-20). Surface downmodulation of either target enhances viral infectivity by promoting particle release and preventing interactions between CD4 and HIV-1 Env (118). Downmodulation of CD4 appears so important that HIV-1 relies on Vpu, Nef and potentially Env to restrict the receptor, although only Nef and Vpu appear to actively degrade CD4 (33, 246). Restriction is thought to occur through two related steps: ER retention followed by ERAD mediated dislocation and degradation (136). Vpu has multiple regions within its topology contributing to CD4 and tetherin targeting. Vpu binds CD4 through interactions in the cytoplasmic tail (CT) by recognition of a LSEKKT motif within CD4 (248). Within its own cytoplasmic tail, Vpu houses two critical serines sandwiched within an acidic amino acid hinge region. This motif resembles what is
known as a destruction motif, which are commonly recognized by β-TrCP, a component of an E3 ubiquitin ligase complex (63). During CD4 restriction, casein kinase II (CK-II) is recruited to the hinge region and phosphorylates both serines (202). Subsequently, the Skp1-Cullin1-F-boxβ-TrCP E3 ubiquitin ligase machinery is recruited to the Vpu-CD4 complex where CD4 is subsequently polyubiquitinated and targeted for degradation via the proteasome (142, 200).

It has become increasingly evident that restriction factors, from either the perspective of the virus or the host, work through multiple steps and that degradation of target proteins is often a late stage of restriction. Work investigating Vpu antagonism of tetherin is conflicted on the role of degradation, with several labs demonstrating that degradation is not required. In a similar example, Vif is an HIV accessory gene that counteracts the host anti-viral protein APOBEC. The APOBEC family members are cytidine-deaminases and create lethal G-to-A hypermutations during reverse transcription. Vif binds to APOBEC, recruiting E3 ubiquitin ligase conjugating machinery to ubiquitinate APOBEC and target it for proteasomal degradation (146). However, work from the Strebel lab demonstrated that APOBEC degradation was not required for Vif-mediated restriction, suggesting that interactions between Vif and APOBEC may be sufficient to abrogate the availability of APOBEC active sites (99). These studies, and others, suggest that restriction factors have multiple steps in prohibiting either host or viral proteins and that degradation, when it occurs, is a late stage in restriction.
We and others previously identified the Env protein from gibbon ape leukemia virus (GaLV Env) as a novel target for Vpu-mediated restriction (39, 129). We currently believe that Vpu recognizes GaLV Env as a CD4 analogue due to sequence similarities in the cytoplasmic tails of both targets (92, submitted work). Additional studies from the perspective of Vpu show that Vpu requires similar critical amino acids within its cytoplasmic tail for restriction of both CD4 and GaLV Env (92, 129). Collectively, these findings show a shared molecular pathway for both targets. However, two factors identified in our 2010 study fail to fit the classically defined model for CD4 restriction (reviewed in 20, 162, 187). First, we reported a Gag-specific phenotype for Vpu activity. Second, we found that Vpu-mediated restriction did not require a severe surface downmodulation of GaLV Env as has been reported for CD4. The reduction in surface expression of CD4 is thought to prevent incorporation of CD4 into virus, which compromises viral fitness. Here, we provide additional studies to further elucidate the mechanism of GaLV Env restriction and reconcile our previously reported differences between the Vpu-CD4 and Vpu-GaLV Env models.

MATERIALS AND METHODS

Cells.
The 293FT cell line (Invitrogen) was used in all transfections to produce virus. The 293T mCAT-1 cell line stably expresses a triply HA-tagged receptor murine cationic transporter-1, this target cellular receptor for ecotropic MLV Env, and was obtained from Walter Mothers. Cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM non-essential amino acids, and 0.5 mg/ml G418. The 293T TVA cell line, which expresses the putative RSV receptor, was used for SEM studies because of its non-clumping, flattened phenotype.

**Plasmid constructs.**

The HIV-1 proviral reporter, green fluorescent protein (GFP)-tagged Env construct have been previously described (129). MLV Env and the chimeric MLV/GaLV Env (referred to at GaLV Env) have been previously described (92, 130, 210). The HA-tagged GaLV Env was made by replacing YFP with an HA tag. The Vpu sensitive GaLV Env has been previously described as GaLV Env Δ8 and the Vpu sensitive GaLV Env has been previously described as GaLV Env Δ12 (92). For scanning electron microscopy (SEM) studies, a budding defective and protease defective provirus was used. The SEM viral constructs were generated from a PTAP budding defective provirus. The parental pNL4-3 provirus was obtained from Vineet KewalRamani and was modified by R. Jorgenson by replacing RFP with puromycin+mCherry. The PTAP-Protease defective mutant was generated by two-step PCR by mutagenizing the protease amino acid (site
81-90) sequence from PVNIIGRNLL to PVTIKKNLV and adding 5’ Spel and 3’ AgeI sites. The Vpu containing provirus had Vpu from the previously described Vpu-containing provirus at the EcoRI and BamHI sites (129).

Panel of retroviral Gags: the pCPRDEnv FIV Gag-Pol was obtained from Addgene (Addgene #1732, deposited by Gary Nolan) and the FIV genome was constructed by adding GFP into the pTIGER genome (Addgene #1728, deposited by Gary Nolan). EIAV was from pONY 3.0 and the pONY 3.8 GFP genomic EIAV reporter a kind gift from Greg Towers. MLV Gag-Pol was kindly provided by Walther Mothes. MLV GFP-reporter genome (pQCXIP+GFP) was made inserting GFP into the pQCXIP vector (Clontech) and was used as a reporter in MLV and RD114 infectivity assays. SIV pUPSVOd-psi Gag-Pol and pV1eGFPSVO GFP-genomic reporter were kind gifts from Hung Fan. RD114 Gag-Pol was a kind gift from Manuel Caruso.

The HIV-1 provirus containing intronic Gassausia luciferase was made by S. Janaka by adding the intronic Gassausia luciferase through PCR amplification and insertion at NotI and Nhel. The parent was created by D. Gregory and was derived from proviral pNL4-3+GFP from Vineet KewalRamani with GFP substituted with an intronic GFP (iGFP) reporter obtained from David Dersey. The chimeric MA Gag substitutions were made by D. Gregory. Vpu-GFP, a kind gift from Edward Stephens, is derived from HIV-1 HXB2, group M, subtype B and has been previously described (83). Vpu-Cherry was synthesized by 2-step PCR to replace GFP with mCherry, a kind gift from Roger Tsien. Ubiquitin dominant negatives were a kind gift from Mark Hannink.
Infectivity.

Infectivity work was conducted by transfecting 293FT cells in 6 well plates with 3.5ul PEI transfection reagent per 1ug plasmid DNA. Transfection media was replaced 5-12h posttransfection with fresh media and virus containing media was collected 48h after transfection, and frozen at -80°C overnight as previously described (130). 1ml of viral media was added to 293T mCAT-1 along with 2ul polybrene (Milipore) per 1ml media. 48h post infection, cells were harvested, fixed and analyzed for infectivity by expression of GFP in flow cytometry with an Accuri C-Flow Sampler. For infectivity assays where the endpoint is production of Gaussia luciferase, 293mCAT-1 cells were infected and 48 later, media was harvested. A BioTek plate reader was used to analyze luminescent signal from 25ul collected media, 25ul PBS and 50ul of coelenterazine (221).

Surface labeling.

Surface labeling was done similarly to previously described methods (129). Briefly, 48h posttransfection, 293FTs were resuspended in 10mM EDTA in PBS and then blocked for 1h in 10mM EDTA, 2% goat serum in PBS for 1h. Cells expressing GFP-tagged Env were then antibody labeled with anti-GFP- Alexa Fluor-647 conjugated antibody (1:700, Invitrogen), or anti-CD4-APC conjugated antibody (1:20, Invitrogen) for 1h at 4°C on ice. Cells were then rinsed 2 times in ice cold 10mM EDTA in PBS, with 10m
incubations, were fixed for 20m with 4% paraformaldehyde and analyzed by flow cytometry with an Accuri C-Flow Sampler.

**SEM.**

Viral Env and CD4 were imaged similarly to previously described techniques (98, 129, 130). 293T TVA cells were plated onto gold-labeled glass coverslips and transfected with 1ug total DNA, with 500ug of an HIV-1 proviral construct (with or without Vpu) containing a PTAP defective domain and additional disruptions in the protease cleavage site. 48h posttransfection, cells were lightly fixed for 5m with 4% paraformaldehyde and transfected (fluorescent) cells were mapped. Samples were then incubated with the appropriate antibody: anti-CD4-biotin conjugated antibody (1:25, Invitrogen) or anti-GFP-monoclonal mouse antibody (1:25, Sigma) for one hour. Each sample was then rinsed three times and labeled with the secondary antibody: anti-biotin- 10nm colloidal gold (1:25, Jackson ImmunoResearch) or anti-mouse- 12nm colloidal gold (1:25, Jackson ImmunoResearch). Lastly, cells were hard fixed with 2.5% gluteraldehyde for 20m, ethanol dehydrated, critical point dried, carbon coated and imaged with a Hitachi S-4700 FE-SEM.

**Inhibitors.**

For infectivity studies, 40h posttransfection media was removed from 293FT cells and was replaced with either 10uM MG132, 6uM ALLN, 50nM Concanamycin A, or
100nM Bafilomycin A1 for 1 h, then media was removed and replaced with fresh media and inhibitors for 8h. Viral media was then collected and spinoculated with 293T mCAT-1 cells by centrifugation of cells for 30m at 4°C to permit binding but not entry to virus. Residual media was aspirated off and cells were replated and collected 48h later and analyzed for infectivity by flow cytometry.

For Western studies, the above process was applied to cells transfected with GFP-tagged Vpu and an HA-tagged GaLV Env. Cells were collected 8h post-treatment for PNGase treatment and analyzed by standard Western blotting techniques.

**Western Blots.**

Transfections for westerns were performed as described for infectivity assays. 48h posttransfection, cells were lysed in protease inhibitor as described (39), and either retained for PNGase treatment or prepared in 1% SDS-PAGE loading buffer and approximately 4% of the cell suspension was analyzed in parallel with supernatant. Proteins were transferred to 0.45μm PVC membranes by standard transfer methods. Membranes were blocked with 5% non-fat dry milk, and probed with mouse anti-HA (Sigma) diluted 1:500. Blots were then probed with horseradish peroxidase conjugated anti-mouse diluted 1:10,000 (Sigma).

**PNGase treatment.**
Cellular 293FT lysate was collected and treated with PNGase as previously described (39). Cells were lysed at 4°C for 10m in 200ul lysis buffer (20 mM Tris-HCl, 1% triton X-100, 0.05% SDS, 5 mg/ml sodium deoxycholate, 150 mM NaCl) in the presence of a protease inhibitor cocktail (Roche) and sonicated to further disrupt membranes. Samples were then spun for 10m at 10,000 x g to pellet cellular debris. Purified lysate was transferred to a fresh tube and 16ul was incubated with PNGaseF denaturation buffer for 5m at 95°C. Following heat denaturing, samples were treated with PNGase by manufactures protocol with 1x G7 buffer, 1% NP40, and 2ul PNGaseF for 2h at 37°C (NEB). Samples were analyzed by SDS-PAGE and western blotted.

RESULTS

The role of Gag in Vpu-GaLV Env restriction.

Our previously published work (129) suggests that Vpu’s ability to restrict GaLV Env from viral particles is dependent of the species of Gag employed. We found that when HIV-1 or MLV was pseudotyped with GaLV Env in the presences of Vpu, only HIV-1 was sensitive to the Vpu-mediated restriction while MLV Gag was not and produced infectious particles. This was unexpected, as Vpu has been previously reported to antagonize tetherin and CD4 independently of virus or viral components. We therefore sought to determine if the Gag-specific phenotype was present in other retroviruses or
was unique to HIV-1. First, we tested a panel of retroviral species, including several lentiviral Gags (HIV-1, FIV, SIV, ELAIV) and two gammaretroviruses (MLV and the feline endogenous retrovirus RD114), on Vpu mediated sensitivity of GaLV Env. In the case of lentiviral Gags, Vpu sensitivity was more pronounced than in gammaretroviral Gags (MLV, RD114) (Figure 5-1). These findings suggest that lentiviruses contain unique features that contribute to Vpu’s activity against GaLV Env or, alternatively, the gammaretroviruses have characteristics that protect GaLV Env from Vpu targeting. This was unexpected, as Vpu does not require other components of the virus to downmodulate either CD4 or tetherin.

We then postulated that the MA domain, the region thought to interact with the Env cytoplasmic tail, might contribute to the Gag phenotype. Several studies have demonstrated that the MA region of HIV and other lentiviruses mediates compatibility or incompatibility with some Env proteins during assembly (59, 60, 171). We sought to determine whether the HIV MA contributes to sensitivity or the MLV domain confers protection of GaLV Env from Vpu. To address the possible role of MA in Vpu-mediated restriction, we used a panel of HIV mutants where the MA domain of HIV-1 was replaced with an alternative membrane targeting motif (Lyn or Src) or the MA of MLV was replaced with that of either HIV-1 or MPMV. Other combinations were not tolerated and failed to produce infectious particles possibly due to defects in assembly or maturation of Gag (data not shown). The MA domain of HIV-1 did not appear to contribute to Vpu sensitivity, although the downstream region of Gag-Pol affected
sensitivity (Figure 5-2). Mutants that contained MLV Gag-Pol were still sensitive to Vpu. The MA domain of MLV does not appear to protect GaLV Env nor does the MA domain of HIV appear to promote Vpu sensitivity. These studies suggest that either a region outside of HIV-1 MA confers Vpu sensitivity or alternatively MLV contains a downstream region that promotes Env compatibility. We suspect that MLV more efficiently acquires and/or processes GaLV Env, a closely related gammaretroviruses.

Because MLV Env and other gammaretroviral Envs, such as GaLV Env, go through an additional proteolytic process from other retroviral Envs, we sought to determine if there were inherent differences in the efficiency of both MLV and HIV-1 cores to produce infectious particles when the availability of Env was reduced in the cell. To do this, we transfected 293FT cells with equal amount of either HIV-1-GFP provirus or MLV Gag-Pol with a GFP-genomic reporter and a titrated panel of GaLV Env in the presence or absence of Vpu. Surprisingly, we observed that both MLV and HIV-1 cores became sensitive to Vpu downmodulation of GaLV Env when we restricted the amount of Env available (Figure 5-3). Together, these results suggest that the Vpu-GaLV phenotype is not dependent on the type of Gag used, but rather is symptomatic of how efficient or inefficient either virus is at either acquiring and/or processing GaLV Env (13). We suspect that MLV protease is more effective at processing the R peptide of GaLV Env than HIV-1, as HIV does not have an R peptide. Importantly, our results suggest that Vpu restricts GaLV Env in a manner that is independent of Gag, which is consistent for previous reports for Vpu downmodulation of CD4 or tetherin. Interestingly, different
retroviruses demonstrate unique affinities for effectively acquiring and/or processing their glycoproteins.

**The role of the degradation pathways in restriction.**

As previously reported for CD4, Vpu mediates restriction by the recruitment of E3 ubiquitin ligase machinery to polyubiquitinate CD4 and ultimately targeting it for proteasomal degradation. We therefore sought to determine if there was a role for ubiquitination in our Vpu-GaLV Env system. In order to test this, we employed a series of dominant negative ubiquitins, where either all lysine acceptor sites were mutated to arginine (Ub7R) or any single acceptor ubiquitin site was mutated (K48R, K63R, K6R, K11R, K27R, K29R and K33R). Although polyubiquitination is typically associated with degradation of the tagged protein, some forms like K63 have been shown to mediate protein trafficking. We found that Ub7R enhanced infectivity, alleviating much of the Vpu imposed restriction on GaLV Env (Figure 5-4). However, when we tried individual ubiquitin acceptors, no single species of ubiquitin appeared to be specifically important in the enhancement of infectivity.

Polyubiquitination of proteins is a modification typically associated with proteasomal targeting, although they may also alter trafficking of the ubiquitinated protein. Because we found that Vpu-mediated restriction required polyubiquitination, we questioned whether degradation for required. Therefore, we sought to determine if gross degradation through either the proteasomal or lysosomal route was required for
GaLV Env targeting. If Vpu is in fact targeting GaLV Env in a manner to CD4, we expect that MG132 treatment will alleviate degradation as has been previously reported for CD4. However, previous work by the Cannon lab suggests a unique route for GaLV degradation from CD4. Through biochemical analyses, they found that Vpu targets mature GaLV Env for lysosomal degradation and treatment with lysosomal inhibitors enhanced the gp70 mature Env population (39). To address a requirement for degradation on infectivity, we transfected cells with HIV-1 provirus with a GFP reporter and a Vpu-sensitive GaLV Env or an insensitive GaLV Env missing the last 12 amino acids of the C-terminal CT. At 40h posttransfection, we treated virus producing cells with a DMSO control, the proteasomal inhibitors MG132 or ALLN (a more specific inhibitor of the proteasome) or lysosomal inhibitors (Bafilomycin A1 or Concanamycin A). We found that neither inhibition of the proteasome or lysosome alleviated the Vpu imposed restriction (Figure 5-5). Proteasomal inhibitors did decrease overall infectivity, likely through the depletion of free ubiquitins which are required for monoubiquitination and assembly of Gag (203). Although we expected block either degradation pathways to potentially alleviate Vpu-mediated restriction, the Cannon lab relied on biochemical analyses and did not investigate infectivity of HIV-1 pseudotyped GaLV Env.

We then asked whether we could observe biochemical changes in the mature and immature populations of Env in the presence of these inhibitors. Although we found no relief from Vpu-mediated restriction on infectivity, a previous report with biochemical data suggested that Vpu targets GaLV Env for lysosomal degradation (39).
We therefore sought to revisit their experiment to determine if lysosomal or proteasomal inhibitors could restore the gp70 population in cellular lysate. 8h prior to collection, cells producing virus in the presence or absence or Vpu were treated with a proteasomal inhibitor cocktail consisting of MG132 and ALLN or a lysosomal inhibitor cocktail of Bafilomycin A1 and Concanamycin A. Cell lysate was treated with PNGaseF to better resolve the immature from the mature forms of Env. In all cases where Vpu was present, we observed almost exclusively the immature GaLV Env (Pr85) (Figure 5-6). In the absence of Vpu, SU GaLV Env was present in the immature (Pr85), mature (gp70), and intermediate glycosylated (Pr95) forms. Regardless of the presence of inhibitor cocktails, we observed no rescue of gp70 or presence of the Pr95 intermediate band. Pr95 is suspected to be a glycosylating intermediate observed in the Golgi. Therefore, we agree with previous reports observing an absence of the mature form of Env, however, we do not find degradation to play a major role in Vpu-mediated restriction of infectivity. We postulate that Vpu is acting to prevent the maturation of Env rather than degrading mature Env.

**Surface expression of Env.**

In our previous studies, we found only a modest reduction in the surface expression of GaLV Env despite a dramatic restriction in infectivity. This was surprising considering the known role of Vpu restriction on CD4 expression at the PM. We therefore sought to determine the role of Vpu on GaLV Env surface expression. First we
determined the amount of Vpu required for reduction of GaLV Env expression at the PM in parallel with restriction of activity. We transfected 293FT cells with titrated amounts of Vpu tagged with mCherry, equivalent amounts of GaLV Env tagged with GFP and proviral HIV-1 containing an intronic Gaussia luciferase reporter. We used a luciferase reporter system as opposed to a fluorescent reporter system to avoid the addition of fluorophores into our flow cytometry study. To compare the amount of total GaLV Env to surface GaLV Env, we surface labeled against GaLV-GFP with an APC conjugated antibody. We verified expression of Vpu through mCherry expression. At high Vpu titers, we observed a modest reduction of GaLV Env from the PM (Figure 5-7). However, restriction of infectivity occurred with low amounts of transfected Vpu. These findings collectively suggest that removal of GaLV Env from the cell surface is not required for Vpu-mediated restriction.

If GaLV Env is present at the plasma membrane and yet not incorporated at the plasma membrane, we postulated that it has an altered distribution. To determine if Vpu alters the surface distribution of target proteins, we performed scanning electron microscopy (SEM) on both GaLV Env and CD4 in the presence of budding virus. When Vpu was absent, both GaLV Env and CD4 were robustly recruited to viral budding sites (Figure 5-8, Figure 5-9). However, in the presence of Vpu, GaLV Env and CD4 exhibited a somewhat random distribution and were not colocalized with budding virus. These findings suggest that Vpu-mediated restriction of target proteins does not require surface downmodulation, but rather restricts incorporation of target proteins into viral
particles by altering the distribution of target proteins. Or, alternatively, surface expression data along with western blot data, suggest that population of GaLV Env is an unprocessed (non-furin cleaved) form of the precursor Env.

**DISCUSSION**

Here we report a discrepancy in the Vpu-mediated restriction of CD4 through a CD4 analogue, GaLV Env. We established parallels between the targeting of CD4 and GaLV Env: Vpu-mediated restriction of GaLV Env is not dependent on Gag and that exclusion of GaLV Env from the plasma membrane or degradation of GaLV Env is not required for restriction of GaLV Env pseudotyped virions. Surprisingly, we observed that Vpu could sufficiently redistribute either target away from virus budding locations, a finding which is in conflict with the role of degradation in the established Vpu-CD4 model. We found that Vpu reduced the mature form of GaLV Env and that the population of mature Env was not restored in the presence of proteasomal or lysosomal inhibitors.

The loss of mature Env can be accounted for in two possible ways: 1) Vpu recognizes and degrades gp70, or 2) Vpu prevents the maturation of Pr85 (Figure 5-10). Our findings fail to support the first hypothesis, as proteasomal and lysosomal inhibitors failed to restore infectivity or the presence of gp70 in cellular lysate. We suspect that
the Env present on the cell surface likely represents the immature population. Previously published studies by our lab have demonstrated that furin-cleavage defective MLV Env reaches the PM but fails to colocalize with viral budding sites (130). How does Vpu prevent GaLV Env from being processed by furin? While we currently do not know, we have two hypotheses. Vpu may prevent colocalization of furin and Env within the trans-Golgi. Alternatively, Vpu could prevent recognition of the furin cleavage site by either masking the site directly or preventing trimerization of Env. The trimerization of retroviral glycoproteins occurs in the ER is required for downstream recognition by furin. Indeed, Vpu has precedent for this with another viral glycoprotein, HIV-1 Env. Vpu interacts with HIV-1 Env, sequestering it in the ER and preventing trimerization and subsequent furin cleavage into mature Env (16, 42). So long as Vpu binds to GaLV Env at stoichiometrically equivalent levels, we predict that Vpu can sufficiently inhibit GaLV Env from maturing and reaching budding sites.

Our current understanding of GaLV Env restriction conflicts with CD4 models where degradation precludes CD4 restriction from viral budding sites. Based on SEM images, we can see that both GaLV Env and CD4 are present at the surface; however, their distributions are altered in the presence of Vpu. While CD4 does not undergo furin cleavage or trimerization, it does form either homodimers or heterodimers with Lck. It is possible that Vpu prohibits the CD4 from assembling into either dimeric population, thus disrupting trafficking, targeting or microdomain association at the cell surface. Precedent exists for an HIV accessory gene reducing the dimeric CD4 population; work
done by the Foti lab demonstrated the Nef reduced CD4 dimers but not monomers (58). Further studies will need to determine the exact effects that Vpu has on both GaLV Env and CD4 populations. Our previous work has demonstrated that MLV Env is relocalized on the plasma membrane when the furin-cleavage requirement is not met (130), which may explain the apparent surface expression of CD4 and GaLV Env and the striking redistribution of both proteins at the plasma membrane away from viral budding sites in the presence of Vpu.
Figure 5-1 Lentiviruses demonstrate Vpu sensitivity.

Infectivity assays were performed by transfecting 293 FT cells and 28h later transferring viral media onto 293T mCAT-1 cells. (A) Infectivity for retroviral panel: provirus with a genomic reporter or GagPol along with a GFP genomic reporter without Vpu (dark bar) or with Vpu (light bar) and a panel of retroviral constructs with a genomic GFP reporter. Lentiviruses (HIV-1, FIV, SIV, and EIAV) or gammaretroviruses (MLV or RD114) Gags were tested for infectivity. Infectious units (I.U.) per ml reported on the Y-axis. (B) Relative infectivity was calculated for each Gag from part (A) by comparing infectivity in the presence or absence of Vpu.
Figure 5-1. Lentiviruses demonstrate Vpu sensitivity.
Figure 5-2. The Vpu sensitivity region is outside of the Gag MA domain.

Infectivity assays were performed by transfecting 293 FT cells and 28h later transferring viral media onto 293T mCAT-1 cells. (A) Gag chimeric constructs exchanged the MA domain of HIV with either membrane targeting sequence Lyn or Src, or the MA domain of HIV was placed onto the Gag of MLV or MPMV. Chimeric proviral constructs were transfected without Vpu (dark bar) or with Vpu (light bar). Infectious units (I.U.) per ml reported on the Y-axis. (B) Relative Vpu sensitivity is calculated for each chimeric Gag from infectivity reported in (A).
Figure 5-2. The Vpu sensitivity region is outside of the Gag MA domain.

A.

![Bar chart showing I.U. per ml for different Gag constructs](image)

**Gag construct employed**

B.

![Bar chart showing Vpu sensitivity (Vpu+/Vpu-) for different Gag constructs](image)

**Gag construct employed**
Figure 5-3. Both MLV and HIV-1 Gag become Vpu sensitive with Env availability is restricted.

Infectivity assays were performed by transfecting 293 FT cells and 28h later transferring viral media onto 293T mCAT-1 cells. (A) Cells were transfected with HIV-1 provirus with a GFP reporter or MLV GagPol with an MLV GFP genomic reporter in the presence (dotted line) or absence of Vpu (solid line) with decreasing amounts of GaLV Env (see figure legend). Infectious units (I.U.) per ml reported on the Y-axis. (B) Relative Vpu sensitivity was calculated from data shown in (A) by calculating the ratio of infectivity in the presence or absence of Vpu.
Figure 5-3. Both MLV and HIV-1 Gag become Vpu sensitive with Env availability is restricted.
Figure 5-4. Vpu restricts GaLV Env in a polyubiquitin-dependent manner.

293FT cells were transfected with a GaLV Env, a proviral HIV-1 without (dark bars) or with Vpu (light bars) and a dominant negative ubiquitin and infectivity was measured for each treatment. Ub7R contains mutations in all lysine acceptor sites and fails to form polyubiquitin chains. K11R, K29R, K48R and K63R contain lysine to arginine mutations at the respectively numbered site and are dominant negatives for each type of polyubiquitination. (B) A parallel experiment was performed identical to (A) except using an insensitive GaLV Env construct to identify the effects of ubiquitin dominant negatives.
Figure 5-4. Vpu restricts GaLV Env in a polyubiquitin-dependent manner.

A. 

B. 

I.U. per ml

filler Ub7R K11R K29R K48R K63R

filler Ub7R K11R K29R K48R K53R
Figure 5-5. Vpu-mediated restriction of GaLV Env is not dependent on an active proteasome or lysosome.

Infectivity assays were performed by transfecting 293 FT cells and 40h later treating virus producing cells with inhibitors. 8h later, viral media was collected and spinoculated onto 293T mCAT-1 cells. Proteasomal inhibitors: 6uM ALLN and 10uM MG132. Lysosomal inhibitors: Bafilo= 100nM bafilomycin A1 or CanA= 50nM Concanamycin. (A) 293 FT cells were transfected with HIV-1 provirus with a GFP reporter and Vpu-sensitive GaLV Env and were measured for output of infectious virus. (B) The experiment was performed as in (A), however, an insensitive GaLV Env with a truncation in the CT was used. Infectious units (I.U.) per ml reported on the Y-axis.
Figure 5-5. Vpu-mediated restriction of GaLV Env is not dependent on an active proteasome or lysosome.
Figure 5-6. Vpu depletes the mature Env population regardless of proteasomal or lysosomal degradation.

293FT cells were transfected with HA-tagged GaLV Env and GFP-tagged Vpu. At 40h posttransfection, cells were treated for 8h with either a lysosomal inhibitor cocktail (100nM Bafilomycin A1 and 50nM Concanamycin A) or a proteasomal cocktail (6uM ALLN and 10uM MG132). Cellular lysate was PNGase treated to resolve the Pr85 and gp70 bands and was analyzed by Western blot. The band selectively seen above Pr85 is likely Pr95, a glycosylation intermediate found in the Golgi.
Figure 5-6. Vpu depletes the mature Env population regardless of proteasomal or lysosomal degradation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>None (DMSO)</th>
<th>Lysosomal inhibitors</th>
<th>Proteasomal inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vpu (-/+)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Pr85
gp70
Figure 5-7. In the presence of Vpu, GaLV Env surface expression is minimally reduced relative to restriction in infectivity.

(A) Cells were transfected with HIV-1 with an intronic Gaussia luciferase reporter, GFP-tagged GaLV Env and decreasing amounts of mCherry tagged-Vpu and assayed for surface expression of GaLV Env with an anti-GFP APC conjugated antibody. Y-axis is FL4 mean fluorescent intensity for APC. Background represents non-specific staining for cells that are not transfected with GaLV Env. (B) Virus was collected from the producer cells assayed in (A) and applied to 293mCAT-1 cells. 48h post-infection, media was collected from infected cells and assayed by luciferase activity.
Figure 5-7. In the presence of Vpu, GaLV Env surface expression is minimally reduced relative to restriction in infectivity.
Figure 5-8. Vpu alters the distribution of GaLV Env on the cellular surface.

293TVA cells expressing GFP-GaLV Env were transfected with a protease and late domain-defective HIV-1 provirus without (top) or with Vpu (bottom). Env was labeled with 12-nm gold and imaged by SEM. Left, secondary electron images of HIV-1 assembly site. Right, backscatter electron images of gold-labeled Env. Scale bars, 200nm.
Figure 5-8. Vpu alters the distribution of GaLV Env on the cellular surface.
Figure 5-9. Vpu alters the distribution of CD4 on the cellular surface.

293TVA cells were transfected with a CD4 and a protease and late domain-defective HIV-1 provirus without (top) or with Vpu (bottom). CD4 was labeled with 10-nm gold and imaged by SEM. Left, secondary electron images of HIV-1 assembly site. Right, backscatter electron images of gold-labeled CD4. Scale bars, 200nm.
Figure 5-9. Vpu alters the distribution of CD4 on the cellular surface.
Figure 5-10. Vpu potentially restricts GaLV Env through one of two routes.

Vpu excludes the mature form of GaLV Env (gp70) in the cell through either (1) preventing the maturation of immature Env (Pr85) from being cleaved by furin to produce gp70 and p15E, the mature subunits. (2) Alternatively, Vpu may target gp70 for degradation (39). Our studies support the first model, suggesting a limited role for degradation in restriction.
Figure 5-10. Vpu potentially restricts GaLV Env through one of two routes.
VI. Summary and Discussion

The use of viral pseudotyping systems is of interest in gene therapy and has greatly promoted our understanding the basic biology of retrovirus assembly, including the mechanisms by which viruses actively include or exclude viral and cellular proteins. Retrovirus assembly is a process that is still surprisingly poorly understood and currently no anti-viral drugs are available to target this stage in the HIV-1 lifecycle. Assembly is a complex process dependent on the coordinated multimerization of Gag, incorporation of the dimeric RNA genome and recruitment of Env at the plasma membrane during the budding process. Multiple domains within both Gag and Env likely contribute to direct and indirect interactions during the assembly process (recently review in 32, 96).

Previous work investigating the Env proteins and assembly has suggested a role for the cytoplasmic tail in recruitment. The CT appears to play a role in incompatibility between Env and some retroviral Gags and is the most likely region to physically interact with Gag or cellular factors. I, therefore, sought to investigate the role of Env in retroviral assembly through two venues: (1) to understand the contribution of the MLV Env protein to its recognition and selective incorporation into virus, and (2) to investigate the mechanism by which the HIV-1 accessory gene excludes a non-native viral Env from gibbon ape leukemia virus and the host cell protein CD4.

Viruses employ a series of mechanisms to both recognize self and to exclude host proteins that can be detrimental to viral fitness. Surprisingly, we have limited
understanding of factors contributing to compatibilities and incompatibilities in retrovirus assembly. For example, the glycoproteins from MLV are highly promiscuous in their ability to pseudotype with unrelated retroviral Gags, while others like HIV-1 Env are highly specific and tolerate only their own Gag. Previous research suggests that there are likely multiple reasons dictating compatible and incompatible situations, including physical (steric hindrance), possibly localization and cellular factors. The role of the Env cytoplasmic tail in assembly is particularly attractive for two reasons: (1) this is the only region of Env that is available for interactions with Gag, (2) the CT contains multiple trafficking motifs that may promote appropriate colocalization to viral budding sites.

To address features in MLV Env that mediate recruitment, we investigated a panel of cytoplasmic tail truncations and a processing defective mutant MLV Envs. We found that removal of the entire CT did not abrogate effective recruitment of MLV Env into either HIV-1 or MLV particles. This suggests that the CT is not required for localization with Gag during assembly and that direct interactions do not need to occur between Env and Gag. We have named this type of non-CT mediated recruitment “generic recruitment”. However, the CT does play a role in assembly preference through what we term “specific recruitment”. In cases where two retroviruses, HIV-1 and MLV, infect the same cell and must compete for Env during assembly, MLV outcompetes HIV-1 for acquisition of Env. When we progressively removed regions of the cytoplasmic tail, this competitive edge was progressively eliminated. One possible
hypothesis for MLV robustly sequestering its Env efficiently is that murine retroviruses and endogenous murine retroviruses often encounter each other within the host and potentially must protect their own Env from competing retroviruses (73).

Additionally we analyzed an MLV Env known as “furin-cleavage defective”. Through a single point mutation, this MLV Env is not recognized by furin or furin-like proteases and is not processed from the immature Pr85 to the mature gp70-p15E heterodimer. As reported by others, we also observed that cleavage defective Env is expressed at the plasma membrane and that Env is defective in infectivity (133, 153, 257). Interestingly, SEM reveals a change in the distribution of Pr85 at the surface demonstrating a processing requirement for appropriate targeting and/or colocalization with viral budding sites. Altogether, our findings demonstrate a requirement for appropriate processing of MLV Env and that the cytoplasmic tail can facilitate Gag specific recruitment, but is not essential in the colocalization of Env at viral assembly sites.

During the assembly process, viruses employ a series of strategies to counteract host immune responses and exclude host proteins from assembly sites. The accessory gene Vpu restricts the incorporation of the HIV-1 receptor CD4 from incorporation in the virus and liberates HIV-1 Env from Vpu-Env complexes within the ER. In addition, Vpu counteracts the interferon induced antiviral factor tetherin. Here we report the ability of Vpu to recognize a novel target gibbon ape leukemia virus envelope protein. In some respects, our early findings suggest a distinct but overlapping mechanism for
restriction between GaLV Env and CD4. However, we were surprised to find two distinct phenotypes: (1) GaLV Env restriction exhibited only a mild reduction in surface expression compared to previous reports for CD4, and (2) GaLV Env restriction appeared to be dependent upon the species of Gag employed (i.e.- the Vpu phenotype was observed in the presence of HIV-1 Gag, but not MLV Gag).

We therefore sought to determine if there were shared features within Vpu for counteraction of GaLV Env and tetherin and previous reports for Vpu antagonism of CD4. Previous studies have found that modulation of CD4 requires the Vpu CT, while modulation of tetherin requires both the Vpu CT and TMD. Previous Vpu mutational studies looked at anti-tetherin and anti-CD4 activities independently of one another. We used mutagenic scanning of Vpu to reveal that nearly identical amino acids are required for restriction of GaLV Env and those previously reported for CD4 targeting. Additional work demonstrated that GaLV Env contains a similar recognition sequence to that reported previously for CD4 (92).

The mechanism by which Vpu restricts CD4 from incorporation into viral particles has been well mapped (reviewed in 20, 162, 187). We therefore sought to further investigate mechanistic discrepancies between the Vpu-CD4 model and our working model for Vpu-mediated restriction of GaLV Env. In conflict with the CD4 model, we found that (1) Vpu only modestly reduced surface expression of GaLV Env relative to the robust restriction of infectivity, and (2) Vpu-mediated restriction of GaLV Env was dependent on the species of viral core used (HIV-1 versus MLV). Vpu modulation of CD4
is independent of the virus and restricts surface expression. In addition, the Cannon lab has reported a virus-dependent phenotype for Vpu restriction of GaLV Env (39). In order to address these observed differences, we tested a panel of non-lentiviral retroviruses and lentiviruses for Vpu sensitivity. These initial findings suggested lentivirus sensitivity. However, we found that all retroviruses are sensitive to Vpu when Env availability is restricted. The species specific specificity we previously observed is likely due to the ability of some Gags to acquire and/or activate Env (217).

We further investigated the modest reduction in surface expression by determining where GaLV Env and CD4 were located on the cell surface relative to budding virus. Interestingly, we found that Vpu altered the surface localization of both GaLV Env and CD4, redistributing it away from virus. Work done by the Cannon lab and our own, shows that Vpu reduces the mature Env (gp70) population, but does little to effect immature Env (Pr85) levels (39). We propose that the population of Env observed at the cell surface is immature Env, which we have previously shown to be unable to colocalize at viral budding sites (130).

How is Vpu restricting mature Env from viral particles? Two possible hypotheses can explain the Vpu reduction of mature Env: either Vpu degrades gp70 or Vpu prevents the maturation of GaLV Env. The Cannon lab has shown a role for lysosomal degradation of gp70 (39). However, we did not observe a rescue effect in the presence of either lysosomal or proteasomal inhibitors on either infectivity or the presence of
gp70, suggesting that Vpu prevents the maturation of Env and thus the distribution at
the cell surface.

Through our parallel studies between the known CD4 model and our working
GaLV Env model, we have demonstrated nearly identical overlapping pathways for
restriction. However, in disagreement with the current CD4 model, we observe that
Vpu can restrict incorporation of GaLV Env and CD4 from viral budding sites even when
both target proteins reach the cell surface.
VII. Literature cited


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IIX. Vita

Tiffany Lucas was born on July 7th, 1981 in Springfield, Illinois to an artist mother, Linda Post-Lucas, and an engineer father, Rick Lucas. She received her first grant in high school to monitor honey bee populations. In 1999, she reconciled the left- and right-sides of the brain at Truman State University, earning a Bachelor’s in Biology with honors for her research on ticks under supervision of Dr. Laura Fielden. She then moved to Tucson and completed her Master’s in Entomology at the University of Arizona in 2007 studying honey bee neurobiology under the guidance of Drs. Gloria DeGrandi-Hoffman and Wulfila Gronenburg. In 2007, Tiffany began her PhD in Molecular Microbiology and Immunology as a Life Sciences Fellow.

During the course of her scientific career, she has been fortunate to conduct research both in the laboratory and in the field in Mexico, the Canadian Rockies and the beautiful Sonora desert.