A PLPPV Sequence in the p8 Region of Gag Provides Late Domain Function for Mouse Mammary Tumor Virus

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Abstract

The late (L) domain sequence used by mouse mammary tumor virus (MMTV) remains undefined. Similar to other L domain-containing proteins, MMTV p8 and p14[NC] proteins are monoubiquitinated, suggesting L domain function. Site-directed mutagenesis of p8, PLPPV, and p14[NC], PLPPL, sequences in MMTV Gag revealed a requirement only for the PLPPV sequence in virion release in a position-dependent manner. Electron microscopy of a defective Gag mutant confirmed an L domain budding defect morphology. The equine infectious anemia virus (EIAV) YPDL core L domain sequence and PLPPV provided L domain function in reciprocal MMTV and EIAV Gag exchange mutants, respectively. Alanine scanning of the PLPPV sequence revealed a strict requirement for the valine residue but only minor requirements for any one of the other residues. Thus, PLPPV provides MMTV L domain function, representing a fourth type of retroviral L domain that enables MMTV Gag proteins to co-opt cellular budding pathways for release.

Introduction

Retroviruses assemble into virions that are released from the cell by budding through the cellular membrane, the last step in particle formation. The critical step in this process is the

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pinching off of the neck of the virus bud to release the virion from the cell. This process requires the use of cellular proteins and pathways to resolve this structure (Bieniasz, 2006; Hurley and Cada, 2018; Johnson, Bleck, and Simon, 2018; Martin-Serrano and Neil, 2011; Pincetic and Leis, 2009; Sette et al., 2013; Votteler and Sundquist, 2013).

In the absence of the cellular assistance, the retroviruses remain attached to either their budding membrane, forming lollipop-like structures (Gottlinger et al., 1991; Huang et al., 1995), or to one another in long strings or arrays of virions released from the cell (Demirov, Orenstein, and Freed, 2002). The Gag polyprotein is the only viral protein strictly required to produce particles from the cell and is the major structural protein of the virion. Gag contains one or more late (L) domain sequences that commandeer cellular budding pathways by binding cellular proteins which ultimately sequester components of the endosomal sorting complex required for transport III (ESCRT III) to the site of viral budding. Here they act with vacuolar protein sorting-associated complex protein 4 (VPS4) to complete the budding process by resolving the neck structure and releasing the virion (Baumgartel et al., 2011; Hurley and Cada, 2018; Johnson, Bleck, and Simon, 2018; Jouvenet et al., 2011; Morita et al., 2011; Muziol et al., 2006; Stuchell-Brereton et al., 2007; Votteler and Sundquist, 2013).

There are currently three recognized retroviral L domain motifs by which retroviruses gain access to the release machinery: PPXY recognized by HECT domain containing E3 ubiquitin ligases, including Nedd4 and Nedd4-like ligases (Heidecker et al., 2007; Jadwin et al.,; Medina et al., 2005), PTAP/PSAP bound by Tsg101 (Garrus et al., 2001; Martin-Serrano, Zang, and Bieniasz, 2001; VerPlank et al., 2001), and YPX$_n$L, recognized by Alix (Strack et al., 2003; von Schwedler et al., 2003). In many cases L domains can act in a positionally independent manner (Li et al., 2002; Parent et al., 1995; Xaing et al., 1996) and can also be exchanged between viruses (Li et al., 2002; Medina et al., 2005; Parent et al., 1995; Shehu-Xhilaga et al., 2004). However, heterologous L domain sequences do not always function in HIV-1 Gag (Martin-Serrano, Perez-Caballero, and Bieniasz, 2004; Ott et al., 2005; Shehu-Xhilaga et al., 2004; Xaing et al., 1996).

The study of L domains and their cognate protein partners that allow them to access the cellular budding and release machinery has increased our understanding of the production of both retroviruses (Bieniasz, 2009; Morita and Sundquist, 2004; Weiss and Gottlinger, 2010) as well as of other enveloped RNA viruses (Craven et al., 1999; Martin-Serrano, Zang, and Bieniasz, 2001; Schmitt et al., 2005). Despite the large body of work in this area, the L domain sequence for mouse mammary tumor virus (MMTV) has not been identified. Here, we identify and characterize an L domain-like sequence used by MMTV Gag to produce virus-like particles and show it is able to provide L domain function to equine infectious anemia virus Gag (EIAV).

Materials and methods

Gag expression constructs and mutant construction

All mutations were introduced into construct using either direct PCR or overlap extension (Horton et al., 1990). MMTV Gag mutants were constructed using the MMTV expression

construct pSMt-HYB Pro (Zabransky et al., 2009), a kind gift of Iva Pichova (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic) which contains a large internal deletion that eliminates Pol expression from the modified proviral construct. We modified this construct by adding an OLLAS epitope tag (SGFANELGPRLMGK) to the C-terminus of Gag (Park et al., 2008). Addition of this tag had no discernable effect on virus-like particle (VLP) production (data not shown). Mutants constructed in pSMt proOLLAS are: p8, the PLPPV at amino acid (aa) positions 246–250 changed to ASVA; p14, the PLPPL at aa positions 577–581 changed to ASVA; p8–p14, the p8 and p14 mutations combined; p8PLPPL, valine at aa 250 changed to L; p8PLPPL–p14, the p8PLPPL and p14 mutation combined, p14PLPPV, the leucine at position 581 changed to valine in the p8 mutant; p8YPDL, a replacement of PLPPV (aa 246–250) with a variant EIAV sequence containing the EIAV L domain core sequence (underlined): QNL<u>YPDL</u>SKIK; p8YPDL–p14, the p8YPDL mutant combined with the p14 mutant; p14YPDL, a replacement of PLPPL (aa 577–581) with the EIAV QNLYPDLSKIK sequence; p8P^1–A, proline aa 246 replaced with alanine, p8L^2–A; p8P^3–A, proline aa 248 replaced with alanine; p8P^4–A, proline aa 249 replaced with alanine, p8V–A, valine at aa 250 replaced with alanine; and p8P^1–A/P^3–A, p8P^1–A and P^3–A combined. EIAV Gag expression constructs were engineered using pGag.PRE (Patnaik et al., 2002) that contained a 7-amino acid deletion (p9^{Gag} aa 450–457) and an Sbf1 restriction site in the DNA just 3′ of the region encoding the YPDL that inserts 4 residues between aa 461 and aa 464. The release properties of this modified EIAV Gag were indistinguishable from the parent pGag.PRE (data not shown), consistent with the prior finding, of the p9^{Gag} sequence, that YPDL is a main contributor to L domain function (Li et al., 2002; Puffer, Watkins, and Montelaro, 1998). The EIAV plasmid was then used to produce the following constructs: E-PLPPV, the YPDL (aa 457–461, according to the original EIAV Gag sequence) replaced with PLPPV; E-PLxtnd, the YPDL replaced with aa 243–254 of MMTV, RRKPLPPVGFAG. The YPDL mutant, pGag.PRE with replacement of YPDL with SRSA was a kind gift of Eric Freed (Drug Resistance Program, NCI-Frederick, Frederick, MD) and placed in our EIAV Gag background.

Cell culture and lysate preparation

HEK293T cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 U per ml penicillin and streptomycin. Transfections were carried out using the TransIT-293® transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's recommendations and supernatant and cells were harvested 48 h post-transfection. VLP from cell supernatants were isolated by density centrifugation and prepared for immunoblot analysis as previously described (Ott et al., 1999). Cells were washed once with Dulbecco's phosphate-buffered saline solution without calcium or magnesium and with 1 mM EDTA, then lysed with 10 mM Tris HCl pH 7.5, 0.1 % SDS, and 40 U/ml of Omnicleave® nuclease (Epicentre Biotechnologies, Madison, WI), and incubated for 2 h at 4°C; then treated with an equal volume of SDS-PAGE gel loading buffer (250 mM Tris-Cl pH 6.8, 8% sodium dodecyl sulfate, 20% vol/vol β-mercaptoethanol, 40% vol/vol glycerol, and 0.02% bromophenol brilliant blue).

Near infrared (nIR) immunoblots

Samples were separated by SDS-PAGE electrophoresis and blotted onto PDVF-FL membranes (Millipore, Billerica, MA) using a semi-dry apparatus as previously described (Ott et al., 1999). Blots were blocked for at least 1 h in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), then incubated in blocking buffer with one or two primary antiserum(a)/antibody(ies) for at least 2 h. Blots were washed twice with blocking buffer for 10 min then incubated with the appropriate Donkey IRDye 800CW and/or IR Dye680 LT fluorescently labeled secondary antibodies (LI-COR) at a 1:10,000 ratio vol/vol in blocking buffer for at least 2 h. Blots were washed five times for 10 m in blocking buffer, and then the image was captured with an Odyssey infrared imaging system using a laser intensity of between 1 and 5 and analyzed by Odyssey software. All blotting steps were carried out at room temperature. Goat antiserum against EIAV p15^{MA} was produced internally within our program and goat antiserum against MMTV p27^{CA} was obtained from (ViroMed Biosafety Products, Camden, NJ). The actin monoclonal used was obtained from the former Amersham Life Science company. Release factors for the VLPs were determined by dividing the measured MMTV Gag VLP fluorescence values (arbitrary units) by the total Gag signal (VLP + cellular Gag). The cellular Gag values were corrected for loading and cell extract processing differences by normalizing signals from actin staining with a second color. Immunoblots for figures 2B, 4, 5, 6 and 7, are presented in Fig. S1.

Transmission electron microscopy

Transmission electron micrographs of thin section VLP samples were obtained as previously described (Dussupt et al., 2011).

Results

Our prior studies and those of others have shown that the virions of most retroviruses contain a small amount of a mature Gag protein that is monoubiquitinated near its L domain (Heidecker et al., 2004; Ott et al., 2000; Ott et al., 1998; Ott et al., 2003; Ott et al., 2002). We had previously found that MMTV virions contain small amounts of monoubiquitinated mature p8 and p14^{NC} proteins (Ott et al., 2003), Fig. 1), making these regions in Gag prime candidates to harbor MMTV L domain sequences. Furthermore, since L domain core sequences (and other low affinity protein-binding motifs, e.g., WW and SH3 domain-binding sites) typically rely on the unique structure of proline for specificity (Zarrinpar, Bhattacharyya, and Lim, 2003), we examined the MMTV Gag sequences near p8 and p14^{NC} for sequences rich in proline and identified two PLPPΦ motifs (Φ, any hydrophobic amino acid (Aasland et al., 2002)) in p8 and p14^{NC} (Fig. 1). Additionally, another potential L domain sequence in MMTV Gag is a PSAP sequence in CA, a variant of the PTAP motif that can bind Tsg101 (Pornillos et al., 2002).

To examine the potential L domain function of these sequences, we produced a series of constructs in the MMTV Gag-expressing construct pSMt-HYB Pro (Zabransky et al.), a Pol-deleted version of the MMTV provirus that contains a 5′ Mason-Pfizer monkey virus LTR in lieu of its hormone-dependent LTR. Earlier attempts to carry out these studies in the MMTV-HYB proviral clone (Shackleford and Varmus, 1988) were abandoned, due to the

clone's poor expression in both human and mouse cell line transfections even in the presence of dexamethasone. To test the importance of the PLPPΦ motifs this motif was changed to ASVAT in either p8 (PLPPV, Gag amino acids 246–250) to produce the p8 construct or p14[NC] (PLPPL, Gag amino acids 577–581) to produce the p14 construct (Fig. 1). These two mutations were also combined to produce a double mutant, p8–p14, that removes both PLPPΦ motifs. To determine the effect of these mutations on VLP production, these mutant constructs were transfected into HEK293T cells, and purified VLP and cell lysates were produced and examined by nIR immunoblotting using the LI-COR Odyssey imaging system. The results showed that the signal intensity of the Gag bands in the p8 and p8–p14 mutant VLP samples were markedly reduced from those in the wild-type sample (Fig. 2A). In contrast, the Gag band in the VLP sample from the p14[NC] single mutant, p14, had a similar intensity as the wild-type band (Fig. 2A). The release factor, the amount of Gag signal detected in the VLP sample divided by the sum of the signal in the VLP and actin normalized cell samples, was calculated from the signal density of the nIR immunoblot scans for each sample. Release factors from at least three independent experiments revealed that mutating the PLPPV site in p8, either alone or in combination with p14 in the p8–14 construct, significantly reduced VLP production (Fig. 2A), greater than 5- to 10-fold in this assay system. In contrast, the loss of the PLPPL site in p14[NC] had no discernable effect on release.

The PSAP motif is dispensable for MMTV VLP production

To determine the importance of the PSAP motif, an MMTV Gag mutant, CA P[305]A, was constructed with a proline-to-alanine substitution at position 305 of Gag (Fig. 1), effectively interrupting the potential of this motif to bind Tsg101 and function as an L domain (Garrus et al., 2001; Huang et al., 1995; Pornillos et al., 2002). Our nIR immunoblot analysis of CA P[305]A transfections found only a slight decrease in the relative release factor, compared to wild-type (Fig. 2B). Similar to the results above, the p8-p14 double mutant had a marked reduction in virion release. Thus, this putative Tsg101 binding site is not required for MMTV Gag VLP release.

Defective mutant VLPs exhibit an L domain morphology

A hallmark phenotype of L domain mutations is a marked arrest in the release of budding forms from the surface of the virus-producing cells (Gottlinger et al., 1991; Gottwein et al., 2003; Huang et al., 1995) and, in some systems, accumulation of cell-free structures that contain multiple immature Gag particles held together by plasma membrane (Demirov, Orenstein, and Freed, 2002). To visualize the budding phenotype of our L domain mutant, we analyzed transfected HEK293T cells expressing either wild-type or mutant p8-p14 Gag by thin-section electron transmission microscopy (Fig. 3). Micrographs of cells expressing wild-type MMTV Gag contained mostly free VLPs with a complete concentric electron dense ring (Fig. 3A), typical of immature MMTV virions (Bernhard, 1958; de Harven, 1974; Tanaka and Moore, 1967), with some plasma membrane budding forms with electron dense partial rings (arrow in Fig. 3A). In contrast, micrographs of p8-p14 transfected cells contained only a few observable free VLPs with the vast majority of virus-like structures present as either arrested budding forms attached to the membrane or complexes of cell-free VLPs that appear linked together by membrane (black-bordered white arrow, Fig. 3B). In

many cases the arrested budding forms appeared to be associated with ruffles or cellular filopodia adjacent to the main cell body in the thin sections (Fig. 3B, black arrows). Those few free single VLP forms observed did have a complete well-resolved morphology identical to the VLP in the wild-type samples (grey arrow, Fig. 3B), inconsistent with an assembly defect. Taken together, these results show that the PLPPV mutant exhibits a clear L domain morphology, being arrested late in the assembly process, during budding.

Replacement of PLPPV with EIAV YPDL restores MMTV Gag release

To confirm our electron microscopy results indicating that the mutation of PLPPV results in an L domain effect, we exploited one of the key properties of L domains, the ability to be functionally exchanged for other L domains (Li et al., 2002; Parent et al., 1995; Puffer et al., 1997). We constructed a mutant, p8YPDL (Fig. 4), that contained a replacement of the PLPPV sequence in p8 with an EIAV sequence that contains the YPDL L domain which has been used successfully in L domain exchange experiments (Li et al., 2002; Puffer et al., 1997). The nIR immunoblot analysis of these mutants expressed by transfection of HEK293T cells found that the YPDL exchange mutant dramatically released VLPs better than the p8 mutant (Fig. 4), showing that the EIAV sequences could functionally replace the PLPPV sequences in MMTV Gag though not at wild-type levels. This result rules out the defect being a failure of the p8 mutant to form VLP due to inappropriate Gag-Gag interactions, since it is improbable that the exchanged EIAV sequence would be able to rescue a structural MMTV assembly defect in this context. Interestingly, a p8YPDL–p14 mutant that combined the p8YPDL mutant with the p14 mutation of PLPPL was consistently less efficient at release than just the YPDL exchange (Fig. 4). This was unexpected since mutating the p14 PLPPL sequence had no effect on VLP release in the presence of p8. Perhaps the p14 PLPPL sequence can mildly assist the YPDL-using Gag in a way that is not apparent in the p8 mutation context. To determine if the EIAV sequence could function in the p14^{NC} position, a p8–p14YPDL mutant that combined the p8 mutation and the replacement of PLPPL with the EIAV sequence (Fig. 4) was constructed. The release of p8–p14YPDL from transfected cells was quite low, demonstrating that YPDL was unable to rescue MMTV budding when placed in NC. The ability of a heterologous L domain to functionally replace the p8 PLPPV sequence suggests that the p8 sequence supplies an L domain function rather than a required structural role in particle formation.

PLPPV can replace the EIAV L domain

To determine if PLPPV alone or with some of its flanking sequences could provide L domain function in a heterologous context, we replaced the YPDL sequence in an EIAV Gag expression construct with either PLPPV, ePLPPV, or an extended PLPPV sequence containing p8 flanking sequences (RRKPLPPVGFAG), ePLxtnd. We chose EIAV Gag for these experiments as prior studies have shown that more types of foreign L domains can function it the EAIV context, especially more so than HIV-1, which has limited tolerance in L domain exchange constructs (Martin-Serrano, Perez-Caballero, and Bieniasz, 2004; Ott et al., 2005; Shehu-Xhilaga et al., 2004). Also, EIAV appears to have only one known L domain, while other routinely studied retroviruses contain two or more functioning core L domain sequences that complicate exchange experiments, including HIV-1 (Gottlinger et al., 1991; Huang et al., 1995; Strack et al., 2003), human T-cell leukemia virus (Bouamr et al.,

2003), Mason-Pfizer monkey virus (Gottwein et al., 2003), murine leukemia virus (Segura-Morales et al., 2005), and Rous sarcoma virus (Dilley et al., 2010; Wills et al., 1994). Testing these EIAV-MMTV chimeras in our release analysis system found that PLPPV could readily restore L domain function to EIAV Gag (Fig. 5). However, inclusion of some of the MMTV flanking sequences around the PLPPV sequence reduced the efficiency of particle release over the PLPPV alone, possibly due to an incompatibility of these extra MMTV sequences with EIAV Gag. Nevertheless, these results show that PLPPV can function as an L domain in EIAV.

The MMTV L domain is position-dependent

Even though they are quite similar, both having a PLPPΦ motif, the motif in the p8 sequence provides release function while the motif in p14^{NC} is dispensable. This could be due to either the sequence difference between them– the p8 motif has a valine while the p14^{NC} motif contains a leucine– or the difference in position, its presence in p8 or p14^{NC}. To examine these possibilities, we altered the p8 PLPPV sequence to PLPPL, creating p8PLPPL that expresses Gag with two PLPPL motifs and also introducing the same change in the p14 mutant, forming p8PLPPL–p14 which contains PLPPL only in the p8 position (Fig. 6). Both constructs failed to release particles efficiently, a defect comparable to that of the p8-p14 mutant which lacks both motifs (Fig. 6). Therefore, the p14^{NC} motif fails to function in the p8 position, indicating a requirement for valine in position 5.

To examine positional dependence, we converted the p14 PLPPL to PLPPV in the p8 mutant, forming p8–p14 PLPPV (Fig. 6). Testing of its release properties revealed the same profound defect in release as the other defective mutants (Fig. 6). Thus, the L domain of MMTV is both positionally and sequence-dependent: efficient release requires PLPPV in p8, similar to the p8YPDL chimera.

Fine mapping of the MMTV L domain

To better understand the importance of the individual residues in the PLPPV sequence, we produced a series of mutants that replaced amino acids in this region of MMTV Gag with alanine and examined the release properties of the mutants (Fig. 7). Similar to the results presented above, in which PLPPV and PLPPL were exchanged, a valine-to-alanine substitution eliminated the release function (p8P^{5}V, Fig. 7). However, singularly altering the leucine or prolines in the motif resulted in a slight or modest reduction in release efficiency, revealing a flexibility in the motif. A p8 P^{1}A /P^{3}A double mutant also exhibited a greater reduction than either P^1 or P^3 alone. Thus, the only strictly required amino acid in the motif is valine.

Discussion

Here, we identify that a PLPPV sequence in the p8 Gag protein provides an L domain function for MMTV Gag, enabling efficient release of VLP. This sequence represents a fourth type of L domain motif used by retroviruses for release from the plasma membrane. PLPPV exhibited key properties of L domains. Mutation of the sequence produced an arrested budding phenotype where particles formed but failed to efficiently release from the

cell and separate from one another. However, unlike the case with some retroviruses, the MMTV L domain sequence was positionally dependent in Gag, functioning only within its natural context in p8: ectopic placement of PLPPV at the PLPPL-containing site in p14[NC] failed to provide release function. The PLPPV sequence functionally substituted for the YPDL L domain sequence in EIAV Gag. Similarly, an EIAV sequence containing YPDL was only able to function in the p8 position but not in the p14[NC] position. Thus, the both PLPPV and YPDL sequences only provided release function in the p8 site, suggesting that MMTV Gag has a more stringent positional requirement than other retroviruses.

Other putative L domain sequences present in MMTV, PLPPL in p14[NC] and PSAP in CA, did not appear to provide any significant release function for MMTV. Mutating the p14[NC] PLPPL sequence readily produced particles and placing the PLPPL sequence in lieu of the PLPPV in p8 failed to efficiently release virus-like particles. Furthermore, mutating both p8 PLPPV and p14[NC] PLPPL in MMTV Gag produced only a marginally lower release defect over the p8 PLPPV mutation alone, suggesting, at best, a minor influence of PLPPL on budding. However, we did observe a minor contribution of PLPPL to the release of MMTV Gag with the YPDL L domain sequence exchange mutant. Perhaps PLPPL in its p14 position binds some complex that assists only YPDL-mediated release.

Since both a small amount of p8 and p14[NC] in MMTV virions is monoubiquitinated, it is likely that both sequences interact with complexes that possess ubiquitin ligase activity. However, since only PLPPV is sufficient for release, PLPPL might interact with a different complex or one that is inactive in release function.

While the p8 PLPPV sequence is critical for budding, another unidentified sequence could also be providing some additional L domain function for MMTV. Many retroviruses have two sequences which additively provide L domain (Dilley et al., 2010; Gottlinger et al., 1991; Gottwein et al., 2003; Heidecker et al., 2004; Segura-Morales et al., 2005; Strack et al., 2003), typically with one providing the majority of the release activity, and the other playing an auxiliary role. While the severity of the p8 PLPPV mutation on release is clear, we cannot exclude this possibility

Alanine scanning of the PLPPV found that the valine was the only clearly required sequence in the motif, consistent with our PLPPL motif exchange result. Mutation of any one of the other residues in the motif produced only a mild reduction in VLP release, however mutation of P^1 and P^3 did have some additive decrease on release. Other proline-rich sequences can have relatively flexible sequence requirements for protein binding, where any one proline in a motif seems able to compensate for the loss of any other (Li, 2005), which seems to be the case here.

One of the difficulties in examining Gag mutants by the presence of particles in the supernatant is that a mutation in Gag might result in virus-like particle production defects due to an interruption of Gag-Gag assembly interactions, rather than to a failure to release. It is especially important to differentiate between these two possibilities since the deletion of p8 interrupts particle production (Zabransky et al., 2010). Our data strongly support PLPPV acting purely as an L domain. First, our microscopy reveals an accumulation of a classic

attached lollipop structure on the surface of cells and virions that fail to separate from one another, the latter presumably being released from the cell en masse rather than by individual budding. A purely Gag-Gag interaction mutant would fail to produce these types of arrested forms. Second, the ability of the EIAV L domain-containing sequences to replace the PLPPV sequence in MMTV Gag strongly suggests that the PLPPV motif mutation did not alter any critical Gag-Gag interactions, since it is highly unlikely that EIAV sequences would fortuitously provide an interaction that would complement the missing MMTV sequences to rescue a putative Gag-Gag assembly defect. Third, the ability of PLPPV to provide release function to EIAV clearly demonstrates that this sequence is an L domain.

Zabransky et al. recently reported that the deletion of the PLPPVGFAG sequence in p8 produces both a particle release and spherical particle assembly defect (Zabransky et al., 2010). Together with our results, it appears that this region has both an L domain and an undefined assembly domain. In their report, the authors noted that the deleted sequence contains a PPXXF EVH1 motif which is associated with actin dynamics (Zabransky et al., 2010). The loss of this site could be an important contributor to the assembly defect portion of this region. It is important to note that several of our mutants that interrupt this site had little or only very modest negative effects on release. In contrast, our valine mutants (p8P^{5}V and p8PLPPL) have a severe release reduction, yet do not interrupt the core EVH1 motif. An MMTV Gag construct carrying a phenylalanine-to-threonine mutation, which removes a critical part of the EVH1 motif, appears to be unstable in cells, so we are unable to directly test the significance of this motif in MMTV assembly (data not shown).

The cellular protein that functionally interacts with PLPPV is not clear. This sequence could be a ligand for a SH3 or WW domain-containing protein, binding consensuses PXXP or PΦPΦ, respectively (Li, 2005; Mayer, 2001; Zarrinpar, Bhattacharyya, and Lim, 2003). While PLPPV resembles the PPLP ligand recognized by a specialized WW-domain present in formin-containing proteins (Chan, Bedford, and Leder, 1996; Ermekova et al., 1997), our data show that the required leucine in this motif is dispensable for efficient release in the MMTV sequence. Nevertheless, a WW domain protein might be recognizing this motif. NEDD4-like HECT ubiquitin ligases, which contain WW-domains, have been implicated in the release pathways of retroviruses and Ebola virus (Freed, 2002; Morita and Sundquist, 2004).

At its core, retroviral budding and release requires cellular proteins and systems. Studies with HIV-1 and EIAV have implicated ESCRT III, most notably members of the CHMP4 and CHMP 2 families and VPS4 as key proteins that pinch off the plasma membrane neck allowing for virus release (Baumgartel et al., 2011; Hurley and Cada, 2018; Johnson, Bleck, and Simon, 2018; Jouvenet et al., 2011; Morita et al., 2011; Muziol et al., 2006; Stuchell-Brereton et al., 2007; Votteler and Sundquist, 2013). It seems likely that retroviruses share this common mechanism yet use different cellular pathways to approach this shared point, dictated by their respective L domains (Votteler and Sundquist, 2013). Our identification of a fourth L domain opens up the possibility that MMTV connects to this shared point from a unique direction that does not depend on proteasome function (Ott et al., 2003). Thus, the identification of this pathway should greatly assist our understanding of retroviral budding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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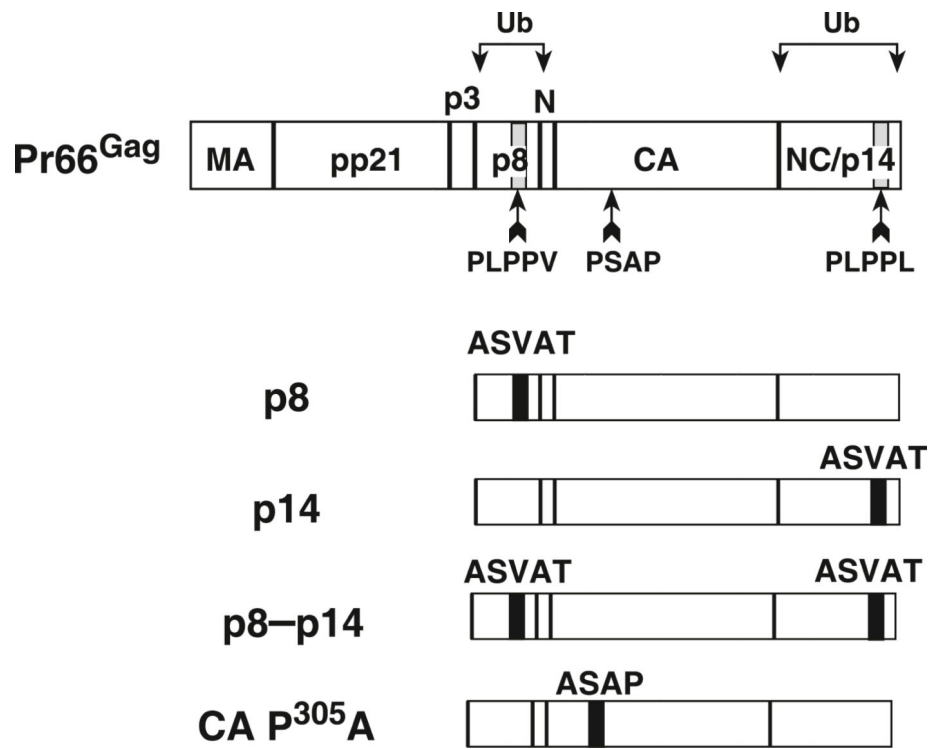
Fig. 1.
A diagram of mutants. MMTV Gag is shown at top with the various PLPPΦ mutations made in the p8-to-NC (p14) to the MMTV expression construct indicated below.
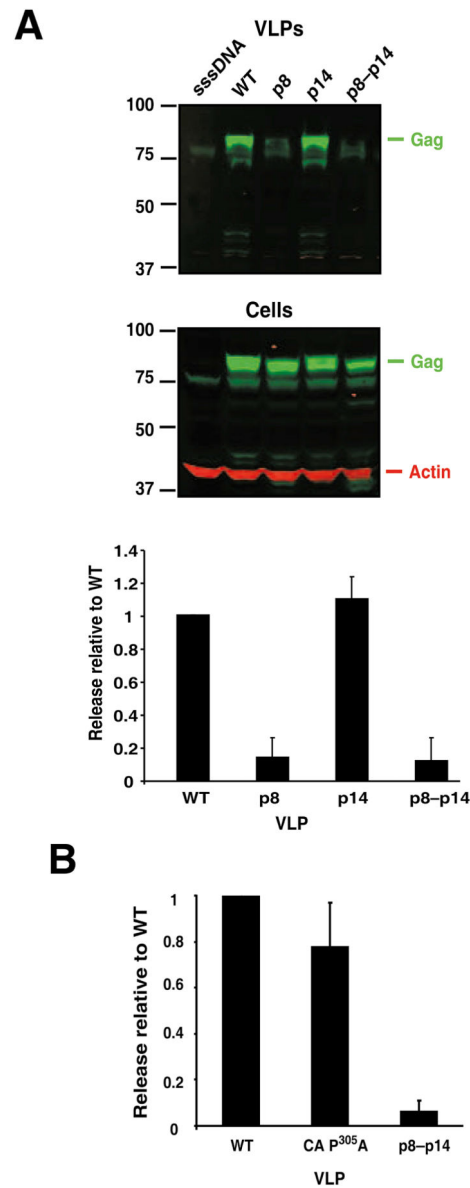
Fig. 2.
Relative release factors of motif mutants. A. An image of an nIR-immunoblot analysis of
VLP and cellular lysates from 293T cells transfected with sheared salmon sperm (sssDNA),
WT, p8, p14, or p8–p14 constructs is presented with Gag staining pseudocolored in green
and actin staining in red. Molecular mass markers are indicated at left and reacting bands
identified at right. A graphical summary of our experiments (at least three independent
experiments) is presented below the blot. B. A graphical summary of average data from 3
independent experiments with the CA P^{305}A is presented. Standard deviations are indicated
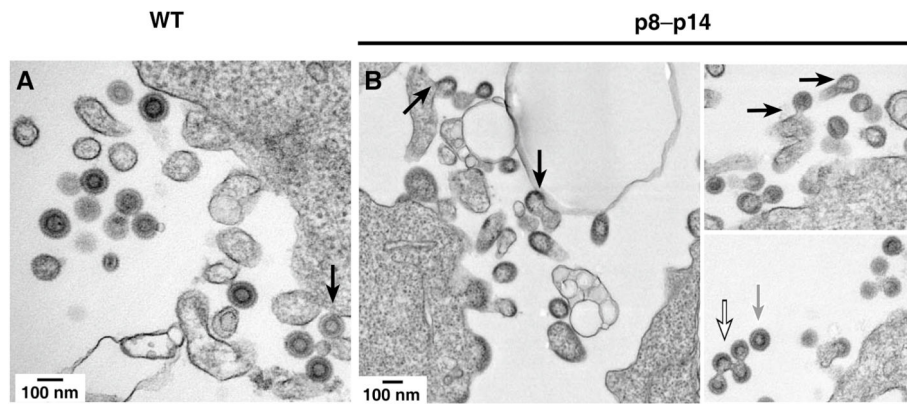by error bars.

Fig. 3.
Thin section transmission electron microscopy. Micrographs of (A) MMTV Gag and (B) p8–p14 mutant VLPs are presented.
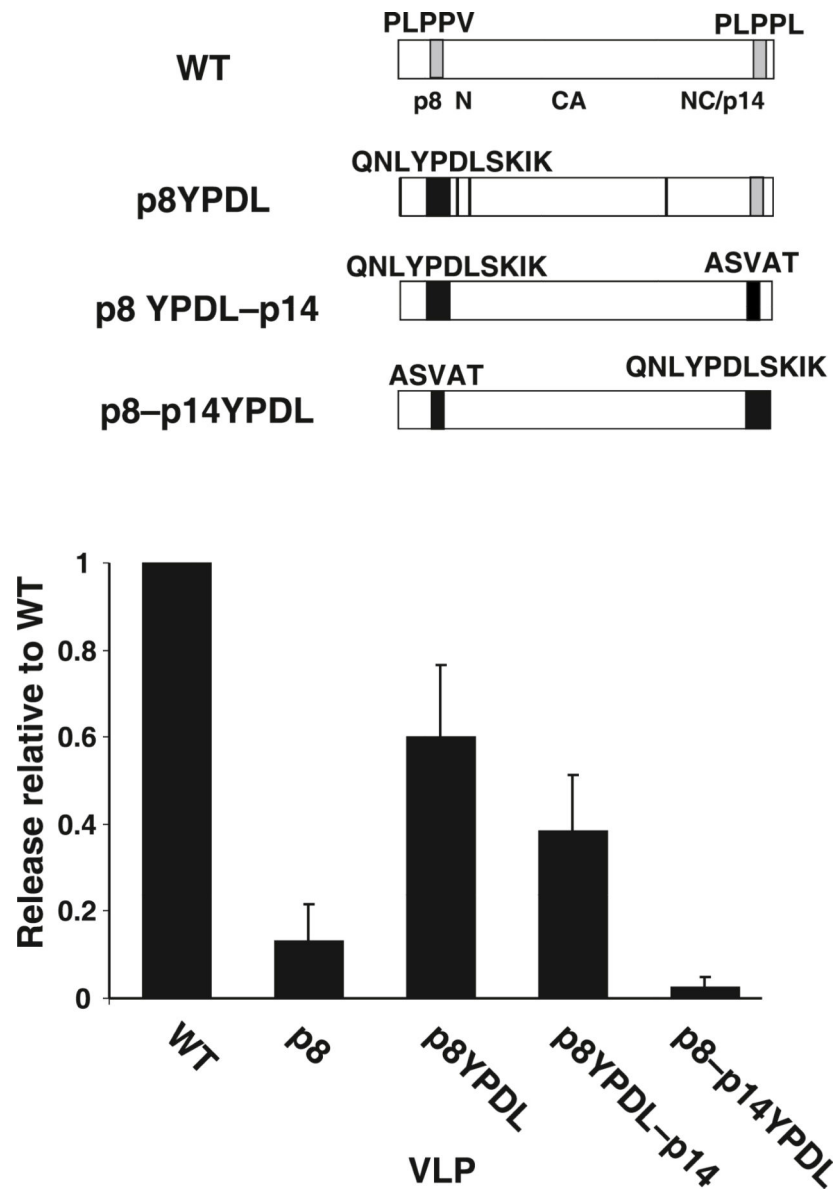
Fig. 4.
Release analysis of YPDL transfer into MMTV Gag. A diagram of mutants tested is presented above a graph of the relative release factors of mutant VLPs from at least 3 independent experiments. Standard deviations are indicated by error bars.
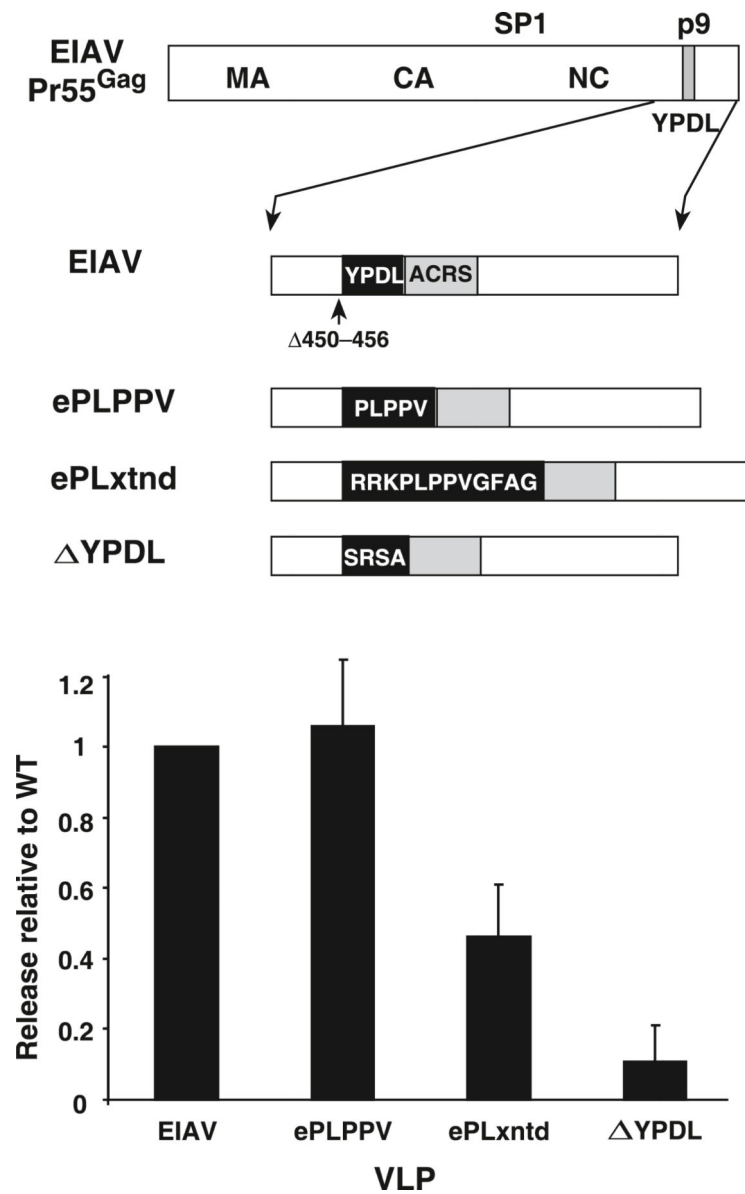
Fig. 5.
Release analysis of PLPPV transfer into EIAV Gag. A diagram of mutants tested is presented above a graph of the relative release factors of mutant VLPs from at least 3 independent experiments. Standard deviations are indicated by error bars.

Fig. 6.
Release analysis of position-dependence mutants. A diagram of mutants tested is presented above a graph of the relative release factors of mutant VLPs from at least 3 independent experiments. Standard deviations are indicated by error bars.
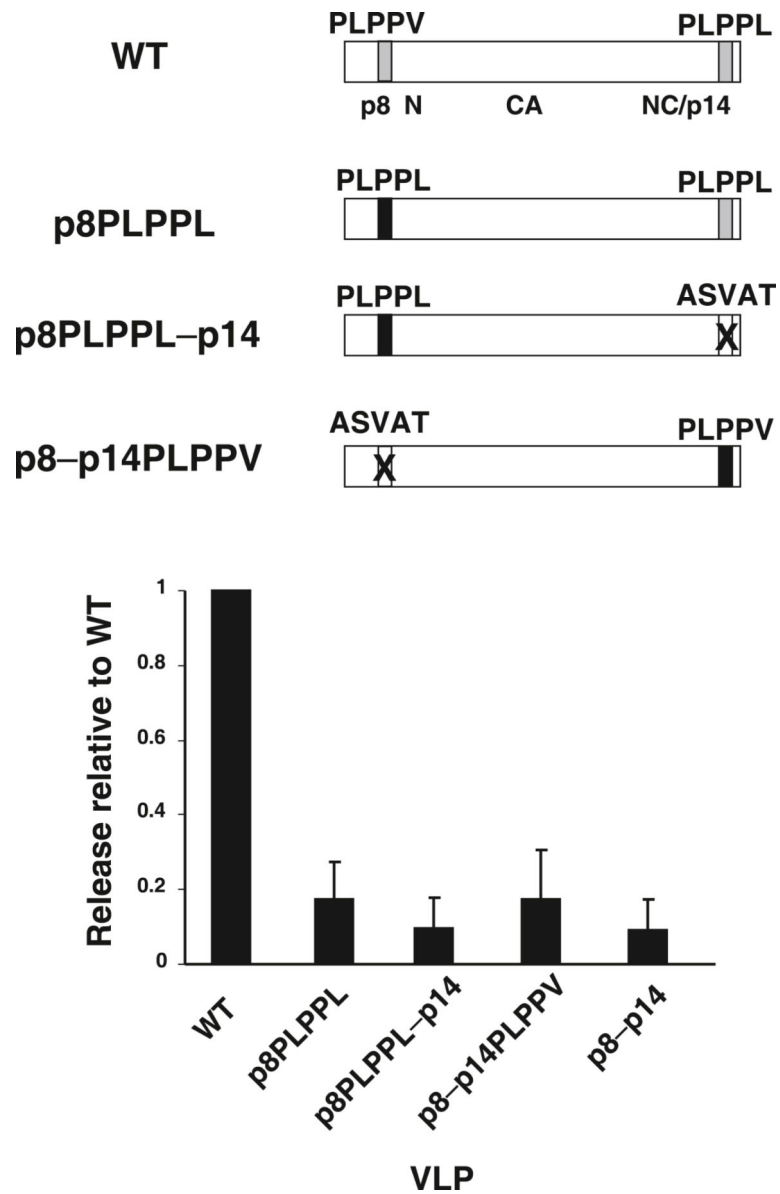
	P^1	L^2	P^3	P^4	V^5
p8P^1A	A^1				
p8L^2A		A^2			
p8P^3A			A^3		
p8P^4A				A^4	
p8V^5A					A^5
p8P^1A/P^3A	A^1		A^3		

Fig. 7.
Release analysis of PLPPV fine-mapping mutants. A diagram of mutants tested is presented above a graph of the relative release factors of mutant VLPs from at least 3 independent experiments. Standa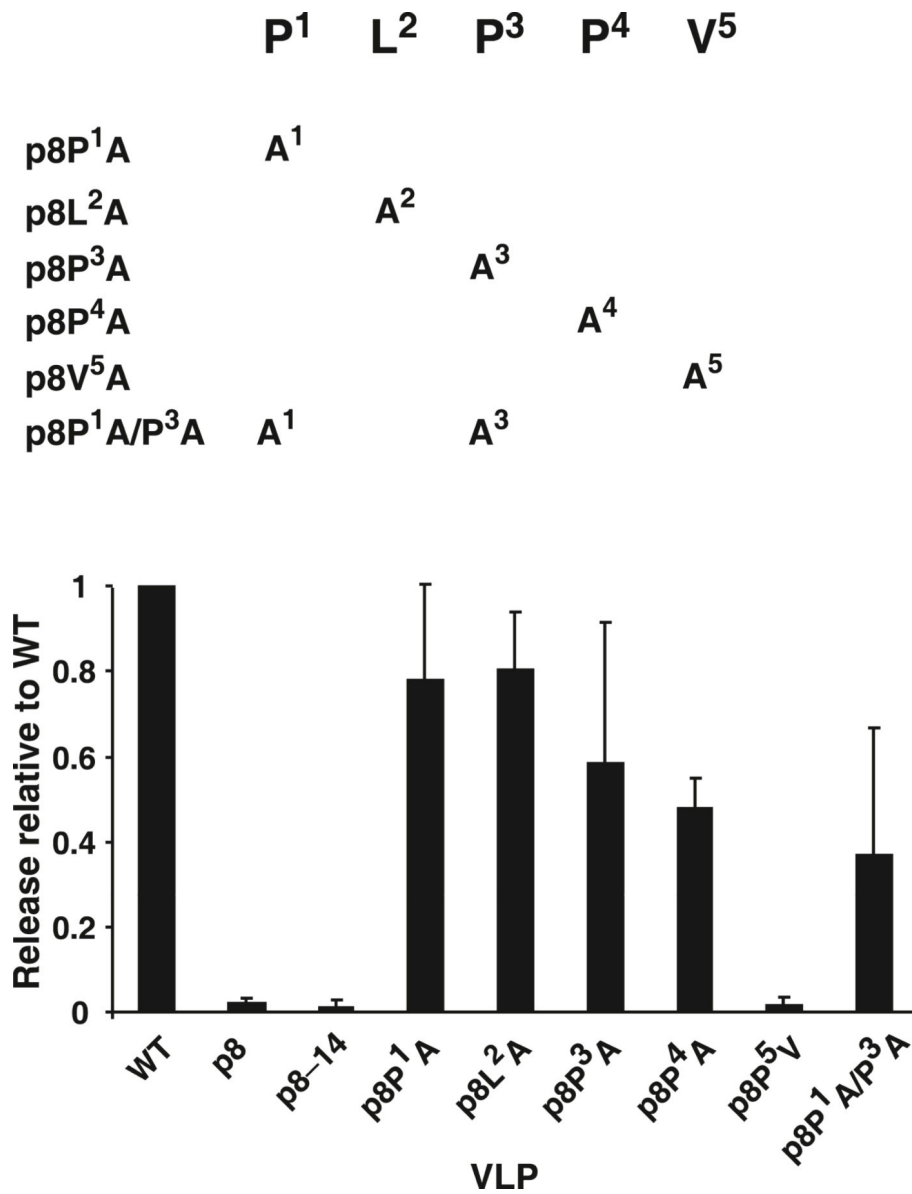rd deviations are indicated by error bars.