Evidence for a New Viral Late-Domain Core Sequence, FPIV, Necessary for Budding of a Paramyxovirus

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Enveloped virus budding has been linked to both the ubiquitin-proteasome pathway and the vacuolar protein-sorting pathway of cells. We show here for the paramyxovirus SV5 that proteasome inhibitors and expression of dominant-negative VPS4(E228Q) ATPase blocks budding. The SV5 matrix (M) protein lacks previously defined late domains (e.g., P[T/S]AP, PPxY, YPDL) that recruit cellular factors. We identified a new motif for budding (core sequence FPIV) that can compensate functionally for lack of a PTAP late domain in budding human immunodeficiency virus type 1 virus-like particles (VLPs). Mutagenesis experiments suggest the more general sequence Ø-P-x-V. The proline residue was found to be critically important for function of this sequence, as substitution of this proline in the SV5 M protein resulted in poor budding of SV5 VLPs and failure of recombinant SV5 virus to replicate normally. Adaptation of mutant virus occurred rapidly, resulting in new proline residues elsewhere in the M protein. We hypothesize that these proline residues act to partially restore virus budding by generation of new motifs that act as suboptimal late domains.

Enveloped viruses are formed by a budding process that occurs following the assembly of viral components at cellular membranes. For several viruses it has been shown that budding occurs in a way that requires the manipulation of host machinery. Protein-protein interaction domains called late domains have been defined in retroviral Gag proteins and in the matrix proteins of some negative-strand RNA viruses. These late domains function to recruit host factors to viral assembly sites, where they assist in virus release (reviewed in references 4 and 24). Disruption of viral late domains often leads to phenotypes in which virus particles assemble normally but fail to be released by membrane fission and instead accumulate as tethered particles on cellular membranes (3, 5, 6, 15).

Several types of late domains which have distinct amino acid sequences have been characterized. P(T/S)AP late domains were found initially in the p6 region of human immunodeficiency virus type 1 (HIV-1) Gag protein (6, 12). A second type of late domain with core sequence PPxY was subsequently identified in the p2b region of Rous sarcoma virus (RSV) Gag protein (48, 49), and a third late domain type having the sequence YP(x)_nL was identified in the p9 region of equine infectious anemia virus (EIAV) Gag protein (32). Additionally, a YRKL sequence has been identified in the matrix protein of influenza virus that has been proposed to act as a late budding domain (13). Each of these late-domain sequences likely functions to bind with a different host factor to facilitate virus budding. P(T/S)AP late domains mediate binding to TSG101 (5, 22, 43), and the host partner protein for YP(x)_nL late domains appears to be AIP1 (21, 40, 44, 46). Both TSG101 and AIP1 are part of the cellular vacuolar protein sorting

(VPS) pathway that allows formation of multivesicular bodies (MVBs), an observation that is significant owing to the fact that virus budding and vesicle budding into MVBs are similar processes in which cytoplasmic cargo is packed into vesicles that bud outward from the cytoplasm. Although the partner proteins for PPxY and YRKL late domains that are relevant to virus budding have not yet been defined unequivocally, PPxY-type late domains have been shown to interact with WW domains from a variety of proteins, such as Nedd4-related E3 ubiquitin ligases (9, 17, 42). It has been proposed that recruitment of Nedd4 family members may allow indirect recruitment of other host proteins, including those involved in MVB formation (40).

Matrix proteins of the negative-strand RNA viruses vesicular stomatitis virus (2, 9, 15) and Ebola virus (8, 19, 20, 22) contain the same P(T/S)AP and PPxY late-domain sequences that were defined originally in retroviruses, and these late domains have been found to be important for the budding of these viruses (8, 15, 19, 20). This suggests that the strategy of recruiting host machinery via late domains is conserved even among distantly related viruses. Other negative-strand RNA viruses, including many of the paramyxoviruses, lack recognizable late-domain sequences in their matrix proteins and, consequently, very little is known for these viruses about how events leading up to membrane fission and particle release occur.

Paramyxovirus particles contain a core of genomic RNA that is encapsidated by nucleocapsid (NP) protein and associated with an RNA-dependent RNA polymerase composed of large (L) and phosphoprotein (P) subunits. This core is surrounded by a lipid envelope that is acquired by progeny virions from host cells during budding. The inner surface of this virion envelope is coated with the soluble matrix (M) protein, which is the most abundant protein in virions and plays key roles in

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virus assembly and budding (reviewed in reference 35). The virion envelope also contains viral glycoproteins (HN and F, mediating attachment and membrane fusion, respectively).

Here, we present evidence that a late budding domain exists within the M protein of the prototype paramyxovirus, parainfluenza virus 5 (SV5). This late domain, which has the core amino acid sequence FPIV, functioned to allow efficient VLP budding of HIV-1 Gag protein lacking its natural PTAP late domain. We show that this new late-domain sequence is important for efficient budding of SV5 particles and is important for replication of a recombinant virus. We also characterize adapted variants (second-site revertants) of this virus that appear to restore late budding function as a result of proline residues introduced into other regions of the M protein.

MATERIALS AND METHODS

Plasmids. Plasmids pCAGGS-SV5 M, pCAGGS-SV5 NP, and pCAGGS-SV5 HN (36), as well as plasmids encoding GFP-VPS4A and GFP-VPS4A E228Q (5), have been described previously. Mutant SV5 M proteins were made by PCR mutagenesis of the M DNA sequence, and their identities were confirmed by DNA sequencing of the entire M genes. SV5 infectious clone pSV5 M.NS is a variant of the previously described pBH276 (10) in which intergenic regions have been modified to facilitate cloning of mutant M genes to generate plasmids pSV5 M.P21A and pSV5 M.V23A. All plasmids encoding Gag proteins were based on the Rev-independent HIV-1_{HXB2} Gag sequence (11). Peptide segments derived from SV5 M protein, Ebola virus VP40 protein, and HIV-1 Gag protein were appended to the C-terminal ends of Gag proteins. For the screening experiments shown below in Fig. 2, annealed oligonucleotides that encode two copies of the candidate peptides were ligated into plasmid WISP02-68, encoding a modified HIV-1 Gag protein in which the PTAP late domain has been changed to LIRL. For the mutagenesis experiments shown below in Fig. 3 and 8, annealed oligonucleotides that encode two copies of the candidate peptides were ligated into plasmid WISP02-69, resulting in Gag proteins that lack p6 and contain the appended peptides in their place. Plasmids were confirmed by DNA sequencing. Additional cloning details will be provided on request.

Assays for virus budding and VLP budding. 293T cells in 6-cm-diameter dishes (70 to 80% confluent) grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum were either infected with SV5, transfected with plasmids encoding SV5 proteins M, NP, and HN to generate SV5 VLPs, or transfected with plasmids encoding HIV-1 Gag protein to generate HIV-1 VLPs. Plasmid DNA amounts were as follows: pCAGGS SV5 M and derivatives, 0.4 μ g; pCAGGS SV5 NP, 15 ng; pCAGGS SV5 HN, 0.5 μ g; pGFP-VPS4A, 100 ng; pGFP-VPS4A E228Q, 100 ng; WISP02-68 VP40LD, 0.6 μ g; WISP02-68 mutVP40LD, 0.6 μ g; WISP02-68 HIV-1LD, 0.6 μ g. Plasmid amounts for the various HIV-1 Gag constructs containing candidate or mutated peptide segments were determined experimentally to achieve equivalent Gag protein expression levels in all cases (details are available on request; there was no correlation between the amount of plasmid used and Gag budding efficiency).

At 16 h postinfection (p.i.) or posttransfection (p.t.), the culture medium was replaced with DMEM containing 1/10 the normal amount of methionine and cysteine and 50 μ Ci of [³⁵S]Promix (Amersham Pharmacia Biotech, Piscataway, N.J.)/ml. At 40 h p.i. or p.t., cells and culture media were collected. For experiments with proteasome inhibitor treatments, cells were pretreated for 30 min with MG-132 at 24 h p.i. or p.t. and then incubated with MG-132 together with Promix for 16 h. For experiments in which VPS4A protein was expressed in virus-infected cells, virus was added to cells at the same time as addition of VPS4A plasmid DNA.

Media fractions containing virions or VLPs were centrifuged at 5,000 $\times$ g for 5 min to remove cell debris and then layered onto 20% sucrose cushions and centrifuged for 2 h at 186,000 $\times$ g. For Gag budding experiments, the VLP pellets were analyzed by immunoprecipitation. For experiments measuring budding of SV5 or SV5 VLPs, particles were further purified by flotation on sucrose gradients as described previously (36).

Immunoprecipitation analysis of proteins was performed as described previously (36). Antisera used were as follows: for SV5 M, HN, and NP proteins, monoclonal antibodies (MAbs) M-h, HN1b, and NP-a (33); for HIV-1 Gag protein, rabbit anti-p24 polyclonal antiserum (Advanced Biotechnologies, Inc., Columbia, Md.); for GFP-VPS4A, Living Colors A.v. polyclonal antibody (Clontech, Palo Alto, Calif.). Polypeptides were analyzed by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels as described elsewhere (28). Quantification was performed using a Fuji BioImager 1000 (Fuji Medical System, Stamford, Conn.). Pulse-chase analysis was performed as described previously (34). Budding efficiency was calculated as the M protein-specific counts in culture media/(counts in culture media + counts in cell lysate) and normalized relative to values obtained in control experiments.

Generation of recombinant viruses from cloned DNA, RNA isolation, and RT-PCR. Recovery of infectious virus from DNA using BSR T7/5 cells was performed as described elsewhere (10) with modifications to avoid use of helper vaccinia viruses (47). The parental virus designated rSV5 and used here as a wild-type (wt) control is recombinant virus generated from plasmid rSV5 M.NS. This virus replicates with the same efficiency as wt SV5. Reverse transcription-PCR (RT-PCR) and nucleotide sequencing of the rSV5 M gene were performed as described previously (10).

Measurements of virus replication kinetics. LLC-MK2 cells in 0.8-cm-diameter wells were infected with viruses at the indicated multiplicity of infection (MOI). After incubation for 1 h at 37°C, the inocula were removed, the cells were washed three times with phosphate-buffered saline, and cultures grown in 0.5 ml of DMEM were supplemented with 2% fetal bovine serum for various periods (0, 1, 2, 3, 4, 5, and 6 days) at 37 or 33°C, as indicated. Culture media were harvested from the cells, and virus titers were measured by plaque assay on BHK-21F cells as described elsewhere (28).

Immunoblotting and electron microscopy (EM). 293T cells in 6-cm-diameter dishes were infected with virus at an MOI of 1.0 PFU/cell. At 48 h p.i., cells and culture media were collected. Virions were purified from media fractions as for VLPs (see above). Virion polypeptides were fractionated by SDS-PAGE on 10% gels, and M protein was detected by immunoblotting with an M protein-specific polyclonal antibody and an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Detection and quantification were performed with a STORM 860 imaging system (Molecular Dynamics, Sunnyvale, Calif.).

Cells were fixed, dehydrated, and embedded in epoxy resin as described previously (36). Sections were examined in a JEOL JEM-100CX II electron microscope. Cell profiles were randomly selected, and 70 to 80 images were acquired of each sample. The length of plasma membrane contained in each image was measured using the public domain ImageJ program (developed at the U.S. National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/ij/>), and the number of viruses was counted. Virions were identified on the basis of shape, surface spikes, and the apparent presence of nucleocapsid.

RESULTS

SV5 budding is sensitive to proteasome inhibitor treatment.

For several enveloped viruses, the late steps of budding have been linked to the ubiquitin-proteasome pathway (reviewed in reference 45). Depletion of the free pools of ubiquitin in cells by treatment with proteasome inhibitors, such as MG-132 or lactacystin, blocks budding of viruses such as HIV-1 (37), RSV (30), and rhabdoviruses (7) at a late stage such that fully assembled particles accumulate at the cell surface, similar to the phenotype observed upon disruption of viral late domain sequences (30, 37). To investigate possible involvement of the ubiquitin-proteasome pathway in paramyxovirus budding, SV5-infected 293T cells were treated with MG-132 and the effect of the drug on virion release was quantified. As shown in Fig. 1A, proteasome inhibitor treatment of cells inhibited SV5 budding. A dose-dependent decrease in budding efficiency was observed, with the highest dose (20 μ M MG-132) resulting in approximately 23% of the normal virus budding efficiency. A similar effect of MG-132 treatment was observed on the budding of SV5 VLPs generated in transfected 293T cells by co-expression of the viral M, NP, and HN proteins (36) (Fig. 1B). The accumulation of viral proteins in infected or transfected cell lysates was not significantly altered by MG-132 treatment of cells, indicating that drug treatment did not adversely affect steps of virus replication that come before the steps of virus assembly and budding. As SV5 budding is sensitive to MG-132 treatment, the data suggest that the mechanism of budding of

FIG. 1. Proteasome inhibitor treatment and dominant-negative VPS4 protein expression block SV5 budding. 293T cells were infected with SV5 (A and C) or cotransfected with pCAGGS plasmids encoding SV5 HN, NP, and M proteins for production of VLPs (B and D). Proteasome inhibitor MG-132 was added at 24 h p.i. or p.t. (A and B). Transfection with VPS4 plasmids was performed at the same time as addition of virus (C) or transfection with plasmids encoding SV5 proteins (D). Cell and culture media fractions were harvested at 40 h p.i. or p.t. Virions and VLPs were purified by sedimentation and flotation centrifugation. Viral proteins were analyzed as described in Materials and Methods. Budding efficiency was calculated as the amount of M protein detected in the culture medium/(amount in culture medium + amount in cell lysate) and normalized relative to values obtained in control experiments.

paramyxoviruses is similar to that of retroviruses and rhabdoviruses.

SV5 budding is sensitive to expression of dominant-negative VPS4A ATPase. Disruption of the MVB formation pathway in cells by expression of dominant-negative versions of class E proteins blocks budding of retroviruses and filoviruses (5, 19, 23, 38, 46). In the case of HIV-1, budding was shown to be arrested at a late stage, possibly even later than the block in virus budding which is observed on late-domain disruption (5, 46). To test the possibility that the MVB formation pathway may be important for paramyxovirus budding, we expressed a dominant-negative version of the VPS4A ATPase (VPS4 E228Q) in SV5-infected 293T cells (Fig. 1C) and in cells transfected to produce SV5 VLPs (Fig. 1D). We found that both SV5 budding and SV5 VLP budding were inhibited by dominant-negative VPS4A protein expression, with budding reduced to about 10% of the control level in each case. These data suggest that SV5 budding may occur in a manner related to the efficient budding of other enveloped viruses that manipulate the host MVB formation pathway to facilitate particle release.

Identification of a segment of SV5 M protein that can function as a late budding domain in the context of HIV-1 Gag protein. Paramyxovirus M proteins appear not to contain the same late-domain sequences that have been defined in retroviral Gag proteins. We hypothesize that paramyxoviruses use an unidentified late domain(s) to recruit cellular machinery to

FIG. 2. Identification of a segment of the SV5 M protein that can function as a late budding domain. (A) Candidate segments of the SV5 M protein were fused to a defective HIV-1 Gag protein that lacks its natural late-domain sequence PTAP, and Gag proteins expressed in VLPs were purified by sedimentation centrifugation and analyzed as described in Materials and Methods. Budding efficiency was calculated as the amount of Gag protein detected in the culture medium/(amount in culture medium + amount in cell lysate) and normalized relative to the value obtained with control Gag protein appended with a segment containing the Ebola virus late-domain sequence. (B) Amino acid sequences of candidate segments that were screened. Eb wt and Eb mut are segments derived from Ebola virus VP40 that contain intact or disrupted versions of the PTAPPEY late domain. HIV-1 wt is a segment derived from HIV-1 Gag protein p6 that contains the late-domain sequence PTAP. (C) Amino acid sequence of SV5 segment B in comparison to corresponding regions of other paramyxovirus M proteins. Full-length paramyxovirus M proteins were aligned using the CLUSTAL W program, version 1.82, with default settings.

virus assembly sites for budding, and that these late domains are likely to be present in the viral M proteins. The functional interchangeability among different types of late domains (1, 2, 18, 27, 49, 50) suggested an experimental approach that could be used to identify new types of late domains. Candidate sequences to be tested for late-domain function were appended to the C-terminal end of a defective HIV-1 Gag protein in which the PTAP late domain had been changed to LIRL, and they were tested for the ability to restore Gag budding function. The validity of this approach is demonstrated in Fig. 2A. Addition of known late-domain sequences derived either from Ebola virus matrix protein VP40 or from HIV-1 Gag protein resulted in restoration of budding function and release of Gag VLPs into the culture medium of transfected cells. Conversely, addition of a mutated version of the Ebola virus late domain (PTAPPEY changed to PTAAAEY) failed to restore budding function and did not elicit efficient release of Gag VLPs. Thus, the HIV-1 Gag VLP assay system could distinguish between a sequence that contains a functioning late domain and one that does not.

Candidate SV5 M protein late-domain sequences were appended to the defective Gag protein. These sequences (Fig. 2B) were chosen on the basis of multiple sequence alignments of paramyxovirus M proteins, with preference given to regions

FIG. 3. Mutagenesis to define a functioning late-domain sequence from SV5 M protein. (A) Scanning mutagenesis of segment B was performed, and the segments were tested for the ability to restore budding to late-domain-defective HIV-1 Gag protein. Budding efficiency was calculated as the amount of Gag protein detected in the culture medium/(amount in culture medium + amount in cell lysate) and normalized relative to the value obtained with control Gag protein appended with wt segment B peptide. (B) VLP budding efficiencies for Gag proteins appended with peptide segments having various modifications in the region 20-FPIV-23.

containing conserved proline residues, as proline residues have been found to be important for the functions of previously identified late domains (12, 15, 22, 49). A segment was identified, segment B, that restored budding function to the defective Gag protein to a level similar to that of the positive controls (Fig. 2A). Other SV5 M protein-derived candidate sequences lacked this ability (Fig. 2A and data not shown),

although partial restoration of Gag budding was observed in some cases (e.g., segment D). The amino acid sequence of segment B of SV5 M protein (residues 15 to 27) was compared to the corresponding regions of other paramyxovirus M proteins (Fig. 2C). Mumps virus and Newcastle disease virus, which are closely related to SV5, contain similar sequences at analogous positions of their M proteins, in particular F20 and P21 (Fig. 2C). The more distantly related Nipah virus, measles virus, and Sendai virus M proteins also contain proline residues in this region; however, these proline residues are not preceded by aromatic residues such as phenylalanine.

Mutagenesis to define a sequence from the SV5 M protein that acts as a late domain for budding of HIV-1 Gag. To determine which amino acid residues within segment B are important for late-domain function in the context of HIV-1 Gag, scanning mutagenesis was performed (Fig. 3A). Three amino acid residues were identified which when altered resulted in reduced late budding function in Gag protein. The most severe defect was observed on deletion of P21, which led to almost no detectable budding of Gag VLPs. Significant, but less severe, defects were observed on alteration of residues F20 and V23. These data suggest that within segment B, the residues most important for budding function are located in the tetrapeptide 20-FPIV-23. This short sequence was further characterized by mutagenesis, as shown in Fig. 3B. For efficient Gag budding, residue F20 could be replaced with tyrosine or tryptophan but not alanine or leucine, suggesting the importance of an aromatic residue at this position. Residue P21 was critical for late-domain function, as changes to alanine, glycine, leucine, or serine residues caused very severe defects in Gag budding. Mutation of residue I22 to alanine, leucine, serine, or lysine had no effect on Gag VLP budding, and mutation of

FIG. 4. Alterations to 20-FPIV-23 in intact M protein cause poor budding of SV5 VLPs. (A) Mutant M proteins were coexpressed with SV5 HN and NP proteins in transfected 293T cells, and the efficiency of VLP release was measured. (B) Quantification of results from three experiments performed as for panel A, with standard deviations indicated. Budding efficiency was calculated as for Fig. 1. (C) Stability of mutant SV5 M proteins. 293T cells were transfected to express the indicated SV5 M proteins. A pulse-label and chase protocol was performed, and proteins were immunoprecipitated and analyzed by SDS-PAGE.

FIG. 5. Amino acid sequence analysis and replication kinetics of second-site adapted viruses. (A) Comparison of amino acid sequence of recombinant SV5 containing P21A and its adapted viruses (ada1 and ada2) that contain new second-site proline residues. (B to D) Replication kinetics of nonadapted and adapted recombinant viruses with alterations to M protein in the region 20-FPIV-23. LLC-MK2

FIG. 6. Adapted viruses containing the M.P21A mutation bud poorly. 293T cells were infected with the indicated viruses at an MOI of 0.4 PFU/cell, and budding efficiency was measured. (A) rSV5 M.P21A-ada1 infection at 37°C led to reduced accumulation of M protein in the cell. (B) Virus infection at 33°C led to normal accumulation of modified M proteins in all cases, and virus budding efficiency was measured as the fraction of total SV5 M protein detected in virions, normalized to the value obtained with rSV5. (C) Quantification of results from three experiments performed as for panel B, with standard deviations indicated.

residue V23 to these same amino acid residues led to moderate budding defects in all cases. These mutagenesis results, though not exhaustive, suggest a tetrapeptide sequence defined as Ø-P-x-V that can function as a late budding domain in the context of HIV-1 Gag protein.

Alteration to 20-FPIV-23 within the SV5 M protein causes poor budding of SV5 VLPs. Mutations that disrupt function of the 20-FPIV-23 late-domain sequence in Gag protein were then introduced into the intact SV5 M protein, and the formation of SV5 VLPs was studied. Mutant M proteins were coexpressed with SV5 HN and NP proteins to generate SV5 VLPs. In each case, mutation of M protein resulted in a significant reduction in VLP release (Fig. 4A and B). Mutation of residues F20 and P21 led to defects in VLP release that were greater than 20-fold, whereas mutation of residue V23 led to a defect that was less severe. Thus, although in some cases late domains can exhibit context dependence in their function (20, 41), the FPIV sequence was found to be important both in the context of HIV-1 Gag protein and in its native context within the SV5 M protein. To rule out the possibility that the poor budding of SV5 VLPs was due to the mutations causing mal-

cells were infected with viruses at the indicated MOI, incubated at 37 or 33°C, and culture media were harvested at various times p.i. Virus titers were determined by plaque assay. Values plotted represent averages of results from two experiments.

folding of the M protein, the stability of the M protein was assessed in a pulse-chase analysis. The mutant M proteins were found to be very stable over a 5-h period, similar to the stability of the wt M protein (Fig. 4C). Mutant M proteins and wt M protein also showed similar reactivities with a panel of conformation-specific MAbs in immunoprecipitation experiments (data not shown). Based on these data, it seems unlikely that the poor VLP budding directed by the mutant M proteins is due to overall M protein misfolding, but more likely is due to a specific function of the 20-FPIV-23 sequence in virus budding.

Recombinant SV5 containing M.P21A adapts quickly, acquiring second-site proline residues in the M protein. To assess further the importance of the 20-FPIV-23 tetrapeptide for SV5 budding, recombinant viruses harboring mutant M proteins were generated by using an SV5 reverse genetics system (10). Recovered virus containing the mutation M.P21A was found initially to be barely viable, but on passaging the virus was able to replicate to a titer of greater than 10^6 PFU/ml. RT-PCR sequence analysis of viral RNA corresponding to the M gene showed that in addition to the engineered M.P21A mutation, the passaged virus (rSV5 M.P21A-ada1) also contained a single nucleotide substitution changing residue S369 to proline (Fig. 5A). rSV5 M.P21A was generated independently a second time from cloned DNA. On this occasion, recovered virus was barely viable, and again, adaptation of virus occurred. This recovered virus (rSV5 M.P21A-ada2) contained a single nucleotide substitution converting residue L336 to a proline residue (Fig. 5A). Thus, in two independent cases, replication of rSV5 M.P21A in tissue culture led to single nucleotide substitutions within four passages, creating two different proline residues in the C-terminal region of the 377 residue M protein, far distant from the engineered N-terminal M.P21A mutation. Although the engineered mutation to convert residue P21 to alanine was a simple C-to-G nucleotide substitution, we have not yet observed a direct reverted virus; both adapted viruses still contain alanine residues at position 21.

The replication kinetics of mutant viruses were analyzed in multiple-step growth curve experiments. For experiments with unadapted rSV5 M.P21A (Fig. 5B), LLC-MK2 cells were infected at an MOI of 0.001 PFU/cell, as this was the highest possible MOI given the low titers of rSV5 M.P21A virus stocks. rSV5 M.P21A barely replicated in these experiments, reaching a maximum titer of less than 10^3 PFU/ml. In contrast, second-site revertant viruses (rSV5 M.P21A-ada1 and rSV5 M.P21A-ada2) reached titers close to 10^7 PFU/ml. None of these viruses replicated as efficiently as the wt virus (10^8 PFU/ml). Additional virus replication studies were performed using an input MOI of 0.01 PFU/cell. These experiments confirmed that the replication efficiencies of second-site revertant viruses were restored only incompletely in comparison to the wt virus. Despite a moderate defect for M.V23A protein in VLP budding assays, recovered virus rSV5 M.V23A showed growth kinetics that were similar to wt SV5 and nucleotide sequence analysis of the M gene showed no additional nucleotide changes.

Adapted viruses having the M.P21A mutation bud poorly. As rSV5 M.P21A-ada1 and rSV5 M.P21A-ada2 are replication defective in comparison to the wt virus (Fig. 5), the efficiency of virus assembly and budding was measured. Parallel bio-

chemical and EM experiments (Fig. 6 and 7) with the unadapted M.P21A mutant virus were not possible due to the very low titer to which this virus replicates. Budding experiments were performed at 33°C (Fig. 6B), as infection with one of the adapted mutant viruses (rSV5 M.P21A-ada1) led to reduced accumulation of M protein in cell lysates at 37°C (Fig. 6A). Quantification of virus budding at 33°C (Fig. 6B and C) showed that budding was reduced to about 25% of wt efficiency in the case of rSV5 M.P21A-ada1. rSV5 M.P21A-ada2 had a less severe defect in which budding efficiency ranged from 50 to 65% of wt both at 33 and 37°C. These data indicate that, despite second-site revertant mutations, both of these viruses still exhibit detectable defects in assembly and/or budding. Quantitation of virus replication kinetics at 33°C (Fig. 5C and D) led to results that were not significantly different from those obtained at 37°C, especially at time points later than 1 day p.i. Thus, underaccumulation of M protein in cells infected with rSV5 M.P21A-ada1 at 37°C may have been transient and did not substantially affect the replication of the virus. rSV5 M.V23A exhibited no defects in assembly and budding (Fig. 6B and C), consistent with virus replication results, possibly indicating that the VLP assay is more sensitive for changes in budding than the virus release assay.

To determine if the assembly-budding defect for rSV5 M.P21A-ada1 occurs at a late step of budding, thin sections of cells were examined by EM. Disruption of late budding domains in some retroviruses, such as HIV-1, causes an increase in the number of budding structures visible at the plasma membrane, tethering of virus particles to cells by thin membrane stalks, and the tethering of particles to each other (3, 5, 6). For rSV5 M.P21A-ada1 both spherical and filamentous budding structures exhibiting typical paramyxovirus morphology were observed at the plasma membranes of infected 293T cells, and there was no obvious change in the frequency of these structures in comparison to wt virus-infected cells (Fig. 7A). A quantitative approach to late budding efficiency was developed to investigate if the SV5-adapted mutants showed a more subtle budding defect than that observed with late-domain-defective HIV-1. Culture supernatants were harvested from the same cells infected with rSV5 M.P21A-ada1 that were subjected to EM analysis, to quantify the amount of released virions. Virions were purified by sedimentation and flotation ultracentrifugation, and the amount of M protein was quantified after immunoblotting (Fig. 7B). The quantity of virions released from rSV5 M.P21A-ada1-infected cells (based on M protein content) was found to be approximately 30% of that released from wt virus-infected cells. Thin sections of the same infected cells were examined by EM to quantify the amount of SV5 budding structures, which were identified by morphological criteria (presence of glycoprotein spikes, visible nucleocapsid strands) and the number of budding structures per unit length of plasma membrane was determined. This number was found to be similar between rSV5 M.P21A-ada1-infected cells (0.26 budding structures per μm) and wt virus-infected cells (0.34 budding structures per μm). Release efficiency (the efficiency with which budding structures on the cell are converted to released virions) was estimated by calculating the ratio of released particles to budding structures per unit of membrane (Fig. 7C), and release efficiency for the mutant adapted virus was 38% of that calculated for the wt virus. These data are

C

	<u>rSV5</u>	<u>rSV5 M.P21A-ada1</u>
Budding structures counted	263	249
µm of membrane	767.4	942.9
Budding structures / µm membrane	0.34	0.26
Amount of released virions	1.0	0.29
Release efficiency ^a	2.9	1.1

^aRelease efficiency defined as released virions / (budding structures / µm membrane)

FIG. 8. Alterations to SV5 M protein found on adaptation of rSV5 M.P21A result in improved late-domain function in HIV-1 Gag protein. Segments of SV5 M protein containing the alterations observed in adapted viruses were fused to late-domain-defective HIV-1 Gag protein. (A) Amino acid sequences of adapted versions as well as the corresponding wt (unadapted) versions of peptide segments that were fused to Gag protein. (B) 293T cells were transfected to express the modified Gag proteins, and VLP budding was measured. Budding efficiency was calculated as the fraction of total Gag protein detected in VLPs, normalized to the value obtained with Gag protein appended with the FPVIV-containing segment B peptide.

consistent with 20-FPIV-23 functioning as a late domain for SV5 budding and that adaptation of rSV5 M.P21A-ada1 failed to restore completely its late budding function.

Second-site mutations to SV5 M protein found on adaptation of rSV5 M.P21A result in improved late-domain function in HIV-1 Gag protein. To test if SV5 M gene sequences created by second-site mutations on adaptation of virus acted as new M protein late domains, we appended segments of SV5 M protein containing these sequence alterations to late-domain-defective HIV-1 Gag protein and measured Gag VLP budding. Amino acid sequences of the adapted and wt M protein segments are shown in Fig. 8A. In both cases, it was found that the adapted segments restored budding function to the late-domain-defective Gag protein more effectively than the unadapted segments (Fig. 8B). Furthermore, alteration L336P (found in adapted virus rSV5 M.P21A-ada2) caused better budding of HIV-1 Gag protein than alteration S369P (found in adapted virus rSV5 M.P21A-ada1). Thus, the budding efficiencies associated with these sequences correlate well between the SV5 budding

FIG. 7. Evidence for a late defect in the budding of rSV5 M.P21A-ada1. 293T cells were infected with rSV5 or rSV5 M.P21A-ada1 at an MOI of 1.0 PFU/cell. At 48 h p.i., cells and culture media were collected. (A) Cells were sectioned and examined by EM. (B) Virions from media fractions were purified and quantified by immunoblotting using an M protein-specific polyclonal antibody. Values represent the amount of M protein-specific signal, normalized to the value obtained for the rSV5 wt control. (C) Estimation of release efficiency. SV5 budding structures were identified by EM using morphological criteria, and the number of budding structures per unit length of plasma membrane was determined. Release efficiency was estimated by calculating the ratio of virions released to the number of budding structures per unit of membrane length.

assay and the Gag budding assay. It is noted that neither of the segments derived from adapted virus M proteins restored Gag budding as effectively as the original segment B. These results are consistent with the possibility that adaptation of rSV5 M.P21A led to creation of new proline-based motifs that partially restored late-domain function to allow virus budding.

DISCUSSION

We describe here an experimental approach using mutant HIV-1 Gag protein fusions that should be generally applicable for discovering novel late domains, based on the idea that many viral late domains are functionally interchangeable and position independent. Using this strategy, we identified a motif having a core amino acid sequence of FPIV from the M protein of the paramyxovirus SV5 that functions as a late domain for the budding of HIV-1 Gag protein. Although addition of this sequence to PTAP-disrupted Gag restored VLP budding levels significantly, these levels were not fully restored to the level observed for wt Gag with PTAP in its native context; furthermore, we were unable to rescue the infectivity of a PTAP-defective proviral clone by coexpression of the FPIV-appended Gag protein. Nonetheless, this FPIV late-domain sequence was found to be important for budding of SV5-like particles and for replication of a recombinant virus generated by SV5 reverse genetics. Virus in which the critical proline residue of this motif was changed to alanine was found to be genetically unstable, and adapted variants arose quickly. None of the adapted viruses characterized to this point has restored the proline residue to regenerate the original FPIV sequence; rather, adaptation of virus resulted in new proline residues introduced near the C terminus of M protein. Replication was greatly improved for adapted viruses compared with the original mutant virus but was not restored to the level of wt virus, and budding defects were still observed that appeared to affect a late step of virus budding judged by quantification of virus budding structures on the cell surface compared with the quantity of released particles. Segments of M protein that were altered by introduction of proline residues in adapted viruses were found to have improved late-domain function compared with the original, unadapted segments based on the ability to restore budding function to late-domain-disrupted HIV-1 Gag protein, suggesting that adaptations may have improved virus replication by creating new, suboptimal proline-based late domains in M protein to compensate for lack of the original FPIV late domain.

Late budding defects for retroviruses and rhabdoviruses have been characterized principally by examination of infected cells using EM. Failure of virus particles to pinch off and be released leads in some cases to overaccumulation of budding structures at the cell surface (3, 6, 15), as well as morphological defects such as visible tethering of particles to the cell surface by thin membrane stalks (3, 5, 6, 40, 46). We did not observe overaccumulation of virus particles at the cell surface or morphological defects in virions in the case of rSV5 M.P21A-ada1. One possible explanation is that the block for this virus is not at the same late step of pinching off, but rather affects an earlier step of virus assembly. Another possibility is that mutant buds are unstable and rapidly collapse back into the cytoplasm rather than accumulate. Alternatively, it is possible

that a late step of budding has been affected, but because the virus has adapted and regained much of its budding function the defect is difficult to detect by EM. Here, we have attempted to quantify late budding efficiency by calculating both the amount of budding structures at the cell surface per unit length of membrane and also the quantity of released virus particles from the same infected cells. Using this method we calculated a 2.6-fold decrease in late budding efficiency for rSV5 M.P21A-ada1 in a situation where we were unable to discern a defect based on examination of infected cells by EM alone. This approach may be generally useful in situations where moderate defects in late budding efficiency are suspected.

Budding of SV5 and SV5-like particles was reduced by treatment of cells with the proteasome inhibitor MG-132. Inhibition of proteasome function prevents recycling of ubiquitin that is attached to proteins targeted for degradation, thereby depleting free ubiquitin levels in the cell (45). Similar effects of proteasome inhibitor treatment on virus budding have been observed for retroviruses such as HIV-1 (37) and RSV (30), which use PTAP and PPxY late domains for budding. In contrast, budding of EIAV, which uses a YPDL late domain, is unaffected by proteasome inhibitor treatment (25, 29, 38). Among the negative-strand RNA viruses, proteasome inhibitor treatment was found to affect the budding of vesicular stomatitis virus (7), which uses a PPxY late domain, but not the budding of influenza virus (14, 16), for which a YRKL sequence has been proposed to function as a late domain (13). Sensitivity of virus budding to proteasome inhibitors has been suggested as being specifically related to the type of late domain used (26, 38), as budding of EIAV becomes sensitive to proteasome inhibitor treatment when its YPDL late domain is replaced with PTAP or PPxY late domains (38). This would suggest that the late domain(s) used by the paramyxovirus SV5 falls into the same category as PTAP and PPxY late domains that function in a way that confers sensitivity of virus budding to proteasome inhibitor treatments.

Budding of SV5 and SV5-like particles was also reduced on expression of a dominant-negative VPS4A ATPase. Interestingly, substantial incorporation of VPS4A E228Q into VLPs was noted, despite the relatively small amount of VLPs produced under these conditions (Fig. 1D). VPS4 mutants disrupt the cellular MVB formation pathway, likely because ATP hydrolysis is required for release of class E proteins from late endosomal membranes. The resulting inhibition of retrovirus budding appears to occur regardless of the specific type of late domain that is used (24). We suspect that VPS4A E228Q was unable to release itself from the sites of SV5 VLP budding and as a result was packaged efficiently into the minor amount of released VLPs, whereas wt VPS4A associated only transiently with VLP budding sites and hence was incorporated less efficiently into VLPs.

The FPIV sequence that we describe here is located near the N terminus of the SV5 M protein. Interestingly, a similar sequence that matches the Ø-P-x-V pattern (YPIV) is present in HIV-1 Gag protein at the junction between the MA and CA domains, although the significance of this sequence in HIV-1 budding is not known. Many well-characterized late-domain sequences are located in unstructured regions of Gag proteins or in the terminal regions of matrix proteins that are likely to be unstructured (4, 31). Adaptation of FPIV-disrupted SV5 to

generate new sequences having late-domain function occurred such that these new sequences were introduced near the C-terminal end of the M protein. Multiple sequence alignment of paramyxovirus M proteins revealed sequences identical or similar to the FPIV sequence near the N termini of M proteins of some paramyxoviruses, such as Newcastle Disease virus and mumps virus, which are closely related to SV5, but no similar sequences were observed at analogous positions of more distantly related paramyxoviruses (Fig. 2C). Further examination of the sequences of other paramyxovirus M proteins revealed in some cases the presence of additional motifs near the C-terminal ends of the proteins that match the Ø-P-x-V pattern. For example, Sendai virus and human parainfluenza virus type 1 (hPIV-1) M proteins each contain YPNV sequences spanning residues 334 to 337 of their 348 residue M proteins. It will be useful to determine if this sequence functions as a late domain in the context of HIV-1 Gag protein as predicted and if it is important for efficient budding of Sendai virus and hPIV-1.

Alteration of a lysine residue (K18) within segment B near the FPIV sequence reproducibly caused enhanced budding of HIV-1 Gag VLPs (Fig. 3). However, a corresponding increase in SV5 VLP budding was not observed when this residue was changed to alanine in the context of the SV5 M protein. To the contrary, VLP budding was reduced in the M.K18A mutant to levels that were 25 to 50% of wt levels (data not shown). In a recent study of RSV budding, efficient budding was found to depend on the presence of one or more lysine residues in Gag protein near the late domain (39). Lysine residues near viral late domains may serve as targets for ubiquitination to allow efficient budding. Further investigation will be required to define the role(s) of lysine residues near the FPIV sequence in SV5 budding.

The Ø-P-x-V amino acid sequence pattern that was defined here in mutagenesis experiments resembles the YPDL motif, or more generally the YP(x)_nL motif, that has been defined in EIAV and, more recently, in HIV-1 Gag p6 (32, 40). However, we believe that the FPIV motif defined here is distinct from the previously characterized YPDL late domain for two reasons: (i) virus budding that is directed by the YPDL late domain was insensitive to proteasome inhibitor treatments, whereas SV5 and SV5-like particle budding were sensitive to treatments of cells with the proteasome inhibitor MG-132; and (ii) we failed to detect an interaction between the FPIV motif and AIP1. YP(x)_nL acts as a protein-protein interaction domain to bind with host protein AIP1 (21, 40, 44, 46), an important component of the MVB formation pathway that links together the ESCRT-I and ESCRT-III protein complexes. We examined possible binding between SV5 M protein and AIP1 and also between the FPIV-containing segment B and AIP1 by glutathione-S-transferase pull-down experiments as well as directed yeast two-hybrid experiments. No evidence was found for an interaction under conditions that detect successfully an interaction between YPDL-containing sequences and AIP1 (data not shown). Consequently, we interpret these data as indicating that the FPIV sequence acts as a late domain that is separate from YPDL and likely recruits a host factor that is distinct from AIP1. However, the net result of recruiting host MVB formation machinery for budding may be the same in both cases, and this notion is supported by the finding that both

EIAV budding (38, 40) and SV5 budding (this study) are blocked when cellular MVB formation machinery is disrupted by expression of dominant-negative versions of class E VPS proteins.

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REFERENCES

- Accola, M. A., B. Strack, and H. G. Gottlinger. 2000. Efficient particle production by minimal Gag constructs which retain the carboxy-terminal domain of human immunodeficiency virus type 1 capsid-p2 and a late assembly domain. *J. Virol.* **74**:5395–5402.
- Craven, R. C., R. N. Harty, J. Paragas, P. Palese, and J. W. Wills. 1999. Late domain function identified in the vesicular stomatitis virus M protein by use of rhabdovirus-retrovirus chimeras. *J. Virol.* **73**:3359–3365.
- Demirov, D. G., J. M. Orenstein, and E. O. Freed. 2002. The late domain of human immunodeficiency virus type 1 p6 promotes virus release in a cell type-dependent manner. *J. Virol.* **76**:105–117.
- Freed, E. O. 2002. Viral late domains. *J. Virol.* **76**:4679–4687.
- Garrus, J. E., U. K. von Schwedler, O. W. Pornillos, S. G. Morham, K. H. Zavitz, H. E. Wang, D. A. Wettstein, K. M. Stray, M. Cote, R. L. Rich, D. G. Myszka, and W. I. Sundquist. 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* **107**:55–65.
- Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine. 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. *Proc. Natl. Acad. Sci. USA* **88**:3195–3199.
- Harty, R. N., M. E. Brown, J. P. McGittigan, G. Wang, H. R. Jayakar, J. M. Huibregtse, M. A. Whitt, and M. J. Schnell. 2001. Rhabdoviruses and the cellular ubiquitin-proteasome system: a budding interaction. *J. Virol.* **75**:10623–10629.
- Harty, R. N., M. E. Brown, G. Wang, J. Huibregtse, and F. P. Hayes. 2000. A PPXY motif within the VP40 protein of Ebola virus interacts physically and functionally with a ubiquitin ligase: implications for filovirus budding. *Proc. Natl. Acad. Sci. USA* **97**:13871–13876.
- Harty, R. N., J. Paragas, M. Sudol, and P. Palese. 1999. A proline-rich motif within the matrix protein of vesicular stomatitis virus and rabies virus interacts with WW domains of cellular proteins: implications for viral budding. *J. Virol.* **73**:2921–2929.
- He, B., R. G. Paterson, C. D. Ward, and R. A. Lamb. 1997. Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. *Virology* **237**:249–260.
- Hermida-Matsumoto, L., and M. D. Resh. 2000. Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. *J. Virol.* **74**:8670–8679.
- Huang, M., J. M. Orenstein, M. A. Martin, and E. O. Freed. 1995. p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. *J. Virol.* **69**:6810–6818.
- Hui, E. K., S. Barman, T. Y. Yang, and D. P. Nayak. 2003. Basic residues of the helix six domain of influenza virus M1 involved in nuclear translocation of M1 can be replaced by PTAP and YPDL late assembly domain motifs. *J. Virol.* **77**:7078–7092.
- Hui, E. K., and D. P. Nayak. 2001. Role of ATP in influenza virus budding. *Virology* **290**:329–341.
- Jayakar, H. R., K. G. Murthy, and M. A. Whitt. 2000. Mutations in the PPPY motif of vesicular stomatitis virus matrix protein reduce virus budding by inhibiting a late step in virion release. *J. Virol.* **74**:9818–9827.
- Khor, R., L. J. McElroy, and G. R. Whittaker. 2003. The ubiquitin-vacuolar protein sorting system is selectively required during entry of influenza virus into host cells. *Traffic* **4**:857–868.
- Kikonyogo, A., F. Bouamr, M. L. Vana, Y. Xiang, A. Aiyar, C. Carter, and J. Leis. 2001. Proteins related to the Nedd4 family of ubiquitin protein ligases interact with the L domain of Rous sarcoma virus and are required for Gag budding from cells. *Proc. Natl. Acad. Sci. USA* **98**:11199–11204.
- Li, F., C. Chen, B. A. Puffer, and R. C. Montelaro. 2002. Functional replacement and positional dependence of homologous and heterologous L domains in equine infectious anemia virus replication. *J. Virol.* **76**:1569–1577.
- Licata, J. M., M. Simpson-Holley, N. T. Wright, Z. Han, J. Paragas, and R. N. Harty. 2003. Overlapping motifs (PTAP and PPEY) within the Ebola virus VP40 protein function independently as late budding domains: involvement of host proteins TSG101 and VPS-4. *J. Virol.* **77**:1812–1819.
- Martin-Serrano, J., D. Perez-Caballero, and P. D. Bieniasz. 2004. Context-dependent effects of L domains and ubiquitination on viral budding. *J. Virol.* **78**:5554–5563.

21. Martin-Serrano, J., A. Yarovoy, D. Perez-Caballero, and P. D. Bieniasz. 2003. Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. *Proc. Natl. Acad. Sci. USA* **100**:12414–12419.
22. Martin-Serrano, J., T. Zang, and P. D. Bieniasz. 2001. HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. *Nat. Med.* **7**:1313–1319.
23. Martin-Serrano, J., T. Zang, and P. D. Bieniasz. 2003. Role of ESCRT-I in retroviral budding. *J. Virol.* **77**:4794–4804.
24. Morita, E., and W. I. Sundquist. 2004. Retrovirus budding. *Annu. Rev. Cell Dev. Biol.* **20**:395–425.
25. Ott, D. E., L. V. Coren, R. C. Sowder II, J. Adams, K. Nagashima, and U. Schubert. 2002. Equine infectious anemia virus and the ubiquitin-proteasome system. *J. Virol.* **76**:3038–3044.
26. Ott, D. E., L. V. Coren, R. C. Sowder II, J. Adams, and U. Schubert. 2003. Retroviruses have differing requirements for proteasome function in the budding process. *J. Virol.* **77**:3384–3393.
27. Parent, L. J., R. P. Bennett, R. C. Craven, T. D. Nelle, N. K. Krishna, J. B. Bowzard, C. B. Wilson, B. A. Puffer, R. C. Montelaro, and J. W. Wills. 1995. Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. *J. Virol.* **69**:5455–5460.
28. Paterson, R. G., and R. A. Lamb. 1993. The molecular biology of influenza viruses and paramyxoviruses, p. 35–73. *In* A. Davidson and R. M. Elliott (ed.), *Molecular virology: a practical approach*. IRL Oxford University Press, Oxford, England.
29. Patnaik, A., V. Chau, F. Li, R. C. Montelaro, and J. W. Wills. 2002. Budding of equine infectious anemia virus is insensitive to proteasome inhibitors. *J. Virol.* **76**:2641–2647.
30. Patnaik, A., V. Chau, and J. W. Wills. 2000. Ubiquitin is part of the retrovirus budding machinery. *Proc. Natl. Acad. Sci. USA* **97**:13069–13074.
31. Pornillos, O., J. E. Garrus, and W. I. Sundquist. 2002. Mechanisms of enveloped RNA virus budding. *Trends Cell Biol.* **12**:569–579.
32. Puffer, B. A., L. J. Parent, J. W. Wills, and R. C. Montelaro. 1997. Equine infectious anemia virus utilizes a YXXL motif within the late assembly domain of the Gag p9 protein. *J. Virol.* **71**:6541–6546.
33. Randall, R. E., D. F. Young, K. K. A. Goswami, and W. C. Russell. 1987. Isolation and characterization of monoclonal antibodies to simian virus 5 and their use in revealing antigenic differences between human, canine and simian isolates. *J. Gen. Virol.* **68**:2769–2780.
34. Schmitt, A. P., B. He, and R. A. Lamb. 1999. Involvement of the cytoplasmic domain of the hemagglutinin-neuraminidase protein in assembly of the paramyxovirus simian virus 5. *J. Virol.* **73**:8703–8712.
35. Schmitt, A. P., and R. A. Lamb. 2004. Escaping from the cell: assembly and budding of negative-strand RNA viruses. *Curr. Top. Microbiol. Immunol.* **283**:145–196.
36. Schmitt, A. P., G. P. Leser, D. L. Waning, and R. A. Lamb. 2002. Requirements for budding of paramyxovirus simian virus 5 virus-like particles. *J. Virol.* **76**:3952–3964.
37. Schubert, U., D. E. Ott, E. N. Chertova, R. Welker, U. Tessmer, M. F. Princiotta, J. R. Bennink, H. G. Krausslich, and J. W. Yewdell. 2000. Proteasome inhibition interferes with Gag polypeptide processing, release, and maturation of HIV-1 and HIV-2. *Proc. Natl. Acad. Sci. USA* **97**:13057–13062.
38. Shehu-Xhilaga, M., S. Ablan, D. G. Demirov, C. Chen, R. C. Montelaro, and E. O. Freed. 2004. Late domain-dependent inhibition of equine infectious anemia virus budding. *J. Virol.* **78**:724–732.
39. Spidel, J. L., R. C. Craven, C. B. Wilson, A. Patnaik, H. Wang, L. M. Mansky, and J. W. Wills. 2004. Lysines close to the Rous sarcoma virus late domain critical for budding. *J. Virol.* **78**:10606–10616.
40. Strack, B., A. Calistri, S. Craig, E. Popova, and H. G. Gottlinger. 2003. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. *Cell* **114**:689–699.
41. Strack, B., A. Calistri, and H. G. Gottlinger. 2002. Late assembly domain function can exhibit context dependence and involves ubiquitin residues implicated in endocytosis. *J. Virol.* **76**:5472–5479.
42. Timmins, J., G. Schoehn, S. Ricard-Blum, S. Scianimanico, T. Vernet, R. W. Ruigrok, and W. Weissenhorn. 2003. Ebola virus matrix protein VP40 interaction with human cellular factors Tsg101 and Nedd4. *J. Mol. Biol.* **326**:493–502.
43. VerPlank, L., F. Bouamr, T. J. LaGrassa, B. Agresta, A. Kikonyogo, J. Leis, and C. A. Carter. 2001. Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). *Proc. Natl. Acad. Sci. USA* **98**:7724–7729.
44. Vincent, O., L. Rainbow, J. Tilburn, H. N. Arst, Jr., and M. A. Penalva. 2003. YPXL/I is a protein interaction motif recognized by *Aspergillus* PalA and its human homologue, AIP1/Alix. *Mol. Cell. Biol.* **23**:1647–1655.
45. Vogt, V. M. 2000. Ubiquitin in retrovirus assembly: actor or bystander? *Proc. Natl. Acad. Sci. USA* **97**:12945–12947.
46. von Schwedler, U. K., M. Stuchell, B. Muller, D. M. Ward, H. Y. Chung, E. Morita, H. E. Wang, T. Davis, G. P. He, D. M. Cimbora, A. Scott, H. G. Krausslich, J. Kaplan, S. G. Morham, and W. I. Sundquist. 2003. The protein network of HIV budding. *Cell* **114**:701–713.
47. Waning, D. L., A. P. Schmitt, G. P. Leser, and R. A. Lamb. 2002. Roles for the cytoplasmic tails of the fusion and hemagglutinin-neuraminidase proteins in budding of the paramyxovirus simian virus 5. *J. Virol.* **76**:9284–9297.
48. Wills, J. W., C. E. Cameron, C. B. Wilson, Y. Xiang, R. P. Bennett, and J. Leis. 1994. An assembly domain of the Rous sarcoma virus Gag protein required late in budding. *J. Virol.* **68**:6605–6618.
49. Xiang, Y., C. E. Cameron, J. W. Wills, and J. Leis. 1996. Fine mapping and characterization of the Rous sarcoma virus Pr76^{gag} late assembly domain. *J. Virol.* **70**:5695–5700.
50. Yuan, B., S. Campbell, E. Bacharach, A. Rein, and S. P. Goff. 2000. Infectivity of Moloney murine leukemia virus defective in late assembly events is restored by late assembly domains of other retroviruses. *J. Virol.* **74**:7250–7260.