JOURNAL OF VIROLOGY, July 2005, p. 9038–9045 Vol. 79, No. 14
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.14.9038–9045.2005

Heterologous Late-Domain Sequences Have Various Abilities To Promote Budding of Human Immunodeficiency Virus Type 1

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Received 30 December 2004/Accepted 29 March 2005

Retroviral late (L) domains present within Gag act in conjunction with cellular proteins to efficiently release virions from the surface of the cell. Three different critical core sequences have been identified as required elements for L-domain function: PPPY, PTAP (also PSAP), and YPDL, with different retroviruses utilizing one or two of these core sequences. The human immunodeficiency virus type 1 (HIV-1) L domain is centered around a PTAP sequence in the p6 region of Gag. To assess the ability of heterologous L-domain sequences to be functionally interchanged for those in full-length HIV-1, we produced a series of constructs that replaced PTAP-containing $p6^{Gag}$ sequences with those of PPPY- or YPDL-based L domains. While previous studies had found that L domains are interchangeable in other retroviruses, most of the sequences introduced into $p6^{Gag}$ failed to substitute for PTAP-mediated L-domain function. One exception was the 11-amino-acid p2b sequence of Rous sarcoma virus (RSV) Gag, which could fully restore HIV-1 budding, while a PPPPY sequence exchange alone did not. This suggests that the RSV L domain consists of more than simply its core L-domain sequence. The HIV-p2b chimera was as infectious as the wild type, produced normal virions, and was sensitive to proteasome inhibitors. These results show that L-domain sequences are not necessarily interchangeable. Thus, HIV-1 Gag might have a more stringent requirement for L-domain function than the other retroviruses previously studied.

Human immunodeficiency virus type 1 (HIV-1) assembly takes place in association with membranes (53) and is clearly observed at the plasma membrane in most cell types (11), although in macrophages assembly occurs primarily at internal membranous structures (42, 46). Assembly is a process that is driven by Gag, the primary structural protein of the virion and the only viral protein strictly required for particle formation (53). Gag is a polyprotein that consists of the mature internal structural proteins, minimally matrix (MA), capsid (CA), and nucleocapsid (NC), that are produced from this precursor by viral protease processing (53). (The organization of HIV-1 Gag and the mature Gag cleavage products are presented on the NL4-3 diagram in Fig. 1.) For HIV-1, processing begins during assembly and concludes sometime after the virion buds from the cell (22, 23).

While retroviruses can assemble and form lollipop-like budding structures driven by Gag interactions, late (L)-domain sequences within Gag act in concert with various members of the class E vacuolar protein-sorting (VPS) pathway to efficiently release these budding virions from the cell (reviewed in references 5, 9, and 34). L domains interact with their cellular partners after Gag assembly begins (41), apparently catalyzing the fusion of membranes that is required for pinching off the distended viral buds to release particles (5, 9, 34). Without these interactions, viruses remain tethered to the plasma membrane, arrested at this rate-limiting budding step (7, 15, 62). While the exact mechanism that provides this release function remains unknown, it is clear that HIV-1 and equine anemia virus (EAIV) L domains interact with the ESCRT I/III (endosomal sorting complex required for transport I/III) pathway during assembly and budding (6, 8, 10, 12, 13, 31–33, 35, 43, 49, 51, 54, 56, 57).

Wills et al. initially discovered that the p2b region of Rous sarcoma virus (RSV) Gag, located between MA and CA, was important for efficient release of virus particles (58) and later coined the term "L domain" (38). The essential core sequence in p2b is PPPY (58, 59), a sequence that was later observed in and found to be part of the L domain for the Gag proteins of Mason-Pfizer monkey virus (16, 60), murine leukemia virus (MuLV) (62), and human T-cell leukemia virus type 1 (19, 26). This PPPY sequence can bind members of the HECT family of ubiquitin ligases and appears to functionally interact with several family members: LDI-1 for RSV (24, 55); WWP1, WWP2, and Itch for MuLV (29); BUL1 for Mason-Pfizer monkey virus (61); and Nedd4.1 and WWP1 for human T-cell leukemia virus type 1 (3, 19). The L domain of HIV-1 is centered around a PTAP core sequence (7, 21, 28) found in the N-terminal region of $p6^{Gag}$, the C-terminal protein of $Pr55^{Gag}$ (Fig. 1). This core sequence binds Tsg101 and works in concert with other members of the ESCRT pathway to promote virus budding (6, 8, 10, 12, 13, 32, 33, 35, 43, 56, 57). In addition to the PTAP-based L domain, there is a secondary sequence in $p6^{Gag}$, $L_{41}XXLF_{46}$, that binds AIP1/Alix in the cell, which in turn appears to recruit ESCRT III proteins to assist in HIV-1 Gag release (28, 51). A third core L-domain sequence, YPDL, within the $p9^{Gag}$ protein of EIAV Gag (apparently the $p6^{Gag}$ homolog for EIAV), is required for its L-domain activity (4, 27, 44). The $p9^{Gag}$ protein can bind the AP-2 adapter protein complex (45)

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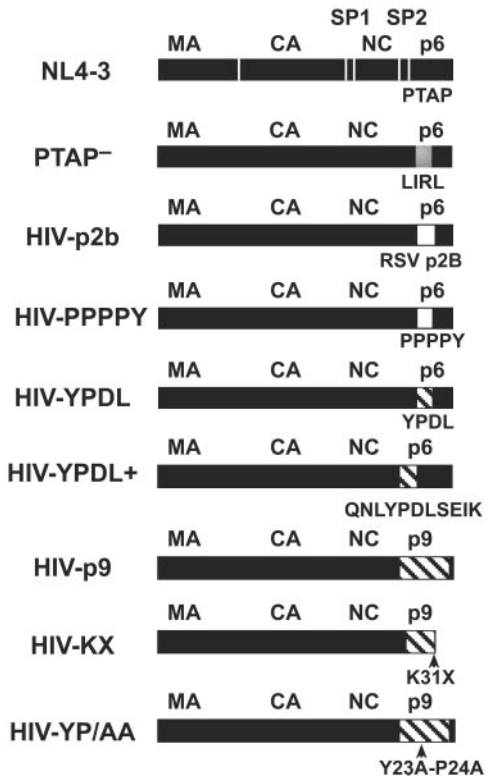

FIG. 1. DNA constructs. Diagrams of the various Gags from the full-length HIV-1 constructs used in this study are presented with HIV-1 sequences denoted in black, RSV in white, and EIAV in hatched shading. Sequence changes are indicated below each diagram. Protease cleavage sites that produce the mature Gag proteins are denoted in the NL4-3 diagram by white lines in Gag.

and recently has been found to bind AIP1 and, ultimately, the ESCRT III complex to mediate particle release (31, 51, 57).

One interesting clue to the nature of viral budding is that L domains can be interchanged between different viruses: HIV $p6^{Gag}$, EIAV $p9^{Gag}$, or a portion of $p6^{Gag}$ that includes PTAP can provide L-domain function for RSV Gag (38). Also, the PPPY sequence in MuLV can be complemented with p2b or $p6^{Gag}$ sequences (62). Similar results were found for EIAV budding: the defect observed by the removal of the $p9^{Gag}$ sequence with its YPDL sequence from EIAV Gag was complemented by $p6^{Gag}$ or p2b sequences (38, 44, 49, 62). In addition to being interchangeable, L domains can act in a positionally independent manner, thereby functioning as true modular domains apart from the remainder of Gag (27, 38, 44, 49, 62). Thus, the various types of Gag appear to exploit different cellular pathways for release with the route taken dictated by the particular type of L domain present. This concept is supported by the ability of Gag L-domain mutants to be rescued in *trans* by coexpression of Gag proteins with heterologous L domains (28, 30, 58).

While the L-domain core sequences are required for function, it is not clear if these four amino acid sequences are entirely sufficient for function, especially when placed within heterologous Gag sequences. Mutating individual sequences flanking the core domain has essentially no effect on L-domain function, indicating that these flanking sequences are not strictly required (21, 38, 44, 59). However, experimental evidence suggests that these core L-domain sequences may not be sufficient for function in heterologous contexts (52).

Although the exchangeability of L domains has been established in several retroviruses, less has been done in the HIV-1 context (38, 44, 62). Recently, HIV-1 Gag with a complete $p6^{Gag}$ deletion was not rescued by coexpression of Gag containing several portions of PPPY-based heterologous L-domain sequences (30). To examine the interchangeability of various L-domain-associated sequences within full-length HIV-1, we tested L-domain sequences from EIAV $p9^{Gag}$ and RSV p2b for their ability to provide L-domain function in an HIV-1 proviral context. Our results show that only the RSV p2b sequence could provide L-domain function for HIV-1, suggesting that heterologous L-domain sequences can have limited ability to promote release of HIV-1.

MATERIALS AND METHODS

DNA mutagenesis. The pNL4-3 infectious molecular clone of HIV-1 (1) was used for these studies and altered by site-directed mutagenesis using PCR-based methods, either by a single amplification with a mutagenic primer method or by two rounds of amplification using the overlap extension procedure (20). The PTAP$^-$ mutant (21), carrying a PTAP-to-LIRL change in $p6^{Gag}$ (amino acids 7 to 10), was provided by Eric Freed (Drug Resistance Program, NCI—Frederick, Frederick, MD). Mutations introduced into $p6^{Gag}$ by this method are as follows: HIV-PPPPY, exchange of $p6^{Gag}$ amino acids 6 to 10 (EPTAP) for RSV p2b amino acids 5 to 9 (PPPPY); HIV-p2b, exchange of $p6^{Gag}$ amino acids 3 to 13 (SRPEPTAPPEE) for RSV p2b (TASAPPPPYVG); HIV-YPDL, exchange of $p6^{Gag}$ amino acids 7 to 10 (PTAP) for EIAV $p9^{Gag}$ amino acids 23 to 26 (YPDL); HIV-p9, exchange of $p6^{Gag}$ amino acids 4 to 36 for EAIV amino acids 4 to 45 of $p9^{Gag}$; HIV-KX, the HIV-p9 construct with a K_{31} to a nonsense codon; HIV-YP/AA, the HIV-p9 construct with the $Y_{28}P_{29}$ of $p9^{Gag}$ mutated to AA; HIV-YPDL+, exchange of $p6^{Gag}$ amino acids 4 to 14 (RPEPTAPPEES) for EIAV $p9^{Gag}$ amino acids 20 to 30 (QNLYPDLSEIK); and HIV-p2b/L45P, which contains an L_{45}-to-P mutation in the remaining $p6^{Gag}$ sequence of the HIV-p2b construct. After construction, the region of DNA that was PCR amplified was sequenced to confirm the mutation and to rule out the possibility of any additional changes introduced during the mutagenesis process.

Cell culture methods. 293T human embryonic kidney cells and HeLa-CD4-LTR-*lacZ* (HCLZ) cell lines were cultured in Dulbecco's modified Eagle's medium, while H9 T-cell leukemia cells were cultured in RPMI 1640. All media were supplemented with 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, 100 U per ml penicillin, and 100 μg per ml streptomycin. All cell culture products were obtained from Invitrogen Inc. (Carlsbad, CA). Transient transfections of 293T cells were carried out using a calcium phosphate mammalian cell transfection (17). Viral protein production was measured by reverse transcriptase assay on cell culture supernatants as previously described (14). The HIV-1 infection assays using HCLZ cells as a *lacZ*-Tat transcomplementation reporter for HIV-1 infection were carried out as previously described (14). The cells were infected with dilutions of transfection supernatants, and the assay was developed for β-galactosidase activity by 5-bromo-4-chloro-3-indolyl-β-galactoside staining 48 h postinfection. Positive-staining cells (those colored blue from infection) were observed by light microscopy and counted to score infection events. Viral replication assays were carried out by infecting 1×10^6 H9 cells with dilutions of virions in a 24-well plate (Costar Corporation, Cambridge, MA), and clarified samples were taken routinely and monitored for the presence of virus by reverse transcriptase activity. All HIV-1 infections were carried out in the presence of 2 μg per ml Polybrene.

Protein analysis. Virions were isolated by centrifugation through a 20% sucrose pad in an SW28 rotor at 112,000 × *g* at 4°C for 1 h. Immunoblot analysis was performed by lysing equal volumes of virion preparations with lysis buffer (125 mM Tris-HCl, pH 7.5, 4% [wt/vol] sodium dodecyl sulfate [SDS], and 1.8% [vol/vol] β-mercaptoethanol) and separating the proteins on a 4 to 20% Tris-glycine gel (Invitrogen). Proteins were electroblotted onto 0.45-μm-pore-size polyvinylidene difluoride membrane (Invitrogen) using a semidry technique (25). Primary goat antisera against $p24^{CA}$ and $p7^{NC}$ were obtained from the AIDS Vaccine Program (National Cancer Institute—Frederick, Frederick, MD). Proteins were detected by development with horseradish peroxidase-conjugated

anti-goat secondary antibody (Biochain Institute, Hayward CA) and the Immunstar horseradish peroxidase substrate kit (Bio-Rad, Hercules, CA) on LumiFilm, Roche Applied Science (Indianapolis, IN). Metabolic labeling of transfected cells with [^{35}S]Met and [^{35}S]Cys was carried out as previously described (47). Briefly, cells were first Met/Cys starved for 1 h before being labeled with Promix, a mixture of 150 μCi of Met and 60 μCi of Cys (Amersham Biosciences, Piscataway, NJ). Virions were isolated by sucrose density centrifugation as described above. Gag was immunoprecipitated from viral and cellular lysates with goat antibodies to p24^{CA} and p7^{NC} and Ezview protein G beads (Sigma). Precipitates were separated on a 10% Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE) gel (Invitrogen) and analyzed with a PMI phosphorimager (Bio-Rad). Signal values were normalized for the relative amount of Met and Cys in the observed products. For proteasome inhibitor studies, 293T cells were transfected 24 h before study. Cells were pretreated with medium containing 10 μM of MG-132 (z-Leu-Leu-Leu-CHO, or zLLL) and lactacystin (Bio-Mol, Plymouth Meeting, PA) for 10 min (immunoblot) or 1 h (during the starvation phase of metabolic labeling) before changing the medium with fresh inhibitor-containing medium for either an 8-h virus collection phase (immunoblot) as previously described (36) or a 6-h metabolic radiolabeling as described above.

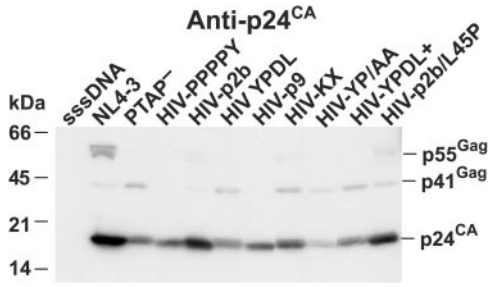

FIG. 2. Immunoblot analysis of virion preparations. A p24^{CA} immunoblot of equal volumes of virions isolated from transfection supernatants is presented. Samples are identified above their respective lanes. Positions of the molecular mass standards are indicated at left, and reactive bands are identified at right. sssDNA, virus preparation isolated from a sheared salmon sperm DNA transfection.

RESULTS

To investigate the interchangeability of L domains within HIV-1, we produced several full-length HIV-1 constructs that replaced portions of p6^{Gag} sequences in the NL4-3 proviral clone with heterologous sequences from either RSV or EIAV L-domain regions to examine them for release-promoting function. The different mutants examined are presented in Fig. 1 and can be classified as either PPPY- or YPDL-based chimeras. Since p6^{Gag} and protease genes overlap, the sequence alterations made in this region were made without introduction of nonsense codons in the *pol* reading frame to maintain protease expression and potential infectivity. Two PPPY-based chimeras were produced with RSV sequences. The HIV-p2b construct had the SRPEPTAPPEE p6^{Gag} sequence in NL4-3 replaced with the complete 11-amino-acid p2b protein sequence (TASAPPPPYVG) that has been shown to provide L-domain function for other Gag proteins (62). To determine if simply the core RSV sequence is sufficient, the HIV-PPPPY construct was produced by replacing the EPTAP p6^{Gag} sequence with the PPPPY RSV core L-domain motif. Five YPDL-based chimeras were made with EIAV sequences. The HIV-YPDL construct was produced by replacing the p6^{Gag} PTAP sequence with the YPDL L-domain core sequence. HIV-YPDL+ was constructed by replacing the RPEPTAP PEES sequence with QNLYPDLSEIK, a p9^{Gag} sequence that previously had been shown to be sufficient for L-domain function (44). The HIV-p9 construct was produced by replacing most of the p6^{Gag} sequence, amino acids 4 to 36, with amino acids 4 to 45 of p9^{Gag}. This portion of p9^{Gag} has been shown to be sufficient for L-domain and other replicative functions in EIAV Gag (4). The N- and C-terminal p6^{Gag} sequences were retained in the HIV-p9 construct to maintain the SP2 (p1^{Gag})-p6^{Gag} cleavage site (GNF^LQS) in the Gag frame and the preprotease leader (p6^{Pol})-protease cleavage site in the *pol* frame, as well as the LXXLF AIP1 binding motif in p6^{Gag} (amino acids 45 to 49). Since the central region of p6^{Gag} is dispensable for HIV-1 replication in vitro (2), this p9^{Gag}-for-p6^{Gag} substitution should not affect HIV-1 Gag functions other than release. Two additional mutants were produced from this HIV-p9^{Gag} construct. The HIV-KX construct is an HIV-p9 derivative that contains a lysine-to-nonsense codon change at p9^{Gag} position 31. In EIAV Gag, this mutation does not affect L-domain function in EIAV (4) but removes a ubiquitin-like sequence that has been proposed to assist EIAV release (39). The HIV-YP/AA construct contains a mutation in the HIV-p9^{Gag} core L-domain sequence, YPDL to AADL, that destroys L-domain function in EIAV (44).

Virion production of mutants. Virions were expressed by transfection of the proviral constructs into 293T cells, and virions were isolated out of 48-h cell culture supernatants. Equal volumes of the preparations were examined by p24^{CA} immunoblot analysis. The intensities of the bands in the PTAP^{-} mutant, all the EIAV sequence exchange chimeras, and the HIV-PPPPY mutant samples were considerably lower than that of the NL4-3 wild-type sample bands (Fig. 2). However, the bands in the HIV-p2b sample had only slightly less intensity than those in the wild-type sample. Values from the reverse transcription assays of supernatants from transfected cells mirrored the immunoblot results (data not shown). None of the viruses had severe protease processing defects (Fig. 2). Wild-type preparations contained unprocessed Gag, Pr55^{Gag}, possibly due to the larger amount of virus present in the sample. While all of the mutants processed Gag, PTAP^{-}, HIV-YPDL, HIV-KX, and HIV-YPDL+ had slight defects in processing, as evidenced by the increased presence of a p41^{Gag} partially processed precursor relative to their respective p24^{CA} levels.

To examine the budding of these mutants further, the release of virions was examined by metabolically labeling Gag in transfected cell cultures with [^{35}S]Met/Cys for 6 h and then immunoprecipitation of Gag from lysates of virion and cytoplasmic preparations with anti-p24^{CA} and anti-p7^{NC} sera. Similar to the immunoblot results, the data showed that nearly all of the chimeric viruses were released at a much lower level than wild type (Fig. 3, Table 1, virions relative to NL4-3). In contrast, the HIV-p2b construct produced nearly wild-type levels of virions. To normalize for potential differences in protein expression, the total amounts of labeled Gag precipitated from the virion preparations and the cell lysates were quantified by phosphorimager analysis (Fig. 3A) and the release factor was calculated: i.e., the amount of virions released in 6 h (Gag pelletable by density centrifugation)/total Gag (pelletable Gag and Gag in cell lysates). (Typical results are provided in Table 1.) Release factors relative to the wild-type NL4-3 construct from three independent experiments revealed that the PTAP^{-}

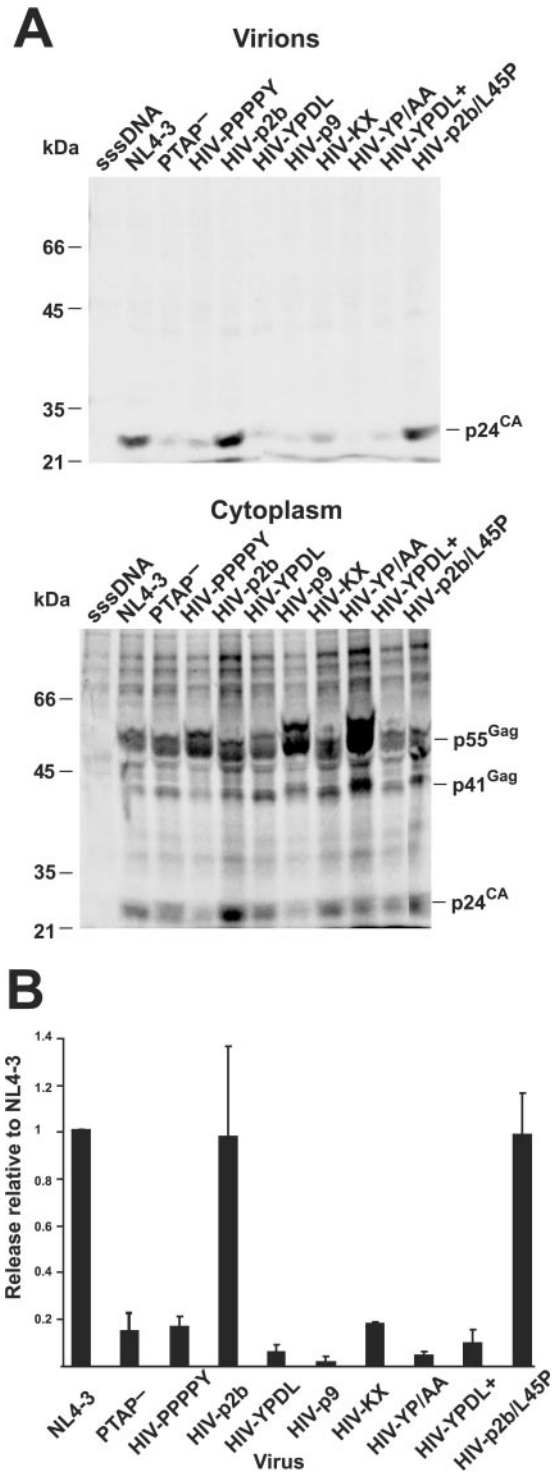

FIG. 3. [^{35}S]Met/Cys metabolic radiolabeling and immunoprecipitation. (A) Phosphorimages of SDS-PAGE gels from a typical radioimmunoprecipitation analysis of metabolically labeled Gag from virion and cytoplasmic lysates are presented. The lysates examined are indicated above each image, and samples are identified above their respective lanes. Positions of the molecular mass standards are indicated at left, and reactive bands are identified at right. sssDNA, virus preparation isolated from a sheared salmon sperm DNA transfection. (B) Graphical results of release factors relative to wild-type virus from three independent experiments are presented.

TABLE 1. Immunoprecipitation of [^{35}S]Met-Cys-labeled Gag

Virus	Amt of virions[a]	Amt of virions relative to NL4-3	Release factor[b]	Release relative to NL4-3
NL4-3	1.9×10^4	1	0.33	1
PTAP$^-$	1.3×10^3	0.07	0.031	0.09
HIV-PPPPY	2.6×10^3	0.1	0.064	0.2
HIV-p2b	2.7×10^4	1	0.27	0.8
HIV-YPDL	1.4×10^3	0.07	0.025	0.07
HIV-p9	7.7×10^2	0.04	0.014	0.04
HIV-KX	4.3×10^3	0.2	0.067	0.2
HIV-YP/AA	1.9×10^2	0.01	0.0014	0.004
HIV-YPDL+	1.4×10^3	0.07	0.035	0.1
HIV-p2b/L45P	2.5×10^4	1	0.32	1

[a] Phosphorimager quantitation of SDS-PAGE gel analysis of Gag immunoprecipitations using p24^{CA} and p7^{NC} antisera in arbitrary phosphorimager pixel units subtracted for local background.

[b] Relative amount of units in particle preparations versus total amount of Gag recovered from particles and cell lysates after 6 h of labeling.

mutant released particles at a 10-fold-lower level (Fig. 3B), a release value similar to that observed by others (15, 21). The core motif exchanges, HIV-PPPY and HIV-YPDL, both had similarly low release values. Therefore, these short core sequences themselves were insufficient to provide L-domain function in this context. Similarly, the HIV-YPDL+ virions failed to release efficiently; therefore, this sequence does not function in our HIV-1 Gag chimera, even though it can provide L-domain function to EIAV (27, 44). The release factors for the HIV-p9 and HIV-YP/AA constructs were considerably lower than those for the wild type and somewhat less than those for the PTAP$^-$ clone. While the HIV-K31X construct also failed to bud efficiently, it released better than HIV-p9, which suggests sequences in the C-terminal region of p9^{Gag} might suppress HIV-1 budding. Taken together, our results show that the EIAV L-domain sequences and the PPPPY core sequence failed to function as L domains in these HIV-1 constructs.

In contrast to most of the HIV chimeras, the HIV-p2b construct budded almost as efficiently as the wild type (Table 1, Fig. 3), revealing that the complete p2b sequence can replace the HIV-1 L-domain function, even though the PPPPY sequences alone could not. Recently, it has been found that an $L_{41}RSLF_{46}$ motif in the C-terminal region of p6^{Gag} binds AIP1 and provides additional L-domain function, although the contribution of this sequence to budding appears to be weaker than that of PTAP (31, 51). Also, this sequence can be deleted with a modest decrease in viral budding (21). The HIV-1 L domain might rely on both of these signals for complete function (28). To test the importance of this sequence in the context of HIV-p2b, we altered a critical AIP1-binding residue in p6^{Gag}, L_{45} to P, to produce HIV-p2b/L45P. This virus was released as efficiently as the HIV-p2b construct in both immunoblot and radioimmunoprecipitation analyses (Table 1, Fig. 2 and 3), showing that an intact AIP binding site was not contributing to the release of HIV-p2b. Thus, the p2b sequence alone appears to be sufficient for L-domain function in this context.

Infectivity of mutants. To determine if these L-domain exchanges affected other aspects of HIV-1 replication, we examined the infectivity of NL4-3, PTAP$^-$, HIV-p2b, HIV-KX, and

TABLE 2. Infectivity and replication of HIV chimeras

Virion	Titer of BCFU[a]	Titer relative to NL4-3	H9 replication (TCID/ml)[b]
sssDNA[c]	1	3×10^{-6}	<1
NL4-3	2.9×10^{5}	1	$\geq 10^{6}$
PTAP^{-}	1.9×10^{3}	7×10^{-3}	<1
HIV-p2b	3.1×10^{5}	1.1	$\geq 10^{6}$
HIV-KX	1.0×10^{3}	3×10^{-3}	$\leq 10^{1}$
HIV-PPPPY	3.4×10^{2}	1×10^{-3}	<1

[a] Titer of blue cell-forming units (BCFU) per ml of clarified transfection supernatant.

[b] Tissue culture infective dose (TCID) per ml as measured by infecting H9 cells with 10-fold dilutions of clarified transfection supernatant. The highest dilution tested was 10^{-7}.

[c] sssDNA, virus preparation from a sheared salmon sperm DNA transfection.

HIV-PPPPY in a single-round Tat *lacZ* complementation assay. The results with transfected cell supernatants revealed that the titer of HIV-p2b was equivalent to that of the wild type (typical results presented in Table 2). Thus, HIV-p2b has wild-type HIV-1 properties. Consistent with this conclusion, HIV-p2b displayed a normal virion morphology by electron microscopy (data not shown). In contrast, the titers of PTAP^{-}, HIV-KX, and HIV-PPPPY were considerably lower than that of wild-type, consistent with their lower release factors (Table 1 and Fig. 3B). Therefore, these L-domain chimeras maintained a degree of infectivity that roughly mirrors their release rate. Replication assays with H9 T cells revealed that the HIV-p2b mutant end-point dilution values paralleled the infectivity results (typical results presented in Table 2). The HIV-KX mutant replicated in H9 cells at a much lower level than the wild type, and the PTAP^{-} and HIV-PPPPY virions showed little capacity to replicate in H9 cells after 5 weeks in culture.

HIV-p2b is sensitive to proteasome inhibitors. We previously have found that the budding of HIV-1 is reduced by proteasome inhibitors (48). However, not all retroviruses are sensitive to proteasome inhibitors: those that use either PPPY or PTAP for L-domain function are sensitive to proteasome inhibitors, while EIAV, which uses a YPDL L domain, or mouse mammary tumor virus, which uses an as yet undefined L domain, release normally under inhibitor treatment (36, 37, 40, 41, 49, 50). Interestingly, rhabdoviruses employing a PPPY L-domain motif are also sensitive to proteasome inhibitors (18). To determine if the release of the HIV-p2b chimera was altered by proteasome inhibitor treatment, we examined p24^{CA} immunoblots of virion preparations produced during 8 h from cells expressing NL4-3 or HIV-p2b in the presence or absence of 10 μM MG-132 and lactacystin. These conditions do not have adverse effects on protein synthesis over this time period (48). The results showed that the proteasome inhibitor-treated cells produced noticeably less HIV, either wild type or HIV-p2b, than the untreated cultures (Fig. 4A). Electronic image capture and signal quantitation analysis of the immunoblot showed that the amount of HIV-p2b virus produced from proteasome inhibitor-treated cells was threefold lower than that of the untreated sample (Table 3). To confirm this observation, we metabolically labeled cells expressing these two viruses and determined the release factor with and without the inhibitors. The results showed that the budding of HIV-p2b was decreased approximately fourfold by the proteasome inhibitors (Fig. 4B). Thus, HIV-p2b maintains a requirement for active proteasomes as predicted (37, 49).

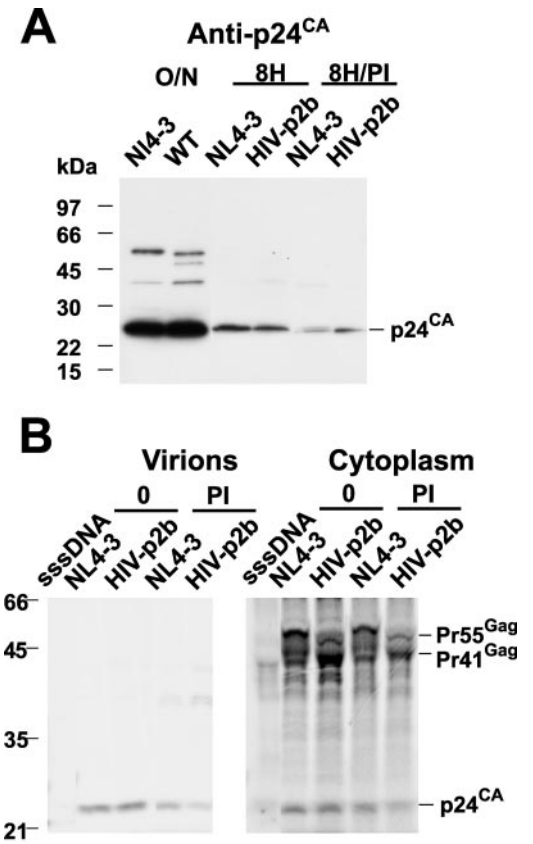

FIG. 4. Proteasome inhibitor studies. (A) Immunoblot of cells treated with or without proteasome inhibitors for 8 h is presented. Samples are identified above their respective lanes. O/N, virion preparations prepared from an 18-h harvest of supernatants from transfected cultures. Positions of the molecular mass standards are indicated at left, and reactive bands are identified at right. (B) Phosphorimages of an SDS-PAGE gel from radioimmunoprecipitation analysis of metabolically labeled Gag from virion and cytoplasmic lysates are presented. Cells were labeled for 6 h in the presence or absence of proteasome inhibitors (MG-132 and lactacystin) as indicated. The lysates examined are indicated above each image, and samples are identified above their respective lanes. Positions of the molecular mass standards are indicated at left, and reactive bands are identified at right. sssDNA, virus preparation isolated from a sheared salmon sperm DNA transfection.

DISCUSSION

We show here that only the p2b sequence of RSV was able to provide L-domain function for HIV-1 out of the seven chimeras tested in our constructs. The other heterologous sequences that we transferred into Gag, the RSV PPPPY core motif or EIAV p9^{Gag} sequences, were unable to functionally replace the PTAP sequence. Thus, they do not function as L domains in these chimeric constructions (i.e., these sequences are not sufficient to mediate efficient release in this context). Other groups have found that the complete HIV-1 L domain appears to involve other sequences in addition to its PTAP sequence, the LXXLF motif (28, 51, 57), and sequences within the NC-SP2 (p1) region (52). It is important to note that our

TABLE 3. Proteasome inhibitor sensitivity of HIV-p2b

Virus[a]	Value by:					
	Immunoblotting			[^{35}S]Met/Cys radioimmunoprecipitation		
	Virion[b]	Value vs untreated	Virion[c]	Value vs NL4-3	Release factor[d]	Release relative to untreated
NL4-3	1.0×10^3	1	1.4×10^4	1	0.23	1
HIV-p2b	7.3×10^2	1	2.1×10^4	1	0.38	1
WT + PI	1.7×10^2	0.2	7.7×10^3	0.5	0.16	0.68
HIV-p2b + PI	2.1×10^2	0.3	2.5×10^3	0.2	0.09	0.24

[a] WT, wild type; PI, proteasome inhibitor treatment.
[b] Arbitrary imager units by Versa-Doc image capture.
[c] Phosphorimager quantitation of SDS-PAGE analysis of Gag immunoprecipitations using $p24^{CA}$ and $p7^{NC}$ antisera in arbritrary phosphorimager pixel units subtracted for local background.
[d] Relative units in particle preparations versus total amount of Gag recovered from particles and cell lysates after 6 h of labeling.

constructs maintained these sequences, so failure to complement the PTAP function seems to arise more from fundamental differences between the exchanged sequences.

One important caveat to this study is that the failure of heterologous sequences to provide L-domain function could be due to subtle factors in the constructs: e.g., the extent of the foreign sequence introduced, the amount of native sequence removed, or the region in the provirus chosen for the sequence exchange. To minimize these complications, the heterologous sequences were placed in the L-domain region of HIV-1 Gag, while maintaining important $p6^{Gag}$ sequences: e.g., *pol* and the AIP1-binding sequences. We also introduced L-domain sequences that can function in other Gag proteins. Additionally, our data showed that these mutant viruses were infectious and that Gag was processed. Thus, the L-domain-deficient virions have no gross defects in other Gag functions. Despite these precautions and results, we cannot eliminate the possibility that these sequences could not provide L-domain function in other HIV-1 contexts.

The failure of the EIAV constructs to supply PTAP function might be due to a difference in factors used by these two L domains. The EIAV YPDL sequence interacts with AIP1 and enters the ESCRT pathway by associating with the ESCRT III complex (31, 51, 57). In contrast, the PTAP sequence in HIV-1 Gag interacts with Tsg101 and enters the pathway at the ESCRT I stage (6, 8, 10, 12, 13, 32, 33, 35, 43, 56, 57), two steps upstream of ESCRT III (5, 34). Thus, these EAIV sequences might not function in HIV-1 due to a requirement for HIV-1 Gag targeting to VPS components found in the pathway before ESCRT III (likely present in ESCRT I and/or II). Consistent with this idea, EIAV can use the HIV-1 pathway: either PTAP-containing sequences (49) or those of the Vps28 protein, a component of the ESCRT I complex, can provide L-domain function when introduced into EIAV Gag (54). Proteasome inhibitor studies also suggest that the cellular mechanism used for EIAV release might be fundamentally different from HIV-1 and the PPPY L-domain-containing retroviruses (36, 37, 39).

While the PPPY sequence has been shown to be required for L-domain function in p2b (59), this core motif alone was insufficient to provide L-domain function in our HIV-PPPPY chimera. This implies that the p2b sequences flanking PPPPY in p2b are also required for L-domain function in this HIV-1 context. This is consistent with the previous interpretation that core L-domain sequences are required but not sufficient for function (52).

Currently, the cellular pathway for candidate retroviral PPPY-binding proteins in budding is not as well defined as the PTAP-mediated pathway. Experiments have functionally linked MuLV budding with the VPS system (29), though not directly with the ESCRT complexes. Thus it is likely that HIV-p2b enters the VPS system the same way as MuLV and presumably RSV. Since the AIP1 site was not required for efficient virus release, a direct interaction between HIV-p2b and ESCRT III does not appear to be responsible for L-domain function in this chimera.

As predicted based on our previous findings (36, 37, 40, 48–50), HIV-p2b budding was reduced by proteasome inhibitors, consistent with this type of L domain requiring proteasome function (37). This agrees with results showing that EIAV proteasome inhibitor sensitivity is dictated by its L domain (49). Taken together, these results confirm a linkage between proteasome activity and PTAP- or PPPY-based L-domain function, likely due to disruption of important VPS components that interact with these particular domains (36, 37, 40, 48–50).

Previously, Martin-Serrano et al. found that short PPPY-containing sequences from either Ebola virus or MuLV were unable to provide L-domain function in the context of full-length HIV-1 (30). There are significant differences between RSV p2b and the Ebola virus or MuLV PPPY-containing sequences examined that could be responsible for the difference between our results and theirs. In the same report, *trans*-complementation experiments that coexpressed a proviral clone that contained a $p6^{Gag}$ deletion with Gag proteins carrying heterologous L-domain substitutions found that EIAV $p9^{Gag}$ but not RSV p2b could rescue the budding of the $p6^{Gag}$-deleted provirus in *trans* (30). The discrepancy between those results and our results using proviral constructs may be due to experimental approaches. Similar to our findings, Shehu-Xhilaga et al. mentioned that EIAV sequences were unable to provide L-domain function for HIV-1 $p6^{Gag}$ proviral chimeras (49). Despite these differences, our data support the general conclusion of Martin-Serrano that heterologous L domains are not simply interchangeable in the HIV-1 context, consistent with a previous proposal that the HIV-1 L domain is more complex than other viruses (52).

While many of the chimeric virions budded poorly, these L-domain-deficient virions still could undergo at least one round of replication: the infectivity of the mutants was roughly proportional to the amount of virus released. Therefore, the L domain is not strictly required for other Gag functions. Thus, the L-domain sequences appear to truly act like domains, functioning apart from the other functions of Gag.

The limited interchangeability of L domains and HIV-1 Gag we observe appears to underlie an important aspect of HIV-1 budding that is not yet clear. Understanding the complex interactions between retroviruses and cellular machinery is important, and our results point to important differences between HIV-1 and other retroviral budding pathways. Future studies should assist our understanding of this process.

ACKNOWLEDGMENTS

We thank Eric Freed for the gift of the PTAP$^-$ mutant and Robert Gorelick for assistance in the preparation of the manuscript.

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-12400.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organization imply endorsement by the U.S. Government.

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