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Distal Leucines Are Key Functional Determinants of Alix-Binding Simian Immunodeficiency Virus SIV_{smE543} and SIV_{mac239} Type 3 L Domains▽

Nana F. Bello, Fan Wu, Paola Sette, Vincent Dussupt, Vanessa M. Hirsch, and Fadila Bouamr*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

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In addition to PTAP L domains, primate lentiviruses carry Alix-binding motifs that include the recently described type 3 SREKPYKEVTEDLLHLNSLF sequence. We examined the requirements for the type 3 sequence motif in simian immunodeficiency virus SIV_{smE543} and identified the $_{499}$LNSLF$_{503}$ sequence as a key functional determinant. Mutation of distal leucines $_{499}$L and $_{502}$L (LL mutant) caused an inhibitory effect on Alix-dependent SIV_{smE543} release that was quantitatively similar to that observed following disruption of the type 3 L domain or RNA interference (RNAi) depletion of Alix. Similar results were obtained with the SIV_{mac239} LL mutant. Thus, distal leucines are key determinants of SIV_{smE543} and SIV_{mac239} type 3 L domains.

Retroviruses acquire their envelopes at the host cell membrane of infected cells. To this end, they utilize short sequences designated L (late) domains to recruit members of the *e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport (ESCRT) to catalyze membrane modeling events that lead to virus release (1, 2, 5, 11, 16). Three types of L domains have so far been identified: the PT/SAP, PPXY, and LYPX$_n$L motifs. They bind the host proteins Tsg101, Nedd4-like ubiquitin ligase family members, and Alix, respectively (6, 13, 15, 23, 25, 26). These interactions lead to the recruitment of members of the ESCRT pathway and require the activity of the AAA ATPase VPS4 (14, 23, 24, 26).

All retroviral Gag proteins contain at least one L domain, though most carry multiple L domain motifs, believed to function synergistically and ensure efficient viral release (4, 7, 8, 13, 21, 27). Structural studies have been used to determine L domain sequence and functional requirements. Only limited sequence variability was noted in motifs that bind Tsg101, including PTAP, PSAP, and PXXP motifs found in Hrs, the natural partner for Tsg101 (3, 10, 18–20, 28). However, more sequence divergence was observed in L domains binding Alix, with the identification of three sequences: YPDL in equine infectious anemia virus (EIAV) (23, 26, 29), LYPLAS*L*RS*LF* in human immunodeficiency virus type 1 (HIV-1), and the recently described SREKPYKE*V*TED*L*LHLNSLF sequence in the simian immunodeficiency virus SIV_{mac239} (30). They have been dubbed types 1, 2, and 3, respectively. The last type contains an anchoring tyrosine, followed by one or two hydrophobic residues (valine and/or leucine) (proximal leucines), and both residues are key functional determinants for Alix (30). We observed that the Alix-binding type 3 L domain also contains a downstream LXXLF sequence carrying additional leucines (distal leucines) that are present in SIV_{mac239}, $SIV_{agmTan-1}$, and SIV_{smE543}. Similarly, the HIV-1 p6 domain also harbors an LXXLF motif in its LYPLAS*L*RS*LF* L domain (underlined), which is critical for Alix binding and function (23, 26). In this study, we examined the role of distal leucine residues in the LNSLF sequence within the Alix-binding type 3 L domains of both SIV_{smE543} (9) and SIV_{mac239} (12).

The $_{499}$LNSLF$_{503}$ sequence is required for PTAP-independent SIV_{smE543} release. L domain function has recently been assessed in simian retroviruses and has led to the discovery of a new Alix-binding L domain in SIV_{mac239} (30). We examined the L domain function in SIV_{smE543} (9). The Gag protein of this virus contains a total of two PTAP L domains located in the SIV_{smE543} MA and p6 domains, respectively. Substitution of LTAA for the PTAP motif in p6 ($_{sm}$L2 mutant) eliminated ~50% of virus release from 293T (Fig. 1A, lane 3) and CEM cells (data not shown), whereas substitutions in MA had little effect on release ($_{sm}$L1 mutant), indicating that the p6 motif plays a dominant role in virus release. SIV_{smE543} devoid of both motifs ($_{sm}$L1L2 mutant) retained ~50% of wild-type (WT) virus release (Fig. 1A, lane 4), suggesting that it harbors an additional L domain sequence(s). We searched for an Alix-binding L domain in the C-terminal region of the SIV_{smE543} p6 domain and found a $_{488}$PYKEVTED*L*LH*L*NS*LF*$_{503}$L sequence, which is similar to the recently described Alix-binding type 3 L domain in SIV_{mac239} (Fig. 1B). To investigate the function of this motif, we replaced distal leucines $_{499}$L, $_{502}$L, and $_{503}$F in the $_{499}$LNSLF$_{503}$ motif with prolines and a serine ($_{sm}$LL mutant) (Fig. 1B) and examined the effect of such changes. As expected, an $_{sm}$LL mutation in the context of WT SIV_{smE543} had only a minimal affect on virus release (Fig. 1C, lane 2). Conversely, when the LL mutation was combined with L1 and L2 mutations ($_{sm}$L1L2/LL), virus release was obliterated (Fig. 1C, lanes 6 and 8). The effect of these mutations was comparable to that seen following the substitution of serines for the $_{489}$Y and $_{496}$L residues in the type 3 L domain (Fig. 1D, lanes 6 and 8), thus underscoring the importance of distal leucines in virus release. Similar results were obtained when

* Corresponding author. Mailing address: Laboratory of Molecular Microbiology, NIAID, NIH, 4 Center Drive, Bethesda, MD 20892. Phone: (301) 496-4099. Fax: (301) 402-0226. E-mail: bouamrf@mail.nih.gov.

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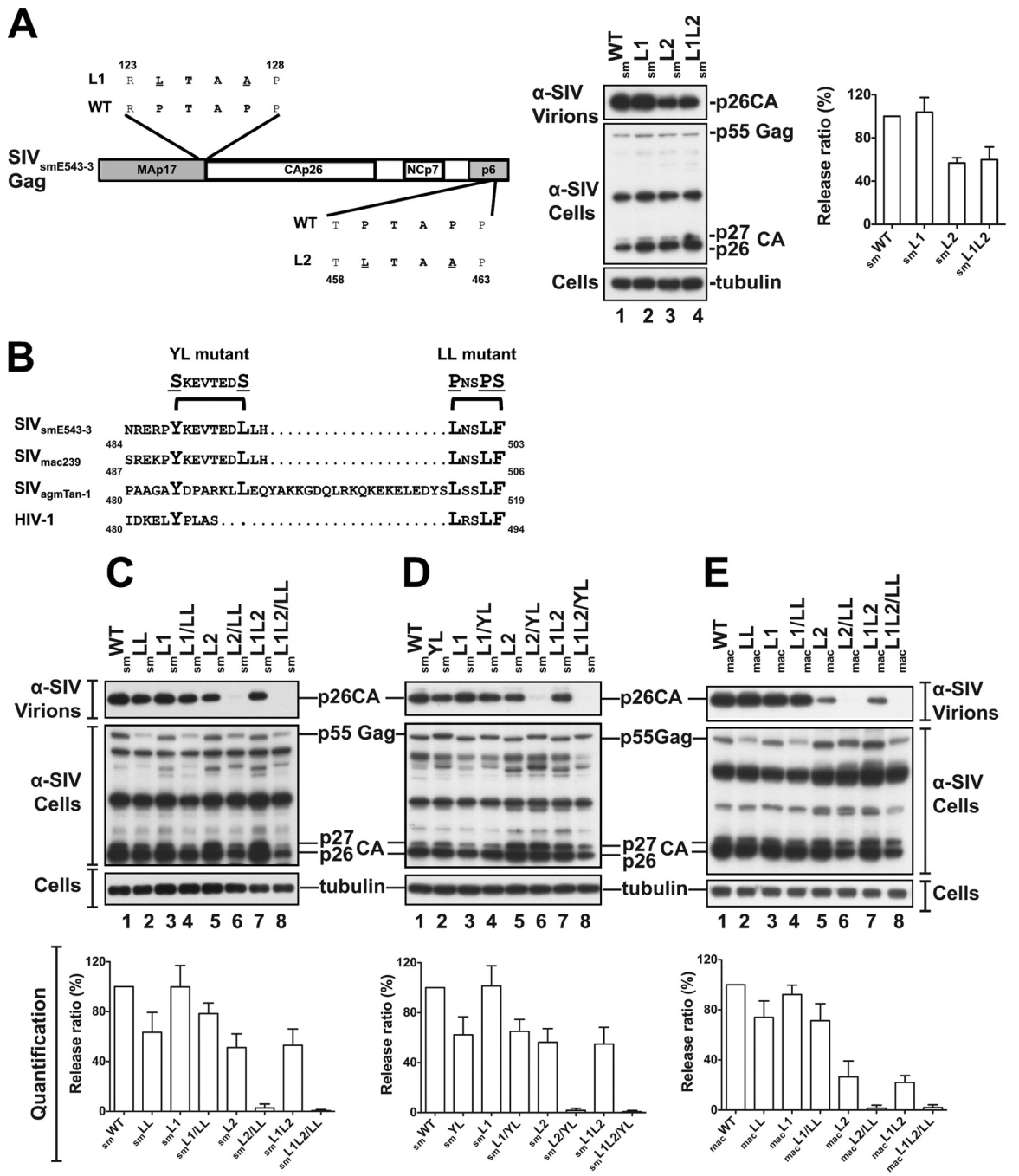

FIG. 1. Distal leucines in the type 3 L domain are key participants in the release of SIV_{smE543} and SIV_{mac239}. (A) PTAP L domain motifs drive SIV_{smE543} release from 293T. (Left) Schematic representation of SIV_{smE543} Gag showing the positions of PTAP motifs. (Middle) 293T cells were transfected with WT SIV_{smE543} proviral DNA (lane 1), the MA-PTAP motif mutant ($_{sm}$L1) (lane 2), the p6-PTAP mutant ($_{sm}$L2) (lane 3), or the double mutant ($_{sm}$L1L2) (lane 4). (Right) Quantification of the release of L domain mutants. (B) Sequence comparison of Alix-binding type 3 L domains in SIV_{smE543}, SIV_{mac239}, and $SIV_{agmTAN-1}$. The three lentiviruses carry LXXLF motifs (boldface) downstream of their defined type 3 L domains (anchorage tyrosine in boldface). Dots indicate absent residues. Positions of YL and LL mutants are indicated. (C) The distal leucine $_{sm}$LL mutant is as defective as the $_{sm}$YL mutant in Alix-mediated release. 293T cells were transfected with WT SIV_{smE543} proviral DNA (lane 1), the $_{sm}$LL mutant (lane 2), the MA-PTAP $_{sm}$L1 mutant (lane 3), the p6-PTAP $_{sm}$L2 mutant (lane 5), or the indicated double mutant. (D) Cells were transfected with the YL mutant (lane 2) or the indicated single or double mutants. (E) Analysis of the SIV_{mac239} $_{mac}$L1, $_{mac}$L2, and $_{mac}$LL mutants. Cells were transfected with the SIV_{mac239} PTAP motif single mutants $_{mac}$L1, $_{mac}$L2, the LXXLF motif single mutant $_{mac}$LL (lanes 2, 3, and 5), or the indicated double mutants. Cells and viruses were collected 24 h posttransfection, and their protein content was analyzed by SDS-PAGE and Western blotting using an anti-SIV serum. Virus release efficiencies (values in percentages) were quantified as the ratio of virion-associated Gag and cellular Gag from three independent experiments and are shown in the panels under the blots.

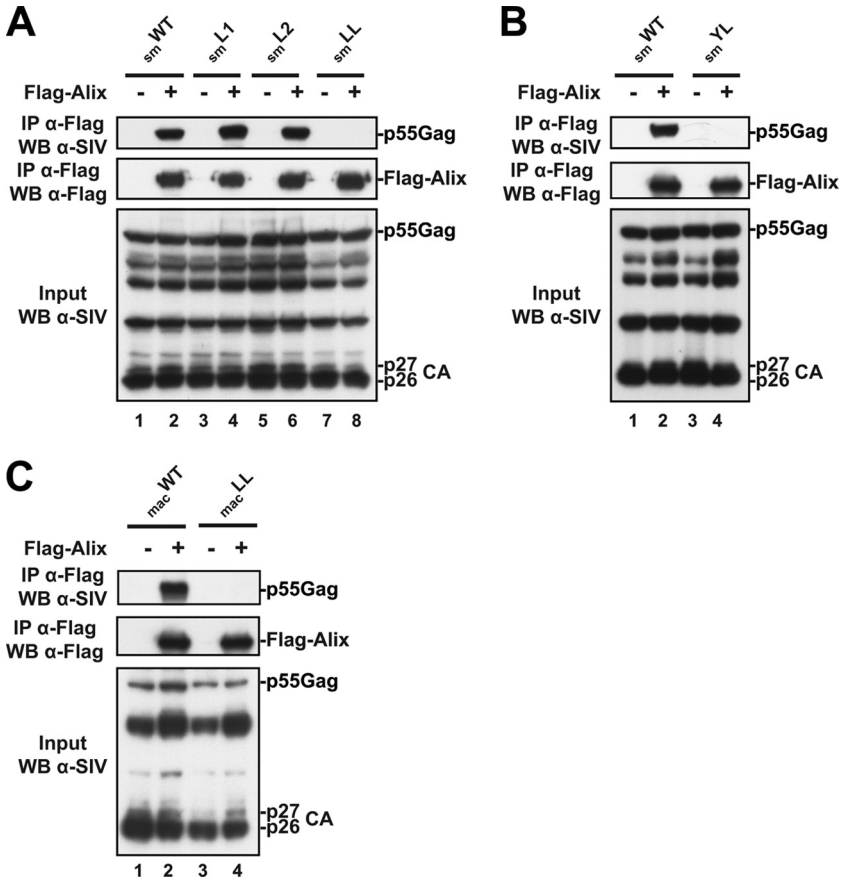

FIG. 2. Distal leucine residues in the Alix-binding type 3 L domain are required for SIV_{smE543} and SIV_{mac239} Gag-Alix interactions. 293T cells were transfected with either WT SIV_{smE543} (A and B) or WT SIV_{mac} (C) proviral DNA, or with the $_{sm}$L1, $_{sm}$L2, or $_{sm}$LL mutants (panel A, lanes 3 to 8), the $_{sm}$YL mutant (panel B, lanes 3 and 4), or the $_{mac}$LL mutant (panel C, lanes 3 and 4) in the presence or absence of Flag-tagged Alix. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and lysates were incubated with anti-Flag antibody-conjugated beads. Both input and immunoprecipitated complexes were analyzed by SDS-PAGE and Western blotting using the indicated antibodies.

distal leucines were mutated in the SIV_{mac239} LNSLF motif ($_{mac}$L2/LL and $_{mac}$L1L2/LL mutants) (Fig. 1E). These results, which were observed in three independent experiments (see quantification panels under the blots), indicate that distal leucines are an active part of the type 3 L domain and critical for Alix-dependent SIV_{smE543} and SIV_{mac239} release.

Leucines in the LNSLF motif are critical for SIV_{smE543} and SIV_{mac239} Gag interactions with Alix. Next, we assessed whether the $_{499}$L and LF_{503} residues are involved in Gag interactions with Alix. Cell lysates from 293T cells expressing SIV_{smE543} $_{sm}$L1, $_{sm}$L2, and $_{sm}$LL mutants were tested for their ability to interact in immunoprecipitation assays with Flag-tagged Alix in the presence of 1% NP-40 detergent. Similar to observations with WT SIV_{smE543} Gag, the $_{sm}$L1 and $_{sm}$L2 mutants were captured by Flag-Alix proteins (Fig. 2A, lanes 2, 4, and 6). In contrast, SIV_{smE543} and SIV_{mac239} LL mutants ($_{sm}$LL and $_{mac}$LL) were not captured (Fig. 2A, lane 8, and C, lane 4). Likewise, substitutions of alanines for $_{489}$Y and $_{496}$L residues within SIV_{smE543} and SIV_{mac239} ($_{sm}$YL and $_{mac}$YL mutants) eliminated binding to Alix (Fig. 2B, lane 4) (30). These results indicate that the Y and L residues are as critical as the distal leucines for the SIV_{smE543} and SIV_{mac239} type 3 L domain binding to Alix.

Alix enhances the release of SIV_{smE543} and SIV_{mac239} in an LXXLF-dependent manner. To examine whether distal leucines $_{499}$L and $_{502}LF_{503}$ are involved in Alix-mediated virus release, we used a virus rescue assay to test the effect of Flag-tagged Alix overexpression (22) on the release of a SIV_{smE543} mutant that lacked the PTAP motif activity. Alix overexpression enhanced the release of SIV_{smE543} lacking either the p6-located dominant PTAP motif ($_{sm}$L2 mutant) or both PTAP motifs in Gag ($_{sm}$L1L2 mutant) (Fig. 3A, lanes 2 and 6). Virus release augmentation was inhibited by the mutation of $_{499}$L and $_{502}LF_{503}$ residues in the $_{499}LNSLF_{503}$ motif ($_{sm}$L2/LL and $_{sm}$L1L2/LL mutants) (Fig. 3A, lanes 4 and 8) in a manner similar to that seen following the substitution of serines for $_{489}$Y and $_{496}$L (Fig. 3B). A similar result was obtained when the LNSLF motif was disrupted in SIV_{mac239} (Fig. 3C). These results were confirmed in three independent experiments, as shown in the release quantification panel in Fig. 3A. Together, they indicate that SIV_{smE543} and SIV_{mac239} are less responsive to Alix overexpression when distal leucines $_{499}$L and $_{502}LF_{503}$ are mutated.

LNSLF-dependent SIV_{smE543} release requires cellular Alix. When both PTAP L domains were disrupted in SIV_{smE543} ($_{sm}$L1L2 mutant), ~50% of virus release was retained, as long

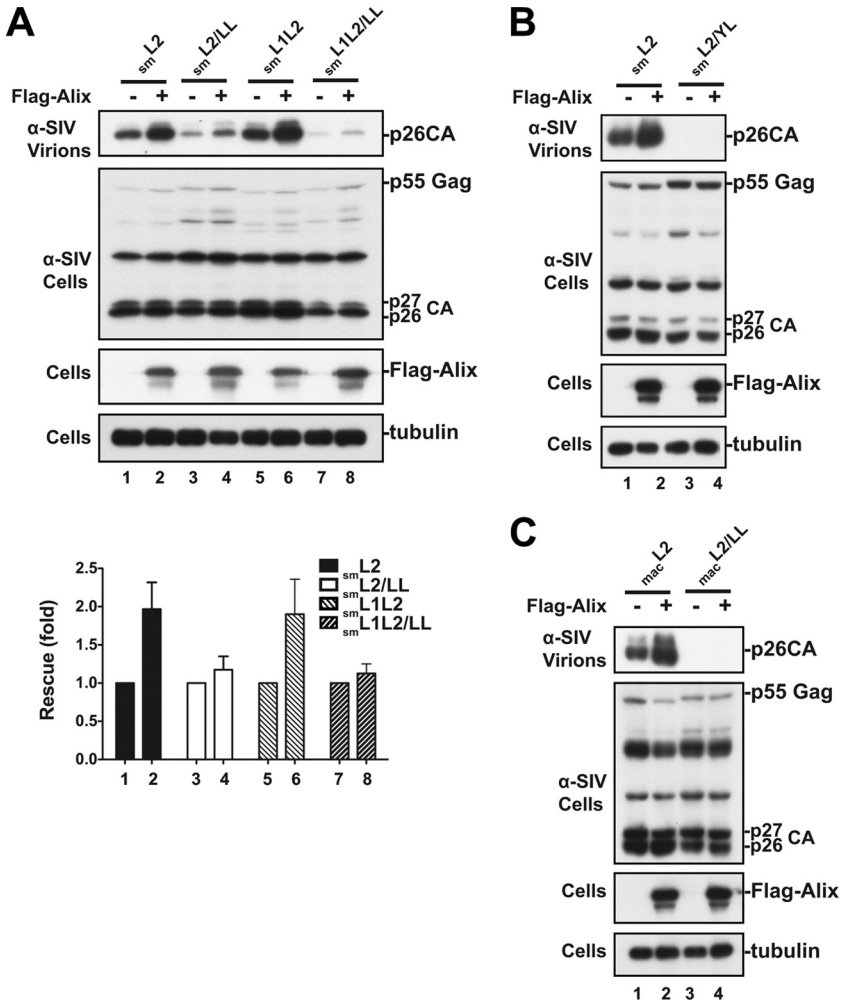

FIG. 3. Alix enhances the release of SIV_{smE543} and SIV_{mac239} in an LNSLF-dependent manner. 293T cells were transfected with either SIV_{smE543} carrying disrupted PTAP motifs ($_{sm}$L2 or $_{sm}$L1L2 mutants) or SIV_{smE543} carrying mutations in the PTAP and LXXLF motifs ($_{sm}$L2/LL or $_{sm}$L1L2/LL mutant) (A), SIV_{smE543} carrying mutations in both the p6-bound PTAP and the Alix-binding YL motifs ($_{sm}$L2YL mutant) (B), or SIV_{mac239} carrying mutations in the PTAP and LXXLF motifs ($_{mac}$L2 and $_{mac}$L2/LL mutants) (C) in the presence or absence of Flag-Alix. Cells and viruses were collected 24 h posttransfection, and their protein content was analyzed by SDS-PAGE and Western blotting using an anti-SIV serum. Expression of Alix was detected using an anti-Flag antibody, and cellular tubulin levels were analyzed using a mouse monoclonal antitubulin antibody. Alix-mediated virus enhancement was quantified from three independent experiments, and the results are summarized in panel A (under the Western blot in panel A).

as the Gag possessed an intact $_{488}$P*Y*KE*V*TED*L*LH*L*NS*L*F motif (Fig. 1 and 3). These findings suggest that the SIV_{smE543} Gag utilizes the type 3 L domain sequence to recruit the host cell Alix and promote virus release. To examine whether distal leucines in the $_{499}$LNSLF motif are involved in the utilization of the host Alix, we knocked down Alix using RNAi and examined its effect on the WT or mutant viruses carrying either one ($_{sm}$L1 and $_{sm}$L2 mutants) or no ($_{sm}$L1L2 mutant) PTAP motif. As expected, RNAi depletion of cellular Alix had only a modest effect on the release of WT or L1 mutant viruses because of the second PTAP L domain dominance in the p6 region of Gag (Fig. 4A, lanes 3 and 6, and the release quantification below the blots). Conversely, depletion of cellular Alix decreased the release of SIV_{smE543} L2 and L1L2 mutants to nearly undetectable levels (Fig. 4B, lanes 2 and 5), demonstrating that SIV_{smE543} LNSLF is critical for the utilization of the host cell Alix during virus release.

Conclusions. Three types of Alix-binding L domains have been identified: YPDL, LYPLAS*LRSLF* and SREKPYKEVT EDLLHL*L*NS*LF* motifs (key residues underlined) found within EIAV, HIV-1, and $SIV_{mac239,}$ respectively (23, 26, 30). These motifs share key binding and functional determinants. A tyrosine considered an "anchorage" of Alix (29, 30) is followed by hydrophobic residues, often leucines, which make contact with the Alix V domain (30). Here we report that two leucines and a phenylalanine residue in the SIV_{smE543} $_{488}$PYKEVTED LLH*L*NS*LF*$_{503}$ L domain (italics) play a key role in Alix function (Fig. 1 to 3), since substitutions at these residues resulted in loss of both binding and function. We conclude that in addition to the tyrosine and proximal hydrophobic residues in the type 3 L domain, additional relatively distant residues (distal leucines) are critical functional determinants of Alix. Interestingly, both leucines are found within an LXXLF sequence that is part of the type 3 L domains of SIV_{smE543},

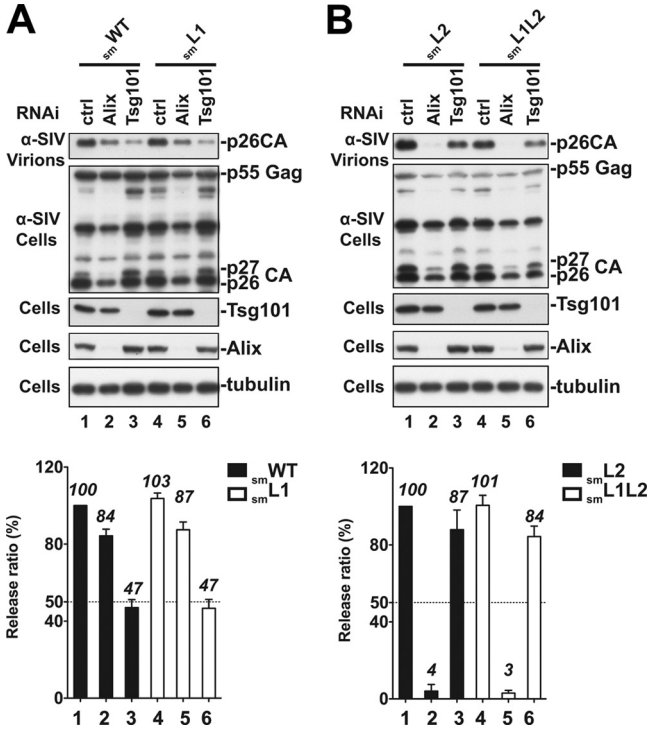

FIG. 4. (A and B) LNSLF-dependent SIV_{smE543} release requires cellular Alix. 293T cells were transfected with control RNAi (lanes 1 and 4), RNAi directed against Tsg101 (lanes 3 and 6), or Alix (lanes 2 and 5) at 24-h intervals. At the second transfection, cells were cotransfected with $_{sm}$WT SIV_{smE543} or the $_{sm}$L1, $_{sm}$L2, or $_{sm}$L1L2 L domain mutants. Cells and viruses were collected 24 h posttransfection, and their protein content was analyzed by SDS-PAGE and Western blotting using an anti-SIV serum. Cellular Alix and Tsg101 were detected using a rabbit anti-Alix and mouse anti-Tsg101 antibody, respectively. Alix-mediated virus release rescue was quantified from three independent experiments, and the results are summarized in the panels below the blots.

SIV_{mac239}, and $SIV_{agmTAN-1}$ (30). An LXXLF motif is also found in HIV-1 and is critical for Alix recruitment and Alix-dependent release (23, 26). These observations confirm the conservation of the LXXLF sequence across primate lentiviruses, underscoring its importance in Alix-mediated virus release.

The Alix-binding type 3 L domain shares key structural and functional determinants with types 1 and 2, including the tyrosine (Y) and the hydrophobic proximal leucines that make contact within the V domain in order to stabilize binding with Alix (29, 30). Our data demonstrate that both functional determinants are required, but not sufficient, for the type 3 L domain binding to Alix, suggesting that further stabilization is necessary for recruitment and function. Consistent with this hypothesis, we found that leucines located 10 and 13 residues downstream of the tyrosine (distal leucines) in the P*Y*KEVTE D*L*LH*L*NS*LF* sequence, are required for Alix binding to SIV_{smE543} and SIV_{mac239} Gag proteins (Fig. 2). Similarly, leucines in the HIV-1 LYPLAS*L*RS*LF* L domain (underlined) are also critical for Alix binding (17, 23, 26, 29). In contrast, distant leucines appear to be dispensable for the EIAV-borne type 1 L domain. One possible explanation for these discrepant requirements is the ability of the latter L domain to bind Alix with a high affinity (29). Together, these observations suggest that distal leucines in type 2 and 3 L domains are involved in further stabilization of Alix-p6 interactions, possibly to compensate for their low-affinity binding to p6.

Virus release was completely eliminated when mutation of distal leucines was combined with the disruption of both PTAP L domains in SIV_{smE543}. This suggested that the entire P*Y*KE *V*TED*L*LH*L*NS*L*F sequence functions as an additional L domain in p6 (Fig. 1). Such a notion is further supported by the reliance of the type 3 L domain on cellular Alix to drive virus release in the absence of PTAP motifs (Fig. 4). In summary, our data identify distal leucine residues in the SIV_{smE543} and SIV_{mac239} type 3 Alix-binding L domains as key functional determinants.

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