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

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In addition to PTAP L domains, primate lentiviruses carry Alix-binding motifs that include the recently described type 3 SREKPYKETEDLHNLNSLF sequence. We examined the requirements for the type 3 sequence motif in simian immunodeficiency virus SIV^{smE543} and identified the 499YTEDLHLNSLF sequence as a key functional determinant. Mutation of distal leucines 499L and 502L (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 endosomal sorting complex required for transport (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_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 PXPX_L motifs found in Hrs, the natural partner for Tsg101 (3, 10, 18–20, 28). However, more functional determinant. Mutation of distal leucines 499L and 502L (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.

The 499YTEDLHLNSLF 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 (smL2 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 (smL1 mutant), indicating that the p6 motif plays a dominant role in virus release. SIV^{smE543} devoid of both motifs (smL1L2 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 499YPKEVTEDLLLHNLNSLF 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 499L, 502L, and 503F in the 499YPKEVTEDLLLHNLNSLF motif with prolines and a serine (smLL mutant) (Fig. 1B) and examined the effect of such changes. As expected, an smLL 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 (smL1L2L2), 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 499Y and 499L 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...
FIG. 1. Distal leucines in the type 3 L domain are key participants in the release of SIV \text{smE543} and SIV \text{mac239}. (A) PTAP L domain motifs drive SIV \text{smE543} release from 293T. (Left) Schematic representation of SIV \text{smE543} Gag showing the positions of PTAP motifs. (Middle) 293T cells were transfected with WT SIV \text{smE543} proviral DNA (lane 1), the MA-PTAP motif mutant (\text{smL1}) (lane 2), the p6-PTAP mutant (\text{smL2}) (lane 3), or the double mutant (\text{smL1L2}) (lane 4). (Right) Quantification of the release of L domain mutants. (B) Sequence comparison of Alix-binding type 3 L domains in SIV \text{smE543}, SIV \text{mac239}, and SIV \text{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 \text{smLL} mutant is as defective as the \text{smYL} mutant in Alix-mediated release. 293T cells were transfected with WT SIV \text{smE543} proviral DNA (lane 1), the \text{smLL} mutant (lane 2), the MA-PTAP \text{smL1} mutant (lane 3), the p6-PTAP \text{smL2} 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 \text{mac239}, \text{macLL} and \text{macL2} mutants. Cells were transfected with the SIV \text{mac239} PTAP motif single mutants (\text{macL1}, \text{macL2}, the LXXLF motif single mutant \text{macLL}) (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.
distal leucines were mutated in the SIV<sub>mac239</sub> LNSLF motif (macL2/LL and macL1L2/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<sub>smE543</sub> and SIV<sub>mac239</sub> release.

Leucines in the LNSLF motif are critical for SIV<sub>smE543</sub> and SIV<sub>mac239</sub> Gag interactions with Alix. Next, we assessed whether the 499L and LF 503 residues are involved in Gag interactions with Alix. Cell lysates from 293T cells expressing SIV<sub>smE543</sub> smL1, smL2, and smLL 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<sub>smE543</sub> Gag, the smL1 and smL2 mutants were captured by Flag-Alix proteins (Fig. 2A, lanes 2, 4, and 6). In contrast, SIV<sub>smE543</sub> and SIV<sub>mac239</sub> LL mutants (smLL and macLL) were not captured (Fig. 2A, lane 3). Likewise, substitutions of alanines for 489Y and 496L residues within SIV<sub>smE543</sub> and SIV<sub>mac239</sub> (smYL and macYL 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<sub>smE543</sub> and SIV<sub>mac239</sub> type 3 L domain binding to Alix.

**Alix enhances the release of SIV<sub>smE543</sub> and SIV<sub>mac239</sub> in an LXXLF-dependent manner.** To examine whether distal leucines 499L and 502LF503 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<sub>smE543</sub> mutant that lacked the PTAP motif activity. Alix overexpression enhanced the release of SIV<sub>smE543</sub> lacking either the p6-located dominant PTAP motif (smL2 mutant) or both PTAP motifs in Gag (smL1L2 mutant) (Fig. 3A, lanes 2 and 6). Virus release augmentation was inhibited by the mutation of 499L and 502LF503 residues in the 499LNSLF<sub>503</sub> motif (smL2/LL and smL1L2/LL mutants) (Fig. 3A, lanes 2 and 6). A similar result was obtained when the LNSLF motif was disrupted in SIV<sub>mac239</sub> (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<sub>smE543</sub> and SIV<sub>mac239</sub> are less responsive to Alix overexpression when distal leucines 499L and 502LF503 are mutated.

LNSLF-dependent SIV<sub>smE543</sub> release requires cellular Alix. When both PTAP L domains were disrupted in SIV<sub>smE543</sub> (smL1L2 mutant), ~50% of virus release was retained, as long...
as the Gag possessed an intact SIVsmE543 PYKEVTEDLLHLSLF motif (Fig. 1 and 3). These findings suggest that the SIVsmE543 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 SIVsmE543 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 (smL1 and smL2 mutants) or no (smL1L2 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 SIVsmE543 L2 and L1L2 mutants to nearly undetectable levels (Fig. 4B, lanes 2 and 5), demonstrating that SIVsmE543 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, LYPLASRSLF and SREKPYKEVT EDLLHLSLF motifs (key residues underlined) found within EIAV, HIV-1, and SIVmac239, 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 SIVsmE543 488 PYKEVTEDLLHLSLF 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 SIVsmE543.

FIG. 3. Alix enhances the release of SIVsmE543 and SIVmac239 in an LNSLF-dependent manner. 293T cells were transfected with either SIVsmE543 carrying disrupted PTAP motifs (smL2 or smL1L2 mutants) or SIVsmE543 carrying mutations in the PTAP and LXXLF motifs (smL2/LL or smL1L2/LL mutant) (A), SIVsmE543 carrying mutations in both the L domain and the Alix-binding YL motifs (smL1L2YL mutant) (B), or SIVmac239 carrying mutations in the PTAP and LXXLF motifs (macL2 and macL2/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).
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<sub>sm</sub>E543. This suggested that the entire FYKE/VTED/LH<sub>LNSLF</sub> 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<sub>sm</sub>E543 and SIV<sub>mac</sub>239 type 3 Alix-binding L domains as key functional determinants.

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