Equine Infectious Anemia Virus Utilizes a YXXL Motif within the Late Assembly Domain of the Gag p9 Protein

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We have previously demonstrated that the Gag p9 protein of equine infectious anemia virus (EIAV) is functionally homologous with Rous sarcoma virus (RSV) p2b and human immunodeficiency virus type 1 (HIV-1) p6 in providing a critical late assembly function in RSV Gag-mediated budding from transfected COS-1 cells (L. J. Parent et al., J. Virol. 69:5455–5460, 1995). In light of the absence of amino acid sequence homology between EIAV p9 and the functional homologs of RSV and HIV-1, we have now designed an EIAV Gag-mediated budding assay to define the late assembly (L) domain peptide sequences contained in the EIAV p9 protein. The results of these particle budding assays revealed that expression of EIAV Gag polyprotein in COS-1 cells yielded extracellular Gag particles with a characteristic density of 1.18 g/ml, while expression of EIAV Gag polyprotein lacking p9 resulted in a severe reduction in the release of extracellular Gag particles. The defect in EIAV Gag polyprotein particle assembly could be corrected by substituting either the RSV p2b or HIV-1 p6 protein for EIAV p9. These observations demonstrated that the L domains of EIAV, HIV-1, and RSV were interchangeable in mediating assembly of EIAV Gag particles in the COS-1 cell budding assay. To localize the L domain of EIAV p9, we next assayed the effects of deletions and site-specific mutations in the p9 protein on its ability to mediate budding of EIAV Gag particles. Analyses of EIAV Gag constructs with progressive N-terminal or C-terminal deletions of the p9 protein identified a minimum sequence of 11 amino acids (Q20N21L22Y23P24D25L26S27E28I29K30) capable of providing the late assembly function. Alanine scanning studies of this L-domain sequence demonstrated that mutations of residues Y23, P24, and L26 abrogated the p9 late budding function; mutations of other residues in the p9 L domain did not substantially affect the level of EIAV Gag particle assembly. These data indicate that the L domain in EIAV p9 utilizes a YXXL motif which we hypothesize may interact with cellular proteins to facilitate virus particle budding from infected cells.

The Gag polyproteins of retroviruses have the ability to assemble immature virus-like particles and bud from cells in the absence of all other virus-encoded proteins (27). Gag polyproteins are synthesized in the cytoplasm and targeted to the plasma membrane, where approximately 2,000 polyproteins interact, associate with genomic RNA, form immature budding particles, and release from the cell plasma membrane (23). Maturation of the particle occurs at some point either late in budding or after release of the particle when the virally encoded protease becomes active. The Gag polyprotein of the lentivirus equine infectious anemia virus (EIAV) is cleaved by the virus-encoded protease into the matrix (MA), capsid (CA), nucleocapsid (NC), and p9 virion proteins (9, 12). The p9 gene sequence is located downstream of a putative split to the pol reading frame; thus, when Gag-Pol is synthesized, p9 is not. This genomic position of p9 is analogous to that of the lentivirus human immunodeficiency virus type 1 (HIV-1) p6 protein; however, the two proteins have only minimal amino acid sequence homology or predicted secondary structures. Comparison of the p9 protein sequence to the sequences of Gag proteins of other lentiviruses demonstrates that EIAV p9 has little amino acid sequence similarity with Gag protein of HIV-1, HIV-2, simian immunodeficiency virus (SIV), caprine arthritis-encephalitis virus, feline immunodeficiency virus, or visna virus (13).

Studies of the HIV-1 p6 protein indicate a function in viral particle release from cellular plasma membranes. Mutation of proline residues within the first 12 amino acids of p6 results in immature particles that assemble to a point late in budding but that fail to be released from cells (2, 8). Additional analysis of HIV-1 p6 mutations indicate that single amino acid substitutions within the sequence P7T8A9P10 greatly decrease viral replication (10). The (P/T/S)AP residues identified to be critical for this function are highly conserved among all lentiviruses except EIAV (1, 20, 21). In addition, this (P/T/S)AP sequence is duplicated in some isolates of HIV-2 and SIV encoding either two motifs in the p6 protein or motifs in both p6 and the MA protein.

Mutational analysis of Rous sarcoma virus (RSV) Gag has also identified an oncoviral functional homolog that mediates this late budding function in virus assembly (26). Deletion of a large internal region of the Gag polyprotein encompassing p2, p10, and parts of CA and MA results in loss of particle assembly. Fusion of HIV-1 p6 to the C terminus of this defective RSV Gag polyprotein results in a protein competent for particle assembly and release from cells (16). The RSV late assembly function maps to the p2 cleavage product, now referred to as the late assembly (L) domain. This protein encodes the residues PPyP, a sequence highly conserved among many retroviruses (18). We have previously demonstrated that EIAV p9 can provide the late function during RSV Gag assembly. Fusion of p9 to the C terminus of an RSV Gag protein defective for budding results in a functional polyprotein that can assemble and release Gag particles from the cell plasma membrane (16). EIAV p9 is unique in that while it provides the late
assembly function, it does not contain either of the highly conserved sequences that have been shown to provide the late assembly function in other retroviruses. In this report, we precisely define the L domain of EIAV p9 and demonstrate that it can be replaced with the L domains of HIV-1 p6 and RSV p2b.

MATERIALS AND METHODS

Construction of Gag expression vectors. Gag expression constructs used in these studies are depicted in Fig. 1.

(i) EIAV Gag expression plasmids. The EIAV Gag gene was amplified by PCR from the EIAV PV919-2 proviral clone (17) by using the upstream primer gag/SstI (5′tttgtcgttgctgtgctgttcgagctcctacagttg3′) and downstream primer pBssHII (5′tgagggctgctggcgagtagatgtgtactc3′). This PCR product was digested with SstI and BssHII ligated into the previously described construct pSV.Myr1.D37S (5) cut with the ApaI and BglII sites. The pSV.EG.p9+ construct was generated by using megaprimer PCR amplifying the p2-encoding fragment into pSV.EG by using the upstream primer p9f5 and downstream primer p9BssHII flanked the mutagenic primer p9P1/Bgl (5′ccagaaacaaactaagatcttacaacagaagagt3′) and ligated into the pSV.EG vector by using BglII and ligated into the pSV.EG vector by using BglII and pGemS11. Plasmid pGemS11 was digested with SstI and BglII and ligated into the pSV.EG template. Gel-purified PCR products were digested with BssHII and ApaI and ligated into the pSV.EG vector by using ApaI and an existing downstream BglII site.

(ii) Chimeric Gag proteins. To produce a chimera containing HIV-1 p6 fused to the EIAV p9-deleted Gag polyprotein, pSV.EG.p9+ was digested by digesting the previously described pSV.RHB (16) with BglII and XbaI and ligation this p6-encoding fragment into pSV.EG.p9+ by using the BglII and XbaI sites. The RSV chimeric construct pSV.EGp2 was generated by PCR amplifying the p2 sequence with the upstream primer PARE 41 (5′tggcagcctggcgagtagatgtgtactc3′) and downstream primer PARE 42 (5′atctacaacactcataccataaat3′). This PCR product was cloned directly into pGEM5Zf(+) (Promega) to generate pGem.tools. Plasmid pGem.tools was digested with BglII and MluI, and this fragment was ligated into pSV.EG.p9+ by using the BglII and XbaI sites.

(iii) Deletion constructs of EIAV p9. C-terminal deletion constructs were generated by using the upstream primer p9S11+ (5′tgcagcctggcgagtagatgtgtactc3′) and downstream primer p9N21* (5′ccagacgcgtccgaaataaaaaagg3′), p9K31*/ (5′tgcagcctggcgagtagatgtgtactc3′) and p9V41*/ (5′tgcagcctggcgagtagatgtgtactc3′). pSV.EG.S11/* was constructed by digesting gel-purified PCR product with BglII and ApaI and then ligated into the pSV.EG vector by using BglII and XbaI. The S27,E28,I29,K30 mutations of S27,E28,I29,K30 to alanine were generated by PCR amplification using primers p9f5 and p9BssHII. This product was gel purified, phosphorylated by using T4 polynucleotide kinase, and ligated into the pSV.EG vector by using BglII and XbaI.

(iv) Alanine scanning mutagenesis of p9. Residues Q20, N21, L22, Y24, and P26 were mutated to alanine by using the upstream primer p9S5′ and the mutagenic downstream primers Q20/A (5′attttctggactgctggctgtgctgttcgagctcctc3′), N21/A (5′aatctttcttggttgtgctgtgctgttccgtccgagtagatgtgtactc3′), L22/A (5′agttcagctggctgtgctgttccgtccgagtagatgtgtactc3′), Y24/A (5′tttattttcttggtgtgctgtgctgttccgtccgagtagatgtgtactc3′), and P26/A (5′gagaattcttggtgtgctgtgctgttccgtccgagtagatgtgtactc3′) to amplify from pSV.EG template. Gel-purified PCR products were digested with BssHII and either EcoRI or BglII and ligated into the BglII/BssHII sites of pSV.EG.p9+.

Gag polyprotein particle budding assay. The Gag particle budding assays were performed essentially as described by Parent et al. (16), with minor modifications to optimize the assay conditions for EIAV Gag polyproteins. Briefly, COS-1 cells were grown in 35-mm-diameter dishes in Dulbecco’s modified Eagle medium ( Gibco BRL) containing 3% newborn calf serum and 7% fetal calf serum (Atlanta Biologicals). When cells were approximately 90% confluent, they were transfected with XbaI-digested and ligated plasmids by the DEAE-dextran-clio-
roquine method as previously described (16). Transfected cells were metabolically labeled for 5 h with EXPRE $^{35}$S protein labeling mix (DuPont NEN) for approximately 48 h posttransfection. Cells and culture media were separated, detergents were added to each as previously described, and the Gag proteins were immunoprecipitated by rabbit serum raised against whole disrupted EIAV and treated with Immuno precipitin (GIBCO BRL). Immunoprecipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel, and the radioactive Gag proteins were detected by autoradiography. Densitometry was performed on autoradiograms by using a Personal Densitometer SI (Molecular Dynamics), and budding efficiency was estimated by dividing the value for protein in the medium by the total of labeled protein in both the lysate and the medium fractions.

**Characterization of EIAV Gag particle budding assay.** Our previous work demonstrated that EIAV p9 could provide a late assembly function to an RSV Gag protein lacking its own L domain (i.e., protein p2b). Based on this result, we initially developed a budding assay for EIAV Gag particles that could be used to test the prediction that a late assembly defect would also be observed upon deletion of the p9 region from Gag. Simian virus 40-based expression vectors containing the wild-type gag gene (Fig. 1, construct EG) and a gag allele lacking the p9-coding sequence (Fig. 1, construct EG.p9-) were constructed. These expression vectors were then transfected into COS cells that were subsequently metabolically labeled with radioactive amino acids to label the EIAV Gag polyprotein products. The resultant cell supernatants and cell lysates were separated and subjected to immunoprecipitation with rabbit polyclonal anti-RSV or anti-EIAV serum. The precipitated proteins were then separated by SDS-PAGE, detected by autoradiography, and quantitated by scanning laser densitometry. The sucrose density of each fraction was measured by refractometry.

**RESULTS**

Characterization of EIAV Gag particle budding assay. Our previous work demonstrated that EIAV p9 could provide a late assembly function to an RSV Gag protein lacking its own L domain (i.e., protein p2b). Based on this result, we initially developed a budding assay for EIAV Gag particles that could be used to test the prediction that a late assembly defect would also be observed upon deletion of the p9 region from Gag. Simian virus 40-based expression vectors containing the wild-type gag gene (Fig. 1, construct EG) and a gag allele lacking the p9-coding sequence (Fig. 1, construct EG.p9-) were constructed. These expression vectors were then transfected into COS cells that were subsequently metabolically labeled with radioactive amino acids to label the EIAV Gag polyprotein products. The resultant cell supernatants and cell lysates were separated and subjected to immunoprecipitation with EIAV antiserum to assay the levels of extracellular and intracellular Gag expression, respectively. The SDS-PAGE profiles presented in Fig. 2 indicate that the full-length and p9-deleted Gag polyproteins are expressed to similar levels in transfected cells (Fig. 2A; compare lanes 2 and 3). However, only expression of the complete EIAV Gag polyprotein produced significant extracellular Gag products (Fig. 2B, lane 2); little extracellular Gag product was detectable in the supernatants taken from cells expressing the p9-deleted Gag polyprotein (Fig. 2B, lane 3), consistent with the hypothesis that p9 contains an L domain. Furthermore, equilibrium sucrose density gradient analysis of the extracellular Gag polyprotein produced by cells transfected with the EG vector revealed that the released EIAV Gag polyprotein banded uniformly at a density of 1.18 g/ml, characteristic of retroviral particles; there was no evidence of soluble Gag polyproteins being released by the transfected cells (Fig. 3A). We also determined a density of 1.18 g/ml for the particles resulting from the EG.p9- construct (Fig. 3B). It is important to note that we extended the labeling time in order to obtain enough particles for detection. These observations validate the EIAV Gag budding assay developed for these studies and confirm the role of EIAV p9 in particle assembly. Thus, the budding assay was used to characterize further the L domain of EIAV p9, as described below.

**Identification of functional homologs.** We were interested in determining whether the proline-rich L domains of the lentivirus HIV-1 or oncovirus RSV could supply the late budding function to the EIAV Gag protein. To answer this question, two chimeric Gag proteins were constructed. The EIAV/HIV-1 chimeric Gag polyprotein (Fig. 1, construct EG.p6) was designed to express HIV-1 p6 protein fused to the EIAV Gag polyprotein at the C terminus of nucleocapsid to determine the functional homology of the two lentiviral L domains. The EIAV/RSV chimeric Gag polyprotein (Fig. 1, construct EG.p2) placed the RSV p2b sequence at the C terminus of EIAV NC to determine if an oncoviral L domain could rescue a lentiviral Gag protein. Analyses of these chimeric Gag polyproteins in the standard budding assay revealed that the EIAV/HIV-1 Gag chimera expression yielded levels of intracellular and extracellular protein equivalent to those observed with the wild-type EIAV Gag polyprotein (Fig. 2, lanes 5). Quantitation of the relative amounts of extracellular protein produced by wild-type and EIAV/HIV-1 chimeric Gag polyproteins indicates that the HIV-1 p6 budding efficiency was about 90% of the level observed for EIAV p9, indicating that the HIV-1 p6 efficiently supplied the late budding function to the EIAV Gag polyprotein. Sucrose density gradient analysis of these chimeric particles also demonstrated that these are of typical 1.17-1.21 g/ml density (Fig. 3C). Similarly, the RSV p2b protein was able to mediate assembly of the EIAV Gag polyprotein, although the expression of this chimeric polyprotein appeared to be somewhat less than that observed with wild-type EIAV Gag, perhaps reflecting an instability of the EIAV/RSV chimera (Fig. 2, lanes 4). Thus, these results demonstrate that the late budding domains of RSV p2b and HIV-1 p6 are functionally homologous and interchangeable with EIAV p9, despite the lack of sequence homology between the L domain of EIAV and those of RSV and HIV-1.

**Identification of the L domain within EIAV p9.** L-domain functions have previously been localized to small proline-rich...
peptide sequences in HIV-1 p6 and in RSV p2 (16). In light of the absence of a similar proline-rich peptide sequence in p9 of EIAV, we were interested in mapping the specific L-domain sequence within the 51 residues of the EIAV p9 protein. To provide an initial localization of the p9 L domain, we made progressive C-terminal truncations of the EIAV Gag proteins (Fig. 1, constructs EG.S11/*, EG.N21/*, EG.K31/*, and EG.V41/*). These Gag polyproteins were then assayed for the ability to produce particles in the standard transient expression assay in COS cells as described above. Analyses of the C-terminal Gag deletions in this budding assay revealed that all of these mutants produced levels of intracellular Gag product that were similar to that observed with the wild-type p9 (Fig. 4A). However, the levels of extracellular Gag particles produced by the different deletion constructs indicated that C-terminal deletions retaining at least the first 30 amino acids of p9 produced essentially wild-type levels of extracellular Gag particles (Fig. 4B, EG.K31 and EG.V41) and hence were capable of providing the late budding function. Constructs containing only the N-terminal 11 (Fig. 4B, EG.S11) or 21 (Fig. 4B, EG.N21) residues failed to produce significant levels of extracellular Gag particles, indicating an absence of the late budding function.

A second set of deletion constructs was generated to map the L domain from the N terminus of p9 (Fig. 1, constructs EG.d1-9, EG.d1-19, and EG.d1-28). The results of these assays (Fig. 4B) demonstrated that Gag polyproteins with deletions of p9 residues 1 to 9 (EG.d1-9) or 1 to 19 (EG.d1-19) produced extracellular Gag particles, while budding was lost when residues after position 20 of p9 were removed (EG.d1-28) (Fig. 4, lanes 8 to 10). Once again, the absence of extracellular particles in the latter case was not due to a lack of production of these truncated polyproteins, as analyses of the intracellular Gag protein reveals similar levels of expression between the Gag polyproteins containing N-terminal truncations and the wild-type p9 (Fig. 4A, lanes 8 to 10).

Taken together, the experiments with progressive N-terminal and C-terminal truncations map the putative EIAV late domain to residues 20 to 30 of the p9 protein. To determine if this sequence alone could indeed provide L-domain function, we generated an EIAV Gag polyprotein construct that expresses only residues 20 to 30 (Q20N21L22Y23P24D25L26S27E28I29K30) of p9 directly fused to the C terminus of NC (Fig. 1, construct EG.20-30). Analysis of this polyprotein in the budding assay demonstrated wild-type levels of protein expression in transfected cells (Fig. 4A, lane 11) and production of extracellular Gag particles (Fig. 4B, lane 11), although at a somewhat reduced level. Thus, these data indicate that residues 20 to 30 of EIAV p9 constitute the L domain and that this peptide sequence can provide the late budding function during the assembly of EIAV Gag polyproteins.

Characterization of critical residues within the EIAV L domain. Having identified the L-domain peptide sequence of EIAV p9, we next used alanine scanning to determine which residues are critical for providing the late function. Thus, p9 residues Q20, N21, L22, Y23, P24, D25, L26, S27, E28, I29, and K30 were individually changed to alanine in the context of the wild-type Gag polyprotein and assayed in our standard EIAV...
Gag particle budding assay. The results of these assays are shown in Fig. 5. All of the mutant polyproteins containing p9 mutations were expressed in transfected cells at levels similar to those of Gag constructs containing wild-type p9 (Fig. 5A). Examination of the media revealed that p9 proteins with alanine substituted for Q \( ^{20} \), N \( ^{21} \), L \( ^{22} \), D \( ^{25} \), S \( ^{27} \), E \( ^{28} \), I \( ^{29} \), and K \( ^{30} \) produced extracellular Gag particles, indicating that these individual residues are not critical for the late budding function (Fig. 5B, lanes 4, 5, 6, 9, 11, 12, 13, and 14, respectively). In distinct contrast, however, those Gag proteins with alanine replacing residues Y \( ^{23} \), P \( ^{24} \), or L \( ^{26} \) completely lost L-domain activity (Fig. 5B, lanes 7, 8, and 10). These results identify a putative YXXL motif (underlined) within the sequence Q\( ^{20}N^{21}L^{22}Y^{23}P^{24}D^{25}L^{26}S^{27}E^{28}I^{29}K^{30} \) as the functional module within the novel EIAV late domain.

DISCUSSION

We report here the development of a transient Gag expression assay to characterize the role of EIAV p9 in virus particle assembly and release from cells. This type of assay has been used extensively to characterize functional domains involved in assembly of other retroviral particles and to identify functional homologs via construction of chimeric Gag polyproteins. Using this new EIAV Gag budding assay, we were able to identify a late assembly function in p9 that is crucial for the efficient release of particles from the plasma membrane. Late assembly defects identified in other retroviruses have been functionally mapped to two highly conserved sequence motifs, P(T/S)AP of HIV-1 (8, 10) and PPPPY of RSV (16, 26). We have shown that both of these motifs can supply that late assembly function to a p9-deficient EIAV Gag protein within the context of HIV-1 p6 and RSV p2b. The EIAV Gag polyprotein does not encode similar sequences; thus, a novel sequence encoded by p9 is able to provide the late function. We mapped this late assembly function within EIAV Gag to residues 20 to 30 (Q\( ^{20} \), N\( ^{21} \), L\( ^{22} \), Y\( ^{23} \), P\( ^{24} \), D\( ^{25} \), L\( ^{26} \), S\( ^{27} \), E\( ^{28} \), I\( ^{29} \), and K\( ^{30} \)) of the p9 protein and demonstrated that these residues are sufficient to provide the assembly function in the absence of other p9 sequences. Furthermore, we have shown that residues Y\( ^{23} \), P\( ^{24} \), and L\( ^{26} \) are critical for a functioning L domain, suggesting the involvement of a YXXL motif in EIAV Gag particle budding.

Our previous work showed that the C terminus of the EIAV Gag protein encoded a late assembly function that could restore budding of a defective RSV Gag protein. In this report, we show that p9 is critical for EIAV Gag assembly and particle release. We also show that the L domains of RSV p2b and HIV-1 p6 can replace p9 function in the context of EIAV Gag. This is the first report that the oncoviral p2b protein of RSV can provide the late assembly function to a lentiviral Gag protein. The positional independence of this L domain has been shown previously, however, in studies using an oncoviral Gag protein. Although the three L domains are different with respect to sequence, they appear to be functionally and positionally interchangeable within both oncoviral and lentiviral Gag proteins. The mechanisms by which L domains function remain to be determined.

Examination of the three types of sequences providing the late assembly function provides some insights as to possible L-domain mechanisms. Critical residues identified within the HIV-1 L domain are PTAP, while in RSV they are PPPPY (10, 28). The PTAPP sequence is highly conserved among lentiviruses (with the exception of EIAV), while PPPPY is conserved among oncoviruses. These motifs also fall into categories of ligands known for protein interaction domains. The PTAPP sequence fits consensus of an SH3 ligand, whereas PPPPY has recently been shown to bind the WW domain (3, 7). Both SH3 and WW domains are found in certain cytoskeletal and signal transduction proteins. The YXXL motif within the EIAV late domain may function by binding to the SH2 domain of a cellular protein that facilitates particle release. SH2 domains recognize the general sequence of phosphotyrosine-X-X-hydrophobic, with X indicating variable residues that may dictate SH2 domain specificity (19). We currently have no evidence that the EIAV p9 tyrosine is phosphorylated and have previously reported that high-pressure liquid chromatography-purified virion p9 does not appear to contain a phosphorylated residue (12). An alternative hypothesis can be based on the identification of nonphosphorylated YXXL motifs that interact with adapter proteins to facilitate clathrin-coated pit formation (14, 22). The EIAV YXXL motif could function to recruit proteins normally involved in the internalization of proteins in order to facilitate release of the budding virion.

YXXL motifs have been found in a variety of viral
cellular proteins. A YXXL motif in Nef has been shown to contribute to the pathogenicity of isolate SIVp273 via an interaction with the Src protein (6). The bovine leukemia virus transmembrane protein contains YXXL sequences which are critical for viral infectivity and replication (25). Surface expression of SIV Env glycoproteins appears to be downregulated by a transmembrane protein YXXL motif (11). Cellular proteins containing these motifs are commonly involved in the signaling pathways of hematopoietic cells such as NK cells, B cells, T cells, and macrophages. It has also been shown that clustering of YXXL motifs within hematopoietic cell lines can induce actin polymerization, resulting in an internalization event (4). This may be a clue to the mechanism of retrovirus L domains, as proteins like actin, coflin, ezrin, and moesin (all proteins involved in regulation of actin assembly) are incorporated into HIV-1 particles (15).

While further experiments are necessary to elucidate the precise mechanisms used by retroviral L domains, the results of studies to date suggest a diversity of budding domains that may vary in the specificity of interactions with cellular proteins involved in virion assembly. Identification of the cellular proteins involved in this assembly event may lead to a better understanding of the mechanism by which particles bud from cells.

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