JOURNAL OF VIROLOGY, Sept. 1997, p. 6541–6546 Vol. 71, No. 9
0022-538X/97/$04.00+0

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

BRIDGET A. PUFFER,[1] LESLIE J. PARENT,[2,3] JOHN W. WILLS,[3] AND RONALD C. MONTELARO[1]*

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261,[1] and Department of Medicine[2] and Department of Microbiology and Immunology,[3] Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Received 27 March 1997/Accepted 4 June 1997

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 ($Q^{20}N^{21}L^{22}Y^{23}P^{24}D^{25}L^{26}S^{27}E^{28}I^{29}K^{30}$) capable of providing the late assembly function. Alanine scanning studies of this L-domain sequence demonstrated that mutations of residues Y^{23}, P^{24}, and L^{26} 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 proteins 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 shift 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).

* Corresponding author. Mailing address: Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, W1244 Biomedical Science Tower, Pittsburgh, PA 15261. Phone: (412) 648-8869. Fax: (412) 383-8859. E-mail: rmont@pop.pitt.edu.

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 $P^7T^8A^9P^{10}$ 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 assemby and release from cells (16). The RSV late assembly function maps to the p2b cleavage product, now referred to as the late assembly (L) domain. This protein encodes the residues PPPY, 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

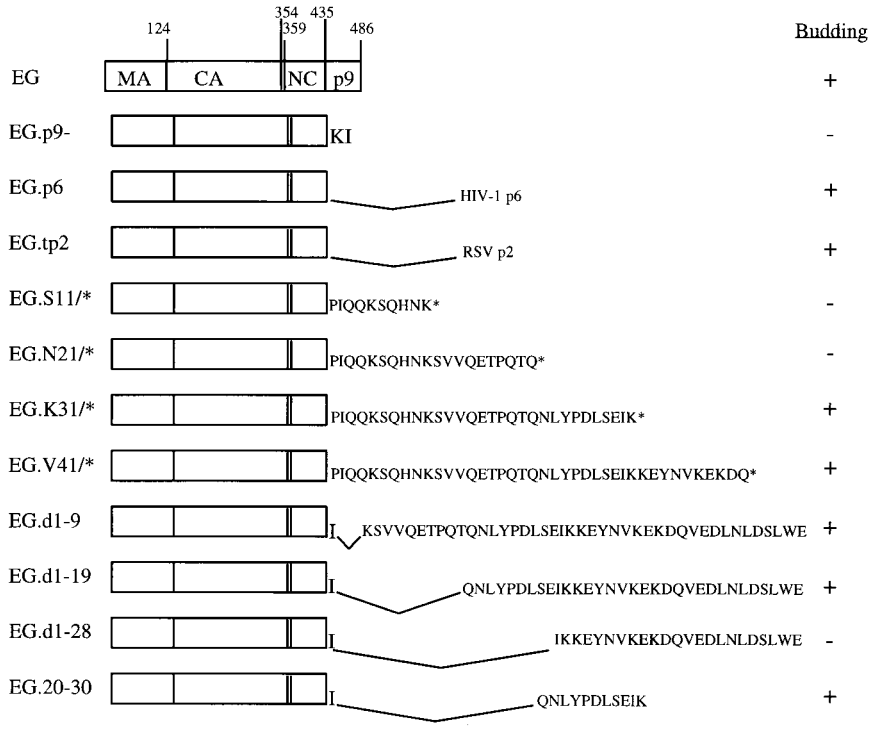

FIG. 1. Schematic diagrams of constructs generated to identify the function provided by EIAV p9 and to localize residues pertinent to that function. Gag polyproteins are diagrammed with designations for the amino acid boundaries of individual cleavage products. The nomenclature for each polyprotein is indicated to the left, and ability to bud is designated to the right.

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 PV19-2 proviral clone (17) by using the upstream primer gag/SstI (5′tttgtctgttcgagctcctacagttg3′) and downstream primer p9BssHII (5′gtagg cctgcgcgctagattatatgttact3′). This PCR product was digested with *Sst*I and *Bss*HII ligated into the previously described construct pSV.Myr1.D37S (5) cut with the same enzymes. The pSV.EG.p9- construct was generated by using megaprimer PCR method to create a *Bgl*II site at the first residue of p9. The upstream primer p9f5′ (5′cagactggtcttgcgggcccattta3′) and downstream primer p9BssHII flanked the mutagenic primer p9P1/Bgl (5′ccagaaacaaactaagatcttacaacagaagagt3′). This PCR product was cut with *Bgl*II and *Apa*I and ligated into the pSV.EG vector by using *Apa*I and an existing downstream *Bgl*II site.

(ii) Chimeric Gag proteins. To produce a chimera containing HIV-1 p6 fused to the EIAV p9-deleted Gag polyprotein, pSV.EG.p6 was generated by digesting the previously described pSV.RHB (16) with *Bgl*II and *Bss*HII and ligating this p6-encoding fragment into pSV.EG.p9- by using the *Bgl*II and *Bss*HII sites. The RSV chimeric construct pSV.EG.tp2 was generated by PCR amplifying the p2 sequence with the upstream primer PARE 41 (5′tggcacatccagatctggcattgcgg aacagctatt3′) and downstream primer PARE 42 (5′ttactacaaacactccccacataa3′). This PCR product was cloned directly into pGEM5Zf(+) (Promega) to generate pGem.tp2. Plasmid pGem.tp2 was digested with *Bgl*II and *Mlu*I, and this fragment was ligated into pSV.EG.p9- by using the *Bgl*II and *Bss*HII sites.

(iii) Deletion constructs of EIAV p9. C-terminal deletion constructs were generated by using the upstream primer p9f5′ and mutagenic downstream primers p9S11/* (5′agtctcttgtacaactcatttgttgtgctgactc3′), p9N21/* (5′cgctcagatctgggta cagttattgagtctgagg3′), p9K31/* (5′gacattgcgcgcctattttatttcgctcaga3′), and p9V41/* (5′ctgtccaggttgcgcgcctctcattgatccttctc3′). pSV.EG.S11/* was constructed by directly cloning the PCR fragment into pGEM5Zf(+) (Promega) to generate pGemS11. Plasmid pGemS11 was digested with *Apa*I and *Mlu*I, and then the fragment was ligated into pSV.EG by using the existing *Apa*I and *Bss*HII sites. pSV.EG.N21/* was created by digestion of the gel-purified PCR product with *Bgl*II and *Apa*I; these fragments were ligated into pSV.EG at the same sites. pSV.EG.K31/* and pSV.EG.V41/* were generated by digesting gel-purified PCR products with *Apa*I and *Bss*HII and ligating the fragment into the *Apa*I-*Bss*HII sites of pSV.EG.

N-terminal deletion constructs were generated by using the downstream primer p9BssHII and mutagenic upstream primers p9d1-9 (5′caacagaagagtcagctg atcaaatctgttgta3′), p9d1-19 (5′gagactcctcggatccaaaatctgtacccaga3′), and p9d1-28 (5′gcgtgatcaaaaaggaatacaatgtcaa3′) to amplify from pSV.EG template. Gel-purified PCR products were digested with *Bss*HII and either *Bcl*I or *Bgl*II and ligated into the *Bgl*II-*Bss*HII sites of pSV.EG.p9-.

(iv) Alanine scanning mutagenesis of p9. Residues Q^{20}, N^{21}, L^{22}, Y^{24}, and P^{24} were mutated to alanine by using the upstream primer p9f5′ and the mutagenic downstream primers Q^{20}/A (5′atttcgctcggatccgggtacagatttgcagtctgagga3′), N^{21}/A (5′tatttcgctcggatccgggtacagcgcttgagtctgagga3′), L^{22}/A (5′tatttcgctcggatccgggtacg cattttgagtctgagga3′), Y^{24}/A (5′ttcgctcagatctggggccagattttga3′), and P^{24}/A (5′ttcgctcagatctgcgtacagatttga3′). These PCR products were gel purified and then digested with *Apa*I and either *Bgl*II or *Bam*HI. The digested fragments were then ligated into the *Apa*I and *Bgl*II sites of pSV.EG. The D^{25} and L^{26} mutations were generated by PCR with *Pfu* polymerase (Stratagene) by using primer D^{25}/A (5′ccagctctgagcgaaataaaaaaggaatacaatg3′) or L^{26}/A (5′ccagacgcgtccgaaataaaaa agg3′) with p9BssHII; a second flanking fragment was amplified by using primers LD/Af (5′gtacagattttgagtctgaggagtctgaggagtctcttgta3′) and p9f5′. Fragments were gel purified, phosphorylated by using T4 polynucleotide kinase, and ligated by using T4 DNA ligase. The ligation reaction was used for a subsequent amplification using primers p9f5′ and p9BssHII. This product was gel purified, digested with *Apa*I and *Bss*HII, and then ligated into the *Apa*I and *Bss*HII sites of pSV.EG. Mutations of S^{27}, E^{28}, I^{29}, and K^{30} to alanine were generated by PCR using the mutagenic primers S^{27}/A (5′tacccagatctggccgaaataaaaaaagg3′), E^{28}/A (5′tacccagatctgagcgcaataaaaaagg3′), I^{29}/A (5′tacccagatctgagcgaagcaaaaaagg3′), and K^{30}/A (5′tacccagatctgagcgaaatagcaaaggaatac3′) and the downstream primer p9BssHII. The PCR fragments were gel purified, digested with *Bgl*II and *Bss*HII, and ligated into pSV.EG by using the existing *Bgl*II and *Bss*HII sites. All vectors were sequenced by using an ABI Prism automated DNA sequencer.

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 *Xba*I-digested and ligated plasmids by the DEAE-dextran-chlo-

roquine method as previously described (16). Transfected cells were metabolically labeled for 5 h with EXPRE^{35}S^{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 treatment with Immunoprecipitin (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.

Analysis of Gag particles by equilibrium density centrifugation. The physical properties (soluble or particle associated) of the Gag proteins released by the transfected COS-1 cells were determined by equilibrium density centrifugation on a sucrose gradient as described previously for RSV Gag particles (24). Briefly, transfected cells in 60-mm-diameter dishes were metabolically labeled for 8 h in serum-free, methionine-free Dulbecco's medium supplemented with 100 μCi of [^{35}S]methionine to produce radiolabeled EIAV Gag particles. Cell supernatants containing radioactive EIAV Gag particles were layered onto a 10 to 50% sucrose gradient and centrifuged at 82,500 × *g* for 18 h at 4°C. As an internal control for particle assembly, radioactive RSV Gag particles were included in each gradient. Gradient fractions were collected 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 p9 functional properties in an 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.

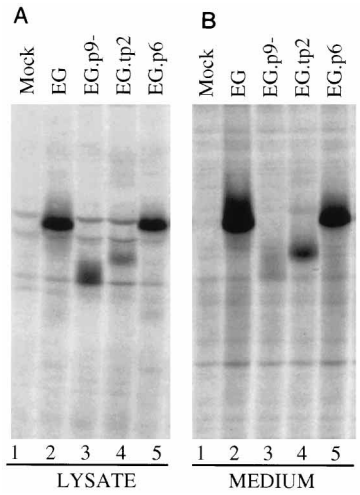

FIG. 2. Budding assay analysis of EIAV Gag polyprotein and characterization of function provided by EIAV p9. A wild-type EIAV Gag expression plasmid was generated to assay the ability of this polyprotein to mediate budding in the absence of other virus-encoded proteins. A p9 deletion EIAV Gag expression plasmid was used to determine if p9 functioned in EIAV particle assembly. Chimeric EIAV Gag polyprotein expression plasmids were designed to substitute either HIV-1 p6 or RSV p2b in place of EIAV p9 (Fig. 1). The expression and budding properties of these Gag constructs were then analyzed by using the standard budding assay. (A) Intracellular expression of Gag polyproteins; (B) extracellular expression of Gag particles.

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.tp2) 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-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

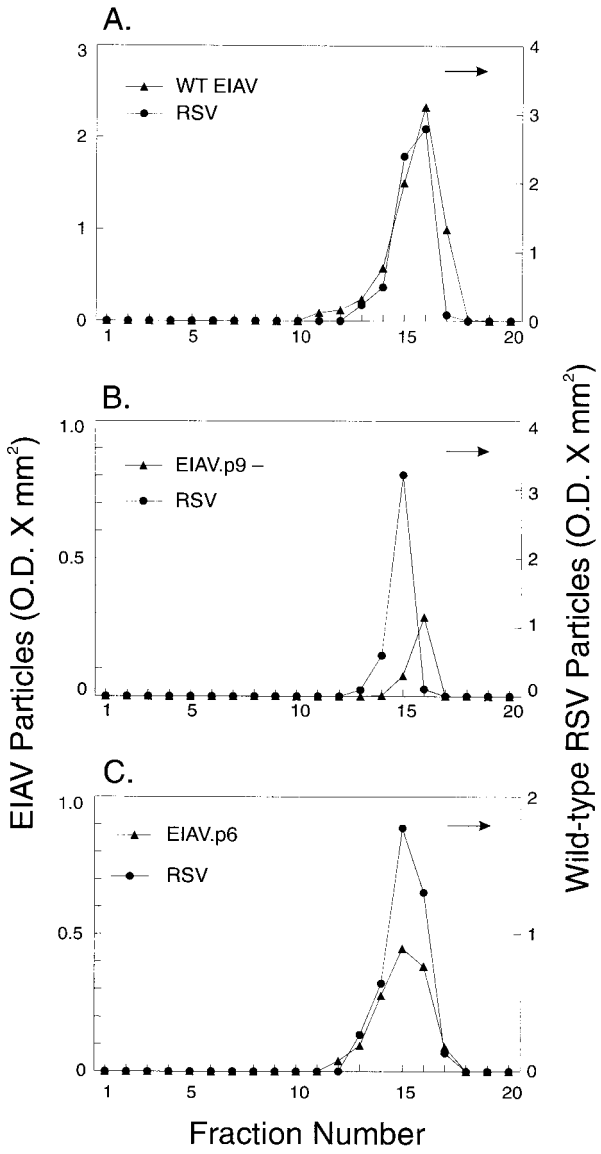

FIG. 3. Sucrose gradient analysis of EIAV Gag particles. Extracellular radiolabeled EIAV Gag particles produced by transfection of COS cells were analyzed by equilibrium density gradient centrifugation as described in Materials and Methods. Radioactive RSV Gag particles were included as an internal standard of 1.18 gm/ml.

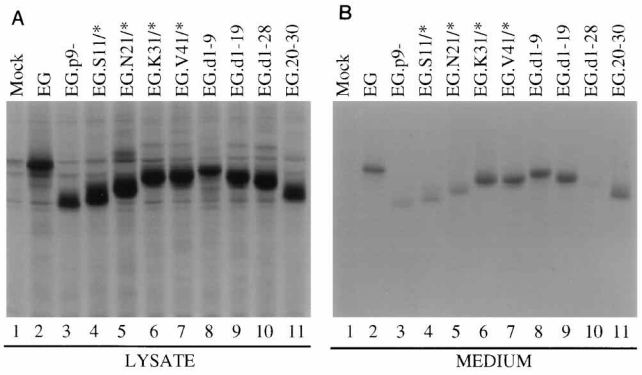

FIG. 4. Budding assay analysis of truncated EIAV Gag polyproteins. COS-1 cells were transfected with the designated Gag expression construct and metabolically labeled with [^{35}S]methionine for 5 h. The intracellular (A) and extracellular (B) levels of expressed Gag proteins were then assayed by immunoprecipitation of the lysate and medium fractions, respectively, with rabbit polyclonal anti-EIAV serum, resolved by SDS-PAGE (12% acrylamide gel), and visualized by autoradiography. Details of Gag constructs are summarized in Fig. 1.

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 ($Q^{20}N^{21}L^{22}Y^{23}P^{24}D^{25}L^{26}S^{27}E^{28}I^{29}K^{30}$) 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 Q^{20}, N^{21}, L^{22}, Y^{23}, P^{24}, D^{25}, L^{26}, S^{27}, E^{28}, I^{29}, and K^{30} were individually changed to alanine in the context of the wild-type Gag polyprotein and assayed in our standard EIAV

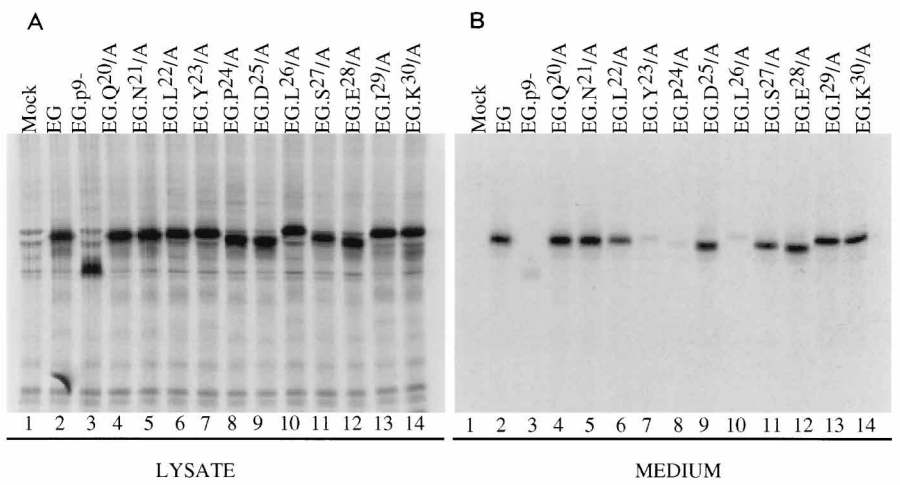

FIG. 5. Analysis of budding properties of EIAV Gag polyprotein constructs containing site-directed mutations in the p9 L domain. To identify the p9 residues critical for EIAV Gag budding, we introduced a series of alanine mutations into the p9 L domain and analyzed the budding activity induced by each of these site-directed mutations as described in the legend to Fig. 2. (A) Intracellular expression of Gag polyproteins; (B) extracellular expression of Gag polyprotein particles.

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 $Q^{20}N^{21}L^{22}\underline{Y^{23}P^{24}}D^{25}\underline{L^{26}}S^{27}E^{28}I^{29}K^{30}$ 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 same 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 PPPY 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 PPPY 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 and

cellular proteins. A YXXL motif in Nef has been shown to contribute to the pathogenicity of isolate SIVpbj 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, cofilin, 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.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants 5R01CA49296 (R.C.M.), R01CA47482 (J.W.W.), and K11A101148 (L.J.P.) and by American Cancer Society grant ACS FRA-427 (J.W.W.).

REFERENCES

1. **Braun, M. J., J. E. Clements, and M. A. Gonda.** 1987. The visna virus genome: evidence for a hypervariable site in the *env* gene and sequence homology among lentivirus envelope proteins. J. Virol. **61:**4046–4054.
2. **Checroune, F., X. Yao, H. G. Gottlinger, D. Bergeron, and E. A. Cohen.** 1995. Incorporation of Vpr into human immunodeficiency virus type 1: role of conserved regions within the p6 domain of Pr55gag. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **10:**1–7.
3. **Chen, H. I., and M. Sudol.** 1995. The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. Proc. Natl. Acad. Sci. USA **92:**7819–7823.
4. **Cox, D., P. Chang, T. Kurosaki, and S. Greenberg.** 1996. Syk tyrosine kinase is required for immunoreceptor tyrosine activation motif-dependent actin assembly. J. Biol. Chem. **271:**16597–16602.
5. **Craven, R. C., R. P. Bennett, and J. W. Wills.** 1991. Role of the avian retroviral protease in the activation of reverse transcriptase during virion assembly. J. Virol. **65:**6205–6217.
6. **Du, A., S. M. Lang, V. G. Sasserville, A. A. Lackner, P. O. Ilyinskii, M. D. Daniel, J. U. Jung, and R. C. Desrosiers.** 1995. Identification of a nef allele that causes lymphocyte activation and acute disease in macaque monkeys. Cell **82:**665–674.
7. **Garnier, L., J. W. Wills, M. F. Verderame, and M. Sudol.** 1996. WW domains and retrovirus budding. Nature **381:**744–745.
8. **Gottlinger, H. G., T. Dorfman, J. G. Sodroski, and W. A. Haseltine.** 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA **88:**3195–3199.
9. **Henderson, L. E., R. C. Sowder, G. W. Smythers, and S. Oroszlan.** 1987. Chemical and immunological characterizations of equine infectious anemia virus *gag*-encoded proteins. J. Virol. **61:**1116–1124.
10. **Huang, M., J. M. Orenstein, M. Martin, and E. O. Freed.** 1995. p6 gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J. Virol. **69:**6810–6818.
11. **LaBranche, C. C., M. M. Sauter, B. S. Haggarty, P. J. Vance, J. Romano, T. K. Hart, P. J. Bugelski, M. Marsh, and J. A. Hoxie.** 1995. A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. J. Virol. **69:**5217–5227.
12. **Montelaro, R. C., N. Lohrey, B. Parekh, E. W. Blakney, and C. J. Issel.** 1982. Isolation and comparative biochemical properties of the major internal polypeptides of equine infectious anemia virus. J. Virol. **42:**1029–1038.
13. **Myers, G., S. Wain-Hobson, B. Korber, R. F. Smith, and G. N. Pavlakis (ed.).** 1994. Human retroviruses and AIDS. Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, N.Mex.
14. **Ohno, H., J. Stewart, M.-C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J. S. Bonifacino.** 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science **269:**1872–1875.
15. **Ott, D. E., L. V. Coren, B. P. Kane, L. K. Busch, D. G. Johnson, R. C. Sowder II, E. N. Chertova, L. O. Arthur, and L. E. Henderson.** 1996. Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. J. Virol. **70:** 7734–7743.
16. **Parent, L. J., R. P. Bennett, R. C. Craven, T. D. Nelle, N. K. Krishna, J. B. Bowzard, C. B. Wilson, B. A. Puffer, R. C. Montelaro, and J. W. Wills.** 1995. Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. J. Virol. **69:**5455–5460.
17. **Payne, S. L., J. Rausch, K. Rushlow, R. C. Montelaro, C. Issel, M. Flaherty, S. Perry, D. Sellon, and F. Fuller.** 1994. Characterization of infectious molecular clones of equine infectious anemia virus. J. Virol. **75:**425–429.
18. **Pepinsky, R. B., R. J. Mattaliano, and V. M. Vogt.** 1986. Structure and process of the p2 region of avian sarcoma and leukemia virus *gag* precursor polyproteins. J. Virol. **58:**50–58.
19. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and L. C. Cantley.** 1993. SH2 domains recognize specific phosphopeptide sequences. Cell **72:**767–778.
20. **Sonigo, P., M. Alizon, K. Staskus, D. Klatzmann, S. Cole, O. Danos, E. Retzel, P. Tiollais, A. Haase, and S. Wain-Hobson.** 1985. Nucleotide sequence of the visna lentivirus: relationship to the AIDS virus. Cell **42:**369–382.
21. **Talbott, R. L., E. E. Sparger, K. M. Lovelace, W. M. Fitch, N. C. Pedersen, P. A. Luciw, and J. H. Elder.** 1989. Nucleotide sequence and genomic organization of feline immunodeficiency virus. Proc. Natl. Acad. Sci. USA **86:**5743–5747.
22. **Trowbridge, I. S., J. F. Collawn, and C. R. Hopkins.** 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. Annu. Rev. Cell Biol. **9:**129–161.
23. **Vogt, V., R. Eisenman, and H. Diggleman.** 1975. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. J. Mol. Biol. **96:**471–493.
24. **Weldon, R. A., Jr., C. R. Erdie, M. G. Oliver, and J. W. Wills.** 1990. Incorporation of chimeric Gag protein into retroviral particles. J. Virol. **64:**4169–4179.
25. **Willems, L., J. S. Gatot, M. Mammerickx, D. Portetelle, A. Burny, P. Kerkhofs, and R. Kettmann.** 1995. The YXXL signalling motifs of the bovine leukemia virus transmembrane protein are required for in vivo infection and maintenance of high viral loads. J. Virol. **69:**4137–4141.
26. **Wills, J. W., C. Cameron, C. B. Wilson, Y. Xiang, R. P. Bennett, and J. Leis.** 1994. An assembly domain of the Rous sarcoma virus Gag protein required late in budding. J. Virol. **68:**6605–6618.
27. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral gag proteins. AIDS **5:**639–654. (Editorial.)
28. **Xiang, Y., C. E. Cameron, J. W. Wills, and J. Leis.** 1996. Fine mapping and characterization of the Rous sarcoma virus pr76^{gag} late assembly domain. J. Virol. **70:**5695–5700.