Functions of Early (AP-2) and Late (AIP1/ALIX) Endocytic Proteins in Equine Infectious Anemia Virus Budding*

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The proline-rich L domains of human immunodeficiency virus 1 (HIV-1) and other retroviruses interact with late endocytic proteins during virion assembly and budding. In contrast, the YPDL L domain of equine infectious anemia virus (EIAV) is apparently unique in its reported ability to interact both with the μ2 subunit of the AP-2 adaptor protein complex and with ALG-2-interacting protein 1 (AIP1/Alix) protein factors involved in early and late endosome formation, respectively. To define further the mechanisms by which EIAV adapts vesicle trafficking machinery to facilitate virion production, we have examined the specificity of EIAV p9 binding to endocytic factors and the effects on virion production of alterations in early and late endocytic protein expression. The results of these studies demonstrated that (i) an ~300-residue region of AIP1/Alix (409–715) was sufficient for binding to the EIAV YPDL motif; (ii) overexpression of AIP1/Alix or AP-2 μ2 subunit specifically inhibited YPDL-mediated EIAV budding; (iii) virion budding from a replication-competent EIAV variant with its L domain replaced by the HIV PTAP sequence was inhibited by wild type or mutant μ2 to a level similar to that observed when a dominant-negative mutant of Tsg101 was expressed; and (iv) overexpression or siRNA silencing of AIP1/Alix and AP-2 revealed additive suppression of YPDL-mediated EIAV budding. Taken together, these results indicated that both early and late endocytic proteins facilitate EIAV production mediated by either YPDL or PTAP L domains, suggesting a comprehensive involvement of endocytic factors in retroviral assembly and budding that can be accessed by distinct L domain specificities.

EIAV p9 protein contains a YPDL late (L) domain that is critical for virus budding. In contrast, the EIAV genome is the simplest among the retroviruses, and EIAV virus particles have been shown to be infectious (5). This suggests that EIAV has evolved a mechanism to facilitate virion assembly and budding. In the light of evidence that MVBs play a central role in retroviral budding, it remains uncertain whether and how components of MVBs contribute to virion budding. It is reported that HLTV-1 Gag polyproteins interact with early endosomes (6), whereas budding into late endosomes is thought to occur at the plasma membrane (7). Therefore, it remains to be determined whether and how components of other enveloped viruses, may have evolved different L domains to exploit redundant cellular endocytic machineries to achieve virus budding and release.

The L-domain-interacting cellular proteins identified to date are endocytic components associated with the formation of either early endosomes (e.g. ubiquitous ligase, AP-2) or late MVBs (e.g. Tsg101, AIP1). Although it is currently assumed that MVBs play a central role in retrovirus budding, it remains uncertain whether and how components of other endosomal vesicles, including early endosomes, contribute to retroviral budding. It is reported that HTLV-1 Gag polyproteins interact first with Nedd4.1 at the plasma membrane and then with Tsg101 in late endosomes/MVB, suggesting a successive assembly and budding process through the endocytic pathway (17, 18). However, neither early nor late endosomes are optimally evolved for viral assembly and release. For example, the formation of early endosomes is topologically opposite that of viral budding, whereas budding into late endosome is topologically correct but imposes subsequent obstacles to virion release from infected cells. Therefore, it remains to be determined whether and how retroviruses combine and/or integrate early and late endosomal machinery to facilitate virion assembly and budding.
previous observations that the EIAV YPDL domain interacts with both AP-2 and AIP1 in different model systems, the current study was designed to examine the specificity of EIAV p9 binding to early and late endocytic factors and the effects of alterations in early and late endocytic protein expression on virion production. The results of these studies demonstrated a previously unrecognized involvement of both early and late endocytic machinery in EIAV virion assembly and budding, whether accessed by YPDL or PTAP L domains.

**EXPERIMENTAL PROCEDURES**

**Plasmids and DNA Mutagenesis**—Construction of CMV\_ct proviral EIAV and the CMV\_ct PTAP derivative was described previously (15). The plasmid encoding LexA\(^{-}\)p9 was constructed by inserting the “p9 fragment of a GST-p9 construct (14) in the polylinker site of pLexA-(1–202)+PL (19). Full-length AIP1, AIP1\(^{272–869}\) or AIP1\(^{409–715}\) were PCR-amplified and inserted into the Ga4 activation domain expression vector pACTII (Clontech). Plasmids encoding GST-p2b, GST-p6, and GST-p9 or mutant derivatives were described previously (14) and confirmed by DNA sequencing. The gene segment encoding AIP1 residues 409–715 was PCR-amplified and inserted in-frame into pET32c(+) (Novagen, San Diego, CA). Plasmids expressing Tsg101\(^{1–204}\) were obtained from Dr. Eric Freed (NIAID, National Institutes of Health, Frederick, MD) with the permission of Dr. Zijie Sun (Stanford University School of Medicine). Vectors expressing HA-tagged full-length and truncated AIP1/Alix proteins were generated by inserting PCR-amplified fragment into pCMV-HA (Clontech). pCDNA3.1 derivative expressing HA-tagged wild type \(\mu_2\) was a gift from Dr. Alexander Sorkin (University of Colorado Health Science Center), and the mutant \(\mu_2\) was generated using standard molecular cloning protocols. pSilencer plasmid was purchased from Ambion (Austin, TX), and sequences of AIP1 (5‘-gcaagatggtgtgataa-3’) or (5‘-gtacagctgctgtcct-3’) were selected to generate pSilencer constructs that produce small hairpin RNA (shRNA), which was then spontaneously processed in vitro into siRNA.

**Cell Culture and Transfection**—COS-1 cells were cultured in minimum essential medium as described previously (20). HeLa and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. GenePorter II (Gene Therapy Systems, San Diego, CA) or FuGENE 6 (Roche Diagnostics) was used to transfect cells according to the manufacturer’s recommendation. SiRNA duplexes were synthesized in vitro and transfected into HeLa cells using GeneEraser\textsuperscript{TM} (Stratagene, La Jolla, CA).

**Yeast Two-hybrid Analysis**—The Saccharomyces cerevisiae strain used for two-hybrid studies was CTY105d (MATa ade2-101 his3-\(\Delta\)200leu2-\(\Delta\)1 trp1-\(\Delta\)901gal4 gal80 URA3:lexAop-lacZ). Yeast cells were grown in synthetic dextrose medium lacking appropriate supplements to maintain selection for plasmids. \(\beta\)-Galactosidase activity was assayed on permeabilized cells grown to mid-log phase in the selective synthetic dextrose medium and expressed in Miller units. Yeast protein extracts for immunoblot analysis were prepared as described previously (21) and analyzed by immunoblotting with a monoclonal anti-HA antibody (Roche Diagnostics).

**GST Pull-down Assays**—GST fusion proteins and N-terminally His-tagged AIP1\(^{409–715}\) were expressed from Escherichia coli BL21(DE3)pLysS (Novagen, Madison, WI). Bacterial extracts were prepared in STE buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) + 1% (v/v) Triton X-100, 5 mM dithiothreitol, and Complete\textsuperscript{TM} protease inhibitor mixture (Roche Diagnostics), as described previously (22). GST-p2b, GST-p6, and GST-p9 or mutant derivatives were incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) at 4 °C for 1 h. After six washes with 1 ml of buffer STE/1% Triton X-100, beads with bound proteins were incubated with bacterial extract (500 µl) containing His-tagged AIP1\(^{409–715}\) at 4 °C for 1 h. After six washes with STE/1% Triton X-100, beads were boiled in sample buffer, and proteins were separated by 10% polyacrylamide SDS-PAGE. Bound His-tagged AIP1\(^{409–715}\) was detected by Western analysis with an anti-His tag antibody and enhanced chemiluminescence with reagents from Amersham Biosciences.

**Reverse Transcriptase (RT) and Western Blotting Assays**—To measure virion production, culture medium collected from 24 to 48 h after transfection was clarified of cell debris by slow speed centrifugation and then centrifuged at 20,000 \(\times g\) for 3 h at 4 °C to pellet virions. Virion pellets were resuspended in 50 µl of phosphate-buffered saline, and 10-µl samples were assayed in RT assays as described previously (20).

To examine EIAV-specific protein content in transfected cells and pelleted virions, total cell lysates prepared as described previously (20) and pelleted virions were resolved by electrophoresis through 4–15% gradient gels (Bio-Rad) and immunoblotted using a reference immune serum from a field-infected horse (20). Horseradish peroxidase-conjugated goat anti-horse IgG F(ab\(^\prime\))\(_2\) (Jackson ImmunoResearch, West Grove, PA) was used as secondary antibody. The immunoblots were developed by incubation with SuperSignal West Pico chemiluminescent substrate (Pierce). Horseradish peroxidase-conjugated high affinity anti-HA antibody (Roche Applied Science) was used to specifically detect HA-tagged proteins.

**RESULTS**

**Interaction between AIP1 and EIAV p9 Proteins**—Interaction between AIP1 and EIAV p9 was previously demonstrated using yeast two-hybrid and GST pull-down assays (12, 13, 23). To serve as a starting point for a comprehensive comparison, we initially examined the p9-AIP1 interaction using similar approaches. We previously demonstrated interaction between the \(\mu_2\) subunit of the AP-2 adaptor protein complex and EIAV p9 using GST pull-down assay (24). In that study, a recombinant protein containing only the N-terminal 30 amino acids of EIAV p9 was fused to GST, in contrast to the full-length p9 protein (51 residues) used in studies of p9-AIP1 binding (12, 13, 23). Therefore, we performed p9-AIP1 binding assays using the N terminus of p9 (designated as “p9”) in this study. Fig. 1 summarizes the yeast two-hybrid results using “p9” as bait to examine binding activities of full-length and two truncated AIP1 proteins. Full-length AIP1 bound to “p9” as expected (Fig. 1A), and interaction of two truncated fragments (AIP1\(^{372–869}\) and AIP1\(^{409–715}\), with “p9” was also detected. The stronger interaction of full-length AIP1 was not due to higher expression of the fusion protein as shown by Western blot analysis (Fig. 1B). The observed binding properties are consistent with previous studies showing that both the N terminus (1–175) and the C-terminal proline-rich domain (717–869) of AIP1 are not required for this interaction (12, 13, 23). Additionally, the current studies further defined the p9 binding domain of AIP1 to residues 409–715. It is interesting to note that the EIAV p9 binding domain of AIP1 (residues 409–715) does not contain the PSAP motif (AIP1717–720) involved in interaction with Tsg101 (13).

To compare the binding properties of AIP1 proteins and the \(\mu_2\) subunit in parallel, we performed GST pull-down assays using GST-“p9” and the AIP1\(^{409–715}\) construct. Our data revealed that similar levels of GST fusion protein were produced from each construct (Fig. 2A) and that “p9” evidenced to bind to the AIP1\(^{409–715}\)-single alanine substitutions in the EIAV L domain at residues Tyr-23, Pro-24, or Leu-26 abolished detectable AIP1\(^{409–715}\) binding, suggesting their critical role for AIP1 interaction, whereas mutation of D25A reduced AIP1\(^{409–715}\) binding by about

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70% when compared with *p9. The D25A substitution in p9 has previously been reported to have no effect on EIAV viral budding (24). Our data confirmed that the YPDL sequence is a critical determinant of AIP1-p9 interaction, consistent with other reports (12, 13, 23). Interestingly, the YPDL sequence specificity for AIP1 binding reported here is similar to that for p9 binding to Tsg101–204 that contains the HIV-1 PTAP binding domain (25). As summarized in Fig. 3, EIAV production was reduced by 50–70% from cells expressing AIP1 proteins when compared with cells transfected with vector DNA (Fig. 3A). Various AIP1 proteins differed slightly in their inhibitory effect on EIAV budding, with the full-length AIP1 apparently the most potent inhibitor despite the relatively low levels of full-length AIP1 detected in transfected cells. Another interesting observation is that co-expression of Tsg101–204 reproducibly enhanced EIAV production by an average of 30–40% when compared with the vector control. Overexpression of AIP1 proteins and Tsg101–204 seemed not to significantly affect the steady state levels of EIAV-specific protein as shown in Fig. 3B (upper panel). Thus, these data demonstrated that EIAV budding mediated by the YPDL domain is specifically inhibited by AIP1 protein overexpression.

To further verify the specificity of the interactions observed above, we next examined the presence of HA-tagged AIP1 proteins in pelleted EIAV virions. Our data showed that all three AIP1 proteins as well as Tsg101 were detected in pelleted virions (Fig. 3B, lower panel). To exclude the possibility that overexpression of these endocytic proteins results in their appearance in the pelletable fraction of the culture medium, we transfected COS-1 cells with the various endocytic protein
expression vectors in the absence of EIAV proviral DNA and centrifuged the culture medium under conditions used to pellet EIAV virions. No AIP1 or Tsg101 could be detected by immunoblotting in these control pelleted preparations in the absence of virion production (data not shown). Therefore, our results further confirmed specific incorporation of AIP1 into EIAV virions. The incorporation of Tsg101 into EIAV virions that we observed also suggested its involvement in EIAV budding, perhaps through indirect interactions mediated by AIP1-Tsg101 interaction (13).

Effects of AIP1 and \( \mu_2 \) Expression on EIAV Budding—Results from the preceding experiments and previous reports have confirmed that both AIP1 and \( \mu_2 \) bind to the YPDL sequence of EIAV p9 protein in GST pull-down assays and that overexpression of AIP1 inhibits EIAV budding from transfected COS-1 cells. We next compared the roles of AIP1 and \( \mu_2 \) in EIAV budding using wild type EIAV (CMVukYPDL) and a mutant EIAV (CMVuk\(_{PTAP}\)) with its YPDL motif replaced by the HIV-1-derived PTAP motif. AIP1 and \( \mu_2 \) inhibited EIAV budding by 60 and 30\%, respectively, as measured by pelleted virion-associated RT activity (Fig. 4A). The specificity of \( \mu_2 \) inhibition was further confirmed by the observation that overexpression of a mutant \( \mu_2 \) (\( \mu_2\_{DM} \)) that is unable to bind to EIAV p9 protein had no significant effect on EIAV production (Fig. 4A). Thus, both AIP1 and \( \mu_2 \) contributed to EIAV budding.

Overexpression of Tsg101\(_{1-204}\) enhanced CMVuk(YPDL) budding by about 30\% (Fig. 4A) as observed in Fig. 3A, and budding of CMVuk(PTAP) was inhibited about 55\% by overexpressed Tsg101\(_{1-204}\) as expected (Fig. 4B). These two proviral constructs only differ from each other in the specificity of the L domain sequences, and both are previously demonstrated to be replication-competent (15). Our data indicated that Tsg101 is involved in both PTAP- and YPDL-mediated retrovirus budding but probably through different interaction mechanisms. Interestingly, expression of both wild type and mutant \( \mu_2 \) reduced CMVuk(PTAP) budding by about 80\% (Fig. 4B), indicating that the AP-2 adaptor protein complex might also participate in PTAP-mediated budding, although direct interaction with the PTAP motif may not be required.

The involvement of both early and late endocytic proteins in retrovirus budding is also supported by the observation that these proteins could be co-pelleted with the EIAV virion (Fig. 4, C and D). It appeared that there was more Tsg101\(_{1-204}\) and AIP1\(_{409-715}\) incorporated into pelleted EIAV virions when compared with the levels of \( \mu_2 \) subunit. There are several possible explanations for the observed differences. Although all proteins were detected via their HA epitope tags, the positions of these tags differ (N-terminal for Tsg101 and AIP1, internal for \( \mu_2 \)), possibly affecting the level of antibody reactivity of each protein to the anti-HA conjugate used in our immunoblots. If equal reactivity is assumed, different incorporation of these proteins might be the result of varied levels of these proteins expressed in the transfected cells (cell lysate data not shown). In particular, AP-2 expression is under such a tight regulation that loss of one subunit leads to complete depletion of AP-2 (26), and robust expression of AP-2 complex is difficult to achieve by overexpressing only one subunit of the complex. Taken together, our results demonstrated that both AIP1 and the \( \mu_2 \) subunit of AP-2 contribute to YPDL-mediated EIAV budding and that AP-2 also participates in PTAP-mediated budding.

Combined Effect of AIP1 and \( \mu_2 \) Overexpression on EIAV Budding—As both AIP1\(_{409-715}\) and \( \mu_2 \) overexpression inhibited EIAV budding, we sought to determine whether they function independently or in cooperation. For this purpose, we individually titrated the minimum amounts of AIP1\(_{409-715}\) or \( \mu_2 \) expression vector for detectable inhibitory effect (data not shown). Based on these assays, we cotransfected 293T cells using 0.8 \( \mu_\)g of \( \mu_2 \) and 0.2 \( \mu_\)g of AIP1\(_{409-715}\) individually or in combination, whereas keeping the total amount of DNA constant with blank vector plus EIAV proviral DNA. Virions released into the culture medium during the period from 24 to 48 h after transfection were pelleted, and RT activity associated with the resuspended pellet was measured to quantify EIAV budding (Fig. 5). Co-expression of \( \mu_2 \) and AIP1\(_{409-715}\) inhibited EIAV budding more than either protein expressed individually (80\% versus 40 and 50\% inhibition, respectively), indicating an additive effect of AIP1 and \( \mu_2 \) and further supporting the concept that both AIP1 and \( \mu_2 \) contribute to EIAV budding. To test whether the combined effect was cell type-dependent, we also carried out the same experiment in COS-1 cells and found similar results (Fig. 5B). Incorporation of AIP1\(_{409-715}\) and \( \mu_2 \) was also detected in pelleted EIAV virions (Fig. 5C).
siRNA and shRNA Silencing of μ2 and AIP1 on EIAV Budding—It has been cautioned that overexpression of certain proteins might nonspecifically affect global cellular functions rather than specifically disturbing the precise cellular pathway under investigation. To complement our overexpression study on the roles of μ2 and AIP1 in EIAV budding, we examined the effects of μ2 and AIP1 down-regulation on EIAV budding. Fraile-Ramos et al. (26) previously tested specific siRNA sequences against the μ2 subunit of AP-2 and demonstrated an average 50% reduction in μ2 expression in HeLa cells at 48 h after transfection. Based on the same siRNA sequence, we generated a pSilencer-derived DNA construct that produces small hairpin RNA (shRNA μ2) upon transfection, which is spontaneously processed into siRNA in the transfected cells. The silencing effect of this construct on COS-1 cells cotransfected with HA-tagged μ2 and AIP1 was also examined (Fig. 6A). Consistent with the results of Fraile-Ramos et al. (26), shRNA μ2 reduced μ2 expression by about 50% at 48 h after transfection. To knock down AIP1 expression, we cloned several sequences targeting various regions of AIP1 into the pSilencer vector and identified a specific sequence (5'/gctcaagatggtgtgataa-3') to achieve efficient silencing (Fig. 6B). At 48 h after co-transfection, expression of full-length AIP1 was reduced by about 80% and expression of AIP1409–715 was reduced by about 70%, respectively.

Our results revealed that 70–80% of AIP1 silencing could be achieved by shRNA AIP1, but only about 50% of μ2 silencing was observed using either siRNA or shRNA at 48 h after transfection. The long half-life of μ2 subunit (24 h) (27) might partially account for the relative inefficiency of knockdown procedures. Therefore, to more rigorously suppress AP-2 expression, assays at later time points (72–96 h after transfection) or two consecutive transfections were used to achieve higher levels of μ2 silencing (28). We therefore designed our experiments to first transfected 293T cells with increasing concentrations of μ2 siRNA and use only EIAV proviral DNA during the second transfection. Under these conditions, EIAV budding was inhib-
It has become increasingly appreciated that retroviruses have evolved to adapt various host cellular machinery for their replication, and the number of identified cellular factors that interact with viral proteins is rapidly increasing (7, 29–31). Recently, cellular proteins that specifically bind to retrovirus L domains have been extensively characterized (9, 10, 23, 24), providing strong evidence to link retrovirus budding with vesicular trafficking. However, the detailed molecular and cellular mechanisms that underlie the adaptation of endocytic proteins by retroviruses to facilitate budding remain to be illustrated.

In the current study, we compared the functions of the μ2 subunit of AP-2 and AIP1 in EIAV budding using complementary approaches. Our results revealed that disruption of μ2 or AIP1 expression, either up-regulation by transient transfection or down-regulation by siRNA silencing, reduced EIAV budding from cells cotransfected with an EIAV proviral construct. Furthermore, the effects of μ2 and AIP1 were cumulative, if not synergistic, as overexpression or knockdown of both resulted in more potent inhibition than that observed for either individual protein. Interestingly, our data demonstrated that HIV-1 PTAP L domain-mediated budding was also inhibited when μ2 subunit was overexpressed in the same cells. Therefore, the current report has provided evidence for a contribution of the AP-2 adaptor protein complex in both PTAP- and YPDL-mediated budding, in addition to the previously reported role of AIP1 in this process.

The μ2 subunit of AP-2 is a critical component for the formation of early endosomes, and identification of its interaction with the EIAV L domain suggested the involvement of the early endocytic pathway in retrovirus budding (24). However, the precise role of early endocytic proteins in viral budding has been controversial as the topology of endocytosis is opposite to that of retrovirus budding. The past few years have seen a collection of evidence that AIP1, a cellular component involved in late endosome/MVB formation, interacts with the EIAV YPDL L domain and an LYP motif located downstream of the PTAP motif in HIV p6 (12, 13, 23). Along with the identification of Tsg101 (a component of the ESCRT I complex essential for MVB formation) as the binding partner of HIV PTAP motif, these results suggest that retroviruses can recruit late endocytic machinery for budding as both processes share the same topology. However, it remains unclear whether and how retroviruses bud into late endosomes before they exit host cells. The current results that both μ2 and AIP1 contribute to YPDL- and PTAP-mediated budding strongly indicated that retrovirus might actually combine both early and late endosomal machinery to facilitate budding. Consistent with this model, MLV Gag polyproteins have been shown to traffic between the plasma membrane and the late endosomes, although the functional significance of this trafficking remains to be defined (17). Here we have reported that PTAP-mediated budding is also sensitive to μ2 overexpression and that both wild type and mutant μ2 had similar effects on budding. These data indicated that the AP-2 adaptor protein complex might contribute to retrovirus budding via a general mechanism that remains to be defined. It is also worth noting that when the μ2 subunit was depleted using high concentrations of siRNA oligonucleotides (25 nM), no further reduction in EIAV budding was observed upon introduction of a second siRNA (siRNA μ2 or siRNA AIP1) by transfection (data not shown). These observations indicated that adaptation of μ2 and AIP1 might be sequential such that complete blockade of one interaction abolishes the entire chain. Further characterizations of the apparently complex interactions between the Gag proteins of different retroviruses and endocytic proteins involved in assembly and budding can provide further insights into the exact role of the endocytic machin-
ery in Gag trafficking, assembly, and budding. In addition, the characterization of these critical Gag-endocytic protein interactions can provide new targets for the development of antiviral drugs.

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