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Interaction of HIV-1 Gag with the clathrin-associated adaptor AP-2

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Abstract

The envelope glycoprotein (Env) of HIV-1 interacts with the clathrin-associated adaptor complex AP-2 during the late phase of the viral replication cycle. Upon its synthesis, Env, therefore, is retrieved from the cellular surface unless internalization is inhibited by viral Gag. Here we demonstrate that not only Env, but also HIV-1 Gag, specifically binds to AP-2. Gag–AP-2 association was found to depend on tyrosine residue 132 and valine residue 135 at the matrix–capsid junction in the Gag polyprotein. Results of a morphological analysis of viral egress from cells expressing dominant-negative AP-2 suggest an involvement of AP-2 in confining HIV-1 exit to distinct microdomains. Further, particle release from AP-2-mutant cells was enhanced compared to release from wild-type cells but the infectivity of virus released from these cells was moderately reduced. Together these data attribute a role to the AP-2 complex in the regulation of HIV-1 assembly/release.

Keywords: HIV-1; Gag; Assembly; Release; Adaptor; AP-2

Introduction

HIV-1 particles are not shed continuously from infected cells and virus budding does not take place uniformly all over the cell. Instead, budding appears to be restricted to certain membrane domains. Such spatially restricted release of viral particles is likely to ensure efficient dissemination within the infected organism. The mechanisms that confine lentivirus release to distinct areas are not yet understood. However, viral budding from discrete sites coincides with the accumulation of the viral components, particularly Gag and the envelope glycoprotein (Env), in these areas. The observed non-random localization of the viral structural components is probably a prerequisite for directional particle release (e.g., Lodge et al., 1997).

To characterize signals that control the routing of the structural components of HIV-1, it is necessary to analyze their trafficking and to investigate how they interact with elements of the cellular protein sorting machinery. Previous analyses have allowed to determine sequences in the cytosolic domain of HIV-1 Env which are crucial for the interaction with two components of the cellular sorting machinery. Specifically, it was shown that viral Env interacts with the clathrin-associated adaptor complexes AP-1 and AP-2 during the late phase of the viral replication cycle (Berlioz-Torrent et al., 1999; Boge et al., 1998; Ohno et al., 1997; Wyss et al., 2001). AP-2, a heterotetrameric complex, recruits membrane proteins into coated pits via specific interactions of its medium chain μ2 with signals in the cytosolic domains of these proteins (e.g., Bonifacino and Traub, 2003). Upon its synthesis, HIV-1 Env therefore is retrieved from the cellular surface

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unless such internalization is inhibited by viral Gag (Egan et al., 1996). It has been suggested that the endocytosis of newly synthesized Env helps protect the infected cells from immune attack (Fultz et al., 2001; Marsh and Pelchen-Matthews, 2000). In addition, it is now known that HIV-1 exits directly through late endosomal membranes in macrophages and that it can egress through membranes belonging to, or derived from, the endocytic compartment in other cell types (Nguyen et al., 2003; Nydegger et al., 2003; Ono and Freed, 2004; Pelchen-Matthews et al., 2003; Raposo et al., 2002; Sherer et al., 2003; von Schwedler et al., 2003). Hence, interactions of viral components with the cellular internalization machinery, which provides guidance into the endocytic pathway, may assist in targeting the formation of HIV-1 particles to these subcellular sites.

Like other retroviral Gag proteins, HIV-1 Gag can form virus-like particles (VLPs) in the absence of other viral components and thus directs particle assembly and release (Swanstrom and Wills, 1997). Efficient release of retroviruses depends on the presence, within Gag, of a so-called late (L) domain that recruits the cellular machinery necessary for viral budding (Freed, 2002; Morita and Sundquist, 2004). The L domain of HIV-1 is located in p6 at the COOH-terminal region of Gag (Gottlinger et al., 1991; Huang et al., 1995). It contains two distinct motifs, a PTAP and an LYPLTSL sequence for the recruitment of cellular vesiculation machinery, whereas a PPPY motif in the L domain of other retroviruses appears to be critical for particle release (Freed, 2002; Morita and Sundquist, 2004). The L domain of equine infectious anemia virus (EIAV) was reported to be centered at Y23 within p9 of EIAV Gag (Puffer et al., 1997) and the same Y23 was shown to be part of a YxxL motif that recruits the μ2 subunit of AP-2 (Puffer et al., 1998), suggesting that AP-2 may contribute to promoting release of this virus. However, so far no functional data support this hypothesis, whereas results reported by three groups demonstrated that AIP1/ALIX, a human VPS class E protein, mediates EIAV L domain function via its recruitment by the LY23PDL sequence (Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003), as predicted (Vincent et al., 2003).

Here we report that HIV-1 Gag, like EIAV Gag, binds AP-2. Because AP-2 has been considered a cellular factor mediating efficient release of EIAV, we tested whether abrogation of Gag–AP-2 interaction resulted in reduced particle release. However, we found the opposite: HIV-1 release was enhanced when the Gag–AP-2 association was interrupted. Interestingly, the subcellular distribution of Gag in cells expressing dominant-negative AP-2 differed markedly from the distribution in AP-2-wild-type cells, implying that AP-2 plays a role in the late phase of the HIV-1 replication cycle, perhaps by targeting the external and the internal structural components of this virus to the sites where particles exit from cells.

Results

HIV-1 Gag binds the clathrin-associated adaptor complex AP-2

Upon its biosynthesis and transport to the cell surface, HIV-1 Env is rapidly internalized through a specific interaction with the clathrin-associated adaptor complex AP-2 (Boge et al., 1998; Marsh and Pelchen-Matthews, 2000; Rowell et al., 1995). As co-expression of HIV-1 Gag reduces the level of Env internalization, Gag was postulated to compete with the cellular endocytosis machinery for the acquisition of Env. However, Gag itself contains six sequences conforming to the AP-2 binding motifs YxxΦ (Φ being a bulky, hydrophobic residue) or LL (Fig. 1A; for a recent review, see Bonifacino and Traub, 2003). Fig. 1B, third lane, shows that full-length Gag, in the context of a GST fusion protein, precipitates

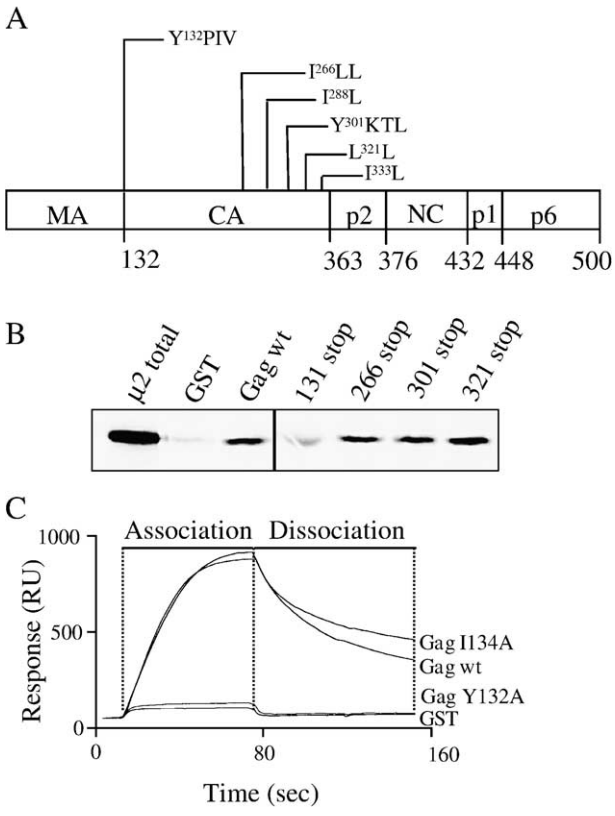

Fig. 1. Binding of purified μ2 and AP-2 to Gag. (A) HIV-1 Gag protein organization, potential AP-2 binding sites. (B) Binding of μ2 to GST-Gag fusion proteins. Gag wt or Gag deletion mutants were used in a pulldown assay with ^{35}S-labeled μ2 subunit of AP-2. Two sections of the same gel are shown (same exposure time). (C) Binding of GST-Gag fusion proteins to either purified AP-2 complex or to μ2 subunit. GST-Gag fusion proteins were coupled to SPR sensor chips via an anti-GST antibody at equal densities. Purified adaptor complexes or the purified adaptor subunit μ2 were passed over the Gag-derived chip surfaces. Sensograms of Gag–adaptor/μ2 interaction recorded in real time were used to calculate k_a (on rate), k_d (off rate), K_D (k_a / k_d). Shown is the binding of AP-2 injected at a concentration of 2 μM. The kinetic data of all Gag variants tested are summarized in Table 1.

the medium chain μ2 of AP-2. C-terminal deletion mutants were engineered such that Gag lacked the potential AP-2 binding sites at positions 322 and 333 (321 stop), or the potential binding sites downstream of, and including, 301 (301 stop), 267 (266 stop), or all six sites (131 stop) (Fig. 1A). Binding to μ2 was affected for the 131 stop mutant in the GST pull-down experiments but not for the other deletion mutants (Fig. 1B), suggesting that Y132 might be part of a YxxΦ motif.

To test the possibility that the sequence Y132PIV135 was involved in Gag–μ2 binding, we made mutants with single amino acid changes at positions 132 through 135. We also sought to determine the relative affinities for μ2 and for the entire AP-2 complex of the different Gag mutants, as done previously for HIV-1 Env (Wyss et al., 2001). Gag–adaptor interactions were thus quantified by surface plasmon resonance (SPR) analysis, a technique that allows real-time analysis of μ2 or purified adaptor complex association with GST-Gag that has been coupled to a sensor chip via an anti-GST antibody. Association and dissociation curves (Fig. 1C) were determined by adding different concentrations of μ2 or purified AP-2 complex and served as basis for calculating the kinetic rate constants that are displayed as relative values in Table 1. This analysis revealed that residues Y132 and V135 are important for μ2/AP-2 binding, as the relative affinities (K_D's) of the Y132A and the V135A mutant were indistinguishable from background values measured for GST. In contrast, binding of μ2/AP-2 to Gag was only marginally affected, if at all, when Y132 was exchanged for a phenylalanine, if residues P133 or I134 were replaced by alanine, or if the dileucines at positions 267, 282, 322, and 333 or the Y302-based motif were deleted. Overall, the SPR analysis demonstrates that distinct residues within the YxxΦ motif at the MA-CA junction are critical for Gag–μ2/AP-2 interaction.

Table 1
Rate constants for binding of AP-2 or μ2 to GST-Gag fusion proteins

	A			B		
	k_a (1/M×s)	k_d (1/s)	K_D (μm)	k_a (1/M×s)	k_d (1/s)	K_D (μm)
GST	NB	NB	NB	NB	NB	NB
WT	2.7×10^3	4.0×10^{-3}	1.5	2.5×10^3	5.5×10^{-3}	2.2
Y132A	NB	NB	NB	NB	NB	NB
Y132F	2.5×10^3	4.0×10^{-3}	1.6	2.1×10^3	5.0×10^{-3}	2.4
P133A	3.3×10^3	3.9×10^{-3}	1.2	2.8×10^3	5.5×10^{-3}	2.0
I134A	3.3×10^3	3.8×10^{-3}	1.4	2.5×10^3	5.5×10^{-3}	2.2
V135A	NB	NB	NB	NB	NB	NB
131 stop	NB	NB	NB	NB	NB	NB
266 stop	2.9×10^3	4.0×10^{-3}	1.4	2.4×10^3	5.1×10^{-3}	2.1
301 stop	2.8×10^3	3.9×10^{-3}	1.4	2.5×10^3	5.5×10^{-3}	2.2
321 stop	2.9×10^3	4.0×10^{-3}	1.4	2.4×10^3	5.2×10^{-3}	2.2

Binding of AP-2 (A) or μ2 (B) was monitored in real time with an SPR-based biosensor as outlined in Materials and methods. At least 4 concentrations were passed over the GST-Gag derivatized surfaces. The resulting sensorgrams were then used to calculate the rate constants of the interaction assuming a 1:1 type of interaction. NB: binding indistinguishable from background.

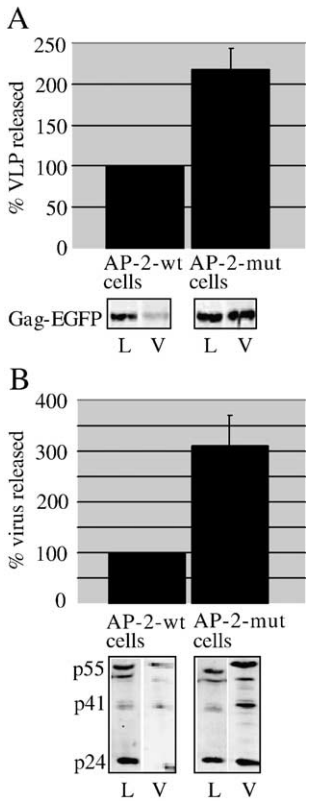

Fig. 2. VLP and of HIV-1 Gag release is increased in AP-2-mutant cells. (A) Gag-EGFP VLP release in AP-2-wild-type and AP-2-mutant cells. (B) HIV-1 particle release from AP-2-wild-type and AP-2-mutant cells. ^{35}S-labeled cells expressing wild-type or mutant AP-2 (Nesterov et al., 1999) were transfected with either Gag-EGFP expressor plasmids or pNL43 for the production of either VLPs or the full-length virus. VLPs or viruses (V) were harvested and Gag was immunoprecipitated from the lysate (L) as described in Materials and methods and the amounts of released particles were quantified by phosphoimager. Different sections of the same gel are shown (same exposure time). The relative amount of released particles was determined by dividing the amount of Gag-EGFP in released particles by the amount of total Gag-EGFP (i.e., cell-associated and released Gag-EGFP together). Data are representative of at least six experiments. Error bars represent standard error of mean (SEM).

Disruption of Gag–AP-2 interaction leads to increased HIV-1 particle release

A μ2 binding site is situated at the center of the EIAV L domain (Puffer et al., 1998). The AP-2 complex thus had to be considered a candidate cellular factor that mediates efficient lentivirus particle release (for a review, e.g., Freed, 2002). Because AP-2 specifically interacts with HIV-1 Gag, we tested whether Y132 was part of a L domain, i.e., whether it was involved in mediating proper HIV-1 particle release by acting as a receptor for AP-2. We made use of a HeLa-derived cell line in which the wild-type μ2 subunit of AP-2 can be replaced by mutant μ2 that no longer associates with tyrosine-based internalization motifs (for a description of this inducible, functional knockout cell line see Nesterov et al., 1999). AP-2-wild-type and AP-2-mutant cells transiently expressing Gag-EGFP were radiolabeled and the

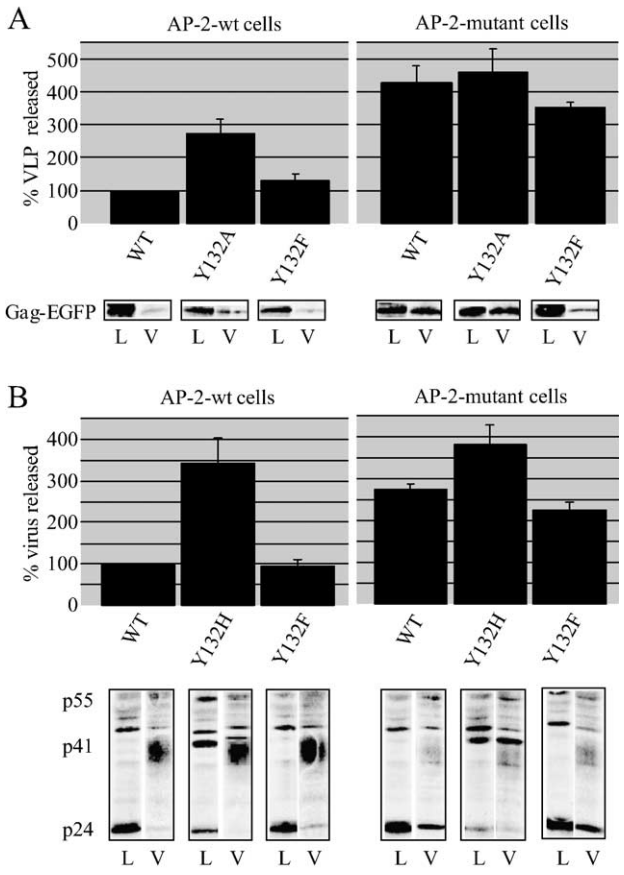

Fig. 3. Non-conservative mutation of Y132 in Gag leads to increased particle release. (A) Mutant Gag-EGFP release from wild-type and mutant AP-2 cells. ^{35}S-labeled cells expressing wild-type or mutant AP-2 were transfected with either wild-type Gag-EGFP or Gag-EGFP with either a non-conservative (Y132A) or a conservative (Y132F) mutation at position 132. (B) Release of mutant HIV-1 Gag from wild-type and mutant AP-2 cells. ^{35}S-labeled cells expressing wild-type or mutant AP-2 were transfected with either wild-type pNL43 or pNL43 with a non-conservative (Y132H) or a conservative (Y132F) mutation in Gag. The relative amount of VLPs or released virus was determined by dividing the amount of Gag in released particles by the amount of total Gag (i.e., cell-associated and released Gag together). Different sections of the same gel are shown (same exposure time). Data are representative of four experiments. Error bars represent SEM.

relative amounts of released virus-like particles (VLPs) were determined by phosphoimager analysis of pelleted VLPs and immunoprecipitated Gag from cell lysates. As shown in Fig. 2A, cells expressing non-functional AP-2 released more than twice as many particles than cells expressing wild-type AP-2. This inequality was not due to clonal differences between the cell line expressing wild-type μ2 and the line expressing mutant μ2, as both cell lines release virus with the same efficiency in the uninduced state (data not shown). To determine whether release of full-length virus (NL-43) was also enhanced in cells expressing mutant AP-2, the amounts of shed virions were determined as described above for the VLPs. Fig. 2B documents that lack of functional AP-2 in virus-producing cells leads to a threefold enhancement of viral particle release. Together, these experiments suggested that AP-2, rather than promoting particle release, limits viral egress.

To further confirm that the HIV-1 Gag–AP-2 interaction is involved in restricting particle release, we analyzed whether mutations of Y132, one of the residues in Gag that is critical for Gag–AP-2 interaction (see Fig. 1C, Table 1), positively affected virus release. Release experiments as described above were performed using a previously described mutant virus with a conservative amino acid exchange at position 132 (Y132F) (Freed et al., 1997) and another virus from the same laboratory with a non-conservative mutation (Y132H) which causes a partial processing defect (see Fig. 3B, middle lanes of both panels).

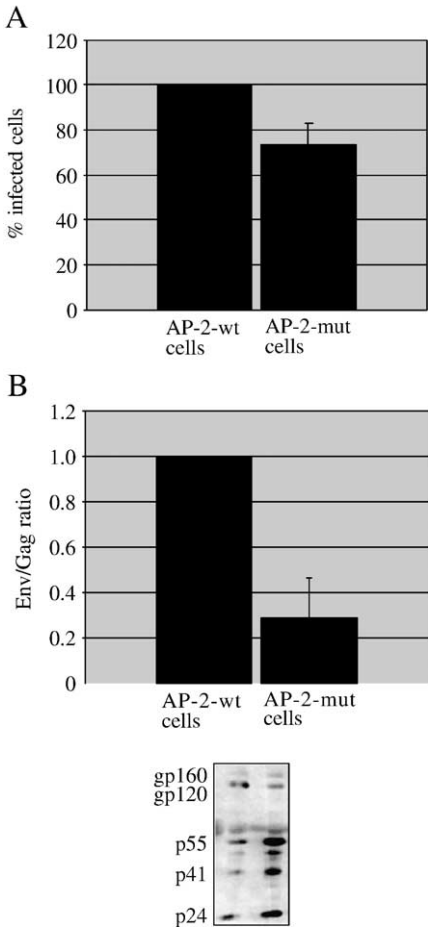

Fig. 4. Virus released from AP-2-mutant cells is less infectious and incorporates less Env than virus released from wild-type cells. (A) Infectivity of virus released from AP-2-wild-type or AP-2-mutant cells. Cells expressing either wild-type or mutant AP-2 were transfected with pNL43. The amount of released virus was quantified by p24 ELISA and equal amounts were used to infect MAGI cells. Infected MAGI cells were counted by an unbiased investigator. Data are the means of three experiments. Error bars represent standard deviation (P value < 0.01). (B) Env incorporation. AP-2-wild-type and AP-2-mutant cells were transfected with pNL43. Released virus was harvested and its composition analyzed as described in Materials and methods. Data are the means of three experiments. Error bars represent standard deviation (P value < 0.01). The representative gel shows virus released from AP-2-wild-type cells (left lane) and released from AP-2-mutant cells (right lane).

We also tested how the release of Gag-EGFP VLPs was affected if Y132 was replaced by either phenylalanine or alanine. While the release of VLPs (Fig. 3A, left panel) and virus (Fig. 3B, left panel) was only slightly affected by the conservative mutation, release of the VLP and of virus carrying the non-conservative mutation was enhanced at least twofold and threefold respectively as compared to wild-type (Figs. 3A and B, respectively), in agreement with the hypothesis that the Gag–AP-2 interaction contributes to limiting VLP/virus release from cells. The combination of mutations in AP-2 and in Gag in the context of the whole virus but not in the context of Gag-EGFP lead to a further enhancement of particle release (compare first to second bars in Figs. 3A and B, right panels).

Lack of functional AP-2 in virus-producing cells affects the infectivity of HIV-1 particles

To assess whether the enhancement of release affected the infectivity of the virus, we incubated the indicator cell line MAGI (Kimpton and Emerman, 1992) with equal amounts of wild-type virus released from either AP-2-wild-type or AP-2-mutant cells. Fig. 4A documents that the infectivity of virions released from AP-2-mutant cells was moderately affected. Virus produced in AP-2-mutant cells was found to incorporate less Env than particles produced in wild-type cells (Fig. 4B), presumably contributing to this reduced infectivity.

The subcellular distribution of viral Gag is altered if Gag–AP-2 association is disrupted

Because AP-2, like other adaptor complexes, forms only low-affinity interactions with its cargo (Kirchhausen, 1999), at any given time only a small percentage of cargo molecules bind to this adaptor. Consequently, AP-2 interacting proteins do not colocalize extensively with adaptors and in immunofluorescence studies the patterns for AP-2 and its various ligands typically look quite different. Nevertheless, when examining AP-2 and Gag localization, we found that a small fraction of Gag colocalized with AP-2 in cells expressing wild-type μ2. Gag–AP-2 colocalization was reduced by about a third in cells expressing mutant μ2 (Fig. 5). More strikingly though, the overall distribution of viral Gag appeared altered in cells expressing mutant μ2 (see below).

As HIV-1 egress normally is confined to distinct spots at the plasma membrane (e.g., Hermida-Matsumoto and Resh, 2000; Ono et al., 2000; Sandefur et al., 1998), and also given the altered distribution of Gag in cells expressing mutant μ2 (Fig. 5B), it seemed conceivable that AP-2 limits the amount of virus release (Figs. 2 and 3) by restricting the interaction of HIV-1 Gag and the cellular extravesiculation machinery. As a first step towards analyzing this hypothesis, we utilized fluorescence microscopy to compare the localization of surface Gag clusters in situations where Gag and AP-2 are allowed to associate efficiently with the localization of Gag in cells where such an interaction was prohibited either because μ2 was mutated or because residue Y132 in Gag was exchanged for an alanine. To rigorously quantify the data, an investigator who was unaware of the nature of the samples scored 100 randomly mixed pictures of cells (50 each of wild-type and AP-2-mutant cells combined in a common pool of images). Criteria for the analysis were the size of Gag clusters and their subcellular distribution (see legend of Fig. 6 for a description of how cell phenotypes were scored and the results of this quantitative analysis). Figs. 6A and B show representative wild-type AP-2 and mutant AP-2 expressing cells, respectively. While the overall distribution of wild-type and mutant AP-2 complex was judged to be similar in both cell types (left panels), HIV-1 Gag-EGFP accumulated more peripherally and in fewer but larger clusters in cells expressing non-functional AP-2 adaptor complex (right panels). To confirm that a lack of Gag–AP-2 interaction alters the localization of Gag clusters, either Gag Y132A (Fig. 6C) or Gag Y132F (Fig. 6D) was expressed in AP-2-wild-type HeLa cells. Similar to the situation shown for wild-type Gag in AP-2-mutant cells in Fig. 6B, right panel, Y132A mutant Gag accumulated in fewer but larger peripheral clusters and also at the outer edge of the cell-substrate interface. The Y132F mutant showed wild-type phenotype. Finally, the distribution of Gag Y132A and Y132F clusters in AP-2-mutant cells was analyzed. Like in all the previous experiments the investigator scored

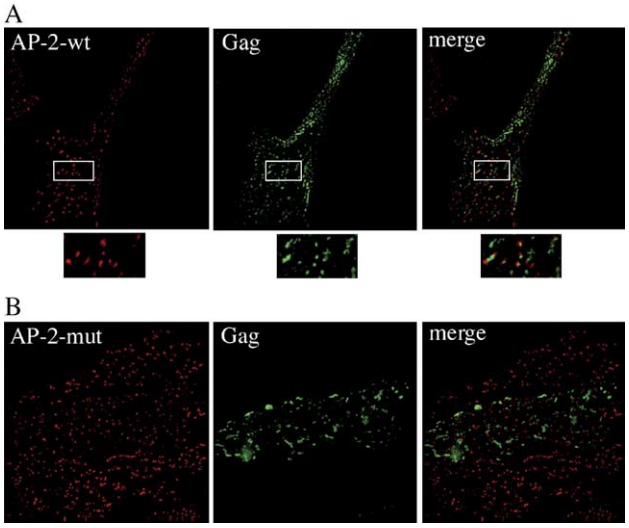

Fig. 5. Colocalization of Gag and AP-2 in wild-type and AP-2-mutant cells. (A) AP-2-wild-type cells or (B) AP-2-mutant cells expressing Gag. Cells expressing Gag were fixed, permeabilized, and stained with antibodies against p6 and the α-chain of AP-2 and with an anti-mouse secondary antibody conjugated to AlexaFluor 594 or an anti-rabbit secondary antibody conjugated to AlexaFluor 488, as described in Materials and methods. The bottom section of cells is shown. Ten pictures of each situation were taken and Gag–AP-2 colocalization was quantified using Volocity (Improvision) colocalization software. Gag colocalization with AP-2 in AP-2-mutant cells is reduced by 38% as compared to colocalization in wild-type cells (colocalization in wild-type cells: 20.4%, SD = 2.65; colocalization in AP-2-mutant cells: 12.5%, SD = 3.98; P value < 0.001).

ungrouped pictures of similarly shaped cells that were part of a pool containing both AP-2-wild-type or mutant cells expressing either mutant or wild-type Gag. The distribution of the mutant Gag surface clusters in AP-2-mutant cells clearly showed the most pronounced aberration (Fig. 6E). Altogether, these experiments are compatible with the hypothesis that AP-2, via a specific interaction with HIV-1 Gag, is involved in controlling the subcellular site of virus release.

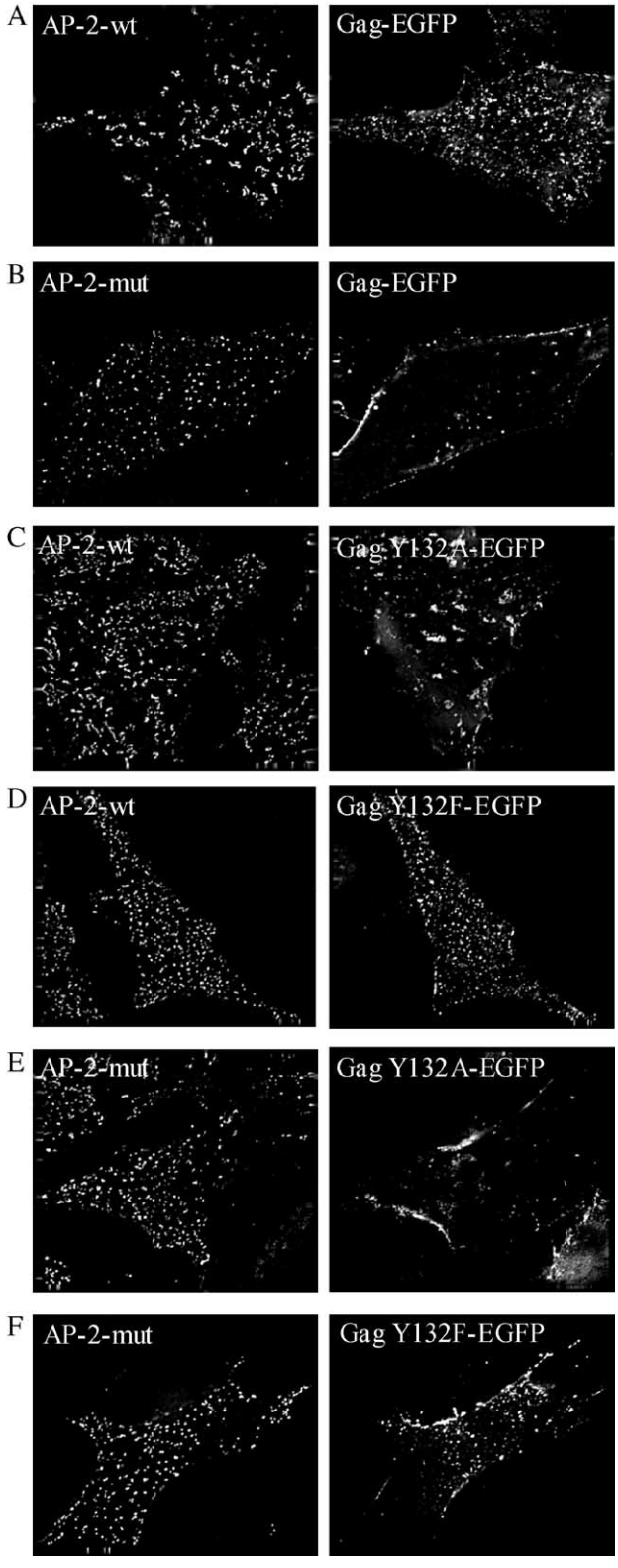

Fig. 6. Gag-EGFP distribution in wild-type and AP-2-mutant cells. Localization of Gag-EGFP expressed in AP-2-wild-type cells (A) and in AP-2-mutant cells (B). Localization of Gag-EGFP containing a mutation at Y132 in AP-2-wild-type cells. Gag-EGFP with a non-conservative mutation, Y132A (C), or a conservative mutation, Y132F (D). AP-2-mutant cells expressing GagY132A-EGFP (E) or GagY132F-EGFP (F). Cells were fixed and permeabilized and stained with an anti-α chain antibody (left panels) and with an AlexaFlour 594-conjugated anti-mouse secondary antibody as described in Materials and methods. Fifty pictures each of secondary mutant scenarios (B–F) were pooled together with fifty pictures of wild-type scenario (A) and were analyzed by an investigator unaware of the nature of the samples. The cells were scored on a scale of 1–5 with 1 displaying the most wild-type phenotype and 5 displaying the most mutant phenotype with regard to Gag distribution. The numbers shown are the average of 50 cells counted, a representative cell is shown for each phenotype: (A) AP-2-wild-type cells/wild-type Gag: 1.8; (B) AP-2-mutant cells/wild-type Gag: 3.2; (C) AP-2-wild-type cells/Y132A mutant Gag: 3.2; (D) AP-2-wild-type cells/Y132F mutant Gag: 1.8; (E) AP-2-mutant cells/Y132A mutant Gag: 4.0; (F) AP-2-mutant cells/Y132F mutant Gag: 3.4.

Discussion

HIV-1 Env interacts with AP-2 during the late phase of the viral replication cycle. We now show that this cellular sorting complex also associates with HIV-1 Gag. Based on the finding that the central motif in the L domain of EIAV Gag binds AP-2, the possibility that this complex is involved in promoting lentiviral release had to be considered. Our data suggest that under the circumstances tested in this paper, rather than furthering particle release, this adaptor limits HIV-1 egress.

The cellular internalization machinery recruits cargo for clathrin coated pits via interactions with adaptors such as arrestin or AP-2 (for recent reviews, see Bonifacino and Traub, 2003; Traub, 2003). Cargo typically consists of integral membrane proteins that are retrieved from the cell surface. HIV-1 Env, for example, may have evolved to be retrieved from the cell surface upon synthesis in order to prevent immune attack (Marsh and Pelchen-Matthews, 2000) and to prevent fusion of the producer cell and the target cell in situations where virus is transmitted at the virological synapse (Johnson and Huber, 2002; Jolly et al., 2004; Phillips, 1994). Retroviral Gag, however, is not exposed at the surface. As a peripheral membrane protein, it anchors at the cytoplasmic side of cellular membranes via a myristic acid that is post-translationally attached to its N-terminus. We were surprised, therefore, when we first noticed that full-length HIV-1 Gag associates with AP-2. However, as documented now in this report, HIV-1 Gag and AP-2 interact specifically with each other. The GST pull-down experiments and the SPR experiments reported in Fig. 1 and in Table 1 demonstrate that residues Y132 and V135 at/near the MA–

CA junction are critical for the Gag–μ2/AP-2 association. Together with the finding that the 131 stop mutant no longer binds μ2 and AP-2, these data also strongly suggest that this association is significant only during the late phase of the viral replication cycle and not when the virus enters into cells, as maturation during viral budding cleaves the Gag precursor between the residues Y132 and P133. As a consequence of this developmental switch, the N-terminus of the p24 capsid refolds into a β-hairpin/helix structure (von Schwedler et al., 1998), thus destroying the AP-2 binding site.

Comparable to our Env-AP-2 and Env-AP-1 studies, we found that mutations that decrease binding of the μ subunit (μ1 or μ2) also show lower affinity for the whole complexes (AP-1 or AP-2). However, compared with our previous studies, in the current SPR analysis we used a buffer containing higher salt concentrations because we recently recognized that such conditions minimize non-specific protein-protein interactions (Honing et al., 2005). The fact that affinities of Gag for μ2 and AP-2 do exactly parallel each other provides strong evidence for the importance of the Y132-centered region for Gag–AP-2 interaction. Still, it should be pointed out that the SPR method provides measurements of relative affinities only. It is now well established that post-translational modifications such as phosphorylation of the adaptors and interactions of both cargo and adaptors with other components of the cellular internalization modulate the adaptor-cargo interaction in vivo (Conner and Schmid, 2002; Ricotta et al., 2002; for a review, see Kirchhausen, 1999).

As the sequence Y23PDL that is responsible for EIAV Gag–AP-2 interaction was shown to be critical for EIAV release (Puffer et al., 1997, 1998), it seemed conceivable that AP-2 is a cellular factor mediating L domain function, i.e., promoting viral particle release, but so far no functional studies support that hypothesis. As demonstrated in Figs. 2 and 3, we find that mutations in either AP-2 or HIV-1 Gag that inhibit their interaction, rather than decreasing viral budding, lead to increased particle release in HeLa cells. Our data therefore suggest that AP-2 is involved in restricting HIV-1 particle release. Nevertheless, the small but still significant decrease in infectivity of virus released from AP-2-mutant cells (Fig. 4A), possibly due to reduced Env incorporation in virus produced in AP-2-mutant cells (Fig. 4B), is also compatible with a potentially positive role for AP-2 in HIV-1 egress. Interestingly, Lamb and colleagues recently identified a ΦPxV motif being the center of an L domain in a paramyxovirus (Schmitt et al., 2005). AIP1/ALIX apparently is not the partner for that motif. Whether AP-2 binds to this sequence is currently under investigation (A. Schmitt and R. Lamb, personal communication).

Consistent with the hypothesis that AP-2 plays a positive role in virus release, we found that AP-2 is involved in proper Gag targeting (Figs. 5 and 6). While newly synthesized wild-type HIV-1 Gag localizes to relatively small but distinct areas at the surface of HeLa cells (Fig. 6A), compatible with previous reports (Hermida-Matsumoto and Resh, 2000; Ono et al., 2000; Sandefur et al., 1998), Gag is targeted to fewer but larger and more peripherally localized zones at the plasma membrane if the Gag–AP-2 interaction is disrupted (Figs. 6B–F). Also, as in the experiments where we analyzed the effects on particle egress of mutations in AP-2 and Gag (Fig. 3), we noticed that the effects on targeting of release were additive, i.e., that the distribution of viral budding sites was distorted most dramatically if Y132A mutant Gag was expressed in AP-2-mutant cells (Fig. 6E). The findings that the phenotypes of mutant virus (i.e., its release, as shown in Fig. 3 and Gag targeting, as documented in Fig. 6) are further enhanced in AP-2-mutant cells suggest that the exchange of Y132 for an alanine does not completely abrogate Gag–AP-2 binding in situ (in cells), or that sequences in Gag other than Y132 or other cellular or viral components (e.g., Env) contribute to the Gag–AP-2 interaction and release of virus. Also, note that binding to μ2 of the 131 stop mutant is not completely abrogated in the GST pull-down experiments (Fig. 1B), perhaps due to the presence of such hypothetical cellular factors in the reticulocyte lysate in which μ2 was produced.

So far AP-2 is regarded as one key component of receptor mediated endocytosis linking cargo concentration to the process of coat formation. Recent evidence suggests that HIV-1 Gag, if anchored to the membrane via the transmembrane and the extracellular domains of a cellular integral membrane protein, can be retrieved from the plasma membrane (Lindwasser and Resh, 2004). It remains to be analyzed whether AP-2 contributes to the observed retrieval. Also, the purpose of such a retrieval of Gag during the HIV-1 assembly/release process is not immediately evident. HIV-1 and other viruses may have evolved to have Gag endocytosed in order to prevent excess budding which could be detrimental to the health of the producer cells, thus allowing for sustained shedding of viral particles. Alternatively, such down regulation may be restricted to misfolded and/or mistargeted Gag.

Rather than promoting Gag internalization, the transient interaction with AP-2 may serve to target Gag, and thus virus release, to distinct microdomains. Recently published data suggest that determinants in Gag other than the $p6^{Gag}$-localized L domain, which is responsible for the recruitment of the vesiculation machinery, must exist that assist in targeting Gag to those sites where morphogenesis takes place (Ono and Freed, 2004). The Y132-centered motif would be particularly well suited to serve as a targeting signal because it is destroyed upon viral budding and thus inactive in newly infected cells. Based on the findings that (1) the release of HIV-1 particles is mediated by distinct components of a cellular outward vesiculation machinery and (2) not only Gag but also Env interacts with AP-2, it would appear plausible that the observed Gag–AP-2 interaction assists in targeting Gag to those subcellular sites, where the vesiculation machinery and viral Env are hosted. Studies are underway in our laboratory to determine whether Gag and Env can associate with AP-2 simultaneously. Finally, even though Gag and AP-2 also affect each

other, it is possible that AP-2 affects HIV-1 release indirectly, e.g., via an interaction with other cellular proteins involved in Gag trafficking. This seems all the more likely given that AP-2 serves as membrane-based hub for many proteins (e.g., Praefcke et al., 2004).

The current analysis presents a first step only in our investigation aimed at elucidating AP-2's function(s) during HIV-1 assembly and release. However, together with a recently published study that reports the incorporation of AP-2 into Moloney murine leukemia virus (Mo-MuLV) (Wang et al., 2003) and a paper describing competition between cellular factors involved in inward budding (AP-2) and outward budding (AIP1/ALIX) (Geminard et al., 2004), our study also raises the intriguing possibility that retroviral budding may be controlled by components of the early endocytic pathway.

Materials and methods

Mammalian cell culture, plasmids, and transfections

Wild-type and mutant AP-2 HeLa cells expressing an inducible μ2 subunit (Nesterov et al., 1999) were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 400 μg/ml G418 (Invitrogen), 100 U/ml penicillin/streptomycin (Invitrogen), 200 ng/ml puromycin (Sigma), and 2 μg/ml tetracycline (Calbiochem). The cells were planted in either chambered coverslides (Lab-Tek) or T25 flasks, in media without tetracycline, and grown to approximately 90% confluency. The following plasmids were used: pNL43, pGag-EGFP (Sandefur et al., 1998), and pGagopt (Rudner et al., 2005). Transfections were performed 3 days after removal of tetracycline from the media using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Analysis of cells was done 24 h after transfection with pGag-EGFP or pGagopt and 48 h after transfection with pNL43.

Recombinant proteins

All constructs used in the in vitro binding assays were made by ligation of polymerase chain reaction (Pwo DNA polymerase; Boehringer Mannheim)-amplified DNA fragments into pGEX-2TK (Amersham Pharmacia Biotech). The 5′-primer sequence was the same for all GST-Gag constructs: 5′-GATGGGTGCGAGAGC-3′. The 3′-primer sequences were as follows: GST-Gag: 5′-TTATTGTGACGAGGGGTC-3′; GST-GagL321Stop: 5′-TTAGGTTTCTGTCATCCAA-3′; GST-GagY301Stop: 5′-TTAGAACCGGTCTACATAG-3′; GST-GagI266-Stop: 5′-TTACCATCTTTTATAAATTTCTCC-3′; and GST-GagN131Stop: 5′-TTATTGGCTGACCTGATTG-3′. The polymerase chain reaction products were phosphorylated and inserted into *Sma*I-linearized pGEX-2TK.

Site-directed mutagenesis was performed in the GST fusion constructs using the Quick ChangeTM system (Stratagene). The following primer was used: 5′-CAGGTCAGCCAAAATGCCCCGATCGTGCAGAA-CATC-3′. The mutations were confirmed by DNA sequencing.

Binding assay: GST pull-down experiments

GST fusion proteins were produced in *Escherichia coli* strain BL21 and purified over glutathione Sepharose 4B beads (Amersham). Precultures were grown in 100 ml of LB +Amp overnight at 37 °C. The cultures were diluted into 700 ml of LB +Amp and protein production was induced by adding 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). Purification over glutathione Sepharose 4B beads was performed according to the manufacturer's instructions including a heat shock step with 50 mM Tris–HCl (pH 7.4), 10 mM $MgSO_4$, and 2 mM ATP for 10 min at 37 °C. Concentration and quality of the fusion proteins was monitored on an SDS–PAGE gel after staining with Coomassie blue (Sigma Chemicals) not only after preparation of the probes but also at the end of each experiment, after precipitation of μ2.

^{35}S-labeled μ2 proteins (Boge et al., 1998) were made by in vitro transcription and translation of the 3M9 μ2 plasmid (Ohno et al., 1995) from a T7 promoter using a TNT kit (Promega), according to the manufacturer's protocol. 4 μl of ^{35}S-labeled μ2 and equal molar amounts of GST-fusion proteins were added to beads and incubated for 1 h at 37 °C. The beads were washed twice with PBS then heated in sample buffer for 5 min at 100 °C. The precipitates were resolved by SDS–PAGE, gels were dried, exposed to X-ray film and analyzed by phosphoimaging.

Binding assay: surface plasmon resonance (SPR) analysis

Binding of purified AP-2 and recombinant μ2 (residues 157–434) to immobilized GST-Gag fusion proteins was monitored by surface plasmon resonance. AP-2 was purified from pig brain. μ2 was purified from the *E. coli* strain BL21 transfected with the pET28a vector (Novagen) containing N-terminally 6× His-tagged rat μ2 (residues 158–9435) and was a gift from V. Haucke (Free University Berlin, Germany). Protein production was induced by addition of IPTG for 3 h at 30 °C followed by purification of the μ-chain protein from 1 L of bacteria. The purification was performed according to a standard protocol (Qiagen) using Ni-NTA agarose as an affinity matrix (the complete protocol is available upon request). The purity of the μ2 which were stored in 10 mM HEPES–KOH pH 7.4, 500 mM NaCl, 10 mM ß-mercaptoethanol was controlled by SDS–PAGE. Before any SPR experiment, all proteins were centrifuged 30 min/100,000 × *g* for removal of possible protein aggregates.

For SPR analysis monoclonal anti-GST (Upstate) was immobilized on all 4 flow-cells of a CM5 sensor-chip resulting in shift of the baseline of 8.000 resonance units. Variation in immobilization between flow-cells was less than 4%. The running buffer was 10 mM HEPES–KOH pH 7.4 and contained 500 mM NaCl to minimize non-specific electrostatic interactions with the negatively charged carboxymethylated sensor surface. The GST-Gag fusion proteins were injected at equal concentrations at a flow rate of 5 μl/min to allow capture by the anti-GST antibodies. After capturing was saturated, AP-2 or μ2 was injected at a flow rate of 20 μl/min for 1 min at concentrations ranging from 0.4 to 3 μM. Dissociation was monitored after switching back to running buffer. The surface was regenerated by 3 consecutive pulse injections of 50 mM NaOH for 5 s each. The determination of the rate constants was calculated assuming a 1:1 first order kinetics after subtraction of the binding of μ to GST which was regarded as background binding. The sensorgrams shown in Fig. 1C are the original sensorgrams recorded simultaneously on the 4 individual flow cells.

Release experiments

One million wild-type or mutant AP-2 cells were planted in T25 flasks in tetracycline minus medium to induce expression of either wild-type- or mutant-tagged μ2, as described (Nesterov et al., 1999). Three days later the cells were transfected with 4 μg of pGag-EGFP or pNL43 DNA. The cells were labeled 24 h post-transfection with 100 μCi/ml of Easy Tag Express ^{35}S-Protein Labeling Mix (NEN) in DMEM without Met and Cys (Invitrogen) and incubated at 37 °C for 12–16 h. The supernatant was removed and filtered through a 0.45-μm filter followed by centrifugation at 16,000 × g for up to 90 min at 4 °C. The viral pellet was resuspended in 30 μl of RIPA buffer (50 mM Tris–HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) to which an equal volume of sample buffer was added before boiling for 5 min at 100 °C. The cells were lysed in RIPA buffer and centrifuged at 16,000 × g for 90 min at 4 °C. To visualize cellular Gag-EGFP, the lysates were immunoprecipitated by adding 4 μg of anti-EGFP antibody (Molecular Probes) and 50 μl of Protein G sepharose (Pharmacia) to 100 μl of lysate and incubated overnight at 4 °C. To visualize cellular HIV-1 Gag proteins, the lysates were immunoprecipitated with a rabbit anti-CA antibody (1:100; ABI) and 50 μl of protein A sepharose (Pharmacia) and incubated overnight at 4 °C. The beads were washed three times with RIPA and heated in 20 μl of sample buffer for 5 min at 100 °C. The precipitates were resolved by SDS–PAGE; gels were dried, exposed to X-ray films, and analyzed by phosphoimager. Bands representing uncleaved or partially cleaved Gag and thus contributing to the Gag signal in either VLP/viral and cell lysates (i.e., p55, p41) were included in the calculations throughout this analysis.

Infectivity experiments

AP-2-wild-type or -mutant cells were planted in T75 flasks. Tetracycline was removed from the media three days before transfection with 12 μg of pNL43 DNA. Released virus was collected 48 h post-transfection and centrifuged for 10 min at 805 × *g*. The amount of p24 was quantified by ELISA (NEN Perkin Elmer). The CD4-positive long terminal repeat galactosidase-expressing HeLa (MAGI) cell indicator line (Kimpton and Emerman, 1992) was obtained from the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and maintained in DMEM supplemented with 10% FBS, 100 U/ml of penicillin G sodium, 0.1 mg/ml of streptomycin sulfate. Cells were seeded at a density of 1.6×10^5 per well in a 6-well plate 24 h prior to infection. Samples of virus were adsorbed to cells in the presence of 20 μg/ml DEAE-dextran for 2 h at 37 °C prior to the addition of 1 ml of medium. Following incubation for 48 h at 37 °C, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) as previously described (Kimpton and Emerman, 1992).

Protein profile of released virus

One million wild-type or mutant AP-2 cells were planted in T25 flasks in tetracycline minus medium to induce expression of either wild-type- or mutant-tagged μ2. Three days later the cells were transfected with 4 μg of pNL43 DNA. Forty-eight hours post-transfection the supernatant was centrifuged for 10 min at 805 × g to clear cellular debris. The virus was harvested by a 90-min centrifugation at 16,000 × g. The viral pellet was resuspended in 30 μl of RIPA buffer to which 10 μl of 4× sample buffer was added before boiling for 5 min at 100 °C. To visualize the viral proteins, lysed virions were loaded onto a 10% SDS–PAGE gel and transferred to nitrocellulose membrane (Bio-Rad). The membrane was probed with a mixture of monoclonal antibodies obtained from the AIDS Research and Reference Program, α-p24 AG3.0 at 1:500 and α-gp120 at 1:500. A secondary α-mouse antibody conjugated to HRP was used at 1:2000. The amounts of virus-associated Gag and Env were quantified by CCD Imager (Bio-Rad).

Immunocytochemistry, imaging analysis by fluorescence microscopy

AP-2-wild-type and -mutant cells expressing pGag-EGFP or pGagopt were grown on chambered coverslides (Lab-Tek). The cells were washed with PBS and fixed with 3.7% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After blocking with 1% BSA, the fixed cells were incubated in primary antibody for 1 h at 37 °C, washed with PBS, blocked for 10 min in 1% BSA, and incubated in secondary antibody for 30 min at room temperature. The stained cells were washed with

PBS and stored in 1% BSA at 4 °C. The following primary antibodies and serum were used; undiluted AP-6 supernatant (ATCC), rabbit p6Gag antiserum (AIDS Vaccine Program, NCI Fredrick, Frederick, MD). The following secondary antibodies were used; anti-mouse Texas red-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA), anti-mouse, or anti-rabbit antibodies conjugated to either AlexaFluor 488, 594, or 647 (Molecular Probes).

To obtain high-resolution images of the cells, a DeltaVision imaging workstation consisting of a high resolution CCD CH350 camera (Kodak KAF1401E, Full Frame Chip) and a Nikon inverted wide-field fluorescence microscope equipped with a *z*-step motor (Applied Precision, Inc.) was used. EGFP and AlexaFluor 488 were detected using a standard FITC filter set, Texas red and AlexaFluor 594 fluorescence were detected using a standard rhodamine/Texas red filter set.

For quantification of Gag–AP-2 colocalization described in Fig. 5, ten pictures of cells expressing Gagopt (Rudner et al., 2005) with either wild-type or mutant AP-2 were taken. 0.2-μm *z*-stacks from the bottom of each cell were imaged and deconvolved using the constrained iteration method on the DeltaVision software. Using the DeltaVision software, SoftWoRx, the bottom *z*-stack of each cell was chosen for further analysis. Gag–AP-2 colocalization was quantified using Volocity (Improvision) colocalization software.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2005.08.001.

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