The matrix domain of the Gag protein from avian sarcoma virus contains a PI(4,5)P₂-binding site that targets Gag to the cell periphery

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The Gag protein of avian sarcoma virus (ASV) lacks an N-myristoyl (myr) group, but contains structural domains similar to those of HIV-1 Gag. Similarly to HIV-1, ASV Gag accumulates on the plasma membrane (PM) before egress; however, it is unclear whether the phospholipid PI(4,5)P₂ binds directly to the matrix (MA) domain of ASV Gag, as is the case for HIV-1 Gag. Moreover, the role of PI(4,5)P₂ in ASV Gag localization and budding has been controversial. Here, we report that substitution of residues that define the PI(4,5)P₂-binding site in the ASV MA domain (reported in an accompanying paper) interfere with Gag localization to the cell periphery and inhibit the production of virus-like particles (VLPs). We show that co-expression of Sprouty2 (Spry2) or the pleckstrin homology domain of phospholipase Cδ (PH-PLC), two proteins that bind PI(4,5)P₂, affects ASV Gag trafficking to the PM and budding. Replacement of the N-terminal 32 residues of HIV-1 MA, which encode its N-terminal myr signal and its PI(4,5)P₂-binding site, with the structurally equivalent N-terminal 24 residues of ASV MA created a chimera that localized at the PM and produced VLPs. In contrast, the homologous PI(4,5)P₂-binding signal in ASV MA could target HIV-1 Gag to the PM when substituted, but did not support budding. Collectively, these findings reveal a basic patch in both ASV and HIV-1 Gag capable of mediating PM binding and budding for ASV but not for HIV-1 Gag. We conclude that PI(4,5)P₂ is a strong determinant of ASV Gag targeting to the PM and budding.

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) is an essential lipid in eukaryotic cells that functions in many different cellular processes. It is enriched in the inner leaflet of the plasma membrane (PM) (1), where it regulates motility, phagocytosis, exocytosis, and cell signaling (2). It is established that a critical early event in the assembly of the HIV-1 particle is the targeting of the structural precursor polyprotein, Gag, to the inner leaflet of the PM (3, 4). Studies by Ono and co-workers (5) revealed that upon overexpression of phosphoinositide 5-phosphatase IV (5ptaseIV), which reduces PI(4,5)P₂ levels by hydrolyzing the phosphate at the D5 position of PI(4,5)P₂, HIV-1 Gag proteins no longer accumulated on the peripheral cell membrane and that virus production was severely impaired.

Gag–membrane binding is mediated by the matrix (MA) domain of Gag, which, in the case of HIV-1, contains an N-terminal myristoyl (myr) group that can adopt sequestered and exposed conformations (3, 4). Myr exposure is known to promote membrane binding (6, 7). Saad et al. (8) demonstrated that PI(4,5)P₂ binds directly to HIV-1 MA, inducing a minor conformational change that triggers myr exposure. Subsequently, other laboratories, including ours, employed structural analysis and an enzymatic depletion strategy to evaluate the role of the phospholipid in the assembly of other retroviruses. Some retroviruses, such as equine infectious anemia virus (EIAV) and human T-cell leukemia virus 1, appear to be less dependent on PI(4,5)P₂ than HIV-1 (9, 10), whereas other retroviruses, such as HIV-2 and murine leukemia virus, exhibit PI(4,5)P₂ dependence (4, 11-13).

The PI(4,5)P₂ dependence of the avian sarcoma virus (ASV) MA and Gag for membrane binding is less clear. The ASV MA domain lacks a myr group, but its structure is similar to that of other retroviruses, including HIV-1 (14, 51). The MA protein possesses several basic residues that have been shown to be important for membrane binding. Mutation of these residues disrupted membrane binding and budding (15, 16). Increasing the concentration of phosphatidylinerine (PS) was also shown to enhance Gag association with liposomes (17). In that study, 5ptaseIV-mediated depletion of susceptible phospholipids in vivo did not significantly alter ASV Gag PM localization or virus release under the same conditions in which HIV-1 PM localization and budding were knocked down. However, in another study employing a similar enzyme-mediated depletion approach,

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2 The abbreviations used are: ASV, avian sarcoma virus; EIAV, equine infectious anemia virus; MA, matrix; myr, myristoyl; PI(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate; PM, plasma membrane; PH, pleckstrin homology; PH-PLC, pleckstrin homology region of phospholipase Cδ; Spry2, Sprouty2; 5ptaseIV, phosphoinositide 5-phosphatase IV; PS, phosphatidylinerine; VLP, virus-like particle; HA, hemagglutinin.
PI(4,5)P₂ depletion was observed to reduce virus release (18). In a third study, an ASV Gag construct with a leucine zipper substituted for the nucleocapsid domain to permit assessment of the competitive effect of MA binding to RNA versus PI(4,5)P₂, an effect of 5ptaseIV overexpression was observed but was less severe than that on HIV-1 Gag (19). Thus, in contrast to HIV-1, the role of PI(4,5)P₂ in ASV Gag-PM binding and budding remains unclear.

In an accompanying paper (51), we show that, like several other retroviral MA proteins, ASV MA can bind to PI(4,5)P₂. We have employed NMR, biophysical methods, liposome assays, and mutagenesis to identify a specific PI(4,5)P₂-binding pocket. Here, we show that mutations in key residues comprising the binding site diminished PM localization in the avian cell line DF-1. We employed co-expression of Sprouty2 (Spry2), a protein that localizes to membranes enriched in PI(4,5)P₂ and cholesterol, to test the importance of PI(4,5)P₂-targeted localization. Spry2 has been shown to inhibit localization of HIV-1 Gag on the PM and reduce virus-like particle (VLP) release (20). Our results indicate that Spry2 expression can interfere with ASV Gag accumulation on the PM and affect ASV Gag VLP production. Similarly, co-expression of PH-PLC, a protein that specifically targets PI(4,5)P₂, resulted in loss of VLP release. We constructed a chimera in which HIV-1's PI(4,5)P₂-binding determinant alone was substituted for that of ASV Gag and demonstrated its ability to target both PM binding and budding of the ASV protein, suggesting that the HIV-1 PS-binding determinants are not essential. In contrast, whereas ASV’s PI(4,5)P₂-binding site when substituted for that in HIV-1 Gag was sufficient to target Gag to the PM, additional HIV-1 MA determinants were required for its budding. Collectively, these results support the identification of a basic patch in the MA domain of ASV Gag as a specific PI(4,5)P₂-binding site (51) and demonstrate that PI(4,5)P₂ is an important determinant of ASV Gag targeting to and budding from the PM.

**Results**

**Mutations in Gag MA residues important for PI(4,5)P₂ binding in vitro inhibit Gag localization and virus release from cells**

As described in the accompanying paper (51), NMR studies of a MA construct encoding the membrane-binding domain (residues 1–87; MA87) identified Lys⁶, Lys¹³, Lys²³, and Lys²⁴ as important residues for lipid and liposome binding (Fig. 1A). These residues, along with other Lys residues throughout the MA gene, have been previously implicated in ASV Gag PM binding, virus assembly, and release (15). Herein, Ala and Glu substitutions were introduced at the Lys⁶, Lys¹³, Lys²³, and Lys²⁴ sites, and the resulting ASV Gag mutants were transfected into the DF-1 chicken fibroblast cell line. Both the VLPs and cellular Gag proteins were collected at 24 h post-transfection followed by analysis by Western blotting. In all cases, the efficiency of particle production was reduced (Fig. 1, B and C). For Lys⁶ mutants with Ala and Glu, VLP production was severely affected but reproducibly less than at the other sites. For Lys¹³, Lys²³, and Lys²⁴, VLP production was virtually eliminated by Ala or Glu substitutions.

Examination by fluorescence microscopy of the DF-1 cells expressing the mutated Gag-GFP proteins provided an explanation. Instead of the dispersed punctate distribution characteristic of the WT Gag protein (Fig. 2), the altered Lys⁶, Lys¹³, Lys²³, and Lys²⁴ Gag proteins were detected as large aggregates located near the nucleus. This indicates perturbation of both the appropriate assemblages and their localization for both Ala and Glu substitution. The disruption caused by the Lys⁶ → Ala mutation was reproducibly less, consistent with the results of the Western blotting analyses (Fig. 2). To ensure that the observed results were not due to the presence of the GFP tag on Gag, the effect of the Lys⁶ → Glu mutation was retested, employing a mixture of tagged and untagged Gag proteins. Identical results were obtained (Fig. S1). These results confirm and extend previous studies demonstrating that Lys⁶, Lys¹³, Lys²³, and Lys²⁴ are critical residues for ASV Gag membrane binding and particle release.

**Mutations in putative MA residues important for PS binding interfere with membrane targeting and budding in some cells**

In the accompanying paper (51), Vlach et al. show that PI(4,5)P₂ and PS have a strong synergetic effect on MA–membrane binding. Therefore, affecting either lipid could have some effect on ASV Gag. Doktorova et al. (21) suggested that Lys¹⁸, Lys⁶⁷, and Lys⁷² might be involved in PI–interaction. Their individual contributions to the overall binding were small (51), and Callahan et al. observed only a partial effect on particle production with single mutants (15). To assess the importance of the PS interaction relative to that of PI(4,5)P₂, we substituted Ala for Lys¹⁸ and Lys⁶⁷ and Thr for Lys⁷², leaving the PI(4,5)P₂-binding site intact, and compared the effect on Gag localization. As shown in Fig. 3, disruption of the presumptive PS-binding site in ASV Gag interfered with Gag targeting to the PM in the majority of cells, suggesting that binding to these lipids also contributes to PM targeting. Nevertheless, it appears that the PI(4,5)P₂-binding determinant in ASV Gag may contribute to a greater extent, as one change in the PI(4,5)P₂-binding pocket was sufficient to disrupt PM targeting (Fig. 2), whereas even with three changes, ASV-Gag-GFP's distribution in some cells was punctate and similar to WT (Fig. 3, A and B). Finding the WT punctate distribution pattern in 22% of the cell population indicated that proper localization is possible in the absence of the PS contribution. Analysis by Western blotting indicated that, despite the appearance of a WT cellular distribution in 22% of the population, ASV Gag with the Lys¹⁸, Lys⁶⁷, and Lys⁷² mutations had greatly reduced budding efficiency (Fig. 3D). ASV Gag levels in all lysates were equivalent (Fig. 3E).

**Sequestration of PI(4,5)P₂ by Spry2**

Previous studies employing an enzymatic approach (5ptaseIV) to inquire whether depletion by an enzyme that dephosphorylates phospholipids with a 5′-PO₄ including PI(4,5)P₂ interferes with ASV egress produced conflicting results (17, 18). We reexamed this question employing the cellular factor Spry2 as a probe. Spry2 is a member of a family of PI(4,5)P₂-binding proteins that localize in cholesterol-rich, lipid raft-containing invaginations of the PM through a determinant in the
C-terminal region of the protein that directs interaction with caveolin-1 (22) and in endosomal and secretory vesicles (23). Mutation of a single residue in the protein, Arg252 to Asp (R252D), essentially abrogates Spry2 binding to PI(4,5)P2 (24). This mutation does not necessarily prevent Spry2 co-localization at the cell periphery, but PI(4,5)P2 co-localization is disrupted (22). We previously demonstrated that overexpression of Spry2 interfered with PI(4,5)P2, exhibiting the same effect as

Figure 1. Mutation of residues in ASV MA that interact with PI(4,5)P2, *in vitro* inhibit VLP production. A, residues in the ASV MA structure predicted to be important for recognition of PI(4,5)P2 (51) are shown in blue sticks. The impact of these residues was assessed by mutating the Gag MA residues Lys6, Lys13, Lys23, and Lys24 to Ala (B) or Glu (C) and testing their effect on Gag stability (cell lysate) and the VLPs in the avian cell line DF-1. As demonstrated by the Western blot analysis, Gag accumulation in the cell lysate was not decreased by the mutations, but VLP production was inhibited. VLP efficiency values are normalized to WT. Error bars, S.D.
inhibitors of PLC hydrolysis (20). The interference required Spry2 interaction with PI(4,5)P

PI(4,5)P and hemagglutinin (HA)-tagged Spry2 were visualized by indirect immunofluorescence microscopy in COS-1 cells. The COS-1 cell line was chosen to facilitate comparison with HIV-1, as both ASV and HIV-1 Gag have been studied in this cell line (25, 26), and the effect of Spry2 expression on HIV-1 Gag localization was examined in this cell line as well (20). As shown in Fig. 4A1, an antibody directed against PI(4,5)P detected the phospholipid at the cell periphery; some cells also exhibited an intracellular pool. A construct encoding the PI(4,5)P lipid-selective PH domain of PLCδ tagged with GFP (27) exhibited signal primarily at the PM (Fig. 4A2). An antibody against the HA tag on HA-Spry2 detected the protein in vesicles throughout the cytoplasm and near the cell periphery (Fig. 4B). In all cases, the observed localization is consistent with results obtained in previous studies (20, 28). Consistent with localization in cell surface invaginations, co-staining revealed regions where PI(4,5)P and Spry2 co-localized at or near the cell periphery (Fig. 4, C–E). Moreover, the detection of this proximity depended on residue Arg252 because substitution of Asp for Arg at this site in the protein resulted in loss of the co-localization (Fig. 4, F–H). These observations support previous studies indicating that Spry2 has the ability to translocate to PI(4,5)P-enriched regions at the cell periphery through the Arg252 determinant in its so-called C-terminal translocation domain (24).

Spry2 interferes with ASV Gag localization; Spry2’s R252D mutation restores PM localization

Like HIV-1 Gag, ASV Gag expressed alone localizes predominantly to the cell periphery, evident as dispersed puncta distributed throughout the cell in z section planes above the nucleus (Fig. 5A) (25). To determine whether (Spry2) expression had an effect on Gag localization, COS-1 cell cultures were co-transfected with ASV Gag-GFP and HA-Spry2 or the Spry2 R252D mutant. Samples were fixed, stained, and examined by deconvolution microscopy at 24 h post-transfection. In ~40% of cells, Gag was detected at or near the cell periphery (z = 0–0.4 µm), and there was no apparent effect of Spry2 co-expression on Gag distribution. In the remaining ~60% of cells, a sensitive Gag subpopulation was apparent (Fig. 5, compare F with A and B). In these cells, Gag was detected in the cell interior (z > 2.0 µm), where it co-localized with the Spry2 protein.
Determinants of ASV Gag plasma membrane targeting

Figure 3. Mutation of MA residues implicated in PS binding alter PM targeting and budding. Shown is the effect on Gag distribution pattern for WT ASV Gag-GFP (A) and ASV Gag-GFP with Lys^{18}, Lys^{67}, and Lys^{72} mutated to Ala, Ala, and Thr, respectively (B and C). Three different isolates for the mutants were transfected, and 20–60 cells were analyzed for each construct and WT (scale bars, 10 μm). D, relative VLP efficiency for WT and triple mutants (values were normalized to WT). Error bars, S.D. E, Western blotting of WT ASV Gag-GFP (lane 1) and three separate isolates of the ASV Gag with mutations at Lys^{18}, Lys^{67}, and Lys^{72} (lanes 2–4).

Figure 4. Spry2 co-localizes with PI(4,5)P₂ on the PM; substitution of Asp for Arg^{252} inhibits the interaction. A1, localization of PI(4,5)P₂ using antibody directed against PI(4,5)P₂; A2, localization of PI(4,5)P₂ by the PI(4,5)P₂-specific probe PH-PLC-GFP. B, localization of Spry2 expressed alone visualized using antibody against the HA tag on HA-Spry2. C–E, Spry2 co-localizes with PI(4,5)P₂; F–H, Spry2-R252D mutant does not co-localize with PI(4,5)P₂. Scale bars, 10 μm.

(representative images are shown in Fig. 5 (F–H)). Fig. 5 (E and I) shows software-generated three-dimensional renderings of cross-sections through the cells in Fig. 5 (D and H, respectively) co-expressing Gag and HA-tagged Spry2. As observed, the dispersed distribution pattern evident in control cells (i.e. cells expressing Gag alone) was restored in most, although not all, cells by co-expression with the R252D mutant (Fig. 5, J–L) and was evident in the three-dimensional rendering (Fig. 5, compare M with I). Possibly, Spry2 directly caused Gag sequestration in the cell interior; alternatively, the loss of PI(4,5)P₂-binding sites for ASV Gag on the PM redirected Gag to the cell interior. The reduction in the range of Pearson’s coefficients of correlation for Gag–Spry2 co-localization is shown in Fig. 5N. The apparent partial sensitivity of the ASV Gag population to
Spry2-mediated interference with PI(4,5)P2 could explain the conflicting results obtained by different laboratories following enzyme-mediated interference with PI(4,5)P2.

Spry2 inhibits budding when Gag is modified by ubiquitin

We previously provided evidence that the dose-dependent expression of the WT Spry2 protein inhibits VLP release from cells expressing HIV-1 Gag (20). The inhibition was relieved by the R252D mutant. As PI(4,5)P2 is a major determinant of HIV-1 Gag localization to the PM (8, 29), a likely explanation is that the sequestration of PI(4,5)P2 by Spry2 interferes with HIV-1 Gag targeting to the PM to form productive assembly sites and, thereby, disrupts VLP release. As an indication of sensitivity to interference with PI(4,5)P2, we determined whether ASV Gag was similarly affected. As shown in Fig. 6 (top panels), co-expression of ASV Gag-GFP with Spry2 in DF-1 or COS-1 cells did not diminish the amount of VLPs detected in the media or reduce the accumulation of intracellular Gag (Fig. 6, middle panels). Quantitative analysis (Fig. 6, bottom panels) indicated that VLP release efficiency was unaffected. Thus, unlike HIV-1, co-transfection with Spry2 did not affect ASV budding efficiency.

Spry2 trafficking to the cell periphery is signaled by the occurrence of internalized and ubiquitin-modified epidermal growth factor receptor at the PM (30, 31). We therefore speculated that its inhibitory effect might be augmented if ASV Gag were modified by ubiquitin. It has been observed that increased modification by ubiquitin was induced by mutations in HIV-1 Gag’s L domains (32–35), either the primary (PTAP) or the secondary (LYPXnL). ASV Gag possesses a primary (P172PPPY or “PY”) motif recognized by Nedd4 (36) and a secondary (LY181PXL) motif recognized by Alix (37). Supporting the hypothesis, mutation of either of the ASV Gag L domains resulted in Spry2 dose-dependent inhibition of budding (Fig. 7, A and B, lanes 3–6 and 7–10) compared with the budding level of the WT ASV Gag protein expressed alone (lane 1). As expected, deletion of the region housing the L domains, p2b, blocked budding completely (lane 2). To determine the effect of Spry2 on subcellular localization of the ubiquitin-modified proteins, cells co-expressing Spry2 and the PY mutant (Fig. 7, E–G)
or the mutant alone (Fig. 7D) were examined by fluorescence microscopy. When expressed without Spry2, the mutant exhibited a more dispersed punctate pattern (compare boxes in Fig. 7, D and F). When co-expressed with Spry2, the mutant was sequestered in the cell interior, where it co-localized with the Spry2 protein in 100, 85, and 67% of cells in three independent trials (n = 14, 6, and 9 cells examined, respectively). The results demonstrate that specific recruitment of Spry2, facilitated by its ability to target PI(4,5)P2-enriched membranes, can augment an inhibitory effect on budding.

**The pleckstrin domain of phospholipase Cδ interferes with ASV Gag budding**

The observed change in localization/inhibition of budding following Spry2 expression was likely influenced by other cellular factors. To more selectively test the lipid dependence of ASV budding, we determined the effect on budding when cells were exposed to the pleckstrin homology domain of phospholipase Cδ (PH-PLC). This domain binds PI(4,5)P2 with high specificity and affinity (38, 39). DF-1 and COS-1 cells were transfected with DNA encoding a GFP-tagged PH-PLC and ASV Gag-HA or HIV-1 Gag-HA. HIV-1-Gag-HA served as a positive control because its trafficking to PI(4,5)P2 is well established (8, 29). VLP and cell lysates were analyzed by Western blotting (Fig. 8, A and B). The results show that for both ASV Gag and HIV-1 Gag, budding was inhibited in a dose-dependent fashion. For ASV Gag, Western blotting revealed that it was cut, as evidenced by the low-molecular weight band recognized by the anti-MA antiserum. This cleavage possibly reflects a more “open” structure due to displacement by PH-PLC. Relative VLP efficiency was decreased for ASV in COS-1 but not in DF-1, implying that DF-1 cells were more effective at budding the remaining full-length Gag. In summary, both HIV-1 and ASV Gag were affected by PH-PLC. This outcome supports the hypothesis that ASV targeting is driven mainly by the PI(4,5)P2 lipid interaction.

**The N-terminal 24 residues of ASV Gag are sufficient for ASV but not HIV-1 Gag localization and budding**

Based on studies employing chimeric proteins containing HIV-1 and ASV Gag residues, it was previously concluded that the N-terminal 32 residues of HIV-1 Gag could fully substitute
for the N-terminal 100 residues of ASV Gag for budding of VLPs and weakly substitute for the N-terminal 10 residues (40). The N-terminal 32 residues of HIV-1 Gag contain three membrane-binding signals: the myr group, the conserved basic region, and a \( \mathrm{PI(4,5)P}_2 \)-binding pocket (41, 42). ASV Gag lacks an N-terminal myr group. However, as is evident in the model for ASV MA87 proposed by Vlach et al. (51), residues 1–24 of ASV MA and 1–32 of HIV-1 MA share nearly identical structural features (Fig. 9A).

To examine the functional significance of this apparent structural conservation, we constructed a chimeric protein in which the N-terminal 32 residues of HIV-1 Gag substituted for the N-terminal 24 residues of ASV Gag (H32A25; Fig. 9B). Examination by microscopy revealed that H32A25-Gag exhibited a punctuate subcellular distribution pattern indistinguishable from that of WT ASV Gag or \( \Delta p2b \) Gag, an ASV Gag mutant that binds to the PM but is defective for budding (36) (Fig. 9C). In contrast, the H32R chimera containing the same N-terminal HIV-1 32 residues substituting for only the first 10 ASV Gag residues accumulated in noticeably larger internal structures and was also more diffuse in the cytoplasm (Fig. 9C). We noted that the expression or stability of H32R was lower than that of the WT protein and also lower than H32A25-Gag. Nevertheless, Western blot analysis indicated comparable VLP release efficiency (Fig. 10A). This is consistent with the model proposed by Vlach et al. (51), that ASV and HIV-1 conserve the \( \mathrm{PI(4,5)P}_2 \)-binding structural element in MA. This element is sufficient to confer the PM binding function necessary for ASV budding. The N-terminal 10 residues in ASV Gag, which include Lys6, target the protein to the PM inefficiently compared with the N-terminal 24 residues.

To determine whether the \( \mathrm{PI(4,5)P}_2 \)-binding determinant alone is sufficient for HIV-1 Gag targeting and budding, as with ASV Gag, two additional chimeras were constructed: A24H33, which has ASV’s N-terminal 24 residues attached to HIV-1 Gag at residue 33, and A24H133, with ASV residues attached at HIV-1 residue 133 (i.e. no HIV-1 MA sequences) (Fig. 9B). When analyzed by fluorescence microscopy, only A24H133 exhibited a punctate distribution similar to that of WT Gag (Fig. 9C). Interestingly, despite this apparently normal appearance, the A24H133 chimera was unable to bud, as determined by Western blot analysis (Fig. 10B). Thus, whereas the ASV PI(4,5)P2-binding determinant is sufficient to drive PM targeting and budding for ASV Gag (Fig. 10, A and B, lane 1), it was not
sufficient to drive PM targeting of HIV-1 Gag unless the HIV-1 MA sequences were absent. Also, it was not sufficient to drive HIV-1 budding whether the MA sequences were there or not. We conclude that the N-terminal region of the MA domain of ASV Gag targets the protein to the PM, where it interacts with PI(4,5)P₂, and that binding the phospholipid is a major determinant of ASV Gag budding.

Discussion

This study was guided by the findings of Vlach et al. (51), in which ASV MA protein binding to lipids and liposomes was characterized using NMR methods. That study defined the molecular details of PI(4,5)P₂ binding to ASV MA₈₇ and revealed a previously unidentified membrane-binding basic patch in which residues Lys⁶, Lys¹³, Lys²³, and Lys²⁴ in particular contribute to binding. Here, we mutated these residues in the context of the Gag precursor and assessed their impact on Gag localization within cells, overall VLP production, and budding efficiency. We found that Lys¹³, Lys²³, and Lys²⁴ consistently and significantly altered Gag localization and budding (Figs. 1 and 2). For Lys⁶ mutants, VLP production was severely affected, but reproducibly less than the other sites for both Ala and Glu substitutions. These results from the targeted mutations and their effect in an avian cell line support the in vitro findings and provide validation of the structural model derived from the in vitro analyses.

Previous studies demonstrated that the HIV-1 tripartite membrane-binding signal, housed in residues 1–32, can fully replace the membrane-targeting function of the N-terminal 100 residues of ASV Gag, which is naturally nonmyristoylated (40). This was especially found to be the case when the central MA region was removed. The ASV MA sequence in Gag is highly basic and binds phosphatidylserine-containing membranes through electrostatic interactions (21, 43–45). Such membrane interaction is blocked by RNA binding to this region (19). Examination by fluorescence microscopy of the previously described chimeric H₃₂R revealed that, although it recapitulated WT virus release efficiency, it localized aberrantly within the cell (Fig. 9). In contrast, replacing the first 24 rather than the first 10 N-terminal ASV Gag residues produced a protein whose stability and distribution pattern resembled that of WT ASV Gag much more closely, suggesting that proper folding and Gag interaction had been attained. These observations support the view that the structures formed by the N-terminal 24 and 32 residues of ASV and HIV-1, respectively, comprise a conserved functional element that constitutes the PM-targeting domain.

The reverse chimera, wherein ASV provides the N-terminal PI(4,5)P₂-binding sequences attached to HIV-1 Gag with or without MA sequences, showed two distinct distributions in the cell. Whereas the Δ₃₃H₃₃ chimera was aberrant, deletion of the HIV-1 MA sequence from the chimeric ASV-HIV Gag restored the typical plasma membrane pattern. In both cases, however, budding was significantly inhibited. This finding provides evidence that PI(4,5)P₂ targeting is sufficient for ASV Gag budding.

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The reverse chimera, wherein ASV provides the N-terminal PI(4,5)P₂-binding sequences attached to HIV-1 Gag with or without MA sequences, showed two distinct distributions in the cell. Whereas the Δ₃₃H₃₃ chimera was aberrant, deletion of the HIV-1 MA sequence from the chimeric ASV-HIV Gag restored the typical plasma membrane pattern. In both cases, however, budding was significantly inhibited. This finding provides evidence that PI(4,5)P₂ targeting is sufficient for ASV Gag budding.
budding but not HIV-1 Gag budding. Residues 18, 67, and 72 in ASV Gag have been implicated in PS binding in studies reported by Doktorova et al. (21). The effects on targeting or budding of single (15) or triple substitutions were only partial (this report). It appears that these residues have a less pronounced affect on the recognition of the PI(4,5)P_2-binding site needed for Gag localization and budding than Lys_6, Lys_13, Lys_23, and Lys_24.

To perturb the intracellular PI(4,5)P_2 homeostasis and investigate the impact on ASV Gag targeting to the PM, we employed Spry2, a member of a family of cellular factors that are known to bind PI(4,5)P_2 and to interfere with its PLC-mediated synthesis (24, 46, 47). Interestingly, ASV Gag appeared to be more resistant than HIV-1 Gag to Spry2-mediated interference with the phospholipid. This does not reflect a greater relative affinity of HIV-1 Gag for PI(4,5)P_2, as the apparent affinity of the two Gag proteins for the phospholipid is on the same order of magnitude, with ASV Gag affinity somewhat weaker (51). It should be noted that whereas co-expression of Spry2 inhibited HIV-1 VLP release (20), it had no apparent effect on ASV VLP release efficiency (Fig. 6) even though ASV Gag localization was altered in ~60% of the cells examined (n = 40). Moreover, although mutation of the PI(4,5)P_2-binding determinant in Spry2 restored Gag localization in most cells, internalization was still apparent in others. We previously demonstrated that Spry2 binds the endocytic sorting complex required for transport (ESCRT)-II component Eap20 (28). As this ESCRT-II factor is required for ASV Gag release (26) but may (48) or may not (26, 49) be required for HIV-1 budding, the impact of Spry2 co-expression on the Gag proteins is likely influenced by other assembly events. The determinants of Spry2 binding to ESCRT-II reside in the N-terminal half of the protein (28), whereas the determinants of Spry2 binding to PI(4,5)P_2 and caveolin-1 lie in the C-terminal region of the protein (22, 24). Thus, Spry2 binding to ESCRT-II is not expected to diminish the population avail-

Figure 9. Effect of exchanging N-terminal sequences from ASV Gag and HIV-1 Gag on PM targeting. A, model of the ASV and HIV-1 MA structures highlighting the similar N-terminal structural motifs and basic residues. B, schematic representation of HIV-1 Gag, ASV Gag, and the chimeric proteins H_32A_25 and H_32R, wherein HIV-1 N-terminal sequences replace residues in the MA domain of ASV Gag, and A_32H_13 and A_32H_ΔMA, wherein ASV N-terminal sequences replace HIV N-terminal sequences. C, fluorescence imaging of DF-1 cells expressing WT ASV Gag-GFP, Δp2b Gag-GFP, H_24A_25 Gag-GFP, and H_24R Gag-GFP (top panels) and A_32H_13 and A_32H_ΔMA (bottom panels). Between 20 and 200 cells were examined for each construct, and the percentages of cells with only punctate GFP signal were as follows: WT (85%), H_32A_25 Gag-GFP (90%), H_32R Gag-GFP (0%), Δp2b Gag-GFP (100%), A_32H_13 (2%), and A_32H_ΔMA (48%). Scale bars, 10 μm.
able to interact with the lipid. Indeed, Spry2 appeared to recruit ESCRT-II to the PM (28).

We observed that, in contrast to HIV-1, but similar to EIAV (50), the intracellular localization, production, and budding efficiency of ASV VLPs were not as significantly diminished by interference by Spry2. This appeared to be due, at least in part, to the size of the susceptible ASV Gag population, as we demonstrated that modification of Gag by ubiquitin increased its sensitivity to Spry2-mediated interference. The impact of Spry2 on ASV Gag may be influenced by other assembly-related events. The interference mediated by PH-PLC, a PI(4,5)P2-selective probe, rather than Spry2, a PI(4,5)P2-binding signaling modulator, demonstrated the PI(4,5)P2 dependence of ASV Gag budding.

We conclude that a basic patch in the MA region of ASV Gag forms a specific PI(4,5)P2-binding site and that the phospholipid is a strong determinant of PM targeting. Our findings also suggest that although PI(4,5)P2-mediated targeting to the PM is a critical factor in assembly and release of ASV Gag, the collective contribution of additional facilitating or antagonistic cellular factor–trafficking events likely accounts for differences observed between HIV-1 and ASV membrane targeting and budding.

**Experimental procedures**

**Plasmids and reagents**

pCMV-ASV Gag-GFP expresses the ASV Gag gene with a terminal GFP tag (36). Mutations in the ASV MA gene were introduced by site-directed mutagenesis (Roche Applied Science). Plasmids encoding full-length human Spry2 N-terminally tagged with HA (pCGN-Spry2) and its R252D mutant were generous gifts of D. Bar-Sagi (New York University) (23). PH-PLC-GFP plasmid was a generous gift of J. Donaldson (National Institutes of Health, Bethesda, MD). HA-tagged proteins were detected by a mAb directed at the HA tag (Bionlegend). Gag proteins tagged with GFP were detected with a mAb against GFP (Clontech). Antibody to ASV MA was developed by David Boettiger and was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa (Iowa City, IA). Actin levels were detected by using a mAb against actin (Sigma-Aldrich). Monoclonal antibodies against PI(4,5)P2 were obtained from Abcam, Inc. (Cambridge, MA).

**Transfection and protein analysis**

COS-1 and DF-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum and antibiotics and transfected using XtremeGene reagent (Roche Applied Science). At 24 h post-transfection, the culture medium was removed, and the cells were washed in PBS followed by lysis in buffer (20 mM Tris, pH 8, 1% Triton X-100, 160 mM NaCl, Roche Complete mini protease inhibitor). Lysates were centrifuged for 15 min at 1,000 × g, and the supernatants were saved for analysis. Culture medium was filtered through a 0.45-µm pore size filter, and the VLPs were isolated by ultracentrifugation through a cushion of 20% sucrose at 160,000 × g for 90 min at 4 °C using a Beckman SW41 rotor. Lysates and
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VLP preparations were separated on 9% polyacrylamide-SDS gels and identified by Western blotting and detection with antibodies as described in the figure legends. The secondary antibodies used to detect protein expression were Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes; 1:10,000) and IRDyeTM800-conjugated affinity-purified goat anti-rabbit IgG (Rockland; 1:10,000). The Western blots were analyzed on a LI-COR Odyssey CLx IR imaging system, and the bands were quantified with LI-COR Image Studio Lite version 5.2. VLP release efficiency was defined as the ratio of the signal intensity value for the VLP-associated Gag to the sum of the values for VLP-associated Gag plus cell lysate–associated Gag (VLP/ (VLP + Gag from cell lysate)).

Deconvolution microscopy

DF-1 cells grown on lysine-treated coverslips were transfected with pCMV-ASV Gag-GFP alone or together with pCGN-Spry2 using XtremeGene (Roche Applied Science). After 24 h, cells were fixed in 4% formaldehyde (Sigma) and permeabilized in 0.1% Triton X-100. Spry2 was detected in the samples by indirect immunofluorescence using anti-HA antibody (BioLegend) and Texas Red anti-mouse IgG (Rockland Immunochemicals Inc.). Nuclei were stained with Hoechst.

All images were captured on an inverted fluorescence/deconvolution microscope: differential interference contrast Zeiss Axiovert 200M deconvolution fluorescence microscope operated by AxioVision version 4.5 (Zeiss) software and deconvolution image processing by using the constrained iterative method (AxioVision software). Protein co-localization was assessed in cells by determining the role of phosphatidylinositol-(4,5)-bisphosphate in membrane targeting. J. Mol. Biol. 382, 434–447

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