The matrix domain of the Gag protein from avian sarcoma virus contains a PI(4,5)P$_2$-binding site that targets Gag to the cell periphery

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ABSTRACT
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)P$_2$ 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)P$_2$ in ASV Gag localization and budding has been controversial. Here, we report that substitution of residues that define the PI(4,5)P$_2$-binding site in the ASV MA domain (reported in a companion manuscript), 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 Cd (PH-PLC), two proteins that bind PI(4,5)P$_2$, 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)P$_2$-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)P$_2$-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)P$_2$ is a strong determinant of ASV Gag targeting to the PM and budding.

Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] 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 human immunodeficiency virus type 1 (HIV-1) particle is the targeting of the structural precursor polyprotein, Gag, to the inner leaflet of the PM (3,4). Studies by Ono et al. revealed that upon overexpression of phosphoinositide 5-phosphatase IV (5-tpsase IV), which reduces PI(4,5)P$_2$ levels by hydrolyzing the phosphate at the D5 position of PI(4,5)P$_2$, HIV-1 Gag proteins no longer accumulated on the peripheral cell membrane and that virus production was severely impaired (5).
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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 (HTLV-1), appear to be less dependent on PI(4,5)P2 than HIV-1 (9,10) while other retroviruses such as HIV-2 and MLV 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 (joint manuscript) (14). 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 phosphatidylserine (PS) was also shown to enhance Gag association with liposomes (17). In that study, 5-ptide IV-mediated depletion of susceptible phospholipids in vivo did not significantly alter ASV Gag PM localization or virus release under the same conditions that HIV-1 PM localization and budding were knocked-down. However, in another study employing a similar enzyme-mediated depletion approach, 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 (NC) domain to permit assessment of the competitive effect of MA binding to RNA versus PI(4,5)P2, an effect of 5-ptide IV 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 manuscript (Vlach et al.), we show that, like several other retroviral MA proteins, ASV MA can bind to PI(4,5)P2. We have employed nuclear magnetic resonance (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, while 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 (accompanying manuscript) 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 manuscript (Vlach et al.), 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 Lys\textsuperscript{6}, Lys\textsuperscript{13}, Lys\textsuperscript{23}, and Lys\textsuperscript{24} 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 hr post-transfection followed by analysis by Western blotting. In all cases, the efficiency of particle production was reduced (Fig. 1B and 1C). For Lys\textsuperscript{6} mutants with Ala and Glu, VLP production was severely affected but reproducibly less than the other sites. For Lys\textsuperscript{13}, Lys\textsuperscript{23}, and Lys\textsuperscript{24}, 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 Lys\textsuperscript{6}, Lys\textsuperscript{13}, Lys\textsuperscript{23}, and Lys\textsuperscript{24} Gag proteins were detected as large aggregates located near the nucleus, indicating perturbation of both the appropriate assemblages and their localization for both Ala and Glu substitution. The disruption caused by the Lys\textsuperscript{6} to Ala mutation was reproducibly less, consistent with the results of the Western blot 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 Lys\textsuperscript{6} to Glu mutation was re-tested employing a mixture of tagged and untagged Gag proteins. Identical results were obtained (Supplementary Figure S1). These results confirm and extend previous studies demonstrating that Lys\textsuperscript{6}, Lys\textsuperscript{13}, Lys\textsuperscript{23}, and Lys\textsuperscript{24} 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, Vlach et al. show that PI(4,5)P\textsubscript{2} 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 Lys\textsuperscript{18}, Lys\textsuperscript{67} and Lys\textsuperscript{72} might be involved in PS interactions. Their individual contributions to the overall binding were small (accompanying manuscript) 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)P\textsubscript{2}, we substituted Ala for Lys\textsuperscript{18} and Lys\textsuperscript{67} and Thr for Lys\textsuperscript{72} leaving the PI(4,5)P\textsubscript{2}-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)P\textsubscript{2}-binding determinant in ASV Gag may contribute to a greater extent, as one change in the PI(4,5)P\textsubscript{2} binding pocket was sufficient to disrupt PM targeting (Figure 2), while even with 3 changes, ASV-Gag-GFP’s distribution in some cells was punctate and similar to WT (Fig. 3A 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 wild type cellular distribution in 22% of the population, ASV Gag with the Lys\textsuperscript{18}, Lys\textsuperscript{67} and Lys\textsuperscript{72} mutations had greatly reduced budding efficiency (Fig. 3D). ASV Gag levels in all lysates were equivalent (Fig. 3E).

**Sequestration of PI(4,5)P\textsubscript{2} by Spry2**

Previous studies employing an enzymatic approach (5-ptase IV) to inquire if depletion by an enzyme that dephosphorylates phospholipids with a 5’-PO\textsubscript{4} including PI(4,5)P\textsubscript{2}, interferes with ASV egress produced conflicting results (17,18). We re-examined this question employing the cellular factor Spry2 as a probe. Spry2 is a member of a family of PI(4,5)P\textsubscript{2}-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, Arg\textsuperscript{252} to Asp (R252D), essentially abrogates Spry2 binding to PI(4,5)P\textsubscript{2} (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 inhibitors of PLC hydrolysis (20). The interference required Spry2 interaction with PI(4,5)P₂.

PI(4,5)P₂ 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)P₂ detected the phospholipid at the cell periphery; some cells also exhibited an intracellular pool. A construct encoding the PI(4,5)P₂ lipid selective PH domain of PLC8 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)P₂ and Spry2 co-localized at or near the cell periphery (Fig. 4C-E). Moreover, the detection of this proximity was dependent on residue Arg²⁵² since substitution of Asp for Arg at this site in the protein resulted in loss of the co-localization (Fig. 4F-H). These observations support previous studies indicating that Spry2 has the ability to translocate to PI(4,5)P₂-rich 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 hr 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 (compare Fig. 5F to 5A and 5B). In these cells, Gag was detected in the cell interior (z = >2.0 µm), where it co-localized with the Spry2 protein (representative images are shown in Fig.5F-H). Figs.5E,1 show software-generated 3-dimensional renderings of cross sections through the cells in panels 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 5J-L) and was evident in the 3-D rendering (compare Fig. 5M to Fig 5I). 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)P$_2$, 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 (EGFR) 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 ASV Gag’s L domain, either the primary (P$_{172}$PPPY or “PY”) motif recognized by Nedd4 (36) or the secondary (LY$_{181}$PXL) motif recognized by Alix (37). Supporting the hypothesis, Spry2 dose-dependent inhibition of budding was evident (Fig. 7, panels A and B, lanes 3-6 and 7-10) compared to 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 (panels E-G) or the mutant alone (panel D) were examined by fluorescence microscopy. When expressed without Spry2, the mutant exhibited a more dispersed punctate pattern (compare boxes in Fig. 7D and 7F). 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)P$_2$-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)P$_2$ 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 since its trafficking to PI(4,5)P$_2$ is well established (8,29). VLP and cell lysates were analyzed by Western blotting (Fig. 8, panel 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 blot revealed that it was cut as evidenced by the low molecular weight band recognized by the anti-MA antisera. 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)P$_2$ 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
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PI(4,5)P₂-binding pocket (41,42). ASV Gag lacks an N-terminal myr group. However, as is evident in the model for ASV MA₁₋₄₇ proposed by Vlach et al. in the accompanying manuscript, residues 1 through 24 of ASV MA and 1 through 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 Δp2b Gag, an ASV Gag mutant which 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. that ASV and HIV-1 conserve the PI(4,5)P₂-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 to the N-terminal 24 residues.

To determine whether the PI(4,5)P₂-binding determinant alone is sufficient for HIV-1 Gag targeting and budding, as with ASV Gag, two additional chimeras were constructed: A₂₄H₁₃ which has ASV’s N-terminal 24 residues attached to HIV-1 Gag at residue 33 and A₂₄H₁₃MA with ASV residues attached at HIV-1 residue 133 (i.e., no HIV-1 MA sequences) (Fig. 9B). When analyzed by fluorescence microscopy, only A₂₄H₁₃MA exhibited a punctate distribution similar to that of WT Gag (Fig. 9C). Interestingly, despite this apparently normal appearance, the A₂₄H₁₃MA chimera was unable to bud as determined by Western blot analysis (Fig. 10B). Thus, while the ASV PI(4,5)P₂-binding determinant is sufficient to drive PM targeting and budding for ASV Gag (Fig. 10, panels 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., accompanying manuscript, 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 MA₁₋₄₇ 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 non-myristoylated (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 phosphatidylycerol-containing membranes through electrostatic interactions (21,44,45). 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. While the A33H3 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 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). These residues have a less pronounced impact on Gag localization and budding than Lys6, Lys15, Lys23, and Lys24 form the PI(4,5)P2 binding site.

To perturb the intracellular PI(4,5)P2 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)P2 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)P2 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 (accompanying manuscript). It should be noted that while 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)P2-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) while the determinant of Spry2 binding to PI(4,5)P2 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 available 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 was 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 factors trafficking events likely account 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). Plasmids encoding full-length human Spry2 N-terminally tagged with hemagglutinin (HA) (pCGN-Spry2) and its R252D mutant were generous gifts of D. Bar-Sagi (NYU, NY) (23). PH-PLC-GFP plasmid was a generous gift of J. Donaldson (NIH, Bethesda). HA-tagged proteins were detected by a monoclonal antibody directed at the HA tag (Biolegend). Gag proteins tagged with GFP were detected with a monoclonal antibody against GFP (Clontech Laboratories). Antibody to ASV MA was developed by David Boettiger and was obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Development and maintained by the University of Iowa (Iowa City, IA). Actin levels were detected by using a monoclonal antibody 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). At 24 hr 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 1,000xg and the supernatants 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 160000xg for 90 min at 4°C using a Beckman SW41 rotor. Lysates and VLP preparations were separated on 9% polyacrylamide/SDS gels and identified by Western blotting and detection with antibodies as described in the Figures. The secondary antibodies used to detect protein expression were Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, 1:10,000) andIRDyeTM800-conjugated affinity purified goat anti-rabbit IgG (Rockland, 1:10,000). The Western blots were analyzed on a Li-Cor Odyssey CLx infrared imaging system and the bands quantified with Li-Cor Image Studio Lite v 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 cover slips were transfected with pCMV-ASV Gag-GFP alone, or together with pCGN-Spry2 using XtremeGene (Roche). After 24 hr, 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 (Biolegends) and Texas Red anti-mouse IgG (Rockland ImmunocOm chemicals Inc.). Nuclei were stained with Hoechst. COS-1 cells were transfected and fixed using a similar protocol but with cold Triton X-100 or saponin permeabilization in the experiments for PI(4,5)P₂ detection. PI(4,5)P₂ was detected with mouse monoclonal antibodies 2C11 and 2335 (Abcam). All images were captured on an inverted fluorescence/differential-interference contrast (dic) Zeiss Axiosvert 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 determination of Pearson’s coefficient of correlation using NIH Image J software. GraphPad Prism 7 software was used.
to generate the scatter plots and the t-test analysis of Pearson’s values.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.
Determinants of ASV Gag Plasma membrane targeting

References

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FOOTNOTES:

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Abbreviations: ASV, Avian sarcoma virus; HIV-1, human immunodeficiency virus type 1; EIAV, equine infectious anemia virus; MA, matrix; myr, myristoyl; PI(4,5)P2, phosphatidylinositol (4,5)-bisphosphate; PM, plasma membrane; PH-PLC, pleckstrin homology region of phospholipase Cδ; Spry2, Sprouty2.
Figure 1. Mutation of residues in ASV MA that interact with PI(4,5)P$_2$ in vitro inhibit VLP production. (A) Residues in the ASV MA structure predicted to be important for recognition of PI(4,5)P$_2$ (accompanying manuscript, Vlach et al.) are shown in blue sticks. The impact of these residues was assessed by mutating the Gag MA residues Lys$^6$, Lys$^{13}$, Lys$^{23}$, and Lys$^{24}$ to Ala (B) or Glu (C) and testing their effect on Gag stability (cell lysate) and the production of virus-like particles (VLP) 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; the error bars represent 1 SD.
Figure 2. Mutations in MA residues important for PI(4,5)P₂ binding in vitro prevent Gag localization to the cell periphery in DF-1 cells. In this fluorescence imaging analysis, ASV Gag-GFP WT exhibited its typical dispersed punctate signal. For the K6A mutant, ASV Gag cellular localization was altered but much of the signal remained punctate. Substitution of Ala for Lys₁³, Lys₂³, and Lys₂⁴ or substitution of Glu for Lys₆, Lys₁³, Lys₂³, and Lys₂⁴ residues severely affected protein distribution with Gag appearing as aggregates located primarily near the nucleus. For each construct, 10 to 40 cells were analyzed; only cells transfected with WT ASV Gag-GFP displayed signal that was exclusively punctate. Scale bars represent 10 μm.
Figure 3. Mutation of MA residues implicated in PS-binding alter PM targeting and budding. Effect on Gag distribution pattern for (A) WT ASV Gag-GFP; (B and C) ASV Gag-GFP with Lys18, Lys67, and Lys72 mutated to Ala, Ala, and Thr, respectively. Three different isolates for the mutants were transfected and 20 to 60 cells were analyzed for each construct and WT (scale bars represent 10 μm). (D) Relative VLP efficiency for WT and triple mutants (values were normalized to WT); the error bars represent 1 SD. (E) Western blot of WT ASV Gag-GFP (lane 1) and three separate isolates of the ASV Gag with mutations at Lys18, Lys67, and Lys72 (lanes 2-4).
Figure 4. Spry2 co-localizes with PI(4,5)P_2 on the PM; substitution of Asp for Arg^{252} inhibits the interaction. (A1) Localization of PI(4,5)P_2 using antibody directed against PI(4,5)P_2; (A2) Localization of PI(4,5)P_2 by the PI(4,5)P_2-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)P_2. (F – H) Spry2-R252D mutant does not co-localize with PI(4,5)P_2. Scale bars represent 10 μm.
Figure 5. Spry2 expression interferes with ASV Gag localization. Cells were transfected with DNA encoding ASV Gag-GFP alone (A) or with DNA encoding Gag and WT HA-Spry2 (B to I) or HA-Spry2-R252D (J to M). At 24 hr after transfection, the COS-1 cells were examined by microscopy. Spry2 was detected in the samples by indirect immunofluorescence using anti-HA antibody and Texas Red anti-mouse IgG. (M) Axiovision software 3-dimensional renderings of cells in cultures expressing Gag and WT Spry (E and I) or Spry2- R252D mutant. (N) Scatter plot of Pearson’s coefficients of correlation for cells co-transfected with Gag and HA-tagged WT- or Spry2 R252D (Pearson’s coefficients were calculated for entire cells). The distribution of the WT and R252D populations was significantly different (p<0.05) as assessed by a t-test analysis of the Pearson’s coefficient for co-localization of positive cells (NIH Image J software). Scale bars represent 10 μm.
**Figure 6.** Lack of effect of Spry2 WT or Spry R252D mutant expression on ASV Gag VLP release. (A) DF-1 or (B) COS-1 cells were transfected with DNA encoding ASV Gag-GFP alone (lane 1) or with increasing amounts of DNA encoding WT HA-Spry2 or HA-Spry R252D. At 24 hr after transfection, cells were harvested and media analyzed. Media (top panels) and cell lysates (middle panels) were examined for VLP production or for expression of Gag-GFP, HA-Spry2 and actin, respectively, by Western analysis. Bottom panels, quantitative analysis of relative VLP release efficiency normalized to WT; error bars represent 1 SD.
Figure 7. Spry2 inhibits VLP release when ASV Gag is modified by ubiquitin. ASV Gag WT (lane 1), Δp2b (lane 2), P172A (lanes 3-6) and Y181S (lanes 7-10) were transfected alone or with increasing amounts of DNA encoding WT HA-Spry2. At 24 hr after transfection, cells were harvested and media analyzed. Media (A) and cell lysates (B) were examined for VLP production and for lysate levels of Gag-GFP, HA-Spry2 and actin, respectively, by Western blotting. (C) Quantitative analysis of relative VLP release efficiency normalized to WT; the error bars represent 1 SD. Gag P172A was further analyzed by microscopy when transfected alone (D) or in the presence of WT HA-Spry2 (E, F, G). Scale bars represent 10 μm.
Figure 8. Co-transfection with PH-PLC, a protein that binds PI(4,5)P₂, inhibits ASV and HIV-1 Gag budding in a dose-dependent fashion. (A) COS1 cells were transfected with ASV Gag HA (lanes 1–4) or HIV-1 Gag-HA (lanes 5–8) and PH PLC-GFP (PH-PLC:Gag ratio 0, 0.5, and 1.5). (B) DF-1 cells were transfected with ASV Gag-HA (lanes 1–4) and PH-PLC-GFP (PH-PLC:Gag ratio 0, 0.5, 1, and 2). Cells were processed 24 h post-transfection and Western blot analysis of cell lysates and VLP in the media was performed. Graphs show the relative VLP release efficiency normalized to zero PH-PLC. For ASV Gag, VLP efficiency was calculated based on the full-length protein. Bars represent 1 SD.
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 H32A25 and H32R, wherein HIV-1 N-terminal sequences replace residues in the MA domain of ASV Gag, and A24H33 and A24HΔMA, 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, H32A25 Gag-GFP, and H32R Gag-GFP (upper panels); A24H33 and A24HΔMA (lower panels). Between 20 to 200 cells were examined for each construct and the percentages of cells with only punctate GFP signal were: WT (85%), H32A25 Gag-GFP (90%), H32R Gag-GFP (0%), Δp2b Gag-GFP (100%), A24H33 (2%) and A24HΔMA (48%). Scale bars represent 10 μm.
Figure 10. Effect of exchanging N-terminal sequences from ASV Gag and HIV-1 Gag on budding. DF-1 cells were transfected with the indicated chimeric Gag; media and cell lysates were analyzed by Western blot to determine the relative VLP efficiency. (A) Western blot analysis of ASV WT-GFP (lane 1), H32A25 (lane 2), Δp2b-GFP (lane 3) and H32R (lane 4). (B) Western blot analysis of ASV WT-GFP (lane 1), A24H33 (lane 2), ASV-WT-GFP (lane 3), and A24HΔMA (lane 4). The bar graphs illustrate the relative VLP release efficiency normalized to WT; error bars represent 1 SD.
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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