Traffic 2006; **7:** 966–977
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doi: 10.1111/j.1600-0854.2006.00448.x

Foamy Virus Capsid Assembly Occurs at a Pericentriolar Region Through a Cytoplasmic Targeting/Retention Signal in Gag

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Foamy viruses (FV) are unusual retroviruses that differ in many aspects of their life cycle from the orthoretroviruses such as human immunodeficiency virus. Similar to Mason–Pfizer monkey virus (MPMV), FV assemble into capsids intracellularly. The capsids are then transported to a cellular membrane for acquisition of envelope (Env) glycoproteins and budding. However, unlike MPMV, budding of FV is dependent upon the presence of Env. Previous work suggested that FV Env proteins are localized to the endoplasmic reticulum (ER) where budding takes place. However, very little was known about the details of FV assembly. We have used immunofluorescence and electron microscopy to visualize the intracellular location of FV assembly and budding. We have found that, as in the case of MPMV, FV capsids assemble at a pericentriolar site in the cytoplasm. Surprisingly, FV Env is mostly absent from this site and, contrary to expectations, FV capsid structural protein (Gag) is absent from the ER. Gag and Env only co-localize at the *trans*-Golgi network, suggesting that Env–Gag interactions that are required for viral egress from the cell, occurs at this site. Finally, inhibitor studies suggest an important role of microtubule networks for foamy viral assembly and budding.

Key words: centrioles, Env, foamy virus, Gag, MTOC, retrovirus, virus assembly, virus budding

Received 30 November 2005, revised and accepted for publication 5 May 2006, published on-line 5 June 2006

Foamy viruses (FV) are an unusual group of retroviruses that comprise the only genus in the subfamily *Spumaretrovirinae*. Aspects of their life cycle are similar to the major retroviral subfamily, the orthoretroviruses, but in others they are more similar to the Hepadnaviruses such as human hepatitis B virus (HBV). In the case of orthoretroviruses, two distinct assembly pathways have been described [reviewed in Ref. (1)]. In both gammaretroviruses such as murine leukemia virus (MLV) and lentiviruses such as human immunodeficiency virus (HIV), the viral structural proteins (Gag) are targeted to the plasma membrane through a bipartite signal that involves positive charges and a myristylation signal (type C assembly). Particles are assembled at the membrane, and subsequently released. HIV can also bud into late endosomes or multivesicular bodies (MVB) in some cell types such as macrophages and monocytes (2,3). Release of HIV capsids is initiated when a specific motif (PTAP) in the L domain of Gag allows interaction with Tsg101, an ESCRT-1 (endosomal sorting complex required for transport) component (4,5). A second assembly pathway is that of the type B/D retroviruses, such as Mason–Pfizer monkey virus (MPMV). In MPMV, the Gag precursors are directed by a cytoplasmic targeting-retention signal (CTRS) to a pericentriolar region in the cytoplasm where capsid assembly occurs (6). Capsids are then transported to the plasma membrane for budding. Despite the presence of a myristylation signal at the N-terminus of Gag, the CTRS functions as a dominant signal for cytoplasmic assembly of capsids. However, if the CTRS is mutated, the myristylated Gag is targeted to the plasma membrane where assembly can take place in a manner similar to that of the type C retroviruses (7).

Foamy viruses are complex retroviruses that have a replication pathway distinct from the other retroviruses (1). One unusual feature is the reliance on the envelope (Env) glycoproteins for budding. In the absence of Env, intracellular capsids are assembled, but these particles are not released from the cells (8,9). In the case of HIV and other orthoretroviruses, infectious viral particles can be obtained if the Env protein is substituted by surface proteins of other viruses such as vesicular stomatitis virus (VSV), a process called pseudotyping (10,11). However, FV cannot be pseudotyped with heterologous viral glycoproteins, even from other retroviruses (12). This supports the idea of a requirement for a specific interaction of FV Env with Gag for budding. Indeed, sequences at the amino-terminus of the FV Env leader peptide (LP) have been shown to be essential for viral budding (13). A biophysical study has clearly demonstrated a direct and specific binding of LP to the amino-terminal domain of Gag in feline FV (14,15).

Foamy virus use the retroviral B/D type assembly pathway. The Gag protein of the prototype primate foamy virus (PFV) contains a CTRS motif homologous to that of MPMV, but lacks a myristylation signal. Unlike MPMV, FV is completely dependent upon its CTRS for assembly.

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When the CTRS is mutated, Gag is not redirected to the plasma membrane, as found for MPMV, but instead capsid formation is completely abolished (16). The reliance upon envelope glycoproteins for viral budding is reminiscent of the situation for the Hepadnaviruses, which cannot bud from the cell without synthesis of its glycoproteins. HBV is thought to mature by budding into the endoplasmic reticulum (ER)–Golgi compartments and exiting through the cell secretory pathway [reviewed in (17)]. The host protein adaptin, a clathrin adaptor-related protein, interacts with the HBV L glycoprotein, and could be involved in assembly of viral particles and/or export (18).

The main goals of our current study were to determine the location of PFV structural protein (Gag) assembly and the site of Gag interaction with the Env glycoproteins to allow budding. Using immunofluorescence analysis and electron microscopy, we have identified a pericentriolar region as the site of PFV capsid assembly. Our data suggest that PFV Gag is targeted through its CTRS signal to the pericentriolar location for synthesis of capsids, an assembly pathway reminiscent of MPMV. However, MPMV Env is strongly co-localized with Gag at the assembly region and is believed to play an important role in assisting export of capsids out of the area (6). In contrast, we have found that PFV Env is absent from the pericentriolar assembly site, suggesting a separate subcellular compartment required for Gag–Env interaction. Previous work described an endoplasmic reticulum retention signal (ERRS) in the FV Env protein (19–21), a motif also found in the glycoprotein of HBV. It was therefore surprising to find that co-localization of Gag and Env of PFV was not detected in ER or in late endosomes/MVB, but in the *trans*-Golgi network (TGN), suggesting a possible location for envelopment and/or budding of FV capsids. Finally, our inhibitor studies strongly suggest the importance of a functional microtubule network for foamy viral assembly and budding.

Results

The intracellular distributions of PFV Gag and Env proteins differ

During the PFV life cycle, Gag proteins are transiently translocated to the cell nucleus (22,23), although the biological significance of the nuclear Gag remains unclear. Using electron microscopy, immature capsids are primarily found in the cytoplasm, and detection of electron-dense cores in the nucleus or inside the nuclear membrane has not been reported. Thus, it is likely that following nuclear localization, unassembled Gag proteins are exported to the cytoplasm where they assemble into capsids, which are then transported to membrane location(s) for Env association and budding. To better understand intracellular trafficking of the FV structural proteins, we analyzed the subcellular distribution of Gag and Env in PFV-infected HT1080 cells (Figure 1) and BHK-21 cells (data not shown; similar patterns were found in both cell types). Cells were harvested at different times after wild-type (wt) virus infection and subjected to indirect immunofluorescence analysis (IFA) using rabbit polyclonal anti-Gag and mouse monoclonal anti-Env antibodies. The result from IFA reveals different cellular localizations for Gag and Env. Both proteins are first detected 14–16 h post-infection (p.i.). Initially, Gag is found throughout the cytoplasm, with translocation to the nucleus detected by 20 h (Figure 1A,B). In contrast, Env proteins are detected only in the cytoplasm at all times (Figure 1A–E). At late times after infection, large numbers of vacuoles are found in the cytoplasm (from which FV derive their name), and multinucleate syncytia caused by cell fusion are apparent. In the multinucleate cells, it appears that cytoplasmic staining of Gag intensifies whereas nuclear Gag staining decreases (Figure 1C–E).

A distinctive feature of these PFV-infected cells is a transient perinuclear localization of Gag with accumulation in a distinct 'spot' adjacent to the nuclear membrane which we are calling SGA (site of Gag accumulation; white arrows; Figure 1A–C). The SGA is detectable as early as 16–20 h p.i. in approximately 30% of cells, and in 60% of total infected cells at 24–30 h p.i. (data not shown). Once multinucleate syncytia form, Gag staining at the SGA is less apparent (Figure 1D,E). Notably, the SGA does not contain detectable levels of Env protein at any time (white arrows; Figure 1A–C).

Next, Deltavision microscopy was used to identify specific co-localization of Gag and Env in PFV-infected cells. Several Z sections from one typical cell are shown in Figure 2. Each Z section represents a 0.2 μm thick section of the cell whose thickness is approximately 10 μm. Co-localization of Gag and Env is seen in the area outside the SGA, and develops horizontally or apically towards the edge of the cell (Figure 2A,B). However, the co-localization signals are not as extensive as might be expected, given the requirement of Env for budding both at the plasma membrane and intracellular membrane structures. Notably, Env is absent from the SGA (denoted by white arrows). There are small dots of co-localization but given the large amount of Gag and Env staining, these may not be significant.

PFV Gag proteins accumulate near a pericentriolar region

To determine the subcellular site associated with the SGA, cells were co-stained with anti-Gag antibody in conjunction with various subcellular markers or antibodies. We find that the Gag staining at the SGA is detected near the centrioles, as identified by antibody to γ-tubulin (white arrows; Figure 3A). γ-Tubulin is known to be specifically located at the centrioles where it mediates the interaction between microtubules and centrosomes (24). The Gag proteins found at this pericentriolar location could be either in assembled capsids or as free molecules. When PFV-infected cells were treated with nocodazole,

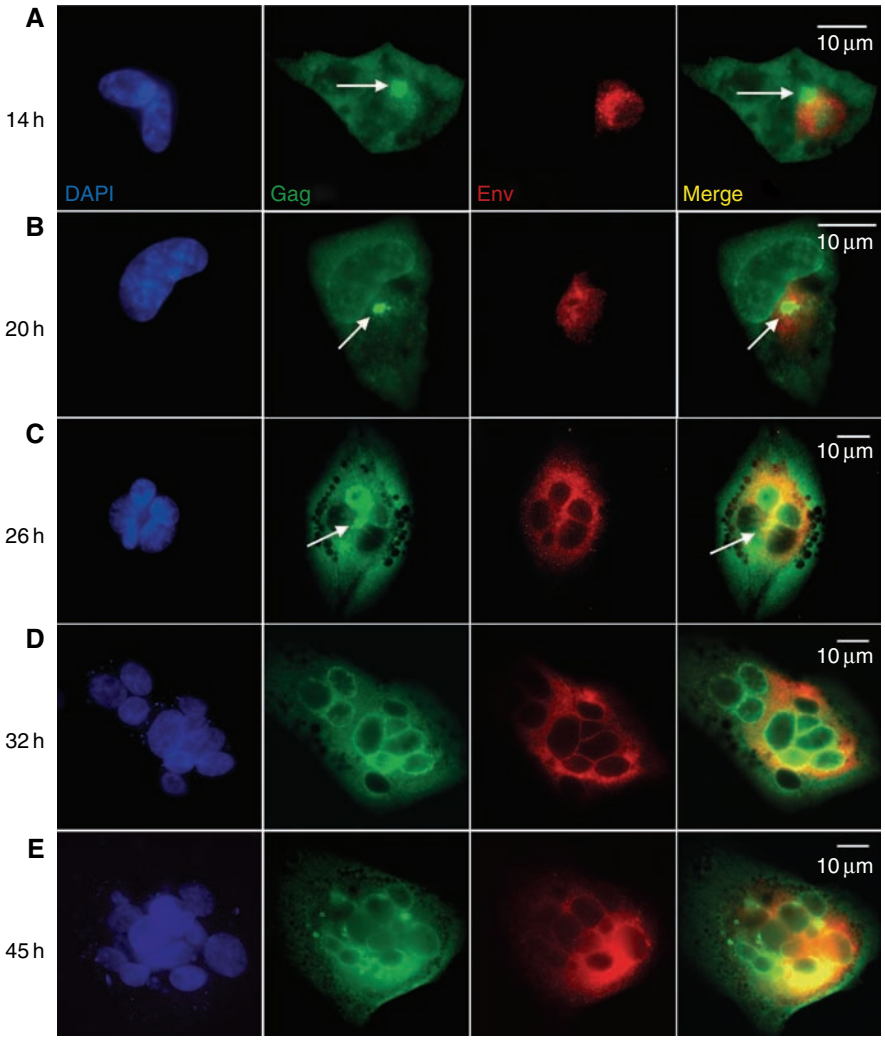

Figure 1: Gag and Env proteins localize differently in PFV-infected cells. HT1080 cells were infected with wt virus at an m.o.i. of 2 and co-stained at different times with rabbit anti-Gag (green) and mouse anti-Env antibody (red). Cell nuclei were labeled with DAPI (blue). Overlapped images from Gag and Env stainings in each panel are shown in the 'Merge' lane. The white arrows indicate the location of the SGA (site of Gag accumulation). All images were captured using a Nikon E800 microscope.

which depolymerizes microtubules (25), very little Gag protein is found near the centrioles (white arrow, Figure 3B), indicating a strong association of the microtubule network with Gag accumulation near the centrioles.

It has been previously reported by Saib et al. (26–28) that microtubules are required early after infection to transport FV particles to the proper intracellular compartment for processing and integration. However, when the cells were infected with a multiplicity of infection (m.o.i.) of as high as 50 wt PFV under our conditions, very little or low accumulation of Gag was found at the centrioles at 4–8 h after infection (data not shown). Additionally, Gag accumulation was readily detected at the pericentriolar area in ΔEnv-transfected cells (data not shown). In this case, intracellular capsid assembly is normal but re-infection cannot occur because the particles do not bud (8). Thus, we believe that what we have detected in this study is pericentriolar accumulation of Gag that occurs during viral assembly.

To determine if the microtubule network plays any significant role in PFV capsid assembly and budding, cells were treated with nocodazole at various times after infection of PFV and left on cells until cell-free supernatants and lysates were harvested at 48 h. Samples were analyzed by Western blot for Gag expression in both cells and extracellular particles. As shown in Figure 4, extracellular viral production is dramatically decreased when either HT1080 or HEL cells were treated with 5 μg/mL nocodazole at 18 h (lanes 3, 7), 24 h (lanes 4, 8), or 30 h (lane 9) after PFV infection, suggesting that viral assembly and/or budding is dependent upon microtubules. Nocodazole treatment does cause a small decrease in Gag synthesis in cells (Figure 4B, lanes 3, 7–9). More importantly, when nocodazole was added to cells at 18 h after infection, only p71 Gag precursor was detected in the lysates (lanes 3, 7). This is a lack of viral assembly that leads to the generation of the p68 Gag cleavage products (29). Our earlier results from time courses have shown that Gag accumulation at the SGA is usually detected around 14–18 h after infection (see Figure 1). Therefore, these results support the idea that Gag trafficking to the SGA is required for particle assembly and demonstrate the importance of a functional microtubule network for PFV viral production. Whether

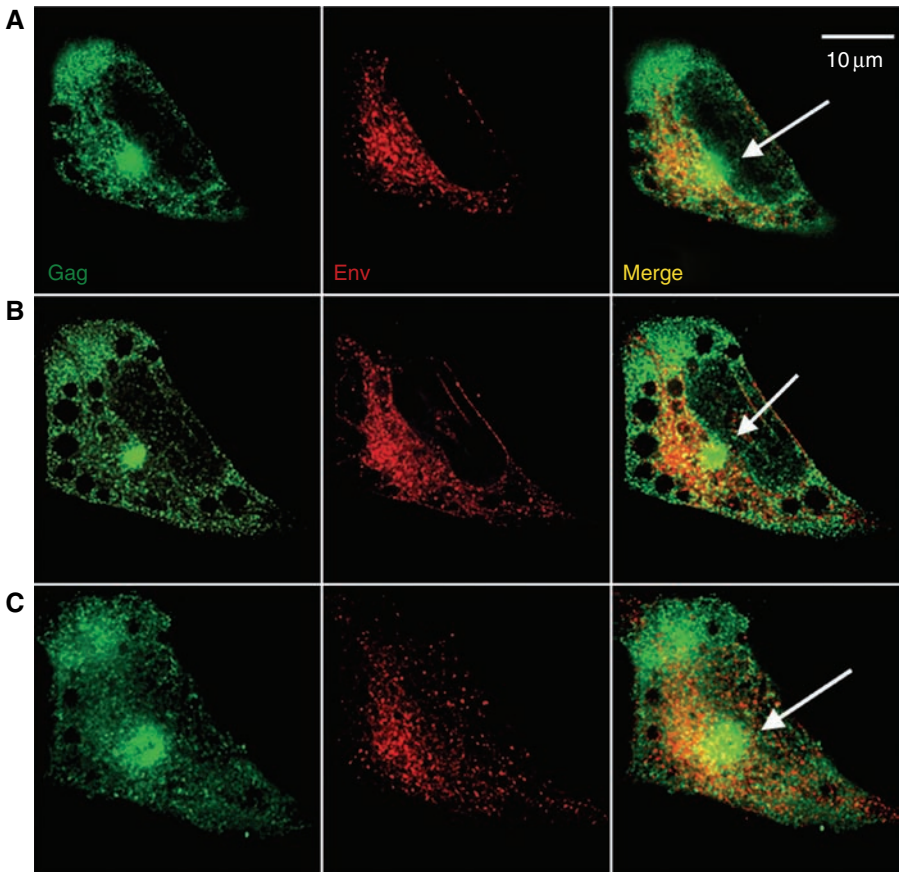

Figure 2: Analysis of the site of co-localization of Gag and Env. HT1080 cells were infected with wt PFV at an m.o.i. 0.5. At 42 h p.i. cells were co-stained using rabbit anti-Gag (green) and mouse anti-Env antibody (red). All images were captured using Deltavision microscopy. Each panel represents a single 0.2 μm/step Z stack from different sections of the cell (A, apical; B, middle; C, basolateral). White arrows indicate the location of SGA.

microtubule movement is required for Gag trafficking to the assembly site, and/or capsid transport to the budding location, remains to be determined.

PFV capsids assemble at a pericentriolar region

To determine if Gag proteins assemble into capsids at the pericentriolar region, we examined PFV-infected cells using transmission electron microscopy (Figure 5). We consistently find large amounts of naked capsids in the area adjacent to one or both of the centriole pairs (indicated by the closed arrows in Figure 5A,C), strongly suggesting that the pericentriolar area is a major site for FV capsid assembly. However, enveloped virions or virions budding at a cell membrane are rarely, if ever, detected at the pericentriolar site. Instead, capsids are seen budding from the plasma membrane (Figure 5D) or into membranous vacuole-like structures (Figure 5E,F). In both of these locations, viral glycoprotein spikes can clearly be seen inserted in the membrane (open arrows in Figure 5D,E). These spikes are not seen when virus lacking Env are examined [data not shown and Ref. (8)]. Although it is reasonable to assume that the enveloped particles found in the vacuole-like structures (Figure 5E,F) are released from cells by fusion of the vacuoles with the plasma membrane, we have never detected such fusion events.

These results are consistent with a requirement for at least two different intracytoplasmic locations for Gag assembly and budding. First, Gag proteins are specifically targeted to the pericentriolar area for capsid assembly, a process probably initiated by an intrinsic signal within the Gag proteins. Subsequently, capsids are transported to either the plasma membrane or intracellular membranous structures where they interact with Env for further budding. The identity of the intracellular organelles for PFV budding is not known, nor is there an explanation for the two different fates of virions.

The CTRS is required for correct Gag trafficking

Mason–Pfizer monkey virus uses the CTRS to direct Gag to a pericentriolar region in the cytoplasm, where they assemble into capsids (6). MPMV Env is found to co-localize with Gag at this pericentriolar site, and is thought to mediate capsid transport out of the assembly site through recycling endosomes (30). The Gag protein of PFV also contains a CTRS motif. When the CTRS is mutated, intracellular assembly of PFV capsid is completely abolished (16). To investigate the functional role of the CTRS in PFV assembly, cells were transfected with plasmids encoding wt PFV or a CTRS(–) mutant and subjected to IFA (Figure 6). Generally, the time courses of Gag expression, distribution, and accumulation at the SGA in

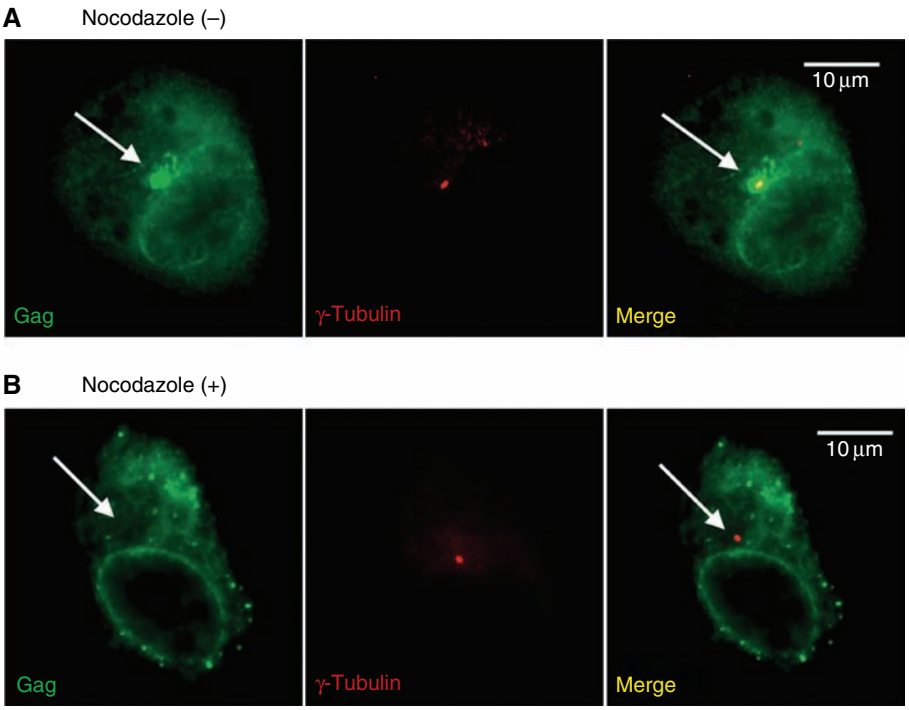

Figure 3: Gag proteins accumulated near the centrioles and the effect of nocodazole on pericentriolar Gag accumulation. PFV-infected HT1080 cells were treated with 5 μg/mL nocodazole at 12 h after infection. At 28 h after infection, cells were co-stained with rabbit anti-Gag antibody (green) and mouse anti-γ-tubulin antibody (red). White arrows indicate the location of centrioles. All images were captured using a Nikon E800 microscope.

the wt-transfected cells (Figure 6A–C) are parallel to those infected with wt virus (as shown in Figure 1). In the case of the CTRS(–) mutant, even at 18 h after transfection Gag is found predominantly in the nucleus (Figure 6D), where it remains at later times (Figure 6E,F). The Gag protein of the CTRS(–) mutant is never detected at the pericentriolar site at any time after transfection. Because the CTRS(–) mutant does not assemble (16), we infer that the CTRS signal of PFV specifically targets Gag to the pericentriolar region for capsid assembly. Interestingly, in the CTRS(–) mutant, much less Env staining is seen at all times after transfection (Figure 6D–F), for reasons that are not yet apparent.

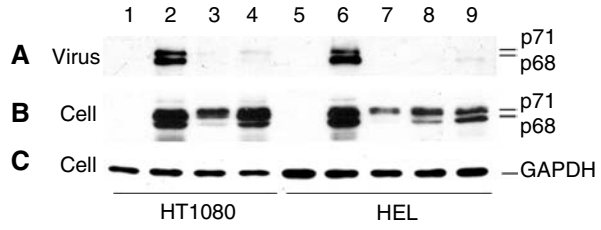

Figure 4: Inhibition of extracellular virus production by nocodazole. HT1080 or HEL cells were mock infected (lanes 1 and 5) or infected with wt PFV (lanes 2–4 and 6–9). At 18 h (lanes 3 and 7), 24 h (lanes 4 and 8), or 30 h (lane 9) after infection, 5 μg/mL nocodazole was added to the cells and left on cells until 48 h after infection. Infected cells in the lanes 2 and 6 were treated with an equal amount of DMSO as in the nocodazole-containing media. At 48 h after infection, culture supernatants were collected and pelleted to concentrate virus particles. Cell lysates were normalized for the level of GAPDH (C) and assayed for intracellular Gag using rabbit anti-Gag by Western blot (B). Normalized levels of culture supernatants were then assayed by Western blot for Gag in viral particles, shown as p71 precursor and p68 cleavage products (A).

PFV Gag and Env proteins co-localize in the TGN but not in the ER

To identify the nature of the membranous structures in which capsids become enveloped and bud, subcellular markers or antibodies were used in conjunction with anti-Gag or anti-Env antibody to detect co-staining. Because PFV Env contains an ER retention signal (19–21), it is logical to speculate that the ER is the site of the Gag–Env interactions required to produce enveloped particles. Thus, it is surprising that Gag is not detected in the ER, using anti-calnexin antibody, an integral ER membrane protein (Figure 7A). In contrast, we find that the Env glycoproteins are strongly associated with the ER (Figure 7B).

Next, PFV-infected cells were co-stained using rabbit anti-Gag and mouse anti-p230 *trans*-Golgi antibodies. p230 is a peripheral membrane protein associated with the cytosolic face of TGN and recycles between the cytosol and TGN (31). As shown in Figure 7C, the Gag antigens, which are detected outside of the centriolar region (SGA) co-localize with p230. Three-dimensional (3-D) reconstructed images from the entire Z stacks of co-stained cells were then used to quantitate the extent of co-localization. The ratio of the area co-stained by both Gag and p230 to the total area stained by each individual antibody indicates the degree of co-localization. Such analyses (using Volocity software) indicate that only 5% of PFV Gag co-localizes with p230, whereas 40% of p230 co-localizes with Gag. This is

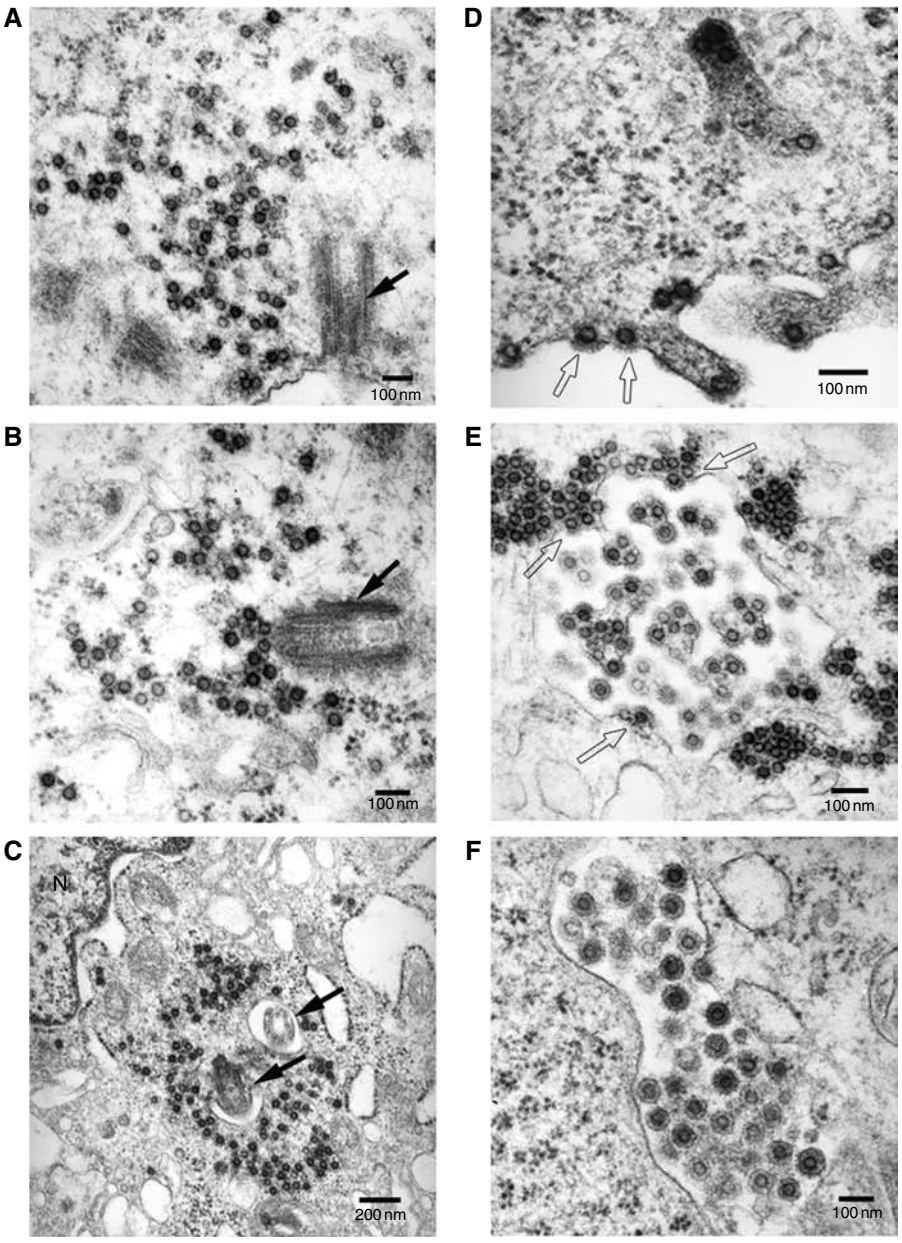

Figure 5: Transmission electron microscopy of PFV-infected cells. (A–C) Pre-assembled capsids are found to accumulate in a pericentriolar area. Closed arrows indicate the centrioles. N denotes the nucleus. (D, E) Naked capsids are shown budding from the plasma membrane (D) or into membranous vacuole-like structures (E). Open arrows indicate virus budding from the membranes. (F) Fully assembled enveloped particles are found in vacuole-like structures.

consistent with the results in Figure 7C, which show very little co-staining of Gag and p230 in the cytoplasm, and that only the Gag located at the border of the SGA co-localizes with p230. As 40% of the p230 overlaps with Gag, this might indicate that Gag is in excess of p230 and that only small amounts of Gag can transit through the TGN at any time. Because there was no rabbit anti-p230 antibody suitable for this study, a tagged TGN marker, TGN38-YFP, was used to co-stain cells with mouse anti-Env antibody. TGN38 is the rat homologue of human TGN46. Both are integral membrane proteins, which recycle between the TGN and plasma membrane via endosomes. At steady state, TGN38 is localized predominantly in the TGN (32). We did not find any gross difference in the localization of TGN38 in cells transfected with the marker alone or with the marker and PFV plasmid (data not shown). Only the Gag proteins surrounding the SGA co-localize with TGN38 (Figure 7D), although Env and TGN38 co-localize to a great extent (Figure 7E). Volocity sofware analyses show that 5% of the total Gag co-localizes with TGN38 and 36% of the total TGN38 marker co-localizes with Gag, as in the case of p230. In contrast, 78% of total Env co-localizes with TGN38-YFP and 44% of total TGN38-YFP overlaps with Env. These results indicate that both Gag and Env are associated with the TGN, especially at the area adjacent to the SGA. However, a greater fraction of Env than of Gag is found in this compartment at steady state.

Co-staining of Gag with early endosomes markers, such as Rab4 and Rab5, is detected, but at a relatively low level

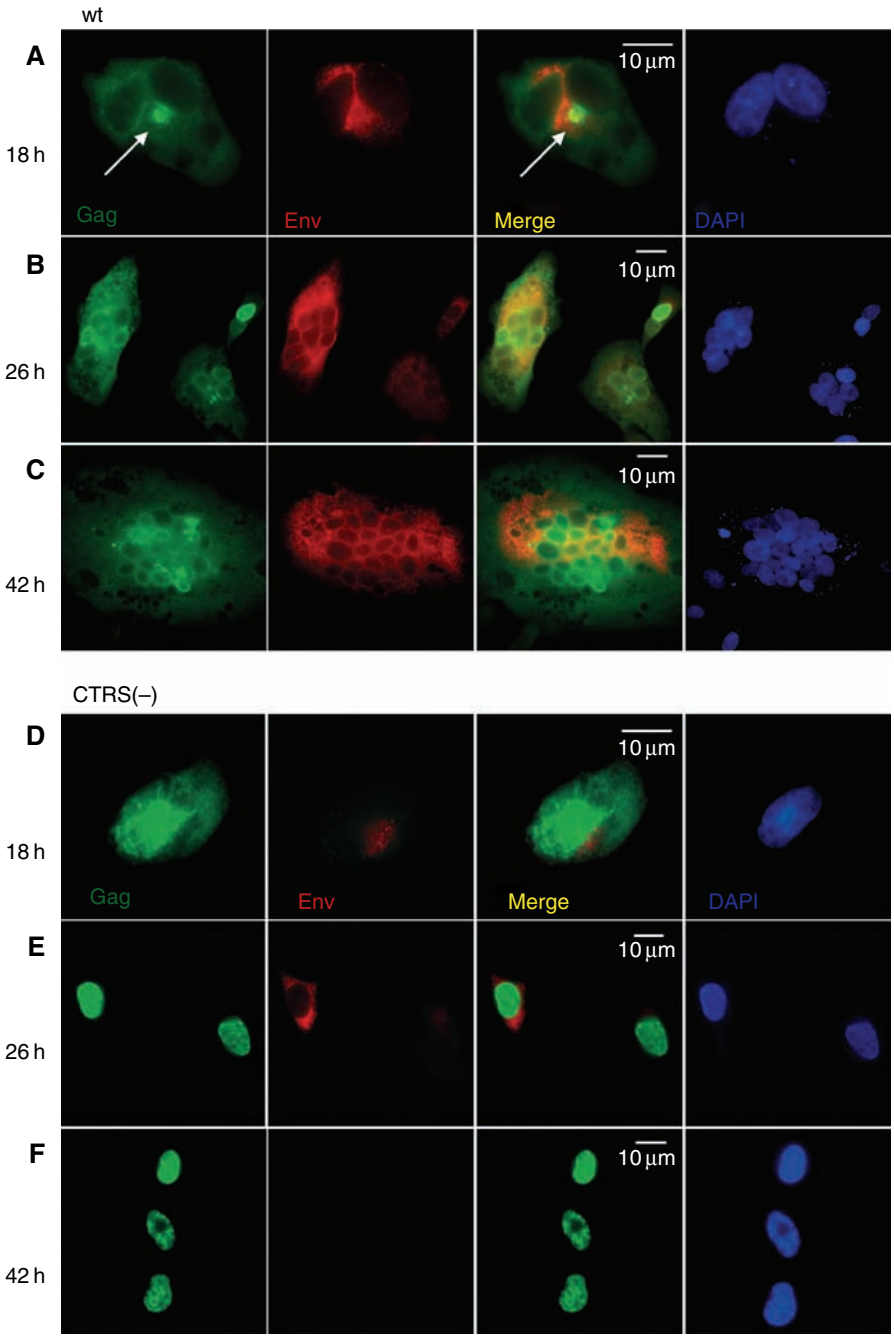

Figure 6: Localization of Gag in cells containing the CTRS(–) mutant or wt plasmids. HT1080 cells were transfected with wt (A–C), or CTRS (–) DNA (D–F). At the indicated times after transfection, cells were stained using rabbit anti-Gag (green) and mouse anti-Env antibody (red). Nuclei were labeled with DAPI (blue). All images were captured using a Nikon E800 microscope.

(Figure 8A,C). There is more extensive Env co-staining with these markers of early endosomes (Figure 8B,D). Very little Gag is localized in lysosomes (Lamp1, Figure 9A) while a good amount of Env is detected (Figure 9B). Very little Gag or Env is seen in the late endosomes/MVBs (Figure 5C–F). The lack of Gag and Env in the MVB is also surprising, as recent work has shown an association of PFV Gag, through its late (L) domain, with TSG101, a component of the vacuolar protein sorting (VPS) machinery of the endocytic pathway (33,34). Thus, these studies provide no direct evidence that PFV uses the MVB pathway for viral egress. Combined with the results shown in Figures 7–9, the only subcellular sites in which we see both Gag and Env localization is the TGN and, to a lesser extent, the early endosomes. It is possible that after assembly at a pericentriolar site, preformed capsids are transported to the Golgi or Golgi-derived vacuoles where capsids associate with viral glycoproteins and subsequently bud.

Discussion

Although many enveloped viruses assemble and bud at the plasma membrane, others assemble intracellularly, often in a specialized compartment where they acquire

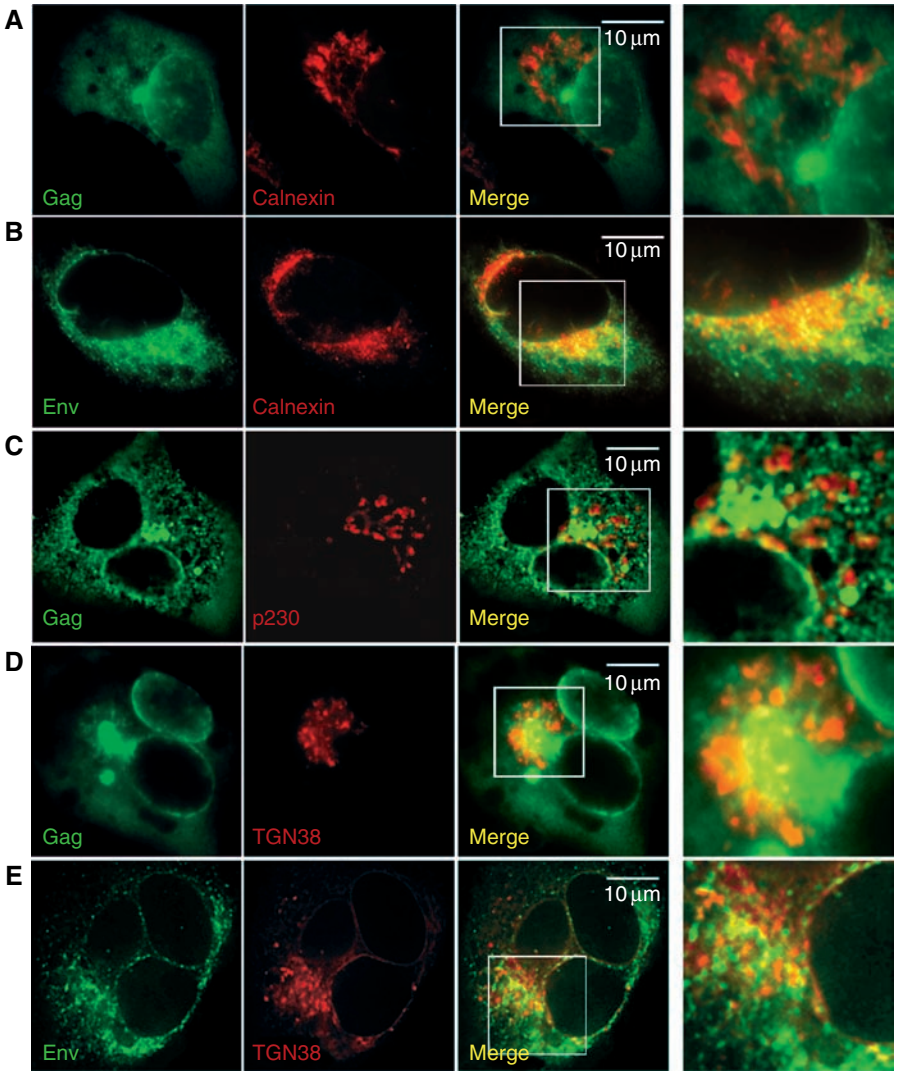

Figure 7: Localization of Gag or Env in the endoplasmic reticulum (ER) or *trans*-Golgi network (TGN). (A, C) PFV-infected HT1080 cells were co-stained using rabbit anti-Gag and mouse monoclonal antibody specific to ER (A; calnexin) or TGN (C; p230). (B) Infected cells were co-stained with mouse anti-Env and rabbit anti-calnexin antibody. (D, E) HT1080 cells were co-transfected with pcHFV and TGN38-YFP DNA. At 28 h after transfection, cells were stained using either rabbit anti-Gag or mouse anti-Env and a secondary antibody conjugated with Alexa Fluor 594. To keep the presentation consistent, Gag or Env staining is indicated by green and subcellular antibodies or markers are indicated by red. The last column in each row shows an enlarged image from the boxed area of each set of merged pictures. All panels represent reconstructed 3-D pictures that were captured using Deltavision SA3.1 microscopy. All images present individual Z sections taken through the center of the cell. Quantitation of marker co-localization in cells stained as in C–E were done as described in *Materials and Methods*.

their envelopes. For example, flaviviruses assemble at the ER (35), coronaviruses assemble at an ER–Golgi intermediate compartment (36), bunyamwera viruses (37) and rubella viruses (38) assemble in the Golgi, and human cytomegaloviruses bud into Golgi-derived vacuole compartments (39). Recent work has focused on understanding the mechanisms used by different viruses to achieve the same goal of producing extracellular particles. Viruses differ in how they exploit normal cellular pathways for their capsid assembly and budding, and how they use the cytoskeletal system for directional transport. For example, a protein encoded by African swine fever virus (ASFV), binds directly to the light chain of dynein, a motor complex involved in minus-end transport (40), to assist transport of the structural proteins to its pericentriolar assembly site. Vaccinia viruses are recognized as cargos by binding to kinesin, a motor protein for plus-end microtubular transport, to facilitate viral exit (41–43).

In this report, we have demonstrated that the major site of PFV capsid assembly is located near the centrioles. This strategy is similar to that described for many DNA viruses, such as ASFV (41,44), iridoviruses (45), vaccinia virus (42,46), and herpes simplex virus type 2 (47), which use

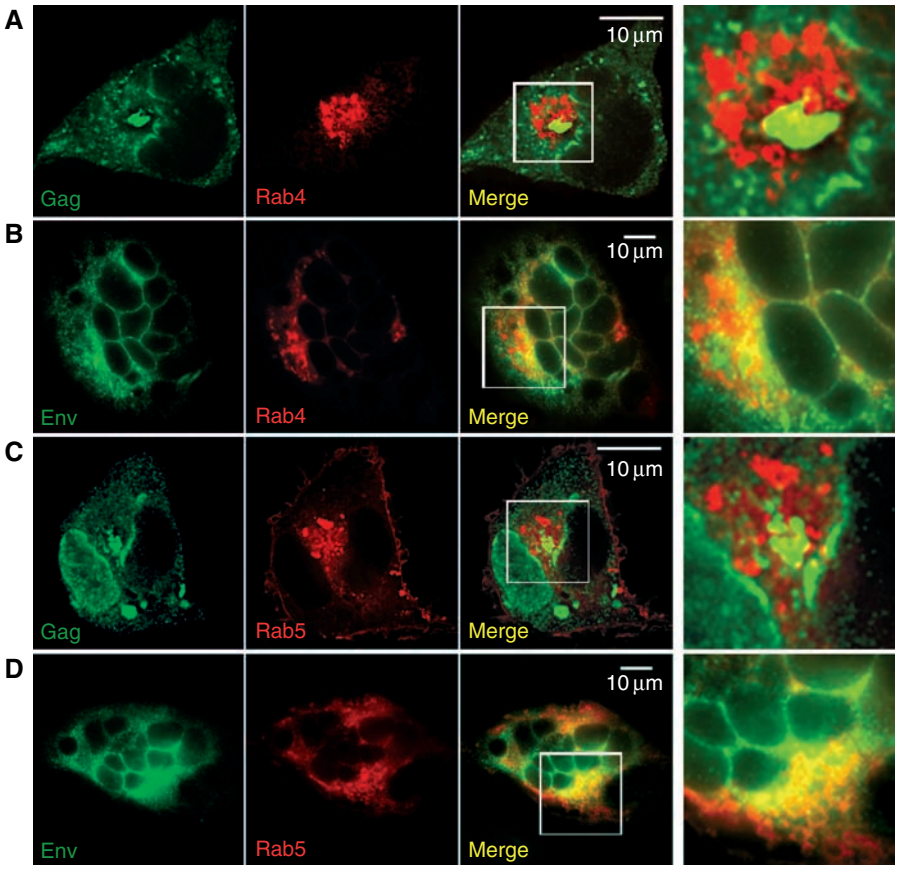

Figure 8: Localization of Gag or Env in the early endosomes. HT1080 cells were co-transfected with pcHFV and YFP-Rab4 or YFP-Rab5 DNA. At 28–42 h after transfection, cells were stained using either rabbit anti-Gag or mouse anti-Env antibody. All panels represent reconstructed 3-D pictures that were captured using Deltavision SA3.1 microscopy. All images present individual Z sections taken through the center of the cell.

a 'virus factory', an aggresome-like structure at a juxtanuclear domain near the centriole. These viruses initiate assembly at the pericentriolar area by recruiting both viral structural proteins and cellular factors to the virus factory. After synthesis, capsids either acquire their envelopes derived from the ER at the same location as in the case of ASFV (48,49), or leave the assembly site and travel to the TGN and endosomes for envelopment, as in the case of vaccinia viruses (50). Enveloped particles are then transported to the cell surface and can exit from cells by exocytosis or after cell lysis, while in other instances remain attached to the cell surface or membrane structures and can only be spread by cell-to-cell contacts. In the case of PFV, viral exit from cells is inefficient and approximately 90% of infectious virus remains cell associated (51).

In our studies, we expected PFV capsids to be transported to the ER, the site, which has previously been suggested to play a role in viral egress. Surprisingly, we found that Gag did not co-localize with Env in the ER. Instead, the only site of co-localization was the TGN and, to a lesser extent, the early endosomes. This is consistent with the fact that deletion of the ER retention signal does not decrease the amount of extracellular infectious viruses (21). Thus, the significance of either the Env ERRS or of the function of the Env proteins which are found in the ER (Figure 5D) is not known.

A second unexpected finding was that little Gag or Env is located in the MVB compartments, comprised of late endosomes. Retroviruses contain L domains which are required for viral egress [reviewed in Ref. (52)]. The PSAP motif in the L domain of PFV Gag can be functionally substituted by heterologous L domains of HIV and other retroviruses (33,34). In HIV, the release of capsids is initiated when the analogous PTAP motif in the Gag p6 protein interacts with Tsg101 (4,53). A separate ubiquitin-binding domain located at the amino-terminus of Tsg101 binds to the PTAP sequence; this binding is enhanced if Gag p6 is ubiquinated (5,54). It is believed that HIV virus particles enter into the lumen of late endosomes/MVBs in some cell types, and that subsequent virus release from cells occurs via the exosomal pathway, in which the MVBs fuse with the plasma membrane. Dominant negative mutants of Tsg101 inhibit HIV release (55). Similarly, a 10–35-fold reduction of PFV particle release was found upon overexpression of dominant negative mutants of Tsg101, as well as Vps4A, or Vps4B, which are components of the ESCRT pathway (33,34). This would imply that the PFV L domain functions by recruiting cellular sorting proteins to move capsids into the MVBs. There is no co-localization of PFV Gag and Env in the late endosomal/MVB compartments in our IFA studies, and very little or no fusion of vesicles containing virions with the plasma membrane detected by electron microscopy.

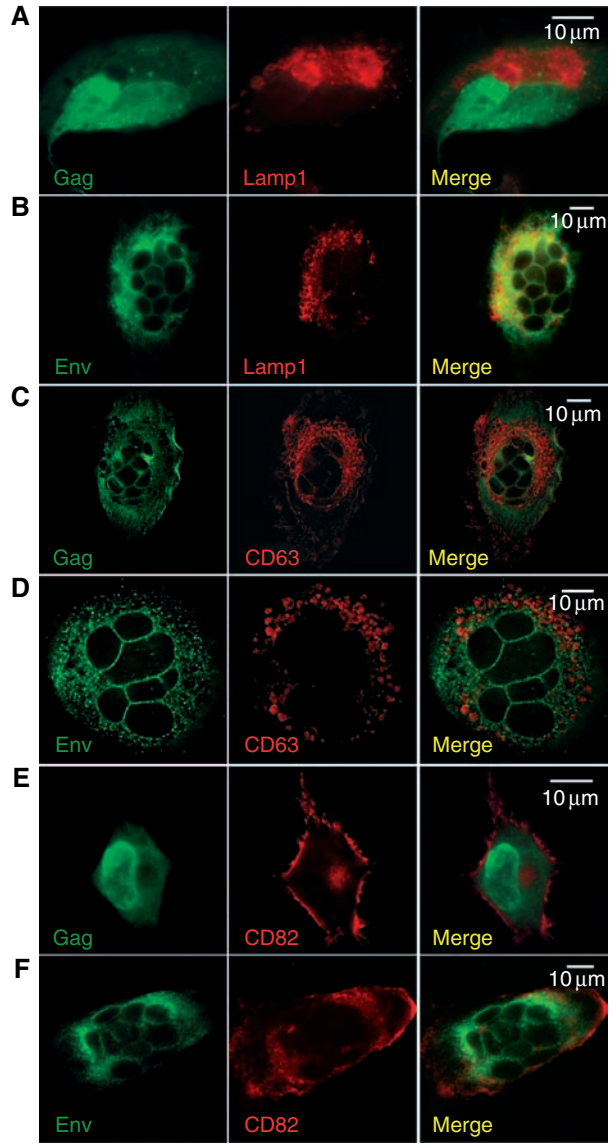

Figure 9: Localization of Gag or Env in the lysosomes or late endosomes. (A, B, E, F) HT1080 cells were co-transfected with pcHFV and Lamp1-YFP or YFP-CD82 DNA. At 28–42 h after transfection, cells were stained using either rabbit anti-Gag or mouse anti-Env antibody. (C, D) PFV-infected HT1080 cells were co-stained using rabbit anti-Gag (C) or mouse anti-Env IgG1 (D) and mouse monoclonal anti-CD63 IgG2a. All images were captured using Deltavision SA3.1 microscopy.

However, it is possible that FV Gag could recruit the ESCRT proteins away from the MVB, to an as yet unidentified site of virus budding.

During MPMV assembly, Env co-localizes with Gag at the pericentriolar assembly site, and aids in the transit of capsids to the plasma membrane. This is not the case for PFV. We find little Env associated with Gag at the SGA. After testing a series of cellular markers, we identified the TGN and, to a lesser extent, early endosomes, as sites where Gag and Env co-localize. The TGN is the major site from which newly synthesized products are sorted to their destinations, including endosomes, secretory vesicles, lysosomes, or the plasma membrane. The Rab proteins are a group of regulatory molecules localized to distinct subsets of membrane structures in the secretory and endocytic pathway (56–58). Rab4 is associated with early endosomes and recycling vesicles and is involved in membrane recycling from early endosomes to the plasma membrane. Rab5 is associated with early endosomes and plasma membranes and is believed to mediate incoming vesicle traffic from the plasma membrane. As both the Golgi and early endosomes are located near the centrioles adjacent to the nucleus, there may be some spatial association of Gag with Rab4 and Rab5. It is possible that after assembly at a pericentriolar site, naked PFV capsids are transported to the TGN where they acquire envelope glycoproteins via budding through the membrane vesicles derived from Golgi. How capsids transit to the plasma membrane is unknown, as we have not detected any vesicles fusing with this membrane. More likely, there is another pathway in which naked capsids are transported to the plasma membrane, possibly via the Golgi-early endosome network mediated by microtubules. However, we do not detect much Env at the plasma membrane, nor do we find enveloped capsids in the cytoplasm, so the actual route is not defined. It should be noted that the antibody we have used was made to the central SU domain of the Env protein and may fail to recognize some conformations of Env.

There are many questions left to be answered about PFV morphogenesis. A major unknown is why Env contains an ER retention signal, and why there is a significant amount of Env protein at this location. While the amino-terminal portion of Env (the leader peptide or LP) interacts with Gag and the LP domain has been mapped (13), the region of Gag with which it interacts is unknown. Antibodies specific to both LP and the amino-terminal portion of Gag may shed light on the location of interaction of Env with assembled capsids.

Materials and Methods

Cells and plasmids

HEL (human embryonic lung fibroblasts), HT1080 (human fibrosarcoma cells), and BHK-21 (baby hamster kidney cells) were cultivated in Dulbecco's modified Eagle's media (DME) supplemented with 10% fetal bovine serum.

pGEX-SU, a bacterial expression vector for PFV Env, was constructed by inserting an 1172-bp *Bsp*EI–*Stu*I fragment of pHSRV13 into pGEX-2T DNA to produce a GST fusion protein containing the central region in the surface (SU) domain of PFV Env. The infectious molecular clone, pcHFV, was used for transfections with wt virus and to generate virus stocks (59). pcHFV-ΔEnv, an Env deletion mutant, and pcHFV-R50W, a CTRS(–) mutant, were described previously (16). The plasmids TGN38-YFP, YFP-Rab4, YFP-Rab5,

Lamp1-YFP, and YFP-CD82 were provided by Walther Mothes (Yale University) (2).

Infection and transfection

For infections, 2.5×10^4 HT1080 cells were plated per well of a 12-well plate, each containing an 18 mm cover slip. The next day, cells were infected with a cell-free virus stock at an m.o.i of 0.5, 2, or 50. Six to eight hours later, cells were washed and incubated with fresh media.

For transfections, 1×10^5 HT1080 cells were plated per well of a 12-well plate and the next day were transfected with 0.7 μg plasmid DNA and 6 μL PolyFect (QIAGEN, Valencia, CA, USA). For co-transfections, 0.6 μg pcHFV DNA was combined with one of the following plasmids: 25 ng TGN38-YFP, 200 ng YFP-Rab4, 200 ng YFP-Rab5, 25 ng Lamp1-YFP, or 200 ng YFP-CD82.

Nocodazole treatment

HT1080 or HEL cells were infected with wt virus at an m.o.i. of 2. At 18, 24, or 30 h after infection, cells were washed and replaced with growth media containing 5 μg/mL nocodazole (Sigma, St Louis, MO, USA) from a 10 mg/mL stock dissolved in DMSO. Mock-treated cultures received the same amount of DMSO. Nocodazole was left on cells until 48 h after infection. Culture supernatants were then collected and filtered to remove cell debris, and pelleted through a 20% sucrose cushion for Western blot analysis as described previously (16).

Antibodies

Mouse monoclonal antibody (IgG1 subtype) against PFV Env was generated using the GST-SU fusion protein described above. This anti-Env antibody was able to react to both the gp130 Env precursor proteins and the gp80 SU cleavage product as detected by Western blot and radioimmunoprecipitation (data not shown). Rabbit polyclonal antiserum specific to the central domain (aa 92–278) of PFV Gag has been previously described (8). Mouse monoclonal anti-γ-tubulin, mouse monoclonal anti-calnexin, and rabbit polyclonal anti-calnexin were purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal p230 *trans*-Golgi was purchased from BD Biosciences (San Jose, CA, USA). Mouse monoclonal anti-CD63 (IgG_{2a} subtype) was purchased from Zymed (South San Francisco, CA, USA). Goat anti-mouse or anti-rabbit secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 were purchased from Molecular Probes (Eugene, OR, USA).

Immunofluorescence microscopy

Infected or transfected cells were fixed with freshly made 3% paraformaldehyde at room temperature (RT) for 15 min, or with methanol at −20 °C for 5 min. Cells were quenched with ammonium chloride and permeabilized with 0.5% TritonX-100 in PBS at RT for 5 min. Samples were blocked with PBS containing 2% bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) and incubated with the primary antibodies at RT for 1 h. After washing, cells were incubated with Alexa Fluor-conjugated secondary antibodies at 1:5000 at RT for 45 min. Samples were then washed in PBS, rinsed with H_2O, and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Fluorescence patterns were visualized using a fixed-stage Nikon Eclipse E800 microscope. 3-D reconstructed images were captured using a Deltavision SA3.1 wide-field deconvolution microscope. Quantitative analysis of co-localization was performed by Volocity software (Improvision Ltd, Conventry, England), using the whole Z stacks (0.2 μm/step) from the stained cells.

Electron microcopy

Approximately 3×10^6 HEL cells or 5×10^6 HT1080 cells were infected with PFV at an m.o.i. of 1. The medium was removed at 42 h after infection and replaced immediately with 1/2 strength Karnovosky's fixative. After incubation for 2 h at RT, cells were scraped from the plate and pelleted in a microcentrifuge tube. The pellet was then resuspended in 1 mL fixative and incubated overnight at 4 °C. The fixed cells were dehydrated, embedded, and stained using standard techniques (51). Thin-sectioned samples were examined using a JEOL 1010 transmission electron microscope.

Acknowledgments

We thank Jon Cooper and Shannon Murray for their critiques of the manuscript. We thank Walter Mothes, Yale University, for generously sharing reagents, and the personnel at the Image Analysis and Electron Microscope facilities for all of their assistance. This research was supported by NIH grant R01 CA 18282 to MLL. SWE was partially supported by T32 CA 09229.

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