Foamy Virus Envelope Glycoprotein Is Sufficient for Particle Budding and Release

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Foamy viruses (FVs) are classified in the family *Retroviridae*, but recent data have shown that they are not conventional retroviruses. Notably, several characteristics of their particle replication strategies are more similar to those of hepatitis B virus (HBV) than those of typical retroviruses. Compared to conventional retroviruses, which require only Gag proteins for budding and release of virus-like particles (VLPs), both FV and HBV require Env proteins. In the case of HBV, Env (S protein) alone is sufficient to form subviral particles (SVPs). Because FVs also depend on Env for budding, we tested whether FV Env alone could produce SVPs. The Env proteins of FV and murine leukemia virus (MuLV) were both released into cell culture supernatants and migrated into isopycnic gradients; however, unlike MuLV Env, FV Env displayed characteristics of SVPs. FV Env particles were of greater density than those of MuLV (1.11 versus 1.07 g/ml, respectively), which strongly suggested that the released proteins of FV Env were particulate. When we examined FV SVPs by immunoelectron microscopy, we found particles that were consistent in morphology, size, and staining with gold beads, similar to FV VLPs and unlike the particle-like structures of MuLV Env, which were more consistent with vesicles produced from nonspecific membrane “blebbing.” Taken together, our results demonstrated that FV Env alone is sufficient for particle budding. This finding is unique among retroviruses and further demonstrated the similarities between FV and HBV.

Glycoproteins of enveloped viruses mediate viral entry into cells by specific receptor binding, membrane fusion, and subsequent release of viral cores into the cytoplasm. Type B and D retroviruses, such as mouse mammary tumor virus and Mason-Pfizer monkey virus, assemble viral capsids in the cytoplasm and later acquire glycoproteins and a lipid envelope during budding at the plasma membrane (PM). Type C retroviruses, such as human immunodeficiency virus type 1, assemble capsids on the inner leaflet of the PM and acquire envelope glycoproteins concurrently with budding. For all retroviruses, the information necessary for the proper formation of virus-like particles (VLPs), through steps such as assembly, membrane targeting and binding, budding, and release, is inherent to the Gag protein, negating the need for other viral proteins in these processes (14). Retroviral envelope proteins, which are required for infecting cells, may enhance particle budding (6, 37) but are not necessary for this process to occur.

Foamy viruses (FVs), or *Spumaviruses*, are classified as B/D-type retroviruses yet remain the least characterized viruses of this family. FVs are complex retroviruses that have typical *gag*, *pol*, and *env* genes as well as the genes for at least two accessory proteins. Human foamy virus (HFV) was originally isolated in 1971 from a nasopharyngeal carcinoma cell line of human origin (1), but because of its high homology to a chimpanzee variant of Simian foamy viruses (19), it is believed to be of chimpanzee origin. Therefore, the nomenclature SFVcpz(hu)

has been used to indicate that HFV is a chimpanzee virus isolated from human cells (27).

FVs are not typical retroviruses but share replication similarities with the reverse transcriptase (RT)-encoding hepadnaviruses, such as hepatitis B virus (HBV) (23, 27). HBV is a DNA virus that is probably evolutionarily linked to retroviruses because both virus families encode RT (41). However, hepadnaviruses utilize RT at a different stage of the viral replication cycle than retroviruses.

The FV envelope protein is a type I membrane glycoprotein with structural characteristics that are similar to those observed in other retroviruses (44); however, the FV glycoprotein also shares similarities with HBV in the behavior of their envelope proteins. For example, the envelope proteins (Envs) of both viruses are localized to the endoplasmic reticulum (ER) because of an inherent ER retrieval signal (ERRS) (17, 21). This localization directs budding of FV and HBV to intracellular membranes. The only exception to this intracellular site of budding is seen with equine foamy virus, for which budding occurs predominantly at the plasma membrane (40). Interestingly, equine foamy virus is the only FV that does not encode for an ERRS in the C terminus of the glycoprotein. For FV and HBV, ER retrieval of Env is not necessary for virion formation in transfected cells (8, 16).

Another interesting envelope characteristic that FV shares with HBV is that both viruses strictly require Env for intracellular and extracellular budding (4, 13); in the absence of envelope glycoproteins, FV Gag proteins are not released extracellularly. This feature is unusual for a retrovirus because all other retroviruses are capable of extracellular particle release by Gag proteins alone. In the case of FV, it is believed that Env provides a critical membrane-targeting function that is inher-

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ently lacking in FV Gag (11). Additionally, the interaction of Gag and Env is critical for budding of particles containing Gag, and this interaction is dependent upon two conserved tryptophan (Trp) residues located within the signal peptide (SP) of Env (26). These results indicate that, unlike other retroviral Envs, the Env protein of FV plays a critical role in budding and release of VLPs.

In the case of HBV, the envelope proteins L and S are necessary for budding of particles containing core proteins (7), and as in FV, N-terminal sequences within L are required for L to interact with HBV core proteins (24). However, it is the S protein that is responsible for driving budding because S, in the absence of all other viral proteins, is sufficient for forming extracellular subviral particles (SVPs) (10). This has been demonstrated by expressing S in a number of different vertebrate cells, which results in the assembly and secretion of morphologically normal SVPs (10, 22, 32, 34, 38).

In general, the organization of SFVcpz(hu) Env is typical of other retroviral glycoproteins; it is synthesized as a precursor molecule which is subsequently cleaved by cellular proteases to yield the mature surface (SU) and transmembrane (TM) subunits (15). Although the SFVcpz(hu) Env is structurally different from the S protein of HBV, because the roles of SFVcpz(hu) and HBV Envs in particle budding and release are similar, we wondered whether the FV Env by itself was capable of forming SVPs. Using transient expression in mammalian cells, we demonstrated that SFVcpz(hu) Env alone, in contrast to the ecotropic murine leukemia virus (MuLV) Env, was capable of forming and budding morphologically distinct particles that were similar to FV VLPs. Our data demonstrated that for SFVcpz(hu), the Env protein, as opposed to the Gag protein in other retroviral systems, is the predominant protein responsible for driving particle budding and release. The observed SVP of FV is unique among retroviruses and further aligns FVs with the hepadnaviruses despite the significant structural differences between these two families of viruses.

MATERIALS AND METHODS

Cells. 293T, COS-1, and BHK-21 cells were grown in complete medium (Dulbecco's modified Eagle medium with 7% fetal bovine serum and antibiotics).

Expression constructs. The eukaryotic expression plasmids pCgp1, pCE-K, and pCE-S all use the human cytomegalovirus (CMV) immediate-early promoter-enhancer to direct protein expression (5). pCgp1 (kindly provided by A. Rethwilm) has been previously described (13). pCE-K and pCE-S were generated from pCMV β (Clontech) by removing the β -galactosidase coding region at the flanking *NotI* sites and replacing it with the entire coding region for Env using the flanking *EcoRI* sites from pHSRV(mod). pCE-S carries Ser residues in place of the three Lys residues located in Env at amino acid positions -3, -4, and -5 relative to the C terminus. These changes abolish the defined ERRS of the protein (17). As described previously, pCME-wt is a CMV immediate-early promoter-enhancer-directed eukaryotic expression vector for the ecotropic MuLV Env protein (35). R572T contains a point mutation that abolishes cleavage of the FV Env precursor, and its construction was previously described (5).

Protein expression. 293T cells were seeded to 5×10^5 to 7.5×10^5 in 35-mm-diameter plates for 18 to 24 h and transfected with Lipofectamine reagent (Gibco-BRL) in Opti-MEM (Gibco-BRL) per the manufacturer's instructions, with no DNA (mock) or with a total of 1.5 μ g of plasmid DNA using 0.75 μ g of pcDNA1.1 (Invitrogen) as empty plasmid in single-protein expression samples (i.e., Env only). For single-protein expression samples, cells were labeled at 12 h posttransfection, and for two-protein expression samples (i.e., Gag and Env), cells were labeled at 30 to 36 h posttransfection. Metabolic labeling was performed for 12 to 20 h with [35 S]methionine-[35 S]cysteine (100 μ Ci/ml). Cell culture supernatant was harvested by filtering through a 0.45- μ m-pore-size syringe filter, and lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM

NaCl, 50 mM Tris [pH 7.4], 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS]) was added at a final concentration of $1 \times$. Cell lysates were prepared in lysis buffer and clarified by centrifugation. Proteins were analyzed by radioimmuno-precipitation assay (RIPA) as previously described (17) using either SFV-positive chimp serum (courtesy of P. Fultz), which detects all major bands of SFVcpz(hu), or goat anti-MuLV antiserum (serum ID #71S000126; NCI/BCB Repository); proteins were then resolved by SDS-9 or 12% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. All experiments were performed by adding 1 μ l of the stated antibody to each RIPA reaction.

For detection of particle-associated proteins, 2.5×10^6 293T cells were seeded in 100-mm-diameter plates 18 to 24 h before transfection. Cells were transfected with 7.5 μ g of DNA using 75 μ l of Lipofectamine reagent and were labeled with 600 μ Ci of 35 S under the conditions described above. The supernatant was filtered through a 0.45- μ m-pore-size syringe filter and separated into two aliquots, and particle-associated proteins were spun through 2 ml of standard phosphate-buffered saline (PBS; pH 7.2) containing 20% sucrose (sucrose cushion) in the presence or absence of 0.5% Nonidet P-40 (NP-40) for 3 h in an SW41Ti rotor (Beckman Instruments) at $130,000 \times g$. The pelleted proteins were resuspended in lysis buffer and subjected to RIPA and SDS-PAGE (12% gel for MuLV proteins and 9% gel for FV proteins), and protein bands were visualized by autoradiography as described above.

For the experiments to determine the effect of Gag on particle release, 10^6 293T cells were seeded in 60-mm-diameter plates 18 to 24 h before transfection. Cells were cotransfected, using Lipofectamine reagent, with either 1.5 μ g of pcDNA1.1 and 1.5 μ g of pCE-K or 1.5 μ g of pCgp1 and 1.5 μ g of pCE-K. At the indicated time points (12 or 30 h) posttransfection, cells were metabolically labeled for 20 h with [35 S]methionine-[35 S]cysteine (100 μ Ci/ml). The supernatant was filtered through a 0.45- μ m-pore-size syringe filter, and particles were spun through a 20% sucrose cushion for 3 h in an SW41Ti rotor (Beckman Instruments) at $130,000 \times g$. The pelleted proteins were resuspended in lysis buffer and subjected to RIPA and SDS-PAGE as described above. Quantitative protein band measurements were made with the Cyclone phosphorimager (Packard Bioscience Co.) and measurements were corrected for the number of Met and Cys residues found in the measured protein.

Isopycnic gradient analysis. MuLV and FV samples were prepared as described above for analysis of particle-associated proteins. However, after particle concentration through 20% sucrose, instead of undergoing the addition of lysis buffer, the FV and MuLV samples were gently resuspended in 400 μ l of standard PBS, pH 7.2, and both samples were layered over the same 10 to 50% step iodixanol (Optiprep; Nycomed Pharma) gradient prepared in a 10-ml centrifuge tube by sequential layering of 2 ml each of 10, 20, 30, 40, and 50% Optiprep solutions (diluted in 0.8% NaCl-10 mM HEPES-NaOH [pH 7.4]). The gradient was spun to equilibrium at $130,000 \times g$ in an SW41Ti rotor for 12 to 18 h. Fractions of equal volume were sequentially removed from the top of the gradient, and for each fraction, the density was determined, lysis buffer was added to a final concentration of $1 \times$, and samples were analyzed by RIPA and SDS-PAGE and visualized by autoradiography. Quantitative protein band measurements were made as described above.

Sucrose gradient analysis. BHK-21 cells (2×10^5) were split into a 100-mm-diameter plate 18 to 24 h before infection. Cells were infected with wild-type (WT) SFVcpz(hu) at a multiplicity of infection of 1.5 for 2 h in serum-free medium. WT FV was obtained by transfection of BHK-21 cells with pHSRV-(mod). Serum-free medium was removed and replaced with complete medium. Cells were incubated for 4 days to establish a productive viral infection followed by metabolic labeling with 900 μ Ci of 35 S under the conditions described above. The supernatant was collected and filtered, and virus was concentrated through a 20% sucrose cushion as described above. The resulting pellet was resuspended in 500 μ l of standard PBS, pH 7.2, and placed on top of a continuous 20 to 50% sucrose gradient prepared in standard PBS. The gradient was spun and analyzed using the same procedure as for the Optiprep gradient described above. Quantitative protein band measurements in each fraction were performed as described above.

Electron microscopy (EM). 293T cells (5×10^5 to 7.5×10^5) were seeded in 35-mm-diameter tissue culture plates 18 to 24 h before transfection. The cells were transiently transfected with plasmid DNA using Lipofectamine reagent as described above.

Immunogold labeling of cells was performed as follows. Cells were washed with cold PBS (pH 7.2) and fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PBS for 30 min. (All incubations and washes were performed at 4°C with rocking.) Cells were washed (two times in cold PBS for 5 min each) and then pre-label blocked with 1% bovine serum albumin (BSA) in PBS for 10 to 30 min. Cells were incubated with primary polyclonal rabbit anti-HFV antiserum (dilution of 1:200 in 1% BSA; kindly provided by A. Saïb) or goat anti-MuLV

antisera (dilution of 1:7,000) for 1 h. Cells were washed (four times for 5 min each with cold 1% BSA) and incubated for 1 to 2 h with either secondary gold-conjugated (particle size, 10 nm) goat anti-rabbit immunoglobulin G (1.0 µg/ml in 1% BSA; EY Laboratories, Inc.) or gold-conjugated (particle size, 10 nm) rabbit anti-goat immunoglobulin G (0.25 µg/ml in 1% BSA; EY Laboratories, Inc.). Cells were washed (four times for 5 min each with cold PBS), postfixed with 1% glutaraldehyde in PBS, pH 7.2, for 30 min, and prepared for ultrathin-section EM. Samples were examined on a Hitachi H-7000 instrument at 75 kV.

For sample preparation of cell-free particles, 3.75×10^6 293T cells were seeded in each of seven 100-mm-diameter plates per sample 18 to 24 h before transfection. A total of 7.5 µg of plasmid DNA was transfected to each plate using Lipofectamine as described above. At 12 h posttransfection, the medium was changed and particles were allowed to collect in the culture supernatant for 24 to 36 h. The supernatant was harvested, filtered, and spun over 2 ml of 50% Optiprep (diluted in 0.8% NaCl–60 mM HEPES-NaOH [pH 7.4]) for 45 min at $100,000 \times g$ in an SW28 rotor (Beckman Instruments). All supernatant was removed by pipetting, except for 2 ml immediately on top of the iodixanol cushion, and the remaining liquids (4 ml total) were mixed. The sample, composed of the particles in a 25% Optiprep solution, was adjusted to volume with 25% Optiprep (diluted in 0.8% NaCl–10 mM HEPES-NaOH [pH 7.4]) and spun to equilibrium for 12 to 18 h at $350,000 \times g$ in a Vti65.2 rotor (Beckman Instruments) to generate a continuous gradient. Aliquots of 400 µl were removed sequentially from the bottom, and densities for each fraction were determined. Fractions having a density of 1.11 to 1.15 g/ml for FV proteins (3) and 1.08 to 1.11 g/ml for MuLV proteins were pooled, diluted with standard PBS (pH 7.2), and spun for 30 min at $135,000 \times g$ in a TLA 100.3 rotor (Beckman Instruments). The final pellet was gently resuspended in 100 µl of PBS (pH 7.2), and 20 µl was used as the sample of concentrated particles for immunogold labeling.

For immunogold labeling of cell-free particles, 20 µl of concentrated particles was applied to a carbon-coated Formvar nickel grid (Electron Microscopy Sciences) for 2 min. Excess sample was removed, and the grid was fixed for 30 min with 2% paraformaldehyde in PBS (pH 7.2). The cell-free particles were immunogold labeled as described for cells except that following the postfix step with 1% glutaraldehyde, samples were negatively stained with 1% uranyl acetate for 15 s before analysis.

RESULTS

FV TM-associated protein is released into cell culture supernatant and is membrane associated. Expression of some retroviral envelope glycoproteins in cell culture results in the release of these proteins into cell culture supernatant. This phenomenon, commonly referred to as “shedding,” results from the dissociation of the SU protein from the membrane-anchored TM protein when the two are not covalently linked following cleavage of the precursor protein (20). The released SU protein is, therefore, soluble and usually not associated with membranes. In order to study the properties of released SFVcpz(hu) Env proteins, we transiently transfected 293T cells with DNA plasmids pCE-K, encoding WT SFVcpz(hu) Env, or pCE-S, encoding ERRS mutant Env and carrying a triple serine (Ser) mutation in place of the WT triple lysines (Lys) located at amino acid positions –3, –4, and –5 relative to the C terminus. SFVcpz(hu) Env was expressed in the presence or absence of Gag and Pol proteins provided by cotransfection of the DNA plasmid pCgp-1. Members of our laboratory had previously demonstrated that FV Envs carrying mutations to the critical residues of the KKXX ERRS motif are transported to and expressed at the PM at higher levels than are WT Envs (17). We used immunoprecipitation analysis experiments to analyze cell lysates and supernatant fractions for protein expression and release. Protein bands in the cell lysates demonstrated that Env and Gag cellular expression levels were approximately equivalent among samples (Fig. 1A, lanes 2 to 5). The supernatant fractions demonstrated that, as previously reported (4, 13), Gag is not released extracellularly in the

absence of Env (Fig. 1A, lane 7). As expected, there was more Gag and Env released from cells cotransfected with ERRS mutant Env than from cells cotransfected with WT Env (Fig. 1A, compare lanes 8 and 9). The dominant Gag protein released into the supernatant was the precursor (p74), although both p74 and the terminally cleaved product (p70) were seen upon longer exposure of the gel (data not shown). This observation is similar to prior results and may represent less than optimal proteolytic processing in our *in vitro* expression system (35). Interestingly, expression of Env alone resulted in the secretion of four protein bands into the supernatant (Fig. 1A, lane 10). In addition to the release of SU and a small amount of precursor protein, the membrane-associated TM component was also present, and the amount of TM released appeared to be comparable to the amount of SU released. This result suggested that FV Env released into the culture supernatant was not from shedding because of the unusual amount of TM that was also released.

We also detected the presence of an unknown protein of about 45 kDa (Fig. 1A, lanes 9, 10, 14, and 15). Interestingly, this protein appears to be a cellular protein that is loosely associated with the FV Env, as it was present in the mock-infected cell lane as well (Fig. 1A, lane 1). However, it was only present in the supernatant and viral pellets of the cells transfected with pCE-K and pCE-S (Fig. 1A, compare lane 6 to lanes 9, 10, 14, and 15). The identity of this 45-kDa protein awaits further studies.

To be certain that these findings were not cell specific, we also performed identical experiments using COS-1 cells (Fig. 1D), with similar results. We also wanted to rule out the possibility that cellular membrane fragmentation contributed to the presence of extracellular FV Env. We therefore transfected 293T cells with a plasmid encoding a cleavage-defective FV Env (R572T) (5). Expression of R572T in 293T cells is not associated with syncytium formation (data not shown) but did result in the extracellular release of the uncleaved Env precursor protein (Fig. 1E, lane 2), demonstrating that SVP release was not due to membrane fragmentation.

To determine whether proteins released into the cell culture supernatant were particulate, we concentrated and purified extracellular proteins through standard PBS containing 20% sucrose (sucrose cushion) and analyzed the resulting pellets. VLPs, consisting of Gag and Env, migrated through the sucrose cushion as expected (Fig. 1A, lanes 13 and 14). Surprisingly, Env alone (SU and TM components) also migrated through a sucrose cushion (Fig. 1A, lane 15). The 45-kDa protein species did not migrate through the sucrose cushion, indicating that this protein was soluble and was not associated with the higher-molecular-size protein complexes. Because mutation of the ERRS did not disrupt Env's ability to form VLPs and because ERRS mutant Env was released extracellularly more often than was WT Env, we used ERRS mutant Env for all subsequent experiments, except where indicated.

To compare the release of FV Env proteins to that of another retrovirus, we used the MuLV envelope glycoprotein expressed from pCME-wt (35). Expression of MuLV Env from 293T cells produced an 80-kDa precursor protein (gp80) which was cleaved into a 70-kDa SU protein (gp70) and a 15-kDa TM protein (p15E; Fig. 1B, lane 2). In the cell culture supernatant fraction, there was a large amount of extracellular gp70 and a

FIG. 1. Autoradiography of SFVcpz(hu) and MuLV proteins from transiently transfected 293T cells. (A) SFVcpz(hu) proteins. Results for cell lysates, supernatants, and viral pellets from cells transfected with no DNA (mock) or vectors expressing SFVcpz(hu) Gag and Pol (pCgpl) alone, Gag and Pol with WT Env (pCE-K), Gag and Pol with an ERRS mutant Env (pCE-S), or ERRS mutant Env alone and immunoprecipitated with chimp serum are shown. Viral pellets were centrifuged through 20% sucrose. (B) MuLV proteins. Results for cell lysates and supernatants from cells transfected with no DNA (mock) or a vector expressing MuLV Env (pCME-wt) and immunoprecipitated with goat anti-MuLV antisera are shown. (C) NP-40 sensitivity of MuLV and SFVcpz(hu) Env complexes collected from the supernatant of cells transfected with pCME-wt or pCE-S, spun through 20% sucrose, and immunoprecipitated with goat anti-MuLV antiserum or chimp serum, respectively. (D) Viral pellets from COS-1 cells transfected with pCE-K or pCE-S. (E) Cell supernatants from 293T cells transfected with pCE-K or pCE-K/Clv (pCE-K containing the cleavage mutant R572T). PRE, precursor envelope glycoprotein (gp).

faint band of p15E. We also detected a band of approximately 38 kDa, which may represent a product of nonspecific MuLV Env cleavage (Fig. 1B, lane 4). The large amount of SU compared to the small amount of TM released suggested that MuLV Env was released because of shedding. However, when we took into account the number of radioactive methionine (Met) and cysteine (Cys) residues within SU and TM for both FV and MuLV, it was possible that the release ratio of SU to TM was actually the same for both viruses.

To further characterize the nature of MuLV and FV supernatant Env proteins, we repeated the 20% sucrose cushion experiment in the absence or presence of NP-40. NP-40 is a

nonionic detergent that dissolves lipid vesicles but does not usually dissociate oligomeric protein complexes (43). For MuLV Env, a protein species consisting of gp70 and p15E was pelletable through 20% sucrose (Fig. 1C, lane 1). Again, this MuLV Env population appeared to have a higher SU-to-TM ratio than the FV proteins (Fig. 1C, compare lanes 1 and 3). When we added 0.5% NP-40 to the sucrose cushions, the majority of the bands disappeared for both MuLV Env and FV Env samples (Fig. 1C, lanes 2 and 4), indicating that the protein species capable of migrating through 20% sucrose were lipid associated.

The sum of these data indicated that, for both MuLV and

FIG. 2. Comparison of densities of MuLV Env complexes and SFVcpz(hu) Env complexes from Optiprep gradient. The Env complexes from cell culture supernatants were collected from 293T cells transiently transfected with pCME-wt or pCE-S, concentrated through 20% sucrose, layered over the same Optiprep gradient, and spun to equilibrium. Sequential aliquots were removed from the top and immunoprecipitated, first with goat anti-MuLV antiserum and second

with chimp serum. (A) Autoradiographies of gradient fractions (numbered sequentially above each lane) revealing banding pattern of MuLV Env proteins (top panel) and SFVcpz(hu) Env proteins (bottom panel). Densities (grams per milliliter) were determined for the indicated fractions and appear over the corresponding fraction numbers. (B) Quantitative protein measurements of SU and TM proteins from each gradient fraction made by phosphorimaging analysis and plotted as digital light units (DLU).

FV, there were at least two populations of Env that were released from 293T cells. One population was soluble, and the other population consisted of protein complexes that migrated through 20% sucrose and were NP-40 sensitive, suggesting that these complexes represented some form of membrane vesicle. It was difficult to determine the nature of these particles from the initial assays, particularly with regard to the actual ratios of SU to TM between the two viruses. Therefore, we decided to perform isopycnic gradient analysis with quantitative band measurements of both MuLV and FV Env complexes.

Unlike MuLV Env vesicles, FV Env vesicles display characteristics of true particles. Although sensitivity to NP-40 is indicative of lipid association, it is not definitive. Therefore, we wanted to determine the density of the MuLV and FV Env protein complexes in an isopycnic equilibrium gradient. MuLV and FV Env proteins were transiently expressed in 293T cells, concentrated, and layered on top of the same 10 to 50% iodixanol (Optiprep) gradient as that described in Materials and Methods. Fractions from the gradient were immunoprecipitated in two different steps, first with goat anti-MuLV antiserum and second with chimp serum, which detects the major protein species of SFVcpz(hu). Thus, from the same gradient, we could determine where MuLV and FV Env proteins banded relative to each other.

Using this method, the MuLV complexes were predominantly concentrated at fractions with densities of 1.07 and 1.09 g/ml (Fig. 2A, top panel), whereas FV Env complexes were predominantly concentrated at fractions with densities of 1.11 and 1.12 g/ml (Fig. 2A, bottom panel). The densities of the FV Env complexes were less than the densities of SFVcpz(hu) virions, which range from 1.13 to 1.17 g/ml (data not shown). We would expect FV Env particles to be somewhat lighter than virions because they lack Gag cores.

Using a phosphorimager, we also measured the intensity of the protein bands in each fraction for the MuLV and FV samples (Fig. 2B). MuLV Env protein concentration peaked at a density of 1.07 g/ml, whereas FV Env protein concentration peaked at a density of 1.12 g/ml. Interestingly, in the fractions where bands were present, there were approximately equivalent amounts of MuLV TM and SU, whereas for FV Env, there was significantly more TM present than SU. These data demonstrated that although both MuLV Env and FV Env are released into the supernatant as lipid-associated vesicles, the properties of these vesicles were different. The density of FV Env vesicles was much closer to the density of true particles (virions) than was the density of MuLV Env vesicles. Additionally, there were more membrane-associated TM proteins in the FV Env vesicles than extracellular SU proteins. Taken together, these results strongly suggested that vesicles from extracellular release of FV Env were SVPs.

FIG. 3. Electron micrographs of immunogold-labeled 293T cells (A to H) and cell-free, Optiprep-purified Env particles (I to O). 293T cells were transiently transfected with no DNA (A), pCE-S (B to E), pCE-S and pCgp1 (F), or pCME-wt (G and H), incubated with rabbit anti-HFV antiserum for FV proteins or goat anti-MuLV antiserum for MuLV proteins, incubated with appropriate immunogold (10-nm particle size)-labeled secondary antibody, fixed, and thin sectioned. Cell-free Env particles were collected from 293T cells transiently transfected with empty plasmid (I), pCE-S (J to M), pCE-S and pCgp1 (N), or pCME-wt (O), concentrated on a 50% Optiprep cushion, and purified through an Optiprep gradient. The particles were collected from the appropriate fractions, concentrated, and resuspended in 100 μ l of PBS. Twenty microliters of sample was applied to a nickel grid, incubated with primary antiserum, incubated with appropriate immunogold-labeled secondary antibody as described above, and negatively stained with 1% uranyl acetate. Magnification: panels A to D and F to H, 30,000; panels E and I to O, 70,000. Bars, 100 nm.

FV SVPs are similar in size and morphology to FV VLPs. In order to determine the morphology and size of the FV SVPs, we performed immuno-EM studies using immunogold labeling experiments. Thin-section EM analysis of 293T cells showed more intense gold labeling of cell surfaces that were transfected with DNA encoding FV Env than of cell surfaces that were mock transfected (Fig. 3, compare panels B and C to

panel A). In the FV Env-transfected cells, we observed specific labeling of structures at the cell surface that resembled budding particles (Fig. 3B and C). We also found extracellular particles that were specifically gold labeled associated with cells (Fig. 3D and E). The size of these cell-associated particles was approximately 100 nm, similar to the size of FV virions (23).

TABLE 1. Analysis of variations in size and bead count of particles from EM studies

Particle sample	Particle size (nm) ^a	No. of beads/particle	n
FV SVPs	125.78 ± 13.96	65.22 ± 23.39	18
FV VLPs	137.33 ± 10.51	66.14 ± 16.22	21
MuLV vesicles	129.60 ± 29.16	15.00 ± 5.48	7

^a All data are means ± standard deviations.

For comparison, we also examined 293T cells transfected with FV Gag and Env. VLPs could be easily distinguished from SVPs because VLPs had prominent cores (Fig. 3F). Like SVPs, VLPs were specifically stained with gold particles, and they were similar in size and morphology to SVPs. For the MuLV Env-transfected cells, we did not observe any structures budding from the cell membrane that resembled particles but did find rare particle-like objects that were gold labeled (Fig. 3H). However, these objects occurred infrequently and were not uniform in size or shape; therefore, it was likely that these structures simply represented spurious extrusions produced from MuLV Env-transfected cells. Consistent with this observation is the fact that the amount of labeling on these particles was not as abundant as the amount of labeling on the FV particles (Fig. 3, compare panel H to panels D and E).

We next wanted to demonstrate that FV SVPs could be harvested and concentrated from cell culture supernatants and visualized by EM. 293T cells were transiently transfected with pCE-S; extracellular particles were collected, concentrated, and partially purified through an Optiprep gradient. The gradient was fractionated, fractions with densities of 1.11 to 1.15 g/ml, which were expected to contain the FV SVPs, were pooled, and then the SVPs were further concentrated by centrifugation. After the pellet was resuspended in PBS, the sample was applied to a nickel grid, immunogold labeled, and stained with 1% uranyl acetate before examination. For comparison to FV SVPs, we also collected the supernatants of cells that were mock transfected with an empty plasmid or cotransfected with pCgp1 and pCE-S to produce VLPs or pCME-wt to produce MuLV Env vesicles. The results demonstrated that FV SVPs could be harvested and concentrated from cell culture supernatants and that they appeared as round, morphologically distinct structures that were specifically and evenly labeled with gold beads (Fig. 3J to M). These structures were not present in the concentrated supernatant of mock-transfected cells (Fig. 3I), indicating that the SVPs were produced specifically in the presence of FV Env. As in the samples that were thin sectioned, the SVPs were similar in morphology and size to VLPs (Fig. 3N). We could not find any structures that were uniformly round and resembled particles from MuLV Env purified samples. We observed, instead, uneven clumping of beads around amorphous material of different sizes that occurred infrequently (Fig. 3O).

From the thin-sectioned and cell-free samples, we calculated the average size and number of gold beads for each of the different types of particles (Table 1) that were observed. The average size for all three types of particles ranged from 125 to 138 nm, but the MuLV Env vesicles had more variability in size than FV SVPs or VLPs. Additionally, the MuLV Env vesicles

were typically decorated with about four times fewer gold beads than FV SVPs or VLPs. The infrequency at which the MuLV Env vesicles appeared only allowed us to tabulate these results from a few particles, whereas a large number of particles could be used from the SVP and VLP samples.

In summary, the results of the EM data indicated that even though we were able to recover particle-like structures from the MuLV samples, they were different from FV particles because the MuLV vesicles were few in number, not uniform in size or shape, and unevenly and sparsely labeled with gold beads. The properties of the MuLV vesicles were more consistent with products of nonspecific membrane “blebbing.” In contrast, FV SVPs were uniform in shape, size, and gold labeling, similar to FV VLPs. We were also able to recover morphologically similar SVPs from cells transfected with WT FV Env (data not shown), indicating that it was not the presence of the ERRS mutations that conferred the ability to produce SVPs, such as by increased cell surface expression. Our ability to recover and visualize particles from FV Env-expressing cells by EM further demonstrated that, unlike MuLV Env, FV Env formed SVPs.

FV Env is released efficiently from cells in the presence or absence of Gag. Eastman and Linial (11) reported that if SFVcpz(hu) Gag is engineered with a myristoylation signal, then it can be released from cells in the absence of Env, suggesting that despite lacking a PM targeting signal, Gag, too, contains an inherent ability to bud. Therefore, we wanted to investigate whether Gag could stimulate the release of Env. We transiently transfected 293T cells with the plasmid vector coding for WT FV Env, pCE-K, in the absence or presence of FV Gag and Pol encoded by the vector pCgp1. Cells were metabolically labeled for 20 h starting at either 12 or 30 h posttransfection and were lysed; culture supernatants were collected, filtered, and spun over 20% sucrose cushions. The resulting lysates and pellets were immunoprecipitated with chimp serum and analyzed by SDS-PAGE and autoradiography. In order to quantitate and compare the amount of Env released into the supernatant from each of the different transfections, we used a phosphorimager to measure the Env bands.

From representative experiments, protein expression of Env and Gag was approximately equal at 12 and 30 h (Fig. 4A, lanes 2, 3, 5, and 6), and at 12 h posttransfection, Env release was not affected by the presence of Gag (Fig. 4B, compare lanes 1 and 2). This result was confirmed by quantitating the Env bands (Fig. 4C). However, at 30 h posttransfection, Env release increased in the presence of Gag (Fig. 4B, compare lanes 3 and 4), and quantitative measurements of the Env bands determined that the amount of the increase was approximately twofold (Fig. 4C). Results from repetitive experiments were similar except that the amount of Env released at 30 h in the presence of Gag was increased as much as threefold in one experiment (data not shown). Although Gag stimulated the release of Env, this stimulation was not excessive, indicating that Env could be released on its own.

No evidence of SVP in FV-infected cells. For HBV, SVPs are produced in vast excess over virions. These 22-nm-diameter empty particles are easily distinguishable from the 42-nm-diameter infectious Dane particles by size alone (33). To date, there have been no descriptions of SVPs in FV infection, perhaps because FV SVPs are similar in size and density to

FIG. 4. Expression and release of WT SFVcpz(hu) Env in the absence or presence of FV Gag and Pol. Autoradiographies demonstrating cellular expression levels (A) and 20% sucrose pellets of cell culture supernatants (B) collected from 293T cells transiently transfected with pCE-K alone or with pCgp1 are shown. (C) Released Env complexes in 20% sucrose pellets, as shown in panel B, quantified by phosphorimaging analysis and displayed as digital light units (DLUs).

virions. However, because our data demonstrated that FV SVPs peak at a slightly lighter density than virions, we wanted to determine whether we could detect SVPs in infected cells. We infected BHK-21 cells with WT SFVcpz(hu), and concentrated particles were spun to equilibrium in a 20 to 50% continuous sucrose gradient (Fig. 5A). The gradient was analyzed as described above, and quantitative measurements of Gag and Env concentrations in each fraction were made with a phosphorimager (Fig. 5B). Measurements of protein bands from each fraction demonstrated that virions, as determined by the presence of Gag bands, peaked at a density of 1.18 g/ml, which is similar to what has previously been reported (47). Interestingly, two Env peak concentrations were noted, one at 1.18 g/ml, which corresponded with the Gag peak at 1.18 g/ml, and the second at 1.15 g/ml. Although this latter peak constituted the majority of the Env proteins, they were still associated with Gag particles. Since FV Gag particles are not released in the absence of FV Env, these findings suggest that SVP formation does not occur during FV infection.

DISCUSSION

In general, budding of enveloped viruses follows one of the following three paradigms: (i) requirement of core proteins only, (ii) requirement of core and Env proteins, or (iii) requirement of Env proteins only. In the first model, core proteins by themselves can assemble and bud from cells as particles. The retroviruses are a group of viruses that follow this model because expression of Gag protein alone can direct particle envelopment and release (14, 36). In the second model, both core and Env proteins are required, and it is their direct interaction that drives the budding process. Viruses that follow this example include the rhabdoviruses (31) and togaviruses. In the third model, budding is independent of core proteins, and expression of Env proteins alone is sufficient for budding of VLPs. HBV (7), coronaviruses (28, 42), and flaviviruses (2) are examples of this model. Unlike other retroviruses, FVs cannot form extracellular particles solely by the expression of Gag but require the presence of Env (4, 13). This observation indicates that FV Env plays an important and critical role in budding and release of virus particles. Our results indicate that Env alone is sufficient for particle budding and release, which categorizes FVs in the third model of viral budding.

The observed SVPs of FV were different from the unusual vesicles that were recovered from the MuLV-transfected cells. Indeed, the MuLV Env vesicles we observed were reminiscent of the simian immunodeficiency virus (SIV) Env vesicles described by Vzorov and Compans (43). These lipid-associated particles were produced from SIV Env proteins with severely truncated cytoplasmic tails (lengths of 3 and 39 amino acids compared with 185 amino acids for the full-length tail). The authors speculated that truncating the extensive cytoplasmic tail allowed SIV Env to associate more compactly at the PM and enhanced the ability of the proteins to be released in membrane-bound form. Because MuLV Env also has a short cytoplasmic tail (approximately 32 amino acids), a similar phenomenon could be taking place. We believe, however, that this phenomenon was inefficient and differed from SVP formation because, in contrast to FV SVPs, it was difficult to find MuLV vesicles by EM.

FIG. 5. Detection of SVPs in SFVcpz(hu)-infected cells by sucrose density gradient analysis of particles collected from infected BHK-21 cells. (A) Autoradiography of sucrose gradient. BHK-21 cells were infected with SFVcpz(hu) at a multiplicity of infection of 1.5, and particles were collected from the culture supernatant at 5 days postinfection. The SFVcpz(hu) particles were concentrated through a 20% sucrose cushion, layered over a 20 to 50% continuous sucrose gradient, and spun to equilibrium. Sequential aliquots were removed from the top and immuno-precipitated with chimp serum. Densities (grams per milliliter) of the indicated fractions appear over the corresponding fraction numbers. (B) Quantitation by phosphorimager of Env (SU and TM) and Gag proteins from each fraction displayed as digital light units (DLUs).

Pseudotyping of human endogenous retroviruses (HERV) with FV Env could not explain our findings of FV SVPs for several reasons. Although 293T cells were shown to express HERV mRNA, they do not express the resultant protein because the mRNA contains multiple stop codons in all three reading frames (12). Similar findings were demonstrated with COS-1 cells (Fig. 1D), which do not contain HERV sequences and are routinely used because of the lack of these sequences

(18, 29). We have looked by thin-section and negative-staining EM and have never identified the presence of core particles when FV Env is expressed in the absence of FV Gag. Finally, despite extensive effort, it is extremely difficult to use FV Env to pseudotype a heterologous Gag and the use of a chimeric Env was required in order for efficient pseudotyping to occur (25).

One of the functions of FV Env, as suggested by Eastman

and Linial (11), is to target particles to membranes, a function that is lacking in FV Gag, and this deficiency explains why Gag alone cannot bud. Interestingly, if Gag is supplied with the N-terminal Src myristoylation signal for membrane targeting, then Gag becomes budding competent, indicating that despite the lack of a transport signal, FV Gag contains all other inherent abilities required for forming particles and budding. In light of our data, we suggest that, in addition to targeting Gag cores to membranes, Env is also inherently involved in forming and budding particles, because expression of Env alone was sufficient for producing extracellular particles. For rabies virus, interactions between TM spike proteins and core proteins induce the budding process. However, rabies virus particles can bud in the absence of the G glycoprotein (31). In the presence of G protein, the efficiency of budding is increased approximately 30-fold. In this viral system, both envelope and core proteins are required for particle formation because the concerted action of the two ensures efficient budding. This does not appear to be the case for SFVcpz(hu) because in the presence of Gag, Env was released with an efficiency that increased only two- to threefold (Fig. 5). It would make sense for Gag to enhance the release of Env because this would result in more efficient production of virions. However, in our experiments, it was unclear why Env was predominantly released at 12 h posttransfection and was independent of Gag or why Gag was able to stimulate Env release only at 30 h. A study of Env and Gag kinetics and intracellular trafficking may address those questions.

The ability of FV Env to form SVPs indicates that the domains responsible for particle assembly, budding, and release are inherent to the Env protein. A budding domain in the SP of Env which is required for specifically interacting with Gag particles for their egress was recently described (26). Env and Gag interaction required two conserved Trp residues located in the first 15 N-terminal amino acids of the SP. For SFVcpz(hu) and feline FV, the SP was found to be associated with virions, and for feline FV, it was demonstrated that the N-terminal half of the SP was buried inside the virion (46), indicating that the SP is cleaved posttranslationally at a late stage of the replication cycle, during or following particle assembly. It remains to be determined whether the SP is required for SVP formation. However, given the SP's topology within virions, it is tempting to speculate that, in addition to interacting specifically with Gag, it may also function as a membrane anchor during particle assembly and release, which may contribute to SVP formation by enabling Env to associate tightly with membranes. The HBV S protein, unlike most viral glycoproteins, contains four membrane-spanning domains, one of which is the SP.

Compared to those for other retroviruses, the extracellular domain of the FV TM protein is unusually long, contains an extensive number of Cys residues (44), and may also be involved in SVP formation. In this region, there are seven conserved Cys residues that are potentially involved in intra- and intermolecular disulfide bonding. It would follow that protein stability conferred by disulfide bonding would make FV Env oligomers more stable than Env oligomers of other retroviral glycoproteins, which contain a significantly smaller number of Cys residues (human immunodeficiency virus type 1 has two Cys residues [9] and MuLV has three Cys residues [39] in the

equivalent region). Indeed, when Wilk et al. (45) examined negatively stained SFVcpz(hu) particles by EM, they found that Env proteins grouped easily into recognizable trimers that formed a network of hexameric rings. The clarity of these electron micrographs, which had not been seen before for any other retrovirus, was unprecedented and suggested that FV Env was capable of forming complexes that were unusually stable. For HBV, the S protein contains 14 conserved Cys residues involved in intermolecular disulfide bridging, and these residues are necessary for assembly and secretion of HBV SVPs (30). Strong membrane associations and tight protein lateral interactions produced by multiple MSDs and Cys residues, respectively, may be a common theme among Envs capable of forming SVPs.

Using sucrose density experiments we identified the presence of two separate Env peaks (Fig. 5); however, we could not demonstrate the presence of SVPs, since Gag was associated with both Env populations. In contrast, we have noted FV particles that seem to lack an identifiable core by EM experiments (data not shown); however, due to the two-dimensional nature of this experiment, it cannot be taken as evidence of FV SVP formation since not all views analyze the center of the particle. It is still possible that SVPs do occur *in vivo* in an FV-infected host; however, it seems as likely that they are not seen in natural infections. Such a finding would be consistent with the placement of FV as a unique family of retroviruses. More specifically, the formation of SVPs makes FV reminiscent but not identical to HBV since SVPs are clearly present in HBV infections.

The ability of SFVcpz(hu) Env to form SVPs, despite its organization as a typical retroviral envelope protein, demonstrates that FVs are unusual retroviruses. In light of our data, the FV envelope protein, as opposed to the Gag protein in other retroviral systems, is the predominant protein that drives particle budding and release. These observations further align FV with HBV, a comparison that has recently been extensively noted (27) and supports the idea that FVs represent an evolutionary bridge of the two virus families.

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