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Ubiquitination of the Prototype Foamy Virus Envelope Glycoprotein Leader Peptide Regulates Subviral Particle Release

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Foamy virus (FV) particle egress is unique among retroviruses because of its essential requirement for Gag and Env coexpression for budding and particle release. The FV glycoprotein undergoes a highly unusual biosynthesis resulting in the generation of three particle-associated, mature subunits, leader peptide (LP), surface (SU), and transmembrane (TM), derived from a precursor protein by posttranslational proteolysis mediated by furin or furinlike proteases. Previously at least three LP products of different molecular weights were detected in purified FV particles. Here we demonstrate that the higher-molecular-weight forms gp28^{LP} and gp38^{LP} are ubiquitinated variants of the major gp18^{LP} cleavage product, which has a type II membrane topology. Furthermore, we show that all five lysine residues located within the N-terminal 60-amino-acid cytoplasmic domain of gp18^{LP} can potentially be ubiquitinated, however, there seems to be a preference for using the first three. Inactivation of ubiquitination sites individually resulted in no obvious phenotype. However, simultaneous inactivation of the first three or all five ubiquitination sites in gp18^{LP} led to a massive increase in subviral particles released by these mutant glycoproteins that were readily detectable by electron microscopy analysis upon expression of the ubiquitination-deficient glycoprotein by itself or in a proviral context. Surprisingly, only the quintuple ubiquitination mutant showed a two- to threefold increase in single-cycle infectivity assays, whereas all other mutants displayed infectivities similar to that of the wild type. Taken together, these data suggest that the balance between viral and subviral particle release of FVs is regulated by ubiquitination of the glycoprotein LP.

Retroviral glycoproteins (Env proteins) are usually translated as precursor proteins and go through a series of modifications before reaching their mature functional state enabling them to interact with specific cellular receptors and to fuse viral and cellular lipid membranes. In general the Env proteins are cotranslationally inserted into the rough endoplasmic reticulum membrane. During their transport to the cell surface the precursor proteins oligomerize and are proteolytically cleaved by cellular proteases into at least two mature subunits, surface (SU), mainly involved in receptor recognition, and transmembrane (TM), harboring the membrane fusion machinery. In addition, the Env proteins undergo different kinds of posttranslational modifications during their biosynthesis and intracellular transport. For example, retroviral Env proteins are generally modified by attachment of N- and sometimes O-linked carbohydrate chains at asparagine and serine or threonine residues, respectively, although the extent and subunit distribution show variation between individual glycoproteins (reviewed in reference 40). Furthermore, for some retroviral Env proteins the addition of fatty acids via linkage to cysteine residues have been reported (reviewed in reference 31).

Incorporation of the viral glycoprotein at the budding step during the particle release process is one of the last steps in the replication cycle of envelope viruses. This assembly step has long been thought to occur exclusively at the plasma membrane. However, increasing evidence was collected that budding can also take place at intracellular compartments (i.e., the multivesicular body) and that budding sites may depend on the type of producer cell analyzed (reviewed in reference 26). While budding across cellular membranes retroviruses incorporate the viral glycoproteins but also cellular proteins by active or passive mechanisms. Passive incorporation of viral or cellular membrane proteins into retroviral particles occurs in the absence of specific interactions with the viral capsid proteins, as long as they are not sterically incompatible with the viral core and are present at the budding site. Active incorporation requires specific interactions between the viral capsid and viral or cellular membrane proteins at the budding site. At least for some retroviruses (i.e., human immunodeficiency virus [HIV] and Mason-Pfizer monkey virus [MPMV]), there is accumulating evidence for an active incorporation of the cognate envelope protein through Gag-Env interactions (3, 20, 35, 44).

Foamy viruses (FVs), the only genus of the retroviral subfamily *Spumaretrovirinae*, display a replication strategy with features distinct from that of orthoretroviruses but resembling in many aspects those of hepadnaviruses (reviewed in reference 32). The FV glycoprotein plays an important role in the assembly process since its coexpression with the Gag protein is essential for targeting of FV capsids, preassembled in the cytoplasm, to cellular membranes and for budding of viral particles (reviewed in reference 13). In addition, the FV glycoprotein has a highly unusual biosynthesis for a retroviral glycoprotein. It is translated as a full-length precursor protein into the rough endoplasmic reticulum and initially has a type III protein configuration with both its N and C termini located

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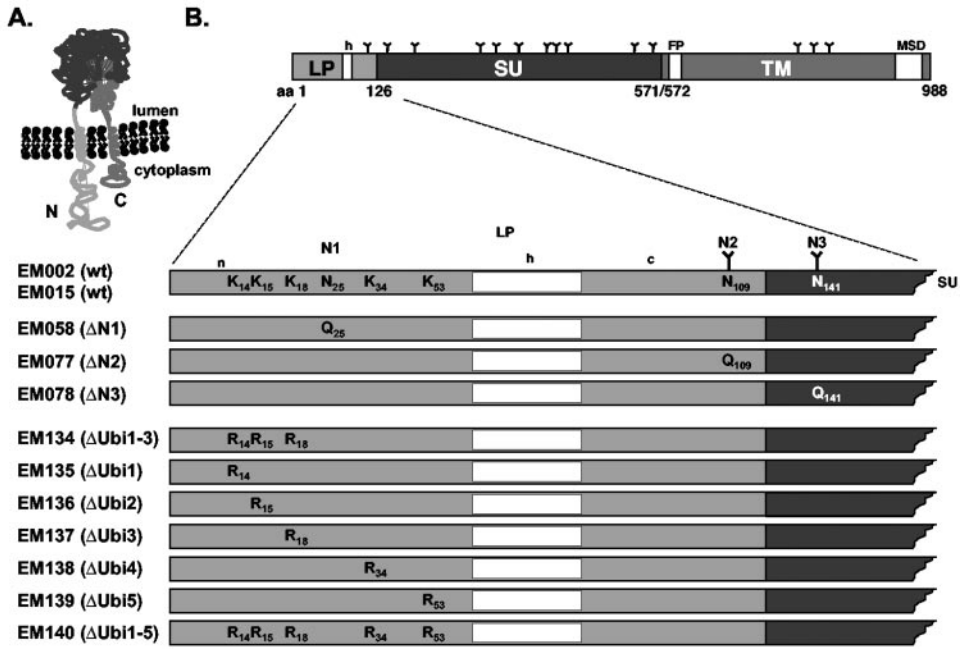

FIG. 1. Schematic illustration of PFV Env membrane topology and expressions constructs. (A) Schematic illustration of the membrane topology of the PFV Env precursor protein as suggested from previous analysis (14, 17). (B) Schematic organization of the PFV Env protein. The N-terminal region spanning the complete leader peptide and partial sequences of the adjacent surface domain is enlarged. Individual lysine residues in the cytoplasmic domain of the LP and the asparagine residues of the first three potential N-glycosylation sites are indicated for the wild-type constructs (wt, EM002 or EM015). N-glycosylation sites used are indicated with a Y. For the individual mutant constructs amino acid sequences deviating at specific positions from the wild-type sequence are indicated. LP: leader peptide; SU: surface subunit; TM: transmembrane subunit; h: hydrophobic region of the LP; FP: fusion peptide; MSD: membrane-spanning domain; N: N terminus; C: C terminus; N1 to N3: potential N-glycosylation sites 1 to 3.

intracytoplasmically (Fig. 1A) (8, 14). Only during its transport to the cell surface is it posttranslationally processed by cellular, most likely furin-like, proteases and not the signal peptidase complex, into at least three subunits (5, 7).

The N-terminal signal or leader peptide (LP) has a type II conformation, whereas the C-terminal TM subunit has a type I conformation. The internal SU subunit presumably associates with extracellular domains of TM on the luminal side (14, 43). Image reconstruction analysis from negative-stained electron microscopy pictures of the characteristic, prominent Env spike structures on FV particles indicates that the Env glycoprotein, as reported for other viral glycoproteins, forms trimeric complexes containing three copies of each of the three individual subunits in every Env spike structure (42). For the FV budding process, two essential interactions between Env and Gag are required (14, 27). One involves the N-terminal region of the putative membrane-spanning domain of the TM subunit (27). The more important, second essential interaction, however, is the contact of the N-terminal cytoplasmic region of the FV Env LP, the so-called budding domain, containing an essential, conserved WXXW sequence motif, with the N terminus of the FV Gag protein (14, 42). Most probably due to the crucial interaction between capsid and the FV Env LP, this cleavage product is particle associated. However, it also suggests that the LP may have additional functions in the FV replication cycle and FV Env function.

We have previously observed at least three different LP products in purified FV particles (14). In the present study we intended to characterize the nature of these different LP forms in more detail and examine their role in glycoprotein function and FV replication.

MATERIALS AND METHODS

Cells. The human kidney cell line 293T (4), the human fibrosarcoma cell line HT1080 (30), and the FV indicator cell line BHK/LTR(HFV)lacZ (33) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics.

Expression constructs. The replication-deficient prototype foamy virus (PFV; formerly known as human foamy virus [HFV]) vector pDWP01 is a variant of the previously described PFV Gag/Pol/Env and enhanced green fluorescent protein (EGFP)-neo fusion protein expressing vector pDL01 (28) having most of the *env* open reading frame (ORF) deleted and all translation initiation codons of the *env* ORF overlapping the *pol* ORF mutated to ACG mutations (5). Thereby the Pol amino acid sequence remained unchanged but expression of residual Env peptides was completely prevented. The glycoprotein expression constructs used in this study are shown schematically in Fig. 1B. The PFV Env expression constructs pczHFVenvEM002 (15); EM015, (5) yielding wild-type proteins; and the constructs pczHFVenvEM058 (ΔN1), EM077 (ΔN2), and EM078 (ΔN3), expressing mutant glycoproteins having the first, second, or third potential N-glycosylation site inactivated by an N->Q exchange, respectively (14), were described earlier. The ubiquitination site mutants pczHFVenvEM134 to -EM140, based on the pczHFVenvEM015 wild-type constructs, were generated by recombinant PCR techniques. The individual cloning strategies and mutagenesis primers are available on request.

In constructs pczHFVenvEM135 (ΔUbi1: $K_{14}R$), EM136 (ΔUbi2: $K_{15}R$), EM137 (ΔUbi3: $K_{18}R$), EM138 (ΔUbi4: $K_{34}R$), and EM139 (ΔUbi5: $K_{53}R$) individual potential ubiquitination sites have been inactivated, whereas in pczHFVenvEM134 (ΔUbi1-3: $K_{14}R$, $K_{15}R$, and $K_{18}R$) the first three and in pczHFVenvEM140 (ΔUbi1-5: $K_{14}R$, $K_{15}R$, $K_{18}R$, $K_{34}R$, and $K_{53}R$) all five potential ubiquitination sites were mutated. The parental human cytomegalovi-

rus immediate-early promoter-driven infectious proviral clone pcHSRV2 has been described previously (19). The mutant pcHSRV2EM026 proviral construct displays a wild-type replication phenotype and has a BsmBI restriction site introduced by silent mutagenesis into the *pol* ORF immediately upstream of the *env* translation start to facilitate introduction of *env* mutants into the proviral backbone. The pcHSRV2EM140 (ΔUbi1-5) mutant provirus construct was generated by replacing a BsmBI/Kpn2I fragment of pcHSRV2EM026 with the same fragment of the pczHFVenvEM140 expression construct. The expression constructs for hemagglutinin (HA)-tagged or wild-type ubiquitin pSG5 HA-Ubi and pSG5 Ubi were a kind gift of H. Göttlinger and were described previously (39).

Generation of viral supernatants and analysis of transduction efficiency. Supernatants containing recombinant viral particles were generated essentially as described earlier (10, 12, 15). FV supernatants were generated by cotransfection of 293T cells with the Gag/Pol-expressing vector pDWP01 and an Env expression plasmid as indicated or by transfection of cells with the proviral expression construct pcHSRV2 and variants thereof. Transductions of recombinant EGFP expressing PFV vector particles were performed by infection of 2×10^4 target cells plated 24 h in advance in 12-well plates for 4 h using 1 ml of viral supernatant or dilutions thereof. The amount of EGFP-positive cells was determined by fluorescence-activated cell sorting analysis 48 h after infection and was in the range of 0.5 to 80% depending on the virus dilution used for infection. All transduction experiments were performed at least three times and in each independent experiment the values obtained with wild-type PFV Env (EM002 or EM015) were arbitrarily set to 100. Viral titers of replication-competent PFV were titrated by endpoint dilution on BHK/LTR(HFV)lacZ cells as described previously (33).

In some experiments intracellular viral particles were artificially released by freeze-thawing of the transfected 293T cells and subsequent centrifugation and filtration of the supernatant through 0.45-μm-pore-size filters to remove cellular debris. The resulting supernatants were then assayed as described above.

Antisera and Western blot expression analysis. Western blot expression analysis of cell- and particle-associated viral proteins was performed essentially as described previously (14) with the exception that blots to be analyzed with some ubiquitin-specific antibodies were autoclaved for 30 min in distilled water using a liquid cycle immediately after blotting to the nitrocellulose to enhance the ubiquitin-specific signal, as suggested previously (18). Subviral particles were isolated similar to FV particles by ultracentrifugation through 20% sucrose. Polyclonal antisera used were specific for PFV Gag (1), the LP of PFV Env, amino acids 1 to 86 (14), the LP and SU subunits, amino acids 87 to 148, or bovine ubiquitin (Sigma U-5379). Furthermore, hybridoma supernatants specific for the HA tag (clone 12CA5), the SU subunit (clone P3E10) of PFV Env (5), or commercially available monoclonal antibodies specific for ubiquitin (Biomol: FK1 and FK2; Covance: P4D1 and P4G7) were employed.

Electron microscopy analysis. Forty-eight hours after transfection, 293T cells were harvested and processed for electron microscopy analysis as described previously (11).

RESULTS

Distinct particle-associated FV Env LP cleavage products arise by differential posttranslational modification. We observed previously that purified FV particles contain different glycoprotein precursor-derived cleavage products recognized by an LP-specific antiserum (14). However, the nature of these distinct forms remained unclear. Basically there are two different potential explanations for their occurrence. The first is that alternative proteolytic cleavage sites are used during FV Env precursor processing. The second is that slower-migrating forms are posttranslationally modified variants of the major cleavage product gp18^{LP}. The recent determination of the LP/SU cleavage site by sequencing of the N terminus of the PFV SU subunit revealed no indication of alternatively processed forms of SU (5).

In order to shed light on the nature of these higher-molecular-weight LP cleavage variants of PFV Env, therefore, we concentrated our efforts on the identification of potential posttranslational modifications of gp18^{LP}. First, we examined if alternative N-glycosylation of gp18^{LP} might account for the observed difference in molecular weight. Therefore PFV particles, purified by ultracentrifugation of cell culture supernatants of 293T cells transfected with the replication-deficient proviral PFV vector pDL01 through a sucrose cushion, were digested with different endoglycosidases or mock incubated (Fig. 2). This analysis revealed the presence of at least three LP cleavage fragments with distinct molecular weights in all samples, although the treatment of PFV particles by either endoglycosidase H (Fig. 2A and B, lane 3) or *N*-glycosidase F (Fig. 2A and B, lane 2) resulted in a similar increase in mobility of all PFV Env LP cleavage products compared to mock incubated particles (Fig. 2A and B, lane 1). This indicated that the higher-molecular-weight forms of gp18^{LP} are not the result of alternative N-glycosylation. The analysis of the particle-associated composition of LP cleavage products of mutant PFV Env expression constructs (Fig. 1) that had individual potential N-linked glycosylation (N-Gly) sites inactivated further supported this explanation (data not shown).

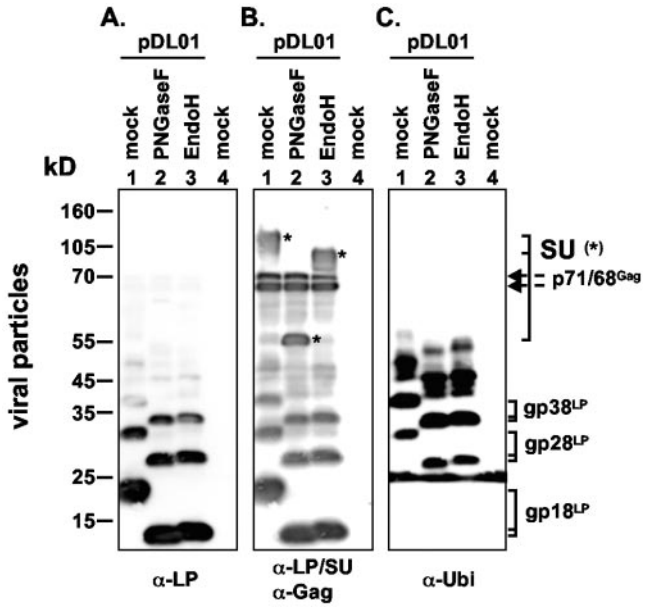

FIG. 2. Glycosylation analysis of PFV particles. PFV particles generated by transfection of 293T cells with the replication-deficient PFV proviral vector pDL01 (lanes 1 to 3) or empty expression vector (lane 4) were purified by ultracentrifugation through 20% sucrose. Subsequently viral particles were digested with *N*-glycosidase F (PNGaseF) or endoglycosidase H (EndoH) or mock incubated (mock) followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using polyclonal rabbit sera specific for (A) PFV Env LP (α-LP), (B) PFV Env LP/SU (α-LP/SU) and PFV Gag (α-Gag), or (C) ubiquitin (α-Ubi). Viral proteins are indicated on the right. The different glycosylated and deglycosylated forms of the PFV SU subunit are marked by asterisks. The band of about 25 kDa in all lanes in panel C represents a staining artifact in this specific experiment that was present even in lanes loaded only with sample buffer.

Taken together, these results are in line with earlier results from our laboratory (5) suggesting that all three products are derived by proteolytic precursor cleavage at amino acid 126 and not at alternative sites. Furthermore, they indicate that the slower-migrating forms of the LP do not arise by differential N-glycosylation of gp18^{LP}.

Ubiquitination of the FV Env gp18^{LP} cleavage product. Another posttranslational modification that leads to a substantial

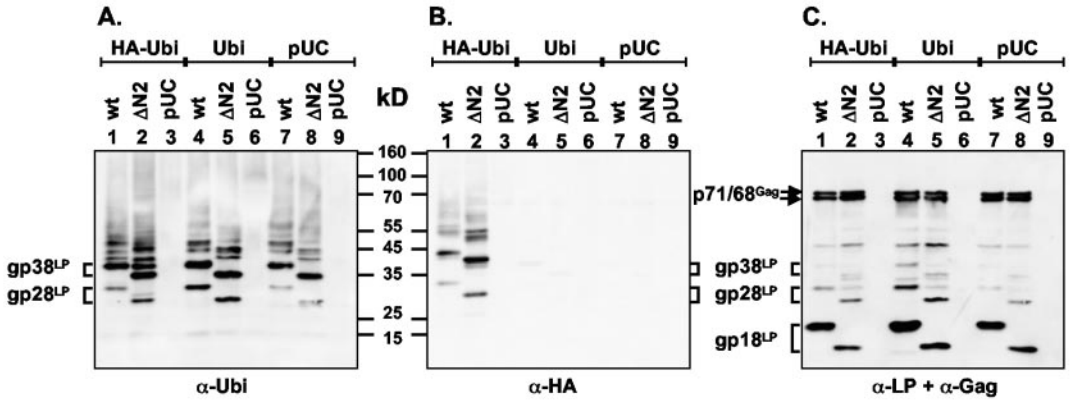

FIG. 3. Labeling of particle-associated PFV Env with HA-tagged ubiquitin. Mutant PFV particles were generated by transient cotransfection of 293T cells with the Gag/Pol-expressing PFV vector pDWP01 and the PFV Env expression construct or empty vector as indicated. In addition, HA-tagged ubiquitin (HA-Ubi), untagged ubiquitin (Ubi), or empty expression vector (pUC) was cotransfected as indicated. Subsequently, lysates of viral particles purified by ultracentrifugation through 20% sucrose were analyzed by Western blotting using consecutive incubation with antibodies specific for (A) ubiquitin (α-Ubi), (B) the HA tag (α-HA), and (C) PFV Env LP (α-LP) and PFV Gag (α-Gag). Viral proteins are indicated on the left and between panels B and C. Cells were transfected with pDWP01 and: lane 1, pczHFV envEM015 (wt) plus pSG5 HA-Ubi (HA-Ubi); lane 2, pczHFVenvEM077 (ΔN2) plus pSG5 HA-Ubi (HA-Ubi); lane 3, pUC19 (pUC) plus pSG5 HA-Ubi (HA-Ubi); lane 4, pczHFVenvEM015 (wt) plus pSG5 Ubi (Ubi); lane 5, pczHFVenvEM077 (ΔN2) plus pSG5 Ubi (Ubi); lane 6, pUC19 (pUC) plus pSG5 Ubi (Ubi); lane 7, pczHFVenvEM015 (wt) plus pUC19 (pUC); lane 8, pczHFVenvEM077 (ΔN2) plus pUC19 (pUC); lane 9, only pUC19 (pUC).

increase in molecular weight and that has recently been associated with retroviral particle release is ubiquitination (41). Therefore, we analyzed PFV particle preparations by Western blot using a ubiquitin-specific polyclonal antiserum. As shown in Fig. 2, digestion of wild-type PFV particles by *N*-glycosidase F or endoglycosidase H resulted in a mobility shift of all ubiquitin-specific proteins bands (Fig. 2C, lanes 2 and 3) that were identical to the higher-molecular-weight bands detected by the LP-specific antiserum (Fig. 2B, lanes 2 and 3). Similarly, only the minor particle-associated $gp28^{LP}$ and $gp38^{LP}$ fragments but not the predominant $gp18^{LP}$ fragments of the wild-type PFV Env protein (data not shown) and the ΔN1 or ΔN3 mutant (data not shown) and the faster-migrating bands of the ΔN2 mutant (data not shown) were detected by this antiserum. Furthermore, at least two to three additional distinct higher-molecular-weight forms, stained by the ubiquitin-specific antiserum, were detectable (Fig. 2C, lanes 1 to 3; data not shown). However, these were only poorly detectable by the LP- or LP-SU-specific antisera (Fig. 2A and B, lanes 1 to 3; data not shown).

In order to exclude detection of the higher-molecular-weight bands as a result of unspecific binding of the ubiquitin specific antiserum we cotransfected in an additional experiment expression constructs for HA-tagged or unmodified ubiquitin together with different combinations of PFV Env and Gag/Pol vector constructs (Fig. 3). Subsequent probing of particle preparations with the ubiquitin-specific antiserum again stained only slower migrating forms other than $gp18^{LP}$, regardless of whether HA-tagged ubiquitin, untagged ubiquitin, or empty expression vectors were cotransfected (Fig. 3A). Using an HA-specific monoclonal antibody, these higher-molecular-weight forms and not $gp18^{LP}$ could be detected only in samples where the HA-tagged ubiquitin expression construct had been cotransfected (Fig. 3B, lanes 1 and 2), whereas in samples cotransfected with untagged ubiquitin (Fig. 3B, lanes 4 and 5) or empty expression vector (Fig. 3B, lanes 7 and 8) no HA-specific signal was detectable. Staining with an LP-specific antiserum resulted in detection of $gp18^{LP}$ and at least two other slower-migrating forms of about 28 kDa and 38 kDa in all three transfection groups (Fig. 3C). In addition, four different types of ubiquitin-specific antibodies specifically stained the $gp28^{LP}$ and $gp38^{LP}$ proteins but not the $gp18^{LP}$ protein (data not shown).

These results showed that the particle-associated PFV Env LP is strongly ubiquitinated, giving rise to differentially migrating proteins. Furthermore, the detection of similarly sized higher-molecular-weight forms by a simian foamy virus (SFVmac) LP-specific antiserum in PFV particles pseudotyped by SFVmac Env (data not shown) suggested that this posttranslational modification of the glycoprotein is not unique to PFV and might be common to all FV species.

Ubiquitination sites in the FV Env leader peptide. Ubiquitin is covalently linked to proteins by attachment to the ε amino group of lysine residues. Inspection of the N-terminal cytoplasmic domain of the PFV Env LP amino acid sequence revealed five potential ubiquitination sites, K_{14}, K_{15}, K_{18}, K_{34}, and K_{53} (Fig. 1B). Mutant PFV Env expression constructs having individual lysine residues or combinations thereof changed to arginine were generated to determine the sites of ubiquitination in PFV Env LP (Fig. 1B). Recombinant PFV particles were produced by transient cotransfection of the individual Env expression constructs and a Gag/Pol and EGFP marker gene expressing the PFV vector (pDWP01) into 293T cells. Western blot analysis of transfected cell lysates showed that all mutants were expressed at similar levels (Fig. 4D), although the K_{14} single mutant (ΔUbi1) and the K_{14}, K_{15}, K_{18}, K_{34}, and K_{53} quintuple mutant (ΔUbi1-5) showed somewhat reduced or increased LP cleavage, respectively (Fig. 4D, lanes 5 and 10).

Examination of particle preparations using a PFV Gag-

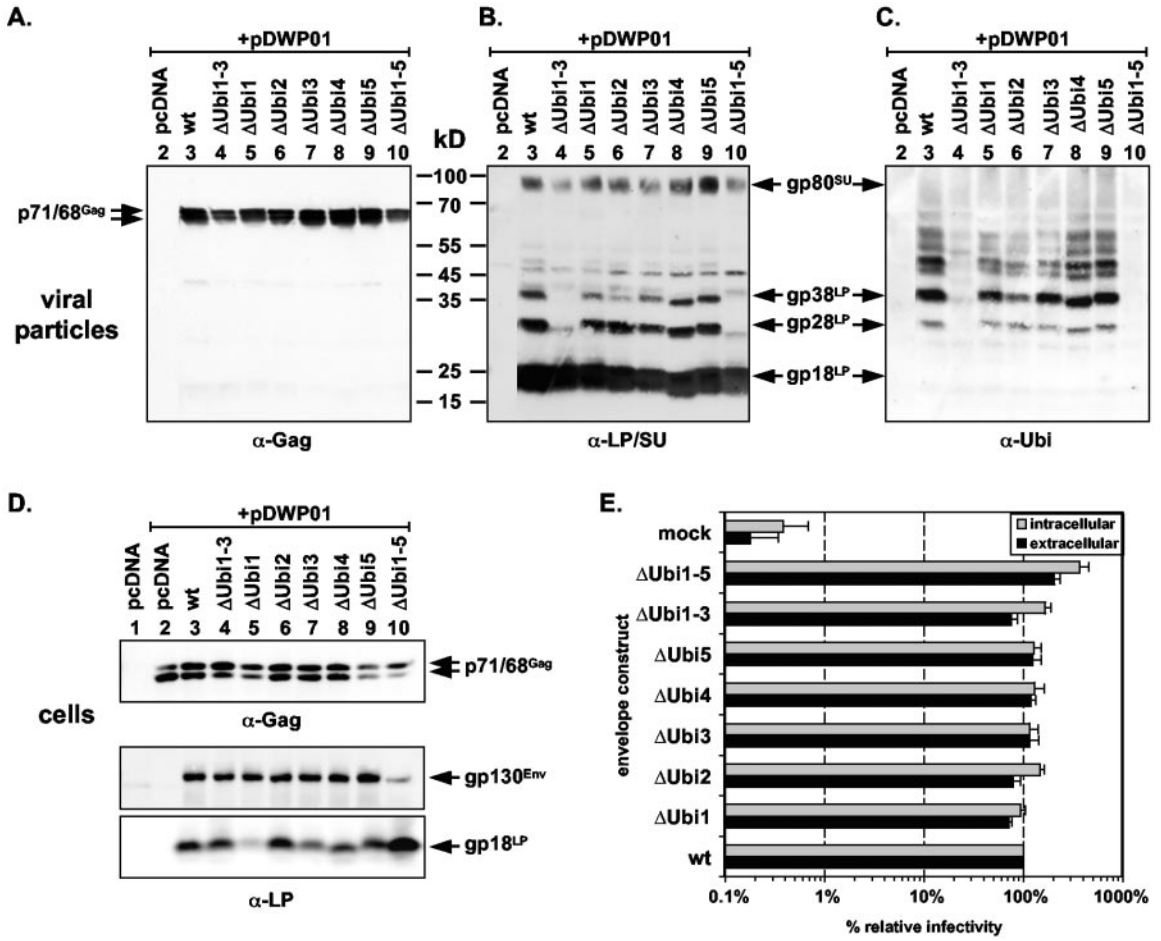

FIG. 4. Analysis of PFV Env mutants with inactivated ubiquitination sites. Mutant PFV particles were generated by transient cotransfection of 293T cells with the Gag/Pol-expressing PFV vector pDWP01 and the PFV Env expression construct or empty vector as indicated. Western blot analysis of purified PFV particles using consecutive incubation with antisera specific for (A) PFV Gag (α-Gag), (B) PFV Env LP/SU (α-LP/SU), or (C) ubiquitin (α-Ubi) or cell lysates using antisera specific for (D) PFV Gag (α-Gag) or PFV Env LP (α-LP). Viral proteins are indicated on the side of the blots. (E) Relative infectivity of 293T cell supernatants (extracellular) and freeze-thaw cell lysates (intracellular) using the GFP marker gene transfer assay on HT1080 target cells. The values obtained using the wild-type PFV Env expression plasmid (wt) were arbitrarily set to 100%. The mean values and standard deviations of at least three independent experiments are shown. Cells were either transfected with pcDNA3.1+zeo alone (lane 1: pcDNA) or cotransfected with pDWP01 and: lane 2, pcDNA3.1+zeo (pcDNA); lane 3, pczHFVenvEM015 (wt); lane 4, pczHFVenvEM134 (ΔUbi1-3); lane 5, pczHFVenvEM135 (ΔUbi1); lane 6, pczHFVenvEM136 (ΔUbi2); lane 7, pczHFVenvEM137 (ΔUbi3); lane 8, pczHFVenvEM138 (ΔUbi4); lane 9, pczHFVenvEM139 (ΔUbi5); lane 10, pczHFVenvEM140 (ΔUbi1-5).

specific (Fig. 4A) or PFV Env LP/SU-specific (Fig. 4B) antiserum revealed that all mutant Env proteins supported FV particle release. However, probing the same blot with the ubiquitin-specific antiserum showed a strong decrease or complete disappearance of ubiquitination for the K_{14}, K_{15}, and K_{18} triple mutant ΔUbi1-3 and the quintuple mutant ΔUbi1-5, respectively (Fig. 4C, lanes 4 and 10). Mutants having only individual lysine residues replaced (ΔUbi1 to ΔUbi5) displayed ubiquitination patterns similar to that of the wild-type protein (Fig. 4C, lanes 3 and 5 to 9). Furthermore, no indication of ubiquitination of other PFV Env domains, in particular the TM subunit containing at least additional four lysine residues in its C-terminal cytoplasmic tail, or the 648-amino-acid PFV Gag protein containing only a single lysine residue, was obtained, even when assaying the PFV Env mutant ΔUbi1-5, which has all potential ubiquitination sites in the LP cytoplasmic domain inactivated (Fig. 4C, lane 10, and data not shown). Interestingly, some additional higher-molecular-weight forms, showing a slightly but clearly different mobility than $gp28^{LP}$ and $gp38^{LP}$, were detectable for all Env proteins analyzed by the PFV LP/SU- (Fig. 4B) or PFV LP-specific antiserum (data not shown) but not by the ubiquitin-specific antiserum (Fig. 4C), although at much weaker intensity. This was most prominent for the ΔUbi1-5 mutant sample, where $gp28^{LP}$ and $gp38^{LP}$ were absent (Fig. 4B, lane 10). The nature of these additional bands is currently unclear.

Single-cycle infectivity analysis of the recombinant mutant particles in a fluorescence-activated cell sorting-based EGFP gene transfer assay revealed wild-type infectivities for cell culture supernatants of most mutants (Fig. 4E). Only supernatants of cells transfected with the ΔUbi1-5 mutant, lacking all potential ubiquitination sites, showed a two- to threefold increase in infectivity (Fig. 4E). However, in the proviral context, no significant difference in viral titers or spread of the mutant virus compared to the wild type was observed (data not shown).

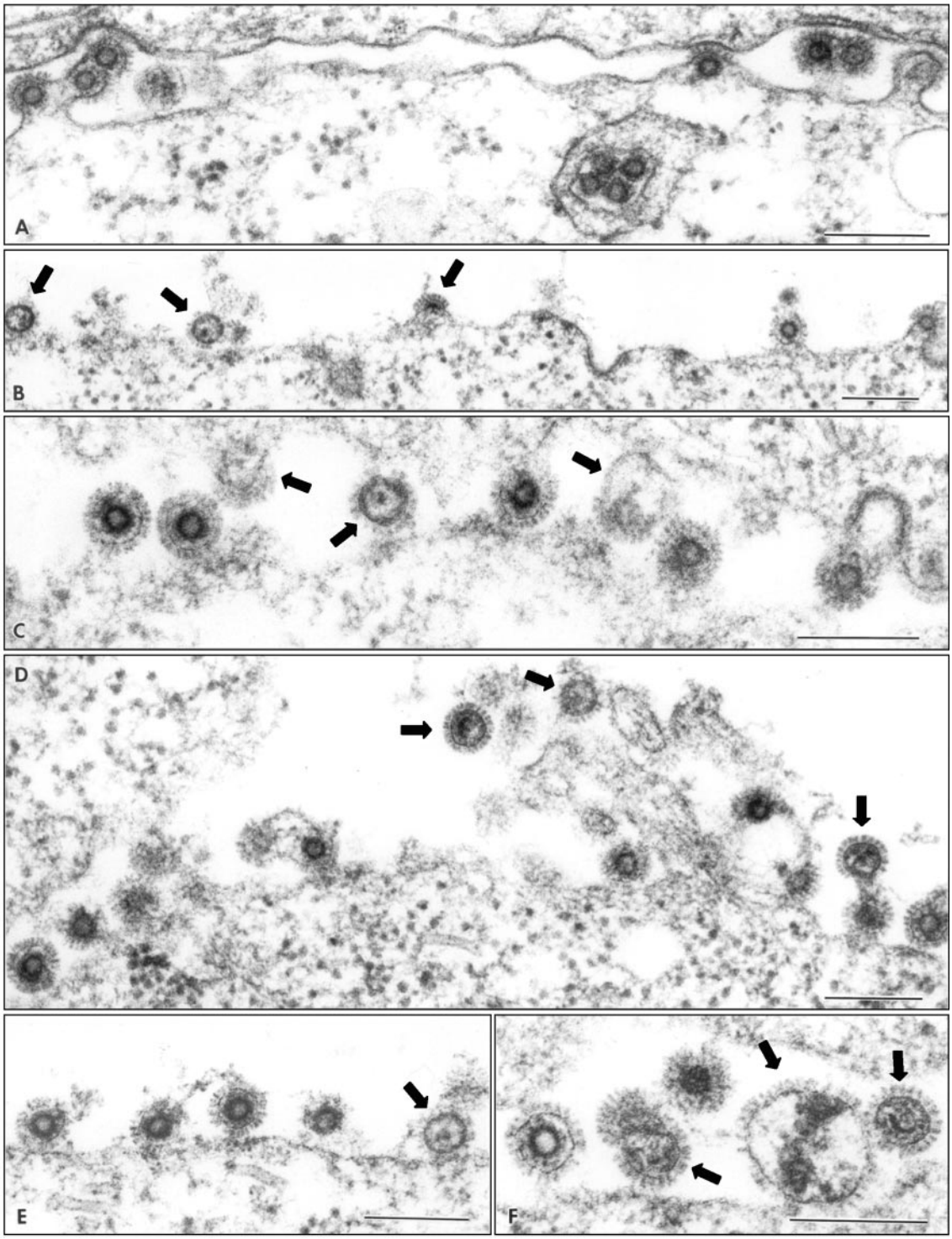

FIG. 5. Electron microscopy analysis of proviral expression clones. Electron micrographs showing representative thin sections of 293T cells transiently transfected with the wild-type PFV proviral expression vector pczHSRV2EM026 (A) or the mutant proviral vector pczHSRV2EM140 expressing the ubiquitination-deficient PFV Env protein ΔUbi1-5 (B to F). Subviral particles are marked by solid arrows. Magnifications: (A) 89,000×; (B) 70,000×; (C) 110,000×; (D) 90,000×; (E) 95,000×; (F) 127,000×. Bar, 200 nm.

These data indicate that all lysine residues of the FV LP cytoplasmic domain can potentially be ubiquitinated, although there seems to be a preference for usage of the first three, as the ubiquitin-specific signal decreased significantly for the triple mutant, ΔUbi1-3, having these lysine residues changed to arginine. Furthermore, the complete absence of ubiquitin-specific signals in the quintuple mutant ΔUbi1-5 indicates that these are the only sites of ubiquitination in the PFV Env LP. In

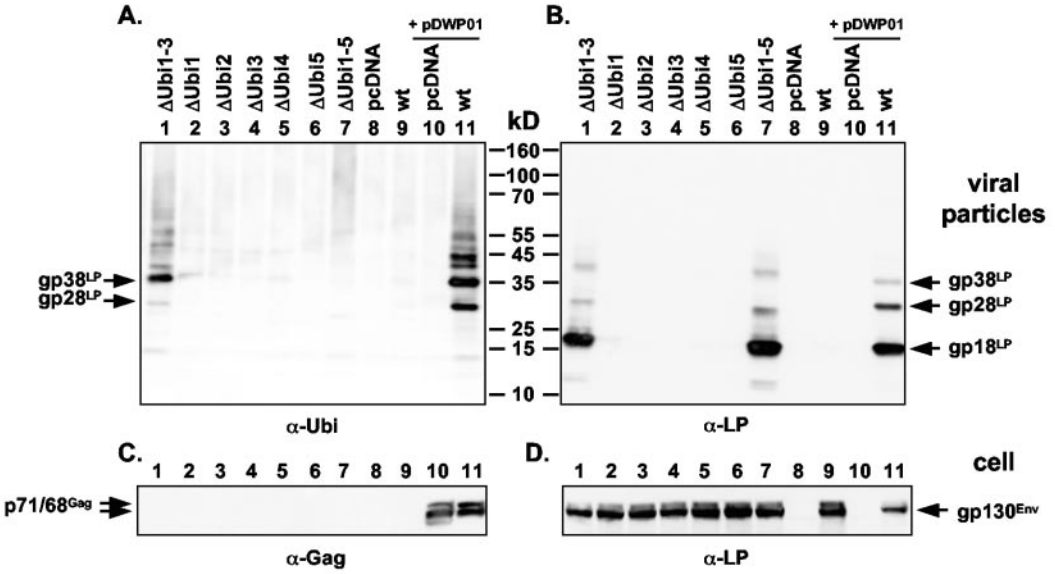

FIG. 6. Analysis of subviral particle release. Subviral particles were harvested by ultracentrifugation through 20% sucrose from supernatants of 293T cells transfected with the individual Env expression constructs or empty expression vector as indicated (lanes 1 to 9). As a control wild-type PFV particles were generated by cotransfecting the PFV Gag/Pol expression vector pDWP01 and the wild-type PFV Env expression construct pczHFVenvEM015 or empty expression vector (lanes 10 and 11). Western blot analysis of particle preparations using consecutive incubation with (A) a ubiquitin-specific monoclonal antibody (α-Ubi, P4D1) and (B) a polyclonal antiserum specific for PFV LP (α-LP). Western blot analysis of the corresponding cell lysates using polyclonal antisera specific for (C) PFV Gag (α-Gag) and for (D) PFV LP (α-LP). Cells were transfected with: lane 1, pczHFVenvEM134 (ΔUbi1-3); lane 2, pczHFVenvEM135 (ΔUbi1); lane 3, pczHFVenvEM136 (ΔUbi2); lane 4, pczHFVenvEM137 (ΔUbi3); lane 5, pczHFVenvEM138 (ΔUbi4); lane 6, pczHFVenvEM139 (ΔUbi5); lane 7, pczHFVenvEM140 (ΔUbi1-5); lane 8, pcDNA3.1+zeo (pcDNA); or lane 9, pczHFVenvEM015 (wt); or cotransfected with pDWP01 and pcDNA3.1+zeo (pcDNA, lane 10) or pczHFVenvEM015 (wt, lane 11).

addition, the results suggest that FV Env LP ubiquitination is not required for viral particle release and has only a minor effect on viral particle infectivity.

Electron microscopy analysis of mutant particle release. In order to analyze the particle morphogenesis of the ubiquitination-deficient PFV Env mutant in further detail, we performed electron microscopy analysis on 293T cells transfected with either different proviral expression constructs or proviral vector constructs expressing Gag, Pol, and Env. In cells expressing PFV Gag/Pol and the wild-type PFV Env protein, viral particles containing typical FV ring-like capsid structures and glycoprotein spikes were readily detectable (Fig. 5A). In contrast, in addition to regular wild-type PFV particles, significant numbers of subviral particles (SVPs) containing the typical glycoprotein spikes but lacking the ring-like capsid structure were detected in cells expressing the mutant EM140 Env in the context of a proviral expression construct (Fig. 5B to F). In corresponding wild-type samples these SVP structures were not detectable (Fig. 5A, and data not shown).

Increased subviral particle release by ubiquitination-deficient PFV Env mutants. Similar to hepadnaviruses, PFV has recently been shown to secrete envelope glycoprotein-containing SVPs, although at much lower levels (36). Therefore, the influence of PFV Env LP ubiquitination on SVP release was analyzed by transfection of 293T cells with the individual mutant glycoprotein expression constructs in the absence of expression of any other PFV protein. Subsequent Western blot analysis of particle preparations using a PFV Env LP-specific antiserum revealed a massive SVP release for the triple mutant ΔUbi1-3 and an even greater SVP release for the quintuple mutant ΔUbi1-5 (Fig. 6B, lanes 1 and 7). In contrast, for wild-type PFV Env as well as for the single mutants (ΔUbi1 to ΔUbi5), displaying a wild-type ubiquitination pattern (Fig. 4C), no SVP release was detectable using the same amount of transfected cell culture supernatant (Fig. 6B, lanes 2 to 6 and 9), although all Env proteins showed similar cellular expression levels (Fig. 6D, lanes 1 to 9).

For wild-type samples, SVP-associated Env was only detectable if using 20- to 50-fold higher amounts of transfected cell culture supernatant than for the ΔUbi1-5 mutant (data not shown). Interestingly, whereas for the triple mutant ΔUbi1-3, ubiquitinated LP forms could be still detected in the SVP preparations (Fig. 6A, lane 1), ubiquitinated LP forms were absent from SVP preparations of the quintuple mutant ΔUbi1-5 (Fig. 6A, lane 7). Based on this biochemical analysis, not surprisingly, in HT1080 cells stably expressing the ΔUbi1-5 (EM140) Env (Fig. 7A and B) or 293T cells transiently transfected with a ΔUbi1-5 (EM140) Env expression vector (Fig. 7C and D) SVPs containing the typical PFV Env spike structures were readily detectable by electron microscopy analysis. Similar structures were not detectable in corresponding samples expressing a wild-type PFV Env protein (data not shown).

Thus, this indicates that ubiquitination of the PFV Env LP regulates SVP formation and interference with LP ubiquitination, in particular at the first three potential attachment sites, leads to a massive increase in PFV Env-mediated SVP release.

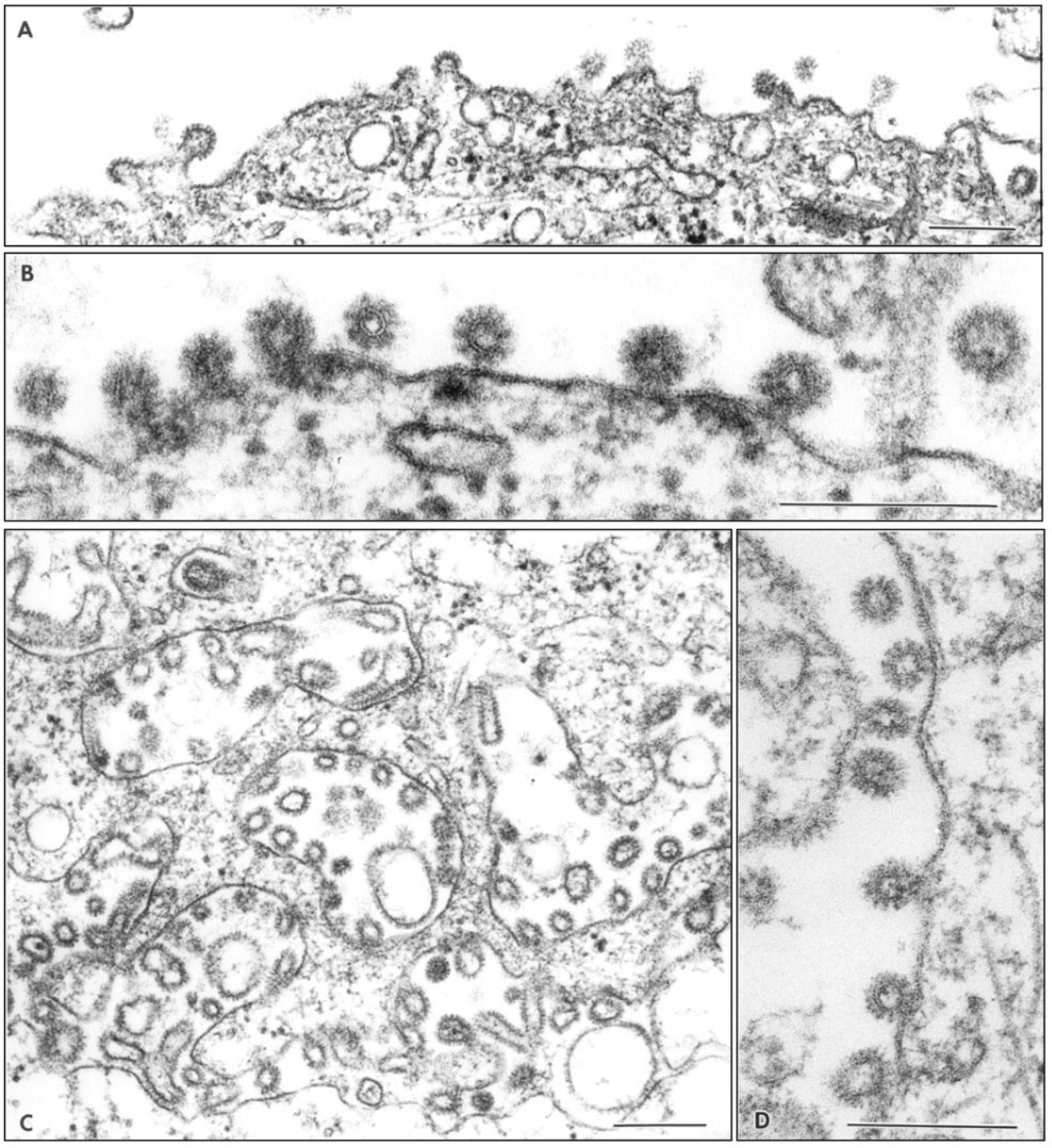

FIG. 7. Electron microscopy analysis of ΔUbi1-5 PFV Env-expressing cells. Electron micrographs showing representative thin sections of (A and B) HT1080 cells stably expressing the mutant ΔUbi1-5 mutant PFV Env protein or (C and D) 293T cells transiently transfected with the mutant PFV Env expression vector pczHFVenvEM140 (ΔUbi1-5). Magnifications: (A) 72,000×; (B) 180,000×; (C) 57,000×; (D) 138,000×. Bar, 200 nm.

DISCUSSION

The cellular ubiquitination machinery is implicated in the budding processes of different types of viruses (41). For different retroviruses monoubiquitination of Gag subunits and the presence of particle-associated free ubiquitin were reported (21–24, 29). Whether there is a specific function of Gag ubiquitination for viral egress, however, remains unclear. Mutation of target lysine residues in HIV-1 p6 or murine leukemia virus p12 had no effect on virus release or replication and did not result in a loss of incorporation of free ubiquitin (21). However, a recent study demonstrated that wild-type HIV-1 Gag and mutants lacking p6 or having the target lysines in p6 replaced are ubiquitinated at different subunits (9). Therefore, the lack of functional phenotypes for the p6 lysine mutants might be explained by compensatory ubiquitination of other

HIV-1 Gag subunits. Furthermore, it is currently unclear whether HIV-1 Gag is ubiquitinated specifically or if Gag ubiquitination is the result of a bystander effect of the nearby presence of an ubiquitin ligase, whose real function is to ubiquitinate a cellular protein at the budding site. In addition, there is no clear correlation in the sensitivity of different viruses to proteasome inhibitors, which is thought to reduce the free pool of ubiquitin within the cells, and the ubiquitination of the viral Gag proteins (23, 24, 34).

We and others have shown recently that PFV, like other viruses, links to the cellular vacuolar protein sorting machinery for particle egress through a PSAP L-domain motif in PFV Gag (25, 38). Interestingly, unlike other retroviral Gag proteins the 648-amino-acid PFV Gag protein contains only a single lysine residue and some primate FV isolates harbor none at all (data not shown). Although we readily detected PFV Env LP ubiquitination in this study, we were unable to detect ubiquitination of the TM subunit harboring additional cytoplasmic lysine residues or of PFV Gag, suggesting that they either are not a substrate for or are not accessible to the cellular ubiquitination machinery associated with viral particle egress.

To our knowledge, this is the first report of viral glycoprotein ubiquitination associated with a function other than protein degradation (2). Ubiquitination of HIV-1 Env at the extracellular domains of the gp41 subunit has been reported previously (2). However, this report suggests that ubiquitination is functionally associated with the probable misfolding and degradation of the majority of the HIV-1 glycoprotein retained within the endoplasmic reticulum.

Unlike the lack of functional consequences of mutation of individual ubiquitination attachment sites in other viral systems, ubiquitination-deficient PFV Env leads to a massive appearance of subviral particles in the subgenomic and proviral context. This might be a consequence of altered intracellular trafficking of ubiquitylation-deficient Env resulting in a less efficient interaction of PFV capsids with the glycoprotein and thereby enhancing the glycoprotein's intrinsic capacity to form subviral particles. In contrast, no significant change in the in vitro infectivity of wild-type and mutant viruses was observed, although subviral and viral particles were generated in similar amounts in the proviral context by the ubiquitination-deficient mutant. However, since there is no good small-animal model for primate foamy viruses, it cannot be easily tested whether such mutant viruses have additional in vivo phenotypes and are able to spread and establish productive infections in animals.

Electron microscopy analysis of cells expressing ubiquitination-deficient PFV Env indicated that SVPs are apparently released at the cell surface but also at intracellular compartments, suggesting that SVP release might take place at different types of cellular membranes. The mechanism underlying the regulation of PFV SVP release by ubiquitination of the glycoprotein leader peptide is unknown at this time. One can envision several different mechanisms. For example, wild-type PFV Env ubiquitinated at the cell surface might be endocytosed at a higher rate than the ubiquitination-deficient mutant protein and thereby SVP release at the plasma membrane is minimized. However, even at intracellular membranes, SVP release was not detectable in samples expressing the wild-type glycoprotein.

Alternatively, a differential intracellular localization of ubiquitinated and nonubiquitinated glycoproteins, and therefore differential access to the cellular machinery associated with viral budding, might account for the different levels of SVP egress observed. Another mechanism might be a selective binding of ubiquitinated glycoprotein to cellular proteins specifically inhibiting SVP release or an increased affinity of ubiquitinated Env for PFV Gag resulting in more efficient incorporation into viral particles. Otherwise, sequence motifs within the PFV Env cytoplasmic domain required for interaction with cellular proteins during SVP budding and release may be masked by ubiquitination, thereby preventing efficient SVP egress. Whether one of these potential mechanisms or a combination of them are involved in the regulation of FV SVP egress by ubiquitination of the glycoprotein LP requires further analysis. Furthermore it would be interesting to examine other viral glycoproteins, in particular retroviral Env proteins (i.e., HIV-1 Env), for potential posttranslational modification by ubiquitination to see whether it serves a functional role in viral or subviral particle egress.

Recently it has been reported that intracellular release of another retrovirus with a B/D-type assembly strategy, MPMV, potentially involves a pericentriolar recycling endosomal compartment and is enhanced by coexpression of the cognate glycoprotein (35). Furthermore, this report demonstrated that intracellular trafficking of the MPMV Env protein through this compartment is important for initiating export of MPMV capsids from the intracellular site of assembly (35). Although a tyrosine-based endocytosis signal motif in the cytoplasmic domain of the MPMV Env TM subunit is implicated in intracellular trafficking of the MPMV glycoprotein (37) it is tempting to speculate that perhaps the MPMV Env protein is also ubiquitinated and its intracellular trafficking and localization are modulated through this type of posttranslational modification.

FV particle egress is unique among retroviruses because coexpression of the cognate glycoprotein is required for budding and particle release (reviewed in references 13 and 16). Unlike other retroviruses harboring all the structural information essential for these processes within the Gag polyprotein, FVs apparently have split these functions between Gag and Env proteins. However, both may contain redundant structural information, since addition of a membrane-targeting signal to FV Gag enables FV Env independent particle release (6) and FV Env expression by itself results in the generation of SVPs (36). With the demonstration of particle-associated, ubiquitinated forms of the PFV Env LP subunit and regulation of the extent of PFV SVP egress by this posttranslational modification of the glycoprotein in this report, we add another unique feature to the already unusual biosynthesis and function of this viral glycoprotein.

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