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Incorporation of Pseudorabies Virus gD into Human Immunodeficiency Virus Type 1 Gag Particles Produced in Baculovirus-Infected Cells

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The human immunodeficiency virus type 1 (HIV-1) Pr55^{gag} precursors were previously shown to assemble and bud efficiently as noninfectious virus-like particles (VLPs) when expressed in baculovirus-infected insect cells. In this study, we examined the abilities of foreign antigens to be incorporated on the outer surface of HIV-1 Gag particles. We have used a dual recombinant baculovirus, expressing the HIV-1 Gag gene and gD gene under the control of the P10 and polyhedrin promoters, respectively, to obtain hybrid VLPs. Transmission electron microscopy of insect cells infected with the dual recombinant revealed very large aggregates of particles budding from the cell membrane. The release of VLPs into the culture medium was clearly different for a recombinant baculovirus producing solely HIV-1 Gag, for which particles were uniformly distributed all around the cell surface. Biochemical analysis of hybrid particles indicated that glycoprotein gD was packaged into HIV-1 Gag VLPs. Moreover, the carboxy-terminal p6 region of Gag polyprotein and the glycoprotein gD intracytoplasmic domain were not required for gD incorporation. The experiments described here clearly demonstrate that glycoprotein gD can be packaged with HIV-1 Gag particles and released from insect cells.

Particulate carrier systems have been developed to increase the immunogenicity of the major immunodominant regions of various viruses for vaccine purposes. In many cases, antigens are genetically fused to a gene encoding a particle-forming protein, and thereby different viral core proteins (2, 10, 13, 30) have been used as carriers for the presentation of heterologous epitopes in a polyvalent particulate structure.

Retroviral Gag proteins are good candidates for antigen presentation. Indeed, this polyprotein precursor drives the budding process in the absence of all other components of the virus (42). Previous reports described the packaging of foreign antigens within Gag particles by fusing the polypeptide of interest directly to the C-terminal domain of Gag polyprotein (39, 40). Assembly of a Gag/V_3 (human immunodeficiency virus type 1 [HIV-1] major neutralizing determinant) fusion protein into hybrid particles induced both humoral and cellular responses (11). However, such particles elicited a strong antibody response against Gag, while that directed to the V3 region was weak. One hypothesis is sequestration of the V3 region within the core particles, preventing interactions with B cells (11). Another promising strategy is to incorporate foreign glycoproteins as antigens on the surface of Gag particles. However, the specificity of viral envelope protein incorporation in the virus envelope as well as host cell surface protein exclusion is poorly understood (32).

In this study, we describe the incorporation of the glycoprotein of one virus on the surface of virus-like particles (VLPs) of an unrelated virus. We have used HIV-1 Gag as the carrier system. Indeed, the unprocessed HIV-1 Pr55^{gag} precursors expressed in recombinant-baculovirus-infected insect cells are targeted to the plasma membrane and assembled into particles of 100 to 120 nm budding from the cell surface (9, 27, 28). As the carried antigen, we have chosen the pseudorabies virus (PRV) glycoprotein gD. PRV is the causative agent of Aujeszky's disease, a problem that mainly affects the pig industry. The gD (gp50) protein is 400 amino acids (aa) long and has an amino-terminal signal sequence, a large external domain, a single transmembrane domain, and a hydrophilic cytoplasmic tail of 25 aa at the carboxy terminus. gD is a major immunogen of PRV and is required only for penetration of the virus into the host cell (23). Previous reports have demonstrated that vaccination with recombinant gD efficiently protects animals from Aujeszky's disease (14, 20, 26).

Here, our results show that PRV gD can be incorporated with HIV-1 Gag particles by using a dual recombinant baculovirus. Moreover, the p6 domain of Gag protein and the glycoprotein gD intracytoplasmic domain are not required for incorporation.

MATERIALS AND METHODS

Virus and cells. *Spodoptera frugiperda* (Sf9) cells (37) were maintained at 28°C in TC100 medium supplemented with 5% fetal calf serum. Wild-type *Autographa californica* multiple nuclear polyhedrosis virus clone 1.2 (5) and recombinant baculoviruses were propagated in Sf9 cells. For infection, cells (4×10^6 per 25-cm^2 flask) were inoculated with a viral suspension (10 PFU per cell). After 1 h of adsorption at room temperature, the viral inoculum was removed, and fresh culture medium was added. Cells were further incubated at 28°C.

Construction of transfer vectors. All dual recombinant baculoviruses were obtained after two successive recombination steps (i) in the p10 locus to introduce the HIV-1 Gag coding sequence downstream of the p10 promoter and (ii) in the polyhedrin locus to insert the PRV gD coding sequence downstream of the polyhedrin promoter. Thus, two transfer vectors, pGm8022Gag and pGmAc118gD, were constructed. A 1,704-bp *Nco*I-*Kpn*I fragment from plasmid pGmAc115TGag (27), encoding the full-length myristylated form of Pr55^{gag}, was blunt ended with Klenow enzyme and ligated to the blunt-ended unique *Bgl*II site of the p10 transfer vector pGm8022 (4). pGm8022GagΔp6 was constucted by addition of a

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12-mer *Nhe*I linker at the unique blunt-ended *Bgl*II site (+1306) of pGm8022Gag, generating an in-frame stop codon upstream of the sequence encoding the $Pr55^{gag}$ p6 domain.

A 1,209-bp *Pst*I-*Bam*HI containing the entire PRV gD coding sequence was inserted in the *Pst*I site of the polyhedrin transfer vector pGmAc118. Plasmid pGmAc118 has a unique *Pst*I linker inserted at a deletion in the polyhedrin gene sequence spanning nucleotides −10 to +483 from the polyhedrin A(+1)TG start codon. pGmAc118gDΔ was constructed by addition of a 12-mer *Nhe*I linker at the unique blunt-ended *Bsu36*I site (+1129) to provide a deletion of the cytoplasmic domain.

pGmAc118gDΔss was constructed by deleting the gD signal sequence of three N-terminal amino acids, using *Bal*31 exonuclease digestion.

Chimeric plasmid pGmAc118gD/Gag, expressing a gD gene fused with the Gag sequence, was constructed. pGmAc118gD was digested with *Rsr*II (+1200) and blunt ended with Klenow enzyme, and a 12-mer *Nco*I linker was inserted. pGmAc115TGag was digested with *Sna*BI (348 bp downstream of the Gag stop codon), and a 12-mer *Nco*I linker was inserted. pGmAc115TGag was then digested with *Nco*I, and the resulting fragment containing the entire Gag coding sequence was then ligated into the unique *Nco*I site of pGmAc118gD. This chimeric plasmid contains the Gag polyprotein coding sequence fused directly in frame to the gD coding sequence.

Cotransfection and purification of recombinant baculoviruses. Sf9 cells were cotransfected with viral DNA and recombinant transfer vector DNA by the lipofection method (7) (DOTAP; Boehringer, Mannheim, Germany). Recombinations in the p10 locus were done by cotransfecting Sf9 cells with DNA prepared from AcSLp10 virus (4). AcSLp10 virus is a modified baculovirus which possesses only one strong late promoter (the p10 promoter), with the polyhedrin coding sequence inserted downstream of this promoter. Thus, unrecombined AcSLp10 has an occlusion body-positive phenotype. Screening and purification of recombinant viruses were carried out by the common procedure described by Summers and Smith (33).

Construction of dual recombinant baculoviruses. HIV-1 Gag polyprotein and PRV glycoprotein gD were expressed under p10 and polyhedrin promoter control, respectively. The construction of dual recombinant baculoviruses is represented in Fig. 1. The AcSLp10-Gag recombinant contains the Gag coding sequence under p10 promoter control. It was isolated as an occlusion body-negative (Ob^-) phenotype virus. The polyhedrin gene was then reintroduced at its natural locus after cotransfection with DNA prepared from AcSLp10-Gag and the transfer vector pAcI (4), containing the wild-type polyhedrin gene. The baculovirus obtained, AcPh-Php10-Gag, was isolated as an Ob^+ phenotype virus. To insert the gD coding sequence under polyhedrin promoter control, we cotransfected cells with DNA prepared from recombinant AcPh-Php10-Gag and plasmid pGmAc118gD. The dual recombinant baculovirus obtained was isolated as an Ob^- phenotype virus.

Analysis of proteins. Infected insect cells were collected and washed with cold phosphate-buffered saline (PBS). Protein samples resuspended in sample-reducing buffer (17) were boiled (100°C for 5 min) and electrophoresed in sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels with a discontinuous buffer system (17). After electrophoresis, proteins were transferred to a nitrocellulose filter (Schleicher & Schuell; BAS 85, 0.45 mm) in a semidry electroblotter apparatus (Ancos). The nitrocellulose membrane was stained with Ponceau red (Ponceau-S concentrate; Sigma, St. Louis, Mo.) and subsequently blocked with a solution of Tris-saline buffer (TS; 0.05 M Tris-HCl [pH 7.4], 0.2 M NaCl) containing 0.05% Tween 20 and 5% dry milk powder (TS-sat). Gag proteins were detected by using a mouse monoclonal antibody against Pr55-p24 (1:2,000 in TS-sat; Du Pont de Nemours) as the primary antibody and rabbit anti-mouse immunoglobulin G (IgG)-peroxidase conjugate as the secondary antibody (1:1,000 in TS-sat; Sigma). Glycoprotein gD was detected by using a polyclonal anti-PRV hyperimmune serum prepared in a pig (1:40 in TS-sat) as the primary antibody and rabbit anti-pig IgG-peroxidase conjugate as the secondary antibody (1:1000 in TS-sat; Sigma). The immunoreactive bands were visualized by using 1-amino-3-ethyl carbazol as a chromogenic agent (Sigma).

HIV-1 $p24^{gag}$ antigen capture ELISA. The HIV-1 p24 capture enzyme-linked immunosorbent assay (ELISA) kit was purchased from Neosystem (Strasbourg, France), and the ELISA was performed as recommended by the manufacter.

Sucrose gradient purification of VLPs. Culture media were harvested at 48 h postinfection, and most of the cellular debris was discarded after self-sedimentation of the supernatant for 3 h at 4°C. The virus-like particles (VLPs) were then precipitated by adding polyethylene glycol 6000 and NaCl to final concentrations of 8 and 2.3%, respectively, and overnight stirring at 4°C. A protein pellet was collected by centrifugation at 9,000 × *g* for 30 min, resuspended in 10 mM Tris (pH 8.0)–100 mM NaCl–1 mM EDTA (TNE), and layered onto a 20 to 60% linear sucrose gradient made in TNE. Particulates were sedimented through the gradients at 30,000 rpm for 1.5 h (Beckman SW41Ti rotor). Fractions were collected (500 to 1,000 μl) and analyzed by Western immunoblot, as described below.

Preparation for electron microscopy. (i) Thin sectioning. At 2 days postinfection, *S. frugiperda* cells (4×10^6) were harvested (by pelleting at 1,000 × *g* for 5 min) and fixed for 1 h in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Cells were then postfixed for 30 min in 2% osmium tetroxide in the same buffer. Following dehydration in graded series of ethanol or acetone solutions for 10 min each, cells were embedded in Epon 812 resin (Fluka). Thin sections were cut with a diamond knife in an LKB Ultrotome III apparatus, mounted on grids, and subsequently stained with uranyl acetate and lead citrate.

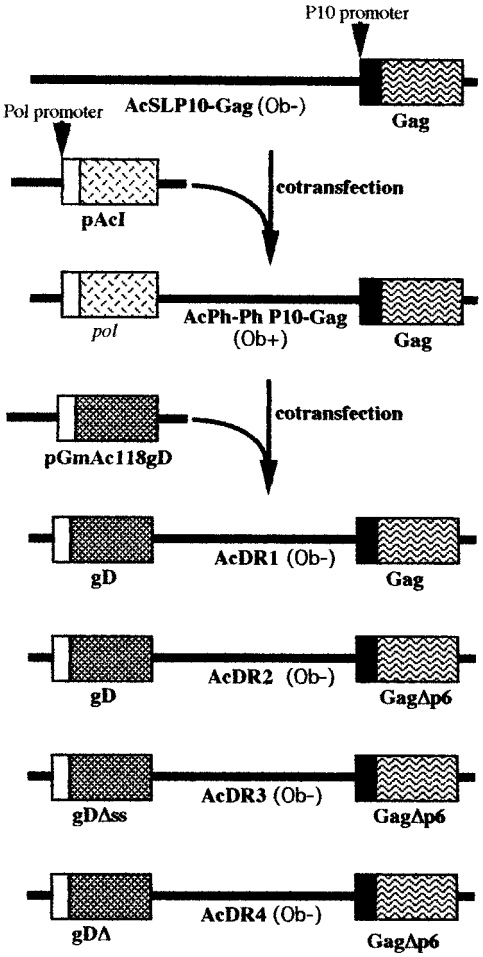

FIG. 1. Construction of dual recombinant baculoviruses AcDR1, AcDR2, AcDR3, and AcDR4. The viral genome (solid lines) is represented as a linear DNA following the orientation proposed by Vlack and Smith (38). The occlusion body (Ob) phenotype character of each virus is indicated in parentheses. Not drawn to scale.

(ii) Immunogold labelling. Infected cells were harvested as previously described and fixed for 1 h in 4% paraformaldehyde–0.2 to 0.5% glutaraldehyde in PBS at 4°C. Following dehydration (ethanol solutions), cells were embedded in LR White resin (Taab). The grids were preincubated twice in NH_4Cl (50 mM in PBS) for 15 min and then saturated for 30 min in PBS containing 1% bovine serum albumin. They were probed with antiserum to Pr55-p24 (Du Pont de Nemours) diluted 1:100 in PBS for 2 h. The grids were then rinsed three times for 10 min each in PBS and probed with a gold-conjugated anti-mouse IgG (10 nm; Biocell) diluted 1:10 in PBS for 45 min. After three rinses of 10 min each in PBS, the grids were stained for 10 min with uranyl acetate. All observations were performed with a Zeiss (Jena) EM 10C/CR electron microscope.

(iii) Scanning electron microscopy. Samples, prepared on Falcon cell culture inserts (Becton Dickinson), were fixed, postfixed, and dehydrated as described above for thin sectioning. The cells were then critical-point dried with liquid CO_2, coated with gold, and examined with a Zeiss DSM 950 electron microscope.

RESULTS

Coexpression of Gag and gD in *S. frugiperda* cells. To try forcing the packaging event between the HIV-1 Pr^{gag} polyprotein and the PRV glycoprotein gD, we constructed a recombinant baculovirus, AcPhgD/Gag, expressing a gD/Gag fusion protein under polyhedrin promoter control. The entire Gag coding sequence was inserted just upstream of the stop codon of the gD coding sequence (see Materials and Methods). West-

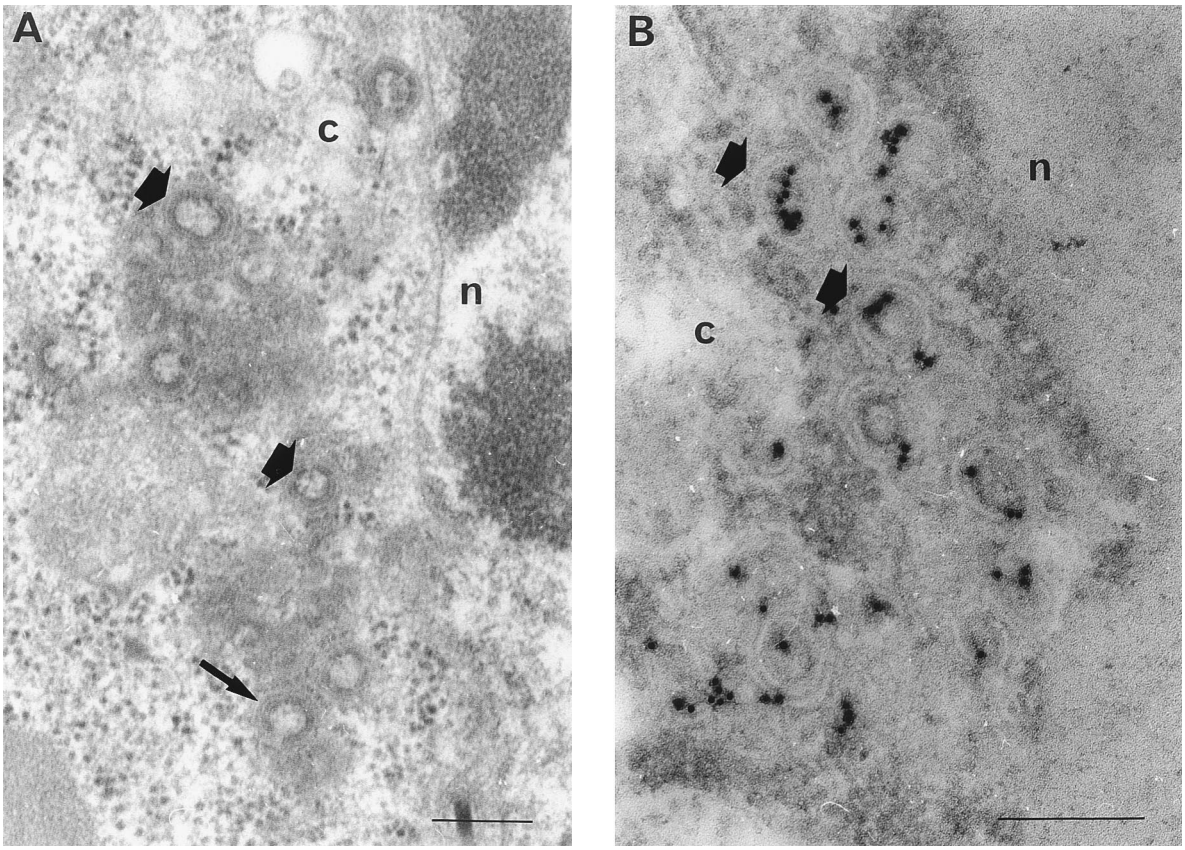

FIG. 2. Thin-section electron microscopy of Sf9 cells harvested at 48 h postinfection with AcPhgD/Gag. Solid arrows indicate intracytoplasmic Gag core-like particles. n, nucleus; c, cytoplasm. (A) Intracytoplasmic Gag particles are seen near the nuclear membrane. The thin arrow indicates the lipid bilayer around Gag particles. Bar, 200 nm. (B) Immunogold staining of intracytoplasmic Gag particles detected with a monoclonal antibody against p24. Bar, 200 nm.

ern blot analysis has shown only trace amounts of a 105-kDa hybrid protein (gD, 50 kDa, and Pr^{gag}, 55 kDa) in cell lysates and a predominance of numerous degradation products (not shown). No immunoreactive product could be detected in the cell culture supernatant.

These data suggest that gD/Gag has been correctly targeted into the endoplasmic reticulum (ER) and glycosylated (gD domain). However, the expressed hybrid protein was severely degraded within the cell. Transmission electron microscopy (TEM) observations showed that the expression of gD/Gag resulted in the accumulation of Gag particles, of various shapes and sizes, only within the cytoplasm near the nuclear membrane (Fig. 2A). Many of these particles contained a lipid bilayer, indicating the budding of Gag polyprotein, probably from the ER membrane. All these particles were specifically gold labelled with monoclonal antibody anti-$HIVp24CA^{gag}$ (Fig. 2B). Taken together, these results showed that fusion between the Gag polyprotein and the glycoprotein gD resulted in cleavage of the hybrid protein, probably by host cellular proteases, leading to budding of Gag particles from the ER or Golgi apparatus membrane.

Because the direct coupling idea did not work, *trans* expression was tried. We constructed a dual recombinant baculovirus, AcDR1, expressing simultaneously the HIV-1 Gag gene and gD gene under p10 and polyhedrin promoter control, respectively (Fig. 1). *S. frugiperda* cells were infected with AcDR1, and at 48 h postinfection, the cell lysates and cell culture medium were characterized by Western blot analysis with monoclonal antibody $HIVp24CA^{gag}$ and a polyclonal anti-PRV serum. As shown in Fig. 3A (lanes 4 and 5), a 55-kDa product was revealed in cell lysates with anti-HIV p24, corresponding to the $Pr55^{gag}$ polyprotein. As previously described (27), additional bands (44 to 45 kDa), representing degradation products by cellular proteases, were observed. A doublet at 67 kDa was also visible, probably a result of ribosomal frameshifting during translation of the Gag open reading frame (21). The electrophoretic profile observed for glycoprotein gD was identical to that obtained with a cellular extract from cells infected with the single PRV gD recombinant AcPhgp50 (Fig. 3B, compare lanes 3 with 4 and 5). The major product with a molecular mass of 50 kDa corresponded to the O-glycosylated form of the protein (35). Several bands with lower molecular masses (32 to 34 kDa) probably resulted from proteolytic degradation. In the infected-cell culture medium, we were unable to detect PRV gD (not shown), suggesting that the glycoprotein was efficiently transported and anchored to the plasma membrane, as observed when gD was expressed from the single PRV gD recombinant.

Examination of cells infected with the dual recombinant AcDR1 by TEM showed abundant production of extracellular budding particles, which appeared very similar to VLPs described previously (9). Infection of cells with AcDR1 led to an efficient release of Gag particles. Quantitative analysis by HIV-1 p24 capture ELISA (Neosystem) showed no significant difference in particle release efficiency between AcDR1 and the single Gag recombinant PhGag (data not shown). However, interestingly, in the case of AcDR1, VLPs were not uniformly distributed on the cell surface. The budding process was

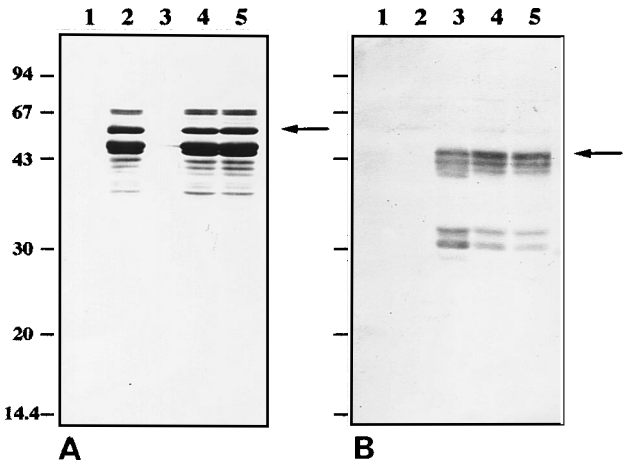

FIG. 3. Analysis of polypeptide patterns of Sf9 cell extracts infected with wild-type *A. californica* multiple nuclear polyhedrosis virus (lanes 1), AcPhPrGag (lanes 2), AcPhgD (lanes 3), and AcDR1 (lanes 4 and 5). Cells were harvested at 48 h postinfection, and cell lysates were analyzed by SDS-PAGE and immunoblotting with a monoclonal anti-p24 Gag antibody (A) and a polyclonal anti-PRV antibody (B). Recombinant proteins are indicated (arrows), as are the positions of markers (in kilodaltons)

limited to well-defined areas of the plasma membrane, forming large VLP aggregates (compare Fig. 4A and B). Moreover, these particles were curiously surrounded with dense knobs of ribosome-like structure, resulting in VLPs presenting a "cogwheel" structure (compare Fig. 4C and D). We also observed such particles when cells were infected with a recombinant baculovirus expressing a truncated form of the Gag polyprotein (27). In this case, Gag polyprotein was deleted of 14 aa in its N-terminal part and of the p6 domain in its C-terminal part (27). This truncated Gag was expressed as a fusion protein with the first 8 aa of the polyhedrin polypeptide. In the absence of the *N*-myristyl group, these Gag core-like particles remained intracellular and were surrounded by knobs, as described here for secreted VLPs (see Fig. 6E and E1). Interestingly, this phenomenon becomes obvious late in infection, when the cytoplasm becomes clear, indicating a firm association between VLPs and ribosomes. Such intracellular particle-ribosome associations were also reported for insect cells infected with recombinant baculovirus expressing bovine immunodeficiency virus Gag polyprotein (25).

Immunogold labelling of thin sections showed specific labelling of VLPs with antibody HIVp24CA^{gag} (Fig. 4E). Unfortunately, because of the high background obtained during immunogold labelling experiments with the polyclonal anti-PRV antiserum, we were unable to detect gD on the VLP structures. However, another anti-PRV polyclonal serum, designated Gλ420 (a kind gift of Hanns-Joachim Rhiza, Tübingen, Germany), provided a weak but specific labelling of VLP aggregates, suggesting colocalization of the Gag and gD proteins (Fig. 4F1). A negative control, realized with cells infected with the single HIV-1 Gag recombinant, showed no labelling of Gag particles (Fig. 4F2). Scanning electron microscopy of AcDR1-infected cells revealed the presence of very large particle clusters budding from the cell membrane, consistent with the TEM observations (compare Fig. 4G and H).

To further characterize the extracellular particles, supernatants from dual-recombinant AcDR1-infected Sf9 cells were sedimented through 20 to 60% sucrose gradients. Fractions were collected from the top of the gradient and examined by Western blot with monoclonal antibody HIVp24CA^{gag} and a polyclonal anti-PRV serum. Retrovirus particles are known to band at a sucrose density of 1.16 g/ml (25). In our case, Gag products band at 1.23 g/ml. Antibodies against glycoprotein gD reacted with a single band corresponding to a molecular mass of 50 kDa in fraction 9 (Fig. 5A). Antibodies against the p24 CA domain reacted with a 55-kDa polypeptide in fractions 6, 7, 8, 9, and 10 (Fig. 5B). In control experiments, sedimentation through a similar gradient of a supernatant prepared from a single PRV gD recombinant-infected Sf9 cells have shown that gD could not be detected at the density corresponding to Gag hybrid particles (not shown). These data indicated clearly that glycoprotein gD was incorporated in VLPs. Whether this event is due to specific interactions between Gag and gD or to nonspecific trapping is unknown.

The carboxy-terminal p6 region is not required for retrovirus-like particle formation in insect cells (27). However, it has been suggested that the p6 domain plays a critical role in the conformation of PrGag polyprotein and might indirectly control both the transport and the assembly of capsid precursors (27). To analyze the role of the p6 domain in the putative gD-Gag interaction, we subsequently constructed a dual baculovirus recombinant, AcDR2, expressing gD and GagΔp6 under polyhedrin and p10 promoter control, respectively (Fig. 1). There is a convenient *Bgl*II site between the p7 and p6 coding sequences, and this was used for truncating the *gag* gene. Western immunoblot analyses have confirmed the expression of gD and GagΔp6 of the expected sizes (Fig. 4C and D). *S. frugiperda* cells were infected with the dual recombinant AcDR2 and examined by TEM. The cell surface was covered with budding particles, as previously described for the dual recombinant AcDR1 (Fig. 5A). In addition, the VLPs obtained were also surrounded with dense knobs (Fig. 6B). Quantitative analysis of Gag production by HIV-1 p24 capture ELISA showed no significant difference between AcDR2 and AcDR1 (data not shown). Sedimentation through 20 to 60% sucrose gradients of supernatants from AcDR2-infected cells (released Gag particle density is 1.23 g/ml) and Western blots of gradient fractions confirmed the incorporation of glycoprotein gD into Gag particles (Fig. 5C and D). These observations clearly indicate that, in this system, the p6 domain is not required for gD incorporation into HIV-1 Gag particles.

Expression of glycoprotein gD with a truncated signal sequence. To ensure that the VLP clustering observed when cells were infected with the dual recombinant is related to the presence of the PRV gD in the cellular membrane, we constructed the dual recombinant baculovirus, AcDR3 (Fig. 1). The PRV gDΔss mutant harboring a nonfunctional signal sequence and GagΔp6 polyprotein were expressed under polyhedrin and p10 promoter control, respectively. In this way, gD transport through the secretory pathway should be significantly reduced.

Western blot analyses of extracts prepared from cells infected with the dual recombinant AcDR3 indicated a very low level of expression of the truncated gD (46 kDa) and the presence of two major additional bands (31 to 32 kDa) due to proteolytic degradation, suggesting that the protein was probably not O-glycosylated (not shown). Furthermore, observations of AcDR3-infected Sf9 cells at 48 h postinfection by TEM showed that the membrane surface was uniformly covered with budding particles, as described for the single Gag recombinant (compare Fig. 4B and 6C). The surface of the VLPs obtained was smooth, with none of the cogwheel structures observed previously with the AcDR1 and AcDR2 recombinants (Fig. 6C). Sucrose gradient sedimentation analysis of infected-cell supernatants showed that AcDR3-induced Gag particles sedimented at a density of 1.16 g/ml, whereas no gDΔss protein could be found, indicating that gDΔss is not incorporated into

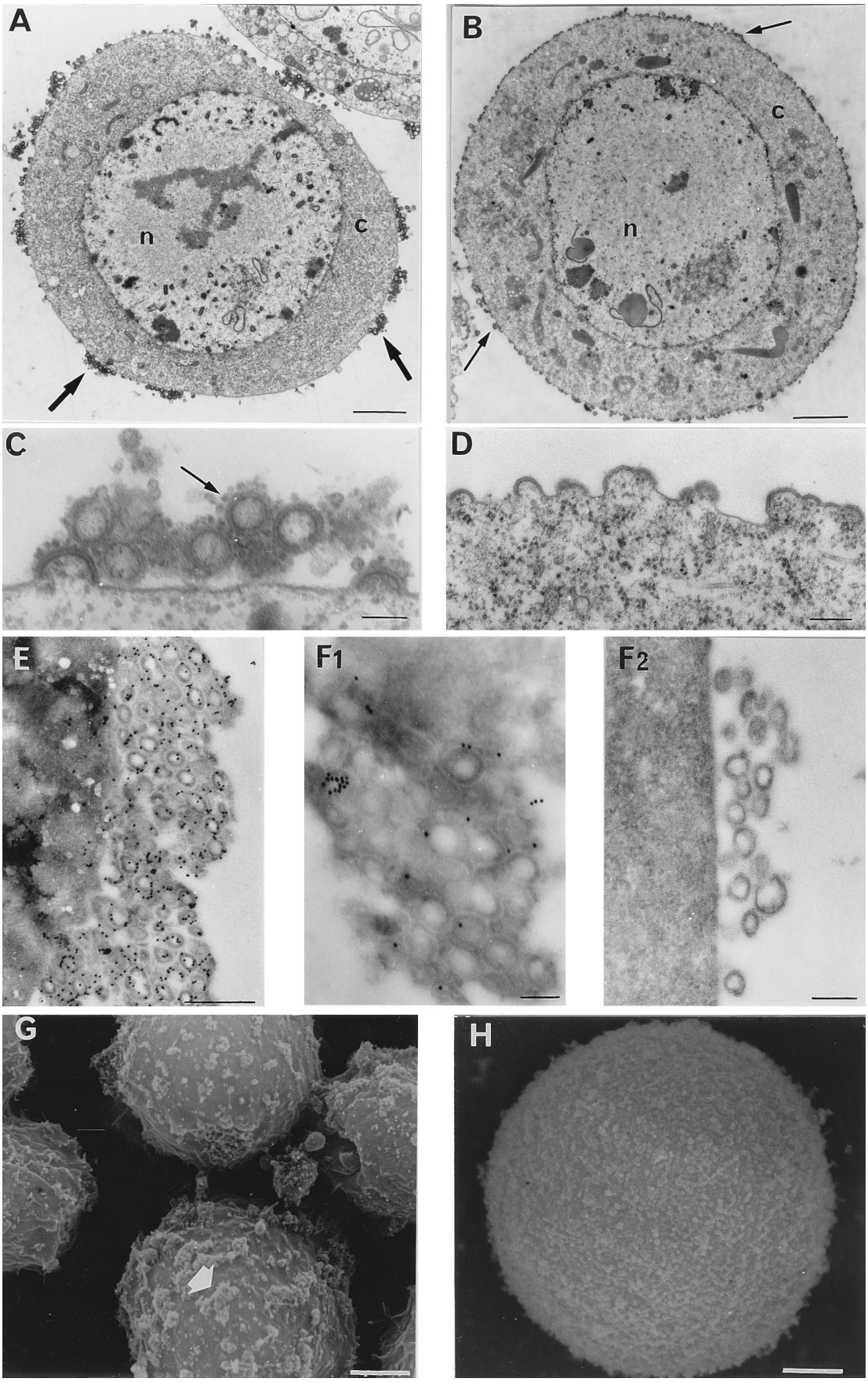
A
n
c
B
c
n
C
D
E
F1
F2
G
H

FIG. 4. Electron miscrocopic analysis of extracellular Gag core-like particles. Sf9 cells were harvested at 48 h postinfection with AcDR1 (A, C, E, F1, and G) or AcPhGag (B, D, F2, and H). n, nucleus; c, cytoplasm. (A, B, C, D, E, F1, and F2) Thin-section electron micscopy. (A) Arrows point to extracellular Gag particle aggregates. Bar, 2 μm. (B) Cell is uniformly surrounded by numerous Gag core-like particles budding from the cellular membrane (arrows). Bar, 2.6 μm. (C) Arrow indicates knobs of ribosome-like structure around extracellular Gag particles. Bar, 100 nm. (D) Budding Gag particles. Bar, 200 nm. (E) Immunogold staining of extracellular Gag particles with a monoclonal antibody against p24. Bar, 500 nm. (F1 and F2) Immunogold labelling with a gD polyclonal antibody, GαPRV (Hanns-Joachim Rhiza, Tübingen, Germany). Bars, 100 nm. (G and H) Scanning electron micrographs. (G) The arrow shows particle clusters budding from the cell membrane. Bar, 2 μm. (H) The surface of the cell is uniformly covered with small particles. Bar, 1.5 μm.

HIV-1 Gag particles (Fig. 5E and F). These results suggest that the gD protein is responsible for the atypical budding of Gag particles in specific areas and that the glycoprotein may determine the sites of VLP release.

Deletion of gD cytoplasmic domain. To analyze the role of the glycoprotein cytoplasmic tail in the putative gD-Gag interaction, we constructed a dual recombinant baculovirus, AcDR4 (Fig. 1). To produce a truncated version of gD, a phase termination codon was inserted into the gene at the *Bsu36*I site, 25 aa from the carboxy-terminal end. The virus recombinant expresses the truncated gD sequence and the GagΔp6 gene under the control of the polyhedrin and p10 promoters, respectively.

SDS-PAGE analysis of infected *S. frugiperda* cells revealed a major product migrating at 45 kDa, corresponding to the O-glycosylated protein lacking 25 aa of the cytoplasmic tail. Several products with lower molecular masses were visible (Fig. 5G).

Insect cells were infected with the dual recombinant AcDR4 and examined at 48 h postinfection by TEM. As previously described for AcDR1-infected cells, the budding in the majority of cells was limited to discrete areas of the plasma membrane (compare Fig. 4A and 6D). However, some cells presented a phenotype similar to that of cells infected with the single Gag recombinant. In all cases, released VLPs did not present the cogwheel structure (Fig. 6D1). Sucrose gradient sedimentation analysis revealed cosedimentation of gDΔ and Gag particles with a density of 1.23 g/ml, indicating that the

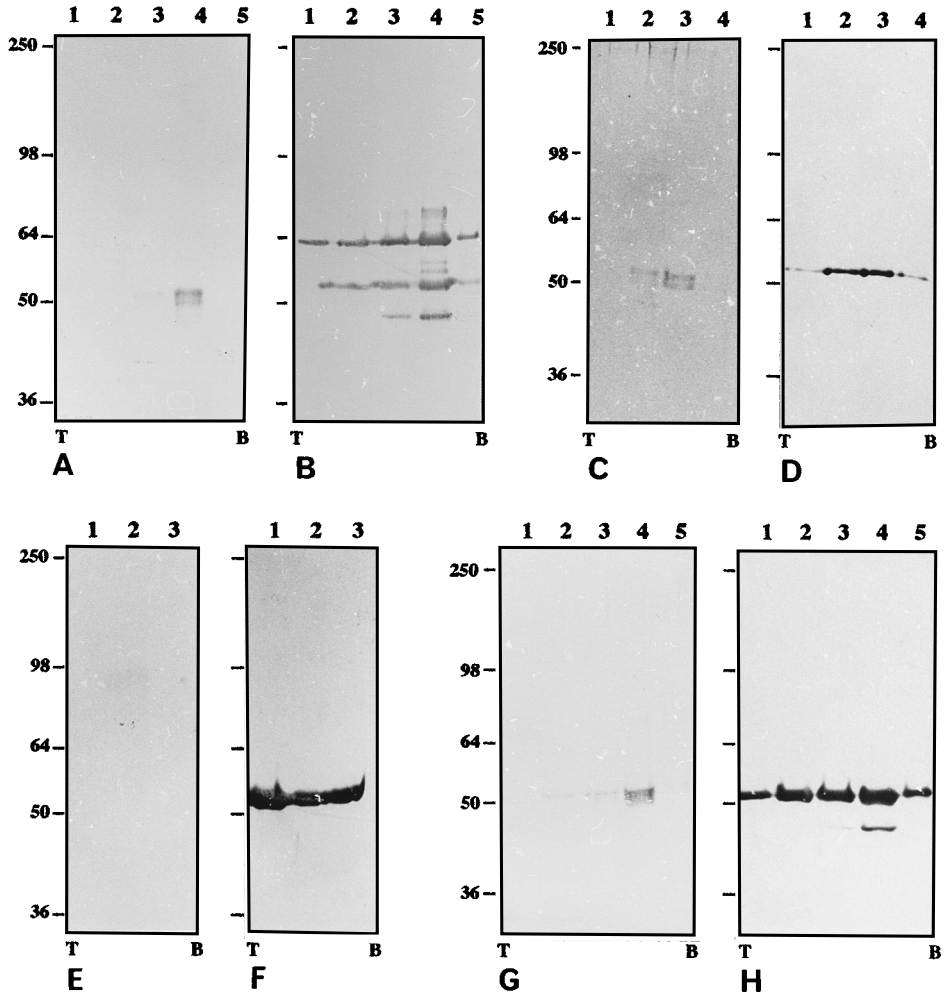

FIG. 5. Western blots after electrophoresis of sucrose gradient-fractionated supernatants from Sf9 cells infected with AcDR1 (A and B), AcDR2 (C and D), AcDR3 (E and F), and AcDR4 (G and H). Blots A, C, E, and G were probed with a polyclonal anti-PRV antibody, and blots B, D, F, and H were probed with a monoclonal anti-p24 Gag antibody. (A and B) Sucrose gradient fractions 6, 7, 8, 9, and 10; (C and D) sucrose gradient fractions 6, 7, 8, and 9; (E and F) sucrose gradient fractions 6, 7, and 8; (G and H) sucrose gradient fractions 5, 6, 7, 8, and 9. The positions of markers are indicated (in kilodaltons). B, bottom; T, top.

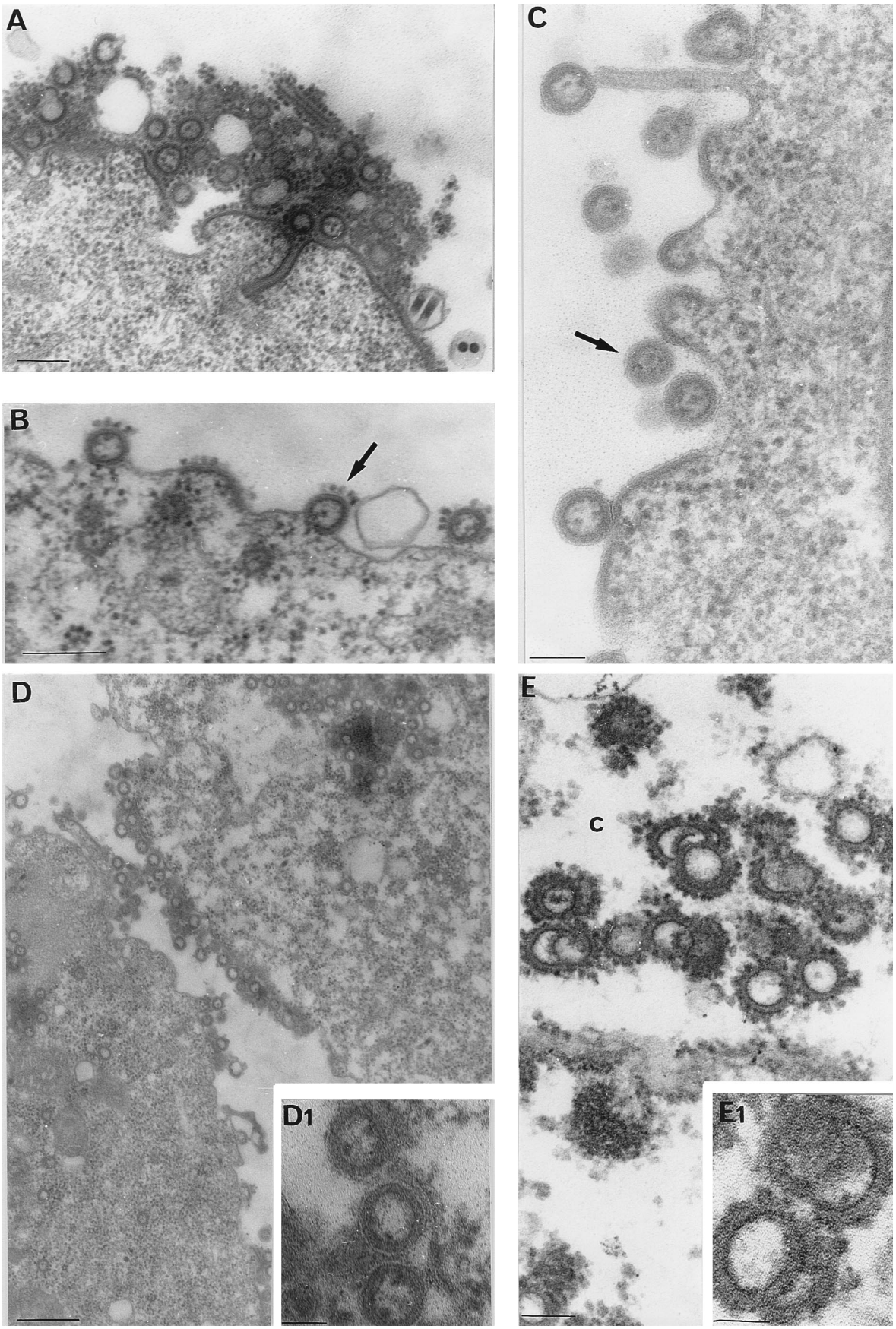
A
B
C
D
D1
E
C
E1

FIG. 6. Thin-section electron microscopy of Sf9 cells infected for 48 h. (A) Cell infected with recombinant AcDR2. Large Gag particle aggregates are visible around the plasma membrane. Bar, 200 nm. (B) Higher magnification of the AcDR2 Gag particles. The arrow points to knobs, as in the text described (Fig. 4C). Bar, 200 nm. (C) Cell infected with recombinant AcDR3. No aggregate is visible. The arrow indicates the extracellular Gag particle smooth surface. Bar, 100 nm. (D) Cell infected with recombinant AcDR4. Budding of Gag particles from the plasma membrane formed large aggregates. Bar, 500 nm. (D1) Higher magnification of extracellular Gag particles, showing no cogwheel structure. Bar, 100 nm. (E) Cell infected with recombinant AcNPV^{gag}170 (27), which produces a truncated protein: the Gag polyprotein is deleted of 14 aa in its N-terminal part and of the p6 domain in its C-terminal part. c, cytoplasm. Intracellular Gag particles are surrounded with dense knobs of ribosome-like structure. Bar, 100 nm. (E1) Higher magnification of intracellular Gag particles. Bar, 50 nm.

cytoplasmic tail is not required for gD incorporation into HIV-1 Gag particles (Fig. 5G and H).

DISCUSSION

The novelty of the HIV-1 VLPs reported in this study is the incorporation of a herpesvirus glycoprotein, PRV gD, into HIV-1 Gag particles in the absence of the other retroviral components. These VLPs were obtained by using a dual recombinant baculovirus which simultaneously expressed the gD protein and the carrier system.

Previously, pseudotypes of HIV-1 were obtained in doubly infected cells (19, 31, 47). Recently, studies have reported that HIV-1 can package the envelope proteins of widely divergent retroviruses in the absence of (i) the homologous envelope proteins and (ii) the heterologous Gag proteins, suggesting the existence of common features between these glycoproteins which might be recognized by both homologous and heterologous assembling core particles (18). However, the reciprocal incorporation of HIV-1 Env glycoprotein into other retroviral capsids has not succeeded (6, 43). Its unusually long cytoplasmic domain may explain these results (12). The selective association of cellular proteins with the surface of retroviral particles along with the viral envelope glycoprotein has also been demonstrated (1, 44).

Specific incorporation of retroviral envelope glycoprotein and exclusion of the majority of cell membrane proteins are controlled by unknown mechanisms (3, 12). However, several data have postulated the requirement of a specific interaction between the transmembrane domain of retroviral glycoproteins and the retrovirus Gag matrix (MA) domain. Previous reports have shown that HIV-1 MA mutants appeared to be defective in Env-associated functions, indicating that the efficient incorporation of HIV-1 envelope glycoprotein into mature virions is dependent on the integrity of MA (6, 45). Moreover, the presence of HIV-1 MA protein sequences is sufficient for the incorporation of HIV-1 Env glycoproteins (gp120 and gp41) into hybrid particles that contain the capsid and nucleocapsid proteins of visna virus (6).

Although our results indicate that the site of Gag particle budding is linked to the presence of the glycoprotein gD, we were unable to demonstrate a specific interaction between the PRV glycoprotein and Gag polyprotein. Interestingly, previous reports also suggested that the HIV-1 Env protein influences the site of budding in polarized epithelial cells (22). Our results showed that a truncated form of the Gag precursor protein, lacking the p6 region, is still capable of incorporating the glycoprotein into budding particles. In addition, the gD cytoplasmic domain is not required for incorporation of the glycoprotein into HIV-1 Gag particles. In our case, the shortening of the cytoplasmic domain might reduce the rate of intracellular transport of the truncated gD glycoprotein, explaining the presence of two types of budding. Previous observations have effectively suggested that the cytoplasmic domains of the Rous sarcoma virus and HIV-1 envelope proteins might (i) play a role in the rate of glycoprotein intracellular transport (24) and (ii) have an effect on the stability of glycoprotein (8). These domains do not appear, by themselves, to be required for the mechanism of incorporation (8, 24, 41). In contrast, some studies indicate that the incorporation of HIV-1 Env glycoproteins into virions required the cytoplasmic domain of the gp41 transmembrane protein (46).

One could hypothesize that glycoprotein gD is able to be incorporated into VLPs without the need for a specific interaction with the Gag polyprotein. Nevertheless, the coinfection of insect cells with two recombinant baculoviruses expressing the feline leukemia virus Gag protein and PRV glycoprotein gD showed that no gD cosedimented with Gag particles (34). Two possibilities might explain this discrepancy: (i) the glycoprotein may interact directly with the HIV-1 Gag polyprotein in a specific manner that allows copackaging, and gD may possess a structural homology with HIV-1 envelope protein, enabling a specific interaction with the Gag matrix or nucleocapsid domain; or (ii) Gag precursors, folded in the cytoplasm, may interact with cellular factors for transport and inclusion in the cell membrane (15). Glycoprotein gD is targeted, after its passage through the secretory pathway, to the plasma membrane and may therefore interact with the same cellular factors.

In this study, the VLPs released were intriguingly surrounded by knobs of ribosome-like structure when insect cells were infected with the dual recombinants AcDR1 and AcDR2. One hypothesis is that interaction with ribosomes might be related to a particular Gag conformation. Such a "competent" folding would naturally occur in the truncated or misfolded forms of Gag polyprotein or be induced during Gag-gD interaction. Thus, the cogwheel structure observed for budding VLPs might be the result of capture of ribosomes liberated into the cell culture supernatant during cell lysis. Alternatively, the knob structures may represent gD complexes, consisting of gD oligomers, as suggested for homolog gD of bovine herpesvirus 1 (36).

The experiments described here also demonstrated that PRV gD glycoprotein cannot be associated with VLPs if the Gag polyprotein is fused directly to its carboxy terminus. The transport of the Gag/gD fusion protein through the secretory pathway is probably stopped in a compartment where it may be sorted into a degradative pathway. Hence, unfolded or misfolded molecules would quickly be degraded inside the rough ER (16).

Further studies are needed to elucidate the incorporation of glycoprotein gD into the VLPs to demonstrate whether specific packaging signals, which might be present on the gD protein, are required. Finally, works are in progress to determine whether the hybrid particles obtained may be useful for efficient protection against PRV.

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REFERENCES

1. **Arthur, L. O., J. W. Bess, R. C. Sowder II, R. E. Benveniste, D. L. Mann, J.-C. Chermann, and L. E. Henderson.** 1992. Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science **258:**1935–1938.
2. **Belyaev, A. S., and P. Roy.** 1992. Presentation of hepatitis B virus $preS_2$ epitope on bluetongue virus core-like particles. Virology **190:**840–844.
3. **Calafat, J., H. Janssen, P. Démant, J. Hilgers, and J. Zàvada.** 1983. Specific selection of host cell glycoproteins during assembly of murine leukaemia virus and vesicular stomatitis virus: presence of Thy-1 glycoprotein and absence of H-2, Pgp-1 and T-200 glycoproteins on the envelopes of these virus particles. J. Gen. Virol. **64:**1241–1253.
4. **Chaabihi, H., M. H. Ogliastro, M. Martin, C. Giraud, G. Devauchelle, and M. Cerutti.** 1993. Competition between baculovirus polyhedrin and p10 gene expression during infection of insect cells. J. Virol. **67:**2664–2671.
5. **Croizier, G., L. Croizier, J. M. Quiot, and D. Lereclus.** 1988. Recombination of *Autographa californica* and *Rachiplusia ou* nuclear polyhedrosis viruses in *Galleria mellonella* L. J. Gen. Virol. **69:**177–185.
6. **Dorfman, T., F. Mammano, W. A. Haseltine, and H. G. Göttlinger.** 1994. Role of the matrix protein in the virion association of the human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. **68:**1689–1696.
7. **Felgner, P. L., and G. M. Ringold.** 1989. Cationic liposome-mediated transfection. Nature (London) **337:**387–388.
8. **Gabuzda, D. H., A. Lewer, E. Terwilliger, and J. Sodroski.** 1992. Effects of deletions in the cytoplasmic domain on biological functions of human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. **66:**3306–3315.
9. **Gheysen, D., R. Yancey, E. Petrovskis, J. Timmins, and L. Post.** 1989. Assembly and release of HIV-1 precursor $Pr55^{gag}$ virus-like particles from recombinant baculovirus-infected insect cells. Cell **59:**103–112.
10. **Griffiths, J. C., E. L. Berrie, L. N. Holdsworth, J. P. Moore, S. J. Harris, J. M. Senior, S. M. Kingsman, A. J. Kingsman, and S. E. Adams.** 1991. Induction of high-titer neutralizing antibodies, using hybrid human immunodeficiency virus V3-Ty virus-like particles in a clinically relevant adjuvant. J. Virol. **65:**450–456.
11. **Griffiths, J. C., S. J. Harris, G. T. Layton, E. L. Berrie, T. J. French, N. R. Burns, S. E. Adams, and A. J. Kingsman.** 1993. Hybrid human immunodeficiency virus Gag particles as an antigen carrier system: induction of cytotoxic T-cell and humoral responses by a Gag:V3 fusion. J. Virol. **67:**3191–3198.
12. **Hunter, E., and R. Swanstrom.** 1990. Retrovirus envelope glycoproteins. Curr. Top. Microbiol. Immunol. **157:**187–253.
13. **Jenkins, O., J. Cason, K. L. Burke, D. Lunney, A. Gillen, D. Patel, D. J. McCance, and J. W. Almond.** 1991. An antigen chimera of poliovirus induces antibodies against human papillomavirus type 16. J. Virol. **64:**1201–1206.
14. **Jestin, A., P. Blanchard, M. Cerutti, T. Foulon, P. Sheldrick, and G. Devauchelle.** 1990. Baculovirus as vector: expression of pseudorabies gp50, abstract P 4-050, p. 160. Abstr. 8th Int. Cong. Virol. 1990.
15. **Klasse, P. J., T. F. Schulz, and K. R. Willison.** 1993. Cyclophilins unfold the gag? Nature (London) **365:**395–396.
16. **Klausner, R. D., and R. Sitia.** 1990. Protein degradation in the endoplasmic reticulum. Cell **62:**611–614.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Landau, N. R., K. A. Page, and D. R. Littman.** 1991. Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J. Virol. **65:**162–169.
19. **Lusso, P., F. D. M. Veronese, B. Ensoli, G. Franchini, C. Jemma, S. E. DeRocco, V. S. Kalyanaraman, and R. C. Gallo.** 1990. Expanded HIV-1 cellular tropism by phenotypic mixing with murine endogenous retroviruses. Science **247:**848–852.
20. **Marchioli, C., R. Yancey, E. Petrovskis, J. Timmins, and L. Post.** 1987. Evaluation of pseudorabies virus glycoprotein gD as a vaccine for Aujeszky's disease in mice and swine: expression by vaccinia virus and Chinese hamster ovary cells. J. Virol. **61:**3977–3982.
21. **Overton, H. A., Y. Fujii, I. R. Price, and I. M. Jones.** 1989. The protease and *gag* gene products of the human immunodeficiency virus: authentic cleavage and post-translational modification in an insect cell expression system. Virology **170:**107–116.
22. **Owens, R. J., J. W. Dubay, E. Hunter, and R. W. Compans.** 1991. Human immunodeficiency virus envelope protein determines the site of virus release in polarized cells. Proc. Natl. Acad. Sci. USA **88:**3987–3991.
23. **Peeters, B., N. de Wind, M. Hooisma, F. Wagenaar, A. Gielkens, and R. Moormann.** 1992. Pseudorabies virus envelope glycoproteins gD and gII are essential for virus penetration, but only gII is involved in membrane fusion. J. Virol. **66:**894–905.
24. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. J. Virol. **61:**2981–2988.
25. **Rasmussen, L., J. K. Battles, W. H. Ennis, K. Nagashima, and M. A. Gonda.** 1990. Characterization of virus-like particles produced by a recombinant baculovirus containing the gag gene of the bovine immunodeficiency-like virus. Virology **178:**435–451.
26. **Riviere, M., J. Tataglia, M. Perkus, E. Norton, C. Bongermino, F. Lacoste, C. Duret, P. Desmettre, and E. Paoletti.** 1992. Protection of mice and swine from pseudorabies virus by vaccinia virus-based recombinants. J. Virol. **184:**417–422.
27. **Royer, M., M. Cerutti, B. Gay, S. S. Hong, G. Devauchelle, and P. Boulanger.** 1991. Functional domains of HIV-1 gag-polyprotein expressed in baculovirus-infected cells. Virology **184:**417–422.
28. **Royer, M., S. S. Hong, B. Gay, M. Cerutti, and P. Boulanger.** 1992. Expression and intracellular release of human immunodeficiency virus type 1 Gag precursors by recombinant baculovirus-infected cells. J. Virol. **66:**3230–3235.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Schlinger, K., M. Mancini, Y. Riviere, D. Dormont, P. Tollias, and M.-L. Michel.** 1992. Human immunodeficiency virus type 1 major neutralizing determinant exposed on hepatitis B surface antigen particles is highly immunogenic in primates. J. Virol. **66:**2570–2576.
31. **Spector, D. H., E. Wade, D. A. Wright, V. Koval, C. Clark, D. Jaquish, and S. A. Spector.** 1990. Human immunodeficiency virus pseudotypes with expanded cellular and species tropism. Virology **64:**2298–2308.
32. **Stephens, E., and R. W. Compans.** 1988. Assembly of animal viruses at cellular membranes. Annu. Rev. Microbiol. **42:**489–516.
33. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin no. 1555. Texas Agricultural Experiment Station, College Station, Tex.
34. **Thomsen, D. R., A. L. Meyer, and L. E. Post.** 1992. Expression of feline leukaemia virus gp85 and gag proteins and assembly into virus-like particles using the baculovirus expression vector. J. Gen. Virol. **73:**1819–1824.
35. **Thomsen, D. R., L. E. Post, and A. P. Elhammer.** 1990. Structure of O-glycosidically linked oligosaccharides synthesized by the insect line Sf9. J. Cell. Biochem. **43:**67–79.
36. **Van Drunen Littel-van den Hurk, S., J. van Donkersgoed, J. Kowalski, S. Tikoo, J. V. van den Hurk, D. Godson, and L. A. Babiuk.** 1994. A subunit gIV (gD) vaccine, produced in tranfected mammalian cells in culture, induces protection against BHV-I in cattle. Abstr. 8th Annu. Bovine Herpesvirus Workshop 1994.
37. **Vaughn, J. L., R. H. Goodwin, G. L. Tompkins, and P. McCauley.** 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro Cell Dev. Biol. **13:**213–217.
38. **Vlack, J. M., and G. E. Smith.** 1982. Orientation of the genome of *Autographa californica* nuclear polyhedrosis virus: a proposal. J. Virol. **41:**1118–1121.
39. **Wang, C. T., J. Stegeman-Olsen, Y. Zhang, and E. Barklis.** 1994. Assembly of HIV GAG-β-galactosidase fusion proteins into virus particles. Virology **200:**524–534.
40. **Weldon, R. A., C. R. Erdie, M. G. Oliver, and J. W. Wills.** 1990. Incorporation of chimeric Gag protein into retroviral particles. J. Virol. **64:**4169–4179.
41. **Wilk, T., T. Pfeiffer, and V. Bosch.** 1992. Retained *in vitro* infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the *env* gene product. Virology **189:**167–177.
42. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral Gag proteins. AIDS **5:**639–654.
43. **Wilson, C., M. S. Reitz, H. Okayama, and M. V. Eiden.** 1989. Formation of infectious hybrid virions with gibbon ape leukemia virus and human T-cell leukemia virus retroviral envelope glycoproteins and the *gag* and *pol* proteins of Moloney murine leukemia virus. J. Virol. **63:**2374–2378.
44. **Young, J. A. T., P. Bates, K. Willert, and H. E. Varmus.** 1990. Efficient incorporation of human CD4 protein into avian leukosis virus particles. Science **250:**1421–1423.
45. **Yu, X., X. Yuan, Z. Matsuda, T. Lee, and M. Essex.** 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J. Virol. **66:**4966–4971.
46. **Yu, X., X. Yuan, M. F. McLane, T. H. Lee, and M. Essex.** 1993. Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions. J. Virol. **67:**213–221.
47. **Zhu, Z., S. S. L. Chen, and A. S. Huang.** 1990. Phenotypic mixing between human immunodeficiency virus and vesicular stomatitis virus or herpes simplex virus. J. Acquired Immune Defic. Syndrome **3:**215–219.