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Hydrophilicity dependent budding and secretion of chimeric HIV Gag-V3 virus-like particles

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Abstract Virus-like particles (VLPs) of numerous viruses have been considered as possible candidates for vaccine development. We have constructed HIV chimeric genes by coupling the *gag* gene of HIV-2 with the V3 domain of the *gp120* gene of either HIV-1 or HIV-2 and expressed the chimeric genes in SF21 cells using the recombinant baculovirus expression system. Although the level of expression of the chimeric HIV-2 *gag* gene with the V3 domain of either HIV-1 *gp120* (*gagC-1V3*) or HIV-2 *gp120* (*gagC-2V3*) was high, the VLP assembly and extracellular release of GagC-1V3 was very poor. In contrast, GagC-2V3 chimeric proteins formed VLPs and released efficiently. We have constructed substitution mutants to investigate the effects of the hydrophobic region of the V3 domain of HIV-1 Gp120 (1V3) in VLP assembly and release. The substitution mutant analyses revealed that in replacing the hydrophobic region of the 1V3 in GagC-1V3 with the hydrophilic sequence of the V3 domain of HIV-2 Gp120 (2V3) enhanced the extracellular VLP. We demonstrate here that disruption of the hydrophobic character of the C-terminus of the chimeric protein improves assembly and release of the VLPs. Our results suggest that the poor GagC-1V3 VLP release was attributed to the hydrophobic region in the V3 sequence of the chimeric protein, and that not only the N-terminal myristylation and positively charged domain of the Gag protein functioned as a targeting signal to direct membrane binding, but also that the C-terminal hydrophobic region affected release of chimeric VLPs.

Keywords Human immunodeficiency virus · Gag virus-like particles · Protein expression · Hydrophilicity · VLP budding

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Introduction

Virus-like particles (VLPs) of numerous virus systems have been considered as possible candidates for vaccine development [1–16]. The expression of unprocessed HIV-1 or HIV-2 Gag proteins in the absence of other HIV proteins leads to the assembly and release of VLPs [1, 2]. Due to its high degree of sequence conservation, Gag protein has been used as an attractive target antigen for cytotoxic-T-lymphocyte-based vaccine development [11, 17].

HIV Gag proteins are targeted to the host cell plasma membrane, and play a crucial role in virion assembly. Although lack of myristylation does not affect particle formation [1, 18], efficient particle budding and release require the N-terminal myristylation of the Gag protein [19]. It has been previously reported that there are two motifs within the matrix (MA) protein of the Gag protein involved in N-terminus plasma membrane targeting. In addition to the N-myristate group, the hydrophobicity of neighboring regions is important for efficient association of the Gag protein with the plasma membrane [20]. HIV

envelope protein Gp120 has been the subject of major investigation for the development of HIV/AIDS vaccine. However, the development of an efficacious HIV/AIDS vaccine based on the Gp120 has been hindered in part by lack of knowledge of the target sites on the proteins that induce potent neutralizing antibodies to primary isolates of the virus. The neutralizing epitopes in HIV-1 Gp120 have been identified at the V2, V3, CD4-binding domain, and the C-terminal region of the V1 loop [21]. The major determinant of entry resides in the V3 region of Gp120 [22] and it is involved in binding to the CCR5 and CXCR4 co-receptors [23]. The V3 loop is also the major determinant of tropism. The C-terminal half of the V3 loop was sufficient to confer on HIV-1_{HXB2} the ability to infect CCR5 expressing cells. The level of infection via CCR5 showed that negatively charged amino acid residues were optimal for interaction whereas hydrophobic residues dramatically reduced interaction [24].

In our previous studies, we have mapped the functional domain for HIV-2 Gag assembly into VLPs. The HIV-2 *gag* gene encodes 519 amino acids (Gag_{519}). Deletion of up to 143 amino acids at the C-terminus of HIV-2 Gag_{519}, leaving 376 amino acids at the N-terminus of the protein, did not affect VLP formation [25]. In this study, the HIV-2 Gag_{425}, deleting 93 amino acids at the C-terminus, was used to fuse with the V3 region. We have analyzed a chimeric *gagC-1V3* gene, which was constructed by coupling the V3 domain (amino acid positions 296–331) of HIV-1 *gp120* (1V3) to the C-terminus of the truncated HIV-2 gag_{425} gene [26, 27]. This chimeric gene was expressed in SF21 cells using a baculovirus expression system, resulting in efficient protein synthesis but poor release. This is due to the hydrophobic region of the 1V3 in the C-terminus of the GagC-1V3 fusion protein, which has been demonstrated by our substitution mutant analyses. Our data demonstrate that the C-terminal hydrophobic region affects budding and release of Gag-V3 chimeric VLPs and results in inefficient particle production.

Materials and methods

Generation of substitution mutants

The *Bgl*II fragments containing the V3 sequences of HIV-1 *gp120* (1V3) and the V3 sequences of HIV-2 *gp120* (2V3) were amplified by PCR, and were inserted into the *Bgl*II site at the 3′ end of the HIV-2 *gag* gene as we have described previously [26]. This recombinant plasmid *gagC-1V3* was used to make an exchange mutation by PCR amplification. A 5′ end primer was designed to create an *Asu*II site at amino acid position 312 of 1V3, and a 3′ end primer was used to introduce a *Ssp*I site at amino acid position 339 of 1V3. The amplified *Asu*II—*Ssp*I fragment of *gagC-1V3* (amino acids 312–339) was used to exchange the *Asu*II—*Ssp*I fragment of *gagC-2V3* (amino acids 331–362). The resultant mutant plasmids were designated as *gagC-1V3E2* for HIV-1 V3 domains with a hydrophilic sequence located at amino acids 331–362 of HIV-2 V3, and *gagC-2V3E1* for HIV-2 V3 domains with a hydrophobic sequence located at amino acids 312–339 of HIV-1 V3.

Generation of recombinant baculovirus

The recombinant baculovirus was generated by co-transfecting SF21 cells with linearized wild-type BacPAK6 viral DNA and transfer vector pBacPAK1 DNA. Liposome-mediated gene transfer was employed, using lipofectin provided in the BacPAK^{TM} Baculovirus Expression System Kit (Clontech Laboratories, Inc.). Several recombinant viral plaques were picked and were verified by DNA sequencing as we have previously described [26]. The newly isolated recombinant virus was purified by a consecutive plaque picking, and used to produce a virus stock of 2×10^8 PFU/ml [2].

Expression analysis and immunoblotting

SF21 cells were infected with either wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) or recombinant AcNPV carrying the chimeric genes at a multiplicity of infection of 5 PFU/cell, and incubated at 27°C. After an appropriate incubation time, cells were harvested and whole cell lysates were prepared by resuspending the cell pellet in water and adding an equal volume of 2× dissociation buffer (10% β-mercaptoethanol, 10% SDS, 25% glycerol, 100 mM Tris–HCI, pH 7.0, 0.04% bromophenol blue). Cell lysates were analyzed in 12% SDS-PAGE, and the protein bands were visualized by staining with Coomassie blue. Western blot analyses were then carried out by using AIDS patient serum provided by the AIDS Research and Reagent Program of the National Institutes of Health and the Bio-Rad Immune Blot AK system to reveal reaction [26, 28].

Isolation of chimeric Gag particle

SF21 cells were infected with recombinant AcNPV at a multiplicity of infection of 5 PFU/cell, and incubated at 27°C for 72 h. The cell culture supernatant was collected after centrifugation at $1{,}000 \times g$ for 20 min. Sedimentable particles in the culture supernatant were collected by ultracentrifugation in an SW 28 rotor at $80{,}000 \times g$ for 1 h, and resuspended in PBS containing 0.1% Tween 20, 10 μg/ml aprotinin and left at 4°C. The chimeric Gag particle band was collected from a 20–60% sucrose gradient,

diluted with PBS, and repelleted in an SW28 rotor at 80,000 × *g* for 1 h. The pellet was gently suspended in PBS and used for further analyses.

Electron microscopy

The infected SF21 cells were detached from the flask by pipetting, pelleted at low speed, and fixed overnight at 4°C with 2.5% glutaraldehyde. The cell pellet was then washed three times in 0.1 M sodium cacodylate buffer (pH 7.4) and then postfixed in 1% osmium tetroxide for 2 h, embedded in Epoxy resin, and stained with 3% uranyl acetate and lead citrate [25, 28]. All grids were examined in a Philip 300 transmission electron microscope (EM).

Results

Construction and expression of chimeric genes

In order to examine whether or not the hydrophobic region in the V3 domain of Gp120 in the GagC-1V3 chimeric protein is responsible for the low level of chimeric VLP secretion, we used the HIV-2 *gag* gene lacking the protease coding sequence [2] to construct two *gag* chimeric genes containing the V3 neutralizing epitope of Gp120 as described previously [26]. As depicted in Fig. 1, *gagC-1V3E2* and *gagC-2V3E1* chimeric genes were constructed by switching the 1V3 hydrophobic region of *gagC-1V3* with the 2V3 hydrophilic sequence of *gagC-2V3*. Both constructs were then used to generate recombinant AcNPV and express the mutated Gag-V3 fusion proteins in SF21 cells. To examine the expression of each chimeric protein, SF21 cells were infected with recombinant AcNPV and whole cell lysates were prepared at day 3 postinfection (p.i.) and analyzed by SDS-PAGE followed by Western blot. Figure 2 shows the proteins produced by infection of recombinant AcNPVs, Ac-*gag*C-1V3E2, and Ac-*gag*C2V3E1. The expected molecular masses of 45 kDa and 55 kDa for the chimeric proteins were observed in the lysates of SF21 cells infected with AcNPV-HIV-2*gag* (lane 1), Ac-*gag*C-2V3E1 (lane 2), and Ac-*gag*C-1V3E2 (lane 3), but were not present in lysates of wild-type infected and uninfected cells. Western blot analyses revealed that the 55 kDa fusion proteins were recognized by HIV-1 positive human sera (Fig. 2 Western blot, lanes 2–3). These results show that the chimeric *gag*-V3 mutants express chimeric proteins at levels, as high as that of recombinant AcNPV-HIV-2*gag* alone.

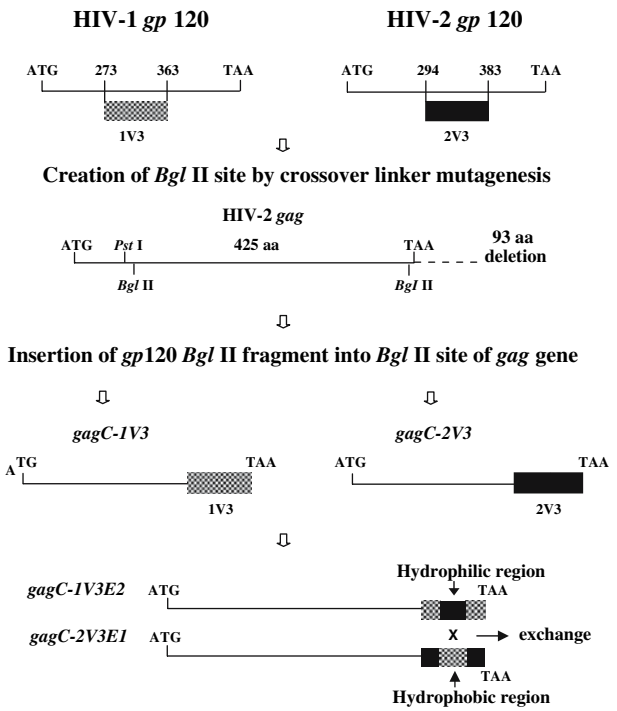

Fig. 1 Construction of chimeric *gag*-V3 gene exchanging the hydrophobic region. *Bgl*II fragments containing the V3 domain (amino acid positions 273–363) from HIV-1 gpl20 (1V3) and the V3 domain (amino acid positions 294–383) from HIV-2 gpl20 (2V3) were amplified by PCR. The V3 fragments were ligated into the *Bgl*II site of the C-terminus of HIV-2 *gag*, as indicated with the checkered box and solid black box. The exchanged hydrophobic and hydrophilic regions from HIV-1 V3 to HIV-2 V3 are indicated with arrows

Substitution of a hydrophobic region with a hydrophilic domain alters the chimeric GagC-1V3 VLP release

The biochemical analyses of *gagC-1V3* and *gagC-2V3* expression have revealed that there are remarkable differences in the secretion of chimeric VLPs. The quantity of released chimeric particles synthesized by the *gagC-2V3*

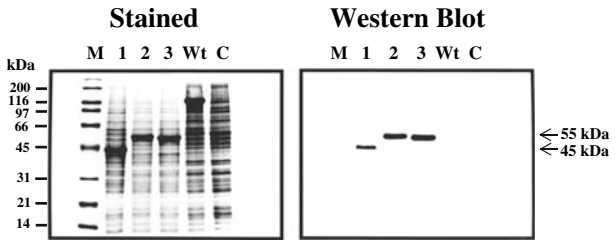

Fig. 2 Expression of chimeric *gag-V3* genes after exchanging the hydrophobic region of HIV-1 V3 with the hydrophilic sequence of HIV-2 V3. Recombinant baculovirus infected SF21 cells were harvested at 3 days p.i., and whole cell lysates were analyzed by 12% SDS-PAGE followed by Coomassie blue staining, and detected by Western blot using HIV positive patient serum. Lanes 1–3, recombinant viruses AcNPV-HIV-2*gag*, Ac-*gag*C-2V3E1, and Ac-*gag*C-1V3E2; M, Marker protein; Wt, wild-type AcNPV-infected cells; C, uninfected cell control. The arrows indicate the positions of immunoreactive Gag, pr45, and chimeric Gag-V3, p55 proteins. The positions of molecular mass standard are given in kilo Dalton (kDa)

gene was about seven-fold higher than that by *gagC-1V3* (data not shown). The C-terminal truncation of the Gag precursor protein was utilized as a carrier to present foreign epitopes; then in comparing the yield of chimeric VLPs from different constructs, we found that low production of the chimeric VLP is due primarily to the high ratio of hydrophobic residues in the V3 region of HIV-1 Gp120. Computer analysis of the hydropathy index of the inserted HIV-1 V3 domain predicts a strong propensity for residues 39–55 (amino acids positions 312–327) to form a hydrophobic region (Fig. 3. *gag*C-1V3). To test whether or not the hydrophobicity affects the budding and release of VLPs, the hydrophobic region of the GagC-1V3 was exchanged for the hydrophilic sequences of GagC-2V3 from residues 37 to 68 located at amino acid positions 331–362 of HIV-2 *gp120* (*gag*C-1V3E2), and the hydrophilic region of GagC-2V3 was replaced with the hydrophobic sequences of GagC-1V3 from residues 39–66 located at amino acid positions 312–339 of HIV-1 *gp120* (*gag*C-2V3E1).

To determine whether or not both mutants had any effect on the synthesis and processing of the chimeric Gag-V3 proteins, SF21 cells were infected with the recombinant AcNPV expressing either GagC-1V3E2 or GagC-2V3E1 proteins. The level of expression of *gagC-2V3E1* and *gagC-1V3E2* genes (Fig. 4A, Cell, lanes 3–4) is similar to those observed in cells expressing the original *gagC-2V3* and *gagC-1V3* genes (Fig. 4A, Cells, lanes 1, 2). To assess the effect of mutations on the VLP assembly and release, the particulate material released into the culture media of the infected cells was pelleted through a 20–60% discontinuous sucrose density gradient and subjected to SDS-PAGE, and detected by Coomassie blue staining. As shown in Fig. 4A and B, the *gagC-1V3E2* shows a remarkable increase in the level of extracellular particle release. In contrast, the quantitation of proteins associated with the released VLPs, measured by densitometry, indicated that the *gagC-2V3E1* reduced the release of chimeric VLPs (Fig. 4B). These results suggest that the hydrophobic domain of the V3 region of HIV-1 Gp120 is responsible for the low level VLP release.

Electron microscopic analyses of SF21 cells expressing gagC-IV3, gagC-2V3, gagC-lV3E2, and gagC-2V3E1

To analyze the VLP formation and release, each of the four recombinant AcNPV-infected SF21 cells were examined by thin-section transmission electron microscopy (EM). First, we compared the assembly and morphogenesis of the original GagC-1V3 and GagC-1V3E2 mutant. In GagC-1V3, the VLPs were found predominantly in the cytoplasm and accumulated as clusters within the nucleus, as indicated by white arrows (Fig. 5A). In contrast, the replacement of the hydrophobic region of 1V3 with the hydrophilic region of 2V3 enhanced the budding of GagC-1V3E2 particles through the cytoplasmic membrane (Fig. 5B). These results indicate that the substitution of the hydrophobic region of 1V3 with the hydrophilic region of 2V3 improved the GagC-1V3E2 particle localization to the cell membrane and resulted in efficient particle release into the culture medium (Fig. 4). The original *gag*C-2V3 produced high yields of secreted chimeric particles (Fig. 6A). However, after the exchange of the hydrophilic sequence of 2V3 with the hydrophobic sequences of 1V3, the GagC-2V3E1 VLPs accumulated in the intravacuolar spaces and in the nucleus. The GagC-2V3E1 VLPs were localized in the nucleus and particles budding through the plasma membrane were dramatically reduced (Fig. 6B). These results are consistent with the biochemical data shown in Fig. 4 and clearly demonstrate that the substitution of

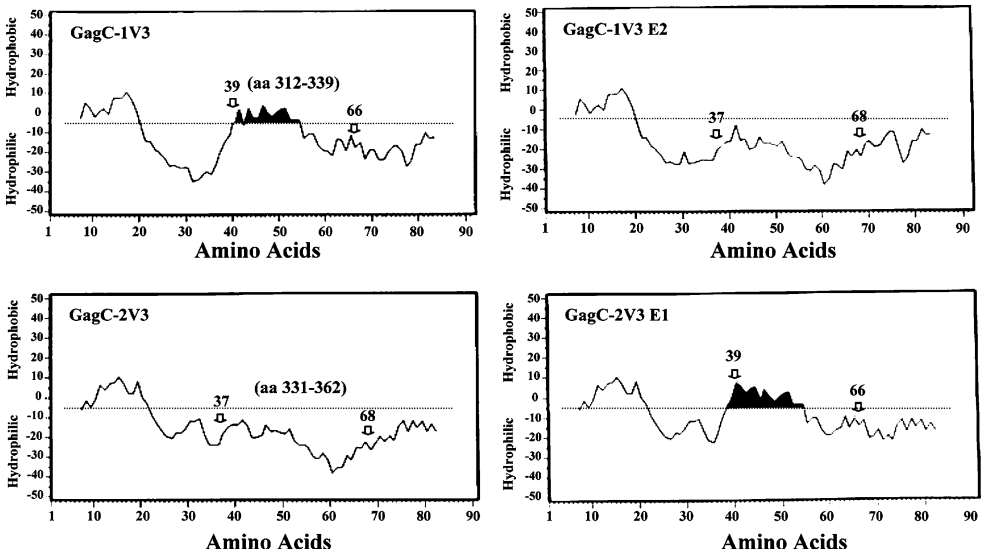

Fig. 3 Hydropathy profiles of the V3 domains of HIV-1 and HIV-2. Hydrophobicity and hydrophilicity plots of the GagC-1V3, GagC-2V3, GagC-1V3E2, and GagC-2V3E1 were generated using the SOAP program in the PC/Gene computer program (version 5.11) with an interval of nine amino acids. A hydrophobic region is indicated in the plots by the peak filling with black. The exchange positions of the amino acids from GagC-1V3 to GagC-2V3 are indicated by empty arrows, and GagC-1V3E2 and GagC-2V3E1 were generated

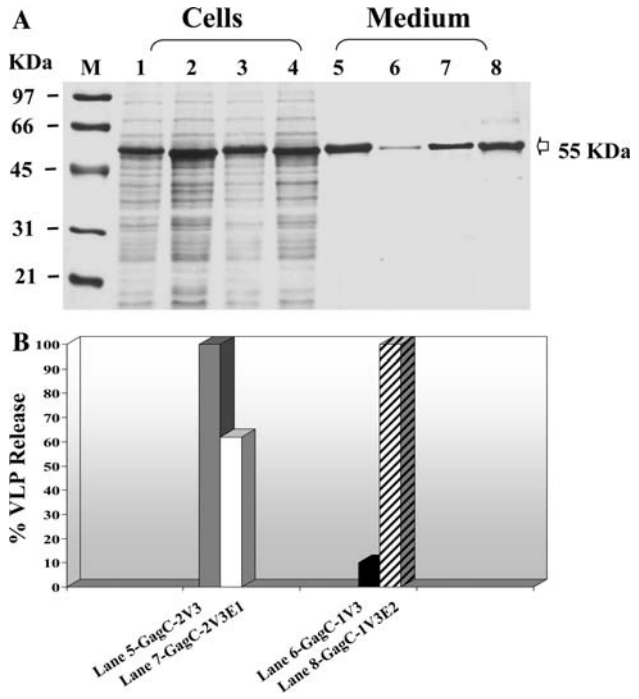

Fig. 4 SDS-PAGE analyses of Gag-V3 exchanging hydrophobic region with hydrophilic sequence. (**A**) Recombinant AcNPV infected SF21 cells and the culture media were harvested 3 days after infection and equal amounts were analyzed in 12% SDS-PAGE. GagC-2V3 (lanes 1 and 5), GagC-1V3 (lanes 2 and 6), GagC-2V3E1 (lanes 3 and 7), and GagC-1V3E2 (lanes 4 and 8). M, molecular marker protein. Expressing chimeric protein are indicated by the arrow. The positions of molecular mass are given in kDa. (**B**) Quantification of chimeric Gag-V3 particle release by densitometry. The 100% represents the VLP release from GagC-2V3 (Lane 5) and the VLP release from GagC-1V3E2 (Lane 8)

hydrophobic amino acids in the 1V3 region with hydrophilic amino acids of 2V3 greatly increases the budding and release of chimeric VLPs. The essential sequence for VLP localization and budding appears to reside in the V3 region of *gag*C-1V3.

Discussion

We have previously suggested that there are many advantages in using the unprocessed HIV-2 Gag precursor protein as a carrier for either the neutralizing epitopes or cytotoxic T cell epitopes of HIV-1 for AIDS vaccine development [25, 26, 28, 29]. Some viral gene products assemble virus-like particles (VLPs) in the absence of other viral structural proteins or the absence of the virus genome. VLPs have been derived from HIV-1 Gag [1, 8, 11], HIV-2 Gag [2], L1 major capsid protein of papillomavirus [3], VP60 of rabbit calicivirus [4], VP2 of bluetunge virus [5], VP2 of parvovirus [7], Norwalk virus VP2 [9], the major structural protein VP1 of polyomavirus [10], ORF2 of hepatitis E virus [12], and co-expression of the glycoprotein GP and matrix protein VP40 of Ebola virus [14]. These VLPs have been produced in insect cells or in mammalian cells and VLPs are highly immunogenic regardless of the expression system used. VLPs can be assembled from either the monomeric capsid protein or a combination of two or more capsid proteins. High-level expression of VLPs is highly desirable in order to produce large quantities of VLPs for vaccine formulation. Accordingly, many different gene expression systems using both animal and insect cell systems have been employed. High-level production of chimeric Gag-Env VLPs will be of great advantage in the production of antigens for HIV/AIDS vaccine development.

In this report, we examined the hydrophobic and hydrophilic sequences in the V3 loop of HIV-1 and HIV-2, which were fused with the HIV-2 Gag protein and incorporated into the C-terminus of the Gag protein in different constructs for protein expression and particle release. According to the four chimeric gene clones used in this study and other *gag-env* clones in a previous study (data not published), the level of yield of chimeric particles was not only determined by the insertion size of the V3 sequence, but also by its C-terminal amino acid composition and arrangement. The structure-function analyses have revealed a remarkable difference between GagC-1V3 and GagC-2V3 production of secreted chimeric particles. The replacement of hydrophobic amino acids in the V3 region of HIV-1 gp120 with the hydrophilic sequences in the V3 domain of HIV-2 gp120 resulted in efficient particle budding and release into the culture media. Our study indicates the importance of C-terminal hydrophobic 12 amino acids in 1V3 in preventing chimeric particles from extruding the plasma membrane and releasing into the extracellular media. This implies that the hydrophobic segment in the V3 region of GagC-1V3 is essential for anchoring the particles to the membrane. This result indicated that the arrangement of the hydrophobic amino acids at the end of the C-terminus of the chimeric Gag-1V3 greatly hampered the release of extracellular chimeric particles. To support these conclusions, we have also examined the myristylated form of the HIV-2 Gag protein, which is used as a carrier to form chimeric VLPs. Previous reports demonstrated that N-terminal myristylation is critical for the association of the Gag protein with the plasma membrane and subsequent release [1, 18, 30, 31]. The nonmyristylated $pr55^{gag}$ protein can assemble into intracellular particulate structures and accumulate in the cytoplasm and nucleus, but virus release failed to occur [32]. To address this question, HIV-2 Gag pr45 was labeled with [H^3] myristic acid and visualized by autoradiography after SDS-PAGE. We found that truncated Gag pr45 is correctly myristylated and released into the culture media. The same results were obtained from

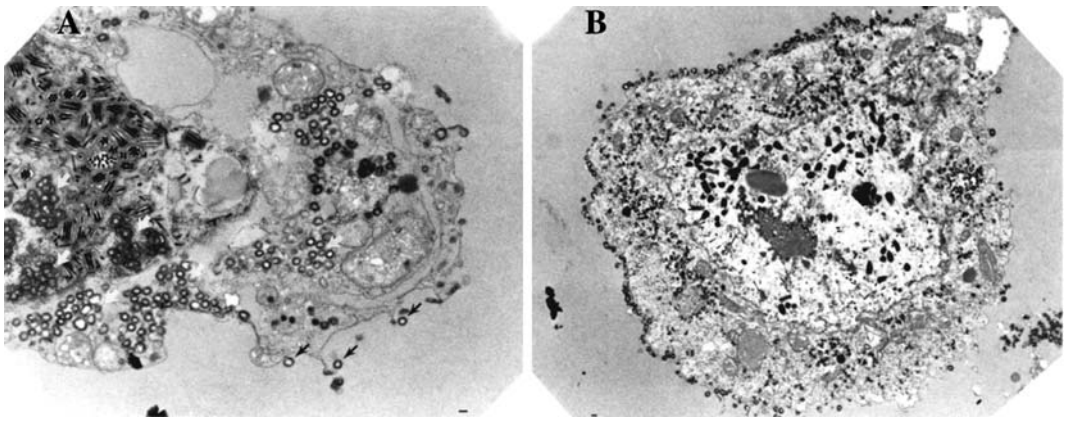

Fig. 5 Electron micrographs of thin sections of SF21 cells infected with recombinant Ac-*gag*C-1V3 and Ac-*gag*C-1V3E2 at 72 h postinfection. (**A**) A number of chimeric particles were found predominantly in the cytoplasm of Ac-*gag*C-1V3-infected SF21 cells. The white arrows point to the accumulating clusters within the nucleus and intravacuols. Only a few extracellular particles have been observed, as indicated by solid arrows. (**B**) After exchanging the hydrophobic region with hydrophilic sequences, a large number of budding and free extracellular VLPs were found adjacent to the plasma membrane of Ac-*gag*C-1V3E2-infected cells. The bars in A (bottom right) and B (bottom left) represent 100 nm

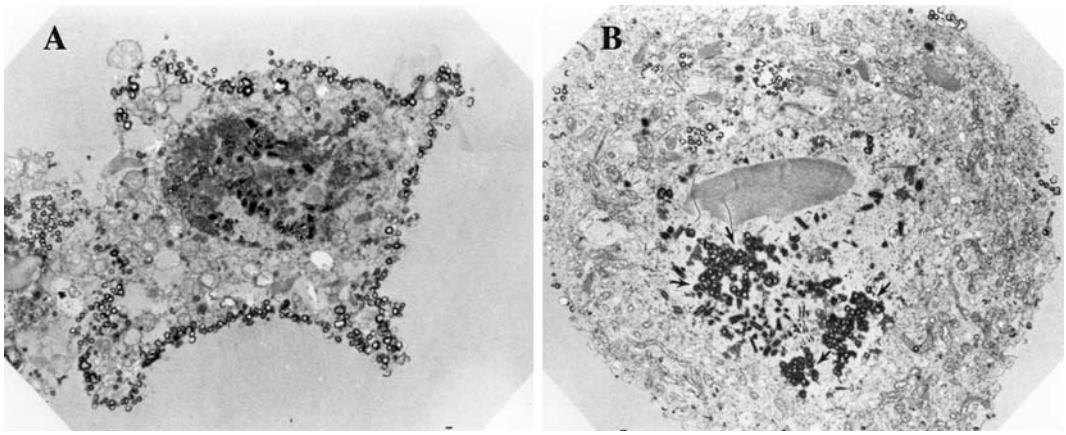

Fig. 6 Electron microscopy of recombinants Ac-*gag*C-2V3 and Ac-*gag*C-2V3E1-infected SF21 cells. The SF21 cell cytoplasmic membrane is covered with budding chimeric VLPs of GagC-2V3 at 72 h p.i. (**A**). The replacement of 2V3 by the hydrophobic region of 1V3 results in the accumulation of chimeric VLPs within the nucleus, and in the intravacuolar space. Only a small number of extracelluar particles can be seen on the cytoplasmic membrane, as indicated by empty arrows (**B**). The bars in A (bottom right) and B (bottom left) represent 100 nm

chimeric GagC-1V3 and GagC-2V3 (data not shown). These results suggest that the defect in extracellular GagC-1V3 VLP release is not due to a lack of Gag myristylation. Instead, the C-terminal hydrophobic sequences in the GagC-1V3 play an important role for markedly impaired extracellular particle production. The result presented in this article is the first to demonstrate that the C-terminal hydrophobic region of fusion Gag-V3 affects VLP release.

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