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Comparative Analysis of the Roles of Simian Immunodeficiency and Bovine Leukemia Virus Matrix Proteins in Gag Assembly in Insect Cells

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The role of the matrix (MA) domain of simian immunodeficiency virus (SIV) and bovine leukaemia virus (BLV) Gag in the assembly of virus-like particles (VLP) in insect cells has been investigated. Wild-type SIV and BLV Gag assembled to form discrete VLP structures typical of many retroviruses analysed by similar systems. When amino acids predicated by the three-dimensional structure to be at the interface of SIV MA monomers were deleted, VLP assembly was abolished consistent with a role for MA multimerization in assembly. When amino acids predicted to be in the analogous positions in BLV MA were mutated, however, VLP assembly was not affected. These data indicate that the models of assembly derived from one model retrovirus may not necessarily apply to more distantly related viruses despite the structural similarity present in equivalent Gag domains. © 2002 Elsevier Science (USA)

INTRODUCTION

The retroviral Gag protein is the minimal particle-forming unit of the virus (Delchambre *et al.,* 1989; Gheysen *et al.,* 1989; Haffar *et al.,* 1990; Mergener *et al.,* 1992; Smith *et al.,* 1990). Particle assembly begins with association of the Gag polyprotein with the host cell plasma membrane and is followed by immature virus-like particle budding from the expressing cell. The particle finally becomes infectious after a maturation reaction which begins at, or soon after, budding and in which the viral protease cleaves the Gag polyprotein into its constituent parts, the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. Of these proteins, MA provides a significant structural component of the virus and has a multifunctional role in viral assembly and morphogenesis.

The three-dimensional structure of the MA proteins of lentiviruses such as simian (SIV) and human (HIV) immunodeficiency viruses (Hill *et al.,* 1996; Rao *et al.,* 1995) have provided structural explanations for the various roles played by MA in virion assembly and associated events. These include incorporation of the envelope glycoproteins, membrane targeting, and morphogenesis (Chazal *et al.,* 1994; Dorfman *et al.,* 1994; Freed and Martin, 1995; Gonzalez *et al.,* 1993; Mammano *et al.,* 1995; Morikawa *et al.,* 1995; Rhee and Hunter, 1991; Yu *et al.,* 1992; Yuan *et al.,* 1993). Both the SIV and the HIV MAs are trimeric (Belyaev *et al.,* 1994; Hill *et al.,* 1996; Rao *et al.,* 1995) and the features of the trimer have provided rational explanations for Gag interaction with the inner viral membrane and with Env (Hill *et al.,* 1996; Rao *et al.,* 1995). The trimeric structure of MA is also consistent with current models of virus assembly (Forster *et al.,* 2000).

The three dimensional solution structure of bovine (BLV) leukaemia virus MA monomers, obtained by heteronuclear magnetic resonance (NMR), reveals a much simpler molecule than the SIV MA monomer but has been modelled, on the basis of the SIV MA trimer, to provide a very similar trimeric structure (Matthews *et al.,* 1996). Similarly, the monomeric MA domain of Mason-Pfizer monkey virus (M-PMV), a type D retrovirus, has been also modelled as a trimer (Conte *et al.,* 1997). The obvious structural relatedness evident in the MA domains from diverse retroviruses, despite low sequence homology, has also been shown to extend to other Gag domains (Turner and Summers, 1999).

In our previous article we have shown that MA and CA domains could efficiently be exchanged between these two members of the lentivirus and leukovirus group of retroviruses without any perturbation of Gag assembly and budding (Kakker *et al.,* 1999). However, the extent to which MA trimerization is an assembly intermediate shared by both groups was not investigated. In the present study, mutagenesis and expression of the SIV MA domain of Gag has been used to confirm the role of the MA trimer in VLP assembly. In addition, based on the proposed trimeric BLV MA structure (Matthews *et al.,* 1996), equivalent mutations in the MA domain of BLV

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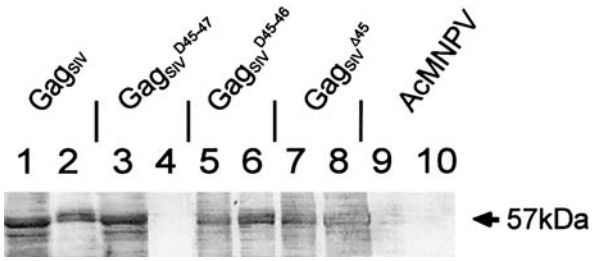

FIG. 1. Western blot analysis of wild-type and mutant SIV Gag expression and particle release in insect cells. Cell lysates and supernatants were resolved by 10% SDS–PAGE and immunoblotted using a monkey anti-SIV serum. The five virus-infected samples, four tests, and a control are indicated above the lanes. Lanes 1, 3, 5, 7, and 9 are cell lysates. Lanes 2, 4, 6, 8 and 10 are supernatants. The identity of SIV $Pr57^{Gag}$ is indicated by the arrowhead.

Gag were constructed and their effect on BLV VLP assembly assessed.

RESULTS AND DISCUSSION

Construction and expression of mutant SIV Gag

The SIV MA trimer has been suggested to be a fundamental assembly intermediate of the Gag shell (Rao *et al.,* 1995). The SIV MA is predominantly helical, consisting of seven helices (H1 to H7) with helix 4 critical for positioning the interacting residues for presentation to the adjacent molecule. The trimer is held together by hydrogen bonds between Gly^{45}-Leu^{46}-Ala^{47} of one monomer and Ser^{72}-Gly^{71}-Thr^{70} of the adjacent monomer. To investigate the role of residues Gly^{45}, Leu^{46}, Ala^{47} in MA oligomerization in context of the full-length SIV Gag, four recombinant baculoviruses were constructed that expressed SIV Gag and mutants thereof. AcS*gag* encoded the wild-type SIV Gag while AcS$gag^{\Delta 45-47}$, AcS$gag^{\Delta 45-46}$, and AcS$gag^{\Delta 45}$ encoded SIV Gag proteins lacking three (Gly^{45}-Leu^{46}-Ala^{47}), two (Gly^{45}, Leu^{46}), or one (Gly^{45}) MA residue, respectively. To ensure all the mutant Gag proteins were expressed at a significant level and to assess assembly and release, *Spodoptera frugiperda* (*Sf*9) cells were infected with each recombinant baculovirus and the expression and distribution of SIV wild-type or mutant Gag protein was examined by SDS–PAGE and Western blot. AcS*gag*-infected *Sf* cells expressed a protein of molecular weight of 57 kDa that was recognised specifically by a monkey anti-SIV serum in Western blot analysis (Fig. 1) and corresponded to a size which agreed with that previously published for unmodified SIV Gag (Delchambre *et al.,* 1989). No SIV-related proteins were detected in control AcMNPV-infected *Sf* cells. Recombinant viruses AcS$gag^{\Delta 45-47}$, AcS$gag^{\Delta 45-46}$, and AcS$gag^{\Delta 45}$ also expressed a 57-kDa protein that was specifically recognised by an anti-SIV serum (Fig. 1) in keeping with the minor deletions of sequence made during mutagenesis.

AcS*gag*-infected cell supernatants showed the presence of abundant SIV $Pr57^{Gag}$ polyprotein typical of the phenotype of VLP competent retroviruses, and absent from the AcMNPV control (Fig. 1). Similarly, AcS$gag^{\Delta 45-46}$ or AcS$gag^{\Delta 45}$-infected cells contained Gag antigen at levels similar to those observed for the parental SIV $Pr57^{Gag}$. In contrast, supernatants from *Sf* cells infected with AcS$gag^{\Delta 45-47}$ showed no evidence of Gag protein (Fig. 1), suggesting removal of MA amino acids 45–47 prevented Gag assembly or release.

SIV $Gag^{\Delta 45-47}$ does not assemble into VLPs

To further characterise the wild-type and mutant SIV Gag polyproteins expressed in this system, insect cells infected with each recombinant virus were fixed at 2 days postinfection and processed for thin-section electron microscopy (Hockley *et al.,* 1994). Numerous VLPs at different stages of assembly and budding were detectable at the plasma membrane of *Sf*9 cells infected with AcS*gag* (Fig. 2A), AcS$gag^{\Delta 45-46}$ (Fig. 2C), or AcS$gag^{\Delta 45}$ (Fig. 2D). However, no retrovirus-like budding particles were detected in sections of AcS$gag^{\Delta 45-47}$-infected *Sf* cells (Fig. 2B), confirming the VLP negative phenotype of this mutation and in marked contrast to deletion of one (AcS$gag^{\Delta 45}$) or two (AcS$gag^{\Delta 45-46}$) residues at the monomer–monomer interface. Low electron density at the plasma membrane of AcS$gag^{\Delta 45-47}$-expressing cells sug-

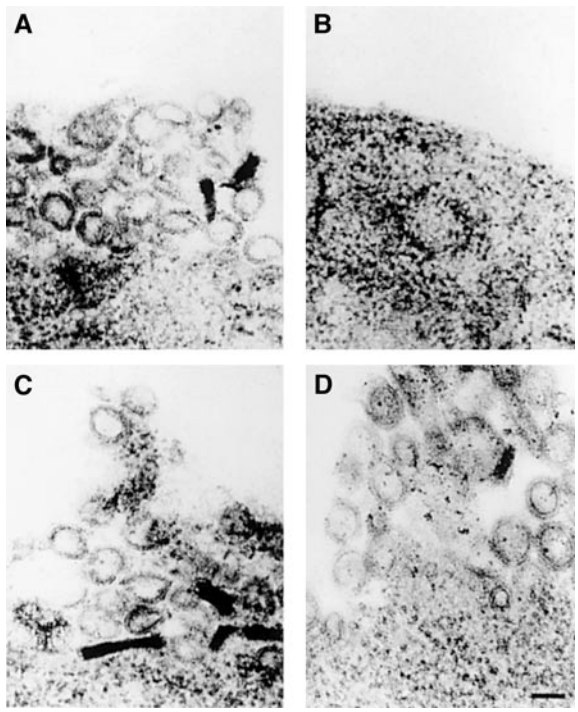

FIG. 2. Electron micrographs of thin sections of *Sf* cells infected recombinant baculovirus expressing SIV $Pr57^{Gag}$ (quadrant A); SIV $Gag^{\Delta 45-47}$ (quadrant B); SIV $Gag^{\Delta 45-46}$ (quadrant C); and SIV $Gag^{\Delta 45}$ (quadrant D). Note the absence of budding or free VLP in quadrant B. The bar represents 100 nm.

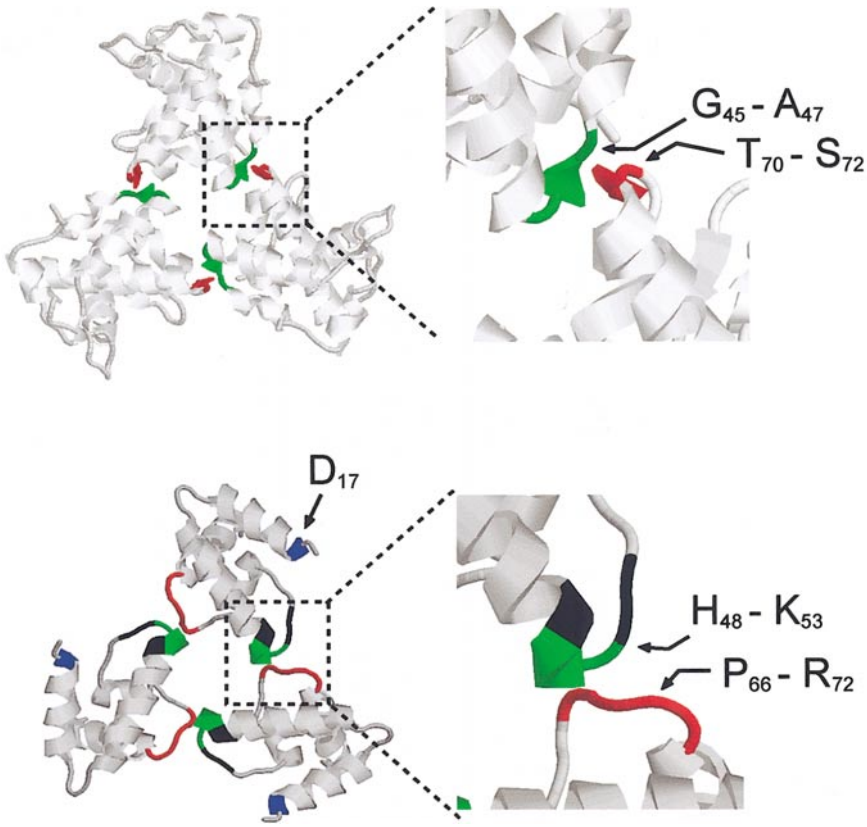

FIG. 3. Backbone structures of the SIV MA trimer (above) and the proposed BLV MA trimer model (below). Areas of monomer–monomer interaction are shown enlarged on the right.

gested that non-membrane-localised Gag may be largely degraded, confirming data obtained recently by pulse chase experiments (Tritel and Resh, 2000). As the positively charged flat surface produced by MA trimerisation has been suggested to be the basis of membrane localisation (Hill *et al.,* 1996; Zhou *et al.,* 1994), an indirect consequence of failure to trimerize could be reduced membrane residency. Thus, mutations constructed on the basis of the SIV MA crystal structure could be interpreted as providing tacit support for a role of MA trimerization, in the context of full-length $Pr57^{Gag}$, in SIV VLP assembly.

Construction and expression of mutant BLV Gag

As the data obtained following targeted mutagenesis of the MA domain of SIV Gag provided a plausible role for the SIV MA trimer interface in VLP assembly, the extent to which this was also the case for BLV Gag was investigated. The BLV MA monomer consists of four α-helices: A (Ser^{14} to Asn^{29}), B (Ser^{32} to Gln^{51}), C (Phe^{57} to Cys^{65}), and D (Val^{73} to Leu^{85}), separated by short, semi-structured loops (Matthews *et al.,* 1996), a topology similar to the first four α-helices of the SIV MA (Fig. 3). In addition, there is a striking similarity between the trimer interface loops (Gly^{45}-Ala^{47} and Thr^{70}-Ser^{72}) of SIV MA and the loops at the junctions of helices B-C and C-D in BLV MA and spanning the regions around Gln^{51}-Lys^{69} (Fig. 3). In the proposed trimeric BLV MA, residues Lys^{49}-Gln^{51} in the loop between helices B and C of one monomer, and Pro^{67}-Arg^{72} between helices C and D of the adjacent monomer, have been postulated to form the trimer interface (Matthews *et al.,* 1996). To assess the role of these residues in BLV VLP assembly, recombinant baculoviruses were constructed in which a deletion window of between three and five amino acids was moved through these proposed interaction domains. In addition, a double deletion, in which key residues on opposing faces of the MA monomer were removed, was also constructed. AcB$gag^{\Delta 49-51}$ lacked Lys^{49}-Gln^{51}; AcB$gag^{\Delta 66-68}$ lacked Pro^{66}-Gly^{68}; AcB$gag^{\Delta 67-69}$ lacked Pro^{67}-Lys^{69}; AcB$gag^{\Delta 68-70}$ lacked Gly^{68}-Phe^{70}; AcB$gag^{\Delta 68-71}$ lacked Gly^{68}-Gly^{71}, and AcB$gag^{\Delta 68-72}$ lacked Gly^{68}-Arg^{72}. The double-deletion AcB$gag^{\Delta 49-51,\Delta 68-72}$ was also produced. Recombinant baculoviruses were isolated and amplified as described before high multiplicity infections of *Sf*9 cells and analysis of their expression profile by SDS–PAGE and Western blot.

Recombinant baculovirus AcB$gag^{\Delta 49-51}$ expressed a recombinant protein of ~44 kDa that was identified specifically in cell lysates on Western blots using a rabbit anti-BLV serum (Fig. 4). The identified band comigrated with wild-type BLV $Pr44^{Gag}$ expressed in BLV-infected

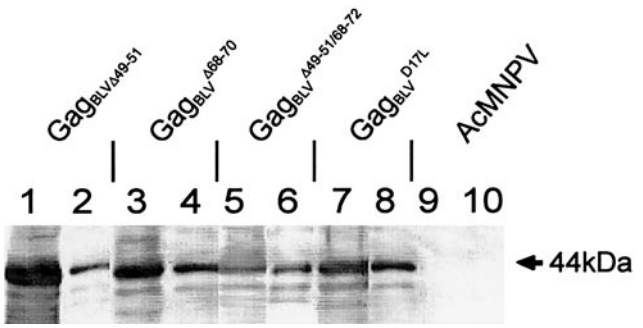

FIG. 4. Western blot analysis of representative mutant BLV Gag expression and particle release in insect cells. Cell lysate and supernatant samples were resolved by 10% SDS–PAGE and immunoblotted using a rabbit anti-BLV serum. BLV Gag$^{\Delta49-51}$ in the cell lysate (lane 1) and supernatant (lane 2); BLV Gag$^{\Delta68-70}$ in the cell lysate (lane 3) and supernatant (lane 4); BLV Gag$^{\Delta49-51,68-72}$ in the cell lysate (lane 5) and supernatant (lane 6); BLV Gag^{D17L} in the cell lysate (lane 7) and supernatant (lane 8); wild-type AcMNPV infected cells (lane 9) and supernatant (lane 10). Presence of BLV Pr44^{Gag} is indicated by an arrowhead. The position of molecular weight markers is indicated on the left.

foetal lamb kidney cells (not shown). Wild-type AcMNPV-infected cells did not express any anti-BLV reactive proteins (Fig. 4). Recombinant baculoviruses AcB*gag*$^{\Delta66-68}$, AcB*gag*$^{\Delta67-69}$, AcB*gag*$^{\Delta68-70}$, AcB*gag*$^{\Delta68-71}$, or AcB*gag*$^{\Delta68-72}$ also expressed a BLV protein of ~44 kDa that was similarly detected by a BLV serum in Western blots and typified by the profiles obtained for AcB*gag*$^{\Delta68-70}$ and AcB*gag*$^{\Delta49-51}$-infected cells (Fig. 4, lanes 3 and 5).

To assess the ability of wild-type and mutant BLV Gag to assemble into VLPs, particulate material in the supernatant from cells infected with each recombinant was concentrated by centrifugation through a sucrose cushion, resuspended, and analysed by SDS–PAGE and Western blot. Each mutant showed evidence of abundant synthesis of Gag antigen that was released into the supernatant of infected cells (Fig. 4, lanes 2, 4, and 6). Further, to confirm assembly and budding of BLV VLPs at the plasma membrane, infected *Sf* cells were fixed, embedded, and processed for thin-section electron microscopy as before. Some tubular forms of Gag were present at the cell surface as observed previously for wild-type BLV Gag (Kakker *et al.,* 1999), but all BLV Gag variants analysed showed evidence of abundant VLP formation at the plasma membrane (representative data from AcB*gag*$^{\Delta49-51}$, AcB*gag*$^{\Delta68-70}$, and AcB*gag*$^{\Delta49-51,68-72}$-infected cells are shown in Fig. 5, panels A–C, respectively). Thus, in contrast to the data obtained with SIV Gag, deletion of three to five residues in one and/or both of the presumed interfaces of the BLV MA trimer did not affect assembly and budding of BLV Gag VLPs.

Mutagenesis at the proposed BLV MA trimer–trimer interface does not affect VLP assembly

The data obtained by mutagenesis of the proposed monomer–monomer contacts in BLV MA cast doubt on the relevance of a trimeric intermediate for BLV Gag assembly. To further investigate the role of any MA dependent trimer, a residue predicted to be at the inter–trimer interface was also mutated and the effect on BLV VLP assembly examined. The BLV MA trimer model features an electrostatic interaction between the side chains of Asp^{17} and Arg^{27} on adjacent trimers. Hydrophobic interactions between Leu^{19}, Leu^{22}, and Leu^{84} located on the surface of the trimer were proposed to further stabilise these interactions (Matthews *et al.,* 1996). To analyse the role of Asp^{17} in assembly, a recombinant virus AcB*gag*^{D17L} was constructed encoding BLV Gag protein incorporating amino acid substitution Asp^{17} to Leu. *Sf*9 cells infected with recombinant virus AcB*gag*^{D17L} was assessed for expression of mutant Gag protein and particle formation as described. Infected cells expressed BLV Pr44^{Gag} with the same electrophoretic mobility as the other mutants when analysed by Western blot (Fig. 4, lane 7). Moreover, antigen was present in the supernatant of infected cells with no apparent diminution in the efficiency of VLP release when compared to other BLV Gag mutants (Fig. 4, lane 8). In addition, thin-section

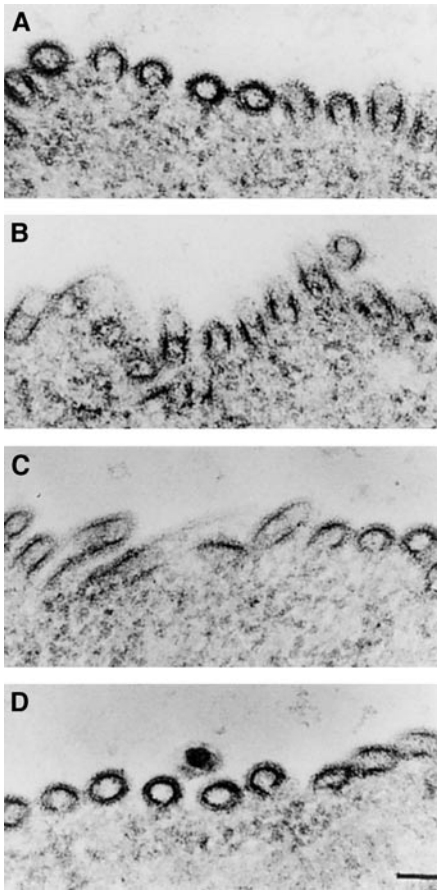

FIG. 5. Thin-section electron micrographs of infected *Sf* cells showing assembly and budding of mutant BLV Gag virus-like particles. The cells were infected with recombinant baculoviruses expressing BLV Gag$^{\Delta49-51}$ (A); BLV Gag$^{\Delta68-70}$ (B); BLV Gag$^{\Delta49-51,68-72}$ (C); and BLV Gag^{D17L} (D). The bar represents 100 nm.

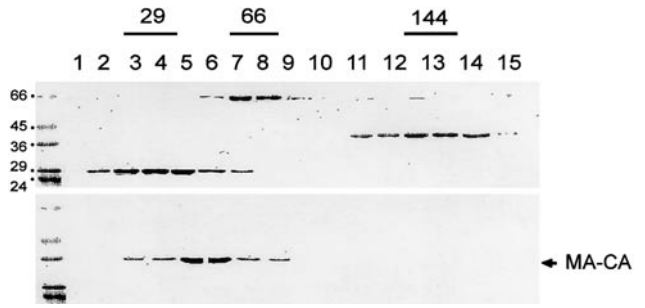

FIG. 6. Gradient analysis of purified BLV MA-CA purified as described. The gradient was fractionated from the top and MA-CA was identified by Western blot of each fraction using a rabbit anti-BLV serum (bottom). Molecular weight markers sedimented in parallel were identified by SDS–PAGE and Coomassie blue staining (top). The peak position of each marker used to calculate the oligomeric form of the MA-CA fragment is shown above the top panel. Numbers to the right of the top panel are molecular weight markers and are measured in kilodaltons.

electron microscopy demonstrated assembly and release of VLPs at the plasma membrane of AcB*gag*^{D17L}-infected cells (Fig. 5D). These results clearly indicate that the identity of residue 17 of BLV MA is not crucial to the assembly and release of Gag VLP.

BLV MA-CA is monomeric in solution

HIV MA and MA-CA have also been shown to exist as a trimer in solution in low (150 mM) salt concentration (Morikawa *et al.,* 1998), providing direct experimental support for the role of trimeric Gag in the assembly of HIV and SIV. To date, however, no data exist on the oligomeric intermediates of BLV MA or MA-CA in solution. In light of the data obtained by site-specific mutagenesis of BLV MA in the present study and to further address the question of the role of MA in BLV Gag oligomerisation, BLV MA-CA was analysed by glycerol velocity gradient sedimentation under conditions in which trimeric HIV MA and MA-CA were reported (Morikawa *et al.,* 1998).

BLV MA-CA was expressed in *Sf* cells as a GST fusion protein following infection with the recombinant baculovirus AcGST.B*ma-ca.* Following cell lysis, recombinant GST-MA-CA protein was captured by glutathione-affinity chromatography and the 36-kDa MA-CA domain released from the immobilised protein by cleavage with thrombin. Purified, soluble MA-CA was concentrated and examined for oligomeric structure by sucrose velocity gradient sedimentation. Gradients were fractionated and analysed by SDS–PAGE and Western blot. BLV MA-CA was found to migrate as a single band between the marker proteins carbonic anhydrase (29 kDa) and bovine serum albumin (66 kDa) with a calculated molecular mass of ~40 kDa, equivalent to the monomeric form (Fig. 6B). These data suggest that, as inferred from the mutagenesis data, the BLV MA-CA Gag protein is monomeric in solution, at least when fused to GST, under conditions in which HIV MA or MA-CA is trimeric.

The results in this article demonstrate that deletion of three amino acids (Gly^{45}, Leu^{46}, Ala^{47}) at the monomer–monomer interface of the SIV MA trimer in the context of the complete Gag severely affect the assembly and release of SIV Gag VLPs from the plasma membrane of expressing cells. Recombinant baculoviruses AcS*gag*$^{\Delta 45-47}$, AcS*gag*$^{\Delta 45-46}$, or AcS*gag*$^{\Delta 45}$ expressing SIV Gag mutants lacking amino acids Gly^{45}, Leu^{46}, Ala^{47}; Gly^{45}, Leu^{46} or Gly^{45}, respectively, expressed levels of a Gag protein equivalent to wild-type encoded by AcS*gag* in cell lysates. However, only the wild-type SIV Pr57^{Gag}, Gag$^{\Delta 45}$, and SIV Gag$^{\Delta 45-46}$ were detected in the supernatant of cells infected with the respective recombinant baculoviruses. Electron microscopic analysis of thin sections of infected cells confirmed the absence of retrovirus-like particle assembly in the case of Gag protein expressed by AcS*gag*$^{\Delta 45-47}$. The association of the monomers within the SIV and HIV trimers is unusual in that the monomer–monomer interactions are held together by main chain hydrogen bonds in which the identity of the amino acid side chain is not crucial (Hill *et al.,* 1996; Rao *et al.,* 1995). Small deletions maybe tolerated as they move the next adjacent residue into the backbone hydrogen bonding configuration. When the deletion removes all key residues, however, the juxtaposition of opposing monomeric faces becomes incompatible with trimer alignment and assembly fails. Although this interpretation of the SIV MA deletions is consistent with a model of lentivirus assembly in which trimerization of Gag via the MA domain is an intermediate (Forster *et al.,* 2000; Rao *et al.,* 1995), other explanations are also valid. Oligomeric intermediates in the cytosol of Gag-expressing cells could represent a pool of Gag that fails to target the plasma membrane and is rapidly degraded (Tritel and Resh, 2000) and the three amino acid deletion of SIV Gag expressed by AcS*gag*$^{\Delta 45-47}$ could preferentially enter this pathway due to significant conformational change. A specific role for the MA driven Gag trimer in the SIV assembly pathway therefore remains unproven and would allow for the observations that viruses with deletions of essentially all of the MA domain of Gag can assemble and bud (Reil *et al.,* 1998; Wang *et al.,* 1998). Interestingly, the crystal structure of a third lentiviral MA domain, that of equine infectious anaemia virus, has been shown recently not to be trimeric (Hatanaka *et al.,* 2002), further suggesting that the role of the SIV/HIV MA trimer in virus assembly may have been overinterpreted.

A model for a BLV MA trimer, based on that of the SIV MA, is plausible based on their similar monomeric structures obtained by NMR (Matthews *et al.,* 1996) and an additional trimeric model has been proposed for the more distantly related M-PMV MA, implying a common mechanism of assembly for type C and B/D retroviruses (Conte *et al.,* 1997). In the case of BLV, however, deletion

of the predicted trimer interface in the context of full-length BLV Gag (Lys^{49}-Gln^{51} in one monomer and Gly^{68}-Arg^{72} in the other) did not prevent BLV Gag VLP assembly. A further substitution of Asp^{17} by Leu in the proposed inter–trimer region also failed to disrupt VLP assembly. These results add further doubt to the generality of an assembly model for retroviral Gag proteins in which the trimerization of MA is an important driver of the process. In *in vitro* virus assembly systems, spherical assemblies of Gag protein with morphologies similar to those seen in budding VLPs occur with CA protein with only a short extension at the amino-terminus in place of the MA domain (Gross *et al.,* 1998; Wilk *et al.,* 2001). Thus, while genetic (Freed *et al.,* 1994) and physical (Morikawa *et al.,* 1995) data support a role for MA in the assembly of HIV virions, alternate pathways of assembly, led by the CA domain, can predominate when MA sequences are deleted (Reil *et al.,* 1998; Wang *et al.,* 1998). Overall, our data confirm that the precise role of the MA domain in retrovirus assembly remains incompletely understood. For example, the production of HIV by rodent cells appears blocked at the level of particle assembly and release (Mariani *et al.,* 2000, 2001), yet can be partially overcome by substitution of the HIV MA domain with the MA of a murine retrovirus (Reed *et al.,* 2002). Our data suggest that studies of the comparative pathways of retrovirus assembly may allow the common underlying mechanisms to be identified.

MATERIALS AND METHODS

Cells and viruses

Sf cells were propagated in suspension or monolayer cultures at 28°C in TC-100 medium (GIBCO BRL, U.K.) supplemented with 5% foetal bovine serum. Baculovirus recombinants were produced using BacPAK-6 viral DNA (Kitts and Possee, 1993) and propagated as described (King and Possee, 1992). Foetal lamb kidney cells infected with BLV (FLK/BLV cells) were kindly supplied by L. Willems, Department of Biochemistry and Applied Biology, University of Brussels, Gembloux, Belgium.

DNA manipulations

DNA manipulations and general molecular biology techniques were as described (Sambrook *et al.,* 1989). DNA fragments encoding mutated *gag* genes were produced by overlapping PCR (Higuchi *et al.,* 1988) and ligated into the Baculovirus transfer vector pAcYM1 (Matsuura *et al.,* 1987) for construction of recombinant baculoviruses. All DNA mutations and junction sites were confirmed by dideoxy nucleotide sequencing (Sanger *et al.,* 1977) prior to use.

Clones

BLV and SIV full-length *gag* or their mutants were amplified from the original clones, pBLVT-15 (Deschamps *et al.,* 1981) and p239SpSp5′ (SIVmac239 isolate), respectively, to generate recombinant transfer vectors as described. Recombinant transfer vector pAcGST-B*ma-ca* was constructed by ligation of the PCR-amplified 972-bp BLV *ma-ca* fragment of BLV Gag into *Bam*H1 restriction enzyme site of pAcG2T (Pharmingen, CA), a baculovirus vector which encodes the cloned protein as a fusion protein with GST, a 26-kDa tag at the N-terminus.

SDS–PAGE and Western blot analysis

Sf cells were harvested at 48 h postinfection (h.p.i.), washed with phosphate-buffered saline, and treated with lysis buffer (2.3% SDS, 10 mM Tris–HCl, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol) as described elsewhere (Hughes *et al.,* 1993). Protein samples were electrophoresed on 10 or 15% SDS–PAGE. For Western blot analysis, proteins resolved by SDS–PAGE were electrophoretically transferred to Immobilon-P membrane (Millipore International) by standard blotting procedure (Towbin *et al.,* 1979). After electroblotting, the membrane was blocked using 3% dried-milk powder in PBS and probed with appropriately diluted specific antiserum (primary antibody) in blocking buffer for 1 h. After three washes, the membrane was incubated with the secondary antibody conjugated with alkaline phosphatase and the bound antibody detected by NBT-BCIP (GIBCO BRL).

VLP purification from infected cell supernatants

Particulate material in the supernatant of infected cells was isolated as described (Hughes *et al.,* 1993). Briefly, infected cell supernatants were harvested at 60 h.p.i. and cells and cellular debris removed by low-speed centrifugation. The clarified supernatant was then layered above a 20% sucrose cushion in TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and centrifuged at 20,000 rpm for 2 h. The pellet from the high-speed centrifugation was resuspended in TE before analysis by SDS–PAGE and Western blot.

Velocity sedimentation analysis of BLV MA-CA

Samples (100 μl) containing approximately 0.1–0.2 mg of purified BLV MA-CA protein were layered onto linear gradients of 15 to 30% glycerol (v/v) in 20 mM Tris (pH 7.4), 100 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA. The proteins were sedimented at 40,000 rpm for 72 h at 4°C in a SW41 rotor. Fractions of 0.5 ml each were collected and numbered from the top. A set of marker proteins were sedimented in parallel; carbonic anhydrase (29 kDa), serum albumin (66 kDa) and lactose dehydrogenase (4×36 kDa $= 144$ kDa). An aliquot of 10 μl of each fraction was analysed by SDS–PAGE.

Electron microscopy

Infected *Sf*9 cells were harvested after 48 h.p.i. and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer,

pH 7.2 for conventional embedding in araldite as described previously (Hockley *et al.,* 1988).

Antisera and enzyme conjugates

Rabbit anti-BLV sera was kindly supplied by D. Portetelle, Department of Microbiology, University of Brussels, Gembloux, Belgium. Monkey anti-SIV serum (ADP 416, antiserum to SIV*mac* 251) was obtained from the NIBSC Centralized Facility for AIDS Reagents. Goat anti-rabbit IgG (A-3687) and affinity-isolated rabbit anti-monkey IgG (A-1929) alkaline phosphatase conjugates for use as second antibodies were obtained commercially (Sigma Inc.).

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