Structural elements in the Gag polyprotein of feline immunodeficiency virus involved in Gag self-association and assembly

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The Gag polyprotein of feline immunodeficiency virus (FIV) assembles at the plasma membrane of the infected cells. We aimed to identify the FIV Gag domains that interact and promote Gag multimerization. To do this we generated a series of Gag subdomains and tested their ability to associate with full-length Gag and be recruited into extracellular virus-like particles (VLPs). Removal of 37 residues from the C-terminus of FIV Gag and deletion of the N-terminal and central regions of the nucleocapsid (NC) domain attenuated but did not abrogate association with wild-type Gag, whereas a Gag mutant protein encompassing the matrix (MA) and capsid (CA) domains interacted poorly with full-length Gag. Association with wild-type Gag was abolished by deleting most of the NC together with the N-terminal 40 residues of the MA, which most likely reflects the inability of this Gag mutant to bind RNA. Notably, the CA–NC Gag subdomain both associated with wild-type Gag and was recruited into particles in a proportion close to 50 % of the total Gag-related protein mass of VLPs. Moreover, both a Gag protein lacking the C-terminal p2 peptide and a nonmyristoylated version of the polyprotein exhibited a transdominant-negative effect on the assembly of wild-type Gag. Analysis of Gag mutants carrying internal deletions within the CA revealed that the N-terminal and the C-terminal domains of the CA are necessary for Gag assembly. Our results demonstrate that the FIV CA–NC region constitutes the principal self-interaction domain of Gag and that the RNA-binding capacity of Gag is necessary for its multimerization.

INTRODUCTION

Feline immunodeficiency virus (FIV) is a lentivirus infecting domestic cats, which induces an immunodeficiency syndrome similar to AIDS caused by human immunodeficiency virus (HIV) (Pedersen *et al.*, 1987). FIV is not only an important cat pathogen, but it is also recognized as a useful model to better understand HIV-1 infection (Burkhard & Dean, 2003; Elder *et al.*, 2010; Yamamoto *et al.*, 2010).

FIV assembles at the plasma membrane of infected cells as the result of the multimerization of the viral Gag polyprotein (Clements & Wong-Staal, 1992). The Gag precursor has all the necessary information to drive the process of particle assembly. Indeed, we have previously demonstrated that expression of the FIV Gag polyprotein in mammalian cells in the absence of the rest of the viral proteins results in the efficient formation of particles that resemble authentic immature virions (Manrique *et al.*, 2001). Moreover, we have established the conditions that allow recombinant FIV Gag expressed in *Escherichia coli* to assemble into spherical particles *in vitro* (Affranchino & González, 2010).

Lentivirus particles are released from infected cells by budding from the plasma membrane (Bieniasz, 2006).

Initially, virions released from the cell surface exhibit an immature morphology. However, concomitantly with or shortly after virus budding from the host cell, the FIV Gag precursor is cleaved by the virus-encoded protease into the structural proteins of the mature virion: matrix (MA), capsid (CA), nucleocapsid (NC), the spacer peptide p1 and the C-terminal peptide p2 (Elder *et al.*, 1993). The MA protein forms a layer beneath the lipid viral envelope, whereas the CA protein assembles into the cone-shaped core of the mature particle that protects the NC–genomic RNA complex (Briggs *et al.*, 2003).

The lentiviral MA, CA and NC proteins differ in both their structural and functional roles in the mature virions and as domains of Gag during the process of particle morphogenesis in infected cells. For the primate lentiviruses HIV-1 and simian immunodeficiency virus (SIV), we and others have demonstrated that the MA domain mediates: (i) Gag transport to and interaction with the plasma membrane (Alfadhli *et al.*, 2009; Chukkapalli *et al.*, 2010; González *et al.*, 1993; González & Affranchino, 1998; Ono & Freed, 1999; Paillart & Göttlinger, 1999; Saad *et al.*, 2006; Zhou *et al.*, 1994), and (ii) envelope glycoprotein incorporation into virions (Affranchino & González, 2014; Dorfman *et al.*,

1994; Freed & Martin, 1996; González et al., 1996; Manrique et al., 2003, 2008; Murakami & Freed, 2000). With respect to the CA domain of Gag, it has been shown for both HIV-1 and SIV that this Gag region promotes the necessary protein–protein interactions that lead to Gag multimerization and particle assembly (Accola et al., 2000; Rauddi et al., 2011; von Schwedler et al., 2003). The main function of the NC is the selective encapsidation of the full-length viral genomic RNA, which is mediated by the association between the two NC zinc-finger motifs and the packaging signal located at the 5′ end of the viral genome (Jewell & Mansky, 2000). In addition, evidence has been presented supporting the concept that the NC–RNA interaction is important for retrovirus assembly as well (Cimarelli et al., 2000; Johnson et al., 2002; Manrique et al., 2004b; Muriaux et al., 2001; Zhang & Barklis, 1997; Zhang et al., 1998). Regarding this, we and others have shown that the in vitro assembly of HIV-1, SIV and FIV Gag proteins is dependent on the presence of RNA in the protein multimerization reaction (Affranchino & González, 2010; Campbell & Rein, 1999; Huseby et al., 2005; Morikawa et al., 1999; Rauddi et al., 2011). Finally, lentiviruses have a PT/SAP motif (HIV-1, SIV and FIV) or an YPX_nL sequence (HIV-1 and equine infectious anemia virus) in the C-terminal domain of Gag that binds, either directly or through an intermediate cellular factor, to components of the endosomal-sorting complexes required for transport (ESCRT) (Bieniasz, 2006). Binding of Gag to ESCRT promotes the release of the lentiviral virions from the plasma membrane of infected cells (Bieniasz, 2006).

We have previously investigated the role of MA and NC domains of FIV Gag in virus assembly and infectivity (Manrique et al., 2001, 2004a, b). Site-directed mutagenesis of the FIV MA led to the identification of domains that are necessary for efficient transport of the Gag polyprotein to the plasma membrane and for particle assembly (Manrique et al., 2001). Moreover, we studied the structural and functional relationship between the MA domains of FIV and SIV by generating chimeric viruses. Interestingly, a SIV carrying the entire FIV MA was incapable of producing particles due to a defect in membrane association of the chimeric Gag polyprotein (Manrique et al., 2004a). By contrast, replacement of the MA-encoding region in the FIV genome with the analogous region of SIV resulted in a chimeric virus that not only assembled into virions as efficiently as wild-type FIV but also replicated in feline T cells in a wild-type manner (Manrique et al., 2004a).

Regarding the FIV NC, we demonstrated that substitution of the first cysteine residue with a serine residue in the proximal zinc finger severely impairs both virion assembly and genomic RNA packaging (Manrique et al., 2004b). Furthermore, disruption of the highly conserved PSAP motif present in the 18 amino acid long p2 peptide abrogates virion production, indicating that this C-terminal FIV Gag peptide is functionally equivalent to the p6 region in the Gag polyproteins of HIV and SIV (Manrique et al., 2004b). The role of p2 in FIV budding was later confirmed and studied in more detail by others (Calistri et al., 2009; Luttge et al., 2008).

Despite the available information on the functions of the MA and NC domains of FIV Gag described above, our knowledge of the process of FIV assembly is still limited. In this regard, the FIV Gag sequences that participate in the homomeric protein interactions that drive virion assembly remained to be identified. To address this issue, we generated a series of FIV Gag subdomains and studied whether they were capable of interacting with wild-type Gag protein and therefore being rescued into Gag-made extracellular particles.

RESULTS

Construction of FIV gag subdomains

In order to map the domains in the FIV Gag precursor that promote its multimerization and assembly into particles, we generated a panel of FIV gag mutants consisting of: (i) truncation mutants in which the FIV Gag protein was truncated at the N- or C- terminus; (ii) two mutants in which the N-terminal and central regions of the NC were deleted alone or in combination with the removal of the first 40 residues of Gag; (iii) the Gag-derived mature proteins, namely MA, CA and NC (Fig. 1).

Ability of the FIV Gag subdomains to interact with wild-type Gag in vivo

We first compared the capacity of the different FIV Gag subdomains to associate with wild-type Gag and be rescued into extracellular virus-like particles (VLPs). We have previously made use of this strategy to study whether chimeric SIV–FIV Gag proteins or SIV Gag subdomains were capable of establishing molecular interactions with wild-type Gag (Manrique et al., 2004a; Rauddi et al., 2011). We and others have demonstrated that Gag multimers are formed prior to Gag transport to the plasma membrane (Lee & Yu, 1998; Manrique et al., 2004a; Ono et al., 2000).

COS-7 cells infected with the recombinant vaccinia virus expressing the T7 RNA polymerase were then cotransfected with plasmids encoding wild-type FIV Gag and each of the Gag subdomains as described in Methods. For control purposes, we analysed in parallel cells expressing only wild-type Gag or each of the Gag subdomains. Cell and VLP lysates were resolved by SDS-PAGE and subjected to Western blotting using an anti-FIV CA mAb.

Deletion of p2 together with the C-terminal 19 residues of the NC (Gag_{1-413}) did not abolish the ability of this Gag subdomain to associate with wild-type Gag as evidenced by its recruitment into VLPs (Fig. 2a). A similar phenotype was observed for $Gag_{\Delta367-413}$, which lacks the N-terminal and central NC regions, and for $Gag_{136-450}$ that comprises the CA and NC domains (Fig. 2b, d). In contrast, low levels of the truncated mutant encompassing the MA and CA domains of Gag (Gag_{1-366}) were detected in VLPs (Fig. 2c).

Quantification of the relative protein levels of wild-type Gag and the deletion mutants in VLPs revealed that Gag_{1-413} and
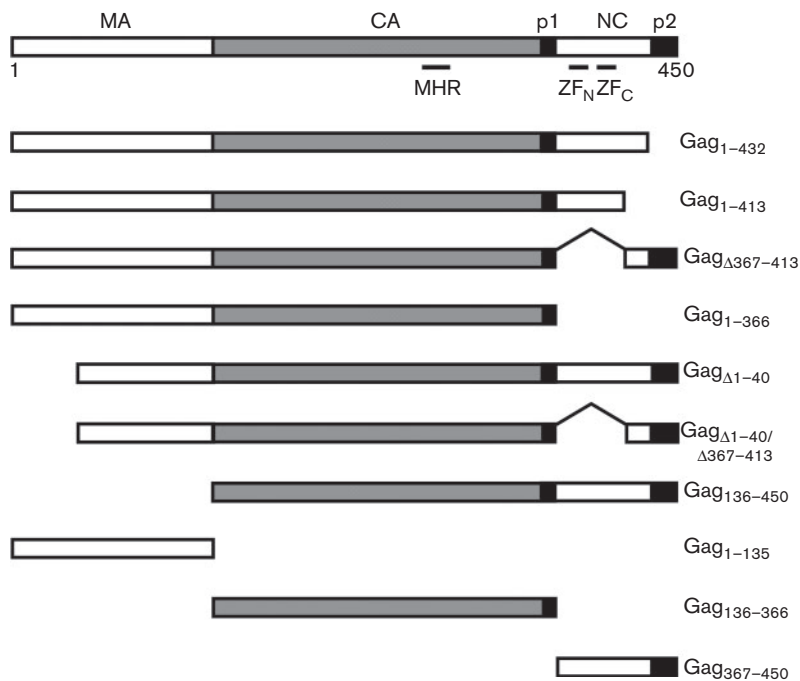
Fig. 1. Construction of FIV *gag* subdomains. Diagram illustrating the domains of the FIV Gag polyprotein and the Gag mutants used in this study. The major homology region (MHR) in the CA and the N-terminal (ZF_N) and C-terminal (ZF_C) zinc-finger motifs in the NC are indicated. The names of the mutants refer to the FIV Gag amino acid positions with residue 1 corresponding to the initiator methionine in the polyprotein.

$Gag_{\Delta 367-413}$ represented 38 and 35 %, respectively, of the total amount of protein, whereas the Gag mutant containing the MA and CA domains (Gag_{1-366}) accounted only for 20 % of the total protein mass in particles (Fig. 2e). Interestingly, the CA–NC Gag region ($Gag_{136-450}$) contributed to the composition of the extracellular particles by 47 % (Fig. 2e).

It should be mentioned that none of the Gag subdomains were capable of assembling by themselves into extracellular VLPs. Mutants Gag_{1-413} and Gag_{1-366} lack the C-terminal p2 peptide that harbours the budding motif PSAP (Manrique *et al.*, 2004b), whereas removal of the entire MA in mutant $Gag_{136-450}$ eliminates the myristate moiety and the N-terminal basic region that mediate Gag interaction with the plasma membrane (Manrique *et al.*, 2001). Interestingly, when we analysed the particulate fraction purified from cells expressing $Gag_{\Delta 367-413}$, we consistently detected trace amounts of this mutant protein (Fig. 2b). This suggests that mutant $Gag_{\Delta 367-413}$ may retain a certain ability to form VLPs. Indeed, this mutant has both the MA and p2 domains of Gag, but lacks the NC region responsible for RNA packaging (Manrique *et al.*, 2004b). It may be speculated that while mutant $Gag_{\Delta 367-413}$ has the capacity to associate with the plasma membrane and bud into the extracellular medium, it is incapable of interacting with RNA and this prevents efficient particle assembly. This issue was further investigated by analysing the phenotype of mutant $Gag_{\Delta 1-40/\Delta 367-413}$ (see below).

Ability of the mature Gag proteins to interact with wild-type FIV Gag

We next examined whether the MA, CA and NC proteins were capable of associating with wild-type Gag. Although these polypeptides were efficiently expressed in the cotransfection experiments, none of them could be detected in VLPs, indicating that they were not rescued by wild-type Gag (Fig. 3 a–c). Only trace amounts of the CA protein were found in the particulate fraction of cells expressing this protein together with wild-type Gag (Fig. 3b).

Analysis of the Gag-binding capacity of mutant $Gag_{\Delta 1-40/\Delta 367-413}$

We showed in Fig. 2(b) that the Gag subdomain lacking the N-terminal and central regions of the FIV NC ($Gag_{\Delta 367-413}$) interacts with wild-type Gag and is recruited into VLPs. Based on our demonstration that the assembly of FIV Gag, both *in vivo* and *in vitro*, is dependent on the interaction of Gag with the viral genomic RNA (Affranchino & González, 2010; Manrique *et al.*, 2004b), we speculated that in mutant $Gag_{\Delta 367-413}$ the highly basic region within the MA domain may non-specifically interact with RNA, thereby mediating the association of this deletion mutant with wild-type Gag. To test this hypothesis, we removed the N-terminal 40 residues of the MA from mutant $Gag_{\Delta 367-413}$, which contains the cluster of basic amino acids (Manrique *et al.*, 2001). As shown in Fig. 4(a), we found that the resulting mutant $Gag_{\Delta 1-40/\Delta 367-413}$ was incapable of interacting with wild-type Gag and was not rescued into VLPs. By contrast, the mutant $Gag_{\Delta 1-40}$, used as a control, was able to associate with wild-type Gag and be recruited into particles in a proportion representing 37.5 ± 5.5 % (mean $\pm$ SD, $n=3$) of the total protein mass of the extracellular particles (Fig. 4b).
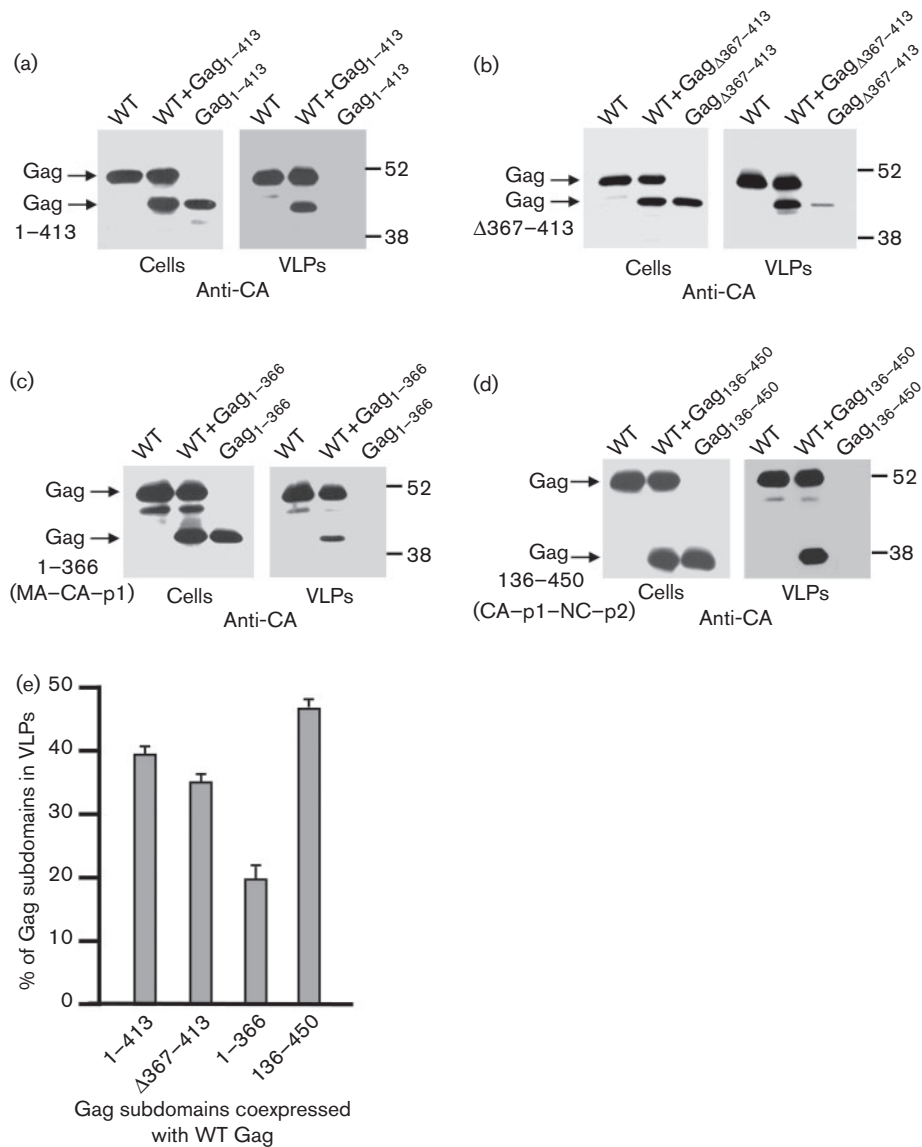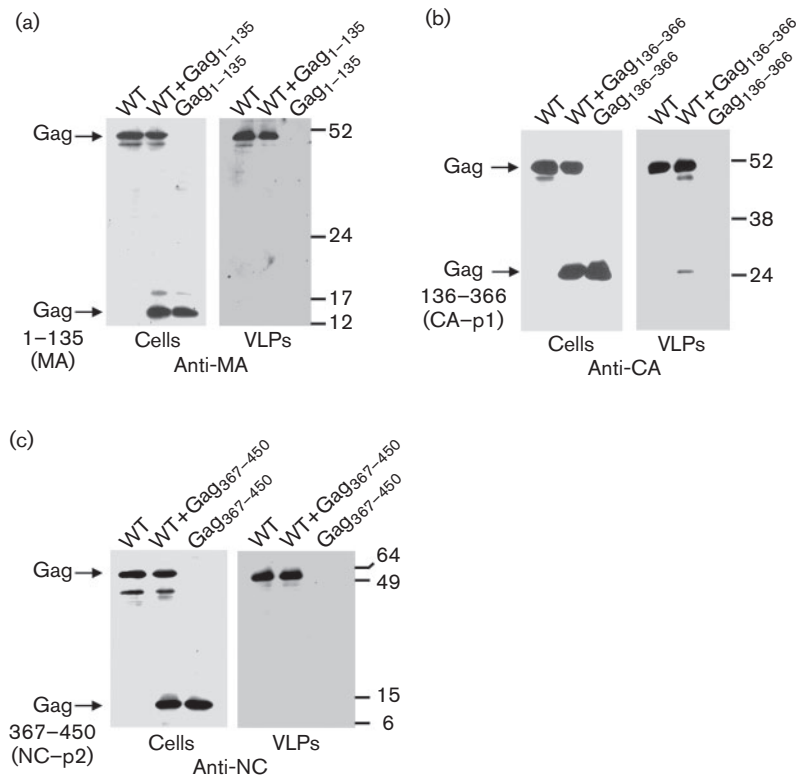
Fig. 2. Ability of the FIV Gag subdomains to interact with wild-type Gag in cell cultures. COS-7 cells infected with the vTF7-3 recombinant vaccinia virus were transfected with plasmids expressing wild-type FIV Gag (WT) or each of the following Gag subdomains: Gag_{1-413} (a), $Gag_{\Delta 367-413}$ (b), Gag_{1-366} (c) or $Gag_{136-450}$ (d). In parallel, cells were cotransfected with wild-type Gag and Gag_{1-413} (a), $Gag_{\Delta 367-413}$ (b), Gag_{1-366} (c) or $Gag_{136-450}$ (d). Thirty hours post-transfection, cells were harvested and VLPs were purified as described in Methods. Gag proteins were detected by Western blotting using the anti-FIV CA mAb. The mobilities of the wild-type FIV Gag and Gag subdomains are shown, as are the positions of the molecular mass standards (kDa). (e) Quantification in VLPs of the amount of each Gag subdomain with respect to the total Gag-related protein mass in particles [Gag subdomain/(wild-type Gag + Gag subdomain)]. Data presented are the mean of three independent experiments ± SD; values are given in text.

Dominant-negative phenotype of FIV Gag proteins lacking p2 or the myristate moiety

Among the panel of Gag subdomains, we generated the construct Gag_{1-432} encoding a FIV Gag protein lacking the entire p2 peptide (Fig. 1). As expected, this mutant is assembly defective because the deletion eliminates the PSAP budding motif (Fig. 5). Interestingly, we found that expression of mutant Gag_{1-432} together with wild-type Gag drastically impaired the ability of the latter polyprotein to produce VLPs (Fig. 5). The levels of wild-type Gag in particles purified from the culture medium of cells coexpressing wild-type Gag and mutant Gag_{1-432} proteins represented $15 \pm 3\%$ (mean ± SD, $n=3$) of those obtained from the supernatant of cells expressing only wild-type FIV Gag.

Fig. 3. Analysis of the association of the mature FIV Gag proteins with wild-type Gag. COS-7 cells infected with the vTF7-3 recombinant vaccinia virus were transfected with plasmids directing the synthesis of wild-type FIV Gag (WT), MA (a), CA (b) or NC (c). Additional cell cultures were cotransfected with the constructs expressing wild-type FIV Gag (WT) and MA (a), CA (b) or NC (c). Cell and VLP lysates were analysed for the presence of Gag and Gag-derived proteins by Western blotting using the anti-FIV MA serum (a), the anti-FIV CA mAb (b) or the anti-FIV NC serum (c). The electrophoretic mobilities of the FIV Gag precursor, and of the MA, CA and NC proteins, are shown, as are the positions of the molecular mass markers (kDa).

We also created a nonmyristoylated version of FIV Gag with an N-terminal 45 amino acid extension including a six-histidine tag (His-Gag) so that it could be easily discriminated from wild-type FIV Gag by SDS-PAGE. Of note, this His-Gag protein was found to assemble into spherical particles *in vitro* (data not shown) under the conditions that we have previously established (Affranchino & González, 2010). We then investigated whether His-Gag was capable of associating with wild-type Gag. As shown in Fig. 6(a), both polyproteins attained comparable levels when coexpressed in COS-7 cells. However, when the particulate fraction of this cell culture was analysed and compared with that purified from cells expressing only wild-type Gag, we found that His-Gag inhibited the production of extracellular VLPs (Fig. 6a). This result was confirmed by coinfecting COS-7 cells with recombinant vaccinia viruses expressing wild-type FIV Gag and a myristoylation-minus FIV Gag (Myr-Gag) carrying a G2A amino acid substitution that prevents both N-terminal myristoylation and extracellular particle formation (Manrique *et al.*, 2001). In line with the results obtained with His-Gag, coexpression of Myr-Gag with wild-type Gag drastically reduced VLP production (Fig. 6b). Indeed, the levels of VLPs purified from the culture medium of cells coexpressing wild-type and Myr-Gag proteins represented only $13.0 \pm 1.6\%$ (mean $\pm$ SD, $n=3$) of those obtained from the supernatant of cells expressing wild-type Gag. Taken together, these results indicate that Gag_{1-432} and myristoylation-defective Gag proteins behave as dominant-negative mutants that severely impair the ability of wild-type FIV Gag to assemble into extracellular particles.

Effect of CA internal deletions on Gag particle assembly

Our results at this point indicated that the Gag truncation mutant containing the CA and NC domains ($Gag_{136-450}$) efficiently interacts with wild-type Gag (Fig. 2e). Given that we have already identified structural elements both in the FIV MA and NC proteins that the mutation of which abrogates virion assembly (Manrique *et al.*, 2001, 2004b), we decided to focus on the CA domain of Gag. As a first step toward defining the role of the FIV CA in Gag multimerization, we introduced six internal in-frame deletions within this Gag domain (Fig. 7a). Alignment of the FIV CA with its HIV-1 counterpart revealed that these proteins share 36 % identity and 59 % similarity at the amino acid level (Fig. 7a). The regions targeted for mutagenesis in the FIV CA were selected on the basis of their conservation among FIV isolates and on their homology with the HIV-1 CA residues that form the α-helices found in the crystal structure of this protein, including the α-helix corresponding to the major homology region (MHR) (Gamble *et al.*, 1997; Momany *et al.*, 1996). The mutated *gag* genes carrying internal deletions in the CA-encoding region were used to generate recombinant vaccinia viruses as described in Methods. We chose to express the CA mutants in the context of Gag in the absence of polyprotein processing by the viral protease so as to solely analyse the functions that the FIV CA exhibits as a domain of Gag.

COS-7 cells infected with the recombinant vaccinia viruses were lysed and the VLPs were purified from the clarified
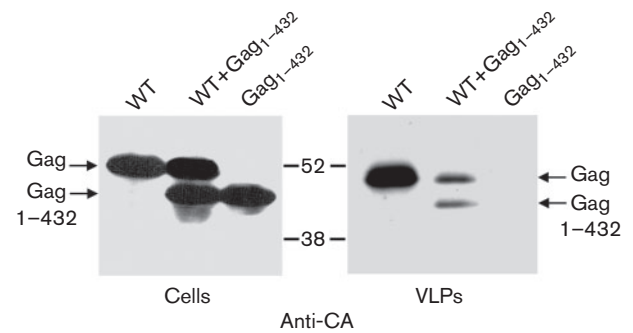
(a)

(b)
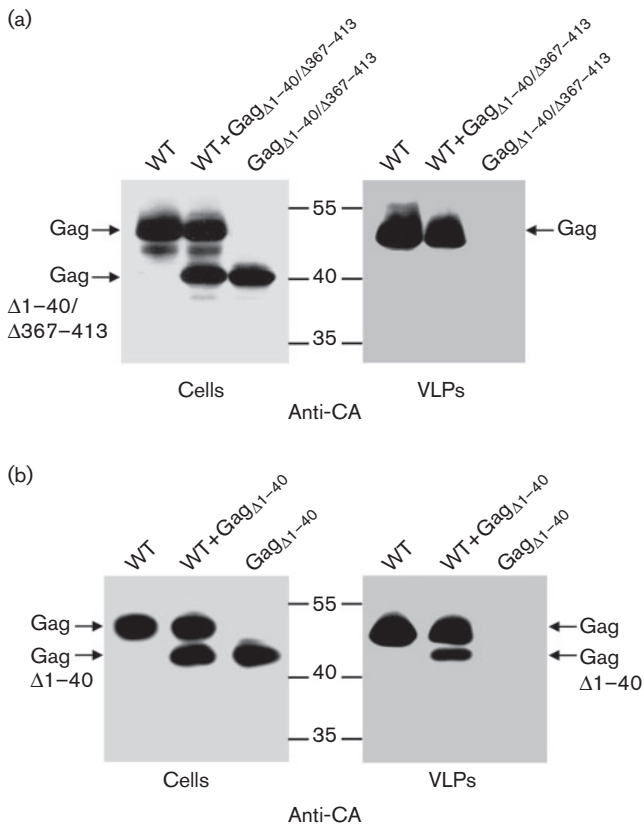
Fig. 4. Interaction of mutant FIV $Gag_{\Delta 1-40/\Delta 367-413}$ with wild-type Gag. COS-7 cells infected with the vTF7-3 vaccinia virus were transfected with plasmids expressing wild-type FIV Gag (WT), $Gag_{\Delta 1-40/\Delta 367-413}$ (a) or $Gag_{\Delta 1-40}$ (b). In parallel, cells were cotransfected with the constructs encoding wild-type Gag and $Gag_{\Delta 1-40/\Delta 367-413}$ (a) or wild-type Gag (WT) and $Gag_{\Delta 1-40}$ (b). Cell and VLP lysates were resolved by SDS-PAGE and the Gag proteins were visualized by Western blotting using the anti-FIV CA mAb. The positions of the molecular mass markers are shown (kDa).

Fig. 5. Effect of the coexpression of FIV mutant Gag_{1-432} with wild-type Gag on the production of extracellular particles. COS-7 cells infected with the vTF7-3 vaccinia virus were transfected with the vectors expressing either wild-type Gag (WT) or Gag_{1-432}, or cotransfected with both plasmids. The wild-type and mutant Gag proteins were detected in both cell and VLP lysates by Western blotting using the anti-CA mAb. The positions of the molecular mass markers are shown (kDa).

culture supernatants as described in Methods. In both the cell and VLP lysates the Gag proteins were detected by Western blot using a polyclonal anti-FIV MA serum. As shown in Fig. 7(b), all the CA deletion mutants were expressed as efficiently as wild-type Gag. Deletions ΔC1, ΔC2, ΔMHR and ΔC5 abrogated Gag particle assembly (Fig. 7b). By contrast, the Gag proteins ΔC3 and ΔC4 were capable of producing VLPs, albeit at lower levels than those of wild-type Gag (Fig. 7b).

DISCUSSION

Although numerous studies have contributed to our current knowledge of retrovirus assembly, the interaction of FIV Gag domains that drive Gag multimerization and assembly remained to be delineated. To this end, we generated a series of FIV Gag subdomains that were

coexpressed with wild-type Gag in cell cultures and determined whether these Gag regions were capable of associating with the full-length viral polyprotein and being recruited into VLPs. By using this approach, we found that FIV Gag mutants lacking either the C-terminal 37 residues of Gag (Gag_{1-413}) or the N-terminal and central regions of the NC ($Gag_{\Delta 367-413}$) still interacted with wild-type Gag and contributed to the composition of the extracellular particles in a proportion close to 40 %. By contrast, the MA–CA subdomain was inefficient at associating with wild-type Gag and represented only 20 % of the total Gag-related protein mass of the VLPs. Of note, the CA–NC region bound to full-length Gag with higher efficiency than the rest of the subdomains tested. In this regard, we have previously shown that the SIV CA–NC polypeptide binds *in vitro* to SIV GagΔp6 fused to the C-terminus of *Schistosoma japonicum* glutathione -*S*-transferase (GST-GagΔp6) more efficiently than wild-type SIV Gag (Rauddi et al., 2011). Moreover, a small SIV Gag subdomain comprising only the C-terminal third of the CA and the entire NC binds to GST-SIVGagΔp6 in pull-down assays at wild-type levels and is also recruited *in vivo* by full-length SIV Gag into extracellular particles (Rauddi et al., 2011). Interestingly, pull-down experiments conducted with GST-HIV-1 Gag as bait also demonstrated that the CA–NC region is the major HIV-1 Gag interacting domain (Burniston et al., 1999). Our results therefore indicate that the CA and NC domains of FIV Gag are as essential for Gag-Gag interactions as those of their primate lentivirus counterparts.

When we analysed the FIV Gag-binding ability of the mature proteins MA, CA and NC none of them was found to interact in cell cultures with the Gag precursor. Using the same methodology for SIV as well as GST-pull down assays, we could not detect any association between the SIV
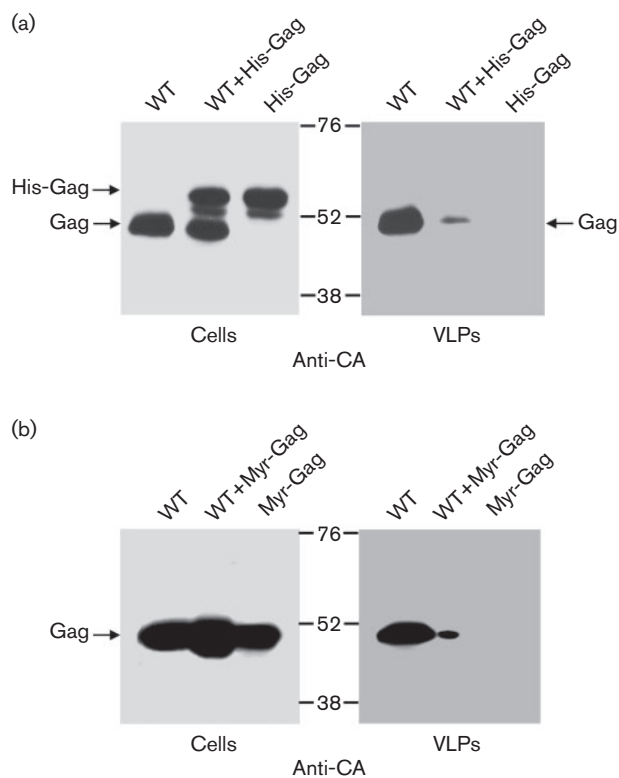
Fig. 6. Effect of the coexpression of non-myristoylated FIV Gag proteins with wild-type Gag on the formation of VLPs. (a) COS-7 cells infected with the vTF7-3 vaccinia virus were transfected with plasmids expressing wild-type FIV Gag (WT) or His-Gag. In parallel, cells were cotransfected with both constructs. (b) COS-7 cells were infected with recombinant vaccinia viruses expressing wild-type FIV Gag (WT) or myristoylation-minus Gag (Myr- Gag) or coinfected with both vaccinia viruses. Cell and VLP lysates were resolved by SDS-PAGE and the Gag proteins were visualized by Western blotting using the anti-FIV CA mAb. The positions of the molecular mass markers are shown (kDa).

CA and wild-type Gag (Rauddi *et al.*, 2011). However, we did detect in *in vitro* assays interaction between GST-SIVGagΔp6 and both the SIV MA protein (20 % with respect to wild-type Gag) and the SIV NC polypeptide (40 % of the wild-type value) (Rauddi *et al.*, 2011).

It is interesting to compare the phenotype of FIV Gag$_{\Delta367-413}$, which lacks the N-terminal and central regions of the NC domain, with that of Gag$_{\Delta1-40/\Delta367-413}$, which, in addition to the internal deletion in the NC, exhibits an N-terminally truncated MA domain. FIV Gag$_{\Delta367-413}$ was found to interact with wild-type Gag, whereas Gag$_{\Delta1-40/\Delta367-413}$ failed to associate with the full-length Gag protein. One possible explanation for the different behaviour observed for these mutants is that the inability of Gag$_{\Delta367-413}$ to bind viral RNA (due to the absence of the NC region) is compensated by the RNA-binding capacity of the basic region of the MA domain, whereas removal of both the NC domain and the MA basic region abrogates the association of Gag$_{\Delta1-40/\Delta367-413}$

with RNA thereby impairing the interaction of this mutant with wild-type Gag. In support of the role of RNA in promoting FIV Gag–Gag multimerization, we have previously shown that: (i) mutation of the first cysteine in the proximal NC zinc finger is sufficient to block both genomic RNA packaging and virion production (Manrique *et al.*, 2004b), and (ii) the *in vitro* assembly of recombinant FIV Gag is strictly dependent on the presence of viral RNA (Affranchino & González, 2010). Furthermore, in other retroviruses such as HIV-1, Rous sarcoma virus and murine leukemia virus, compelling evidence favours the concept that the NC–RNA interaction plays a central role in initiating the process of Gag assembly into particles (Alfadhli *et al.*, 2005; Cimarelli *et al.*, 2000; Johnson *et al.*, 2002, Muriaux *et al.*, 2001; Zhang & Barklis, 1997; Zhang *et al.*, 1998).

An interesting finding from our studies is the observation that the sole removal of the C-terminal p2 peptide is sufficient to generate a dominant-negative Gag mutant (Gag$_{1-432}$) that interacts with wild-type Gag forming nonfunctional protein multimers that are incapable of budding from the plasma membrane. Given that mutant Gag$_{1-432}$ exhibits such a strong dominant-negative effect on wild-type Gag release, it is most likely that this mutant associates with wild-type Gag with high efficiency and therefore makes a major contribution to the composition of the nonfunctional multimeric complexes. Therefore, these structures may contain a number of active ESCRT-recruiting motifs lower than that required for efficient budding of VLPs. In marked contrast, we have previously observed that SIV Gag lacking the p6 late domain not only is recruited into VLPs by full-length SIV Gag but also is capable of assembling *in vitro* into spherical particles (Rauddi *et al.*, 2011). We show here that, in addition to GagΔp2, the nonmyristoylated FIV Gag precursor also behaves as a dominant-negative mutant when coexpressed with wild-type Gag. We have previously demonstrated that Myr-FIV Gag assembles into intracellular particles (Manrique *et al.*, 2001). It is therefore possible that nonmyristoylated FIV Gag recruits wild-type Gag into these structures instead of being rescued by full-length Gag into extracellular VLPs.

In the present study, we initiated the identification of the CA assembly domains by characterizing the phenotype of a panel of mutant FIV Gag proteins carrying internal deletions within the CA region. Only deletions ΔC3 and ΔC4 (located in the N-terminal and C-terminal domains of FIV CA, respectively) did not affect the assembly of Gag into VLPs, which suggests that these CA regions are not essential for Gag multimerization. By contrast, mutations ΔC1, ΔC2, ΔMHR and ΔC5 abrogated VLP assembly. The phenotype of mutant ΔMHR was expected as we and others have shown that mutations affecting the MHR of both HIV-1 and SIV CA domains abolish particle production (Mammano *et al.*, 1994; Rauddi *et al.*, 2011). The fact that domains in both the N- and C-terminal domains of the FIV CA are important for VLP assembly is in keeping with the results from
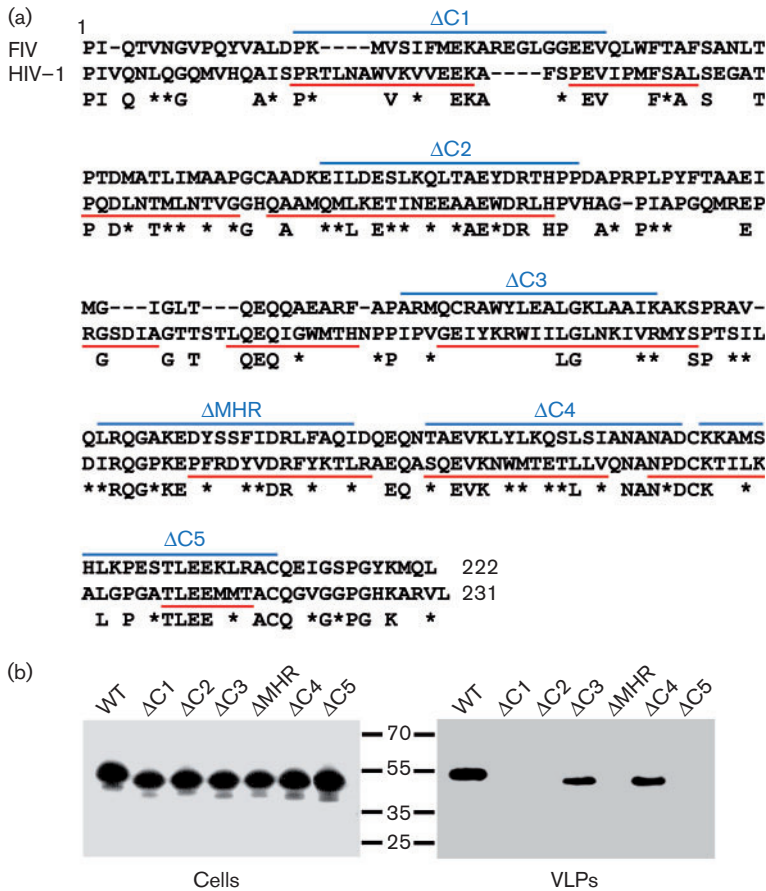
Fig. 7. Effect of internal deletions in the CA domain of FIV Gag on VLP production. (a) Amino acid sequence alignment of the CA proteins of FIV (Petaluma isolate) and HIV-1 (NL4-3 strain). Residues are numbered according to their position relative to the first residue for each CA protein. The internal deletions introduced into the FIV CA are indicated above its sequence: ΔC1 (residues 16–35), ΔC2 (residues 66–85), ΔC3 (residues 117–136), ΔMHR (residues 145–164), ΔC4 (residues 170–189) and ΔC5 (residues 191–210). Red lines denote the eleven α-helices of the HIV-1 CA. Identical amino acids present at the same position in both sequences are highlighted. Asterisks denote conservative substitutions. (b) COS-7 cells were infected with the recombinant vaccinia viruses expressing the mutant Gag proteins. The wild-type (WT) and mutant Gag proteins were detected in cell and VLP lysates by Western blotting with the anti-FIV MA poly-clonal serum. The electrophoretic mobilities of the wild-type and mutant Gag proteins are shown, as are the positions of the molecular mass markers (kDa).

site-directed mutagenesis analysis of the HIV-1 CA (von Schwedler et al., 2003) and with the conclusions drawn from the cryo-electron tomography of immature HIV-1 particles (Briggs et al., 2009). Indeed, it has been proposed for HIV-1 that the N- and C-terminal domains of the CA contribute to the formation of the continuous hexameric Gag lattice found in immature virions and that within this arrangement of Gag, CA residues 153–159 (IRQGPKE) of one CA molecule is involved in interactions with residues 212–219 (EEMMTACQ) of the neighbouring CA molecule (Briggs et al., 2009). Of note, the mutations ΔMHR and ΔC5 in the FIV CA that abolish Gag assembly remove the residues LRQGAKE and EEKLRACQ, respectively (Fig. 7a) and these are highly homologous to the HIV-1 CA sequences pinpointed by Briggs et al. (2009).

Further studies will be necessary to identify the key residues in the FIV CA that are necessary for the Gag homomeric associations that drive particle assembly.

METHODS

Cells and viruses. African green monkey kidney COS-7 cells and rat embryo thymidine kinase-negative Rat2 cells (used for the selection of recombinant vaccinia viruses) were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10 % FBS (Hyclone). The parental vaccinia virus used to generate

recombinant vaccinia viruses was the WR strain. The vaccinia virus vTF7-3 expressing T7 RNA polymerase was kindly provided by Dr B. Moss (NIAID, NIH, Bethesda, Maryland, USA). The generation of the recombinant vaccinia viruses expressing wild-type and myristoylation-minus FIV Gag proteins has previously been described (Manrique et al., 2001).

Plasmid constructs. The gag sequences for all the expression plasmids were derived from the infectious molecular clone FIV-14 of the Petaluma isolate (GenBank accession no. M25381; Olmsted et al., 1989). The FIV gag gene (nucleotides 628–1980 of the FIV-14 genome), as well as the gag subdomains, were obtained by PCR amplification using the elongase enzyme high-fidelity PCR mix (Invitrogen) and cloned into the pcDNA3.1(+) vector. The constructs were verified by DNA sequencing. The sequences of the primers used in the PCR, as well as the cloning strategies, are available upon request. To express a non-myristoylated version of the FIV Gag protein exhibiting lower electrophoretic mobility than wild-type Gag, the gag gene was PCR amplified and first cloned into the EcoRV and SalI sites of the pET-30b(+) vector so as to produce an FIV Gag protein with a six-histidine tag at the N-terminus. The gag clone in the pET vector was linearized with NdeI, blunt-ended with T4 DNA polymerase (New England Biolabs) and further treated with SalI. The DNA fragment excised from this plasmid was cloned into the NheI (blunt ended) and XhoI sites of the pcDNA vector. The resulting construct, pcDNA-His-gag, directs the expression of FIV Gag with an N-terminal extension of 45 amino acids.

Construction of recombinant vaccinia viruses. To introduce six internal in-frame deletions into the coding region of the CA domain of FIV Gag, we amplified by PCR two fragments using the wild-type

gag clone in pcDNA as the template. One fragment extended from the *gag* initiation codon to that corresponding to the amino acid located immediately upstream of the deletion to be created. The second fragment encompassed the region from the first codon downstream of the deletion to the *gag* stop codon. The 5′ and 3′ DNA fragments were ligated as the antisense primer used in the amplification of the 5′ sequences and the sense oligonucleotide used to generate the 3′ DNA fragments were phosphorylated. The resulting *gag* constructs harbouring CA internal deletions were cloned into the vaccinia transfer vector pMJ601 as previously described (González *et al.*, 1993). The recombinant vaccinia viruses were obtained, selected and purified as previously reported (González *et al.*, 1993). The mutated FIV *gag* genes were subjected to DNA sequencing to confirm the presence of the desired deletions and the absence of nucleotide misincorporations due to the gene amplification steps. Several independent recombinant vaccinia viruses for each Gag mutant were initially screened to ensure that their protein products exhibited the same phenotype.

Analysis of protein expression in mammalian cells. Expression of wild-type FIV Gag and Gag subdomains in COS-7 cells was carried out using the vaccinia T7 system (Fuerst *et al.*, 1987) as the *gag* constructs were cloned into the pcDNA vector under the control of the T7 RNA polymerase promoter. Confluent monolayers of COS-7 cells (35 mm diameter dishes) were infected with the vTF7-3 recombinant vaccinia virus at a multiplicity of 10 for 1 h at 37 °C as previously described (Rauddi *et al.*, 2011). After infection, the cells were washed twice with DMEM and then transfected with the plasmid constructs using Lipofectamine 2000 (Invitrogen). COS-7 cells were either transfected with the wild-type FIV *gag*-expressing plasmid or each of the *gag* subdomains constructs. In addition, cotransfection experiments were carried out in order to simultaneously express wild-type FIV Gag and each of the different Gag subdomains. Initial cotransfection assays were carried out so as to establish the appropriate ratio of wild-type *gag* plasmid to *gag* subdomain construct that ensures similar intracellular levels of both proteins. Thirty hours post-transfection, cells were washed twice with ice-cold PBS solution and lysed at 4 °C in buffer containing 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) nonidet P-40, 0.1 % (w/v) SDS, 0.5 % (w/v) sodium deoxycholate, 1 mM PMSF and 10 μg aprotinin ml^{-1}. The culture supernatants from the infected/transfected cells were filtered through 0.45 μm syringe filters and the VLPs were pelleted from the clarified media by ultracentrifugation through a 20 % (w/v) sucrose cushion as previously described (Manrique *et al.*, 2001, 2004b). Cell- and VLP-associated proteins were resolved by SDS-PAGE, blotted onto nitrocellulose membranes and analysed by Western blotting coupled to an enhanced chemiluminescent–chemifluorescent assay (ECL; GE Life Sciences). FIV Gag-related proteins were detected using the anti-FIV CA mAb (PAK3-2C1; NIH AIDS Reference and Reagent Program), or anti-MA or anti-NC mouse polyclonal sera obtained in our laboratory. Quantification of Western blot signals was performed as previously described (Manrique *et al.*, 2003).

To express the Gag proteins carrying internal deletions in the CA domain, confluent monolayers of COS-7 cells were infected with the recombinant vaccinia viruses at an m.o.i. of 5 as previously described (Manrique *et al.*, 2001). Cells were harvested 24 h post-infection; cell and VLP lysates were prepared as described above. Wild-type and mutant Gag proteins were detected in both fractions by Western blotting using the mouse polyclonal serum directed against the FIV MA domain.

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