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Integrase deletion mutation impairs the Gag cleavage in prototype foamy virus

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Summary. – Integrase (IN) is an essential enzyme that catalyzes integration of viral DNA into host cellular genome. Among retroviruses, foamy virus is the first in which the X-ray crystal structure of IN has been reported. Compared with other retroviruses, this virus has a unique expression pattern since Gag and Pol are produced independently from each other. For the successful maturation of prototype foamy virus (PFV), Gag should be cleaved into a 68/71-kDa Gag doublet. Here, we induced deletion mutations in PFV IN and characterized their effects on the Gag cleavage. Among the generated serial mutants, only those with deletions of up to two amino acids showed the wild-type Gag-cleavage pattern and viral titer level. These results were confirmed via transmission electron microscopy and site-specific mutagenesis. Taking together, maturation and morphogenesis, the deletion mutations of more than two amino acids in catalytic core domain (CCD) region are able to compromise the viral reproduction.

Keywords: integrase; prototype foamy virus; deletion mutant; gag cleavage

Introduction

Since the X-ray crystal structure of IN was characterized for the first time in retroviruses (Hare *et al*., 2010; Maertens *et al*., 2010), foamy viruses (FVs) have been intensively studied in the last decade. These viruses have been identified in various mammalian hosts, including non-human primates, cattle, cats, and horses. FVs are unique in the sense that their infection does not cause any serious disease. Therefore, FVs are promising vector candidates for gene therapy (Geiselhart *et al*., 2003).

For successful retroviral replication, the viral cDNA must integrate into the host genome. To this end, the cDNA forms a nucleoprotein complex, termed the pre-integration complex (PIC), with host and viral proteins in the host cytoplasm (Li *et al*., 2006). Integrase (IN), one of the retrovirus-specific enzymes, has two significant roles in the retrovirus life cycle. First, it catalyzes viral-DNA integration into the host genome (Bushman *et al*., 1990). Second, it is one of the PIC components, and its nuclear localization signal thus enables nuclear translocation of the viral cDNA (Craigie, 2001). IN is composed of three domains**:** the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD) (Drelich *et al*., 1992; Kulkosky *et al*., 1992). The NTD is a highly conserved domain containing a pair of cysteine and histidine residues that serve as a zinc-binding domain (Woodward *et al*., 2003). The CCD domain mediates the catalytic reaction during integration into the host genome by using conserved amino-acid residues composed of a DDE motif (Asp, Asp-35-Glu) (Goldgur *et al*., 1998). The CTD is a less conserved domain than the other two domains and is considered to constitute a site for nonspecific DNA binding (Engelman *et al*., 1994).

Retroviral enzymes are translated by the Pol gene, and the IN gene resides on the 3' segment of the Pol. In most retroviruses, including HIV-1, Pol and the viral structural protein Gag are synthesized as polyprotein precursors. Retroviral protease plays a key role in the proteolytic

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Abbreviations: CCD = catalytic core domain; DDE = Asp, Asp-35-Glu motif; FAB assay = foamy virus activated beta-gal expression assay; FVs = foamy viruses; IN = integrase; PFV = prototype foamy virus; TEM = transmission electron microscope

cleavage of these precursors into their mature forms. Regulation of this cleavage is critical for proper capsid assembly and virion infectivity (Kohl *et al.*, 1988). Unlike other retroviruses, FVs follow a unique strategy for the viral maturation. These viruses express Pol independently of Gag (Enssle *et al.*, 1996). FV translate the Gag precursor protein that is encoded by the (pre-) genomic or full-length viral RNA, like all retroviruses. In the case of PFV, this is a 71 kD size protein (Heinkelein *et al.*, 2000). Unlike orthoretroviruses the FV Gag undergoes only a limited proteolytic cleavage upon maturation (Flügel and Pfrepper, 2003). During capsid assembly, the FV Gag precursor (p71) is cleaved near the C-terminus resulting in a larger (p68) and a smaller (p3) Gag cleavage product (Fig. 1a, Cartellieri *et al.*, 2005). However, some studies have demonstrated that the C-terminal residue of the Gag open reading frame affects the splicing of the Pol transcript (Lee *et al.*, 2008). In addition, synthetic FVs with in-frame Gag-Pol fusion proteins are infective, unlike ortho-retroviruses (Lee *et al.*, 2008). Nevertheless, FV Gag-Pol interactions remain unclear. Here, we generated prototype foamy virus (PFV) mutants, in which the IN gene was mutated with deletions, to study Gag-Pol interaction. We observed that deletions of more than two amino acids in PFV IN could impair the Gag maturation, indicating that FV Gag and Pol should interact during the viral maturation and this interaction is necessary for the viral infectivity. Results from serial deletion mutagenesis, immuno-blotting, and TEM confirmed these conclusions.

Materials and Methods

Cloning of the PFV IN deletion mutants. The PFV IN point and deletion mutants were constructed using the PFV cDNA base plasmid pcHFV (molecular clone DNA of PFV) digested with SwaI and BspEI. For subcloning, oligonucleotide containing SwaI and BspEI cleavage sites was ligated into pGEM-5zf (+) plasmid. The oligonucleotides used for cloning are listed in Table 1. The successfully generated subcloning vector (intermediate vector) was ligated into pcHFV DNA digested with SwaI and BspEI. The point mutations of the PFV IN DNA were constructed as follows: first, the DNA fragments encoding the PFV IN mutants were constructed using the overlap PCR method (Horton *et al.*, 1993). For each mutation, two DNA fragments were amplified using sequence-specific primers and pQE-PFV IN as the template. After purification, the two PCR products were mixed and used as the template in a second-round PCR to amplify the mutant DNA by using PFVIN-1S and PFVIN-371A as the primers. All the PCR reactions were performed using pfu DNA polymerase (Bioneer, Korea) to avoid errors in DNA synthesis. The sequences of the PCR-amplified DNA fragments were verified via restriction enzyme analysis and sequencing based on dideoxynucleotide chain termination. The confirmed PFV IN mutants were inserted into pGEM-5zf containing SwaI

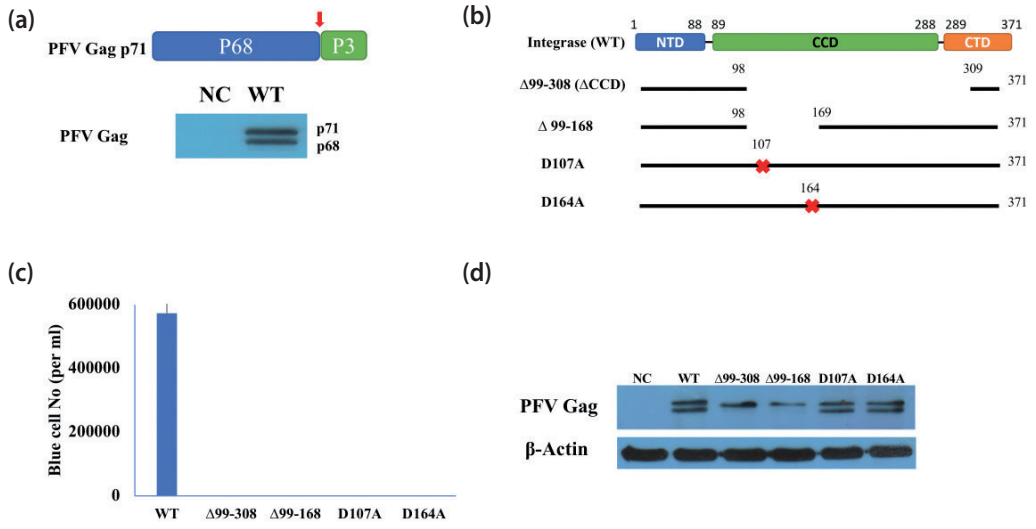

Fig. 1

PFV IN CCD deletion impairs the Gag cleavage

(a) Schematic progress of PFV Gag cleavage and western blotting of wt PFV-infected cell lysates. **(b)** Construction of the IN mutants. Two deletion mutants were designed to compromise the function of IN. The construction of point mutants D107A and D164A were based on the well-known DDE motif of PFV IN. The sites of the point mutations were denoted with red X marks. Wt IN is also shown. **(c)** Viral titers of the PFV IN mutants. Each titer was measured using the FAB assay. All the data are representative of three independent experiments with triplicate samples. **(d)** Western blotting of Gag. Cells were lysed three days after transfection and subjected to western blotting. β-actin was used as an internal loading control. NC = non-transfected Cos-7 cell lysate.

Table 1. DNA sequence of primers used in this study

Primer name	Sequence (5'–3')	Purpose
pGEM-5zf SalI-NsiIS	5'- TCGACATTTAAATTTAATTAACAATTGTCCGGAATGCA -3'	For intermediate vector cloning
pGEM-5zf SalI-NsiIA	5'- TTCCGGACAATTGTTAATTAAATTTAAATG -3'	
PFVIN-1S-NdeI	5'- GTCACATATGAAAGGATATCCCAAACAATATAC-3'	For point mutant cloning
PFVIN-371A-BamH1	5'- GTCAGGATCCTTATTCATTTTTTTCCAAATGATC -3'	
PIN D107AS	5'- CAGGAGAGGGTGTACAGCTCGTCACAATTAAGG -3'	
PIN D107AA	5'- GTGACGAGCTGTACACCCTCTCCTGGACTAGCTG -3'	
PIN-D164AS	5'- GATTCACTCTGCTCAAGGTGCAGCATTCACTTC-3'	
PIN-D164AA	5'- CACCTTGAGCAGAGTGAATCACCTTTGGAATTG-3'	
PIN 164S-StuI	5'- GATCAAGGTGCAAGGCCTACTTCTTCAACCTTTG -3'	For deletion mutant cloning
PIN 311A-StuI	5'- GAAGCAGGCCTAGCCACCCTCTCCTG -3'	
PIN 95S-StuI	5'- GATAGGCCTCAAAGGCCTTTTGATAAATTC -3'	
PIN 107S-StuI	5'- CTATATTGGACCTTTGAGGCCTTCACAGGGATAC -3'	
PIN 135S-StuI	5'- CCCCACTAAGAGGCCTTCTACTAGCGCAAC -3'	
PIN 152S-StuI	5'- CACTAGTATTGCAAGGCCTAAGGTGATTCACTC -3'	
PIN 102S-StuI	5'- GATAAATTCAGGCCTGACTATATTGGACCTTTG -3'	
PIN 98S-StuI	5'- CAAAAACCTAGGCCTAAATTCTTTATTGACTATATTG -3'	
PIN 100S-StuI	5'- CTTTTGATAGGCCTTTTATTGACTATATTGGAC -3'	
PIN 97S-StuI	5'- CTCAAAAAAGGCCTGATAAATTCTTTATTGAC -3'	
PIN 93S-StuI	5'- GACCAGATAGGCCTCAAAAACC -3'	
PIN-235A_2D	5'- GGTGTTGTTTAAAGCTACAACAGGCAATAG -3'	
PIN-223S_2D	5'- CTATTGCCTGTTGTAGCTTTAAACAACACC -3'	
PIN-237A_3D	5'- CTATAGGTGTTGTTTAATACAACAGGCAATAG -3'	
PIN-223S_3D	5'- CTATTGCCTGTTGTATTAAACAACACCTATAG -3'	
PIN 244A_10D	5'- GAGTATATTTTAATACTACAACAGGCAATAG -3'	
PIN 223S_10D	5'- CTATTGCCTGTTGTAGTATTAAAATATACTC -3'	

and BspE1 restriction sites via restriction digestion. Finally, the confirmed intermediate vectors encoding PFV IN were cut with SwaI and BspEI and ligated into pcHFV.

Virus production and titer estimation. Wild-type and mutant viral particles were produced via transient transfection of the pcHFV and mutant viral DNA by using the polyethylenimine transfection reagent as described previously (Müllers *et al.*, 2011). Briefly, Cos-7 cells (3×10^5) were seeded onto 60-mm tissue culture dishes. Once the culture was at approximately 50% confluency the cells were transfected with 5.8 μg of either pcHFV or mutant DNA. The transfected cells were then incubated at 37°C with 5% CO_2. At 3 days post-transfection, the culture supernatants were collected and centrifuged at 22,000 g for 15 min at 4°C, and the pellets were discarded. The virus titer of each supernatant was measured using the FAB assay as described previously (Hamid *et al.*, 2017; Löchelt *et al.*, 1993). Approximately 1.5×10^5 FAB cells were infected with serially diluted supernatants. After 48 h, the cells were fixed with the fixing solution (0.2% glutaraldehyde and 1% formaldehyde in PBS). After washing with PBS, the fixed FAB cells were incubated for 4 h with an X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining solution. The blue cells were then counted using an inverted microscope. All the data are representative of three independent experiments with triplicate samples.

Western blotting. Cos-7 cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, and 0.5% sodium deoxycholate) as described previously (Hossain *et al.*, 2014). The cell lysates were mixed with the SDS-loading dye prior to electrophoresis. Equal amounts of samples were resolved using 12.5% SDS-PAGE at 120 V for 1.5 h. The resolved proteins were then transferred onto nitrocellulose membranes (GE Healthcare UK Ltd., Hammersmith, UK) at 40 V for 1.5 h in a semi-dry–transfer apparatus (Hoefer, Inc., USA). The membranes were blocked for 3 h at 25°C with PBST blocking buffer (0.1% Tween 20, 5% nonfat dry milk in PBS). The membranes were then probed with an in-house rabbit polyclonal antibody against PFV-Gag NC protein (1: 3,000 dilution) in PBST solution overnight. After three washes with PBST, the membranes were incubated with a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1: 10,000 dilutions, Sigma-Aldrich) in PBST for 1 h at 25°C. The membranes were washed three times with PBST and developed using a chemiluminescence (ECL) detection kit (BioFACT, Korea). As an internal control, β-actin was probed using a mouse monoclonal antibody against β-actin (1: 5000 dilution, Thermo Fisher Scientific, USA).

Transmission electron microscope (TEM) imaging. To prepare the samples for TEM imaging, WT and mutant viruses were produced by transfection in Cos-7 cells. After intended time of period, the supernatants were harvested and centrifuged at 2,000 g for 10 min. Next, each supernatant was collected and then filtered through a filter with a pore size of 0.45 μm (Millipore Sigma, USA). The filtered supernatants were concentrated with Vivaspin 20 (Sartorius AG, Germany). Finally, supernatants were centrifuged at 30,000 g for 2 h in 4°C. Each pellet was

resuspended with 0.9% NaCl. The TEM images were captured by an energy-filtering transmission electron microscope (Carl Zeiss, Germany) in NICEM (Seoul, Korea).

Statistical analysis. All the data are expressed as mean ± SEM. Statistical significance was analyzed using the two-paired Student's t-test; * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Results

IN CCD residue deletion impairs the Gag cleavage

First, we constructed whole or partial deletion and DDE point mutants of PFV IN CCD (Fig. 1b). According to the FAB assay result, deletion mutants of the viral DNA could not produce infectious PFV particles. In addition, no productive viruses were observed in the DDE point mutants of D107A and D164A infection FAB assay, as expected (Fig. 1c). We observed that PFV-Gag immunoblots of the deletion mutants showed unique patterns suggestive of defective Gag cleavage (Fig. 1d), while D107A and D164A mutants did not significantly differ from the wild-type (wt) PFV in Gag cleavage.

PFV IN mutants with intact purine-rich regions are defective in Gag cleavage

We have found out, that we have, in previous study, accidently deleted the four purine-rich regions that regulate the Gag/Pol (Fig. 2) (Moschall *et al.*, 2017). To avoid those region deletions, we constructed 4 deletion mutants (Fig. 3a). The deletion site in each mutant was particularly selected to consist of the StuI restriction enzyme digestion site "CCT" to facilitate cloning. The long deletions in three mutants (Δ99–114, Δ99–140, and Δ99–158) compromised the production of infectious virions, based on the results of the FAB assay (Fig. 3b). Additionally, these mutants were defective in Gag cleavage (Fig. 3c). However, surprisingly, the mutant without only the two amino acids on positions 99 and 100 (Δ99–100) still produced infectious viruses and showed normal Gag cleavage as in the wt (Fig. 3b and 3c, Δ99–100 mutant).

Although the deletion mutants retained the purine-rich regions, Gag maturation was still impaired. Therefore, we hypothesized that the PFV IN amino acids between positions 99 and 114 affect Gag maturation and constructed deletion mutants lacking 3, 4, 6, or 8 amino acids (Fig. 4a).

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1   aaaggatatcccaaacaatatacatatttttagaagatggcaaagtaaaagtttccagacctgaaggggttaaaattattccccctcagtcagacagacaaaaaattgtgcttcaagcc  120
    K  G  Y  P  K  Q  Y  T  Y  F  L  E  D  G  K  V  K  V  S  R  P  E  G  V  K  I  I  P  P  Q  S  D  R  Q  K  I  V  L  Q  A

121 cacaatttggctcacaccggacgtgaagccactcttttaaaaattgccaacctttattggtggccaaatatgagaaaggatgtggttaaacaactaggacgctgtcaacagtgtttaatc  240
    H  N  L  A  H  T  G  R  E  A  T  L  L  K  I  A  N  L  Y  W  W  P  N  M  R  K  D  V  V  K  Q  L  G  R  C  Q  Q  C  L  I
                                                                                                  107
241 acaaatgcttccaacaaagcctctggtcctattctaagaccagataggcctcaaaaacctttgataaattctttattgactatattggacctttgccaccttcacagggatacctatat  360
    T  N  A  S  N  K  A  S  G  P  I  L  R  P  D  R  P  Q  K  P  F  D  K  F  F  I  D  Y  I  G  P  L  P  P  S  Q  G  Y  L  Y

361 gtattagtagttgttgatggaatgacaggattcacttggtataccccactaaggctccttctactagcgcaactgttaaatctctcaatgtactcactagtattgcaattccaaaggtg  480
    V  L  V  V  V  D  G  M  T  G  F  T  W  L  Y  P  T  K  A  P  S  T  S  A  T  V  K  S  L  N  V  L  T  S  I  A  I  P  K  V
          164                                                                 A                                          200
481 attcactctgatcaaggtgcagcattcacttcttcaacctttgctgaatgggcaaaggaaagaggtatacatttggaattcagtactccttatcacccccaaagtggtagtaaggtggaa  600
    I  H  S  D  Q  G  A  A  F  T  S  S  T  F  A  E  W  A  K  E  R  G  I  H  L  E  F  S  T  P  Y  H  P  Q  S  G  S  K  V  E
     B
601 aggaaaaatagtgatataaaacgacttttaactaaactgctagtaggaagacccacaaagtggtatgacctattgcctgttgtacaacttgctttaaacaacacctatagccctgtatta  720
    R  K  N  S  D  I  K  R  L  L  T  K  L  L  V  G  R  P  T  K  W  Y  D  L  L  P  V  V  Q  L  A  L  N  N  T  Y  S  P  V  L
                                                                                                  C
721 aaatatactccacatcaactcttatttggtatagattcaaatactccatttgcaaatcaagatacacttgacttgaccagagaagaagaacttttctcttttacaggaaattcgtacttct  840
    K  Y  T  P  H  Q  L  L  F  G  I  D  S  N  T  P  F  A  N  Q  D  T  L  D  L  T  R  E  E  E  L  S  L  L  Q  E  I  R  T  S
                                                                                 D
841 ttataccatccatccacccctccagcctcctctcgttcctggtctcctgttgttggccaattggtccaggagagggtggctaggcctgcttctttgagacctcgttggcataaaccgtct  960
    L  Y  H  P  S  T  P  P  A  S  S  R  S  W  S  P  V  V  G  Q  L  V  Q  E  R  V  A  R  P  A  S  L  R  P  R  W  H  K  P  S

961 actgtacttaaggtgttgaatccaaggactgttgttattttggaccatcttggcaacaacagaactgtaagtatagataatttaaaacctacttctcatcagaatggcaccaccaatgac 1080
    T  V  L  K  V  L  N  P  R  T  V  V  I  L  D  H  L  G  N  N  R  T  V  S  I  D  N  L  K  P  T  S  H  Q  N  G  T  T  N  D

1081 actgcaacaatggatcatttggaaaaaaatgaa 1113
     T  A  T  M  D  H  L  E  K  N  E
```

Fig. 2

Purine-rich regions in PFV IN

The four purine-rich regions (a, b, c, and d; indicated in red font) were determined from a reference study (modified from Moschall *et al.*, 2017). PFV IN DDE-motif residues D107 and D164A are also indicated in red. The DNA sequence is based on human foamy virus proviral DNA complete genome HFV (corresponds to PFV) (Genbank: Y07725.1).

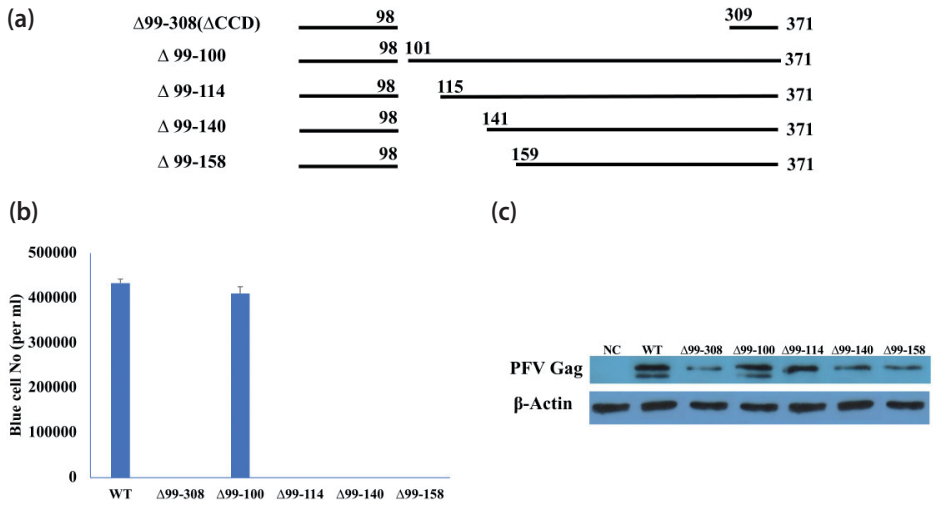

Fig. 3

The long deletion mutants are defective in Gag cleavage, unlike the mutant lacking the two amino acids on positions 99 and 100
(a) Construction of second IN deletion mutants. This time, the deletion mutants were designed to retain the purine-rich regions described in Fig. 2. **(b)** Viral titers of the PFV IN mutants. Each titer was measured using the FAB assay. All the data are representative of three independent experiments with triplicate samples. **(c)** Western blotting of Gag. β-actin was used as an internal loading control. NC = non-transfected Cos-7 cell lysates.

None of these mutants yielded any viral titer, in the FAB assay, and Gag cleavage (Fig. 4b,c). These results indicate that deletion of more than two amino acids in the CCD region impairs the Gag cleavage, compromising the production of infectious virions.

For investigation of viral structure and maturation in detail, transmission electron microscopy (TEM) was used. As shown in Figure 5, the mutant lacking the two amino acids (Δ99–100) and the point mutant (D164A) showed an intact virion structure akin to that of the wt PFV, whereas the mutants lacking three or four amino acids (Δ99–101, Δ99–102) did not form any virion like structure. The long deletion mutants (Δ99–168 and Δ99–308) did not show any clumping structure (Fig. 5).

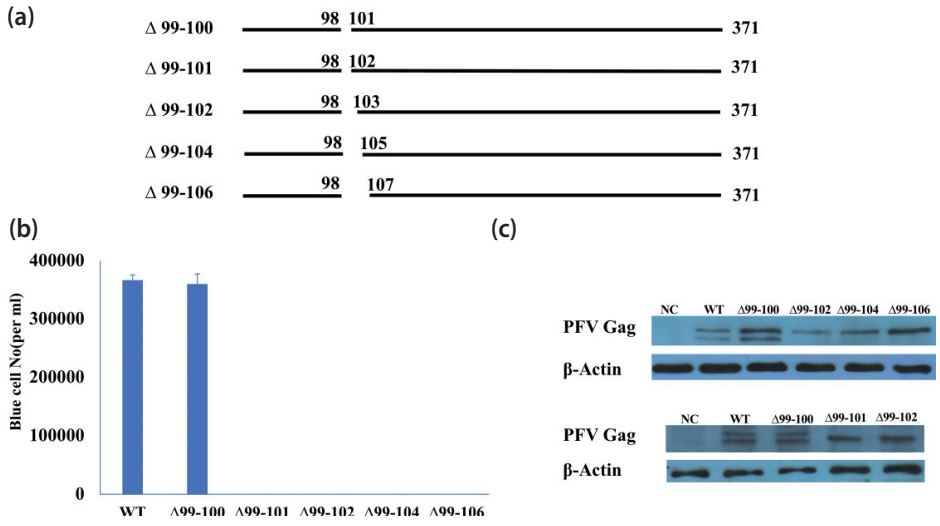

Fig. 4

Deletion of more than two amino acids impairs Gag cleavage
(a) Construction of the IN deletion mutants. **(b)** Viral titers of the PFV IN mutants. Each titer was measured using the FAB assay. All the data are representative of three independent experiments with triplicate samples. **(c)** Western blotting for Gag. β-actin was used as an internal loading control. NC = non-transfected Cos-7 cell lysates.

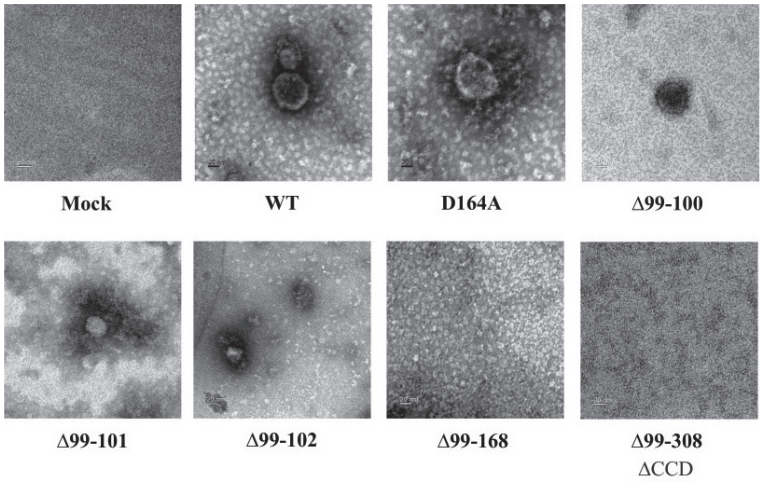

Fig. 5

Transmission electron microscopy images of the wild-type (wt) virus and IN mutants
Cos-7 cells were transfected with the wt and mutant DNAs. TEM images were captured using an energy-filtering transmission electron microscope (Carl Zeiss) in NICEM (Korea). The scale bar denotes 20 nm. NC = non-transfected Cos-7 cell lysates.

Same viral titer and blotting patterns were observed in 2 amino acid deletion mutants at another region of PFV IN

Given the results described above, we hypothesized that the 3D structural characteristics of IN is affected by small deletions, independent of amino acid sequence. To address this hypothesis, another region of PFV IN was mutated. Three mutants, Δ229–230, Δ229–231, and Δ229–238, were designed to mimic Δ99–100, Δ99–101, and Δ99–114, respectively (Fig. 6a). As expected, the viral titer and western blotting pattern were similar to that in the previous results. In detail, only the mutant lacking the two amino acids showed successful Gag cleavage and wt level of viral titer (Fig. 6b,c). However, the mutants without the three or 10 amino acids were defective in Gag cleavage and viral reproduction.

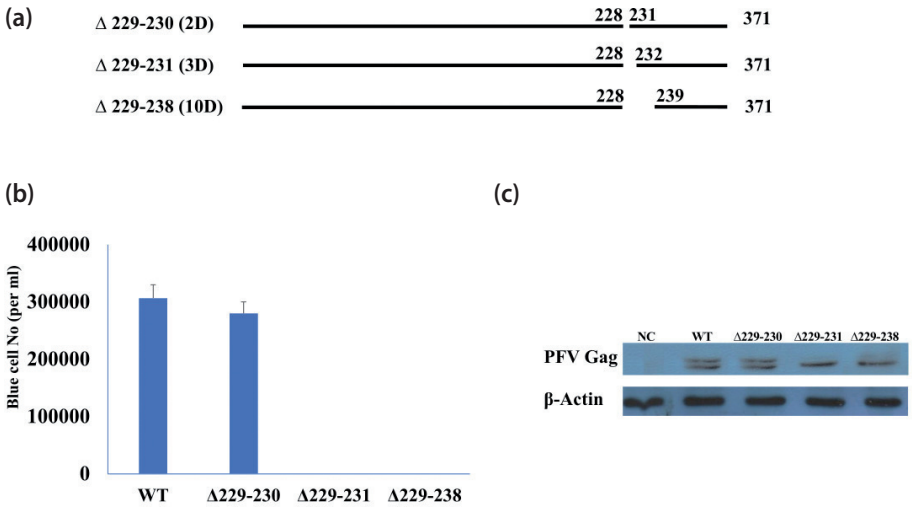

Fig. 6

Same viral titer and western-blotting pattern were observed in mutant deleted with two amino acids at another region of PFV IN
(a) Construction of the mutants lacking residue 229. Three deletion mutants, mimicking Δ99–100, Δ99–101, and Δ99–114 mutants, were designed. **(b)** The viral titers of the PFV IN mutants. Each titer was measured using the FAB assay. All the data are representative of three independent experiments with triplicate samples. **(c)** Western blotting for Gag. β-actin was used as an internal loading control. NC = non-transfected Cos-7 cell lysates.

Discussion

To study PFV Pol and Gag interaction, we constructed various IN deletion mutants. Initially, we intended to make IN CCD deletion mutant for other researches but new phenotype has shown. In the first experiments, we thought that deletion of purine-rich regions might affect the Gag cleavage pattern. However, in the second set of experiments, the mutants lacking two amino acids showed the same cleavage pattern like in the wt. The Gag cleavage is required for the switch of reverse transcriptase (Spannaus *et al*., 2013), RNA packaging, and Pol expression (Stenbak and Linial, 2004). Therefore, the Gag cleavage is necessary for successful viral maturation. To date, there has been no study about the exact cleavage pattern. Interestingly, it has been known that PFV Pol and Gag are expressed independently of each other. Thus, we focused our studies on residues in PFV IN that cause phenotype of deletion mutants. We observed that only the mutation lacking the two amino acids showed wt level of viral production and Gag cleavage pattern. TEM results also demonstrated that the morphology of this mutant virus is the same as that of the wt and D164A point mutant, whereas the mutants lacking more than three amino acids were defective. We first thought that the defective Gag cleavage might be due to the absence of site-specific interaction between Gag and Pol during the maturation process, since the deletion mutants lack some residues of IN. However, we observed the same results in the deletion mutants lacking residue between 229 and 238 (Fig. 6). Therefore, the defective Gag cleavage in the IN mutant is not due to a specific interaction between the Gag and Pol proteins of PFV. The exact mechanism of this results is yet unclear.

Recently, it has been demonstrated that viral IN and RNA interaction affects virion morphogenesis (Elliott *et al*., 2020; Kessl *et al*., 2016). There are two possible explanations for this observation. Cellular proteins are involved in host cell infection and viral maturation. In HIV-1 life cycle, integrase interactor (INI1)/hSNF5 is one of the host factors that interact with HIV-IN in post entry events (Mathew *et al*., 2013). Clathrin is also known as a host factor associated with the morphogenesis of retrovirus particles (Zhang *et al*., 2011). In detail, the HIV-1 Pol protein is required for clathrin incorporation. Point mutations in the Pol domains could impair this incorporation. Clathrin incorporation has been studied in other retroviruses, such as the gamma-retrovirus (murine leukemia virus, MLV) and beta-retrovirus (Mason-Pfizer monkey virus, M-PMV) genera, as well as the lentivirus (simian immunodeficiency virus, SIVmac) genus. FVs have been studied somewhat less than other retroviruses, and thus further research on host factors associated with viral maturation is required. Second possibility is the association with the viral protein inside the foamy virus particles. In lentivirus HIV-1, the cleavage of Gag-Pol precursor protein is required for viral protease (PR), and this event is critical for virion infectivity (Haraguchi *et al*., 2012). In FVs, a small change in IN 3D structure may abolish the interaction between Gag and other enzymes, such as the PR. Besides, it has been known that retroviral IN directly binds to the viral RNA (vRNA) and participates in the vRNA packaging (Elliott and Kutluay, 2020; Fontana *et al*., 2015; Kessl *et al*., 2016). This is likely to be the case with FVs. Additional studies of IN are needed for various perspectives.

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