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Biochemical characteristics of functional domains using feline foamy virus integrase mutants

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We constructed deletion mutants and seven point mutants by polymerase chain reaction to investigate the specificity of feline foamy virus integrase functional domains. Complementation reactions were performed for three enzymatic activities such as 3′-end processing, strand transfer, and disintegration. The complementation reactions with deletion mutants showed several activities for 3′-end processing and strand transfer. The conserved central domain and the combination of the N-terminal or C-terminal domains increased disintegration activity significantly. In the complementation reactions between deletion and point mutants, the combination between D107V and deletion mutants revealed 3′-end processing activities, but the combination with others did not have any activity, including strand transfer activities. Disintegration activity increased evenly, except the combination with glutamic acid 200. These results suggest that an intact central domain mediates enzymatic activities but fails to show these activities in the absence of the N-terminal or C-terminal domains. [BMB Reports 2013; 46(1): 53-58]

INTRODUCTION

Foamy viruses (FVs) belong to the family *Retroviridae* of the *Spumaretrovirinae* subfamily with only one genus (*Spumavirus*) (1). Spumaviruses are representative prototype foamy viruses (PFV) previously called human foamy virus and feline foamy virus (FFV). The foamy viruses (FVs) are endemic to most non-human primates (NPS), cats, cattle, and horses as well as humans who have acquired the infection from NPS (2, 3), which was first isolated in 1971 (4). FVs are also anticipated to be an available candidate as a gene therapy vector (5).

The virally encoded protein integrase (IN) is involved in the integration event, which is a highly ordered three-step process similar to reactions mediated by other members of the polynucleotidyl transferase family (6). Retroviral IN carries out of order DNA cleavage and joining reactions during viral integration, 3'-end processing (or endonucleolytic) activity, and strand transfer (integration or 3'-end joining) activity, respectively. In the first step of the 3'-end processing reaction, the highly conserved CA dinucleotide from the 3'-end of linear viral DNA exposes the 3'-hydroxyl group at both ends (7, 8). The second step, called the strand transfer reaction, is a concerted cleavage-ligation reaction during which IN makes a staggered cut in the chromosomal targeted DNA and ligates the recessed 3'-ends of the pre-processed viral DNA into opposing strands at the target DNA cleavage site (9, 10). The final step, called disintegration (or 5'-end joining), resolves the gapped intermediate of the intact double-stranded DNA, which is repaired by host cell enzymes to produce a stable provirus (11).

Retroviral IN proteins share distinctively conserved organization of their functional domain, and each contains a central catalytic core domain (CCD) flanked by N- and C-terminal domains (NTD and CTD) (12, 13). The NTD comprises the HHCC region (14) and mediates multimerization of IN and formation of a stable complex with viral DNA. The NTD is also necessary for recognizing and binding viral DNA (15, 16). Deletion mutants of this domain lose endonucleolytic and disintegration activities (17). The CCD harbors the most conserved region of INs and bears close structural homology to prokaryotic transposases (18). The enzyme active site is comprised of three invariant acidic residues (D, $DX_{35}E$ motif) (19). The CTD is the least conserved region and is able to bind DNA non-specifically and is likely contained at the site for binding target DNA during integration (20, 21).

The biochemical characteristics of oncoretroviral and lentiviral integration reactions *in vitro* have been well reported and documented using synthetic duplex oligonucleotide substrates mimicking the U5 and U3 ends of retroviral DNA, whereas only a few studies on foamy viral IN have been reported (22, 23). We have characterized the biochemical properties of FFV IN using several metals containing the Mn^{2+} cation, which is a cofactor in the integration reaction (24). However, the FFV IN functional domains have not yet been clearly characterized. In this study, we constructed deletion and point mutants of FFV IN to analyze these functional domains. A complementation analysis indicated that the CCD of FFV IN has potential enzymatic activity but was unable to recognize viral or target DNA without the help of the NTD or CTD.

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RESULTS

Construction, expression, and purification of IN mutants

FFV IN is larger than HIV-1 or PFV INs, with a molecular mass of 45 kDa and 383 amino acid residues. It has three domains called the CCD, NTD, and CTD. DNA constructs for preparing the point mutated INs were prepared by overlap extension polymerase chain reaction (PCR) (Fig. 1A).

Wild-type, deletion mutants, and point mutants were expressed in a vector containing the His-tag upstream of the multiple cloning site. The protein was purified using a Talon column and heparin or Ni-NTA column chromatography (Fig. 1B). Initially, we tried to purify FFV IN and its mutants using a Talon column, but some non-specific nuclease activity remained. So, we loaded the protein onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel, cut the desired band, and prepared a gel slice. The slice was mixed with SDS-running buffer prior to electro-elution. We purified the protein again by nickel-chelated chromatography with a small bed volume (0.25 ml) after dialyzing against the S10 buffer and collecting the supernatant. Finally, the FFV IN and mutant proteins were prepared from 2 L of an *E. coli* culture.

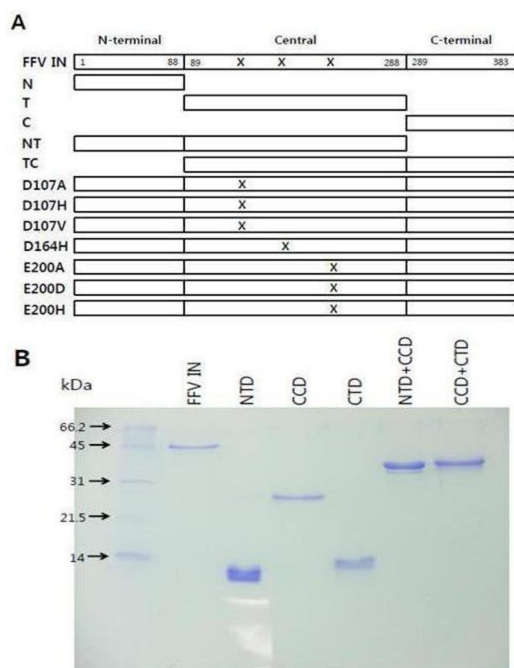

Fig. 1. Structure and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the integrase deletion mutant. (A) Primary structures. The feline foamy virus (FFV) integrase is divided into N-terminal, central, and C-terminal domains. We named N: N-terminal domain (NTD); T: central domain; C: C-terminal domain; NT: N-terminal domain + central domain; TC: central domain + C-terminal domain. Substitutions in the FFV integrase point mutants are marked with an X. (B) SDS-polyacrylamide gel electrophoresis of FFV deletion mutant integrases. Purified wild-type and deletion mutant integrases were separated by 16.5% SDS-polyacrylamide gel electrophoresis, and the gel was stained with Coomassie Blue (low molecular weight standards; 14, 21.5, 31, 45, and 66.2 kDa from the bottom of the gel).

Complementation of FFV IN point mutants

Complementation reactions were performed to confirm whether the enzymatic activity of the CCD could be complemented with each point mutant. Seven kinds of point mutants were produced by overlap extension PCR (Fig. 1A), and D107A, D164H, and E200D were used in the complementation reaction. The combination of D107A and D164H showed all enzymatic activities. We speculate that the D164H point mutant had strong activity by itself. In contrast, the combination with E200D had no activity. These results suggest that if the second aspartic acid at 164 of the DDE motif in the CCD was defective, it can be supplied by adding a defective aspartic acid to 107 of the DDE motif, but that mutated glutamic acid at 200 strongly inhibited all enzymatic activities (data not shown).

Functional complementation of the defective deletion and FFV IN point mutants

The CCD is important for catalysis during IN activity. We combined the deletion mutants with other mutants and tested all possible combinations to investigate whether deletion mutants containing the CCD could increase enzymatic activity. The 3'-end processing reactions using the FFV U5 substrate are shown in Fig. 2A. Except the activity present in the NTD (N) and CCD (T), the other combinations did not increase activity. A similar pattern was observed for strand transfer activity (Fig. 2B). All combinations with CCD showed strong disintegration reaction activity. In particular, the combinations of N + TC, C + NT, and NT + TC showed strong activity. However, no 3'-end processing or disintegration activities were observed for the defective NTD, TC, or the combination of T + C (Fig. 2C). It seemed that the defective deletion mutant INs recover catalytic activity if combined with a different defective IN that compensates for its structural defects. Therefore, we suggest that the CCD of the FFV IN was not completely defective.

Several studies have shown that the DD(35)E mutants of retroviral INs lose enzymatic activities, as these residues are critical for activity. This was presumably the case for FFV IN, although this has not been tested directly. The lack of activity by the FFV IN deletion mutants may be due to an intact but non-functional CCD due to a lack of essential cooperation from the CTD with an appropriate conformation. We constructed seven FFV IN point mutants and five deletion mutants to investigate differences between the deletion and point mutants.

The point mutants D107A, D107H, and D164H alone had weak activity for 3'-end processing, whereas most of the other point mutants did not show any activity. Activities were inhibited or increased weakly when combined with the deletion mutants. However, some combinations such as NT + D107H increased activity (Fig. 2D), and, particularly, the complementation reactions between the NT and TC deletion mutants and the D107V point mutant increased activity significantly (Fig. 2E). All of the point mu-

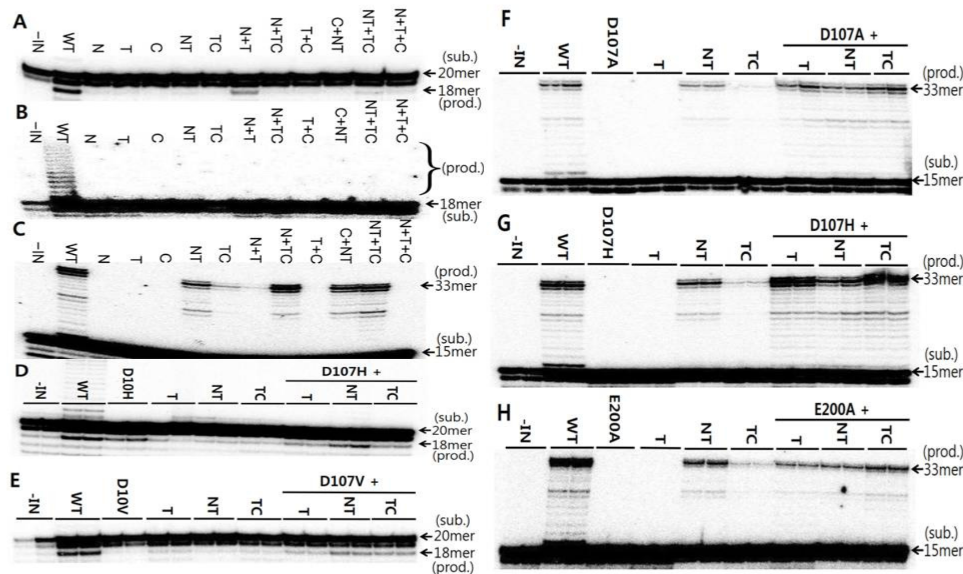

Fig. 2. *In vitro* complementation assays for 3'-end processing, strand transfer, and disintegration activities of the feline foamy virus (FFV) integrase deletion and point mutants. (A) 3'-end processing reactions of the deletion mutant integrases on an FFV U5 substrate. (B) Strand transfer activity of the deletion mutant integrases on an FFV U5 substrate. The strand transfer products (integration) appeared as bands larger than the 18mer. (C) Disintegration activities of the deletion mutant integrases on the FFV U5 substrate. (D, E) Complementation of 3'-end processing activities between deletion and point mutants. The 3'-end processing of D107A, D164H, E200A, E200D, and E200H data are not shown. (F) Complementation of disintegration activity between the deletion and D107A point mutant. (G) Complementation of disintegration activity between the deletion and D107H point mutants. (H) Complementation for disintegration activity between deletion and E200A point mutants. Disintegration data of D107V, D164H, E200D, and E200H are not shown.

tants alone, the deletion mutants alone, and complementation reactions between the point and deletion mutants did not show strand transfer activity (data not shown). As expected, disintegration activity increased in the following combinations: T + D107A/D107H/E200H and TC + D107A/D107H/ E200H (Fig. 2F-H), but no complementation reactions showed increased activity between deletion mutants and E200D or E200H, As their activities were inhibited regardless of all other combinations (data not shown). The results of the complementation reactions are shown in Table 1.

DISCUSSION

Purified retroviral INs have three kinds of *in vitro* enzymatic activities such as 3'-end processing, strand transfer, and disintegration. Several studies have shown that the 3'-end processing and strand transfer reactions require intact IN proteins and specific DNA substrates (22, 25), and that the retroviral IN CCD plays a major role in binding viral DNA and in target site selection (26). We constructed five FFV IN deletion mutants and seven point mutants to investigate the function of the three FFV IN domains.

The deletion mutants with the FFV IN CCD had several disintegration activities. We observed that only the CCD was incapable of performing the disintegration reaction; however, its combination with other mutants resulted in disintegration activity. In addition, we confirmed that the lack of the NTD resulted in no 3'-end processing or disintegration activities (except disintegration activity by TC). We also observed that different combinations of the CCD, NTD, or CTD had stronger disintegration activity compared to that of wild-type IN. This observation indicates that an intact FFV IN structure is not necessary for disintegration activity.

The point mutants lacking the CCD had no enzymatic activity, as the CCD, which is important for catalysis, was mutated. Loss of function of the DDE motif did not complement any of the point mutants. However, a mutated glutamic acid at 200 of the DDE motif resulted in inhibition of all enzymatic activities. This result indicates that the DDE motif glutamic acid at position 200 is more important for enzymatic activity than that of aspartic acid 107 or aspartic acid 164.

The complementation reactions using the point and deletion mutants also produced similar data as those reported previously. Almost all results showed a similar pattern with that of previous results, and the complementation reaction between D107V and the deletion mutants resulted in increased 3'-end processing activity. However, the E200A and E200H mutants did not show any enzymatic activities, as observed previously. But, E200A had disintegration activity when combined with the deletion mutants. This was probably due to structural changes by converting from a negatively charged amino acid to a nonpolar amino acid.

Several studies on FFV IN have been reported. However, the ac-

Table 1. Complementation of defective deletion and point mutants of FFV integrase

	D107A			D107H		
	3'-end processing[a]	Strand transfer	Disintegration	3'-end processing	Strand transfer	Disintegration
FFV IN	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
T	−	−	+ + +	−	−	+ + +
NT	−	−	+ +	+	−	+ +
TC	−	−	+ + +	−	−	+ + +
	D107V			**D164H**		
	3'-end processing	Strand transfer	Disintegration	3'-end processing	Strand transfer	Disintegration
FFV IN	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
T	+/−	−	+ +	−	−	+/−
NT	+ +	−	−	−	−	+
TC	+ +	−	+ +	−	−	+ +
	E200A			**E200D**		
	3'-end processing	Strand transfer	Disintegration	3'-end processing	Strand transfer	Disintegration
FFV IN	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
T	−	−	+ +	−	−	−
NT	−	−	−	−	−	−
TC	−	−	+ +	−	−	+/−
	E200H					
	3'-end processing	Strand transfer	Disintegration			
FFV IN	+ + +	+ + +	+ + +			
T	-	-	-			
NT	-	-	-			
TC	-	-	-			

[a]The enzymatic activities of the wild-type integrase on its own DNA substrate were set at 100%, and -, +/−, +, + +, and + + + indicate 0-2, 2-5, 5-20, 20-70, and 70-100%, respectively of this activity.

tions of the FFV IN functional domains have not yet been clearly elucidated. Other researchers have assumed that the PFV IN NTD is required for all enzyme activity (27). We conclude that the FFV IN is required for enzymatic activity. The three-dimensional structures of each domain have been reported using X-ray crystallography or nuclear magnetic resonance (18, 21). However, it is difficult to establish the exact three-dimensional structures of the IN protein and DNA substrate, and it has not yet been demonstrated. The three-dimensional structure is surely a clue to unravel these questions. Therefore, our biochemical FFV IN studies may help to understand how to identify the enzyme activities of each functional domain and how to develop efficient chemotherapeutic agents.

MATERIALS AND METHODS

Construction of the expression vector

The FFV IN expression vector was constructed by amplifying the FFV IN cDNA isolated from the FFV *pol*-gene and by ligating the PCR product into the *Bam*HI and *Hind*III sites of the pQE9 expression vector. The resulting recombinant (pQE-FF) construct was characterized to contain six histidine codons upstream of the IN sequence. The presence of six histidines in the expressed protein provides for a simple purification method based on the selective affinity for cobalt-chelated adsorbent (24).

Cloning of the FFV IN deletion and point mutants

Five plasmids encoding deletion mutants were constructed by amplifying the IN coding region by PCR, digesting the amplified DNA with *Bam*HI and *Hind*III, and ligating the cut DNA with *Bam*HI-*Hind*III-digested pQE9 vector DNA. The oligonucleotides used to construct the mutants INs and the sequences of each primer are shown in Supplement Table 1. All PCRs were performed with *pfu* DNA polymerase (Beams Biotechnology, Chiang Mai, Thailand) to minimize DNA synthesis errors.

DNA fragments encoding point mutated INs were constructed using the overlap PCR method (28). The primer sequences are pro-

vided in Supplement Table 1. The first part of FFV IN containing the D107A mutation was amplified using FFVIN-1Val and FFVIN D107AA as primers and pQE-FF as the template. The second part of FFV IN containing the D107A mutation was amplified using FFVIN D107A and FFVIN-383SA as primers and pQE-FF as the template. The purified PCR products were mixed and used as a template in the second-round PCR to amplify the full-length D107A DNA, with FFVIN-1Val and FFVIN-383SA as primers. FFVIN-1Val and FFVIN D107HA and FFVIN D107H and FFVIN-383SA were used as primers for the first and second parts, respectively, to construct D107H. The procedures for D107V, D164H, E200A, E200D, and E200H used the same methods. The sequences of PCR-amplified DNA fragments were verified by restriction analysis and the dideoxynucleotide chain termination method.

Expression and purification of IN proteins

The DNA constructs were transformed into *E. coli* XL-1 blue, and the transformants were grown at 37°C in 2 L of LB medium containing 50 μg ampicillin/ml. Isopropyl-1-thio-β-D-galactopyranoside (0.3 mM) was added at an optical density of 0.8 to induce expression, and the culture was grown for an additional 4 h. After harvesting, the cell pellet was frozen at −80°C, and the His-tagged proteins were purified by standard procedures and stored at −80°C. Frozen bacterial pellets were thawed and resuspended in 64 ml of S1 lysis buffer (50 mM Tris・HCl (pH 7.6), 20 mM β-mecaptoethanol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 10 mM imidazole). The cell suspension was kept on ice for 30 min. Then, 16 ml of 5 M NaCl and 8.8 ml of 100 mM CHAPS were added. The suspension was sonicated for 3 min on ice and centrifuged at 40,000 × g for 20 min at 4°C. The supernatant was directly loaded onto a Talon-column (bed volume of 1 ml, Clontech, Palo Alto, CA, USA) pre-equilibrated with S10 buffer (1 M NaCl and 10 mM CHAPS). The resin was washed four times with 3 ml of S10 buffer. Protein was eluted ten times with 0.5 ml S100 buffer (1 M NaCl and 100 mM CHAPS). Two methods were alternatively selected after purification. For the first method, fractions containing the protein were collected and diluted with nine volumes of buffer A [50 mM Tris・HCl (pH 7.6), 200 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol] before loading onto a column containing 0.25 ml heparin agarose. The column was washed with 6 ml of buffer A. The protein was eluted 12 times with 0.2 ml of buffer C [50 mM Tris・HCl (pH 7.6), 1 mM DTT, 0.1 mM EDTA, 1 M NaCl, 10% glycerol], and stored at −80°C. In the other method, the fractions were gathered and loaded on 12.5% or 16.5% SDS-polyacrylamide gels. Then, the gel containing the desired band was cut, sliced, and was added to SDS-running buffer (35 mM SDS, 1.5 M glycine, 250 mM Tris) prior to electro-elution using dialysis tube. The mixture was centrifuged at 2,500 × g for 10 min at 4°C. The supernatant was directly concentrated and dialyzed against S10 buffer using a concentrator (Thermo Scientific, Rockford, Il, USA). The remaining supernatant was collected and loaded onto a column of nickel-chelated nitrilotriacetic acid agarose (bed volume of 0.25 ml, Clontech) pre-equilibrated with S10 buffer. The resin was washed four times with 1 ml of S10 buffer, and the protein was eluted 12 times with 0.2 ml of S100 buffer, and stored at −80°C.

***In vitro* FFV IN enzymatic activities**

The 3'-end processing, strand transfer, and disintegration activities of the deletion and point mutants were assayed as described previously (29). The following oligonucleotides were used as DNA substrates: FFVU5/20A, 5'-ACTGTCGTGGCCTATACCTG-3'; FFVU5/20S, 5'-CAGGTATAGGCCACGACAGT-3'; FFVU5/ 20S-2, 5'-CAGGTATAG-GCCACGACA-3'; FT1, 5'-CAGCAACGCAAGCT T-3'; FD4, 5'-CAGGTATAGGCC-ACGACAGGGCTGCAGGTCG ACTAC-3'; FT2, 5'-GTAGTCGACCTGCAGCCCAA-GCTTGCGTT GCTC-3'. The oligonucleotides were purified by electrophoresis on a 15% denaturing polyacrylamide gel. The substrates used to assay the 3'-end processing activity of the wild-type and the deletion and point mutants were double-stranded oligonucleotides containing sequences derived from the U5 end of FFV (FFVU5/20A and FFVU5/20S). The substrate was prepared by labeling the 5'-end of FFVU5/20S with [γ-^{32}P]ATP and T4 polynucleotide kinase and then annealing the labeled strands with their complementary oligonucleotides. Substrate for analyzing strand transfer was prepared by annealing the 5'-end labeled FFVU5/20S-2 with their complementary oligonucleotides. The FFV disintegration substrate (Y-oligomer) was prepared by annealing the 5'-end-labeled FT1 strand with the oligonucleotides FD4, FT2, and FFVU5/20A. The annealed Y-oligomer was purified on a 15% native polyacrylamide gel. In all assays, 0.1 pmol of DNA substrate was incubated with 3 pmol of IN for 90 min at 33°C in 10 μl of reaction buffer containing a final concentration of 20 mM HEPES (pH 7.5), 5 mM $MnCl_2$, 30 mM NaCl, 10 mM DTT, 0.01 mM EDTA, 1 mM CHAPS, and 0.05% Nonidet P40. One pmol of each of the two INs was premixed and added to 0.1 pmol of substrate for the complementation reactions. The reaction was stopped by adding 10 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF), and heated at 90°C for 3 min before electrophoresis on 15% polyacrylamide gels with 7 M urea in Tris-taurine EDTA buffer. The products were quantified with a Molecular Dynamic PhosphoImager (Cyclone, PerkinElmer, Waltham, MA, USA).

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