Characterization of Biochemical Properties of Feline Foamy Virus Integrase

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In order to study its biochemical properties, the integrase (IN) protein of feline foamy virus (FFV) was overexpressed in Escherichia coli, purified by two-step chromatography, (Talon column and heparin column), and characterized in biochemical aspects. For the three enzymatic reactions of the 3'-processing, strand transfer, and disintegration activities, the Mn$^{2+}$ ion was essentially required as a cofactor. Interestingly, Co$^{2+}$ and Zn$^{2+}$ ions were found to act as effective cofactors, whereas other transition elements such as Ni$^{2+}$, Cu$^{2+}$, La$^{3+}$, Y$^{3+}$, Cd$^{2+}$, Li$^{+}$, Ba$^{2+}$, Sr$^{2+}$, and V$^{3+}$ were not. Regarding the substrate specificity, FFV IN has low substrate specificities as it cleaved in a significant level prototype foamy virus (PFV) U5 LTR substrate as well as FFV U5 LTR substrate, whereas PFV IN did not. Finally, the 3'-processing activity was observed in high concentrations of several solvents such as CHAPS, glycerol, Tween 20, and Triton X-100, which are generally used for dissolution of chemicals in inhibitor screening. Therefore, in this first report showing its biochemical properties, FFV IN is proposed to have low specificities on the use of cofactor and substrate for enzymatic reaction as compared with other retroviral INs.

Keywords: Integrase, feline foamy virus, transition element, cofactor, specificities

Foamy viruses (FV), also known as spumaviruses or spumaretroviruses, are classified as one of the genera of the retroviridae. The FVs have been found in non-human primates (NPS), cats, cows, and horses as well as in humans who have acquired the infection from NPS [19]. The FVs were first described in 1954 and isolated in 1971 [1, 12]. Feline foamy virus (FFV) is a widespread retrovirus of cats. It is generally thought to be nonpathogenic and potentially a useful candidate as a gene therapy vector [14].

Distinguished steps of retrovirus replication are reverse transcription and provirus integration. Like other retroviruses, FVs essentially depend on integration into the host cell genome mediated by virally encoded protein, called integrase (IN), whereas DNA viruses integrate by relying solely on cellular machinery [21]. Integration carried out by IN occurs in two steps, referred to as the 3'-processing (or endonucleolytic) reaction and strand transfer reaction, respectively. In the first 3'-processing reaction, IN removes the two terminal nucleotides following conserved CA bases at each 3' end of the linear viral DNA [9]. The second step, strand transfer reaction, is a phosphoryl-transfer reaction using the 3'-OH end of the viral DNA as the attacking nucleophile, resulting in covalent joining of the two nucleotides [7]. Retroviral IN has one more enzymatic activity in vitro, called disintegration, which cuts off the inserted viral DNA from target DNA. It is an apparent reversal of the strand transfer reaction [6].

IN protein consists of three functional domains, which are the N-terminal, central core, and C-terminal domains [10, 18]. The N-terminal domain contains a pair of highly conserved histidine and cysteine residues that are composed of Zn-finger motifs that include in coordinating metal ions. The functional role of the C-terminal domain is as a nonspecific DNA-binding site. The central core domain has three conserved acidic residues, an Asp, Asp-35-Glu (D, D-35-E) motif, that are important for catalysis [28].

For enzymatic reactions of retroviral IN, it is known that Mn$^{2+}$ or Mg$^{2+}$ is obviously necessary as a cofactor [13]. It is also reported that Ca$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ can bind in the active site of the catalytic domain including Mn$^{2+}$ or Mg$^{2+}$. Moreover, Zn$^{2+}$ can be used as a cofactor for the 3'-processing activity of avian sarcoma virus (ASV) IN, but not for that of other retroviral INs [3]. So far, various biochemical properties of oncoretroviral and lentiviral INs in vitro have been well documented [11]. However, only a few studies on foamy virus IN have been reported [15, 23, 24]. In the case of FFV IN, we have characterized here some of its biochemical properties by investigating cofactors for enzymatic reactions and comparing its reactivity to three different viral U5 LTR substrates.
Materials and Methods

Construction of Expression Vector

The FFV IN expression vector was constructed by PCR amplification of FFV IN cDNA isolated from the FFV pol gene (kindly donated by Dr. Maxine Linial, Fred Hutchinson Cancer Research Center, Seattle, U.S.A.) and by ligation of the PCR product to the expression vector pQE9. The resultant recombinant construct was characterized to contain a 6-histidine codon in front of the integrase sequence. The expressed protein including 6-histidines provides a simple purification based on the selective affinity for a cobalt-chelated absorbent [5].

Expression and Purification of IN Protein

The DNA construct was used to transform into E. coli XL-1 blue. The cells were grown at 37°C in LB medium containing 50 µg ampicillin/ml. At an optical density of 0.7, isopropyl-1-thio-β-D-galactopyranoside was added to 0.3 mM for expression induction, and cultured for an additional 4 h. After harvesting, the cell pellet was 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-mercaptoethanol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 10 mM imidazole] including 64 mg of lysozyme. 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 [15]. The supernatant was directly loaded onto a Talon column (bed volume of 1 ml; Clontech, U.S.A.) and the resin was washed four times with 6 ml of K10 buffer [50 mM Tris•HCl (pH 7.6), 20 mM β-mercaptoethanol, 0.1 M EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM imidazole, 1 M NaCl, and 10 mM CHAPS]. IN protein was eluted by increasing of imidazole concentration to K10 buffer, gradiently. The fractions containing the protein were collected and diluted with 9 volumes of buffer A [50 mM Tris•HCl (pH 7.4), 200 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol] before loading onto a column containing 0.25 ml of heparin agarose. The column was washed with 6 ml of buffer A. The protein was around 100 mg of protein/mL. The purity was prepared from a 2 l culture of E. coli.

Preparation of Duplex Oligonucleotide Substrates

For in vitro assay of the 3'-processing activity, the radiolabeled duplex oligonucleotide substrate was prepared as follows. First, two 20-mer terminal sequences of FFV U5 LTR were synthesized (Ahram, Inc., Korea): FFVU5/20S (U5 LTR, +strand: 5'-CAGGTATAGGCCACGACA-3'), was labeled with γ-32P-ATP at its 5' end, and annealed with its complementary oligonucleotide, FFVU5/20A. Subsequent treatments were the same as for preparation of the substrate for 3'-processing activity. For in vitro assay of the disintegration activity, a Y-shaped oligonucleotide substrate was prepared with four different oligonucleotides. A 15-mer oligonucleotide, FT1 (5'-GAGCAACGCAAGCTT-3'), was labeled with γ-32P-ATP at its 5' end, and then annealed with three other oligonucleotides, FD4 (36-mer, 5'-CAGGTATAGGCCACGAC AGGGCTGCAGGTCGACTAC-3'), FT2 (33-mer, 5'-GIAAGTGAC CTGCAGCCCCAAGCTTGCTGTC-3'), and FFVU5/20A. The rest of the steps were the same as for preparation of the substrate for 3'-processing activity [15].

Assay of FFV IN Activities

A standard reaction of IN activities contained 0.1 pmol of the DNA substrate and 3 pmol of FFV IN in 10 µl of reaction buffer including a final concentration of 5 mM MnCl2, 20 mM Hepes (pH 7.6), 10 mM DTT, and 0.05% NP-40, incubated for 60 min at 37°C. The reaction products were mixed with an equal volume of loading buffer (98% deionized formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), and heated at 90°C for 3 min before analysis by electrophoresis on a 15% polyacrylamide gel with 7 M urea in Tris-borate EDTA buffer. Analysis of the products was carried out with a Molecular Dynamics PhosphorImager (GS525, BioRad).

Results

Purification of FFV IN

An expression vector, pQE-FFVIN, was constructed by ligating the FFV IN DNA fragment to pQE9 vector (Fig. 1A). The expression vector produced FFV IN protein containing six histidines at its N-terminus in the bacteria treated with 1 mM IPTG (Fig. 1B, lane 2). The protein was purified via Talon column and heparin column chromatographies (Fig. 1B, lanes 3 and 4). Initially, we had tried to purify FFV IN through a nickel-chelated column and subsequently SP-Sepharose column, but some activities of nonspecific nuclease were still found in the final preparation. As an alternative way, we used the Talon column using a histidine tag, which is a cobalt-based immobilized metal affinity chromatography and has stronger binding capacity for histidine-tagged proteins than a Ni-chelated column does [5]. However, one-step purification via Talon column chromatography was not enough to completely remove nonspecific nuclease activities, so we brought into play an additional heparin column chromatography whose anionic sulfate groups have the ability to function as a high capacity Y-shaped affinity exchanger [27]. Finally, about 2.5 mg of FFV IN protein (a molecular mass of 40,000 dalton) of over 95% purity was prepared from a 2 l culture of E. coli.

Enzymatic Reactions of FFV IN

To date, several retroviral INs have been studied from various points of view, including its three enzymatic
Properties of FFV IN

However, the biochemical properties of FFV IN have not been reported yet. In this study, the 3'-processing activity of FFV IN was found in the presence of Mn$^{2+}$ ion of 0.1, 1, 5, and 10 mM, respectively, by cutting a dinucleotide off at the 3' end of the 20-mer substrate (Fig. 2A). Its activity was highest near 5 mM Mn$^{2+}$, but some substrates were lost at 10 mM Mn$^{2+}$. In contrast, the 3'-processing activity was weakly observed only at 10 mM in the case of Mg$^{2+}$ as a cofactor. These data indicate that enzymatic properties of FFV IN are very similar to those of other retroviral INs such as HIV-1 IN, FIV IN, MoMuLV IN, and RSV IN, in that they prefer to use Mn$^{2+}$ more than Mg$^{2+}$ as cofactor in the enzymatic reaction [13, 29].

Strand transfer activity is an enzymatic activity by which the cleaved substrate DNA is incorporated to target DNA. In in vitro assay, the radiolabeled oligonucleotide substrate (18 mer) is converted to the longer oligonucleotide (18+n mer). The strand transfer products appeared as 18+n mer. C. Disintegration activity. The Y-shaped oligonucleotide complex composed of 4 different oligonucleotides, where a 15-mer oligonucleotide was radiolabeled, was prepared. The reaction products were observed as 30 mer. Reintegrated products following disintegration were also observed under a 30-mer band.

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target DNA. In this study, the activity was also shown to increase with increment of Mn\(^{2+}\) ion up to 10 mM, like strand transfer activity reactions (Fig. 2C).

Co\(^{2+}\) and Zn\(^{2+}\) as Cofactors for FFV IN Activity

Mn\(^{2+}\) or Mg\(^{2+}\) ion is known to be required as cofactors of retroviral IN activities. In general, retroviral IN activities appear most efficiently in the presence of Mn\(^{2+}\). However, Zn\(^{2+}\) as well as Mn\(^{2+}\) was reported to act as a cofactor for the 3'-processing activity of Avian Sarcoma Virus IN [3]. This fact implied that other transient elements could work as a cofactor if some retroviral IN may adapt them in the metal binding sites. Therefore, in order to investigate which metals can have an influence on enzymatic reactions of FFV IN, we tested various metals such as Co\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Cu\(^{2+}\), Y\(^{3+}\), Zr\(^{2+}\), Cd\(^{2+}\), La\(^{3+}\), Li\(^{+}\), Rb\(^{+}\), Cs\(^{2+}\), Ca\(^{2+}\), K\(^{+}\), Sr\(^{2+}\), Ba\(^{2+}\), V\(^{5+}\), Ru\(^{4+}\), Fe\(^{2+}\), Pd\(^{2+}\), and Cr\(^{3+}\) for whether or not they could act as a cofactor (data not shown). Among the tested metal elements, only Co\(^{2+}\) and Zn\(^{2+}\) ions were found to act as cofactors for 3'-processing activity of FFV IN as they induced enzymatic activity in the absence of Mn\(^{2+}\) ion, respectively (Fig. 3A and 3B). In addition, their inductions of enzymatic activities were quite metal ion concentration-dependent, as the activities increased with increment of their concentration, even though both of the metal ions were less effective than Mn\(^{2+}\). The same results were observed for the strand transfer and disintegration activities (data not shown).

Substrate Specificity of FFV IN

The long terminal repeat (LTR) sequence at the viral DNA ends is the only viral DNA region that is essential for recognition by retroviral IN and significant for exact integration of viral DNA [4, 26]. To investigate the substrate specificity of FFV IN, we performed 3'-processing reactions by incubating different LTR substrates to HIV-1, PFV, and FFV INs, respectively (Fig. 4). In the results, HIV-1 IN cleaved well two nucleotides off at the HIV-1 LTR substrate, whereas PFV and FFV INs did not cut as nearly (Fig. 4B; HIV-1 lines). PFV IN also showed a similar pattern in which PFV LTR was cleaved well but HIV-1 LTR and FFV LTR were not cleaved much (Fig. 4B; PFV lines). In contrast, FFV IN cleaved PFV LTR substrate as well as FFV LTR substrate (Fig. 4B; PFV lines). In addition, it cleaved HIV-1 LTR substrate at a significant level. Therefore, it is likely that FFV IN has low substrate specificity compared with HIV-1 and PFV INs.

Effect of Detergents on the 3'-Processing Activity of FFV IN

The 3'-processing activities were monitored in several solvents which are generally used for dissolution of chemicals in inhibitor-screening. The 3'-processing activities were observed in standard reactions including CHAPS, Glycerol, Tween 20 and Triton X-100, respectively (Fig. 5). CHAPS, one of the nonionic detergents, revealed little inhibition of the

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**Fig. 3.** Effects of Co\(^{2+}\) and Zn\(^{2+}\) ions on the 3'-processing activity of FFV IN.

A 20-mer oligonucleotide mimicking FFV US LTR was labeled with γ\(^{32}\)-P-ATP and annealed with its complementary oligonucleotide. The substrate of 0.1 pmol was incubated with the IN of 3 pmol at 37°C for 60 min in the presence of Co\(^{2+}\) (A) or Zn\(^{2+}\) (B) ions as final concentrations. Conversion of the 20-mer oligonucleotides to the 18-mer oligonucleotides was analyzed in a 15% polyacrylamide gel. – IN, Substrate only; -Mn & - Co, no addition of MnCl\(_2\) and CoCl\(_2\); + Mn & - Co, addition of MnCl\(_2\) without CoCl\(_2\); - Mn & + Zn, no addition of MnCl\(_2\) and ZnCl\(_2\); + Mn & - Zn; addition of MnCl\(_2\) without ZnCl\(_2\).

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**Fig. 4.** Substrate specificity of HIV-1, PFV, and FFV INs.

A. Sequences of HIV-1, PFV, and FFV US LTR ends. B. 3'-processing activities using HIV-1, PFV, and FFV US LTR substrates in the presence of HIV-1 IN, PFV IN, or FFV IN. The substrates were prepared in the same way as described in Fig. 2A, respectively. Products were analyzed in a 15% polyacrylamide gel. * Numbers on the nucleotides indicate the position distant from the 3’ end of the viral DNA US LTR. The highly conserved sequence, CA, among the retroviral LTR DNA ends is underlined. –IN, no addition of IN; HIV-1, HIV-1 US LTR substrate; PFV, PFV US LTR substrate; FFV, FFV US LTR substrate.
3'-processing reaction at the concentration of 10 mM. Glycerol and Triton X-100 showed no inhibitory effect on the enzymatic reaction of FFV IN when they were used up to the final concentration of 50%, compared with the reaction without any detergents. In the case of Tween 20, however, it was too viscous to completely suspend reaction components in the standard condition at the concentration more than 20%. Thus, it was hard to judge whether or not Tween 20 has any inhibitory effect on the enzymatic reaction in the high concentration. Consequently, it was found that glycerol and Triton X-100 did not decrease the enzymatic activity of FFV IN up to the level of 50% in solvent concentration. However, 5 to 10 mM CHAPS showed inhibitory activities on the FFV IN reaction.

**DISCUSSION**

In this report, experimental data show that FFV IN has generalized catalytic activities like other retroviral INs do. FFV IN performs as a site-specific endonuclease to get rid of a dinucleotide from the 3' end of oligonucleotide substrates mimicking the termini of linear viral DNA. Moreover, it catalyzes the insertion of the pre-cleaved DNA into the other DNA using strand transfer activity, and the reverse reaction using disintegration activity *in vitro*.

Various retroviral INs have been reported to show similar reactivity when divalent cations such as Mn$^{2+}$ or Mg$^{2+}$ were added [3, 10, 16]. In the case of FFV IN, we ascertained its reactivity in the presence of metal ions that had been investigated as a cofactor because there were no reports about FFV IN yet. Our results indicated that Mn$^{2+}$ or Mg$^{2+}$ is required as a cofactor of the three different enzymatic reactions of FFV IN, like other retroviral INs. However, our results showed that some other metal ions such as Zn$^{2+}$ and Co$^{2+}$ could be used as cofactors. Previously, Zn$^{2+}$ was reported to be used as a cofactor for 3'-processing activity on ASV IN [3], and another group also mentioned that the HIV-1 IN was active with Ca$^{2+}$ even though other groups could not find such activity [10]. Even though Zn$^{2+}$ was reported as a cofactor for ASV IN, the experimental data that Co$^{2+}$ can act as a cofactor have not been reported yet. Therefore, our results suggest that other bivalent cationic transient elements may act as cofactors for retroviral INs, depending on the size of metal binding sites. It has been reported that retroviral IN has two metal binding domains. One of them is the N-terminal domain including zinc finger motif, which binds Zn$^{2+}$. Secondly, the central core domain, containing a conserved D, D-35-E motif, has been known to bind Mn$^{2+}$, Mg$^{2+}$, or metal cofactors [17, 18]. Although it has not been tested what kinds of metal can make a complex with the D, D-35-E motif of FFV IN, it is likely that Zn$^{2+}$, Co$^{2+}$, Mn$^{2+}$ as well as Mg$^{2+}$ might bind to the catalytic core domain of FFV IN. To understand the reactivity between metal ions and FFV IN, it is necessary to study structures of FFV IN using crystallographic procedures, and have knowledge about the biochemical properties of metal ions.

We also tested the substrate specificity of FFV IN, comparing with reactions of HIV-1 and PFV INs. It is well known that the invariable sequence CA, which is located as the third and fourth nucleotides at the 3' end of all retroviral DNA, is absolutely necessary for the IN activities [20, 25]. The sequences internal to the CA dinucleotide were also reported to determine substrate specificity of retroviral INs [22]. However, because the functional nucleotides of FFV U5LTR have not been investigated in detail, we had tried to compare reactivity to the different viral substrates in order to study substrate specificity. The internal six nucleotide positions in front of the CA dinucleotide are the same between PFV U5LTR and FFV U5LTR substrates, whereas the nucleotides at the corresponding positions are quite different between HIV-1 U5LTR and FFV U5LTR substrates (Fig. 4A). Therefore, it was likely that PFV and FFV INs cleaved well when they are reacted with the different substrates. As a result, HIV-1 IN could not show IN activities with PFV and FFV substrates, respectively. Interestingly, PFV IN successfully carried out 3'-processing activity with the substrate of FFV IN. In contrast with PFV IN, FFV IN showed very weak enzymatic reaction with PFV substrate. In our previous study [15], it was suggested that the nucleotides, located at the fifth, sixth, eighth, and ninth positions at the 3' end of PFV U5LTR, are indicated to mark for specific recognition. Therefore, it seems that the fifth, sixth, eighth, and ninth positions at the 3' end of PFV U5LTR and FFV U5LTR substrates may be used for specific recognition of PFV and FFV IN activity. Although we realized that FFV IN has low substrate specificity compared with HIV-1 and PFV INs, questions about the functional nucleotides of FFV U5LTR are still to be answered with further work.

In conclusion, it is suggested in our study that FFV IN shows the three well-known catalytic activities as other...
retroviral INs. However, it is also shown that FFV IN has its own biochemical properties in lieu of the fact that Co²⁺ and Zn²⁺ can act as cofactors, and it has lower substrate specificity. As this study is the first report of the biochemical properties of FFV IN, it will be useful for understanding the mechanism of FFV viral replication and life cycle.

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References