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Biochemical Properties of Second Site Mutation of Human Immunodeficiency Virus Integrase

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A highly conserved amino acid, glutamic acid (Glu), present at position 152 in the catalytic domain of the human immunodeficiency virus type 1 (HIV-1) integrase (IN) protein has been known to be critical for enzymatic function since substitution of Glu 152 with other residues results in a complete loss of enzymatic activities. In order to better understand the role of Glu 152 as a conserved residue in enzymatic action, intragenic second site mutations have been introduced around residue 152 of a mutant IN (E152A), and their biochemical properties were analyzed in terms of enzymatic activities. Disintegration activities were found to be significantly restored in several second site mutant INs, while integration activities were only recovered weakly. However, endonucleolytic activities were not discovered in all the mutant INs. These findings indicate that the second site mutations can partially restore the catalytic structure of the active site disturbed by the E152A mutation and lead to the regaining of integration and disintegration activities. In addition, it is also suggested that endonucleolytic activity requires a more accurate structure of the catalytic site than that for the integration and disintegration activities.

Keywords: Immunodeficiency, Integrase, Mutation, Second Site.

Introduction

Retroviral replication requires the integration of a linear double-stranded DNA copy of the viral RNA genome into a chromosome of an infected cell. The integration process consists of two enzymatic reactions. The first is an endonucleolytic cleavage of two nucleotides from the 3′ end of the viral DNA. The second is an integration of the viral DNA into a chromosome. The hydroxyl group of the processed 3′ ends of the viral DNA is connected to the phosphate group of the processed 5′ ends of the target DNA. This integration process is mediated by a specific viral enzyme, integrase (IN) (Colicelli and Goff, 1988; Panganiban and Temin, 1983). The purified INs have been shown to have three enzymatic activities *in vitro*. The *in vitro* assays of the enzymatic activities are well developed on the basis that retroviral INs make specific cleavage or ligation on the oligonucleotide substrates whose sequences are equal to those of the 3′ ends of the viral DNA (Brown *et al.*, 1989; Sherman and Fyfe, 1990; Chow *et al.*, 1992). The endonucleolytic activity of INs induces the cleavage of two nucleotides from the 3′ ends of the oligonucleotide substrate. Its integration activity catalyzes the insertion of the pre-processed oligonucleotide substrate into the target DNA. The disintegration activity induces cleavage of the branched strand of the Y-shaped oligonucleotide *in vitro* (Chow *et al.*, 1992). Several studies have revealed that the HIV-1 IN protein consists of three distinctive domains such as the N-terminal, central, and C-terminal domains (Engelman and Craigie, 1992; Kulkosky *et al.*, 1992; Vink *et al.*, 1993). In particular, the central domain is known to be a highly conserved region where the invariant residues of two aspartic acids (Asp) and one glutamic acid (Glu) are present at positions 64, 116, and 152, respectively (Engelman and Craigie, 1992; Shin *et al.*, 1994). A single amino acid substitution among the conserved amino acids impaired the enzymatic activities of the HIV-1 IN. In particular, Glu 152 was proposed to be critical for catalytic function because the HIV-1 IN carrying a mutation at the position 152 exhibited a complete loss of enzymatic activities (Leavitt *et al.*, 1993). Recently, a second site mutation (T125A) against the mutant IN (P109S) defective in its endonucleolytic and integration activities but displaying 5 to 10% of the wild type disintegration activity, was reported to rescue enzymatic activities and infectivity to the P109S IN mutant virus (Taddeo *et al.*, 1996).

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Therefore, it has been an interesting question to investigate whether the enzymatic activities of the defective IN, E152A, can be restored by the intragenic second site mutation. In this work, 12 intragenic second site mutants of the HIV-1 IN E152A IN protein were produced and characterized *in vitro* by studying the recovery of enzymatic activities.

Materials and Methods

Materials NTA-resin was purchased from Qiagen (Valencia, USA). Restriction enzymes, T4 DNA-polynucleotide kinase, and T4 DNA ligase were obtained from New England Biolabs (Beverly, USA). Sequenase version 2.0 DNA polymerase and kit were obtained from the United States Biochemical Corporation (Cleveland, USA). Radionucleotides of [γ-^{32}P]ATP and [α-^{32}P]ATP were purchased from Amersham (Richmond, USA) at a specific activity of 3,000 Ci/mmol. Oligonucleotides used for preparation of the integrase substrates and as PCR primers were obtained from Bioneer Inc. Other chemical agents were of molecular biology reagent grade from Sigma (St. Louis, USA).

Site-directed mutagenesis The oligonucleotides used as PCR primers to construct mutant INs were gel-purified just before use and their sequences are shown in Table 1. DNA fragments encoding site-directed mutation were generated by a two-step PCR protocol, using a pfu polymerase according to the manufacturer's guidelines. Primers were designed to generate DNA fragments containing a *Bam*HI site at the 5′ termini and a stop codon flanked by a *Hin*dIII site at the 3′ termini. The PCR fragments were produced in a two-step procedure (Kim *et al.*, 1999). In the first round of PCR, DNA fragments were amplified from a plasmid (pQEIN E152A) containing the E152A mutation as a template DNA (Oh *et al.*, 1997). Amplification using the 5′ (*Bam*HI site) primer and a 3′ primer containing a mutation produced a PCR half fragment. Separate amplification with the 3′ (*Hin*dIII site) primer and a 5′ primer containing a mutation produced the other half-fragment. In a second round of PCR, the two overlapping half-fragments, after gel purification, were mixed and used as templates to produce IN DNA fragments of the full-length containing a second site-mutation (Fig. 1). After gel purification and cleavage with *Bam*HI and *Hin*dIII, the full-length DNA fragments were ligated into pQE9. Some of the multiple site mutant INs were constructed by using the two-step PCR two times with the second site mutant IN DNA as a template. The mutated nucleotide sequences of the constructs were verified by dideoxy sequencing with a Sequenase kit from the United States Biochemical Corp.

Protein purification HIV-1 IN proteins were purified from *Escherichia coli* as described previously with the following modifications (Oh and Shin, 1996; Oh *et al.*, 1997). The cell pellets obtained from the overexpressed culture were resuspended in buffer A (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 2 mM β-ME, 0.1 mM EDTA, 5 mM imidazole, 6 M Guanidine-HCl). The suspension was centrifuged at 40,000 × *g* for 20 min at 4°C, and supernatant was collected and loaded over a Ni-NTA column

Table 1. Oligonucleotide sequences of the primers used to create the second site mutations.

Oligo	Sequence	Type[a]
V79E	5′-GCAGTTCAT**GAA**GCCAGTGGATATATAG-3′[b]	5′primer
	5′-CTATATATCCACTGGCTTCATGAACTGCTACC-3′	3′primer
N120E	5′-GACAATGGCAGC**GAA**TTCACCGGTGCTACG-3′	5′primer
	5′-AGCACCGGTGAA**TTC**GCTGCCATTGTCAG-3′	3′primer
V151E	5′-GTCAAGGAGTA**GAAGC**ATCTATGAATAAAG-3′	5′primer
	5′-ATTCATAGA**TGCTTC**TACTCCTTGACTTTG-3′	3′primer
S153E	5′-CAAGGAGTAGTA**TCCGAA**ATGAATAAAGAATTA-3′	5′primer
	5′-CTTTATTCAT**TTCGGA**TACTACTCCTTGAC-3′	3′primer
M154E	5′-GGAGTAGTA**GCA**TCT**GAG**AATAAAGAATTAAAG-3′	5′primer
	5′-TAATTCTTTATT**CTC**AGA**TGC**TACTACTCCTTG-3′	3′primer
N155E	5′-GTAGTA**GCA**TCTATG**GAA**AAAGAATTAAAGAAA-3′	5′primer
	5′-CTTTAATTCTTT**TTC**CATAGA**TGC**TACTAC-3′	3′primer
V151E /S153E /M154E	5′-GGAGTA**GAAGCAGAAGAG**AATAAAGAATTAAAGAAA-3′	5′primer
	5′-TAATTCTTTATT**CTCTTCTGCTTC**TACTCCTTGACT TTGGGG-3′	3′primer
HN	5′-GCAGGTACCATGGGATCCTTTTTAGATGGAATAGATAAGGCCCAAGAT-3′	N-terminal
HC	5′-TGCCTGCAGAAGCTTTTACTAATCCTCATCCTGTCTACTTGCCACACA-3′	C-terminal

[a] Indicates orientation of the oligonucleotides used in PCR constructing the second site mutations.
[b] Mutated regions are described in boldface.

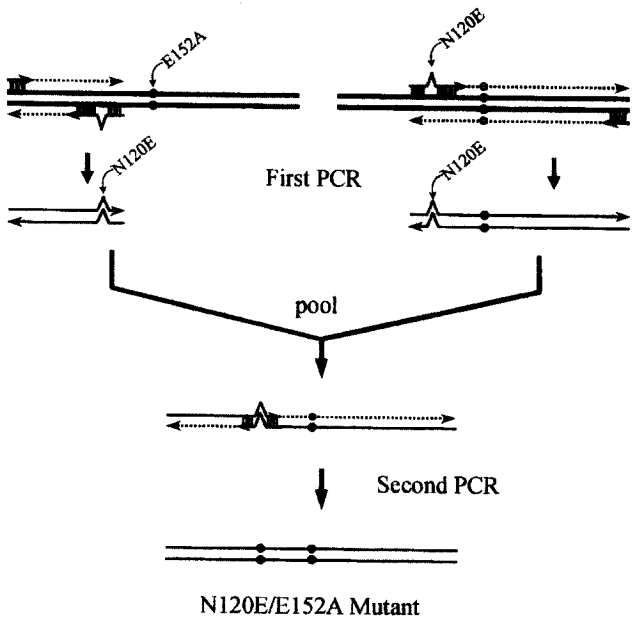


Fig. 1. Schematic illustrations of two-step PCR method for construction of the second site mutations.

(Qiagen). Protein was eluted with a 100 mM and 600 mM imidazole in buffer A. The fraction was diluted with an equal volume of buffer containing 1 M NaCl, 20 mM Tris·HCl (pH 7.4), 2 mM β-ME, 2 mM EDTA. The protein was sequentially dialyzed for 1 h at 4°C against the three different solutions which are buffer I (20 mM Tris·HCl, pH 7.4, 2 M urea, 0.5 M NaCl, 5 mM EDTA, 10 mM β-ME, 10 mM CHAPS), buffer II (20 mM Tris·HCl, pH 7.4, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 10 mM CHAPS, 10% glycerol), and buffer III (20 mM Tris·HCl, pH 7.4, 0.5 M NaCl, 1 mM EDTA, 1 mM DTT, 10 mM CHAPS, 10% glycerol). The dialysate was centrifuged at 19,000 × *g* for 10 min and the supernatant containing IN was saved in liquid nitrogen (Bushman *et al.*, 1993; Taddeo *et al.*, 1996).

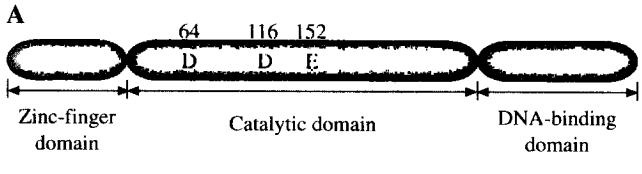
***In vitro* enzymatic activities of HIV-1 IN** For *in vitro* assay of the integration activity, the substrate DNA was prepared by labeling the 5′-end of the oligonucleotide CA104 (18mer, 5′-TGTGGAAAATCTCTAGCA-3′) with γ^{32}P-ATP and annealed with the complementary oligonucleotide 6917 (20mer, 5′-ACTGCTAGAGATTTTCCACA-3′). The target DNA of double stranded oligonucleotide was prepared by annealing two oligonucleotides, IGA (32mer, 5′-TCGAGAAAAAAAAAACTTAAGCCCCCCCCCCC-3′) and IGB (32mer, 5′-TCGAGGGGGGGGGGGCTTAAGTTT-TTTTTTTC-5′). An integration reaction contained 0.1 pmol of oligonucleotide substrate, 10 pmol of target DNA, and 20 pmol of IN protein in 50 mM Bis-tris-Propane (pH 6.5), 4 mM Mn^{2+}, 100 mM NaCl, 2 mM, β-ME, 2.5 mM CHAPS, 0.1 mM EDTA, 0.1 mM PMSF, 1% glycerol, and 10 mM imidazole in a total reaction volume of 10 μl. Reactions were incubated 33°C for 90 min and stopped by the addition of 4 μl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF. The reaction products were visualized by autoradiography of the wet gel.

For *in vitro* assay of the endonucleolytic activity, the oligonucleotide 6916 (20mer, 5′-TGTGGAAAATCTCTA-GCAGT-3′) as substrate was labeled at the 5′-end and annealed with the complementary oligonucleotide 6917. Endonucleolytic reactions were performed in 15 mM Tris·HCl (pH 7.4) and 1 mM $MnCl_2$ in the absence of target DNA. For *in vitro* assay of the disintegration activity, the disintegration substrate (Y-oligomer) was prepared by labeling the oligonucleotide T1 (16mer, 5′-CAGCAACGCAAGCTTG-3′) and annealed with two-fold amounts of the oligonucleotides T2 (32mer, 5′-TGTGGAAAATCTCTAGACGGCTGCAGGTCGAC-3′), T3 (30mer, 5′-GTCGACCTGCAGCCCAAGCTTGCGTTGCTG-3′), and 6917. Disintegration reactions were performed in 15 mM Tris·HCl (pH 7.4) and 10 mM $MnCl_2$.

Results and Discussion

Mutagenesis strategy The retroviral IN mediates incorporation of viral DNA into the host genome, which is essential for viral replication and production (Goff, 1992). The human immunodeficiency virus type 1 (HIV-1) IN protein comprises of three independently folding domains such as the N-terminal (zinc-finger), central (catalytic), and C-terminal (DNA-binding) domains as illustrated in Fig. 2A. In particular, HIV-1 IN is characterized by the presence of invariant acidic amino acids referred to as the D, D(35)E motif that includes several conserved residues in the central domain. Recent studies of the crystal structure of the central domain suggests that a triangle

A



B

Mutant	Mutated amino acid
Wild type	
E152A	E152A
Oh-1	N120E, E152A
Oh-2	V151E, E152A
Oh-3	E152A, N155E
Oh-4	V151E, E152A, S153E, M154E
Oh-5	V79E, N120E, E152A
Oh-6	V79E, V151E, E152A
Oh-7	V79E, E152A, M154E
Oh-8	V79E, V151E, E152A, S153E, M154E
Oh-9	N120E, E152A, N155E
Oh-10	N120E, V151E, E152A
Oh-11	N120E, V151E, E152A, S153E, M154E
Oh-12	N120E, E152A, M154E

Fig. 2. Construction of the second site mutations. (**A**) HIV-1 IN protein domains. The wild type IN protein containing 288 amino acids can be dissected into three functional domains: the N-terminal dimerization domain, the central catalytic domain, and the C-terminal DNA-binding domain. (**B**) List of the HIV-1 IN proteins containing the second site mutations.

configuration composed of Asp 64, Asp 116, and Glu 152 in the D, D(35)E motif play a key role in enzymatic function (Engelman *et al.*, 1993; Dyda *et al.*, 1994). In particular, Glu 152 is critically important since substitution of the amino acid leads to complete loss of all enzymatic activities such as the endonucleolytic, disintegration and integration activities. In order to understand the roles of Glu 152 in catalytic action it was studied whether the enzymatic activities could be recovered by introducing glutamic acid as a second site mutation into the defective, mutant IN, E152A. In consideration of the catalytic structure of the triangle configuration composed of Asp 64, Asp 116, and Glu 152 in a tertiary structure, Val 79, Asn 120, Val 151, Ser 153, Met 154, and Asn 155 which are just beside to Glu 152 were selected as the intragenic mutation sites.

Protein expression and purification By using a two-step PCR method once or twice as described in Materials and Methods, the mutant DNA fragments containing one or more glutamic acid sequences were produced and cloned into the expression vector, pQE9 (Oh and Shin, 1996; Oh *et al.*, 1997). The mutant IN proteins were expressed as a fusion protein linked to an amino-terminal affinity tag with six adjacent histidine residues which facilitated purification of the IN proteins on a nickel-chelated NTA affinity chromatography. Most of the mutant INs were less soluble in renaturing condition. Therefore, the mutant IN proteins were purified under denaturing condition and were subsequently refolded in the presence of detergent (Taddeo *et al.*, 1996). However, some of the mutant INs (including a single second site mutation of V79E or M154E) with a bad solubility even in denaturing condition were discarded and not used in further studies. No difference has been detected in the enzymatic activities of the wild type IN purified by using the alternative approaches (Goulaouic and Chow, 1996). The mutant and wild type IN proteins were recovered at about 1 mg/ml. Mutated positions of the mutant INs were described in Fig. 2B.

***In vitro* enzymatic activities** Purified HIV-1 mutant INs were characterized according to aspects of their biochemical activities by studying whether second site mutations restored the enzymatic activities which were lost by the mutation of Glu 152. Retroviral INs possess all three endonucleolytic, integration, and disintegration enzymatic activities *in vitro* (Brown *et al.*, 1989; Sherman and Fyfe, 1990; Chow *et al.*, 1992). They require only short oligonucleotide DNA substrates of proper structure in biochemical reactions *in vitro*. The integration activity of retroviral IN mediates *in vitro* nonspecific insertion of the prep-processed oligonucleotide substrate into the target DNA, producing an oligonucleotide longer than the original substrate (Fig. 3A). Several of the second site mutants (Oh-1, Oh-4, Oh-6, Oh-8, Oh-9, and Oh-10) restored a weak integration activity (Fig. 3B). This result

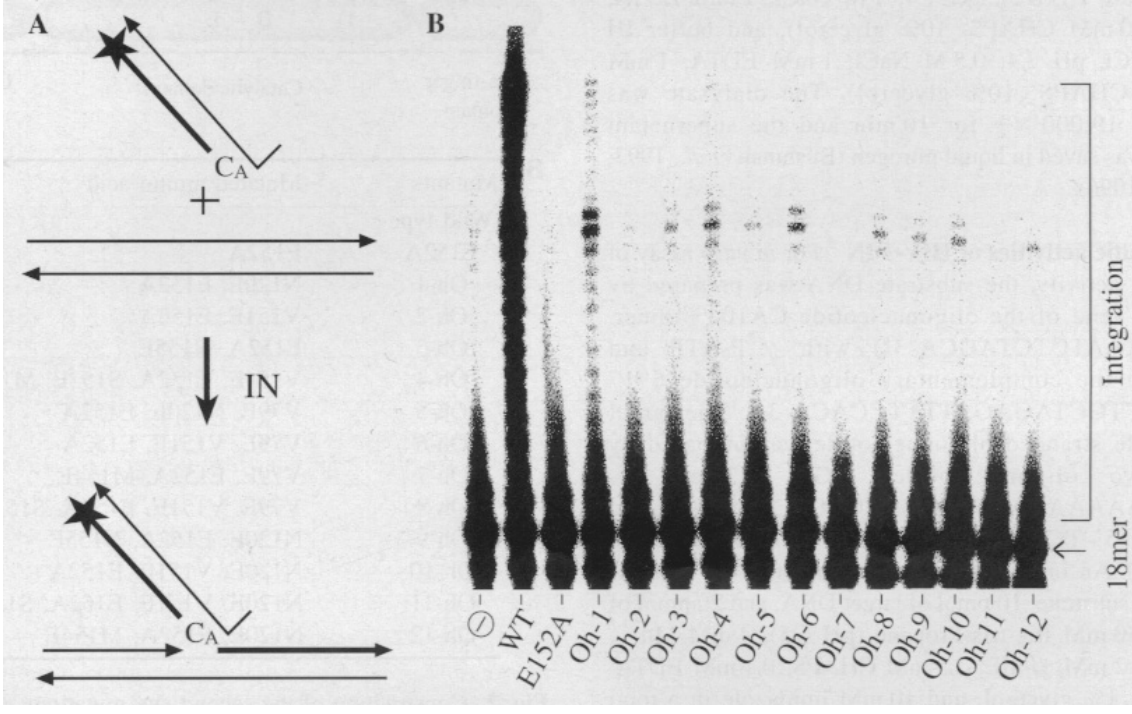


Fig. 3. Integration activities of the second site mutant INs. (**A**) Schematic illustrations of the *in vitro* integration reaction. The substrate prelabeled with γ^{32}P-ATP (★) on a 5′ end of the duplex oligonucleotide is randomly inserted to target DNA by HIV-1 IN. (**B**) Partial restoration of the integration activity of HIV-1 IN mediated by the second site mutations. The integration reactions were performed in 50 mM bis-Tris-propane (pH 6.5) and 4 mM $MnCl_2$ in the presence of 10 μM target DNA, 0.1 μM substrate, and 20 μM IN at 33°C for 90 min. The reaction products were analyzed in a 20% polyacrylamide gel and visualized by autoradiography of the wet gel.

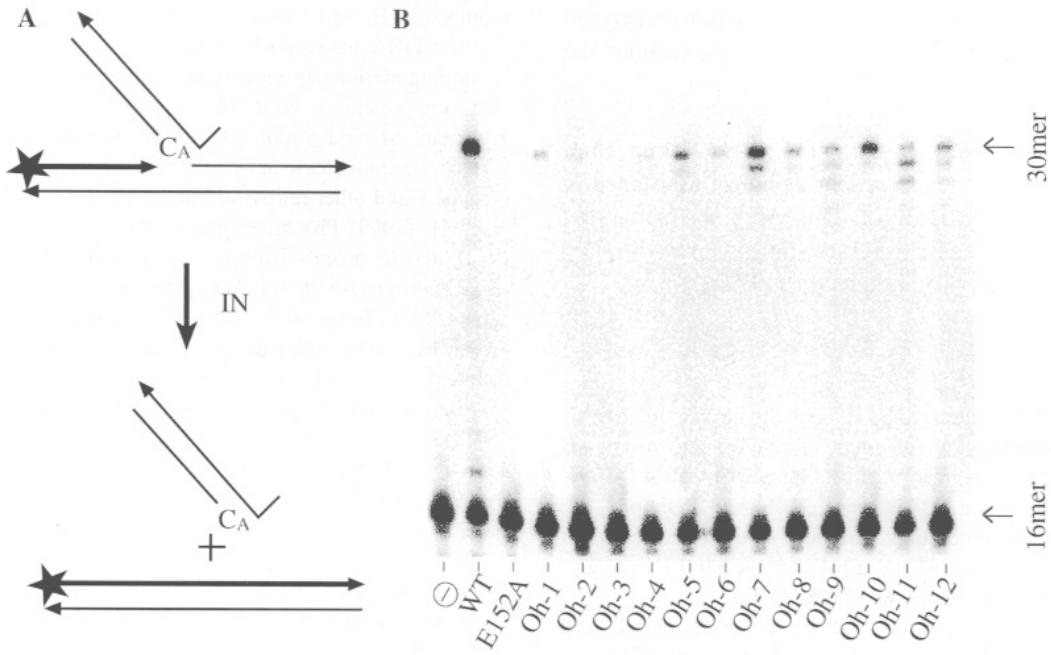


Fig. 4. Disintegration activities of the second site mutant INs. (**A**) Schematic diagram of the *in vitro* disintegration reaction. The Y-oligonucleotide substrate was prepared by labeling a 16mer-oligonucleotide with γ^{32}P-ATP (★) on its 5′ end and by annealing with three complementary oligonucleotides. (**B**) Restoration of the disintegration activity of HIV-1 IN mediated by the second site mutation. The disintegration reactions were performed in 15 mM Tris·HCl (pH 7.4) and 10 mM $MnCl_2$ in the presence of 0.1 μM substrate and 20 μM IN at 33°C for 90 min.

indicates that the introduction of a glutamic acid around residue 152 is able to partially replace the role of Glu152 in the enzymatic action of HIV-1 IN.

The disintegration activity catalyzes the *in vitro* cleavage of a branched junction of an Y-shaped oligonucleotide substrate, and then a ligation between the DNA strands (Fig. 4A). Most of the mutant INs were shown to have recovered their disintegration activity significantly when it was compared to that of the wild type IN (Fig. 4B). Specifically, the disintegration activities of the two mutants, Oh-7 and Oh-10, were recovered to 51% and 40% of their wild type activity, respectively. It is probable that the disintegration activity requires a simpler configuration of the catalytic site for enzymatic function (Oh and Shin, 1999). The bands present between the substrate and the disintegration product represent products resulting from the reintegration of the released branched oligonucleotides into the disintegration products or from nonspecific alcoholysis of the disintegration products (Chow *et al.*, 1992; Katzman and Sudol, 1996). The endonucleolytic activity mediates *in vitro* specific cleavage of two nucleotides from the 3′ end of the oligonucleotide substrate that mimics the LTR ends of the viral DNA (Sherman and Fyfe, 1990). None of the mutant INs showed any endonucleolytic activity at all (data not shown). It is likely that endonucelolytic activity requires an elaborate structure of the catalytic domain of retroviral IN in order to lead to the exact interaction between the viral DNA and the IN protein. In addition, this feature of the endonucleolytic activity seems to endow retroviral IN with a substrate specificity.

To better understand the role of Glu 152 known as a critical residue for enzymatic function of the HIV-1 IN protein, we evaluated 12 second site mutants of a defective mutant IN, E152A, with assays for endonucleolytic, integration, and disintegration activities. We have found that the introduction of glutamic acids around residue 152 induced a partial recovery of enzymatic activities that had been lost by the substitution of a glutamic acid with an alanine at position 152. A common defect of IN proteins mutated at the active site is likely to be the improper coordination between viral DNA and the IN protein. For example, recombinant IN proteins containing conservative charge to charge substitutions support partial enzymatic activities under conditions in which conservative charge to neutral substitution destroys activities (Bushman *et al.*, 1993; Goulaouic and Chow, 1996). Though the mode of the exact interaction between viral DNA and IN is not yet known, the three acidic amino acids of Asp 64, Asp 116, and Glu 152 as catalytic residues are suggested to act on the viral DNA (Leavitt *et al.*, 1993). Therefore, substitution of residue Glu 152 in the catalytic domain abolished detective enzymatic activities (Asante-Appiah *et al.*, 1998). Depending on their location, the second site mutations in defective IN may restore enzymatic activity. More high-resolution structures of the IN-substrate complex are required in order to find effective second site mutations.

Meanwhile, biochemical studies, such as those reported here, are expected to reveal more information about the structure and function of HIV-1 IN.

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