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Ile178 of HIV-1 reverse transcriptase is critical for inhibiting the viral integrase

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Abstract

HIV-1 reverse transcriptase (RT) was shown to inhibit *in vitro* the viral integrase (IN). We have reported previously that an RT-derived 20-residue peptide binds IN and inhibits its enzymatic activities. In this peptide, Leu168, Phe171, Gln174, and Ile178 were predicted to be involved in IN inhibition. In the presented study, these residues were mutagenized and the resulting peptides were tested for binding and inhibiting IN activities. Ile178 was found to be the major contributor to IN inhibition, probably by interacting with IN residue Gly149. As Gly149 is a key IN residue, this inhibition probably results from a steric hindrance of the IN active site.

Keywords: HIV-1; Integrase; Reverse transcriptase; Peptides; Inhibition; Mutation

Two viral-encoded enzymes play central roles in the replication of retroviruses and retrotransposons. The first enzyme, reverse transcriptase (RT), converts the single-stranded viral RNA into double-stranded DNA. This process is entirely catalyzed by the two catalytic activities of RT, the DNA-polymerase activity that copies both RNA and DNA into DNA and the ribonuclease H (RNase H) activity that concomitantly cleaves the RNA strand in the DNA–RNA heteroduplex [1]. Subsequently, the newly-synthesized double-stranded DNA is transported into the nucleus, where is intergrated into the genomic target DNA by the second viral enzyme, the integrase (IN). IN interacts with the ends of the linear viral DNA, then it removes two extra nucleotides located 3′ to the highly-conserved CA 3′-termini, and only then IN catalyzes the integration into the target DNA [1–3]. There are several functional linkages between RT and IN. First, the DNA product of RT is the substrate for IN. Second, pre-integration complexes (PICs) contain the viral DNA, IN, RT, and other proteins [4–7]. Third, the INs and RTs of HIV-1 and of murine leukemia virus (MLV) exhibit *in vitro* physical interactions [8–10]. The direct contacts between the IN and RT of human immunodeficiency virus type-1 (HIV-1) were recently confirmed by us by a dot-blot direct binding assay [11] and by employing a surface plasmon resonance technology (unpublished data). Lastly, we and others have shown that RT can inhibit IN, suggesting functional roles for these interactions [12,13].

After the completion of the reverse transcription process in the cytoplasm of virus-infected cells, there is a delay in the process of integration [1]. Since all possible catalytic components for integration are present in the PICs, the viral DNA can serve as both the donor and target DNA for integration. Such a potential auto-integration process might be suicidal for the virus. Several cellular proteins are involved in the integration of HIV-1 cDNA into the cellular DNA [6]; one of them serves also as a barrier to auto-integration in MLV-infected cells [14]. Since RT was shown to inhibit IN [12,13], it is possible that RT is also involved

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in inhibiting auto-integration, by RT, due to the functional interactions between RT and IN.

We have recently studied the specific RT-derived sequences, which are potentially involved in IN binding and inhibition, by screening of a library of HIV-1 RT-derived peptides [11]. The most active peptide, which inhibited all IN activities (3′-end processing, strand transfer, and disintegration), is derived from the DNA-polymerase active-site, with the sequence K_{166}ILEPFRKQNPDIVIY-QYMD_{185}, designated peptide 4286. This peptide is located mostly on the surface of the folded RT, allowing potential interactions of the RT with IN. The docking model has suggested that Leu168, Phe171, Gln174, and Ile178 (underlined in the above peptide sequence) interact with the catalytic-core domain of IN (IN-CCD) and, hence, inhibit IN by a possible steric hindrance [11]. The present study is a direct extension of this previous study, testing which of the above-predicted residues are involved in inhibiting IN. Each of these residues was modified in peptide 4286 and the resulting peptides were tested for IN binding and inhibition. The data suggest that Ile178 is the major contributor to the observed inhibition of IN, as the Ile178Ser mutation completely abrogates both the binding and inhibition of IN and the other three residues had considerably lesser effects on the inhibitory and binding capacities of the peptides. A better understanding of this inhibition can facilitate the design and development of novel and highly-specific drugs directed against HIV INs.

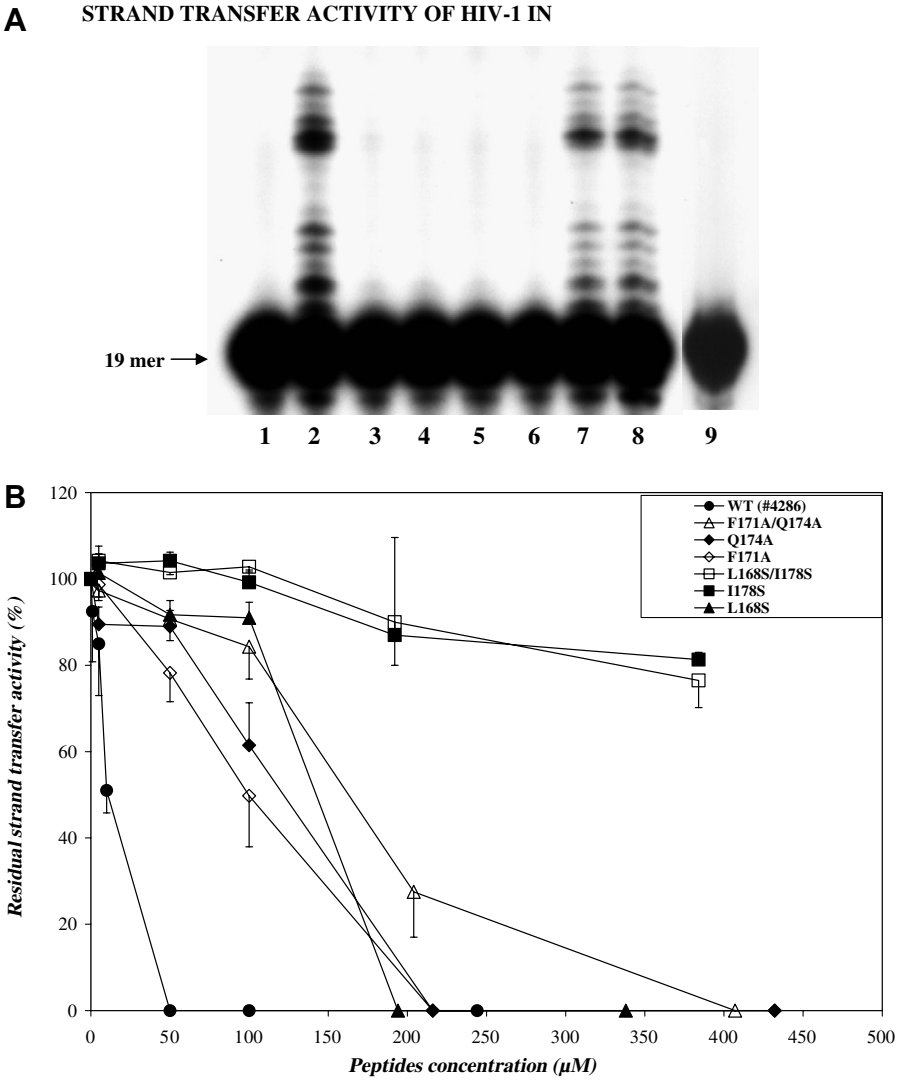

Fig. 1. Effects of mutations in the RT-derived peptide (4286) on inhibiting the strand transfer activity of HIV-1 IN. 240 μM of peptide 4286 and 400 μM of each of the mutated peptides (A) or increasing peptide concentrations (B) were preincubated with a fixed amount of IN, followed by assaying the strand transfer activity, as described (“Materials and methods” and [11,12]). (A) Gel-electrophoresis analysis of the reaction products. Lane 1, substrate only; Lane 2, IN; Lanes 3–9, IN in the presence of the following peptides: 3, peptide 4286 (WT); 4, F171A/Q174A; 5, Q174A; 6, F171A; 7, L168S/I178S; 8, I178S; 9, L168S. (B) Dose–response curves of the residual activities, expressed as percentage of the initial IN activity with no peptide present. Each value is the average of three independent experiments with the standard deviations shown by bars. The apparent IC_{50} values of each peptide are as follows: 4286 (WT), 9 μM; F171A/Q174A, 163 μM; Q174A, 125 μM; F171A, 100 μM; L168S/I178S, >400 μM; I178S, >400, and L168S, 145 μM.

Materials and methods

HIV-1 RT-derived peptides. All peptides used here are 20 residues long. Peptide 4286 with the sequence K_{166}ILEPFRKQNPDIVIYQYMD$_{185}$, derived from the wild-type HIV-1 HXB2R RT, was already studied by us [11]. The numbering of all residues in the peptide was according to their location in the full length HIV-1 RT. All other peptides were custom-synthesized and were derived from the sequence of peptide 4286 with a single or double mutation inserted in each.

Bacterial expression and purification of HIV-1 INs. HIV-1 IN: IN from the HIV-1 BH-10 strain of HIV-1, carrying an amino-terminal six-histidine tag, was expressed and purified as described [15].

HIV-1 IN-catalytic-core domain (W131E, F185K): This large IN fragment (designated IN-CCD), carrying an amino-terminal six-histidine tag, was expressed and purified as described [16].

In vitro assays for the enzymatic activities of HIV-1 IN versions. All assays and oligonucleotides used were described in detail by us [11,12]. The reactions contained 0.33 pmol of the labeled duplex DNA substrate in 90 mM NaCl, 7.5 mM $MnCl_2$, 10 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 25 mM MOPS (pH 7.2), 5% glycerol, and 3.6 pmol IN or 30 pmol IN-CCD. IN versions were preincubated on ice in the presence or the absence of the RT-derived peptides, followed by addition of the substrate and a further incubation at 37 °C. The reaction products were analyzed by gel electrophoresis as described [11,12].

Results and discussion

We have studied herein specific mutants of the inhibitory RT-derived peptide designated 4286 for their involvement in inhibiting IN. Consequently, four peptides were designed, with each of the four suspected residues mutated (L168S, F171A, Q174A, and I178S), as well as two additional peptides, each with a double mutation (L168S/I178S and F171A/Q174A). All peptides were tested for inhibiting IN activities. First, the inhibition of the strand transfer activity by the peptides was tested (Fig. 1A). As expected, peptide 4286 strongly inhibits this activity. However, two mutated peptides, L168S/I178S and I178S, fail to significantly inhibit IN, in contrast to all other peptides that inhibited IN. Only residues directly involved in inhibiting IN are those whose mutagenesis causes the peptides to lose their inhibitory capacity. Hence, it is likely that the only residue essential for IN inhibition is I178, as the peptides with the I178S and L168S/I178S mutations lost nearly all of their inhibitory capacity, while peptide L168S did not. We have further assessed the effect on the strand transfer activity by increasing peptide concentrations (Fig. 1B). The dose–response curves confirm that the only two peptides that lost almost all the inhibitory capacity were I178S and L168S/I178S. However, the other mutated peptides inhibited some of IN activity, albeit at lower efficiencies than the wild-type peptide 4286. The peptide concentrations that inhibit 50% of the initial IN strand transfer activity (IC_{50} values) were calculated. While the apparent IC_{50} value for peptide 4286 is about 9 μM, those calculated for the mutated peptides were higher and the IC_{50} values for the I178S-containing peptides were too high to calculate (see the legend to Fig. 1B).

We have further tested whether the pattern of the strand transfer inhibition is true also for the 3′-end processing

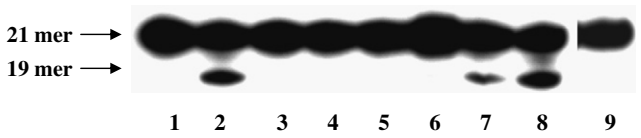

Fig. 2. Effects of mutations in peptide 4286 on inhibiting the 3′-end processing activity of HIV-1 IN. The peptides and IN concentrations used are similar to those described in Fig. 1A, followed by assaying the 3′-end processing activity, as described [11,12]. Lanes 1–9 are exactly as described in Fig. 1A.

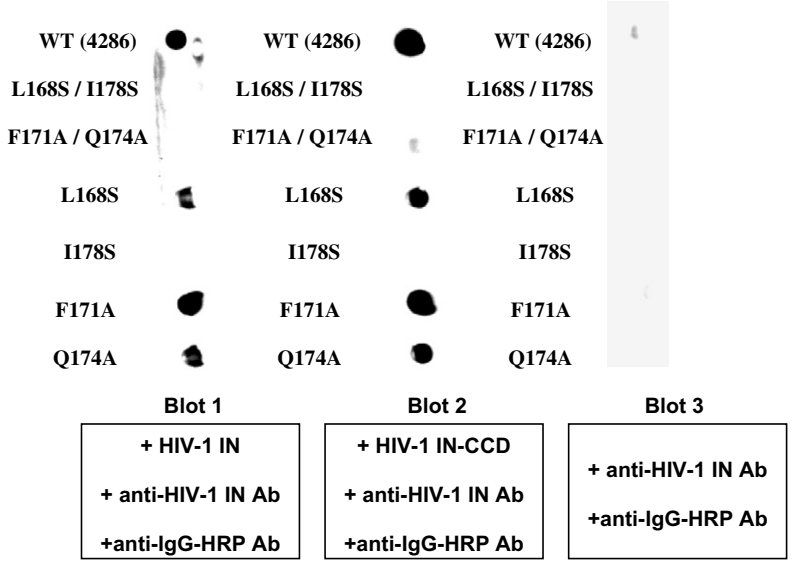

Fig. 3. Dot-blot binding assay of peptide 4286 and its mutated derivatives to full-length HIV-1 IN and to HIV-1 IN-CCD. The designations of each of the tested peptides appear to the left of the dots. The proteins and antibodies (Ab) used and the orders of their additions are indicated. Blot 1, binding of the tested peptides to full-length HIV-1 IN. Blot 2, binding of the tested peptides to HIV-1 IN-CCD. Blot 3, control binding of the tested peptides to the antibodies with no INs present.

activity (Fig. 2). Indeed, peptides I178S and L168S/I178S practically lost all inhibitory capacity, while all other mutated peptides completely inhibited the IN activity. The inhibition of the disintegration activity of both IN and IN-CCD (that exhibits only disintegration activity [2,3,11,17–19]), was also investigated (data not shown). Here again, peptides L168S/I178S and I178S completely lost their inhibitory capacity, implying once again that I178 is cardinal for the inhibition of IN. All other peptides strongly inhibited the disintegration activity.

To evaluate whether the differences in the effects of the mutated peptides on IN and IN-CCD activities reflect variations in the physical interactions with the peptides, we have tested all peptides for binding to both IN versions, by a dot-blot binding assay, previously adapted by us [11]. Since no macromolecules other than INs and the peptides are present in these reactions, the noticeable bindings reflect direct physical interactions between these components. The results presented in Fig. 3 show that peptides I178S and L168S/I178S that have lost their inhibitory ability also lost their capacity to bind both IN versions. The other peptides (except for F171A/Q174A) bind INs to approximately the same extent as peptide 4286. Peptide F171A/Q174A did not show any significant interaction with IN and only a marginal contact with IN-CCD, despite inhibiting all IN activities. As the single mutations F171A and Q174A did not affect the binding to INs, it is not clear why the peptide with the double mutation F171A/Q174A lost almost all binding capacity. It might be that there is a synergistic effect on the reduction in binding that can be seen in this assay only after the two residues are simultaneously mutated. Alternatively, it is possible that, at least with this specific peptide, the binding assay is significantly less sensitive than the activity assays.

Finally, as done previously by us [11], we have performed a docking of peptide 4286 and its I178S mutated version into IN-CCD (Fig. 4), since all crystal structures determined for HIV-1 IN are composed of either only the CCD or CCD fused to one of the other two IN-domains [2,3]. This model suggests that the interaction between Ile178 and Gly149 of IN-CCD is probably lost in peptide I178S due to the smaller radius of the Ser side-chain that substituted the Ile side-chain (Fig. 4B). This can explain why Ile178 is involved in the direct interaction with IN and, consequently, in inhibiting IN. It would be interesting to study in the future a series of mutations in 4286, in which residue 178 will be modified to see whether other factors besides the radius of the side-chain affect the inhibition and binding capacities of the peptide.

In all crystal structures published so far for HIV-1 IN-CCD, there is a surface loop (residues 141–148) that is disordered, due to its flexibility [2]. It is known among other studied enzymes that flexible surface loops are important for substrate binding and catalysis [20–22]. The role of region 140–149 in HIV-1 IN was studied and residues Gly140 and Gly149 (considered potential hinges for the IN flexible loop) were mutated and the

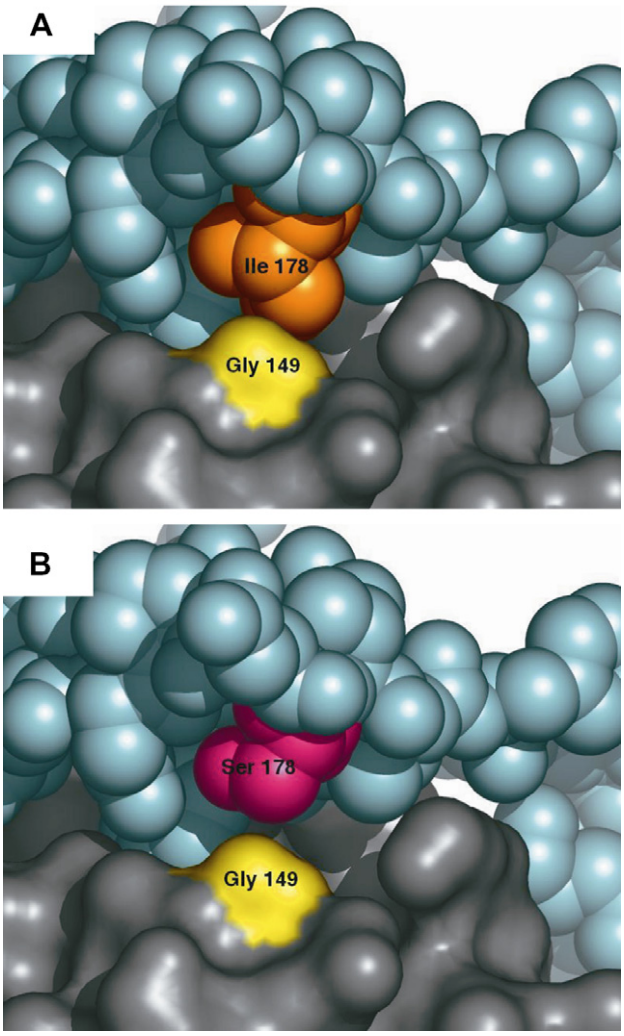

Fig. 4. Molecular docking of peptide 4286 and of I178S mutated peptide into the core domain of HIV-1 IN. The global range molecular matching (*GRAMM*) program [26] was used for docking of the wild-type (4286) and of the mutated (I178S) peptides into IN-CCD. A generic high resolution docking was performed with grid step 2.0 Å and grid size 64 Å. Rotation was calculated in 10° intervals. The repulsion score was 40. The 20 highest scored hits were analyzed visually. The structure of HIV-1 IN-CCD used for docking was based on protein data bank (PDB) entry 1BIS [16]. The peptide conformation was as in PDB entry 1REV [27]. In gray is the space-filling diagram of the surface of IN-CCD. Both peptides are presented as blue spheres. (A) The crucial van der Waals contact between IN-CCD-Gly149 (in yellow) and the peptide Ile178 (in orange). (B) The disruption of the contact between IN-CCD and the RT-derived peptide is apparent when Ile178 is mutated to Ser (in magenta). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effects on the structure and enzymatic activity were tested [23]. The results show that these mutations make the flexible loop more rigid and, consequently, reduce the activity of the mutated IN-CCDs (including one bearing the G149A mutation), without affecting DNA-binding. Therefore, it was suggested that Gly149 of HIV-1 IN is imperative for maintaining the flexibility of the active-site loop that contributes to a post-binding catalytic step of IN. The importance of the flexibility of the active-site loop

for the activities of IN was also established for avian sarcoma/leukosis virus IN, as rigidification of its flexible loop (residues 144–154), by lowering the pH (rather than by mutagenesis), correlates with lower activity [24]. Accordingly, another possible explanation for the inhibition of IN by peptide 4286 is that the binding of Ile178 to the IN-Gly149 interferes with the flexibility of the active-site loop, causing a substantial decrease in IN activities. Furthermore, multiple alignments of HIV-1 sequences of 441 isolates (taken from http://hiv-web.lanl.gov/content/index) show that Gly149 was present in all, substantiating the high importance of this residue.

Multiple alignments conducted among 442 HIV-1 isolates show that Ile178 is present in 73.2%, 15.2% have a Met, 4.1% have a Val, and 7.5% have Leu. Thus, Ile (or other similar hydrophobic residues) at position 178 in HIV-1 RT are well conserved among HIV-1 isolates. Indeed, sequences around Ile178 of HIV-1 RT were suggested to be important for RT activities [25]. In all, it is possible that these specific predicted interactions between RT-Ile178 and IN-Gly149 cannot be evaded *in vivo*, since mutagenesis of these residues is likely to result in impaired enzymes and, hence, in non-infectious virions. We do hope that a better understanding of the peptide sequences that inhibit IN can lead to the development of novel specific peptide-based inhibitors of HIV-1 integrase.

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References

[1] J.M. Coffin, S.H. Hughes, H.E. Varmus, Retroviruses, Cold Spring Harbor Laboratories Press, Cold Spring Harbor, NY, 1997.
[2] K.C. Thang, D.R. Davies, Structure and function of HIV-1 integrase, Curr. Top. Med. Chem. 4 (2004) 965–979.
[3] R. Craigie, Retroviral DNA integration, in: N.L. Craig, R. Craigie, M. Gellert, M. Lambowitz (Eds.), Mobile DNA II, ASM Press, Washington, DC, 2002, pp. 613–630.
[4] M.I. Burkinsky, N. Sharova, T.L. McDonald, T. Pushkarskaya, W.G. Tarplay, M. Stevenson, Association of integrase, matrix and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection, Proc. Natl. Acad. Sci. USA 90 (1993) 6125–6129.
[5] M.D. Miller, C.M. Farnet, F.D. Bushman, Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition, J. Virol. 71 (1997) 5382–5390.
[6] B. Van Maele, K. Busschots, L. Vandekerckhove, F. Christ, Z. Debyser, Cellular co-factors of HIV-1 integration, Trends Biochem. Sci. 31 (2006) 98–105.
[7] R.A.M. Fouchier, M.H. Malim, Nuclear import of human immunodeficiency virus type-1 preintegration complexes, Adv. Virus Res. 52 (1999) (1999) 275–299.
[8] S.C. Hu, D.L. Court, M. Zweig, J.G. Levin, Murine leukemia virus *pol* gene products: analysis with antisera generated against reverse transcriptase and endonuclease fusion proteins expressed in *Escherichia coli*, J. Virol. 60 (1986) 267–274.
[9] T. Ishikawa, N. Okui, N. Kobayashi, R. Sakuma, T. Kitamura, Y. Kitamura, Monoclonal antibodies against the minimal DNA-binding domain in the carboxyl-terminal region of human immunodeficiency virus type 1 integrase, J. Virol. 73 (1999) 4475–4480.
[10] X. Wu, H. Liu, H. Xiao, J.A. Conway, E. Hehl, G.V. Kalpana, V. Prasad, J.C. Kappes, Human immunodeficiency virus type 1 integrase protein promotes reverse transcription through specific interactions with the nucleoprotein reverse transcription complex, J. Virol. 73 (1999) 2126–2213.
[11] I. Oz Gleenberg, O. Avidan, Y. Goldgur, A. Herschhorn, A. Hizi, Peptides derived from the reverse transcriptase of human immunodeficiency virus type 1 as novel inhibitors of the viral integrase, J. Biol. Chem. 280 (2005) 21987–21996.
[12] I. Oz, O. Avidan, A. Hizi, Inhibition of the integrases of human immunodeficiency viruses type 1 and type 2 by reverse transcriptases, Biochem. J. 361 (2002) 557–566.
[13] T. Tasara, G. Maga, O.M. Hottiger, U. Hübscher, HIV-1 reverse transcriptase and integrase enzymes physically interact and inhibit each other, FEBS Lett. 507 (2001) 39–44.
[14] M.S. Lee, R. Craigie, A previously unidentified host protein protects retroviral DNA from autointegration, Proc. Natl. Acad. Sci. USA 95 (1998) 1528–1533.
[15] E.V. Barsov, W.E. Huber, J. Marcotrigiano, P.K. Clark, A.D. Clark, E. Arnold, S.H. Hughes, Inhibition of human immunodeficiency virus type 1 integrase by the Fab fragment of a specific monoclonal antibody suggests that different multimerization states are required for different enzymatic functions, J. Virol. 70 (1996) 4484–4494.
[16] Y. Goldgur, F. Dyda, A.B. Hickman, T.M. Jenkins, R. Craigie, R.D. Davies, Three new structures of the core domain of HIV-1 integrase: an active site that binds magnesium, Proc. Natl. Acad. Sci. USA 95 (1998) 9150–9154.
[17] F.D. Bushman, A. Engelman, I. Palmer, P. Wingfield, R. Craigie, Domains of integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding, Proc. Natl. Acad. Sci. USA 90 (1993) 3428–3432.
[18] J. Kulkosky, R.A. Katz, G. Merkel, A.M. Skalka, Activities and substrate specificity of the evolutionary-conserved central domain of retroviral integrase, Virology 206 (1995) 448–456.
[19] A.B. Hickman, I. Palmer, A. Engelman, R. Craigie, P. Wing-field, Biophysical and enzymatic properties of the catalytic domain of HIV-1 integrase, J. Biol. Chem. 269 (1994) 29279–29287.
[20] S.A. Weston, A. Lahm, D. Suck, X-ray structure of the DNase I-d(GGTATACC)$_2$ complex at 2.3 Å resolution, J. Mol. Biol. 226 (1992) 1237–1256.
[21] M. Newman, T. Strzelecka, L.F. Dorner, I. Schildkraut, A.K. Aggarwal, Structure of Bam HI endonuclease bound to DNA: partial folding and unfolding on DNA binding, Science 269 (1995) 656–663.
[22] P.A. Rice, T.A. Steitz, Refinement of $\gamma\delta$ resolvase reveals a strikingly flexible molecule, Structure 2 (1994) 371–384.
[23] J. Greenwald, V. Le, L.S. Butler, F.D. Bushman, S. Choe, The mobility of an HIV-1 integrase active site loop is correlated with catalytic activity, Biochemistry 38 (1999) 8892–8898.
[24] J. Lubkowski, Z. Dauter, F. Yang, J. Alexandratos, G. Merkel, A.M. Skalka, A. Wlodawer, Atomic resolution structures of the core domain of avian sarcoma virus integrase and its D64N mutant, Biochemistry 38 (1999) 13512–13522.
[25] B. Grimison, J. Laurence, Immunodominant epitope regions of HIV-1 reverse transcriptase: correlations with HIV-1+ serum IgG inhibitory to polymerase activity and with disease progression, J. Acquir. Immun. Defic. Syndr. Hum. Retrovirol. 9 (1995) 58–68.
[26] E. Katchalski-Katzir, I. Shariv, M. Eisenstein, A.A. Friesem, C. Aflalo, I.A. Vakser, Molecular surface recognition: determination of geometric fit between proteins and their ligands by correlation techniques, Proc. Natl. Acad. Sci. USA 89 (1992) 2195–2199.
[27] J. Ren, R. Esnouf, E. Garman, D. Somers, C. Ross, I. Kirby, J. Keeling, G. Darby, Y. Jones, D. Stuart, D. Stammers, High-resolution structures of HIV-1 RT from four RT inhibitor complexes, Nat. Struct. Biol. 2 (1995) 293–302.