Peptides Derived from the Reverse Transcriptase of Human Immunodeficiency Virus Type 1 as Novel Inhibitors of the Viral Integrase*§

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Recent studies have shown that the integrase (IN) of HIV-1 is inhibited in vitro by HIV-1 reverse transcriptase (RT). We further investigated the specific protein sequences of RT that were involved in this inhibition by screening a complete library of RT-derived peptides for their inhibition of IN activities. Two 20-residue peptides, peptide 4286, derived from the RT DNA polymerase domain, and the one designated 4321, from the RT ribonuclease H domain, inhibit the enzymatic activities of IN in vitro. The former peptide inhibits all three IN-associated activities (3′-end processing, strand transfer, and disintegration), whereas the latter one inhibits primarily the first two functions. We showed the importance of the sequences and peptide length for the effective inhibition of IN activities. Binding assays of the peptides to IN (with no DNA substrate present) indicated that the two inhibitory peptides (as well as several non-inhibitory peptides) interact directly with IN. Moreover, the isolated catalytic core domain of IN also interacted directly with the two inhibitory peptides. Nevertheless, only peptide 4286 can inhibit the disintegration activity associated with the IN core domain, because this activity is the only one exhibited by this domain. This result was expected from the lack of inhibition of disintegration of full-length IN by peptide 4321. The data and the three-dimensional models presented suggested that the inhibition resulted from steric hindrance of the catalytic domain of IN. This information can substantially facilitate the development of novel drugs against HIV INs and thus contribute to the fight against AIDS.

Two viral encoded enzymes play central roles in the early stages of the replication of retroviruses and retrotransposons. The first one, reverse transcriptase (RT),1 converts the single-stranded viral RNA into double-stranded DNA in a relatively complex process, reverse transcription. This step is catalyzed by the two catalytic activities of RT, the DNA polymerase (capable of copying both RNA and DNA into DNA) and the RNase H activity, which concomitantly hydrolyzes the RNA strand in the DNA-RNA heteroduplex formed (1). Subsequently, the RT-produced double-stranded DNA is transported into the nucleus, as part of the nucleoprotein complex (designated the preintegration complex), where it integrates into the genomic target DNA by the second viral enzyme, the IN. IN identifies the ends of the linear viral DNA, trims them (by removing two or three extra nucleotides located 3′ to the highly conserved CA 3′ termini), and then accompanies the DNA into the nucleus to catalyze integration into the target cellular DNA (1–5). There are several examples for potential linkages between RT and IN. First, the DNA product of RT is the substrate for IN, the next enzyme in the line of the viral replication cycle. Second, both proteins are proteolytic products of the same polyprotein precursor encoded by a single retroviral gene, the pol (1). In some cases, as in avian sarcoma leukemia virus, the IN sequence appears in two forms, one as part of the large β-subunit of the RT and the other as a free IN protein designated pp32 (1, 4). Moreover, PICs, which are capable of performing in vitro integration, contain the viral DNA, IN, RT, and other proteins (5–8). Third, the INs and RTs of HIV-1 and MLV were shown to exhibit physical interactions (9–11). These direct contacts between the IN and RT of HIV-1 were recently confirmed by us by using surface plasmon resonance technology.2 Finally, we and others (12, 13) have shown recently that RT can inhibit in vitro the enzymatic activities of IN, suggesting functional roles for these interactions.

After the completion of reverse transcription in the cytoplasm of the infected cell, there is a significant delay in the process of integration depending on the rate by which the PICs are transported into the nucleus (1). Because all possible catalytic components for integration are likely to be present in the PICs, the viral DNA can serve as donor DNA as well as the target DNA for integration. Such a potential auto-integration process is suicidal for the virus, as it destructs the viral genome. For that reason, it is imperative to understand the mechanisms that regulate the integration of the retroviral genome and learn how to control it. Several cellular proteins are known to be involved in the integration process in HIV-infected cells (14–17). Most interestingly, one of these factors serves also as a barrier to auto-integration in MLV-infected cells (18).

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1 The abbreviations used are: RT, reverse transcriptase; IN, integrase; IN-CCD, integrase catalytic-core domain; HIV-1, human immunodeficiency virus, type 1; AIDS, acquired immunodeficiency syndrome; RNase H, ribonuclease H; PIC, preintegration complex; MLV, murine leukemia virus; DDW, double distilled water; Me2SO, dimethyl sulfoxide; MOPS, 4-morpholinepropanesulfonic acid.

2 I. Oz Gleenberg, A. Herschhorn, and A. Hizi, unpublished data.

This paper is available on line at http://www.jbc.org
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Because RT can also inhibit IN activities (12, 13), it is possible that an alternative pathway of inhibiting auto-integration without the involvement of cellular proteins exists as well. This course of action involves the functional interactions between the authentic viral proteins, RTs and INs, prior to integration into the cellular DNA.

Here we have extended our previous in vitro study on the inhibition of IN by RT (12) by studying specific RT-derived sequences that are involved in binding and inhibiting IN. This was done through a systematic in vitro screening of a library of HIV-1 RT-derived peptides for their capacity to inhibit HIV-1 IN activities and to interact directly with IN. We have identified two 20-residue-long peptides that inhibit IN activities in vitro. The first peptide is derived from the DNA polymerase active site, and the second peptide is derived from the RNAse H domain of RT. In addition, we have shown by a novel dot-blot analysis that the full-length RT, as well as several RT-derived peptides, bind directly to HIV-1 IN. The localization of the two inhibitory peptides in the three-dimensional structure of the RT and the putative docking (of the DNA polymerase-derived peptide) into IN-CCD suggest that the inhibition of IN activities results from a steric hindrance. These findings can lead to insights into the development of novel peptide-based, specific, and highly potent IN inhibitors. Such inhibitors are expected to interfere with HIV infectivity and, thus, serve as novel prodrugs for the treatment of AIDS.

EXPERIMENTAL PROCEDURES

The sequences of all studied peptides are given in the Supplemental Material.

HIV-1 RT-derived Peptides

Two sets of synthetic peptides libraries were generous gifts from the NIH-AIDS Research and Reference Reagent Program. The first set was the HIV-1 HXB2R Pol peptides complete set (catalog number 4558). These peptides are each 20 residues in length with 10 amino acid overlaps between the sequential peptides. All peptides derived from the DNA polymerase domain were prepared to a final concentration of 900 μM, by dissolving in either DDW or in a minimal amount of 100% Me2SO and then diluted with DDW to 6.5% Me2SO, all according to the recommended instructions (www.aidsreagent.org). All peptides derived from the RNase H domain were dissolved in a minimal amount of 100% Me2SO and then diluted with DDW to 6.5% Me2SO (yielding final concentrations of 400 μM). The second peptide set was the complete set of the HIV-1-CCD consensus pol peptides (catalog number 6208). These peptides are each 15 residues in length with 11 amino acid overlaps between the sequential peptides. They were also dissolved as above to a final concentration of 400 μM each in 6.5% Me2SO. All peptides were designated according to the numbering given by the NIH-AIDS Research and Reference Reagent Program (www.aidsreagent.org).

Peptides 4286-1 (15-mer) and 4321-1 (14-mer) were custom-synthesized. The first one was diluted to a final concentration of 3.5 mM in DDW and the second one as described above to a final concentration of 5.7 mM in 6.5% Me2SO.

Recombinant Proteins

**Bacterial Expression and Purification of HIV-1 IN and HIV-1 RT Proteins**—All HIV-1 IN and RT versions used were highly purified as judged from their pattern after analysis by SDS-PAGE (data not shown).

**HIV-1 IN**—HIV-1 IN from the BH-10 strain of HIV-1, carrying amino-terminal His6 tag, was expressed and purified as described in detail (20).

**HIV-1 IN-Catalytic Core Domain (the Double Mutant W131E, F185K)**—This large IN fragment (designated IN-CCD) was expressed as an amino-terminal His6-tagged protein and purified as described in detail (21).

**HIV-1 RT**—The expression and purification of heterodimeric (p66/p51) HIV-1 RT with a His6 tag, attached to the carboxyl-terminal end of the p66, were described in detail previously (22). The expression and purification of a non-tagged heterodimeric HIV-1 RT were described previously (23, 24).

**Oligonucleotides**

Oligonucleotide A, designated IN-CCD, represents a fragment of the gag gene (25). Oligonucleotides A–C correspond to the U5 end of the HIV-1 long terminal repeat (25). Boldface letters indicate the highly conserved CA/TG dinucleotide pair.

**Assays for Enzymatic Activities of HIV-1 IN and HIV-1 RT-derived Peptides**

The following gel-purified oligonucleotides were used in the enzymatic assays of HIV-1 IN: A (21-mer), 5’-GTGTGAAATCTTAGCAGT-3’; B (21-mer), 5’-ACTGGCTAGGATTTTCCACAC-3’; C (19-mer), 5’-CTGGTGAAATCTCTAGCA-3’; D (38-mer), 5’-TGCTAGTCTCAGGAGCCCCTGGCCGTCGTCGC-3’. Oligonucleotides A–C correspond to the U5 end of the HIV-1 long terminal repeat (25). Boldface letters indicate the highly conserved CA/TG dinucleotide pair. Oligonucleotide C is identical to A, after the removal of the GT dinucleotides from its 3’-end and thus after annealing to oligonucleotide B, creating a dinucleotide overhang at the 5’-end of oligonucleotide B. Oligonucleotide D, termed “dumbbell,” folds to form a structure mimicking the integration intermediate. This substrate, used for assaying the disintegration activity, has 5 bp of a viral sequence and 10 bp of a non-viral sequence, as described earlier (26). In order to test the 3’-end processing and the resulting strand transfer activity of IN, the 5’-end-labeled oligonucleotide A, annealed to its complementary strand, oligonucleotide B (both 21 nucleotides long), was used. We have used the duplex of oligonucleotides C and B and for assaying the 3’-end processing activity (12).

**5’-End Labeling and Substrate Preparations**—Fifty pmol of oligonucleotides A, C, or D were 5’-end-labeled using 1 unit of T4 polynucleotide kinase and 50 μCi of γ-32P]ATP, in a final volume of 50 μl of the appropriate buffer (supplied by the manufacturer) for 30 min at 37 °C. The samples were then heat-inactivated. The 5’-end-labeled oligonucleotides A or C were annealed each to an equimolar amount of oligonucleotide B in 55 mM Tris-HCl (pH 7.5) and 0.27 mM NaCl. For the disintegration assay, we have used 5’-end-labeled oligonucleotide D, which forms a dumbbell structure after self-annealing (12).

**Assays of the 3’-End Processing, Strand Transfer (or DNA Joining), and Disintegration Activities**—In the strand transfer assays described, the labeled 5’-end substrate employed served as both the target and donor DNA leading to an increase in the molecular size of the substrate, whereas in the 3’-end processing and disintegration assays, we have followed the unique cleavage of the labeled substrates (12, 26). All reactions were performed in 10-μl reaction mixtures with 0.33 pmol of the labeled DNA substrate and the reaction buffer, containing 1 mM HEPES (pH 7.5), 54 mM NaCl, 2.5 mM MnCl2, 50 μM EDTA, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.1 mM spermidine, 25 mM MOPS (pH 7.2), 5% glycerol, and 1% Me2SO (12). We have assayed 250 ng of HIV-1 IN (which equals 4 pmol, assuming IN dimers of the 32-kDa subunits) or 1 μg of HIV-1 IN-CCD (which equals 28 pmol, assuming dimers of the 18-kDa subunits). This difference in the amounts of proteins assayed results from the lower activity of IN-CCD relative to the full-length IN. The two versions of HIV-1 IN were preincubated on ice for 5 min in the presence or the absence of HIV-1 RT or the HIV-1 RT-derived peptides. Reactions were initiated after adding the labeled DNA substrate in the reaction buffer, incubated for 30 min at 37 °C, and then stopped by adding 10 μl of formamide loading buffer (90% formamide, 10 mM EDTA, 1 mg/ml bromphenol blue, 1 mg/ml xylene cyanole). The samples were heat-denatured, cooled on ice, and loaded onto 6 M urea, 14% polyacrylamide denaturing gels, followed by electrophoresis (urea-PAGE). The gels were dried and subjected to autoradiography at ~80 °C or at room temperature to obtain close to linear exposures.

**Quantitative Analyses of the Inhibition of HIV-1 IN by HIV-1 RT and by RT-derived Peptides**

The films were scanned, and the levels of IN activity were calculated using the densitometric software TINA (version 2.07d; Raytest Isotopenmessgeräte, GmbH). The activities were determined as a percentage of the total 5’-end-labeled DNA oligonucleotides converted to 19-mer DNA. Strand transfer (or DNA joining) activity was calculated as a percentage of the total amount of labeled DNA found in DNA bands of 22 nucleotides or more in length. Disintegration activity was calculated as a percentage of the total 5’-end processing activity was determined using the densitometric software TINA (version 2.07d; Raytest Isotopenmessgeräte, GmbH). The activities were determined as a percentage of the total amount of labeled DNA found in DNA bands of 22 nucleotides or more in length. Disintegration activity was calculated as a percentage of the total 5’-end-labeled 38-mer DNA oligonucleotide converted to residual DNA. All activities were relative to the control activity of the IN (with no RT or RT-derived peptides present) and were expressed as percentage of the initial IN activities.

**Dot-blot Binding Assay**

**HIV-1 RT-derived Peptides Bound to HIV-1 IN**—Nitrocellulose filters were soaked in DDW followed by transfer buffer, containing 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, for 5 min and then transferred to a dot-blot apparatus (Bio-Rad). Aliquots of RT-derived peptides or...
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RESULTS

We have shown previously (12) that all three enzymatic activities performed by HIV INs, namely the 3'-end processing, the strand transfer (termed together integration), and the reverse process, the disintegration, are inhibited in vitro by RTs. Subsequently, the goal of this study was to identify the sequences in HIV-1 RT that inhibit and interact with the full-length HIV-1 IN, as well as with the IN catalytic core domain. To this end, we have used a large set of synthetic peptides that cover the whole pol gene of HIV-1 (the HXB2R isolate). The peptides were each 20 residues long with 10 amino acids overlaps between the sequential peptides. These peptides were tested for their ability to bind IN and inhibit in vitro the enzymatic activities of HIV-1 IN and of IN-CCD.

DNA Polymerase Domain-derived Peptide 4286 Inhibits in Vitro the Integration Activity of HIV-1 IN—It was already shown by us that HIV INs are inhibited by the p51 subunit of HIV-1 RT, which encompasses the DNA polymerase domain (12). Consequently, we first tested the sequences located within this domain of HIV-1 RT. A total of 42 peptides, designated from 4269 up to 4310 (starting from the amino-terminal peptide, see Supplemental Material), were screened for their ability to inhibit HIV-1 IN. These peptides were first tested in groups, each containing a mixture of three contiguous and partially overlapping peptides (every one at a final concentration of 90 μM). All mixtures were preincubated on ice for 5 min with 0.4 μM purified HIV-1 IN. The enzymatic assays were then initiated by adding the DNA substrate, used for testing the combined 3'-end processing and DNA joining activities of IN (oligonucleotides A and B, see under “Experimental Procedures”), followed by incubation at 37 °C for 30 min. The results obtained show that only the mixture containing peptides 4284, 4285, and 4286 inhibited the integration activity of IN (data not shown). Consequently, each of these peptides was then tested separately (at a final concentration of 270 μM) for its ability to inhibit the integration activity. The results indicated that the inhibition of IN was caused primarily by a single peptide, 4286. This peptide inhibits both IN-mediated strand transfer and 3'-end processing activities (Fig. 1). It is apparent that both activities were substantially inhibited by the peptide at a final concentration of 27 μM. As a result, the extent of inhibition was also quantified to determine the peptide concentrations inhibiting 50% of the initial IN activity (IC50 value, Table I). The value, calculated for the inhibition of the strand transfer, was about 4.5 μM, and the one for the 3'-end processing was ~4.8 μM (Table I).

Because every two adjacent peptides share 10-residue overlaps, we have also calculated the IC50 values for peptides 4285 and 4286 (that share sequences with the inhibitory peptide 4286). The analyses of the reaction products, which led to the calculations of the IC50 values (summarized in Table I), indicate that both 4285 and 4286 were considerably less effective than peptide 4286. Furthermore, we have also checked whether the two peptides 4285 and 4286, when tested together, demonstrate a synergistic effect by increasing their IN inhibiting capacity to a level as efficient as peptide 4286. The results showed that this is not the case, and their combined effect is still far below that of peptide 4286 (data not shown). This indicated that the inhibitory sequence of 4286 can be active only as one continuously linked sequence, which is likely to be structurally different from that of the other two peptides.

Peptide 4221, Derived from the RNase H Domain, Inhibits the Integration Activity of HIV-1 IN—We have found previously that the p51 subunit of HIV-1 RT inhibits the enzymatic activities of HIV INs, without testing the residual RT p15 domain, corresponding to the RNase H domain (12). This does not
necessarily exclude the possibility that the p15 by itself can also exhibit inhibitory effects. To address this issue, we have tested all 15 RNAse H domain-derived 20-mer peptides (peptide numbers from 4311 up to 4325). In each test, the peptides were analyzed in groups of two adjacent and partially overlapping ones (each at a final concentration of 60 μM). Only the mixture containing peptides 4321 and 4322 inhibited IN activities. Consequently, we have tested each peptide individually, and we found that only peptide 4321 was responsible for inhibiting both the 3'-end processing and strand transfer activities of HIV-1 IN (Fig. 2). The quantitative analyses indicated that the apparent IC_{50} values calculated for both activities were quite similar (Table I). These values are close to those calculated for peptide 4286. It should be noted that neither peptide 4320 nor peptide 4322 (that share sequences with peptide 4321) inhibited the enzymatic activity of HIV-1 IN (Fig. 2). The quantitative analyses indicated that the apparent IC_{50} values calculated for both activities were quite similar (Table I). These values are close to those calculated for peptide 4286. It should be noted that neither peptide 4320 nor peptide 4322 (that share sequences with peptide 4321) inhibited the enzymatic activity of HIV-1 IN.

The Complete 20-Residue Sequences of Peptides 4286 and 4321 Are Required to Inhibit HIV-1 IN—To determine the minimal sequence requirements for the inhibition of HIV-1 IN, we have tested short peptides derived from the 20-residue original 20-mer peptides in inhibiting IN enzymatic activities. In addition, we have tested for IN inhibition a battery of 15-mer peptides (with sequences that partially cover those of peptides 4286 and 4321) for their ability to inhibit IN. These peptides were part of a library of 15-mer synthetic peptides that covered the whole pol gene of HIV-1 clade B (see Supplemental Material). Eight peptides (from 5538 to 5545) covered the sequence of 4286, and eight peptides (from 5626 up to 5633) overlapped 4321. Of all 16 peptides tested, only peptide 5628 (related to peptide 4321) moderately inhibited the strand transfer activity of IN with an apparent IC_{50} value of ~60 μM (as opposed to 5 μM of 4321) without showing a significant inhibition of the 3'-end processing (Table I). These results indicated the importance of the full 20-residue sequences (and potential structures of peptides 4286 and 4321) for the in vitro inhibition of HIV-1 IN.

Peptide 4286 Strongly Inhibits the Disintegration Activity of HIV-1 IN—All studied retroviral INs possess in vitro, in addition to the 3'-end processing and the forward strand transfer activities, the reverse activity of disintegration. The authentic disintegration activity was already studied extensively as a major IN activity (26–28). The disintegration activity involved a specific endonuclease excision of the “donor viral” DNA (with the conserved 3'-end “CA” dinucleotide) from its target DNA. Given that HIV-1 RT also inhibited this activity of HIV-1 IN (12), it was of interest to extend the study by testing the inhibitory effects of peptides 4286 and 4321 on the disinte-

| HIV-1 RT-derived peptide | Position in HIV-1 RT (residue no.) | 3'-End processing (IC_{50}) | Strand transfer (IC_{50}) | Disintegration (IC_{50}) |
|--------------------------|-----------------------------------|-----------------------------|---------------------------|--------------------------|
| 4286 (20-mer), KILEPFRKQNPDIVIYQQMD | Palm (166–185) | 4.8 | 4.5 | 9.4 |
| 4285 (20-mer), SPAIQQSMTKILEPFRQN | Palm (156–175) | 35 | 270 | >100 |
| 4287 (20-mer), PDIVIYQQMDDIVLYVGSDELI | Palm (176–195) | 22 | 54 | >120 |
| 4321 (20-mer), ELVQIIIEQLIKKKEKYLAW | RNAse H (516–535) | 6.9 | 5 | 100 |
| 4320 (20-mer), IQAQPDQSESELVQIEKQL | RNAse H (506–525) | >120 | >120 | >120 |
| 4289 (20-mer), IKKEKVLYAWPAPAHHG | RNAse H (526–545) | >120 | >120 | >120 |
| 4286-1 (15-mer), PFRQNPDIVIYQQMD | Palm (171–185) | 119 | 97 | >240 |
| 4321-1 (14-mer), NQIEQILIKK | RNAse H (519–532) | >240 | >240 | >120 |
| 5628 (15-mer), ESILVSQIEQIKKK | RNAse H (514–528) | >120 | 60 | >120 |

Fig. 2. Effects of the RNAse H-derived peptide 4321 (ELVQIIIEQLIKKKEKYLAW) on the activities of HIV-1 IN. Increasing concentrations of RT-derived peptide 4321 were preincubated with a fixed amount of HIV-1 IN followed by assaying the IN activities and urea-PAGE analysis. The peptide concentrations are indicated at the bottom of each panel. A, analysis of the strand transfer activity of IN. B, analysis of the 3'-end processing activity of IN.
tained at a concentration of 27 μM (Fig. 3A). However, peptide 4321 was less effective, because full inhibition was not accomplished even at a high peptide concentration of 120 μM (Fig. 3B). Accordingly, the quantitative analysis showed that the apparent IC_{50} values for 4286 and 4321 were ~9.4 and 100 μM, respectively (Table I).

**HIV-1 RT and RT-derived Peptides Interact Directly with HIV-1 IN**—In order to evaluate whether the inhibitory effect of RT-derived peptides 4286 and 4321 involved physical interactions with IN, we have tested all 20-residue peptides that span the whole 560-residue-long RT molecule for their direct IN binding capacity. Because no macromolecules other than IN and the peptides (or RT) were present in these reactions, every binding result was likely to reflect direct physical interactions between these components. To this goal, we have employed a dot-blot binding assay that involved the pre-binding of HIV-1 RT, or the RT-derived peptides, to a nitrocellulose filter, followed by a reaction with HIV-1 IN. Specific anti-IN antibodies were used to detect the bound IN (see “Experimental Procedures”). The observed interaction between the whole HIV-1 RT and HIV-1 IN (Fig. 4A) supported previous findings (9–11). Out of the 57 peptides tested, only 13 have shown a significant binding. The bindings by the positive peptides and two non-binding peptides (4269 and 4300), along with their test for nonspecific bindings, are shown in Fig. 4A. As three of these peptides (4321, 4316, and 4308) showed some nonspecific binding to the secondary antibodies used in this particular experiment, we have confirmed the specificity of interaction of the inhibitory peptide, 4321 (along with a positive control of peptide 4286) with IN, by using a different set of antibodies (Fig. 4B). Among the peptides shown to bind IN (Fig. 4A and Table II), only three peptides (4286, 4321, and to a lesser extent

**FIG. 3.** The effect of peptides 4286 and 4321 on the disintegration activity of HIV-1 IN. Increasing concentrations of RT-derived peptides 4286 and 4321 were preincubated with a constant amount of HIV-1 IN, followed by assaying disintegration, using oligonucleotide D (“Experimental Procedures”). The peptides concentrations are indicated at the bottom of each panel. A, the effect of peptide 4286 on the disintegration activity of HIV-1 IN. B, the effect of peptide 4321 on the disintegration activity of HIV-1 IN.

**FIG. 4.** Dot-blot binding assay of HIV-1 RT or HIV-1 RT-derived peptides to HIV-1 IN. The experiment was conducted as described under “Experimental Procedures.” The analysis was performed for the peptides found to be positive in a preliminary binding screening (data not shown) in addition to two non-binding peptides (4269 and 4300), which serve as negative controls. The designations of the tested peptides appear either to the left or to the right of the dots. The antibodies (Ab) used and the orders of their additions are indicated. A, blot 1, binding of full-length HIV-1 RT or RT-derived peptides to HIV-1 IN. Blot 2, control binding to the antibodies with no IN present. B, blot 1, binding of RT-derived peptides 4286 and 4321 to HIV-1 IN. Blot 2, control binding to the horseradish peroxidase (HRP)-conjugated anti-His6 antibodies with no IN.
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Summary of the RT-derived peptides that bind HIV-1 IN and the inhibition data

| HIV-1 RT-derived peptides (20-mer) | Position in HIV-1 RT (residue no. and subdomain) | Level of binding to HIV-1 IN | Level of inhibition to HIV-1 IN |
|-------------------------------------|-----------------------------------------------|-----------------------------|-----------------------------|
| 4277, DFRLENKRTQDFWVEQGLGIP          | (75–94) Fingers/Palm                          | Very low                    | –                           |
| 4286, KILEPFKQNPIDIVIQYMD            | (166–185) Palm                               | High                        | ++                          |
| 4287, PD1YIVYDLDIVSGSDLEI            | (173–185) Palm                               | High                        | +                           |
| 4295, DQKLVGKLWANASIQYPIG            | (256–275) Thumb                              | Low                         | –                           |
| 4302, IAIEKQKQGGQWTVQYIQEPE          | (326–345) Connection                        | High                        | –                           |
| 4306, KQIITAYQKIKIVVESIVVYGI        | (366–385) Connection                        | High                        | ND                          |
| 4308, TPFFKLPQIKETWETWETEY           | (386–405) Connection                        | High                        | –                           |
| 4315, GYVTNRGRQKVTLTIDTNQ           | (456–475) RNase H                           | Very low                    | –                           |
| 4316, VVTTLDTTNQKTELQAIYLA           | (466–485) RNase H                           | High                        | –                           |
| 4317, KTELQAIYALQDGSLENVI           | (476–495) RNase H                           | Low                         | ND                          |
| 4319, VTDSQYALGHIQACPQDSSES         | (486–515) RNase H                           | Low                         | –                           |
| 4321, ELVNGHEQILKKREVYLLAW          | (516–535) RNase H                           | High                        | ++                          |
| 4324, EKQDLKLYSAGIRKLFDG           | (546–560) end of RNase H plus 5 residues     | High                        | –                           |

4287) also inhibited IN enzymatic activities (Tables I and II). The fact that there are additional RT-derived peptides that bind IN but do not inhibit any of the IN activities indicated that not all peptide-IN interactions necessarily led to inhibition of catalysis. Because we have assumed (see below) that the inhibition was mediated primarily by steric hindrance of the IN catalytic domain, it is likely that these non-inhibitory peptides bound IN sites that were not directly involved in catalysis.

Both HIV-1 RT and Peptide 4286 Inhibit the Disintegration Activity of HIV-1 IN-CCD—In addition to the inhibition of the 3'-end processing and forward strand transfer activities, peptide 4286 and to a lesser extent peptide 4321 were also shown to inhibit the disintegration activity of HIV-1 IN (Fig. 3). As the isolated core domain of HIV-1 IN exhibited, in vitro, a disintegration activity (2, 3, 27, 29, 30), it was possible that the two peptides interacted with IN-CCD and interfered with its activity. This recombinant segment of HIV-1 IN was 163 residues in length and was derived from residues 50–212 of the 280-residue full-length HIV-1 IN. The disintegration activity of IN-CCD was known to be substantially lower than that of the full-length enzyme (27, 30).

Because it was never tested whether the complete HIV-1 RT molecule inhibited HIV-1 IN CCD, we have first tested whether RT inhibits the disintegration activity of this IN domain. It is apparent that such an inhibition does exist (Fig. 5A) with full inhibition achieved at molar ratios of RT over IN-CCD between 2:1 and 4:1. The inhibition of IN-CCD-associated activity by peptides 4286 and 4321 was evaluated, and peptide 4286 was found to inhibit the disintegration activity of IN-CCD with an apparent IC_{50} value of about 22 μM peptide (Fig. 5B). On the other hand, peptide 4321 had no effect on this activity, even at a concentration as high as 270 μM (Fig. 5B).

HIV-1 RT and RT-derived Peptides 4286 and 4321 Physically Interact with IN-CCD—The direct interactions between HIV-1 IN-CCD and the full-length RT or either peptide 4286 or 4321 (along with peptide 4274 as a negative control) were tested. The results show specific binding of IN-CCD to the full-length RT and the two peptides tested (Fig. 6). In all, it is possible that the interactions and, consequently, inhibitions of IN activities observed with RT (or RT-derived peptides) are mediated for the most part through direct binding to the core domain of the IN.

DISCUSSION

HIV-1 IN is considered one of the major targets for the development of novel anti-HIV-1 inhibitors. Among these are antibodies (10, 31–34), mono-, di-, and oligonucleotides (25, 35–37), various natural and synthetic compounds (35, 37–40), proteins (41), and peptides. The peptides found to be active against HIV-1 IN are those derived from sequences within IN (42–45), from random sequences identified in synthetic libraries (45–47), from combinatorial libraries of chemically synthesized peptides (45, 48), or from cellular IN-interacting proteins (45, 49, 50). In contrast to many anti-RT drugs used in the treatment of AIDS patients, there is only one group of non-peptide anti-IN drugs that has entered clinical trials (39, 51), and none is used routinely so far in patients.

It was recently reported that the HIV-1 IN activities are inhibited by HIV-1 RT (12, 13). Moreover, we have proposed that this phenomenon has a critical biological significance, as it is possible that the self-destructive process of auto-integration of the viral DNA into itself is blocked in the cytoplasm of infected cells by the observed effect of RT on IN. In the present study, we have defined the precise sequences within HIV-1 RT that inhibit and interact with HIV-1 IN. We show that two peptides inhibit in vitro the enzymatic activities of IN and, in addition to few other peptides, can bind IN directly.

Two inhibitory 20-residue peptides, 4286 and 4321, derived from the RT DNA polymerase and RNase H domains, respectively, were identified in the synthetic peptides library that spans the whole pol protein of HIV-1 (Figs. 1–3 and Table I). The extent of inhibition of the strand transfer and 3'-end processing activities by both peptides are similar (Table I). On the other hand, the disintegration activity of IN is by far more sensitive to inhibition by peptide 4286 than by peptide 4321. This suggests that the mechanisms of IN inhibition by the two peptides might be different. For that reason, we have checked whether the inhibitory effects of these two peptides are coupled, namely whether their combined effect is stronger than the effect of each separately. The results of this experiment show no such an apparent augmentation (data not shown), suggesting that inhibitions by both peptides might be still interrelated.

We have previously predicted that the inhibition of IN by RT results from direct interactions between the proteins (12). To confirm this, we have conducted the dot-blot experiment, described in Fig. 4. Similar to the whole RT protein, the two peptides 4286 and 4321 interact also with HIV-1 IN. The fact that the in vitro binding assay was a direct one indicates that the observed interactions are not mediated by nucleic acids or other cofactors, further supporting our hypothesis that the inhibition of IN by RT-derived peptides is caused by physical interactions. However, the same assay also shows that non-inhibitory peptides can also bind IN. This may indicate that the regions containing the sequences of these specific peptides (in
the context of the RT molecules) also interact with IN, consequently increasing the affinity of the RT toward IN and enhancing the efficiency of the inhibition. This might also explain why the inhibitory peptides 4286 and 4321 are less efficient than the full-length RT in inhibiting IN activities. For that reason, full inhibition of the strand transfer activity of IN by the full-length RT was apparent at around equimolar ratios (12), whereas molar ratios, between IN and peptides 4286 or 4321, of −1:70 and 1:300, respectively, were required for the complete inhibition of activity (as deduced from Figs. 1A and 2A, respectively). An alternative explanation can result from the potential differences between the folding of free peptides and the structures of the same sequences in the context of the RT protein. It is likely that only a fraction of the free peptide molecules can mimic the folding within the whole protein, and only this portion is effective in inhibition. Moreover, the proper conformation that leads to inhibition by peptides 4286 and 4321 depends on the length of the peptides. It is apparent that the full 20-residue sequences of both inhibitory peptides are crucial for the inhibition of HIV-1 IN, as shorter peptides, derived from sequences of the same peptides (or peptides with partial sequence overlaps with these peptides), exhibit a substantial reduction in the inhibitory effects (Table I).

Peptide 4286 is derived from a sequence within the DNA polymerase active site of HIV-1 RT, which is located in the “palm” subdomain (as parts of α-helix E and β-sheet 9 (52)). This subdomain contains the highly conserved YXDD motif, typical of polymerases (53). This sequence exists almost entirely in peptide 4286 (except for the last D residue). This may explain our previous findings that HIV-1 and HIV-2 INs are inhibited by both HIV-1 and HIV-2 RTs and by the RT from another retroviral family, MLV RT (12).

Peptide 4321 (residues 516–535 in HIV-1 RT) is derived from the RNase H domain (across α-helix D and β-sheet 5’ (52)). The fact that this peptide interacts with IN seems not to be in line with a very recent study (54), which suggests that the free RNase H domain of HIV-1 RT (residues 422–560, p15) is not capable of binding IN. This may result from differences in the methodologies used in the two studies. We have tested the binding of merely peptides, employing the dot-blot analysis, a test confirmed by IN inhibition. In contrast, Hehl et al. (54) employed for the p15 protein fragment a pull-down experiment, which might be less sensitive than our dot-blot assay. It is also important to note that, apart from peptide 4321, we have found that five additional RNase H-derived peptides bind directly HIV-1 IN (Fig. 4A and Table II).

An additional potential aspect, relating to the inhibition of IN by the RNase H-derived peptide, can stem from the three-
Inhibition of HIV-1 Integrase by HIV-1 RT-derived Peptides

Dimensional similarities between IN-CCD and the RNase H of HIV-1 RT and Escherichia coli (55, 56). This can explain why RNase H inhibitors were found to inhibit IN activities as well (57). It is then possible that the RNase H-derived peptide 4321 interacts with IN due to similarities in structure, thereby interfering with the dimerization of IN. A similar finding (that peptides derived from IN-CCD interfere with IN dimerization and, consequently, inhibit IN activity) was already reported (42, 43).

Another issue of interest is to identify the locations of the sequences of peptides 4286 and 4321 in the surroundings of the globular structure of the heterodimeric p66/p51 RT protein. The DNA polymerase domain of the p66 subunit of RT resembles a right-hand structure, with fingers, thumb, and palm subdomain, connected to the RNase H domain by the connection (52, 58). Fig. 7 shows that both peptide segments (peptide 4286 in the p66 and p51 subunits and peptide 4321 in the RNase H domain of p66) are largely facing outwards, as they are on the surface of the protein, thus allowing potential interactions with other proteins. Yet the polymerase-derived peptide also penetrates from the “backhand” into the base of the palm subdomain (in the “forehand”), where the polymerase active site is located (Fig. 7B). This model supports the validity of the findings presented in this work about the inhibitory peptides, as only segments facing outwards of the RT can potentially interact with the IN molecule.

The DNA polymerase-derived peptide 4286 effectively interacts with IN-CCD and inhibits its disintegration activity, which is the only enzymatic activity expressed in vitro by this protein (Figs. 5 and 6). So far there are no crystal structures of the full HIV-1 IN, and most structural studies of INs involve the core domain. Therefore, we have docked peptide 4286 into the three-dimensional structure of HIV-1 IN-CCD. Among the published crystal structures of the catalytic core domain of HIV-1 IN, several represent an inactive form of the protein (55, 59), crystal form I (21). Crystal structures of the active core domain (crystal forms II and III (21, 60)) are very similar, with the exception of region 141–148. This region comprises a flexible loop, which is close to the active site. The conformation of this loop is strongly affected by the crystal packing and, presumably, by the binding of the substrate or inhibitor. By taking into account the above considerations, we have used the coordinates of HIV-1 IN-CCD from crystal form II (1.95 Å resolution) with the 141–148 region omitted (21). In the docking model (Fig. 8), we assume that the folding of peptide 4286, by which it interacts with IN, adopts a conformation similar to that one in the structure of HIV-1 RT (61). The peptide position, found in the docking solution by the flexible active site loop (residues 141–148). Within this

FIG. 7. The conformations of peptides 4286 and 4321 within the three-dimensional model of HIV-1 RT. A, space-filling model of the three-dimensional structure of the p66/p51 heterodimeric HIV-1 RT as found in Protein Data Bank entry 1FK9 (58). The structure was displayed using the “MOLMOL” program (66). The p66 subunit of HIV-1 RT is shown in yellow, and the p51 subunit is shown in red. The residues of the DNA polymerase-derived peptide 4286 and of the RNase H-derived peptide 4321 are highlighted in cyan and pink, respectively. Both peptides are located on the surface of RT molecule. The different subdomains of the DNA polymerase domain are defined in the figure. The right-hand three-dimensional structure of the DNA polymerase domain is shown from two angles. Peptide 4286 is located at the base of the palm close to the DNA polymerase active site and penetrates outwards into the backhand surface. A, front view of the back of the right-hand conformation of the RT, with the different subdomains indicated. B, after rotating the molecule, an “inside” view of the palm subdomain of the right-hand p66 RT model.

FIG. 8. Molecular docking of peptide 4286 into the core domain of HIV-1 IN. The global range molecular matching (GRAMM) program (19) was used for docking of peptide 4286 into HIV-1 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 the one found in Protein Data Bank entry 1BIS (21). The peptide conformation was as in Protein Data Bank entry 1REV (61). In gray is the space-filling diagram of IN-CCD. The three highly conserved acidic-residues catalytic triad, DX(39–58)DXE, are indicated in red-orange. HIV-1 RT-derived peptide 4286 is represented by the pink stick and ball diagram and is colored by atom types as follows: carbon, magenta; nitrogen, blue; oxygen, red; and sulfur, orange.
segment are residues Tyr-143 and Gln-148, known to be involved in viral DNA binding (62, 63). Tyr-143 was also shown to play a secondary role in catalysis (64). Hence, the binding of peptide 4286 to this region may cause a steric interference with the DNA binding and consequently inhibit IN activities. The proposed docking model (Fig. 8) involves several non-covalent interactions between the peptide and IN-CCD; residue Gln-147 of the peptide (according to the full RT numbering) makes a hydrogen bond with Ser-57 of the IN-CCD complex. The proposed model is correct. Likewise, mutagenesis of IN might inhibit IN, as part of the whole RT molecule. Future site-directed mutagenesis of RT should indicate which residues are involved in viral DNA binding (62, 63). Tyr-143 was also shown to play a secondary role in catalysis (64). Hence, the following reagents were the generous gifts of the following reagents were the generous gifts of the following reagents were the generous gifts of the following reagents were the generous gifts of Craig, N. L., Craigie, R., and Lambowitz, M., eds) pp. 613–630, American Society for Microbiology, Washington, DC, 1994.

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