Targeting HIV-1 integrase with aptamers selected against the purified RNase H domain of HIV-1 RT

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Abstract

Several in vitro strategies have been developed to selectively screen for nucleic acid sequences that bind to specific proteins. We previously used the SELEX procedure to search for aptamers against HIV-1 RNase H activity associated with reverse transcriptase (RT) and human RNase H1. Aptamers containing G-rich sequences were selected in both cases. To investigate whether the interaction with G-rich oligonucleotides (ODNs) was a characteristic of these enzymes, a second in vitro selection was performed with an isolated RNase H domain of HIV-1 RT (p15) as a target and a new DNA library. In this work we found that the second SELEX led again to the isolation of G-rich aptamers. But in contrast to the first selection, these latter ODNs were not able to inhibit the RNase H activity of either the p15 domain or the RNase H embedded in the complete RT. On the other hand, the aptamers from the first SELEX that were inhibitors of the RT-associated RNase H did not inhibit the activity of the isolated p15 domain. This suggests that the active conformation of both RNase H domains is different according to the presence or absence of the DNA polymerase domain.

HIV-1 RNase H and integrase both belong to the phosphotransferase family and share structural similarities. An interesting result was obtained when the DNA aptamers initially raised against p15 RNase H were assayed against HIV-1 integrase. In contrast to RNase H, the HIV-1 integrase was inhibited by these aptamers. Our results point out that prototype structures can be exploited to develop inhibitors of two related enzymes.

Keywords: HIV-1; RNase H; Integrase; Aptamers

1. Introduction

The best strategy for AIDS treatment involves a combination therapy using inhibitors of reverse transcriptase (RT) and protease (PR). Despite the multiple clinical benefits of this combination therapy, the emergence of HIV-1 strains resistant to these drugs and their cytotoxicity highlights the need for new anti-HIV agents as well as new strategies and viral targets. Agents able to interfere in the additional steps of viral replication, such as integration of viral DNA in the host genome catalyzed by integrase (IN), would improve antiviral potency of the treatment. Another novel enzymatic target concerns the ribonuclease H (RNase H) activity associated with retroviral RT, since a functional RNase H is essential for retroviral replication.

RT is responsible for the synthesis of proviral DNA starting from viral RNA in infected cells [1]. RT utilizes two activities for this synthesis: DNA polymerase and RNase H. RNase H specifically cleaves the RNA strand of RNA–DNA hybrids. The HIV-1 RT is a heterodimer of two polypeptides: a 66 kDa subunit and a 51 kDa subunit derived from p66 by proteolytic cleavage. The DNA polymerase domain is located in the N-terminal and the RNase H domain (corresponding to p15) is encoded in the C-terminal part of p66.

Integration of viral DNA into the cell genome is a prerequisite for retroviral replication. Integrase is another essen-

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tial enzyme in the life cycle of retroviruses, and thus it constitutes an attractive target for drugs against AIDS [2,3]. At present, only one class of authentic IN inhibitors has been identified, the diketo acids [4], but they are still not used in antiretroviral therapy.

HIV-1 integrase is composed of three domains, an N-terminal region comprising a zinc finger, the catalytic core, and a C-terminal region that binds DNA [5]. Determination of the three-dimensional structures of the catalytic core of HIV-1 IN and RNase H, as well as *Escherichia coli* RNase H and endonuclease RuvC or resolvase, led to the surprising finding that these proteins are folded in a very similar way and have comparable active site geometry [6]. Both HIV-1 RNase H and IN share a similar αβ-fold containing a central five-stranded mixed β-sheet surrounded by α-helices on both sides, which are in the same topological order. These enzymes have remarkably similar active sites with a set of three highly conserved carboxylates that are absolutely required for catalytic activity.

RNase H of human, *E. coli* and HIV-1 [7–9] are closely related. The sequences of the three enzymes can be properly aligned showing strict conservation of all amino acid residues essential for catalysis. Whereas there is only 24% sequence identity between the RNase H domain of HIV-1 RT and the *E. coli* RNase H, they adopt a very similar 3D structure. The main difference is the presence of a basic protrusion in *E. coli* RNase H, which does not exist in the HIV-1 RNase H isolated domain. The purpose of this protrusion is to correctly position the active center on the RNA–DNA hybrid. This role is probably held by the DNA polymerase domain in the complete RT.

In HIV-1 RT, the two domains DNA polymerase and RNase H are interdependent. Mutations in the DNA polymerase domain affect the RNase H activity and vice versa. A comparative in vitro study of the kinetic parameters of the RNase H activity suggests that mutations in residue 294 influence the affinity for the RNA·DNA substrate [10]. On the other hand mutations in the RNase H domain affect the initiation of DNA synthesis [11].

During this last decade, the notion of using synthetic nucleic acids as ligands to modulate the activities of proteins for therapeutic purposes has received considerable attention. Several in vitro strategies have been developed to selectively screen for nucleic acid sequences that bind to specific proteins, including proteins that do not naturally interact with nucleic acids.

The SELEX procedure was previously used to search for aptamers against the HIV-1 RNase H activity associated with RT [12] and the human RNase H1 [13]. Although each selection started from different DNA libraries, aptamers containing G-rich sequences were selected in both cases. Interestingly, DNA aptamers that were initially raised against RNase H were able to inhibit HIV-1 integrase [14]. Some of these inhibitory G-rich aptamers were found to be folded in G-quartet structures [15]. Jing et al. [16] reported that oligonucleotides (ODNs) composed entirely of deoxyguanosine and thymidine were able to inhibit HIV-1 integrase. These G-rich ODNs formed highly stable intramolecular four-stranded DNA structures containing two stacked guanosine-quartets. A different situation was found with G-rich ODNs able to inhibit human topoisomerase 1. In that case only the G-rich domain and not the G-quartet structure was responsible for the inhibition [17].

Conversely, not all polymerase inhibitors identified by SELEX are necessarily able to form G-quartet structures or present G-rich regions. Tuerk et al. [18] isolated high affinity ligands to HIV-1 RT that presented no G-rich regions. These aptamers formed a pseudoknot secondary structure and were inhibitors of the DNA polymerase activity associated with the RT. Other examples of aptamers which do not present G-rich sequences have been selected against HIV-1 RT [19], thermostable DNA polymerases [20], Qβ replicase [21] or the hepatitis C virus RNA-dependent RNA polymerase [22].

The ability of RNase H and integrase to recognize G-rich sequences and G-quartet structures could be a property of these related enzymes. To investigate this hypothesis, an in vitro selection using the isolated RNase H p15 domain (termed p15) of HIV-1 RT as target was performed. Comparison of the newly selected aptamers with the anti-RNase H molecules previously described highlights the interdependence of the RNase H and DNA polymerase domains.

2. Materials and methods

2.1. Materials

The purified yeast AD13-His protein was a gift from Benoît Pinson (IBGC-CNRS 5095, Bordeaux, France). Oligodeoxynucleotides were purchased from MWG Biotech.

2.2. Methods

2.2.1. Selection of aptamers

A SELEX approach was used to select oligodeoxynucleotides with affinity for the isolated RNase H domain p15. A library of single-stranded DNA containing 35 random nucleotides flanked by invariant regions of 25 and 21 nt was used as starting material. ODN1 (5′-GCC TGT TGT GAG CCT CCT GTC GAA-3′) was used as the first primer for PCR amplification. The sequence of the second primer ODN2 was: 5′-GGG AGA CAA GAA TAA ACG CTC AA-3′. The random ssDNA library was dissolved in 20 mM Tris pH 8.0, 6 mM $MgCl_2$, 200 mM KCl (binding buffer) at 5 μM concentration and an aliquot of 500 μl was incubated in the presence of HAWP filters (Millipore) for 30 min at 37 °C. The unbound ssDNA was then incubated with 0.5 μM p15 for 30 min at 37 °C. After filtration, the ODNs bound to the filter were eluted by incubation for 20 min at 37 °C in 300 μl of 7 M urea and 300 μl of phenol. ODNs were precipitated with ethanol and after dissolution in water, they were subjected to two PCR amplification steps. In the first one, PCR was performed with

primers 1 and 2 corresponding to the constant regions of the ssDNA library by using the Goldstar Red DNA polymerase (Eurogentec). In the second one, only primer 1 was used to allow the regeneration of single-stranded DNA. The concentration of ODNs and RNase H p15 (5 and 0.5 μM, respectively, in the first round) was progressively decreased to reach 100 nM ODNs and 1 nM p15 in the last round (round 6). Before rounds 5 and 6, a counter selection with 0.5 μM of AD13-His (an unrelated protein containing a his tail) was performed to eliminate ODNs which could have bound to the histidine tag. Following the last round of selection, ODNs were amplified using primers 1 and 2 and ligated to the pGEM®-T-easy vector system (Promega). After cloning in *E. coli* DH5α, DNAs were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Kit.

2.2.2. Labeling of oligonucleotides

The DNA aptamers (10 pmol) were 5′-end labeled with 50 μCi [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol) and T4 polynucleotidyl kinase by incubation in a total volume of 10 μl for 30 min at 37 °C, according to the manufacturer's instructions (Promega). Unincorporated [γ-^{32}P]ATP was removed by G25 Sephadex spin column (Amersham, Pharmacia Biotech).

2.2.3. Enzyme purification

i) HIV-1 RNase H p15. The plasmid pET-NY427 was used for expression and purification of the isolated RNase H domain [23]. This construct contains the DNA sequence encoding for amino acids 427–560 of HIV-1 RT (HXB2) and a tag of six histidine residues at the N-terminus.

ii) HIV-1 integrase. The enzyme was purified as described previously [24].

2.2.4. Enzymatic assays

a) RNase H activity. The RNA–DNA hybrid substrate was prepared as follows. Calf thymus DNA (10 μg) (Sigma) was incubated in 50 mM Tris–HCl pH 7.8, 5 mM DTT, 5 mM Mg^{2+}, 100 mM KCl, 500 μM ATP, GTP, CTP each, 30 μCi [^{3}H]UTP (31 Ci/mmol) and 1 unit of *E. coli* RNA polymerase for 1 h at 37 °C (final volume 50 μl). The RNase H activity was measured in the presence of 50 mM Tris–HCl pH 6.8, 6 mM of either $MgCl_2$ or Mn^{2+}, 25 mM KCl, and the RNA–DNA hybrid (20,000 cpm), for 15 min at 37 °C in 50 μl final volume. The reaction was stopped by addition of 1 ml cold 10% TCA. Samples were filtered on nitrocellulose filters, washed and the radioactivity was determined with a scintillation counter.

b) Integrase activity. The 3′-end processing reaction was assayed with a double-stranded ODN, containing sequences derived from the U_5 end of the HIV-1 LTR [25]. The 21-mer duplex DNA was formed by annealing the 5′ labeled ODN 1 (5′-GTG TGG AAA ATC TCT AGC AGT-3′) to ODN 2 (5′-ACT GCT AGA GAT TTT CCA CAC-3′). The reaction mixture contained 20 mM HEPES pH 7.5, 10 mM DTT, 7.5 mM $MnCl_2$, 10 mM NaCl, 0.05% Nonidet-P 40, 300 nM IN and 40 nM of 5′ labeled substrate in a final volume of 20 μl. The reaction mixture was incubated at 37 °C for different lengths of time and stopped by adding 5 μl of loading buffer (95% formamide, 20 mM EDTA, 0.005% bromophenol blue) followed by heating at 90 °C for 5 min. The reaction products were subjected to electrophoresis in 12% polyacrylamide–7 M urea gels in Tris borate EDTA (TBE) pH 7.6 and autoradiographied. For inhibition tests, preincubation for 20 min at 37 °C of IN with different concentrations of ODNs was performed.

Strand transfer reaction was performed as described for the processing reaction. The DNA duplex was formed by annealing the 5′ labeled ODN 3 (5′-GTG TGG AAA ATC TCT AGC A-3′) to ODN 2.

2.2.5. Ultraviolet crosslinking experiments

Enzymes (RNase H p15 or integrase) were incubated with ^{32}P-ODNs (10,000 cpm) in 50 mM Tris pH 7.9, 6 mM Mg^{2+} for 10 min at 37 °C in 15 μl final volume. Samples were then irradiated for 10 min at room temperature with a UV transilluminator (254 nM, 2.4 mW/cm^2) [26] and analyzed on 16% SDS-polyacrylamide gels.

2.2.6. Competitive binding assay

Integrase was incubated in the presence of varying concentrations of aptamers for 10 min at 37 °C. Buffer conditions were the same as those for activity tests. ^{32}P-labeled substrate (40 nM final) was added for 15 min at 37 °C. After addition of 1 ml saturation buffer (20 mM Hepes pH 7.6, 10 mM $MnCl_2$, 10 mM NaCl, 100 μg/ml calf thymus DNA), samples were filtered on nitrocellulose filters, and then washed twice with the saturation buffer. Filters were dried and radioactivity was determined using a Wallac scintillation counter.

3. Results

3.1. Selection of aptamers that bind to the RNase H p15 domain

We previously isolated aptamers able to inhibit specifically the RNase H activity associated with the HIV-1 RT [12]. A sequential selection of ODNs was performed using two recombinant forms of the HIV-1 RT as protein ligands: i) the p51/p51 homodimer, which is devoid of the RNase H region and should be able to select ODNs having a high affinity for the DNA polymerase domain; ii) the native recombinant form of the p66/p51 RT in order to isolate those aptamers which have not been able to previously form a stable complex with the p51/p51 truncated homodimer. Various DNA aptamers with a high affinity for the RNase H domain of HIV-1 RT were isolated. They were G-rich oligonucleotides. Most of the selected ODNs were able to inhibit in vitro the HIV-1 RNase H activity, while no effect was observed on human RNase H [12].

Starting from the anti RNase H aptamers we designed shorter oligonucleotides that were able to bind to the HIV-

Fig. 1. Sequences of selected aptamers.
Clones are numbered on the left. G boxes are in red.

1 integrase and which were highly efficient inhibitors of this enzyme [14]. These results led us to investigate whether integrase and RNase H have a particular affinity for G-rich DNA sequences. To develop this study further we utilized an active RNase H domain (termed p15) of RT [23] to select ODNs with affinity for this domain. The p15 was purified from *E. coli* as described in Section 2. After purification, it exhibited robust RNase H activity in the presence of $MnCl_2$. No activity was detected with $MgCl_2$ the preferred cation of the RNase H activity associated with the complete HIV-1 RT. No contamination by the possible presence of *E. coli* RNase H could be detected (assays performed with $MgCl_2$). Using the purified RNase H p15 as a target we performed a new selection starting with a different DNA library to the previous one. In order to ascertain that the new library did not present any bias, cloning and sequencing of the library was performed: 80% of the sequences contained only one or two boxes of two G residues. As the p15 used in the selection procedure contained an N-terminal 6 His-tag, ODNs able to bind a 6-His sequence were eliminated by pre-incubation with an unrelated His-tagged protein (the yeast AD13 protein described in Section 2.1). To increase the stringency of the selection, the p15 concentration and the ODN/p15 ratio were gradually modified from round 1–6. We started with 0.5 μM p15 and a 10/1 ODN/p15 ratio at the first round to reach 1 nM p15 and a 100/1 ODN/p15 ratio at round 6. The PCR-derived products from round 6 were cloned and the nucleotide sequence of 40 clones was determined. One third of these sequences presented several boxes of two or three G (Fig. 1).

3.2. Interaction of ODNs with p15 RNase H, RNase H-RT, and IN

In order to determine whether these G-rich ODNs were able to bind the p15 protein, UV crosslinking experiments were performed. In parallel, interaction with the complete RT (containing the RNase H and DNA polymerase domains) and HIV-1 integrase (because of the structural similarities with RNase H) was investigated. We chose the UV crosslinking technique to assay the three enzymes simultaneously, since other binding experimental approaches such as gel shift or Biacore are extremely difficult to perform with integrase, due to this enzyme's capacity to aggregate.

Various (^{32}P)-labeled aptamers (12, 21 and 60) were incubated in the presence of each enzyme and then irradiated with UV (Fig. 2). In each case, the migration of free labeled aptamers revealed two major bands (lanes 1, 5 and 9). In the presence of p15 RNase H (lanes 4, 8 and 12), RT (lanes 3, 7 and 11) and IN (lanes 2, 6 and 10) additional bands corresponding to crosslinking products appeared that suggest the interaction of the enzymes with the aptamers.

In the case of p15, the retarded band was weaker with aptamer 60 than with the other two. Moreover, the protein-shifted bands (lane 4 on the one hand, lanes 8 and 12 on the other hand) do not always migrate at the same position, suggesting that perhaps more than one 12-aptamer is bound to the p15 enzyme in lane 4.

These results suggest that the isolated RNase H domain interacts with G-rich sequences as was observed before in our previous selection against the RNase H domain of the complete RT.

3.3. Effect of aptamers on the RNase H activity

Since G-rich aptamers against the p15 domain were selected on the basis of their affinity for the protein, we sought whether these aptamers could affect the RNase H activity. As mentioned above we already selected aptamers against the HIV-1 RNase H associated with RT that proved to be inhibitors of the activity [12]. The aptamers selected against the RNase H p15 in the present study gave an unexpected result. Instead of an inhibition we observed a stimulation of the RNase H activity at all concentrations of aptamers tested (Fig. 3). A maximum 2.5-fold stimulation was obtained with

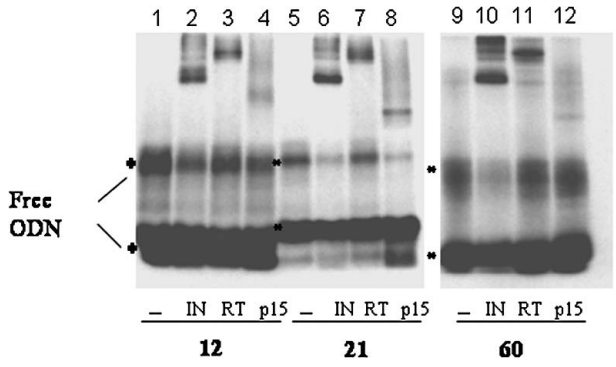

Fig. 2. Crosslinks of integrase, RT and p15 with anti-p15 aptamers.
The 5′-end-labeled ODNs were incubated in the absence (–) or presence of IN, RT or RNase H p15, followed by UV crosslinking. Samples were analyzed on SDS-PAGE followed by autoradiography. Results with aptamers 12 (lanes 1–4), 21 (lanes 5–8) and 60 (lanes 9–12) are shown. Asterisks represent the migration of free ODNs.

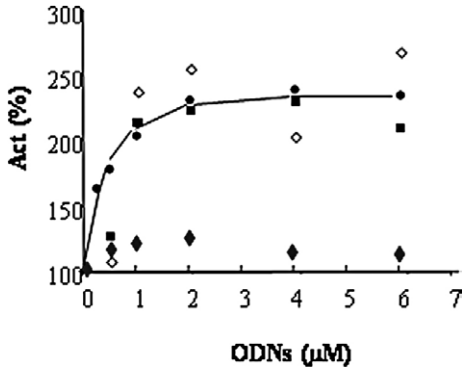

Fig. 3. Effect of aptamers on the RNase H p15 activity.
The RNase H assay was performed as described in Section 2.2. RNase H p15 was preincubated with aptamers for 15 min at 37 °C. Then the labeled RNA–DNA substrate was added and the reaction proceeded for 15 min. ●: ODN 12; ◇: ODN 60; ■: ODN 21; ♦: 93. The RNase H activity without aptamer is taken as 100%.

concentrations higher than 2 µM. The activation of the RNase H activity suggests that conformational changes are induced upon binding of the aptamers. No effect was observed on the RNase H activity of the complete RT under these conditions (data not shown).

We then investigated the effect of aptamer 93 from our previous selection run against the RNase H of the complete RT [12]. While ODN 93 inhibited the RNase H activity of RT, no inhibition of the p15 was observed (Fig. 3). In fact, a minor stimulation was observed that never exceeded more than 10–20% activation.

The results obtained from these cross reactions suggest that aptamers from both selections probably do not bind the same site on the enzymes. Aptamer 93 may bind to the active site of the RNase H-RT enzyme as suggested by its inhibitory effect. In contrast, aptamers raised against p15 might bind outside the active site, inducing a conformational change that increases enzymatic activity. Moreover, the fact that aptamer 93 does not inhibit p15 RNase H suggests that the conformation of the isolated domain is different from the RNase H domain embedded in the RT. This highlights the role of the DNA polymerase domain in the RNase H structure, and as a consequence, its activity.

3.4. Design of ODN 60del

The aptamers previously selected against the RNase H associated with RT were strong inhibitors of this activity [12]. The ODN 93 contained a cluster of five G residues at the 5′-end that were shown to be essential for inhibition. On the basis of mutation experiments and structure prediction, we had shortened the ODN 93 into a 16-mer termed 93del, corresponding to the G-rich region of ODN 93 and the 2 G boxes (Fig. 4A). ODN 93del showed only 30% inhibition of the RNase H activity in RT observed with ODN 93 [14]. Probably other residues were implicated in RNase H inhibition.

As mentioned above, structure and function homologies have been observed between the catalytic core of HIV-

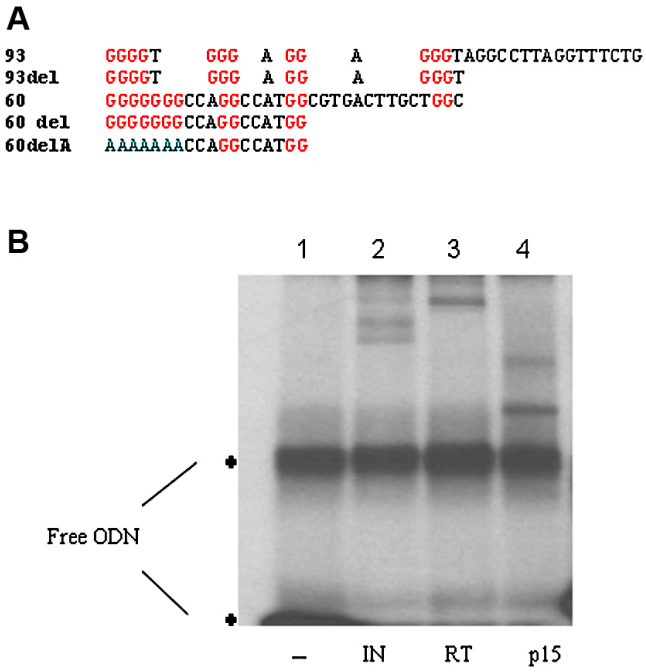

Fig. 4. Crosslink between ODN 60del and IN, RT and p15.
A. Sequences of ODNs 93, 93del, 60, 60del and 60delA. B. The (^{32}P) 5′-end-labeled aptamer 60del was incubated in the absence (–) or in the presence of IN, RT, or p15, followed by UV irradiation. Asterisks show the migration of the free ODN.

1 integrase and the RNase H domain of HIV-1 RT. As described in our previous work, ODN 93del which showed only 30% of the inhibition of RNase H obtained with ODN 93, was a very strong inhibitor of IN [14]. The sequences of anti-p15 aptamers (this work) were compared with the sequences of anti-RNase H aptamers obtained in our previous work [12]. Several ODNs of the new selection contained a G cluster sequence; particularly interesting was ODN 60 which contained a G cluster at the 5′-end and three boxes of two G in the variable sequence (Fig. 4A). We therefore synthesized an oligonucleotide composed of the 18 nucleotides at the 5′-end of ODN 60, keeping the G cluster and the two first G boxes. This ODN was termed 60del. The efficiency of ODN 60del to bind RNase H, RT and integrase was then investigated (Fig. 4B). In the absence of enzymes, the free ODN 60del shows two bands (lane 1). The 60del incubated with the enzymes gave the same bands (Fig. 4B, lanes 2–4) as those obtained with the aptamer 60 (see Fig. 2).

To ascertain the significance of the G cluster in the 60del the first seven G residues were changed into A residues. This mutated ODN was termed ODN 60delA (Fig. 4A). 60delA was not crosslinked to p15 RNase H and to integrase when assayed under the same conditions reported in Fig. 4B (data not shown). These results strongly suggest the importance of some of the 5′-end G residues for the interaction with the three proteins.

On the other hand, no crosslinking was observed when the p15 was incubated in the presence of two ODNs known for

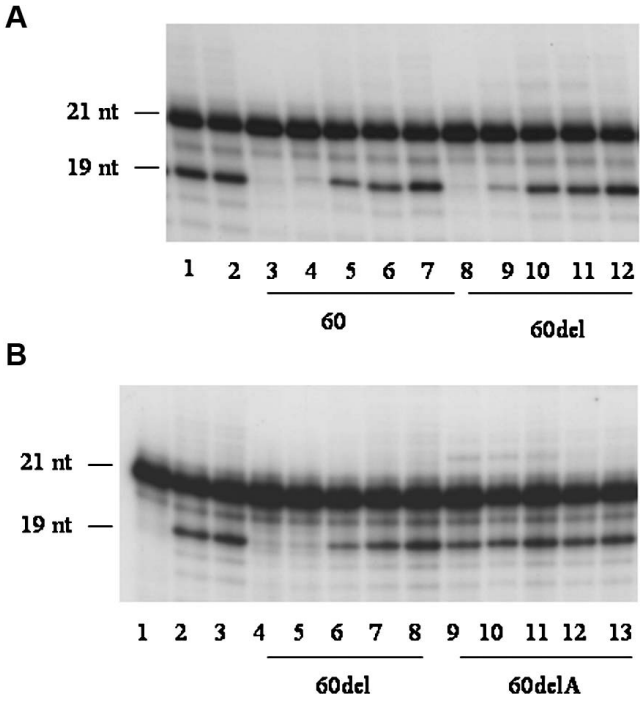

Fig. 5. Effect of aptamers on the 3′-end processing activity.
Integrase was preincubated with ODNs for 10 min at 37 °C. The reaction mixture containing the processing substrate was then added and the reaction proceeded for 1 h. Samples were analyzed on 12% acrylamide/7 M urea gel. **A**. Effect of ODN 60 and 60del. Lane 1 and 2: reaction performed in the presence of integrase; lanes 3–7: processing in the presence of decreasing concentrations of ODN 60 (1000, 500, 200, 100, 20 nM); lanes 8–12: idem with ODN 60del. **B**. Effect of ODNs 60del and 60delA. Lane 1: substrate alone; lanes 2 and 3: in the presence of integrase; lanes 4–8: processing in the presence of decreasing concentrations of ODN 60del (1000, 500, 200, 100, 20 nM); lanes 9–13: decreasing concentrations of ODN 60delA (1000, 500, 200, 100, 20 nM). Processing substrate (21 nt) and processing product (19 nt) are indicated.

strongly binding integrase: the ODN 93del or the T30923 IN inhibitor [16] (not shown).

3.5. Effect of the aptamer 60 and its derivatives on integrase activity

According to the structural similarities described between RNase H and integrase [6] we assayed the effect of the aptamers anti p15 on integrase activity. The ODNs were incubated in the presence of integrase under the conditions used for the 3′-end processing. ODN60 was a good inhibitor of integrase with complete inhibition at 500 nM (Fig. 5A). ODN 60del was almost as potent an inhibitor as ODN 60. In this case residual activity can still be observed at 500 nM. Under the same conditions, ODN 60delA has practically no effect on the processing activity (Fig. 5B), indicating that the 5′-end G residues (some or all of them) are important for the interaction with integrase.

Next, we determined the effect of the aptamers on the strand transfer activity of IN. As for the 3′ processing, ODN 60 and 60del inhibited the strand transfer reaction catalyzed by HIV-1 integrase (Fig. 6), although ODN 60del (lanes 9–13)

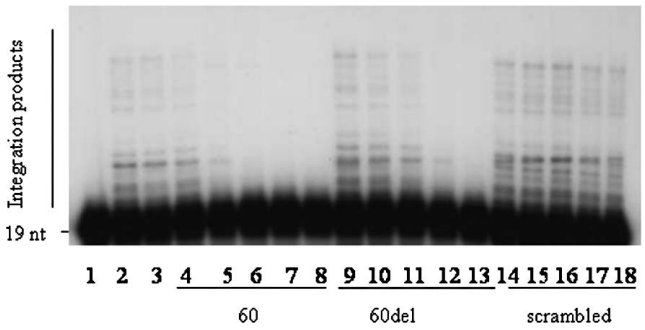

Fig. 6. Effect of aptamers on strand transfer activity.
The reaction was performed as described in Fig. 5, but the strand transfer substrate was used instead of the processing substrate. Lane 1: substrate alone (19 nt); lanes 2 and 3: in the presence of integrase; lanes 4–8: strand transfer reaction in the presence of increasing concentrations of ODN 60 (10, 50, 100, 250, 500 nM); lanes 9–13: increasing concentrations of ODN 60del (10, 50, 100, 250, 500 nM); lanes 14–18: increasing concentrations of scrambled ODN 60del (10, 50, 100, 250, 500 nM). The sequence of scrambled 60del is: 5′ GCGTGCGGGCGGCGAGAG 3′.

was slightly less active as an inhibitor than ODN 60 (lanes 4–8). The effect of a scrambled version of the ODN 60del was also assayed in this reaction. As expected, no inhibition was found with this ODN (Fig. 6, lanes 14–18).

In order to determine the mechanism of integrase inhibition by ODNs, competition experiments with the substrate were performed. Integrase was preincubated with the ODNs followed by addition of the processing substrate and, after filtration, the remaining radiolabeled substrate was determined. Fig. 7 shows the relative ability of each ODN to compete for the substrate-binding site. When comparing these results with those of inhibition, there seems to be a good correlation between the capacity to inhibit integrase (Fig. 5) and the ability of ODNs to compete for the binding of the processing substrate, with the most active inhibitor, ODN60, being the most effective competitor (Fig. 7). ODN 60del showed a competition level similar to ODN 60, while com-

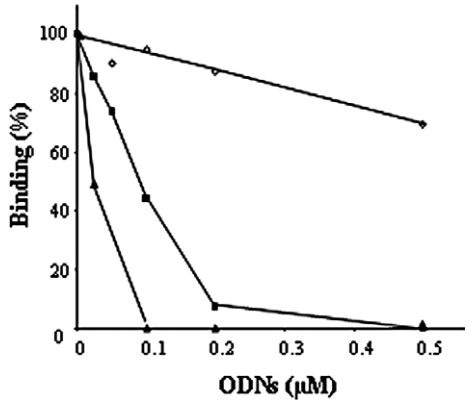

Fig. 7. Competitive binding experiments.
Integrase was incubated at 37 °C for 10 min with different concentrations of aptamers before adding the (^{32}P-labeled) processing substrate for 15 min at 37 °C. Samples were then filtered and the retained radioactivity was counted. ▲: ODN60; ■: ODN 60del; ◇: ODN 60delA.

petition was practically abolished when the G cluster was mutated in A residues in ODN 60delA.

4. Discussion

An in vitro selection performed against the isolated p15 domain of HIV-1 RNase H identified ODNs containing G-rich sequences that were able to bind to the protein. In contrast to aptamers raised against the RNase H embedded in the complete RT [12], anti-p15 aptamers were not inhibitors of the RNase H activity neither of the isolated domain nor of the RNase H-RT. Moreover, aptamers isolated against the RNase H of the complete RT do not inhibit the p15 activity. These results suggest that the active conformation of both RNase H domains is different according to the presence or absence of the DNA polymerase domain. Indeed, there is a large degree of interdependence between the DNA polymerase and RNase H domains of RT. The RNase H and DNA polymerase active sites share a single substrate-binding cleft formed by the DNA polymerase domain. In the case of the isolated RNase H domain the activity is quite low when compared to RNase H-RT. This could be attributed to the absence of the binding site on the polymerase domain. On the other hand, it has been reported that the activity of the isolated RNase H domain was dependent on the histidine tag at the N-terminus [23]. The histidine tag could play a role either in substrate-binding or in stabilization of the structure. Recently a domain consisting of residues 425–560 has been shown to be active in the absence of a His tag.

Interestingly, the incubation of p15 with the aptamers stimulated the p15 RNase H activity. Previous studies have shown that the RT activity can be modulated by nucleic acids. HIV-1 RT is a flexible enzyme and significant conformational changes occur upon primer binding [27]. The binding of primer/template stabilizes the dimeric form of the enzyme [28]. We have previously shown that the binding of the tRNA primer induces structural changes in the RT and that these interactions stimulate DNA polymerase and RNase H activities [29]. In addition to the effect produced by tRNA, synthetic ODNs were also able to stimulate the catalytic activity of RT [30]. All these results indicate that certain ODNs can induce a conformational change of HIV-1 RT which results in a higher specific activity. In spite of the fact that no inhibition of p15 was observed, the stimulation of enzymatic activity induced in the presence of aptamers confirms the interaction of p15 and the aptamers visualized by UV crosslinking. It can be hypothesized that aptamer binding could help to stabilize the structure of the isolated domain.

HIV-1 IN is a member of a family of polynucleotidyl transferase enzymes that share certain functional aspects and topology. The family also includes other retroviral integrases, bacteriophage Mu transposase, RuvC, *E. coli* RNase H and the closely related RNase H domain of HIV-1 RT [6]. To study the relationship between HIV-1 integrase and RNase H the effect of the aptamers selected against the RNase H p15 domain was assayed against IN. The ODNs were able to bind to IN and to inhibit its enzymatic activities. ODN 60 that has a G cluster at the 5′-end was studied in more detail, since an aptamer with a similar sequence at that end was identified as a very good inhibitor in our previous selection. Contrary to the effect on RNase H, ODN 60 inhibited the integrase activity. A truncated version of ODN60 still containing the G-rich region was also able to inhibit IN, while this effect was abolished when the G-rich region was transformed in an A-rich region, or when a scrambled version of the inhibitory ODN was used.

A previous selection run against human RNase H also led to the selection of G-rich sequences [13]. One of them was inhibitory for the RNase H activity and was folded in a G-quartet structure. Formation of G-quartet structures seems a pre-requisite for G-rich ODNs to inhibit integrase [16]. Moreover, the effect of G-quartets and various G-rich ODNs has been assayed on human nuclear DNA topoisomerase I. The results demonstrated that either DNA or RNA G-rich or G-quartet-forming ODNs can bind to topoisomerase and prevent cleavage of duplex DNA [17]. On the other hand, topoisomerase I is known to bind to preformed G-quartet structures and is also able to promote the formation of G-quartets with oligonucleotides containing the polypurine tract (PPT) of the HIV-1 viral sequence [31].

G-quartet structures often interact with enzymes having a nuclease activity. Integrase is an endonuclease that cleaves the cellular DNA to catalyze the integration of viral DNA; RNase H degrades the RNA strand in a hybrid RNA/DNA; topoisomerase controls the topologic state of DNA by transient cleavage of one or two strands of the double helix. An RNase activity has been described for the vaccinia topoisomerase I [32]. Moreover, the G-quartet DNAs are more resistant than other DNAs to nucleases and especially to endonucleases [16,33]. Surprisingly, they are resistant to a cleavage made by the enzyme they bind. For instance, a G-quartet DNA that binds to topoisomerase is not a substrate for this enzyme.

Although the in vivo existence of G-quadruplex structures has not yet been proven, several regions of the human genome with the potential to form such a structure have been described: telomeres [34], immunoglobulin switch region [35], the c-myc promoter [36], and the fragile-X-syndrome triplet repeats [37]. Conserved G-rich sequences are also found in retroviruses (PPT of HIV-1) [38]. G4 DNA resolvase, a DNase specific of G-quartet structures has been described in placenta [39]. This might be a beginning of proof for the existence of G-quartet structures in vivo. The ability of the yeast *KEM1/SEP1* gene to cleave DNA in a G4-DNA-dependent fashion has suggested a crucial role for this structure in meiotic synapsis and recombination [40]. Thus, G-quartets may have a role, through the interaction with proteins, in biological events such as telomere maintenance, promoter activation and immunoglobulin gene rearrangements.

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