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Antiviral Research 46 (2000) 125–134

Antiviral Research

www.elsevier.com/locate/antiviral

Targeted cleavage of HIV-1 coreceptor-CXCR-4 by RNA-cleaving DNA-enzyme: inhibition of coreceptor function

Shrabani Basu, Bandi Sriram, Ritu Goila, Akhil C. Banerjea *

Laboratory of Virology, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110067, India

Received 29 September 1999; accepted 9 February 2000

Abstract

HIV needs the chemokine receptors (HIV-1 coreceptors) to initiate infection and gain entry into a susceptible cell. CCR5 receptor is used by macrophage tropic viruses to establish infection, and CXCR-4 is used by T lymphocyte tropic virus which are usually found at the terminal stages of the disease. These chemokine receptors are, therefore, attractive targets to interfere with the entry as well as spread of HIV-1 in the host. As our antiviral approach, we have earlier assembled a DNA-enzyme-916 against CCR5 (Goila and Banerjea, 1998). We have now designed against the CXCR-4 gene a mono-DNA-enzyme, which showed sequence specific cleavage activity. When CXCR-4-DNA-enzyme was placed in tandem with CCR5-DNA-enzyme, specific cleavage of their respective target sites were observed using a 60 bases long synthetic target RNA which possessed the target sites for both the DNA-enzymes. The cleavage by the CXCR-4 DNA-enzyme was found to be significantly more efficient than by the CCR5-DNA-enzyme. Analyses of the cleaved fragments by mono- and di-DNA-enzyme indicated strongly that hybridization of the CCR5-DNA-enzyme with its cognate target RNA, actually facilitated the cleavage by the CXCR-4 DNA-enzyme. Furthermore, the di-DNA-enzyme was able to cleave the substrate RNA to completion. These DNA-enzymes, when introduced into a mammalian cell line expressing the appropriate chemokine receptor, interfered specifically with the HIV-1 coreceptor functions. Using this strategy, it may be possible to interfere with the infection and spread of R5 as well as X4 viruses.

Keywords: DNA-enzymes; Catalytic DNA; CXCR-4; CCR5; HIV-1 coreceptors; Chemokine receptors

1. Introduction

It is now known that R5 virus predominantly uses the chemokine receptor-CCR5 (HIV-1 coreceptor) and is involved mainly in establishing a new infection and transmission. These viruses are macrophage-tropic. At the later stages of the dis-

Abbreviations: Dz, DNA-enzyme.

* Corresponding author. Tel.: +91-11-6103008; fax: +91-11-6162125.

E-mail address: akhil@nii.res.in (A.C. Banerjea)

0166-3542/00/$ - see front matter
PII: S0166-3542(00)00075-9

ease, one usually finds virus that can use other coreceptors, particularly among them is the CXCR-4 (for recent review see Berger et al., 1999). The appearance of T-cell line-tropic syncytium-inducing (SI) isolates is often associated with the rapid decline of CD4+ cells and speedy progression of the disease. These chemokine receptors play an important role in HIV-1 transmission. Individuals who possess a homozygous deletion of 32 base-pair segment corresponding to the second extracellular loop of the 7-transmembrane G-coupled protein, are usually resistant to HIV-1 infection (Liu et al., 1986; Dean et al., 1996; Huang et al., 1996; Samson et al., 1996), and even heterozygous individuals (for the Δ32 allele of CCR5) show partial protection (Huang et al., 1996). This protection is not absolute (Biti et al., 1997; O'Brien et al., 1997; Theodorou et al., 1997), as CXCR-4 using viruses (X4) have been found in individuals who were homozygous for the Δ32 allele (Nelson et al., 1998). Therefore, it is essential that an antiviral approach based on coreceptors only, should also be directed against CXCR-4 coreceptors. Although a variety of chemokine receptors have been identified that act as HIV-1 coreceptors under in vitro conditions, only the above two coreceptors, namely CCR5 and CXCR-4, have been implicated to have an important physiological role in the viral spread (Zhang and Moore, 1999).

The Δ32 mutation in Caucasian people of European heritage is quite common but extremely rare in Asian and African countries (Huang et al., 1996). We recently reported the presence of this mutation from India (Husain et al., 1998a) and established the complete inheritance pattern (Husain et al., 1998b). This deletion results in the formation of severely truncated protein which results in the loss of both the signal transduction and HIV coreceptor function. Even with this kind of homozygous deletion, people were found to be leading an healthy life. The major reason for this is the redundancy of chemokine receptors that can use overlapping ligands. It is, for this reason, that we had earlier described the function of a hammer-head ribozyme and a DNA-enzyme against the CCR5 gene and showed its intracellular efficacy (Goila and Banerjea, 1998). These general purpose RNA cleaving DNA-enzymes were described by Santoro and Joyce (1997) that were functional under simulated physiological conditions and two kinds of catalytic motifs, 10–23 and 8–17, were identified. We have earlier reported extremely efficient cleavage of HIV-1 envelope RNA by a DNA-enzyme and demonstrated its ability to interfere functionally with the expression of HIV-1 envelope gene (Dash et al., 1998).

In the present study we describe, in detail, the sequence specific cleavage activities of a CXCR-4 DNA-enzyme. We also show that by combining the two DNA-enzymes, directed against CCR5 and CXCR-4, respectively, it is possible to cleave both CCR5 and CXCR-4 RNA and that they can interfere with the coreceptor function when introduced into a mammalian cell. Potential applications of such an approach are discussed.

2. Materials and methods

2.1. Construction of mono- and di-DNA-enzymes

Twenty nine nucleotide long DNA was synthesized in a DNA synthesizer (Applied Biosystems). The target sites in the substrate RNA and the sequence of the engineered DNA-enzyme specifically hybridizing with the target RNA is shown in Fig. 1, panel A. Briefly, seven nucleotides on either side were made complementary to the target RNA sequence, the A nucleotide was left unpaired and the cleavage was predicted to occur after the A nucleotide. The earlier identified 10–23 catalytic motif (Santoro and Joyce, 1997) was placed in between the antisense flanks which possessed the following sequence: 5′ GGCTAGCTACAACGA. A di-DNA-enzyme was synthesized by placing the two DNA-enzymes in tandem, as shown in Fig. 1, panel B.

2.2. Cloning of CXCR-4-CCR5 synthetic DNA for synthesis of target RNA

Chimeric synthetic substrate was synthesized in a manner that 30 nucleotides from the CXCR-4 gene that contained AUG at nucleotide position

337 (Loetscher et al., 1994) was fused precisely with the 28 nucleotides from the CCR5 gene that possessed AUG at nucleotide position 916, as described before (Goila and Banerjea, 1998) (see Fig. 1). The strategy for cloning this 60 nucleotide long CXCR-4-CCR5 chimeric substrate gene was essentially the same as described before (Goila and Banerjea, 1998). Briefly, a 60 nucleotide long DNA was synthesized in a DNA synthesizer. The two terminal primers (sense and antisense) were designed to amplify the 60-base pair fragment by carrying out the polymerase chain reaction using the conditions described earlier (Husain et al., 1998a). The following primers were synthesized:

(1) CXCR-4-CCR5-oligonucleotide:
5′GCAAGGCAGUCC**AUG**UCAUCAACACAGU CAUUCGGUGUCGAA**AUG**AGAAGAGGGC-ACAGG (the two AUG target sites are shown in bold letters).
(2) 5′-terminal sense: GCAAGGCAGTCCATG-TCATC
(3) 5′-terminal antisense: CCTGTGCCTCTTCT-TC

The PCR amplified fragment was cloned into a T-tailed expression vector (pGEM-T-Easy, Promega Biotech., WI) using standard recombinant techniques. The recombinant clones were screened for the insert by *Eco*R1 digestion as these sites are present on either site of the cloned gene. Recombinant plasmid clone was grown to large scale and purified on a Qiagen column (Qiagen, Gmbh, Hilden, Germany) before subjecting it to sequencing, using primers provided by the se-

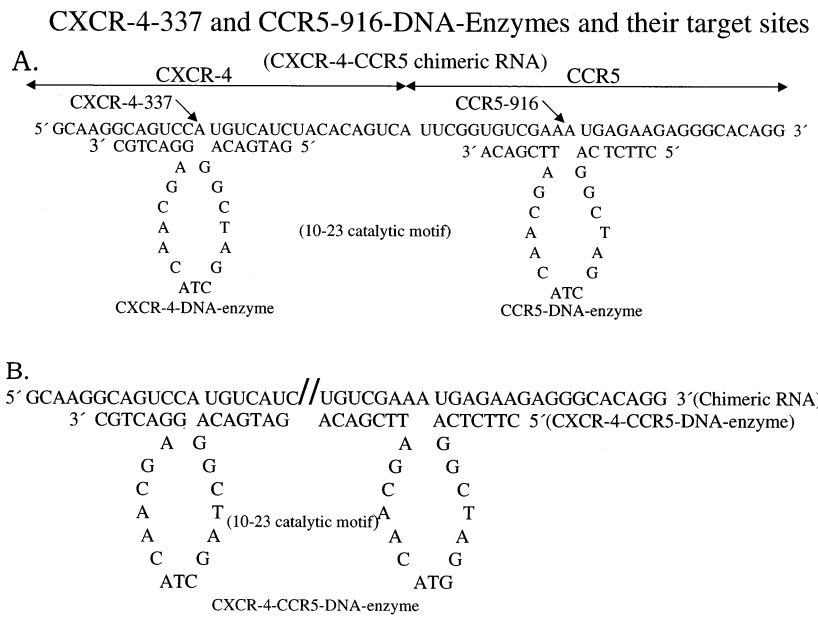

Fig. 1. (A) A 30 base segment specific for CXCR-4 was precisely fused with 30 bases of CCR5 gene to yield a synthetic substrate of 60 bases. The AUGs that have been targeted are shown by arrow and the position of the A nucleotide is 337 (Loetscher et al., 1994) and 916 (Goila and Banerjea, 1998) which are the target sites for mono-DNA-enzymes for CXCR-4 and CCR5, respectively. The cleavage is expected to take place after the A nucleotide which is left unpaired in designing the DNA-enzymes. Both the DNA-enzymes possessed the earlier identified 10–23 catalytic motif (Santoro and Joyce, 1997). These DNA-enzymes were synthesized in a DNA synthesizing machine. The catalytic motif was flanked with seven complementary bases on either side of the unpaired A residue of the target gene. (B) The mono-DNA-enzymes were synthesized by placing them in tandem such that the CCR5-DNA-enzyme followed the CXCR-4 DNA-enzyme. It is only in this order that this di-DNA-enzyme could, potentially, hybridize with the two target sites and bring about the cleavage. This di-DNA-enzyme had no spacer sequence in between them but the 10–23 catalytic motif was common.

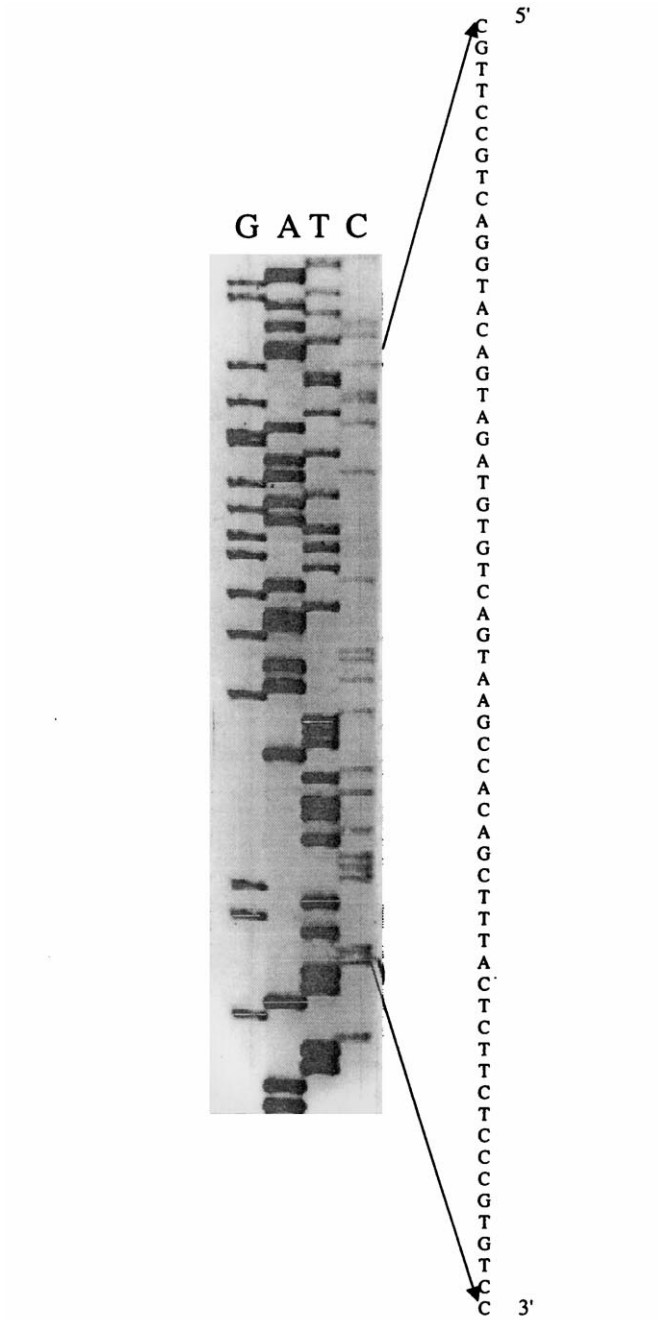

Fig. 2. The 60 nucleotide long CXCR-4-CCR5 chimeric gene was amplified by two terminal primers (see Section 2) and cloned into a T-tailed expression vector (pGEM-T-Easy, Promega). Recombinant clones were checked for the correct size insert by *Eco*R1 digestion. The recombinant clones were grown on a large scale and purified on a Qiagen column. Sequencing was carried out using a kit (Sequenase, Amersham-Life Science). This figure shows the sequence in the 3′ to 5′ direction and corresponds to the complement of the original sequence. The substrate gene was cloned under the SP6 promoter.

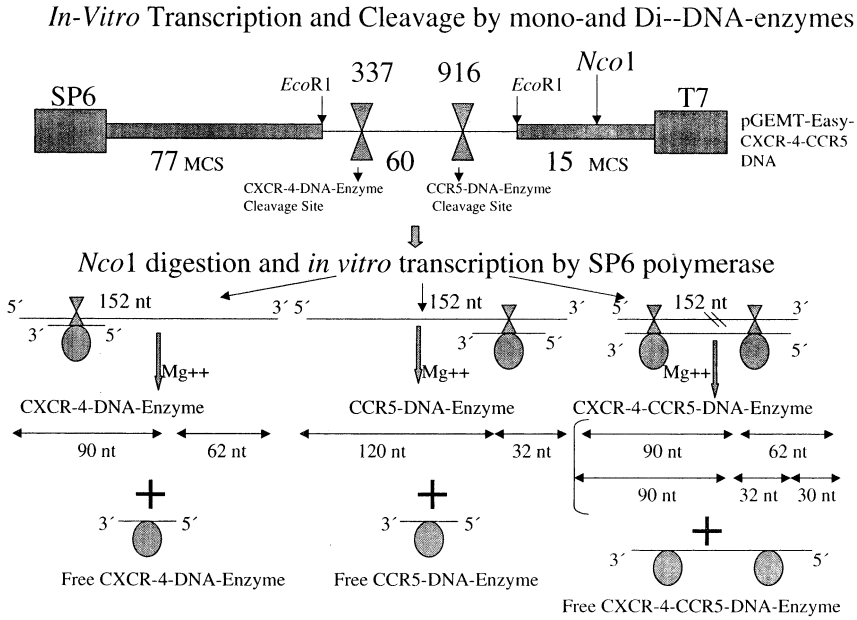

Fig. 3. The recombinant pGEM-T-Easy clone that harbored the 60 base pair of substrate DNA was linearized at the *Nco*1 site which is present in the MCS downstream of the gene, which will direct the synthesis of a 152-base long transcript. Note that this transcript contains extra 77 and 15 nucleotides at the 5′ and 3′ end of the substrate gene, which also gets transcribed with the SP6 polymerase. Mono-DNA-enzymes and di-DNA-enzymes were added to the labeled substrate and in presence of Mg^{2+}, specific cleavage products were expected. The free DNA-enzymes were available to repeat the cycle on a new target RNA.

quencing kit (Sequenase version 2, Amersham-Life Science). The entire sequence of the cloned synthetic substrate is shown in Fig. 2.

2.3. In vitro cleavage reaction

Plasmid encoding the chimeric CXCR-4-CCR5 substrate gene was linearized with an appropriate restriction enzyme site present at the 3′ end of the gene in the multiple cloning sites, *NCo*1. In vitro transcription was carried out in the presence of labeled UTP using SP6 polymerase, as described before (Banerjea and Joklik, 1990; Dash et al., 1998; Goila and Banerjea 1998). Equimolar concentrations of labeled substrate RNA and cold DNA-enzyme were mixed in the presence of 10 mM $MgCl_2$ in appropriate buffer at pH 7.5 and incubated at 37°C for 1 h. The cleaved RNA fragments were analyzed on a 7 M urea-7% polyacrylamide gel using the mini-protein gel system

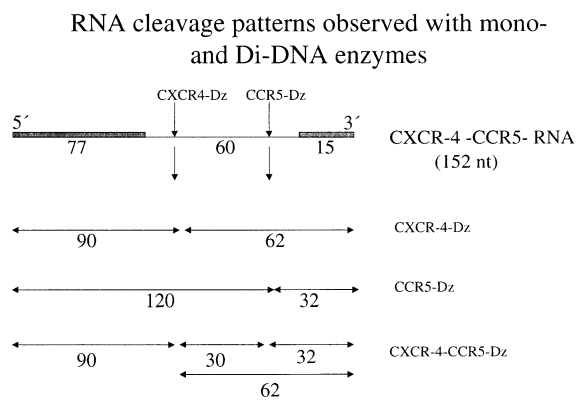

Fig. 4. The expected cleavage pattern is shown when a 152 nucleotide long target RNA is allowed to interact with two mono- and a di-DNA-enzyme. The CXCR-4 DNA-enzyme would yield fragments of 90 and 62 bases, whereas, the CCR5 DNA-enzyme would yield 120 and 32 bases long RNA fragments. A di-DNA-enzyme was expected to generate 90-, 30- and 62-base long fragments only when it simultaneously cleaved at the two target sites.

of Biorad (California). The various steps involved in transcription and cleavage are shown in Fig. 3 and the expected cleavage pattern with mono- and di-DNA-enzyme is shown in Fig. 4.

2.4. Inhibition of HIV-1 envelope HIV-1 coreceptor-CD4 mediated cell fusion by DNA-enzymes

The effectiveness of these DNA-enzymes was assessed by a cell membrane fusion assay, as described previously (Dash et al., 1998; Goila and Banerjea, 1998) and originally described by Nussbaum et al. (1994). Briefly, cell lines expressing either CCR5 or CXCR-4 (U87 cell line, obtained from A. Kinter, LIR, NIAID, NIH, MD) receptor protein were grown to 80% confluency in a 6-well plate (Nunc.) and were treated with varying amounts of DNA-enzyme in serum free medium in 0.5 ml vol. for 15 h and fused with another population of cells that expressed the compatible HIV-1 envelope protein for 15 h (JRFL envelope for CCR5 coreceptor and III B envelope for CXCR-4 receptor) for fusion. A noncompatible HIV-1 envelope served as control. Reporter gene (Luciferase) plasmid DNA and DNA-enzymes were introduced into 80% confluent monolayer in a 6-well plate using Lipofectamine (GIBCO/BRL). The expression of the reporter gene was under T7 polymerase promoter, which was achieved by using a recombinant vaccinia virus expressing T7 RNA polymerase (Fuerst et al., 1987). Recombinant vaccinia virus was grown in HeLa cells according to published procedures (Banerjea et al., 1988) and purified as described before by Joklik (1962).

2.5. Kinetic analysis

Kinetic parameters for the cleavage by DNA-enzyme-CXCR-4-337 were determined using varying concentrations of the substrate RNA (152 bases) in the presence of excess amounts of DNA-enzymes, as reported earlier (Santoro and Joyce, 1997; Dash et al., 1998). The cleaved RNA fragments were quantitated with the help of a phophorimager and kinetic parameters were calculated from a Lineweaver Burk's plot according to the standard procedures.

3. Results

3.1. Target site selection for DNA-enzymes

The AUG trinucleotide sequence present at 337 and 916 in CXCR-4 and CCR5 genes respectively was chosen to assemble the DNA-enzyme (Fig. 1, panels A and B). Sixty nucleotide long synthetic substrate gene encoding indicated portions of CXCR-4 and CCR5 genes, were successfully cloned into an expression vector pGEM-T-Easy (Promega Biotech). The entire sequence from 3′ to 5′ direction is shown in Fig. 2, which corresponds to the complement of the original sequence. This gene was placed under the influence of the SP6 promoter.

3.2. Assembly of DNA-enzymes

Twenty nine nucleotide long mono-DNA-enzymes possessing the common 10–23 catalytic motif were synthesized that were targeted to cleave the CXCR-4 gene after the A nucleotide (position 337) of AUG. The same trinucleotide (AUG) was targeted to cleave the CCR5 gene at position 916, as described before (see Fig. 1). A di-DNA-enzyme was also assembled by placing them in immediate tandem (no spacer) such that CXCR-4 DNA-enzyme was placed down stream of the CCR5-DNA-enzyme. It is clear from Fig. 1 (panel B) that DNA-enzymes when placed in this order alone would be able to cleave the synthetic RNA at two predetermined sites simultaneously.

3.3. In vitro cleavage of the CXCR-4-CCR5 RNA by DNA-enzymes

Recombinant clone of pGEM-T-Easy that harbored the 60 bases long substrate gene was linearized with *Nco*1, which is present in the multiple cloning site (MCS) downstream of the substrate gene. When subjected to transcription in the presence of SP6 polymerase, a 152 nucleotide long transcript could be expected. This also includes 77 nucleotides at the 5′ end and 15 nucleotides at the 3′ end of the cloned gene (see Fig. 4), as they will also be transcribed. As expected, a 152 bases long labeled transcript was synthesized

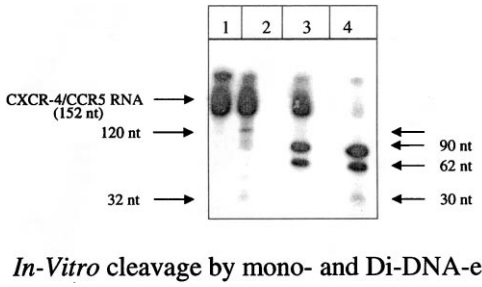

Fig. 5. In vitro cleavage of the substrate RNA by mono- and di-DNA-enzyme. As expected, the *Nco*1 linearized pGEM-T-Easy vector that harbored the target gene, yielded a 152-base long labeled transcript (lane 1). When equimolar (100 pmol) amounts of cold CCR5-DNA-enzyme were added to this labeled transcript, 120- and 32-base long fragments were seen (lane 2). When equivalent amounts of CXCR-4 DNA-enzyme were added, two prominent and expected size RNA fragments were obtained (90 and 62 bases long) (lane 3). Almost complete disappearance of the substrate RNA was achieved with equivalent amounts of di-DNA-enzyme (lane 4). Also the two cleavage products, 90- and 62-base long RNA fragments, became more prominent (compare lane 3 with 4). Note that a prominent 30-base long fragment was seen (lane 4), which could have resulted only if the di-DNA-enzyme had cleaved the target RNA at two sites simultaneously. No cleavage was observed by any of the above DNA-enzymes if the Mg^{2+} was omitted from the cleavage reaction (data not shown).

(Fig. 5, lane 1). When equimolar amounts of substrate RNA (100 pmol) and CCR5 DNA-enzyme were mixed and subjected to cleavage conditions, an expected size cleaved RNA fragment of 120 bases (5′ cleaved product) and 3′ cleaved product (32 bases) could be seen (lane 2). This cleavage was inefficient as most of the substrate RNA was still present. When the same amounts of CXCR-4- DNA-enzyme was added, efficient cleavage was obtained and two prominent cleavage products (5′ cleavage product of 90 bases and 62 bases of 3′ product) could be easily detected (lane 3). When equivalent amounts of di-DNA-enzyme was used, almost complete cleavage of the substrate RNA was achieved (lane 4). This increase in cleavage resulted in more prominent cleavage bands, 90 and 62 bases long, respectively. These bands were about 2-fold more intense when compared with similar bands present in lane 3 as determined by densitometric scanning. Also note that the120 bases long RNA fragment had also been reduced (compare lanes 2 and 4), most likely because this fragment now contains the cleavage site for CXCR-4 DNA-enzyme. A relatively prominent band of 30 bases emerged only in presence of the di-DNA-enzyme, which suggests that simultaneous cleavage at two target sites had been obtained.

3.4. In vitro cleavage of authentic CXCR-4 RNA by CXCR-4 DNA-enzyme

The CXCR-4 plasmid was obtained from the AIDS Repository, NIAID, NIH, MD. Truncated CXCR-4 transcript of ~ 500 bases long was generated by linearizing the transcript at the *Bam*H1 site present in its coding region and subjected to in vitro transcription and cleavage by DNA-enzyme-337. Mono-CXCR-4-337 DNA-enzyme cleaved the CXCR-4 RNA in a sequence-specific manner (Fig. 6). The di-DNA-enzyme also showed efficient cleavage of the same substrate (data not shown).

3.5. Kinetic parameters

The kinetic parameters, K_m (nM) and K_{cat} (min^{-1}) for the mono-DNA-enzyme-337, were 131 and 2.15, respectively. These values are very similar to earlier described ribozymes and DNA-enzymes (Santoro and Joyce, 1997; Dash et al., 1998).

3.6. Inhibition of HIV-1 coreceptor function by DNA-enzymes

The experimental protocol for carrying out HIV-1 envelope, CD4 and HIV-1 coreceptor dependent cell membrane fusion and reporter gene (Luciferase) activation has been described in detail previously (Dash et al., 1998; Goila and Banerjea, 1998; Husain et al., 1998a,b) and others (Nussbaum et al., 1994; Rucker et al., 1996). As expected, CXCR-4 DNA-enzyme specifically interfered with the fusion of cells that harbored the T lymphocytotropic HIV-1 envelope and the extent of inhibition correlated with the amount of the DNA-enzyme (Table 1A). This decrease was specific for CXCR-4 DNA-enzyme as equivalent amounts of CCR5-DNA-enzyme failed to show

any interference (Table 1A). The same was true for the di-DNA-enzyme (Table 1B). Besides, the di-DNA-enzyme was able to interfere with the cells that expressed macrophage tropic HIV-1 envelope-JRFL also (data not shown). We have earlier reported the efficacy of the mono-DNA-enzyme against CCR5 (Goila and Banerjea, 1998). This fusion was dependent upon the presence of all the three components (HIV envelope, CD4 and HIV-1 coreceptors), as omission of any one of these resulted in background levels of fusion. This decrease in fusion activity was specific for the DNA-enzymes that were used, as an unrelated DNA-enzyme failed to show any interfering activity (Table 1A). Also, reporter gene (Luciferase) expression was not detected in the absence of recombinant vaccinia virus infection that expressed T7 RNA polymerase.

4. Discussion

This is the first report of a catalytic DNA or RNA cleaving DNA-enzyme that cleaved the CXCR-4 gene efficiently in a sequence specific manner. This is an important coreceptor for X4 viruses, the appearance of which usually coincides with the later stages of the disease. The cleavage obtained was specific as evident from the expected cleavage pattern that was observed. Ability of the CXCR-4 DNA-enzyme to specifically cleave the target RNA was tested using two kinds of substrates. In the first case we synthesized a CXCR-4-CCR5 fusion gene by using a 60 nucleotide long DNA fragment that was generated by using recombinant DNA techniques and cloned into an expression vector so that labeled transcripts could be generated by in vitro transcription. In the second case, the CXCR-4 encoding DNA, was linearized in its coding region using *Bam*H1 enzyme which could direct the formation of ~ 500 nucleotide long transcript. The CXCR-4-DNA-enzyme cleaved both the transcripts with equal efficiency. Also the kinetics of cleavage was very similar to the one earlier described (Santoro and Joyce, 1997; Dash et al., 1998). With the aim to design DNA-enzymes that could simultaneously cleave the CXCR-4 and CCR5 transcripts, a di-DNA-enzyme was constructed by placing them in tandem. This di-DNA-enzyme possessed the ability to cleave both the targets, CXCR-4 and CCR5, in a sequence-specific manner. We had

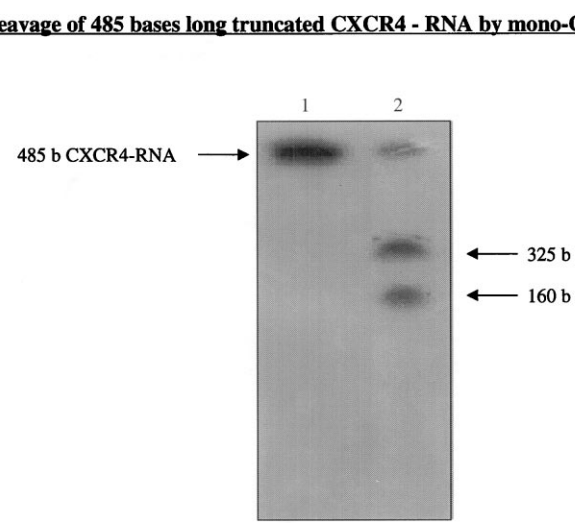

Fig. 6. Plasmid containing the full length CXCR-4 gene (see text) was linearized with *Bam*H1 which is present in its coding region and subjected to in vitro transcription in presence of labeled UTP. As expected, a 485 base-long truncated CXCR-4 transcript was synthesized (lane 1). When the CXCR-4-DNA-enzyme was mixed and incubated as described before (Dash et al., 1998), specific cleavage products (325- and 106-base long RNA fragments) could be observed (lane 2).

Table 1
Inhibition of cell membrane fusion by CXCR-4 DNA-enzyme

Plasmid DNAs used	Luciferase activity[a]
Part A	
Env-T + CD4 + CXCR-4 + UDz[b] (1.00 μg)	844 ± 10
Env-T + CD4 + CXCR-4 + CX-Dz[c] (0.1 μg)	532 ± 40
Env-T + CD4 + CXCR-4 + CXDz (0.5 μg)	327 ± 68
Env-T + CD4 + CXCR-4 + CXDz (1.00 μg)	175 ± 88
Env-T + CD4 + CXCR-4 + CCR5-DZ (1.00 μg)	822 ± 58
Part B	
Env-T + CD4 + CXCR-4 + UDz[b] (1.00 μg)	853 ± 20
Env-T + CD4 + CXCR-4 + Di-Dz[d] (0.1 μg)	421 ± 40
Env-T + CD4 + CXCR-4 + Di-Dz (0.5 μg)	150 ± 68
Env-T + CD4 + CXCR-4 + Di-Dz (1.0 μg)	22 ± 88

[a] Represents the luciferase activity which was determined in a luminometer by following the protocol from Promega. In all these fusion experiments, the concentration of T7-luciferase plasmid and HIV-1 envelope DNA was kept constant at 0.1 μg and only the concentration of DNA-enzyme was varied (0.1–1 μg). This works out to 21 and 210 nM for CXCR-4 DNA-enzyme and 14 and 140 nM for the di-DNA-enzyme. Briefly the cells were grown in a 6-well plate (1×10^6 cells) and subjected to various treatments as described in the text. Cell membrane fusion was carried out in a 96-well plate by combining the two populations of cells (one expressing the receptor and coreceptor and the other expressing the HIV-1 envelope) for 8 h as described before (Dash et al., 1998). Values represent mean ± standard deviation from two separate experiments.

[b] Unrelated DNA-enzyme possessing the same catalytic motif and of similar length was used as control.

[c] CX-Dz represents DNA-enzyme against CXCR-4 gene.

[d] Represents the di-DNA-enzyme that has been explained in Fig. 1B.

earlier reported the construction of DNA-enzyme-916 that was directed against CCR5 gene and we also showed its ability to act intracellularly (Goila and Banerjea 1998) on the full-length authentic CCR5 transcript. Thus, the di-DNA-enzyme that has been constructed, possessed the ability to cleave authentic CXCR-4 and CCR5 target RNAs. It is also remarkable that the di-DNA-enzyme retained the cleavage specificity of the mono-DNA-enzymes.

The efficiency of CCR5 DNA-enzyme to cleave the target RNA was found to be significantly low on a synthetic CXCR-4-CCR5 RNA, as compared to CXCR-4-DNA-enzyme, and this could be due to the specific folding properties of the substrate RNA that we engineered. We then combined the two DNA-enzymes, which now makes it 58 nucleotides long, in a specific order (see text) and addressed the question whether it could cleave the two target sites of the same substrate. The efficiency of the cleavage was significantly enhanced as evident from complete cleavage of the substrate RNA. This could be explained only by entertaining the notion that the di-DNA-enzyme hybridizes with the CCR5 target site first which then opens up secondary structures for the CXCR-4 target site in the substrate RNA. A prominent band of 30 base pair could be obtained only if the cleavage occurred simultaneously. Also the reduction of 120 bases long RNA fragment observed with di-DNA-enzyme could be explained on the grounds that this fragment is a good target for CXCR-4 DNA-enzyme. It can, therefore, be concluded that even when the size of a DNA-enzyme is doubled (as in di-DNA-enzyme), the efficiency of the cleavage was not compromised; on the contrary, it facilitated the cleavage of the other target site. It has been argued that any antiviral or therapeutic strategy that is directed against only one coreceptor, especially CCR5, may allow the virus to adapt quickly to use other coreceptors, namely CXCR-4 or others. Our approach of targeting multiple coreceptors simultaneously, has potential to reduce this possibility.

Finally, we have shown that these DNA-enzymes, when introduced into a mammalian cell, can interfere with the functional expression of the coreceptor genes in a cell membrane fusion assay. The di-DNA-enzyme was more efficient in its ability to interfere with the functional expression of HIV-1 coreceptors. A dose-dependent inhibition was observed only when compatible coreceptors and HIV-1 envelope protein containing cell population were allowed to interact and fuse. The fact that multitarget DNA-enzymes retain excellent cleavage property of the target sites and in some cases, facilitate the cleavage of other target sites, that may not be easily accessible because of rigid secondary structures, could form the basis of designing novel DNA-enzymes and could be exploited for therapeutic purposes in the future.

Acknowledgements

This work was supported from the core funds from Department of Biotechnology, Government of India, to National Institute of Immunology, New Delhi, and to the corresponding author (ACB). Human cell lines (U87) expressing CXCR-4 and CCR5 were obtained as a gift from Dr Audrey Kinter, NIAID, NIH, USA. We thank Dr Sandip K. Basu, Director, NII, ND, for support and encouragement.

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