Sequence specific cleavage of the HIV-1 coreceptor CCR5 gene by a hammer-head ribozyme and a DNA-enzyme: inhibition of the coreceptor function by DNA-enzyme

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Abstract The chemokine receptor CCR5 is used as a major coreceptor for fusion and entry by non-syncytia inducing macrophage tropic isolates of HIV-1, which is mainly involved in transmission. Individuals who are homozygous for the Δ32 allele of CCR5 are usually resistant to HIV-1 infection and continue to lead a normal healthy life. Thus this gene is dispensable and is, therefore, an attractive target in the host cell for interfering specifically with the virus-host interaction. With the aim to develop a specific antiviral approach at the molecular level, we have synthesized a hammer-head ribozyme and a DNA-enzyme. Both ribozyme and DNA-enzyme cleaved the CCR5 RNA in a sequence specific manner. This cleavage was protein independent but Mg^{2+} dependent. The extent of cleavage increased with increasing concentration of magnesium chloride. DNA-enzyme was more effective in cleaving a full length (1376 bases) in vitro generated transcript of CCR5 gene. In this communication, we show that the DNA-enzyme when introduced into a mammalian cell, results in decreased CD4-CCR5-gp160 mediated fusion of cell membranes. Potential applications of these *trans* acting molecules are discussed.

Key words: Ribozyme; DNA-enzyme; Chemokine receptor CCR5; HIV-1

1. Introduction

HIV-1 requires cofactor molecules (chemokine receptors) in addition to CD4 for entry and fusion into target cells (for review [1–3]). These chemokine receptors are 7-transmembrane domain G-protein coupled receptors. Macrophage tropic isolates (R5 viruses based on new classification) of HIV-1 use CCR5 which is mainly responsible for person to person transmission whereas T-lymphocyte tropic-syncytia inducing isolates use CXCR-4 and appear late in the disease ([4,5] and above review articles). The CCR5 coreceptor is used by primary HIV-1 isolates of almost all genetic subtypes, HIV-2 [6] and SIV [7]. Nine other potential chemokine receptors have been identified which can serve as a coreceptor for either HIV or SIV (reviewed in reference [1]). Approximately 1% of the Caucasian population with European heritage is homozygous for the Δ32 allele of CCR5 which afforded protection against HIV-1 infection despite multiple exposure [8–11]. The protection is not absolute as three incidents of infection have been reported recently [12–14]. This deletion corresponds to the second extracellular loop of the 7-transmembrane protein which results in frame shift mutation. A severely truncated protein is expected which can neither act as a coreceptor nor has the ability to transduce any signal [8]. Approximately 15–20% of people of European origin are heterozygous for the Δ32 allele of CCR5 [8–11]. They remain fully susceptible to virus infection but the progression of the disease is delayed [10]. Besides this deletion, many other types of deletion have been reported but they are rare in occurrence [15,16]. While this deleted allele was quite common among European people, it was found remarkably absent among Africans and Asians [8–11]. We recently reported the presence of a normal individual who was heterozygous for Δ32 allele of CCR5 gene from India [17] and studied the inheritance pattern [18]. Since this gene is dispensable, it has become a target for intervention by many groups. We have assembled a hammer-head ribozyme and a DNA-enzyme for selective inactivation of this HIV-1 coreceptor as our antiviral approach. In this communication, we demonstrate their in vitro cleavage properties under variety of experimental conditions and show the effectiveness of the DNA-enzyme in specific intracellular down regulation of CCR5 gene in a functional assay which is based on CD4 coreceptor gp160 mediated cell membrane fusion.

2. Materials and methods

2.1. Plasmids used

We recently reported full length cloning of the CCR5 (wild-type as well as Δ32 allele) gene from a heterozygous individual from India [17,18]. The gene was placed under bacteriophage T7 promoter of the plasmid pGEM-T-Easy (Promega Biotech., Madison, WI). For in vivo studies, pCDNA-CCR5 plasmid was obtained from Marc Parmentier, Belgium. All the plasmids were purified on a Qiagen column (Qiagen, Gmbh, Hilden, Germany) before sequencing or for the purpose of transfection into mammalian cells.

2.2. Target site selection and construction of ribozyme

GUC (541 nt position of G residue) was selected for making the hammer-head ribozyme. The following strategy was used to assemble the ribozyme. A 38 nucleotides long oligonucleotide was synthesized chemically. This was amplified by using two terminal primers and the PCR amplified product was ligated into a T-tailed vector (pGEM-T-Easy). The following primers were synthesized:

(1) Rz-541-5′-CAAGAGTT**CTGATGAGTCCGTGAGGACGAA**A-CACATTG-3′. The sequence of the catalytic motif of the hammer-head ribozyme is shown in bold letters and the sequence of 8 bases on either side of this motif is also shown which provides specificity for the target RNA (Fig. 1).

(2) Sense terminal primer 5′-CAAGAGTTCTGATGAG-3′.

(3) Antisense terminal primer 5′-CAATGTGTTTCGTCCTC.

The PCR conditions were the same as described before [17]. The recombinant plasmids were checked for the ribozyme insert by digestion with *Eco*RI. Recombinant clones were grown to large scale, purified on a Qiagen column before subjecting them to sequencing with forward sequencing and reverse sequencing primers provided in the kit (Sequenase version 2, Amersham, Life Science). The ribozyme

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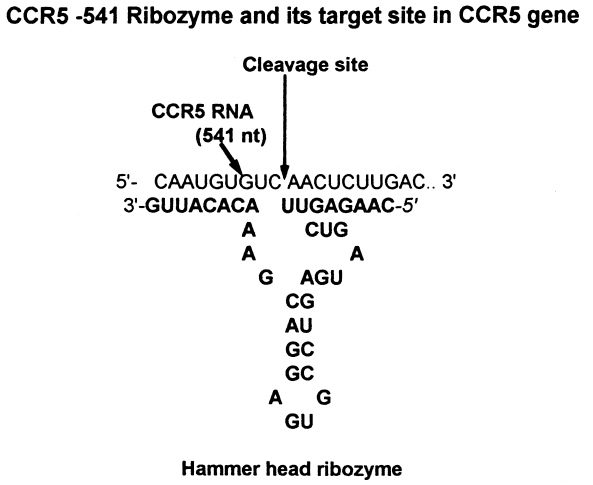

Fig. 1. Hammer-head ribozyme was assembled by standard recombinant DNA methodology as described in the text and described before [29]. 541 nucleotide of the CCR5 gene [32] was targeted. PCR amplified ribozyme DNA was cloned into pGEM-T-Easy (Promega Biotech., Madison, WI) and confirmed by sequencing. A 22 nucleotides hammer-head catalytic motif was placed between the target hybridizing arms on either side of the C residue (which was left unpaired) which were 8 bases long.

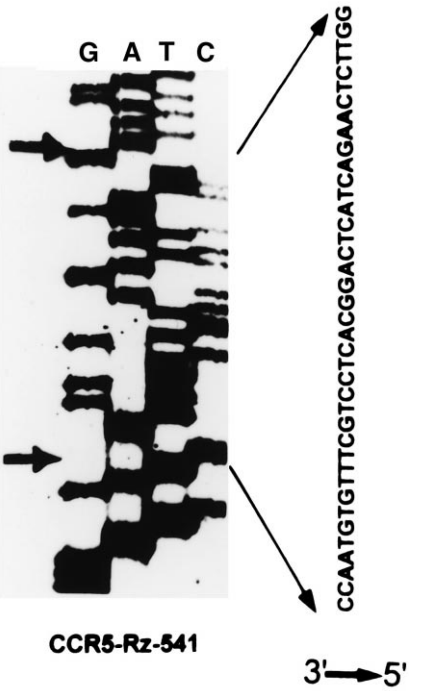

Fig. 2. The ribozyme DNA sequences were amplified by PCR as described in the text and then cloned into pGEM-T-Easy. Clones harboring the ribozymes were grown to large scale in JM 109 cells and the plasmid DNA was isolated according to standard procedures. Before sequencing, the DNA was purified on a Qiagen column. Both the strands were sequenced using forward and reverse sequencing primers provided in the sequencing kit (Sequenase version 2, Amersham, Life Science). Note the presence of *Eco*RI site present (in the polylinker region) on either side of the cloned ribozyme.

was found to be placed under SP6 promoter (Fig. 2). Therefore, ribozyme transcripts were synthesized in vitro by using SP6 polymerase.

2.3. *In vitro transcription of substrate RNA and cleavage by ribozyme and DNA-enzyme*

Qiagen column purified plasmid pCCR5 DNA was linearized by the restriction enzyme *Xba*I for full length transcripts of 1376 nt and by *Bgl*II for 740 nt long transcripts respectively. The ribozyme 541 was linearized at a unique restriction site *Nco*I present in the MCS (multiple cloning sites) of the vector pGEM-T-Easy. Transcription of linearized plasmid DNAs were carried out using either T7 or SP6 polymerase (Promega Biotech) according to the directions of the manufacturer and as described before [19]. RNAs were resolved on a 7 M urea-7% polyacrylamide gel using the mini-protein gel system of Biorad. Various steps involved from plasmid DNA to transcription and cleavage by ribozymes and DNA-enzymes are shown in Fig. 3.

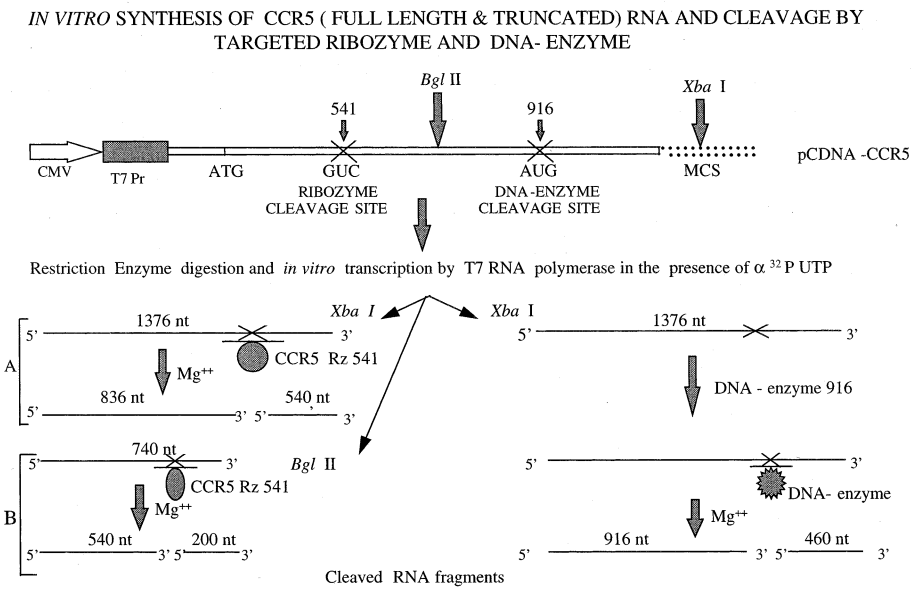

Fig. 3. Plasmid DNA encoding CCR5 gene was linearized by restriction enzymes (*Bgl*II and *Xba*I) to generate two classes of transcripts in presence of labeled UTP using T7/SP6 RNA polymerase as described before [19]. After purifying the labeled transcripts, they were subjected to cleavage by a ribozyme and a DNA-enzyme in presence of Mg^{2+}. The cleaved RNA fragment sizes have been shown. Once the cleavage occurred, the ribozyme (Rz) or DNA-enzyme (Dz) are free to repeat the cycle on a new CCR5 RNA molecule. Diagram is not drawn to scale.

2.4. Selection of target site for DNA-enzyme and cleavage conditions

AUG target site (position of A nucleotide being 916) was selected as a target for DNA-enzyme that contained the recently identified 10–23 catalytic motif [20]. Seven bases on either side of the A residue (which was left unpaired and the cleavage is expected after the A residue) was synthesized which was complementary to the target sequences (by Watson-Crick base pairing) and the catalytic motif was placed in the middle of these hybridizing arms (Fig. 6). Equimolar concentration of the substrate RNA (labeled) and cold DNA-enzyme were added (100 picomoles) and the cleavage was initiated by adding varying amounts of magnesium chloride by using the procedure followed by Santoro and Joyce [20] and Kawabura et al. [21]. The cleaved products were analyzed by gel electrophoresis as described above.

2.5. Inhibition of cell membrane fusion by DNA-enzyme

The effectiveness of the DNA-enzyme was assessed by a cell membrane fusion assay as has been described by us recently wherein we have tested the antisense effect of a portion of CCR5 gene using recombinant vaccinia virus [17]. Fusion dependent reporter gene (luciferase) activation assay as originally described by Nussbaum et al. [22] was used. All the DNAs were used in 0.1 μg quantity for transfection into HeLa-CD4+ cells using lipofectin (GIBCO/BRL) including the various control DNAs. Other ratios of CCR5 DNA and DNA-enzymes were also used and they are explained in the figure legends. HeLa cells were used as control or they were made to express CD4 by DNA transfection with the plasmid expressing it. The recombinant vaccinia virus was grown in HeLa cells and purified according to the published procedures [23]. The infectivity of the virus was determined by plaque assay using BSc-1 cells as described before [24].

3. Results

3.1. Cloning of the CCR5 ribozyme 541

38 nucleotides long oligonucleotide which contained the 22 bases long earlier characterized hammer-head motif and 8 nucleotides on either side of the X residue from GUC target which hybridizes with the target mRNA by Watson-Crick base pairing was successfully cloned into pGEM-T-Easy. The recombinant clones that contained the 38 bases pair insert were grown to large scale and purified on a Qiagen column. They were then subjected to sequencing using forward and reverse sequencing primer provided by the manufacturer (Sequenase version 2, Amersham Life Science, Arlington Heights, IL). The sequence of the ribozyme along with the portion of multiple cloning site is shown in Fig. 2. The ribozyme was cloned in 3′ to 5′ direction with respect to the T7 promoter which was flanked by the *Eco*RI restriction sites. The 5′ to 3′ direction of the coding strand was exactly the same as oligonucleotide #1 that was used as a template to amplify using terminal primers. Thus, the ribozyme RNA could be synthesized using SP6 promoter of the vector.

3.2. Sequence specific cleavage of CCR5 (full length) RNA by ribozyme

Plasmid DNA encoding CCR5 gene was linearized by *Bgl*II and *Xba*I which after subjecting to in vitro transcription is expected to generate a truncated (740 nt) or full length CCR5 transcript of 1376 (Fig. 4A, lane 1) bases. The ribozyme was tested for its ability to cleave these two classes of in vitro generated substrate RNA molecules. The ribozyme specific RNA was generated by linearizing the plasmid pGEM-CCR5-Rz in the polylinker region of the vector using the restriction enzyme *Nco*I. The size of the in vitro generated ribozyme specific transcript is 115 bases (lane 2). Lane 1 shows the synthesis of a full length labeled CCR5 RNA and

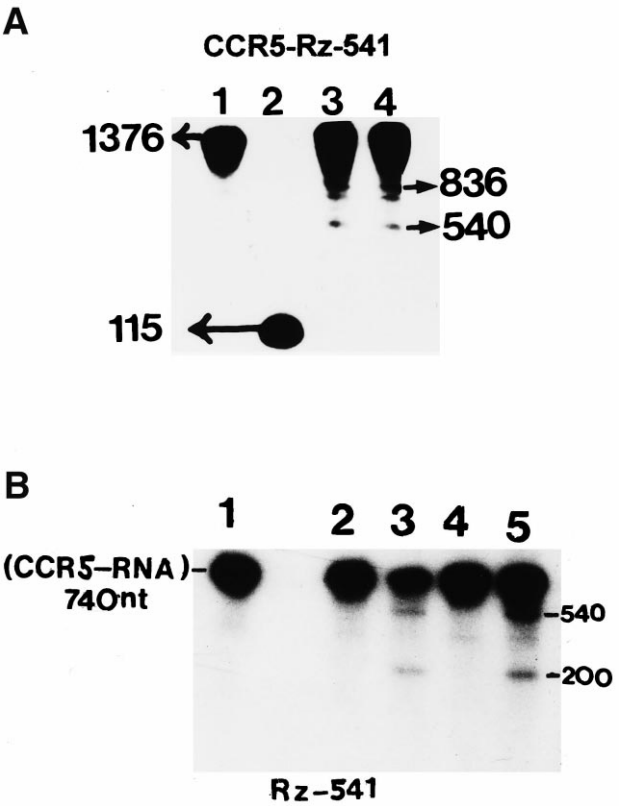

Fig. 4. A: *Xba*I digested CCR5 plasmid DNA when subjected to in vitro transcription using the transcription kit from Promega Biotech., yielded a transcript of 1376 bases (lane 1) and the plasmid containing ribozyme (see text) generated a transcript of 115 bases in presence of labeled UTP (lane 2). The cleavage reactions were carried out with cold ribozyme and labeled CCR5 RNA (substrate). Specific cleavage products were seen in lanes 3 and 4 in the presence of 10 mM $MgCl_2$. The cleavage reaction was carried out for 2 h at 37°C, in one case it was denatured at 90°C for 2 min (lane 3) and in the other case it was omitted (lane 4) before addition of magnesium chloride. The cleaved RNA fragments were analyzed by gel electrophoresis as described in the text. No cleavage was observed in the absence of Mg^{2+} (data not shown). B: The experimental details of this experiment are the same as in the previous one (A). The same ribozyme was used to cleave a truncated CCR5 RNA (740 bases long) which was generated by digesting the CCR5 plasmid by *Bgl*II (see Fig. 3 and text). Lane 1 shows the synthesis of truncated CCR5 RNA. Cleavage reaction was carried out for 2 h at 37°C in the presence of 2 mM $MgCl_2$ (lane 2) and in the presence of 10 mM $MgCl_2$ (lane 3). When an additional heating step (90°C for 2 min) was introduced before initiating the cleavage reaction in presence of 2 mM $MgCl_2$ (lane 4) and 10 mM $MgCl_2$ (lane 5), increased cleavage was obtained. Note the increase in specific cleavage at two different experimental conditions (compare lane 3 with lane 5). Cleavage at 10 mM $MgCl_2$ was significantly better than 2 mM.

115 bases long labeled ribozyme transcript is shown in lane 2. When equivalent amounts of cold ribozyme and labeled substrates were added in equal amounts (100 picomoles each), specific 5′ cleavage (540 bases) and 3′ cleavage products (836 bases) were observed (lanes 3 and 4). Cleavage reaction was continued for 2 h at 37°C in presence of 10 mM $MgCl_2$. In one case the substrate and ribozymes were heated to 90°C for 2 min before incubating at 37°C (lane 3), in the other case (lane 4) the entire cleavage reaction was performed at 37°C. Only about 5% specific cleavage of the input substrate was obtained when full length CCR5 RNA was used. Besides the 1376 bases long CCR5 transcript, there are two additional

minor RNA bands (lane 1), which were most likely because of premature transcription which was seen earlier also with the generation of in vitro transcription of other unrelated genes [9]. Since the intensity of the cleaved RNA bands in lanes 3 and 4 increased with the time and not the premature transcripts in lane 1, we conclude that they are produced as a result of precise cleavage by ribozyme.

3.3. Cleavage of truncated CCR5 RNA (740 bases) by CCR5-Rz-541

The experimental conditions for cleavage were essentially the same except that instead of using a full length in vitro generated CCR5 RNA, 740 bases long labeled CCR5 transcript was used. Lane 1 of Fig. 4B shows the synthesis of 740 bases long CCR5 transcript. Cleavage reactions were carried out for 2 h at 37°C (no prior heating step involved). Lanes 2 and 3 show the extent of specific cleavage obtained in presence of 2 and 10 mM $MgCl_2$ concentrations respectively. Specific cleavage products (5′, 540 bases and 3′, 200 bases) could be easily seen in lane 3 only. Lanes 4 and 5 show the extent of cleavage obtained with 2 (lane 4) and 10 mM $MgCl_2$ (lane 5) when the labeled substrate RNA and ribozyme RNA were heated at 90°C for 2 min before addition of $MgCl_2$. About 5% more cleavage was obtained if the mixture of ribozyme and labeled substrate RNA (CCR5) was subjected to heat treatment before addition of $MgCl_2$ (compare lanes 3 and 5). We conclude from this that the same ribozyme worked more efficiently (~20% specific cleavage) on a truncated CCR5 RNA as compared to full length where the cleavage was ~5%.

3.4. Effect of the concentration of $MgCl_2$ on cleavage efficiency

The effect of varying amounts of $MgCl_2$ on the cleavage efficiency was studied using the 740 bases long in vitro generated labeled transcripts described earlier. Lane 1 shows the presence of 740 bases long CCR5 (Fig. 5, lane 1) transcript in presence of ribozyme which was not cleaved if the Mg^{2+} was omitted from the reaction. The extent of cleavage increased to the extent of 85% in the presence of 100 mM $MgCl_2$ (Fig. 5, lane 6) (shown by arrows). Lanes 2, 3, 4 and 5 show the extent of cleavage obtained in presence of 5, 10, 20 and 50 mM $MgCl_2$ respectively. We conclude that there is a direct correlation of Mg^{2+} with the cleavage efficiency. It is remarkable that the specificity of the ribozyme was not lost over a wide range of concentration of $MgCl_2$.

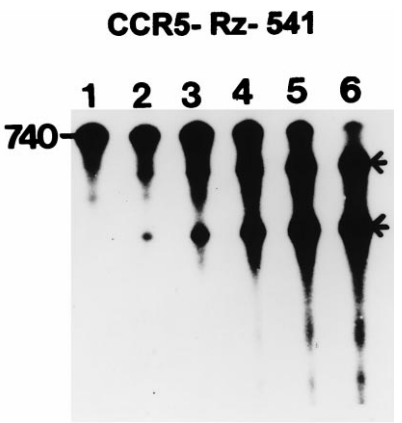

Fig. 5. Effect of varying concentrations of $MgCl_2$ on the cleavage efficiency of CCR5 ribozyme. The CCR5 transcript was 740 bases long which was the same as described in Fig. 4B. Lane 1 shows the presence of intact CCR5 RNA when the cleavage reaction was carried out in the absence of $MgCl_2$. The extent of cleavage increased significantly as the concentration of $MgCl_2$ was increased keeping all other conditions identical, an almost complete cleavage of the CCR5 RNA was achieved at 100 mM $MgCl_2$ (lane 6). Lanes 2, 3, 4, and 5 show the extent of cleavage obtained at 5, 10, 20, and 50 mM $MgCl_2$ respectively.

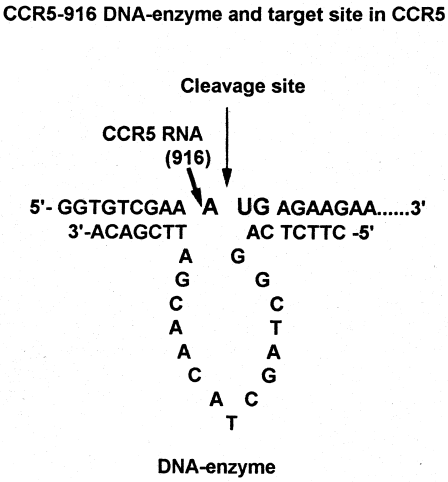

Fig. 6. AUG sequence (position of A being 916) (shown by a diagonal arrow) was used to make the DNA-enzyme using the 10–23 catalytic motif. Cleavage of the CCR5 RNA is expected to take place after the A residue (shown by a long straight arrow). Seven bases on either side of the unpaired A residue were synthesized chemically which were complementary to the target sequence. The catalytic motif was placed between these two hybridizing arms (antisense flanks).

3.5. Target site selection for DNA-enzyme targeted against CCR5 gene

AUG present in the coding region of CCR5 (position of A being 916 nucleotide shown by a diagonal arrow) was chosen for synthesizing a DNA-enzyme which possessed the earlier identified 10–23 catalytic motif [20]. The sequence of the target gene and DNA-enzyme is shown in Fig. 6. The cleavage is expected to take place after the A residue (shown by a long arrow). Seven bases on either side of the A nucleotide are made complementary to the target gene for hybridization by Watson-Crick base pairing (Fig. 6). The catalytic motif [10–23] possessing the sequence 5′-GGCTAGCTACAACGA-3′ was placed in between target hybridizing arms.

3.6. Targeted cleavage of full length CCR5 RNA by DNA-enzyme 916

In vitro synthesized full length CCR5 (1376 bases) transcript was subjected to cleavage by equimolar amounts (100 picomoles) of DNA-enzyme and the cleavage reaction was initiated by adding varying amounts of magnesium chloride as described before [20,21]. Lane 1 of Fig. 7 shows the synthesis of full length labeled CCR5 RNA, lanes 2 and 3 show the cleavage obtained in presence of 2 and 10 mM $MgCl_2$ when samples were denatured at 95°C for 2 min followed by 2 h at 37°C. Lanes 4 (2 mM $MgCl_2$) and 5 (10 mM $MgCl_2$) represent the same conditions except that the heating step was omitted and the entire reaction was carried out at 37°C. Almost 70% of the input substrate was cleaved into specific products of 916 and 460 bases long RNA fragments (lanes 3

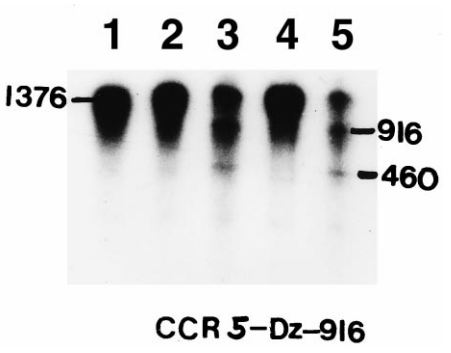

Fig. 7. Sequence specific cleavage of the full length in vitro generated CCR5 RNA (1376 bases) by DNA-enzyme 916. The reaction conditions were the same as described before [20,21] and as described in the text. Plasmid DNA-CCR5 was linearized by *Xba*I digestion (see Fig. 3) and subjected to transcription as described before. Purified labeled transcript was then allowed to react with DNA-enzyme (cold). Lane 1 shows the synthesis of CCR5 transcript. The cleavage reaction was continued for 2 h at 37°C after a 2 min exposure at 95°C for 2 min in presence of 2 mM $MgCl_2$ (lane 2) or 10 mM $MgCl_2$ (lane 3). In another set of experiments, the samples were not subjected to 95°C treatment but the entire reaction was carried out at 37°C in presence of 2 mM $MgCl_2$ (lane 4) and 10 mM $MgCl_2$ (lane 5). Note the specific cleavage products being formed in lane 3 and lane 5. Cleavage at the higher concentration of $MgCl_2$ was significantly better than what was achieved at 2 mM $MgCl_2$ concentration.

and 5). Additional heating step did not improve the cleavage efficiency. We can not use the 740 bases long truncated CCR5 RNA for cleavage by this DNA-enzyme because this target site is not present.

3.7. Inhibition of cell membrane fusion by DNA-enzyme

The detailed procedure for carrying out the gp160 (JRFL)-CD4-CCR5 fusion based activation of a reporter gene assay is based on the method described originally by Nussbaum et al. [22] and also recently described by us [17,18]. Briefly, the DNA-enzyme and the CCR5 encoding DNA (varying ratios) were cotransfected into HeLa cells that were expressing CD4 and fused with HeLa cells expressing HIV-1 Env protein and T7 RNA polymerase. Fusion dependent reporter gene (luciferase gene under T7 promoter) activation is measured. While keeping the DNA constant (0.1 μg each) for JRFL and CD4 plasmid, CCR5 DNA dose dependent increase in the cell membrane fusion activity was observed (Table 1). When DNA-enzyme was used in two different ratios (0.1 and 0.5 μg), not only a dose dependent decrease (from 832 to 508 light units, Table 2) was seen but compared to an equivalent amount of only CCR5 DNA (1655 light units (LU), Table 1), it was significantly lower. Significant drop in the cell membrane fusion activity was seen even when the plasmid DNA-enzyme was five-fold lower (0.1 μg) than CCR5 (0.5 μg) (compare 1832 LU in Table 1 with 985 LU in Table 2). From this, we conclude that the DNA-enzyme in various combinations of DNA-enzymes and CCR5 was able to interfere with the cell membrane fusion. This was a specific effect as variety of control DNAs (pCDNA3, Promega Biotech., USA) as well as control DNA-enzyme which was targeted against the X gene of hepatitis B [25] virus failed to show this effect (data not shown). The sequence of this control DNA-enzyme (HBx-Dz-307) was as follows: 5′-GTTGACA**GGCTAGCTACAACGA**TGCTGGGG-3′. The catalytic motif is the same as before and expressed in bold letters. Also, the oligonucleotides that lacked the catalytic motif but retained the 7+7 bases as antisense flanks, failed to show this kind of effect (data not shown). This suggests that the observed effect is most likely mediated by the catalytic activity of the DNA-enzyme.

Table 1
CCR5-gp160-CD4 mediated cell membrane fusion

Plasmid DNAs used	Luciferase activity
JRFL+CD4+CCR5 (0.01)	673 ± 59
JRFL+CD4+CCR5 (0.10)	1655 ± 93
JRFL+CD4+CCR5 (0.50)	1832 ± 98

CCR5 dependent increase in the cell membrane fusion activity. Values represent average with standard deviation from two separate experiments. For details see text.

Table 2
Inhibition of cell membrane fusion by DNA-enzyme 916

Plasmid DNA used	Luciferase activity[a]
JRFL+CD4+CR5 (0.1) +Dz (0.1)	832 ± 36
JRFL+CD4+CR5 (0.1) +Dz (0.5)	508 ± 29
JRFL+CD4+CR5 (0.5) +Dz (0.1)	985 ± 78

[a]Average mean ± S.D. from two separate experiments. Dz is the abbreviated form for DNA-enzyme 916. The concentration for JRFL, CD4 DNA was kept constant at 0.1 μg for each experiment. Extent of luciferase activity is a direct reflection of the amount of membrane fusion achieved, for details see text.

4. Discussion

With the purpose of selectively down regulating the replication of HIV, we have assembled a hammer-head ribozyme and synthesized a DNA-enzyme which were targeted against the chemokine receptor gene CCR5 (HIV coreceptor), which is now considered essential for HIV-1 entry and fusion. The hammer-head ribozyme was targeted against the 541 nucleotide of the CCR5 gene by making use of the GUC sequence. It is important to mention that most work on ribozymes or DNA-enzymes use very short either synthetic or in vitro synthesized RNA to test the cleavage potential [20,26,27]. This is an artificial situation and for this reason we have tested the cleavage potential of our ribozyme (Rz) or DNA-enzyme (Dz) on in vitro synthesized CCR5 RNA which was either full length (1376 bases) or greater than 50% of its authentic size. Precise cleavage of the substrate RNAs of both sizes were obtained and as expected the cleavage was better with the shorter substrate RNA. The size of the in vitro synthesized ribozyme RNA using SP6 RNA polymerase is 115 bases from the vector pGEM-T-Easy because of the sequences from the multiple cloning site present between the SP6 promoter and the region where we cloned our ribozyme gets transcribed. The efficiency of this ribozyme is likely to improve if a 38 bases long (the minimum size for a functional hammer-head ribozyme) CCR5 ribozyme is placed directly under the SP6/T7 promoter. As expected, the efficiency of cleavage with the same ribozyme was better when the 740 bases long truncated CCR5 transcript was used as compared to the full length. This cleavage was greatly influenced by the concentration of $MgCl_2$ and almost complete cleavage was obtained at 100 mM $MgCl_2$. Similar observations have been made for other unrelated ribozymes earlier [28]. A slight increase in the extent of cleavage was observed with the addition of a denaturing step

before adding the $MgCl_2$ for initiating the cleavage reaction. This is most likely because of the melting of the secondary structures which allows more efficient hybridization. This cleavage was Mg^{2+} dependent and unrelated RNAs were not cleaved by this ribozyme (data not shown). DNA-enzyme that was targeted to cleave the 916th residue (A) of the CCR5 gene, also cleaved the full length CCR5 gene (1376 bases) into specific products and the extent of cleavage was more than 70% and an additional heating step prior to cleavage reaction did not make any difference (Fig. 7, compare lanes 3 and 5). Although direct comparisons can not be made between the ribozyme and the DNA-enzyme since they are targeted to different regions of the CCR5 RNA (full length 1376 bases), nevertheless, DNA-enzyme was found to be more efficient. We then addressed the question whether this DNA-enzyme could act intracellularly in a functional assay described earlier. For this, we first established that CCR5 DNA dependent increase in cell membrane fusion was achieved (Table 1). We then used a variety of ratios of CCR5 and the DNA-enzymes and found in each case a significant drop in the membrane fusion activity (compare Table 1 with Table 2). This decrease was specific as unrelated DNAs, HBx-Dz-370 (unrelated DNA-enzyme control targeted against the X gene of hepatitis B virus) or the oligonucleotides devoid of the catalytic motif, failed to show a significant reduction in cell membrane fusion ($< 10\%$) (data not shown). We conclude that DNA-enzyme was able to interfere with the functional expression of this coreceptor. We wish to subclone our ribozyme under a strong eukaryotic promoter to find out its effectiveness inside the cell. These short RNA and DNA molecules can be explored for their therapeutic ability to interfere with the replication of HIV. It may be possible to make these ribozymes multitarget in nature as has been reported against HIV-1 [29–31]. The same approach could be applied to DNA-enzymes. Combinations of these approaches along with other anti-retroviral strategies may significantly delay the onset of the disease by delaying the appearance of the resistant population of viruses.

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