Targeted cleavage of HIV-1 envelope gene by a DNA enzyme and inhibition of HIV-1 envelope-CD4 mediated cell fusion

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Abstract With the ultimate aim of developing an effective antiviral strategy against HIV-1, a mono-DNA enzyme possessing the 10–23 catalytic motif [Santoro and Joyce (1997) Proc. Natl. Acad. Sci. USA 94, 4264–4266] was synthesized against the HIV-1 envelope gene. We tested the in vitro cleavage efficiency of the 178 bp long truncated HIV-1 Env transcript by DNA enzyme 6339. Protein independent and Mg^{2+} dependent specific cleavage products were obtained. As soon as 5 min after mixing equimolar concentrations of DNA enzyme and substrate RNA, more than 50% cleavage was observed which increased steadily over a period of 4 h. Very little cleavage was obtained at 1 mM $MgCl_2$ concentration which improved significantly when the concentration of $MgCl_2$ was increased up to 20 mM. Specific inhibition of cell membrane fusion caused by the interaction of gp160 and CD4 in HeLa cells was observed when the above DNA enzyme was used. Thus, these chemically synthesized DNA enzymes could prove to be very useful for in vivo application.

Key words: DNA enzyme; HIV-1 Env gene; Cell membrane fusion; Syncytia

1. Introduction

The antisense approach is currently being used for specific down regulation of a target gene for the treatment of many diseases and disorders. Often administration of large amounts of the antisense oligonucleotides (phosphothiorate form) is required which usually leads to toxicity problems [1]. Ribozymes, being catalytic in nature [2–4] and having the potential to cleave a target gene (for review [5–7]), have provided new hope for achieving specific down regulation of the target gene without causing much toxicity. Indeed several mono- and multi-target ribozymes [8–14] have been constructed which showed specific interference of viral or cellular genes. Exogenous application of ribozymes has been a problem because the RNA is very susceptible to degradation inside the cell and efforts are being made to improve the intracellular stability of modified nucleotides [15]. Recently, general purpose RNA-cleaving DNA enzymes have been described which were shown to be functional under simulated physiological conditions and the sequences of the two catalytic motifs, 10–23 and 8–17, were reported [16]. Extreme susceptibility of RNA to intracellular degradation was overcome to quite an extent as the DNA is more stable inside the cell. These DNA enzymes are catalytic in nature and can be engineered to cleave a target gene *in trans* in a sequence specific manner in presence of Mg^{2+}. Besides, they provide more flexibility for the selection of cleavage sites. We constructed a mono-DNA enzyme by targeting an internal AUG sequence (A being the 6339th nucleotide in the pNL4-3 sequence [17]). This AUG (not read as methionine) is present in the coding region of the envelope gene (see Fig. 1). This target site is, therefore, different from what was selected earlier [16]. Another important distinction is that we used a 180 bases long HIV-1 envelope transcript for cleavage which is physiologically more relevant than the synthetic 17 nucleotides long synthetic gene spanning the initiation codon (AUG) of the HIV-1 envelope gene reported earlier [16]. In this communication we show specific in vitro cleavage of the truncated HIV-1 Env gene of HIV-1 by targeted DNA enzyme and demonstrate its ability to interfere with the functional expression of the HIV-1 envelope gene in a cell fusion assay.

2. Materials and methods

2.1. Plasmid DNAs and in vitro synthesis of RNAs

HIV-1 envelope gene (from III B) was placed under T7 promoter [18] and specific ^{32}P-UTP labeled transcripts (full length and truncated variants) were generated after linearizing the plasmid with an appropriate restriction enzyme according to the manufacturer's direction (Promega Biotech, WI, USA) and as described before [19] using T7 RNA polymerase. The steps starting from linearizing the supercoiled plasmid DNA to obtaining the cleaved products of the substrate RNA are diagrammatically shown in Fig. 2. Specific activity of the labeled RNA was determined by standard procedures. These RNAs were resolved in a 7% polyacrylamide-7 M urea gel using the mini-protein gel apparatus from Bio-Rad.

2.2. Construction of DNA enzyme

Twenty-nine nucleotides long DNA was synthesized in a DNA synthesizer (Applied Biosystems). The exact sequence of the target gene and the sequence of the DNA enzyme together with the regions specifically hybridizing with substrate RNA and DNA enzyme 6339 are shown in Fig. 1. Seven nucleotides on either side were made complementary to the target gene and the A nucleotide of the target gene was left unpaired and cleavage is expected to take place after the A nucleotide. The earlier identified catalytic motif (10–23) was placed between the two antisense flanks which possessed the following sequence: 5′-GGCTAGCTACAACGA-3′.

2.3. In vitro cleavage reaction

Equimolar concentrations (100 pmol each) of the ^{32}P labeled substrate RNA and cold DNA enzyme were mixed in 10 μl of 50 mM Tris-HCl, pH 7.5, containing varying amounts of $MgCl_2$ (range 1–20 mM). Samples were heated at 95°C for 2 min before addition of $MgCl_2$ followed by 1 h incubation at 37°C. The cleavage products were analyzed as described before using Tris-Borate-EDTA buffer.

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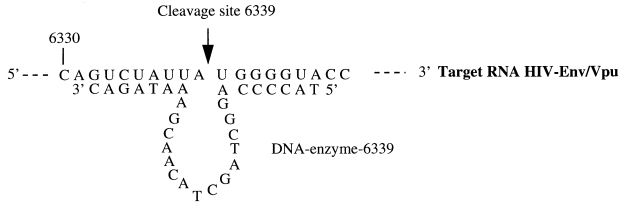

Fig. 1. Sequence of the target site in the HIV-1 envelope gene with the cleavage site (6339 nt). Also shown is the sequence of the DNA enzyme 6339 and the strategy to provide sequence specific cleavage. Seven bases on either side of the nucleotide AUG are synthesized complementary to the target gene (A nucleotide is left unpaired). This DNA enzyme possesses the earlier identified 10–23 catalytic motif [16].

2.4. Analysis of kinetic parameter of DNA enzyme

Kinetic parameters (K_m, k_{cat}) of the DNA enzyme 6339 were determined using ^{32}P-UTP labeled truncated HIV-1 envelope RNA, (178 bases long RNA generated by linearizing HIV-1 envelope plasmid by *Nde*I) in the presence of excess amounts of DNA enzyme using the cleavage conditions described earlier. The same restriction site was earlier used by one of us to synthesize HIV-1 envelope RNA (substrate RNA) to test the cleavage potential of our multitarget ribozyme against the HIV-1 envelope gene [8].

2.5. Construction of antisense devoid of catalytic motif of DNA enzyme

This was synthesized chemically in a similar manner as other oligonucleotide except the 15 bases corresponding to the catalytic motif were omitted. The antisense had the following sequence: 5′- TACCC-CAAATAGAC. In this construction also the A nucleotide of the target gene was left unpaired.

2.6. Inhibition of HIV-1 envelope-CD4 mediated cell fusion by DNA enzyme

This was carried out by HIV-1 envelope and CD4 mediated cell membrane fusion assay as described initially by [20,21] and described in detail by us recently [22]. The HIV-1 envelope gene was cotransfected with 0.01 μg of DNA enzyme and T7-HIV-1 Env DNA along with the reporter gene under T7 promoter (T7-luciferase), which was fused with HeLa cells expressing CD4 glycoprotein (by DNA transfection) and infected with VTf-7. Recombinant vaccinia virus expressing T7 RNA polymerase [23] was used to drive the expression of the luciferase gene which was also placed under T7 promoter. In this kind of assay system only fusion dependent reporter gene activation is measured and the level of reporter gene expression is directly proportional to the extent of cell fusion. Recombinant vaccinia virus was grown in HeLa cells and purified according to the method described by Joklik [24] and as described before [25].

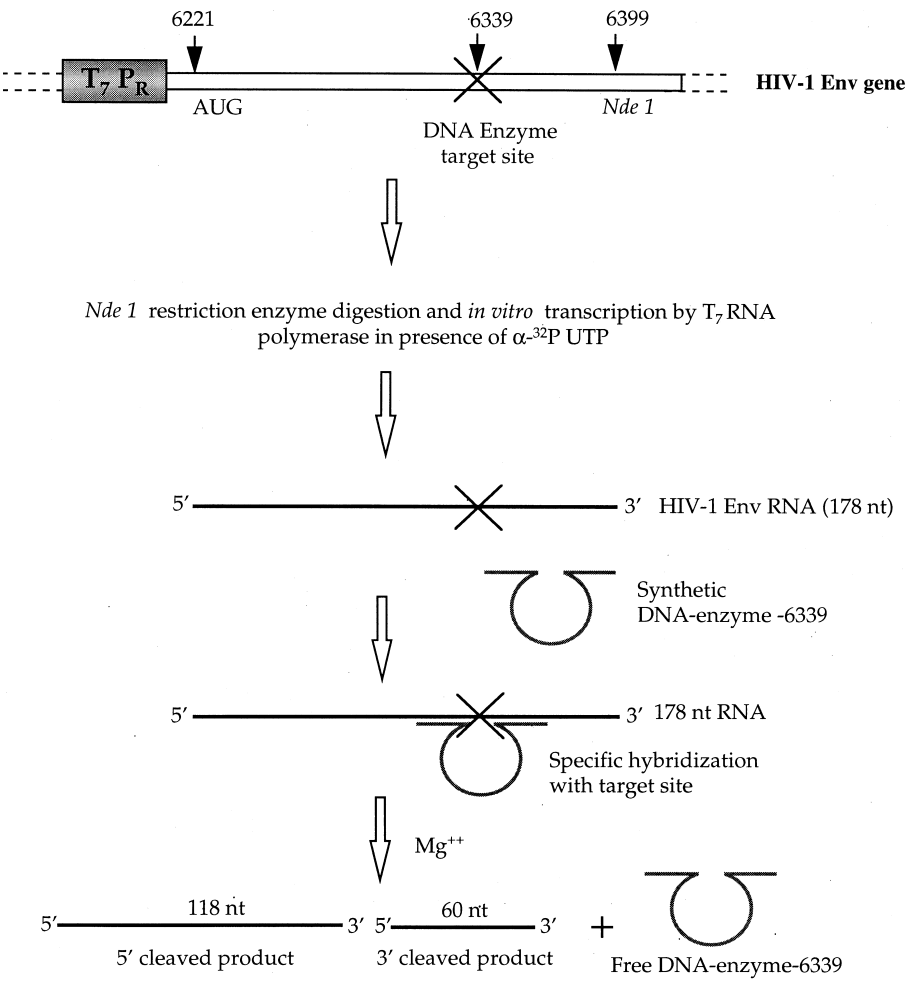

Fig. 2. In vitro synthesis of ^{32}P-UTP labeled 178 bases long HIV-1 RNA transcript using HIV-1 envelope plasmid DNA. The plasmid DNA was linearized with *Nde*I and subjected to in vitro transcription (Promega Biotech) using T7 RNA polymerase. An equimolar concentration of DNA enzyme (cold) was added and the cleavage reaction was initiated by adding varying concentrations of $MgCl_2$ and heating at 90°C for 3 min followed by incubation at 37°c for 1 h. The cleaved products were subjected to analysis on 7% 7 M urea-SDS-PAGE analysis. After cleaving the substrate RNA, the DNA enzyme is available to act on other substrate molecules and repeat the cycle.

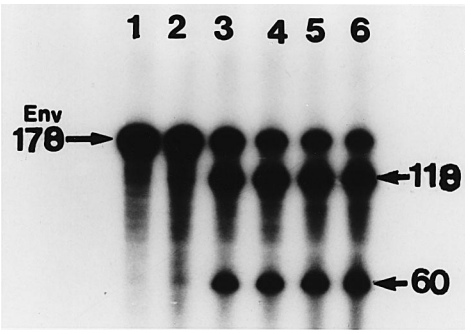

Fig. 3. Effect of magnesium chloride concentration on the cleavage activity. Labeled HIV-1 envelope transcript (178 bases long) was synthesized as described before and equimolar amounts of cold DNA enzyme were added in the presence of varying amounts of $MgCl_2$. The mixture was heated at 90°C for 3 min followed by 1 h incubation at 37°C and subjected to gel electrophoretic analysis as described before. Lane 1 shows the labeled transcript (178 bases) synthesized from the HIV-1 Env DNA that was linearized with *Nde*I. Very little cleavage of the substrate RNA was achieved in the presence of 1 mM $MgCl_2$ (lane 2) but cleavage increased significantly at 5 mM (lane 3), 10 mM (lane 4), 15 mM (lane 5) and 20 mM (lane 6). Also no cleavage was observed in any of these experimental conditions if the magnesium chloride was omitted from the cleavage reaction buffer (data not shown).

3. Results

3.1. In vitro cleavage of the 178 bp HIV-1 Env transcript by DNA enzyme

^{32}P-UTP labeled transcript (178 nt long) was synthesized by the *Nde*I digested HIV-1 envelope DNA (Fig. 3, lane 1) by T7 RNA polymerase. 100 pmol of target RNA and DNA enzyme were added in the presence of 1 mM $MgCl_2$, heated at 90°C for 3 min followed by incubation for 1 h at 37°C in the presence of varying amounts of $MgCl_2$. No cleavage was observed in the absence of Mg^{2+} (data not shown) and very little cleavage was seen at 1 mM $MgCl_2$ concentration (lane 2). The cleavage efficiency increased dramatically to the extent of 50–80% at concentrations of 5, 10, 15 and 20 mM $MgCl_2$, which correspond to lanes 3, 4, 5 and 6 respectively. No cleavage of the substrate RNA was observed when equivalent amounts of unrelated DNA enzyme were used or the antisense nucleotides (14 bases long which lacked only the catalytic motif) (data not shown). The fact that specific cleavage was seen at 1 mM $MgCl_2$ is a significant finding because more cleavage would, in principle, be obtained at 2 mM $MgCl_2$ which is the most likely concentration at physiological levels [16]. The specificity of cleavage of the Env RNA was further confirmed as DNA enzymes (possessing the same catalytic motif 10–23), when targeted for other regions of HIV (Nef and gag), failed to show any activity (data not shown).

Table 1
Kinetic analysis of DNA enzyme 6339

K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
0.416	0.763	1.83×10^9

Kinetic measurements were carried out for the DNA enzyme 6339 by taking varying amounts of labeled substrate RNA in 50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$ under DNA enzyme saturating conditions at 37°C for 1 h. The substrates and the cleaved products were separated on 7% polyacrylamide-7 M urea gels and detected by autoradiography of the dried gels. The extent of cleavage was determined by quantitating the radioactivity present in each band with the help of a phosphorimager. The kinetic parameters were calculated from a Lineweaver-Burk plot. Rate constants are averages from two separate experiments.

3.2. Kinetics of the cleavage reaction

More than 50% specific cleavage was seen by the DNA enzyme 15 min after the cleavage reaction (Fig. 4, lane 3). Cleaved 5′ (118 nt) and 3′ (60 nt) products could be seen as early as 5 min after the DNA enzyme interaction (lane 2). The extent of cleavage increased significantly at 15 min and beyond (lanes 3–7). Complete cleavage of the input substrate was not achieved even after 4 h of incubation (Figs. 4 and 5). The kinetic parameters were very similar to what has been reported earlier for ribozymes and DNA enzymes [16,26]. The K_m and k_{cat} values for this DNA enzyme 6339 are presented in Table 1. They are 0.416 nM and 0.763 min^{-1} and the ratio of k_{cat} over K_m was 1.83×10^9 M^{-1} min^{-1}.

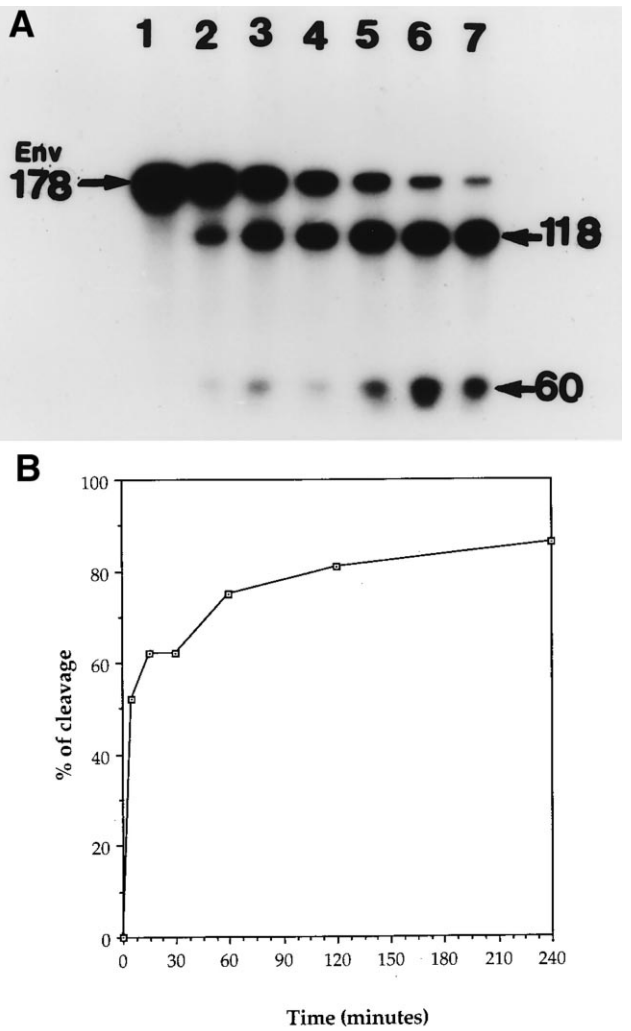

Fig. 4. Kinetics of the cleavage of HIV-1 envelope gene by DNA enzyme 6339. A: Equimolar concentrations of substrate and DNA enzyme were mixed and the cleavage reaction was initiated as described above for varying lengths of time (5 min to 4 h). Lane 1 depicts the synthesis of labeled HIV-1 envelope transcript (178 bases long). Lanes 3, 4, 5, 6 and 7 show the extent of cleavage achieved after 15 min, 30 min, 60 min, 120 min and 240 min respectively. Significant cleavage was obtained after 5 min of interaction (lane 2), which increased to more than 80% when the reaction was carried out for an extended period of time (B).

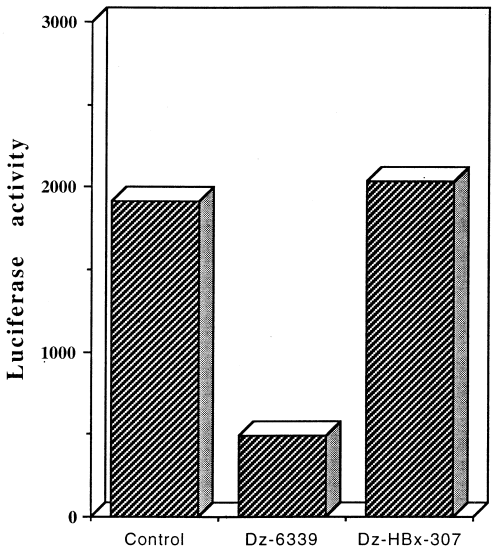

Fig. 5. Inhibition of HIV-1 Env-CD4 cell fusion by DNA enzyme 6339. HeLa cells were grown to 80% confluence in a 12 well plate (Nunc) and various combinations of plasmid DNAs were transfected in a final volume of 100 μl using lipofectin (Gibco-BRL, Bethesda, MD) as suggested by the manufacturer and described earlier [28,29]. One population of HeLa cells that was transfected with T7-luciferase DNA was cotransfected with HIV-1 envelope DNA together with equivalent amounts (0.01 μg) of either DNA enzyme 6339 or control DNA (pGEM-3Z: Promega Biotech). Equivalent amounts of an unrelated DNA enzyme (made against the X gene of hepatitis B virus) were used to compare the specific effects of DNA enzyme 6339. This population was fused with CD4 expressing HeLa cells which were also infected with VTf-7 (recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase). Four hours after fusion, the cell lysates were prepared and assayed for the luciferase activity in a luminometer (Promega Biotech). Values represent a mean of two experiments and the standard deviation was less than 20% of the average values. Dz is the abbreviated form for DNA enzyme.

3.3. Inhibition of cell membrane fusion by DNA enzyme

Significant inhibition of fusion was observed with Dz 6339 but an equivalent amount of unrelated DNA enzyme (targeted to cleave the X gene of hepatitis B) failed to show any inhibition (Fig. 5). The sequence of the DNA enzyme that was targeted to cleave the 307 nt of the X gene [27] of hepatitis B (abbreviated as HBx-Dz-307) virus was as follows: 5′-GTTGACA**GGCTAGCTACAACGA**TGCTGGGG-3′. The catalytic motif is the same as before and is shown in bold letters. This was used as a control DNA enzyme for the sake of comparison under identical experimental conditions. A similar pattern of inhibition was achieved with two other ratios of HIV-1 Env DNA and DNA enzyme (0.05 μg and 0.10 μg each; data not shown). No toxicity towards HeLa cells was observed at these concentrations of plasmid DNAs or oligonucleotides.

4. Discussion

We have shown precise cleavage of a 178 bases long HIV-1 envelope transcript and inhibition of the functional expression of HIV-1 envelope by cell fusion assays. It can be argued that the observed inhibition of the cell membrane could be due to the antisense flanks (14 bases altogether) of the DNA enzymes. We ruled out that possibility to a great extent by synthesizing a 14 bases long oligonucleotide (5′-TACCCCAAATAGAC) which lacked the catalytic motif but retained the antisense flanks. Only 10–15% specific inhibition was observed under similar experimental conditions (data not shown). We conclude that the observed inhibition by this DNA enzyme was most likely because of the catalytic cleavage of the HIV-1 Env RNA. Although we presented targeted in vitro cleavage of 178 bases long HIV-1 RNA transcript (obtained by digesting the HIV-1 envelope DNA by *Nde*I) by the DNA enzyme 6339 and showed its ability to interfere with the functional expression of HIV-1 envelope gene in a cell fusion assay, in vitro cleavage of longer HIV-1 envelope transcripts (819 and 1916 bases long transcripts generated by linearizing the HIV-1 envelope DNA with *Bgl*II and *Hin*dIII restriction enzyme digestions respectively) by this DNA enzyme was also observed but, as expected, the efficiency of the cleavage decreased with increasing size of the substrate RNA (data not shown). As was previously observed [16], the DNA enzyme described in this manuscript possessed a catalytic efficiency (k_{cat}/K_m) of $\sim 10^9$ M^{-1} min^{-1} under multiple turnover conditions.

Our data strongly suggest that these DNA enzymes could prove to be a valuable tool to selectively down regulate HIV replication or alternatively cleave any RNA (cellular or from any pathogen) and should be explored for therapeutic potential. Multitarget DNA enzymes could be used to cleave the target gene at multiple specific sites. This is especially important if the target gene is known to accumulate mutations. Most RNA viruses, including HIV-1, are known to mutate at an extraordinary rate after treatment with anti-retroviral drugs. Using this approach together with other treatment modalities, it may be possible to significantly delay the appearance of the resistant population of viruses.

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