Target sequence-specific inhibition of HIV-1 replication by ribozymes directed to tat RNA

Lun Quan Sun*, Li Wang, Wayne L. Gerlach and Geoff Symonds

The R.W. Johnson Pharmaceutical Research Institute Sydney, Johnson and Johnson Research Pty Limited, GPO Box 3331, Sydney NSW 2001, Australia

Received April 21, 1995; Revised and Accepted June 30, 1995

ABSTRACT

The structural motif formed between a hammerhead ribozyme and its substrate consists of three RNA double helices in which the sequence 5' to the XUY is termed helix I and the sequence 3' to the XUY helix III. Two hammerhead ribozymes targeted to the tat gene of HIV-1SF2 were designed to study target specificity and the potential effect of helix I mismatch on ribozyme efficacy both in vitro and in vivo. The first ribozyme (Rz1) targeted to the 5' splicing region of the tat gene was designed to cleave GUC*A. In HIV-I1IB the A is changed to a G. The second ribozyme (Rz2) was targeted to the translational initiation region of the tat gene which is highly conserved amongst a variety of HIV-1 isolates, including both HIV-1SF2 and HIV-1I1IB. In vitro cleavage studies demonstrated that Rz1 efficiently cleaved HIV-1SF2 substrate RNA, but not HIV-1I1IB, presumably due to the base change from A to G. In contrast, Rz2 cleaved HIV-1SF2 or HIV-1I1IB substrate with equal efficiency. Both ribozymes were cloned into the 3' untranslated region of the neomycin gene (neo) within the pSV2neo vector and transfected into the SupT1 human CD4+ T cell line. Following selection, stable transfectants were challenged with either HIV-1SF2 or HIV-1I1IB virus. While Rz1-expressing cells were significantly protected from HIV-1SF2 infection, they exhibited no protection when infected with HIV-1I1IB virus. In contrast, Rz2 was effective in inhibiting the replication of both HIV-1SF2 and HIV-1I1IB in SupT1 cells. Expression of both ribozymes in these cells was demonstrated by Northern analysis. RT-PCR sequencing analysis confirmed the respective HIV-1 target sequence integrity. These data demonstrate the importance of the first base pair distal to the XUY within helix I of the hammerhead structure for both in vitro and in vivo ribozyme activities and imply that the effectiveness of the anti-HIV-1 ribozymes against appropriate target sequences is due to their catalytic activities rather than any antisense effect.

INTRODUCTION

Ribozymes are catalytic RNA molecules that possess RNA target-specific endoribonuclease activity (reviewed in 1,2). This property leads to a range of potential applications to gene inactivation both in vitro and in vivo. In particular, ribozyme RNAs expressed intracellularly from a ribozyme construct have been successfully applied to inhibit the replication of the human immunodeficiency virus type 1 (HIV-1) in cell culture (3–7), by targeting a variety of sites within the HIV-1 genome. These include the gag gene (3), 5' leader sequence (4,6), tat gene (5) and the viral packaging region (7).

Among several different types of ribozymes, the hammerhead ribozyme has been particularly attractive for application due to its small size in design and a catalytic efficiency both in cis and trans. In the hammerhead structure (8–10), the flanking region found in the ribozyme RNA 5' to the catalytic domain forms helix I in the ribozyme–substrate complex, while the 3' terminal region forms helix III, and a catalytic core is formed by 13 conserved nucleotides and a stem–loop (helix II). The latter structure has been the subject of nucleotide substitution studies to obtain information on the role of nucleotides in the cleavage mechanism (11,12). However, few studies have been conducted regarding the effect of helix I or III nucleotide substitutions on hammerhead ribozyme activity both in vitro and in vivo, despite the fact that a number of kinetic studies have shown that the secondary structure of substrate RNA can be a major determinant of hammerhead catalytic efficiency (13).

In the present study, we describe two anti-tat ribozymes, one targeted near to the 5' splicing region of the tat gene (Rz1); the other to the region near the initiation codon of the tat gene (Rz2). While the target sequence for Rz2 is highly conserved amongst different HIV-1 isolates, sequence variation around the Rz1 target site is frequently observed. This variation is often seen at N in XUY*N in helix III, in which N is A in some cases such as HIV-1SF2, and G in the other cases such as HIV-1I1IB. This provides a model system in which to explore the potential effect of mismatch at the first base pair after XUY in helix I on hammerhead ribozyme in vitro and in vivo efficacy.

MATERIALS AND METHODS

Cell line and virus stocks

The human T lymphocyte cell line SupT1 (ATCC CRL-1942) was grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS, CytoSystem, Sydney). HIV-1I1IB and HIV-1SF2 viruses were propagated by acute infection of HUT78 cells. Virus TCID50 was determined on SupT1 cells using P24 ELISA to detect the end-point as previously described (14).
Construction of anti-tat ribozymes

Two hammerhead ribozymes were designed according to the genomic sequence of the HIV-1SF2 isolate (Fig. 1). Ribozyme 1 (Rz1) was directed to a GUC site in the 5' splicing donor sequence of the tat gene (nucleotides 5784-5801). Ribozyme 2 (Rz2) was targeted to a GUA site within the 5' region of the tat coding sequence (nucleotides 5840-5859). The ribozyme constructs were engineered by cloning synthetic double-stranded inserts into either a SmaI site of pGEM3zf(+) vector (Promega) for in vitro transcription and cleavage assays, or a SmaI site in the 3' untranslated region of the neomycin resistance gene (neo) of the pSV2neo vector (15) by blunt-ended ligation for expression of the ribozymes as chimeric genes within the T cell line. The sequence and orientation of the inserts were confirmed by DNA sequencing.

In vitro cleavage assays

Two 18 residue substrate RNAs were chemically synthesised, based on HIV-1SF2 (AUUGGGUGUCAACAUAAGC) or HIV-IIIIB (AUUGGGUGUCAGACAUAGC) for Rz1. A conserved sequence for both HIV-1SF2 and HIV-IIIIB, GGACCAUGAGAUCUCUAA, was synthesised for Rz2. RNA substrates were end-labelled with [γ-32P]ATP using T4 polynucleotide kinase in the presence of RNase inhibitor RNasin (Promega). The ribozymes were generated as in vitro T7 RNA polymerase transcrits from pGEM3zf(+). The cleavage reaction conditions were 50 mM Tris–HCl (pH 7.5) and 10 mM MgCl2, with a ribozyme to substrate ratio of 2:1. Reactions were initiated by pre-incubating the ribozyme and substrate at 80°C for 2 min, followed by adding 5x cleavage buffer and further incubating at 37°C. Reactions were stopped at various time points by adding formamide loading buffer with EDTA and the mixtures then fractionated by denaturing polyacrylamide gel electrophoresis. The cleavage products were quantified by phosphorimaging (Molecular Dynamics).

Transfection of SupT1 cells with pSV-Rz1 and pSV-Rz2 constructs

The pSV2neo-ribozyme constructs were electroporated into SupT1 cells as described previously (7). Briefly, exponentially growing cells were harvested, washed with PBS and resuspended at a density of 1 x 10^7 viable cells per ml in RPMI-1640 medium without FBS but containing 10 mM dextrose and 0.1 mM dithiothreitol. Mixtures of 0.4 ml cell suspension and 10 μg plasmid DNA were subjected to a single pulse of 960 up, 200 V from a Gene Pulser (BioRad). Cells were then seeded in RPMI medium supplemented with 10% FBS and at 48 h the medium changed to include G418 (800 μg/ml). Selection was conducted for 2 weeks.

HIV-1 challenge assays

Stably transfected SupT1 cells (5 x 10^6) (pooled population) were infected with 1000 TCID50 dose of HIV-1SF2 or HIV-IIIIB virus. Two hours post-infection, the cells were washed and resuspended in 10 ml of fresh medium. Supernatant was taken every 3-4 days for p24 assay using the Coulter HIV-1 p24 ELISA Kit.

Molecular analysis

To confirm integrity of the target sequences for Rz1 and Rz2 within HIV-1SF2 and HIV-IIIIB, a direct RT-PCR sequencing was carried out (16). HIV-1 viral RNA was extracted from the infected
cell cultures by using a silica-based protocol (17). Reverse transcriptase reactions were performed using the primer P1 (Fig. 5A). To generate single-stranded DNA (ssDNA) for sequencing, two rounds of PCR were carried out in the presence of the primers P1 and P3 for the first, and P2 alone for the second reaction using a small aliquot of the first PCR reaction mix (16). The second PCR mixture was purified by passing through a 30 000 MW cut-off ultrafilter (Millipore). ssDNA was sequenced using the primer P4 according to the Sequenase protocol (US Biochemicals). Figure 5A shows the position of each of the primers.

Ribozyme expression in SupT1 cells was also examined by Northern analysis as described (7). A 32P-labelled 22 nucleotide antisense oligomer to the ribozyme core region was utilised to detect ribozyme specific signal.

RESULTS

Design of anti-tat ribozymes

Three primary considerations were used in selecting ribozyme targeting sites within the HIV-1 genome. First, the product of the HIV-1 tat gene is absolutely required for viral replication (18,19). Second, during early HIV-1 gene expression, 11 different HIV-1 exons are present in the multiply spliced mRNAs in which the exon 4 is involved in most of cases, including tat mRNA (20). Therefore, ribozymes targeted to the tat sequence should also cleave all of the mRNA containing the exon 4 as well as pre-mRNA. Finally, previous studies have shown that the 5' end of mRNA, AUG initiation codon region and the sequences involved in RNA splicing and polyadenylation appeared to be more accessible to antisense oligomers (21,22). In the current study, therefore, two sites were chosen around the 5' splicing acceptor region (Rz1) and the 5' end of mRNA (Rz2) of the HIV-1SF2 tat gene. For the Rz2 target sequence, upon alignment with the HIV-1 sequences from GenBank, a complete sequence conservation was observed in all the HIV-1 isolates examined. For the Rz1 site, a lesser degree of conservation was revealed. In particular, the nucleotide 3' adjacent to GUC tends to be an A in some isolates, such as HIV-1SF2, and G in other isolates such as HIV-1IIIB.

For in vitro studies, ribozymes were cloned into the 3' untranslated region of the neomycin-resistance gene (neo), a chimeric molecule containing both the neo mRNA and ribozyme sequences will therefore be produced in the cells. Advantages of this strategy are 2-fold: resistance to G418 indicates the expression of ribozyme; and ribozyme molecules as part of a chimeric gene product could be more stable (7).

In vitro cleavage efficiency of Rz1 and Rz2

Both Rz1 and Rz2 transcripts were tested for their ability to cleave the corresponding substrates in vitro. The cleavage rates were measured as a function of time. As shown in Figure 2, Rz2 efficiently cleaved an identical substrate from either the HIV-1SF2 or HIV-1IIIB at 37°C (45.3% at 30 min) (Fig. 2A). Rz1 showed a similar cleavage efficiency to Rz2 on HIV-1SF2 substrate under the same conditions (40.2%) but it exhibited only very weak cleavage activity on the HIV-1IIIB substrate (7.3%) (Fig. 2B). This data indicates the crucial role of the first base pair after the GUC of helix I in the activity of hammerhead ribozyme.

Inhibition of HIV-1 replication in SupT1 cells

Stable transfected SupT1 cells bearing the ribozyme constructs, pSV-Rz1, pSV-Rz2 and the parent vector pSV2neo, were selected and maintained in culture medium containing G418. The presence of vector DNA in these cells was confirmed by Southern analysis (data not shown). Northern blot hybridisation demonstrated expression of the ribozyme genes in SupT1 cells (Fig. 3). In order to assay the anti-viral efficacy of the ribozyme constructs in vivo, the pooled population of transfected cells was used in HIV-1 challenge experiments. When infected with the HIV-1SF2 isolate, viral replication as estimated by p24 ELISA in both Rz1 and Rz2 expressing SupT1 cells was shown to be significantly inhibited (Fig. 4). By contrast with the HIV-1IIIB infected cells, only the Rz2 expressing cells showed suppression of viral replication (Fig. 4). This observation parallels the in vitro cleavage results in which Rz1 could only efficiently cleave HIV-1SF2 (40.2%), and not HIV-1IIIB substrates (7.3%). However, the weaker cleavage by Rz1 on HIV-1IIIB in vitro and inactivity of Rz1 to HIV-1IIIB in cells may reflect that in vivo cleavage is more sensitive than the in vitro assays.

HIV-1 sequence analysis of the ribozyme target regions in HIV-1SF2 and HIV-1IIIB

To rule out the possibility that mutations in the ribozyme target sequences in the HIV-1 isolates have occurred during the viral infection and replication process, sequencing analysis was performed on RT-PCR products generated from the viral RNA which was collected at day 12 post HIV-1 infection. The results shown in Figure 5B revealed that the target sequences for Rz1 (GUC*Δ in HIV-1SF2, GUC*Δ in HIV-1IIIB) and Rz2 (GUA*G in both HIV-1SF2 and HIV-1IIIB) remained unchanged. This confirmed that the differences observed in Rz1 efficacies both in vitro and in vivo for HIV-1SF2 and HIV-1IIIB were due to the A to G variation after the GUC in helix I.

DISCUSSION

We have described two ribozymes which are targeted to different regions of the tat gene, both of which can significantly inhibit HIV-1 replication in stably transfected T cells. This in vivo effect of the ribozyme constructs is consistent with their ability to cleave the respective substrates in vitro, depending on the hammerhead helix I structure. Since pooled transfecteds were used in HIV-1 challenge assays, this implies that the effect was not due to clonal variation or different copy numbers of the constructs within the transfected population. To the best of our knowledge, this is the first report demonstrating a direct correlation between in vitro and in vivo activities of the hammerhead ribozyme with the mismatch in the first base pair after the GUC in helix I. Previous kinetic studies of hammerhead ribozyme have shown that changes in substrate length and sequence could exert significant impact on ribozyme cleavage, substrate dissociation and product release (13,23). Those changes that eliminated catalytic activity could potentially increase the stability of hammerhead complex. In those experiments, multiple turnover kinetic analyses were performed (23). The reasons why the ribozyme is not active on mismatched substrate could be very complex. One of the possible explanations might be that reduction in the activity of Rz1 on HIV-1IIIB is due to formation of a more stable structure derived from an altered helix I. However, more detailed kinetic study still
The ‘antisense’ complementary interaction is necessary for ribozyme to bind substrate. Unfortunately, to what extent the antisense effect contributes to ribozyme activity is still very difficult to determine. In the present study, the consistency between in vitro and in vivo results strongly argues for the inhibitory effect being due to ribozyme catalytic activity. In a separate study, we have found that a long antisense sequence to the HIV-1 5’ leader region could inhibit a range of the HIV-1 isolates regardless of the mutations within certain regions among the different isolates (24). This reflects less sensitivity to the base change for antisense compared with ribozyme. Furthermore, two recent publications showed similar results to ours (one in vitro study of hammerhead ribozyme, another both in vitro and in vivo study of hairpin ribozyme) (25,26). Taken together, these results indicate that mismatch has a greater effect on ribozyme cleavage activity, rather on the antisense binding.

Ribozyme-based strategies provide a promising gene therapeutic approach towards HIV-1 infection. Although in most cases anti-HIV ribozymes have been shown able to protect T cells from HIV-1 replication in cell culture, attempts to demonstrate in vivo cleavage of viral transcripts have not been successful nor pursued in detail. Therefore, the choice of experimental controls has been

Figure 2. In vitro cleavage study of anti-tat ribozymes. (A) The cleavage efficiency of Rz1 on HIV-1SF2 and HIV-IIIB RNA substrates. (B) Cleavage of HIV-1SF2 or HIV-IIIB RNA substrate (identical in this region) by Rz2. The assay conditions are described in the Materials and Methods.

Figure 3. Expression of ribozyme constructs in transfected SupT1 cells. Total RNA (15 μg) from SupT1 cells (lane 1), SupT1 cells containing pSV2neo (lane 2), pSV2neo/Rz1 (lane 3) and pSV2neo/Rz2 (lane 4) were blotted onto a nylon membrane and hybridised with a ribozyme-specific oligomer probe (TTCGTCCTCAGGGACTCATCAG).
Figure 4. Inhibition of HTV-1 replication in ribozyme-expressing SupT1 cells. Pooled cells transfected with Rz1, Rz2 or pSV2neo constructs were challenged with either HIV-1SF2 or HIV-1IIIB viruses. Samples were collected at days 8 and 12 post-infection. Data are expressed as the percentage of vector-containing cells for p24 production.

Figure 5. Direct sequencing of the target sequences of HIV-1SF2 and HIV-1IIIB. (A) Schematic representation of PCR-amplified regions around the Rz1 target site. The first-round PCR was performed using the primers P1 (TAGCAATGAAACACT) and P3 (TTTAGAGGAGCTTAAGAGAGA). The primer P2 (AGTCCTAGGCTGACTTCCTGG) was used for the second-round PCR to generate ssDNA. P4 was utilized as a sequencing primer (CTTAAGAGAGAAGCTGTTA). (B) RT-PCR sequencing of Rz1 cleavage sites in HIV-1SF2 and HIV-1IIIB. For details, see Materials and Methods.

regarded as a crucial factor in evaluating efficacy of anti-HIV-1 ribozymes. Our data present an alternative means of utilising experimental controls in anti-viral assays, namely, the use of an altered substrate such as an HIV-1 isolate with a mutation at or around the cleavage site. It will be the subject of further investigation to determine whether the present observation can be generalised when applied to other substrates.

In conclusion, the mismatch of the first base pair after the XUY in helix 1 of the hammerhead structure was able to significantly affect ribozyme activity in vitro. It was reflected in protection assays in vivo and is consistent with the notion that in vivo ribozyme efficacy in inhibiting HIV-1 replication is related to cleavage activity.

ACKNOWLEDGEMENTS
We thank Ron Penny, David Cooper for advice and PC3 facilities, and Janet Macpherson for phosphorimaging analysis and preparation of figures. This work was supported by a Research and Development Grant from Gene Shears Pty Ltd.

REFERENCES