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Research paper

Conserved sequences in the current strains of HIV-1 subtype A in Russia are effectively targeted by artificial RNAi *in vitro*

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ABSTRACT

Highly active antiretroviral therapy has greatly reduced the morbidity and mortality of AIDS. However, many of the antiretroviral drugs are toxic with long-term use, and all currently used anti-HIV agents generate drug-resistant mutants. Therefore, there is a great need for new approaches to AIDS therapy. RNAi is a powerful means of inhibiting HIV-1 production in human cells. We propose to use RNAi for gene therapy of HIV/AIDS. Previously we identified a number of new biologically active siRNAs targeting several moderately conserved regions in HIV-1 transcripts. Here we analyze the heterogeneity of nucleotide sequences in three RNAi targets in sequences encoding the reverse transcriptase and integrase domains of current isolates of HIV-1 subtype A in Russia. These data were used to generate genetic constructs expressing short hairpin RNAs 28–30-bp in length that could be processed in cells into siRNAs. After transfection of the constructs we observed siRNAs that efficiently attacked the selected targets. We expect that targeting several viral genes important for HIV-1 reproduction will help overcome the problem of viral adaptation and will prevent the appearance of RNAi escape mutants in current virus strains, an important feature of gene therapy of HIV/AIDS.

1. Introduction

Despite the evident success of the current antiretroviral combination therapy, the emergence of drug resistance and toxicity associated with long-term use of the drugs often leads to treatment failure. There is a great need for new approaches to AIDS therapy. RNAi is a potent natural mechanism that regulates gene expression and provides protection from foreign nucleic acids. RNAi and related mechanisms have been extensively studied in recent years, and RNAi is now one of the most useful tools for gene-specific silencing. The central components of the RNAi machinery are siRNAs, which silence genes by promoting the cleavage of mRNAs with matching complementary sequences (Novina and Sharp, 2004). Libraries of siRNAs, or DNA constructs expressing siRNAs, have been generated to target many human genes to help determine their function (Elbashir et al., 2001; Du et al., 2006). Using this approach, many disease-associated genes have been identified. siRNAs might be even used to treat these diseases. In the case of HIV/AIDS, the genes that could be targeted by siRNA for therapeutic purposes are the viral genes. Other potential targets are the mRNAs for HIV receptors and the host genes CCR5 and CD4 (Boden et al., 2007). The silencing of cellular co-factors by RNAi needs to be balanced with the negative effects that inhibition of essential cellular genes is likely to have on host cell functions (Stevenson, 2003). Whereas targeting CD4 may be deleterious to the cell and the organism due to the critical role of this receptor in host immunity, CCR5, which regulates trafficking of memory/effector T-lymphocytes and some other cells, is a redundant receptor whose inactivation may be tolerated by the organism (Oppermann, 2004; Iordanskiy et al., 2013). Indeed, individuals defective for CCR5 expression are healthy and show resistance to HIV-1 infection (O'Brien and Moore, 2000; Anderson et al., 2003).

Several years ago we selected several new biologically active siRNAs targeting moderately conserved regions in HIV-1 transcripts (Alembekov et al., 2011). In this study, we analyzed reverse transcriptase and integrase domain mRNA targets from clinical isolates of HIV-1 subtype A in Russia, in order to make genetic constructs expressing siRNAs that perfectly match those targets in those current viral isolates. Our data indicate that mutations in the targets are located mostly in the third positions of codons, and that some create premature stop codons that should result in nonfunctional reverse transcriptase and integrase. We observed that genetic constructs producing siRNAs exactly corresponding to the sequences detected in the current strains of the virus efficiently targeted the

Abbreviations: RT, reverse transcriptase domain; *int*, integrase domain; Pol, domain specifying protease, reverse transcriptase, RNase and integrase in HIV-1; A1–A3, about 300-bp regions of pol-domain containing the targets of RNAi.

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viral RNA sequences. We conclude that these data could be used to develop a gene therapy strategy using artificial RNAi, based on the monitoring of the targets in current variants of the virus.

2. Materials and methods

2.1. Collection of samples and virus isolation

RNA preparations were provided by the State Research Center of Virology and Biotechnology Vector (Russia) from their collection of isolates. Five isolates of HIV-1 subtype A, 10RU6587, 11RU6933, 11RU6949, 10RU6483, and 11RU1996, collected by N.M. Gashnikova, were used. RNA was extracted from 500 μL of plasma samples using ViroSeq reagents (Celera Diagnostics) and then treated with DNase using a DNA-free kit (Ambion). Concentration of RNA preparations was measured using NanoDrop 2000.

2.2. RT-PCR

Five RNA preparations were pooled (6 ng of each) and used for RT-PCR. Approximately 15 ng of total RNA and M-MLV reverse transcriptase were used to synthesize cDNAs by use of a DNA-free kit (Ambion) according to the manufacturer's instructions. cDNAs corresponding to two regions in the reverse transcriptase domain and to one region in the integrase domain from the *pol* gene were synthesized using the primers indicated in Table 1. Nested PCR was used for amplification of regions of approximately 300 bp containing the selected RNAi targets (Alembekov et al., 2011). Table 1 shows the primers used for the second PCR. Primers were selected using the Primer Selection Tool (http://biotools.umassmed.edu/). The conditions for PCR for each set of primers were determined in preliminary experiments using a Mastercycler personal PCR instrument (Eppendorf). The identity of amplified DNA fragments was confirmed by cloning and sequencing.

2.3. Cloning procedures

RT-PCR products were cloned into the pGEM-T Easy Vector (Promega) and sequenced. Sequences of hairpins (Table 2) were inserted into the GeneClipU1 Neomycin vector (Promega) according to the manufacturer's instructions. The sequences containing the corresponding RNAi targets (Table 3) were cloned into the psiCHECK-2 vector (Promega) using *Xho* I and *Not* I cloning sites within the 3′ untranslated region (UTR) of the *Renilla* gene. The correctness of the constructs was checked by sequencing. For RNase protection assays, the 50-bp chemically synthesized regions containing centrally located RNAi target sites (Table 3) were cloned into the vector pGEM-1 (Promega). Clone DNA samples were then digested with *Hind*III and used for the synthesis of strand-specific [^{32}P]-labeled RNA probes by T7 RNA polymerase.

Table 1
Primers used for RT-PCR.

№	Text of primer (5′–3′)	Used for	Domain
1	TTTGATATGTCCATTGGTCTAGCCCTTGTT	Primer extension, (−) primer	RT-A1
2	CATCTATTGAGATGGGGATTTACCA	PCR, (+) primer	RT-A1
3	CTCTGCCAATTCCAATTCTG	PCR, (−) primer	RT-A1
4	CTCTGTTAGTGCTTTGGTCCCCCTAAGGAG	Primer extension, (−) primer	RT-A2
5	GACAAAGATCTTAGAGCCCTTTAGA	PCR, (+) primer	RT-A2
6	TATATCATTGACAGTCCAGCTTTCC	PCR, (−) primer	RT-A2
7	TTCTTGGTACTACCTTTATTTCGTTATTGTCTTG	Primer extension, (−) primer	Int-A3
8	AGAATTTGGAATTCCCTACAATCCC	PCR, (+) primer	Int-A3
9	TCTGCTGTCCCTGTAATAAACCC	PCR, (−) primer	Int-A3

Sequences were selected using the corresponding regions in AF316544 sequence.

2.4. Transfection assays

HEK293T culture cells were plated one day prior to transfection (5×10^5 cells per 60 mm culture dish). To prepare liposomes, 10 ng of the experimental DNA constructs containing the targets of RNAi cloned in psiCHECK-2 were mixed with 100 ng of the corresponding DNA constructs expressing RNA hairpins cloned in the GeneClipU1 Neomycin vector in 600 μL of serum-free medium. Next, 1 μL of TransFast reagent (Promega) diluted according to the manufacturer's recommendations was added, and the mixture was incubated at room temperature for 15 min. The cell suspension (0.2 mL, ~5×10^4 cells) was centrifuged at 2000 rpm/min in 1.5-mL Eppendorf tubes for 3 min at room temperature, and the precipitate was mixed with 60 μL of the liposome-containing sample. After incubation for 1 h at 37 °C, the transfected cells were transferred into a 24-well plate containing 0.5 mL per well of the cell culture medium supplemented with serum, and cells were incubated for 72 h. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and a Reporter Microplate Luminometer (Turner BioSystems). The *Renilla* luciferase data were normalized to the firefly luciferase data. Excel and Origin software were used for data analysis and graphing.

2.5. Isolation of RNA preparations from transfected HEK293T cells

Total RNA was isolated as described (Tchurikov et al., 2009). Transfected cells were suspended in DMEM and pelleted by 5 min centrifugation in an Eppendorf 5804 R centrifuge (3 krpm, 2 °C). The pellet was homogenized at 0 °C in Trizol reagent (Invitrogen) using insulin syringes. Next, 1/5 volume of chloroform was added to each sample and, after vortexing for 1 min, the mixture was incubated at room temperature on a rolling wheel. After centrifugation for 10 min in an Eppendorf 5415 R centrifuge (13 krpm, 2 °C), the supernatant was collected and precipitated by the addition of 1.5 volumes of isopropanol. The final pellet was washed three times in 70% ethanol–0.1 M NaCl, dried, and dissolved in milliQ water, and the RNA concentration was measured using a NanoDrop. For digestion of traces of DNA in the total RNA samples, a DNA-free kit (Ambion) was used. Next, 25 μL of solution containing 6 μg of RNA were mixed with 2 μL of DNase-Turbo and incubated for 1 h at 37 °C. Then, 3 μL of DNase-inactivation buffer were added and the mixture was vortexed for 5 s and then incubated for 3 min at room temperature with gentle mixing. After centrifugation at 2 °C for 10 min, at 13 krpm in a MiniSpin centrifuge (Eppendorf), the supernatant containing DNA-free total RNA was collected.

2.6. RNase protection assays

pGEM-1 plasmids containing 50-bp sequences comprising the RNAi targets were digested completely with *Hind*III and used as templates for the synthesis of strand-specific [^{32}P]-labeled RNA probes as previously described (Tchurikov and Kretova, 2011). Next, [^{32}P]-labeled RNA was synthesized in 20-μL reactions containing 1 μg of DNA template, 40 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 U/μL RNasin, ATP, GTP and CTP (500 mM each), 0.75 μM [α-^{32}P]-UTP (6000 Ci/mmol, EIMB), 10 μM unlabeled UTP, and 20 U T7 RNA polymerase (Fermentas). The mirVana miRNA detection kit (Ambion) was used for the purification of the [^{32}P]-labeled RNA probes, for hybridization and for RNase treatment. The [^{32}P]-labeled RNA probes were gel-purified by separation on 52-cm long denaturing 12% polyacrylamide gels, 0.2 mm thick, to isolate the full-length RNA species and to remove shorter fragments. About 2–5 μg of total RNA isolated from transfected HEK293T cells were mixed with about 50,000 cpm of labeled RNA in a 20-μL hybridization mixture (mirVana miRNA detection kit, Ambion), heated for 3 min at 100 °C and hybridized at 42 °C for 16 h. After hybridization, the samples were treated with an RNase A/RNase T1 solution according to the manufacturer's instructions. RNase dilutions were determined experimentally in