Downregulation of HIV-1 vif by a hammerhead ribozyme expressed from a retroviral vector

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HIV infection of $CD4^+$ T helper cells results in a gradual deterioration of immune function and leads to the onset of the Acquired Immune Deficiency Syndrome (AIDS). Current research suggests that HIV infection may be combated with ribozyme therapy. Hammerhead ribozymes are small, catalytic RNAs that can be designed to cleave substrate RNAs at specific sequences, and those targeted to HIV-1 mRNAs have been shown to greatly reduce or inhibit viral replication. The HIV-1 *virion infectivity factor* (*vif*) gene encodes a protein that counteracts an innate, antiretroviral defense mechanism of non-permissive $CD4^+$ T helper cells. This mechanism is mediated by apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G), a cellular cytidine deaminase that is encapsulated into assembling virions in the absence of *vif* and is inhibitory during the next round of viral replication. Vif neutralizes APOBEC3G by reducing its translation and by rapid degradation of the native protein. *Vif* mRNA, therefore, may be a good target for ribozyme mediated inhibition of HIV-1 replication. To test this hypothesis a catalytic hammerhead ribozyme targeted to nucleotide 5113 of the HIV-1 genomic clone NL43 (Accession # M19221) was designed and synthesized. A non-catalytic control, Vif5113Δ was also designed and synthesized. Vif5113 and Vif5113Δ were cloned into the retroviral vector, pSuper.retro.puro (pSRP) to facilitate tissue culture studies. In this study, Vif5113 and Vif113Δ ribozymes were analyzed for their ability to reduce *vif* expression in an intracellular cleavage assay. These studies, as determined by Western blot analysis, suggested that *vif* expression was reduced in the presence of the catalytic ribozyme Vif5113.

Introduction

HIV-1 is a retrovirus that infects $CD4^+$ T helper cells (1;2) resulting in a gradual deterioration of immune function and eventually leading to the onset of the Acquired Immune Deficiency Syndrome (3-6). In December 2007, the World Health UNAIDS Organization estimated that 33 million people worldwide were living with HIV/AIDS. It was also estimated that 14,000 people worldwide become newly infected with the Human Immunodeficiency Virus (HIV) every day (7).

The HIV-1 genome encodes nine viral genes from which fifteen functional gene products are expressed (8). One of these genes, the *virion infectivity factor* (*vif*) encodes a 23 kD protein that counteracts an innate, antiretroviral defense mechanism of $CD4^+$ T helper cells (9), the primary target of HIV (2). This resistance to HIV infection is due to the expression of APOBEC3G (human apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G), which acts to inhibit reverse transcription of retroviruses (8).

APOBEC3G expression is stimulated by certain viral proteins such as HIV-1 Vif. Normally, APOBEC3G is encapsulated into progeny virions, where it remains non-functional until the virion infects its host cell. Upon infection of the host cell and reverse transcription of the viral genome, APOBEC3G induces hypermutation from C to U in the minus strand of viral DNA resulting in G to A mutations in the positive sense DNA strand. These mutations in the viral genome inhibit normal expression of viral genes and render the target cell incapable of producing progeny virions and facilitating a productive infection (8).

Vif counteracts this activity by binding APOBEC3G and targeting the protein for degradation through the ubiquitin pathway (10). We hypothesized that downregulating *vif* expression in infected cells would reconstitute the normal antiviral activity of APOBEC3G. Previous research suggested that HIV infection may be combated with ribozyme therapy (11-13). Hammerhead ribozymes are small, catalytic RNAs that can be designed to target and cleave substrate RNAs at sequence specific sites (14). These ribozymes cleave mRNAs at the target sequence XUX' where X is A, C, G, or U and X' is A, C, or U (15). In this report, a hammerhead ribozyme targeted to HIV-1 vif was designed and cloned into the retroviral vector, pSuper.retro.puro (16;17). This vector was chosen due to its ability to express siRNAs from the RNA Polymerase III H1 promoter, and we hypothesized that it would also efficiently express ribozymes. As a control, a non-catalytic ribozyme targeted to the same HIV-1 *vif* sequence was designed and cloned. These constructs were tested for their antiviral activity in a vif inhibition assay. These results suggested that *vif* expression was reduced in the presence of a hammerhead ribozyme.

Materials and Methods

Cloning

The HIV-1 NL43 *vif* sequence (Accession number M19921) was analyzed for the presence of potential hammerhead cleavage sites (15). One such sequence, a pGUA was located at nucleotide 5113. This sequence along with its immediate flanking sequences were used to generate a hammerhead ribozyme according to the Haseloff and Gerlach model (14). A non-catalytic control ribozyme was generated by an A to G substitution within the ribozyme catalytic core (12) (Figure 1).

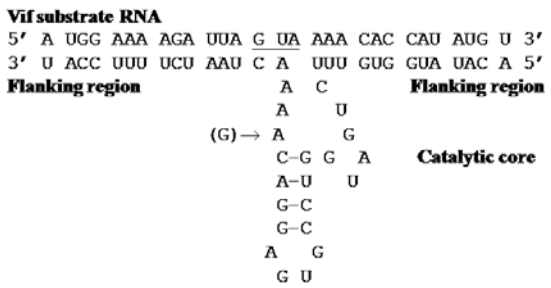

Figure 1. Hammerhead ribozyme design. Vif RNA containing the pGUA cleavage site at nucleotide 5113 is shown annealed to the ribozyme flanking regions. An A to G mutation in the catalytic core converted the ribozyme to a non-catalytic form.

The Vif5113 and Vif5113Δ ribozymes were synthesized (5'ACA TAT GGT GTT TCT GAT GAG TCC GTG AGG ACG A/GAA CTA ATC TTT TCC AT 3') and cloned into the shuttle vector pPCR-Script (Stratagene) as previously described (18). Correct insertion of the ribozyme in the resulting plasmids pVif5113 and pVif5113Δ was verified by sequencing.

To study ribozyme-mediated anti-Vif activity in cell culture, each ribozyme was moved into the retroviral vector, pSuper.retro.puro (Oligoengine). For this ribozyme-specific primers were designed and synthesized to include HindIII and BglII restriction sites: Vif5113BglII Forward (5' ATT AGA TCT ACA TAT GGT GTT TCT GAT GAG 3') and Vif5113HindIII Reverse (5' ATT AAG CTT ATG GAA AAG ATT AGT TTC G 3'). Each ribozyme was amplified using Vent DNA Polymerase (New England Biolabs) and re-cloned into pPCR-Script. The BglII/HindIII ribozyme fragments from each of the resulting plasmids were gel purified and cloned into the similarly digested pSuper.retro.puro vector. Accurate cloning was verified by sequencing and the resulting retroviruses, pSRPVif5113 and pSRPVif5113Δ, were analyzed for their anti-vif activity.

Cells and Transfection

293T cells were maintained in Dulbecco's Modification of Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals) in a humidified 37°C incubator with 5% CO_2. All transfections were done using the calcium phosphate precipitation method of Berkner and Sharp (19). Twenty-four hours prior to transfection, 1.0 x 10^6 cells were plated into 100 mm dishes. The following day, the medium was replaced and the cells were transfected with 10 or 15 μg plasmid DNA in 125 mM $CaCl_2$, 2X HBS (280 mM NaCl/ 1.5 mM Na_2HPO_4/ 50 mM HEPES Buffer (pH 7.05)). After an overnight incubation, the DNA precipitate was removed and replaced with fresh medium. Forty-eight hours after transfection cells were assayed for transgene expression. Transfection efficiency was monitored by observation of GFP expression in control cells transfected with a GFP-expression plasmid.

Vif inhibition assay

To determine the ability of the hammerhead ribozyme Vif5113 to inhibit *vif* expression, a transient inhibition assay was employed. For this, 293T cells were co-transfected with pCMV-VifFLAG and either pSRP5113 or pSRP5113Δ. HIV-1 vif was expressed from pCMV-Vif FLAG, which encodes the HIV-1 NL43 *vif* gene fused to the FLAG epitope (20). pSRP5113 was transfected at a 1:1 (ribozyme to vif) or 2:1 (ribozyme to vif) plasmid ratio. The 1:1 ratio mixtures consisted of 5 μg of pSRP5113 or pSRP5113Δ and 5 μg of pCMV-VifFLAG, for a total of 10 μg DNA. The 2:1 ratio mixtures consisted of 10 μg of either pSRP5113 or pSRP5113Δ and 5 μg of pCMV-VifFLAG, for a total of 15 μg DNA.

Protein Isolation

Total protein was isolated from the transfected cells using RIPA buffer containing protease inhibitors (PBS/0 .1 % SDS/1 % NP40/0.5 % sodium deoxycholate/1 μg/μL aprotinin/1 μg/μL leupeptin/1 μg/μL pepstatin/1 μM NaF/0.1 μM $NaVO_4$/ 0.1 mg/mL PMSF). Forty-eight hours following transfection, the medium was aspirated, and the cells were washed 2X with 4 mL ice cold PBS. One milliliter of ice cold PBS was added to each dish, the cells were scraped from the plate and transferred to a microcentrifuge tube. The cells were centrifuged at 14,000 rpm for 2 minutes, the supernatant was removed, and the pellet was resuspended in 200 μL RIPA buffer. The lysate was homogenized by passing through a 1 mL syringe with a 21 gauge needle. The lysate was incubated on ice for 60 minutes with mixing every 10 minutes. Subsequently, the lysate was spun at 14,000 rpm for 20 min at 4°C, and the supernatant was transferred into a new microcentrifuge tube. The isolated protein was stored at -20°C.

Protein concentrations were determined using the Bradford assay (BioRad). Briefly, 5 μL of each protein sample was added to a microplate well along with 250 μL of room temperature 1X Bradford reagent. The samples were mixed, incubated for five minutes at RT, and read on the microplate reader at 595 nm. Protein concentrations and total protein were determined using the standard curve generated from BSA standards.

SDS PAGE and Transfer

Protein samples from transfected 293T cells were thawed on ice and 50 μg of each were combined with an equal volume of 2X treatment dye (95% formamide/0.025% SDS/0.025% bromophenol blue/0.025% xylene cyanol FF/0.5 mM EDTA). For the positive control generated by transfecting with pCMV-VifFLAG alone, 28 μg of total protein was added due to a smaller yield in this sample. The samples were heated to 100°C for 3 minutes to denature the proteins and immediately quenched on ice. The proteins were separated by molecular weight in a 12 % polyacrylamide gel in 1X Tris-glycine buffer (21). The gel was run at 8 volts/cm (60 V) until the treatment dye reached the resolving gel. The voltage was then increased to 15 volts/cm (105 V) to separate the proteins. The separated proteins were transferred using a semi-dry apparatus (Owl Scientific) to a PVDF membrane. Briefly, a PVDF membrane was cut to equal the size of the resolving gel and activated by soaking in methanol for 3 minutes. Six pieces of similarly sized filter paper were soaked in Towbin buffer (25 mM Tris/192 mM glycine/10 % methanol/ 0.1 % SDS). The gel, membrane, and filter paper were stacked according to the manufacturer's protocol, and the proteins were transferred at

70mA for 2 hours. The membrane was immediately incubated in Ponceau Stain (0.5 g Ponceau-S in 1 % acetic acid) for 5 minutes and subsequently incubated with Ponceau destain (1 % acetic acid) until the bands were visible.

Western Blot

The membrane was blocked in PBS/5% powdered milk overnight at 4°C. The following day, the membrane was washed 2X with PBS for 2 minutes per wash, and incubated in 10 mL PBS containing 20 μg anti-FLAG M2 antibody (Stratagene) at room temperature with gentle rocking for one hour. This was followed by two washes with PBS for 2 minutes per wash. The secondary goat anti-mouse HRP conjugate antibody (Chemicon) was diluted 1:5000 in blocking solution and added to the membrane. The membrane was incubated with the secondary antibody for two hours at room temperature with gentle rocking, and then washed 3X with PBS for 5 minutes per wash. The ECL detection reagent (Amersham) was prepared and added to the membrane. The membrane was incubated at room temperature for 5 minutes, excess reagent was drained away, and the membrane was wrapped in plastic. The blot was placed in a cassette with X-ray film (Kodak) and allowed to expose the film overnight. The film was hand developed using Kodak reagents.

Results

The HIV-1 NL43 *vif* genomic sequence was analyzed for the presence of potential hammerhead ribozyme target sites. One site, a pGUC located at nucleotide 5113 was used to design an anti-vif hammerhead ribozyme. This ribozyme and its non-catalytic control (Figure 1) were synthesized and cloned into the retroviral vector, pSuper.retro.puro. Sequencing was used to verify the ribozyme sequence in the resulting plasmids: pSRP5113 and pSRP5113Δ (Figure 2).

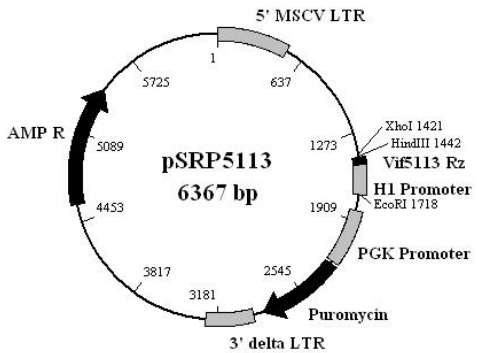

Figure 2. The retroviral vector pSRP5113. An anti-vif ribozyme targeted to nucleotide 5113 of the HIV NL43 sequence was cloned into the retroviral vector, pSuper.retro.puro. The resulting vector is shown. A similar vector was constructed that expresses a non-catalytic form of the ribozyme, pSRP5113Δ.

The ability of the Vif5113 hammerhead ribozyme to reduce expression was analyzed using a vif inhibition assay. For this, two series of co-transfections were carried out in 293T cells using either pSRP5113 or pSRP5113Δ and the HIV *vif* expression plasmid, pCMV-VifFLAG. The first series of co-transfections included 5 μg of pSRPVif5113 or pSRP5113Δ and 5 μg of pCMV-VifFLAG. A second series of co-transfections included 10 μg of either pSRPVif5113 or pSRPVif5113Δ and 5 μg of pCMV-VifFLAG. A 5 μg transfection of a GFP-expressing plasmid served as a negative control.

The intracellular cleavage ability of the anti-vif ribozyme was analyzed by Western blot. Forty-eight hours after each transfection series, total protein was obtained from the cells and 50 μg of each was separated using a 12% polyacrylamide gel. The separated proteins were transferred to a PVDF membrane and probed using an anti-FLAG antibody. The resulting blot was analyzed to determine the relative levels of *vif* expression. A band present at 23 kD was assumed to be HIV Vif (Figure 3).

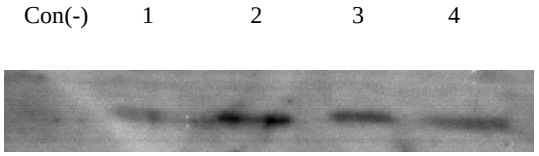

Figure 3. Analysis of vif expression. 293T cells were transfected as follows: Con(-) – GFP; lane 1 – 1:1 pSRPVif5113 to pCMV-VifFLAG; lane 2 – 1:1 pSRPVif5113Δ to pCMV-VifFLAG; lance 3 – 2:1 pSRP5113 to pCMV-VifFLAG; and lane 4 – 2:1 pSRP5113Δ to pCMV-VifFLAG. These results indicated that at the 1:1 ratio, the catalytic ribozyme, Vif5113 reduced vif expression.

Transfection of 293T cells with a 1:1 mixture of the catalytic ribozyme, pSRP5113 and pCMV-VifFLAG indicated a reduction of vif activity (Figure 3, compare lanes 1 and 2). No such decrease in *vif* expression was observed when the ribozyme was transfected at a 2:1 ratio (pSRP5113 to pCMV-VifFLAG). In this instance the level of *vif* expression in cells transfected with the catalytic and non-catalytic ribozymes appeared to be the same. (Figure 3, compare lanes 3 and 4). This produced a confounding result, which has yet to be fully resolved.

Discussion

HIV-1 *vif* encodes a protein that neutralizes an inhibitory host defense mechanism mediated by apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) (8). This protein is a cellular cytidine deaminase that is encapsulated into assembling virions in the absence of vif and is inhibitory during the next round of viral replication. Vif neutralizes APOBEC3G by reducing its translation and by rapid degradation of the native protein (10).

Because Vif inhibits APOBEC3G, its cleavage by hammerhead ribozymes may decrease the infectivity of HIV-1 virions. To test this hypothesis an anti-Vif ribozyme targeted to nucleotide 5113 within the HIV NL43 vif open reading frame and its non-catalytic control were cloned into the retroviral vector pSuper.retro.puro for tissue culture analysis. Our preliminary analysis suggested that the ribozyme was able to decrease *vif* expression in a transient assay. This was supported by a series of co-transfections using a 1:1 ratio of ribozyme and *vif* expression plasmids. However, a similar series of co-transfections using a 2:1 ratio of these plasmids produced conflicting results. In this second transfection series, the samples revealed bands of approximate equal intensity suggesting that the ribozyme had no effect on vif expression.

However, after further analysis it was determined that there were discrepancies in the total amount of protein added to lane 2 (1:1 pSRP5113Δ to pCMV-VifFLAG) and 3 (2:1

pSRP5113 to pCMV-VifFLAG). In both cases the amount of protein was determined to be approximately 50% less than originally calculated. Notwithstanding, when comparing the 1:1 catalytic transfection, the data suggest that the ribozyme may be inhibiting *vif* expression (Figure 3, compare lane 1 and 2). With more protein in the Vif5113 sample (lane 1) as compared to the Vif5113Δ samples (lane 2), the differences are even greater than at first appeared. This also holds true when comparing lane one (Vif5113) with the other sample transfected with the non-catalytic ribozyme (lane 4). Importantly, approximately equal amounts of protein were loaded in these two lanes (1 and 4). These data suggest that the ribozyme may be reducing *vif* expression in this cellular model. However; the relatively equal amounts of *vif* expression observed in lanes 3 and 4 do not support this conclusion. These two samples were obtained from cells that contained catalytic and non-catalytic ribozymes transfected at a 2:1 ratio. Therefore, further analyses are required to reproduce this data and ascertain the efficiency of ribozyme-mediated degradation of vif mRNA

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