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Inhibition of Junín virus replication by small interfering RNAs

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ABSTRACT

Junín virus (JUNV), the etiological agent of the Argentine hemorrhagic fever, has a single-stranded RNA genome with ambisense expression which encodes for five proteins. In previous works we have demonstrated that the Z arenavirus matrix protein represents an attractive target for antiviral therapy. With the aim of studying a new alternative therapeutic mechanism, four Z-specific siRNAs (Z1- to Z4-siRNAs) were tested showing variable efficacy. The most effective inhibitor was Z2-siRNA targeted at the region encompassed by nt 179–197 of Z gene. The efficacy of this Z2-siRNA against JUNV was also demonstrated in virus-infected cells, by testing infectious virus plaque formation (92.8% JUNV yield reduction), viral RNA level or antigen expression, as well as in cells transfected with Z-specific reporter plasmids (91% reduction in expression of Z-EGFP fusion protein). Furthermore, the lack of effect of this Z-siRNA on the expression of other JUNV proteins, such as N and GPC, confirmed the specificity of action exerted by Z2-siRNA on Z transcript. Thus, the present study represents the first report of virus inhibition mediated by RNA interference for a New World arenavirus.

1. Introduction

Viruses are among the most important causes of morbidity and mortality worldwide, but especially in the developing countries where the national health care system is deficient. Particularly, arenaviruses are among the etiological agents that can be considered within the neglected viral infections in South American countries where the human hemorrhagic fevers are often fatal to the infected patients. Junín virus (JUNV) is the agent of Argentine hemorrhagic fever (AHF), with mortality rates ranging from 10 to 20% in the absence of the administration of standardized doses of convalescent plasma that is today the best therapy against AHF (Damonte and Coto, 2002).

JUNV is an enveloped, single stranded, ambisense RNA virus with a segmented genome consisting of two segments, designated large (L) and small (S). The S segment encodes the nucleocapsid protein (N) and a pre-envelope glycoprotein precursor (GPC) which is processed post-translationally into a signal peptide, the external glycoprotein G1 and the transmembrane G2, whereas the L fragment encodes the RNA polymerase L and the matrix protein called Z.

The Z protein has been implicated in several aspects of arenavirus biology. It has been shown that Z exerted an inhibitory effect on viral RNA synthesis (Cornu and de la Torre, 2001, 2002; López et al., 2001). In addition to this regulatory role, Z was also implicated as a virion component with matrix functions, similar to other enveloped negative-strand viruses (Neuman et al., 2005; Perez et al., 2003; Salvato et al., 1992; Salvato, 1993; Strecker et al., 2003). Consistent with its features, Z presents a RING finger and a canonical late domain motif that enable Z to interact with the host cell (Borden et al., 1998; Campbell-Dwyer et al., 2000; Djavani et al., 2005) and viral proteins. Furthermore, Z-mediated budding requires its myristoyl modification (Perez et al., 2004), which likely facilitates Z association with membranes at budding sites. Thus, Z has an essential role in the particle formation.

RNA interference (RNAi) is a post-transcriptional gene silencing process in which double-stranded RNA (dsRNA) initiates specific cleavage of cytoplasmic mRNA. Initially, dsRNA is recognized and processed by DICER, an RNase III enzyme (Hutvágner et al., 2001). The resulting processed dsRNA consists of 21–23 nt fragments with 2–3 nt overhangs at the 3′ end of each RNA strand (Bernstein et al., 2001; Zamore et al., 2000). DICER-processed dsRNA is recognized by the dsRNA-induced silencing complex (RISC) and used as a template to guide degradation of mRNA that is homologous in sequence to the RISC-bound dsRNA fragment, resulting in greatly reduced protein production (Hammond et al., 2000).

Synthetically produced small interfering dsRNA molecules (siRNAs) have been shown to induce the RNAi effect *in vitro* and greatly decrease specifically targeted transcripts (Elbashir et al., 2001). Such efforts have been expanded to include targeting of viral transcripts present in the cytoplasm for degradation by the RISC complex. The list of the human viral pathogens that have

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Table 1
List of siRNAs used in experiments.

siRNA	Sequence	Polarity	Position
Z1-siRNA	5′-CCUGAUAACCUGCAAUGAUuu-3′ 5′-AUCAUUGCAGGUUAUCAGGuu-3′	Sense Antisense	144–162
Z2-siRNA	5′-AACAUAACCUGAUGACACCuu-3′ 5′-GGUGUCAUCAGGUUAUGUUuu-3′	Sense Antisense	179–197
Z3-siRNA	5′-UAUCUACCAUGUAGACUGCuu-3′ 5′-GCAGUCUACAUGGUAGAUAuu-3′	Sense Antisense	92–110
Z4-siRNA	5′-UUGCAGAGAUCUGAAUUCCuu-3′ 5′-GGAAUUCAGAUCUCUGCAAuu-3′	Sense Antisense	199–217
X-siRNA	5′-GACCACAATTCTCGATATACAuu-3′ 5′-TGTATATCGAGAATTGTGGTCuu-3′	Sense Antisense	

been shown to be susceptible to an *in vitro* RNAi-directed attack includes respiratory syncytial virus (Leung and Whittaker, 2005), poliovirus (Gitlin et al., 2005), adenovirus (Chung et al., 2007), human immunodeficiency virus (Hannon and Rossi, 2004; Naito et al., 2007), hepatitis B virus (Li et al., 2007), hepatitis C virus (Liu et al., 2006; Lupberger et al., 2008), influenza A (Ma et al., 2007), Marburg virus (Fowler et al., 2005), human papillomavirus (Jiang and Milner, 2002), and coronavirus (Zhang et al., 2004), among others. To the present, only two reports were published describing the use of siRNAs with the Old World arenaviruses lymphocytic choriomeningitis virus (LCMV) (Sánchez et al., 2005) and Lassa virus (LASV) (Müller and Günther, 2007).

Since the matrix Z protein is involved in processes that are fundamental to the success of viral infection, we selected Z as candidate target gene in order to determine the efficacy of RNAi for inhibition of JUNV gene expression. Here we report the identification of siRNA target regions on Z that are able to silence its expression and impair the production of infectious viral progeny.

2. Materials and methods

2.1. Cell culture and viruses

Vero and BHK-21 cells were grown as monolayers in Eagle's minimum essential medium (MEM) (GIBCO) containing 5% fetal bovine serum (GIBCO) and 50 μg/ml gentamycin (Sigma). Medium was supplemented with 20 μM HEPES buffer (Sigma) when incubated at 37 °C under 5% CO_2. Maintenance medium (MM) consisted of MEM with 1.5% fetal bovine serum. The attenuated strains IV_{4454} and XJCl3 of JUNV (Candurra et al., 1989), the TRLV11573 strain of Tacaribe virus (TCRV), and the Armstrong 53b strain of LCMV were used. Arenavirus stocks were prepared in BHK-21 cells and titrated by plaque formation in Vero cells.

2.2. Design and synthesis of siRNAs

Four double-stranded siRNAs were designed targeting the Z transcript of JUNV-IV_{4454} (GenBank accession no. DQ538136) by using the algorithm programs from different companies. BLAST analysis was performed for ensuring the lack of homology with cellular genes. The siRNAs targeted to Z transcript (Z1- to Z4-siRNAs) and a siRNA without homology to any known gene transcript used as a non-silencing control (X-siRNA) were supplied as annealed duplexes by Invitrogen (Table 1).

2.3. Transfection of siRNA and infection assay

Vero cells grown in six-well plates at approximately 80% confluence were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to Manufacturer's instructions with minimum modifications. Briefly, cell supernatant was removed and 500 μl Opti-MEM (GIBCO) containing 10 μl Lipofectamine 2000 and 1 μM siRNA was added. After 4 h of incubation at 37 °C, supernatant was removed and fresh Advanced-MEM (GIBCO) containing 2% fetal bovine serum was added to the cells. After 24 h incubation at 37 °C, transfected monolayers were infected with JUNV at a MOI of 0.1. At 24 h post-infection (p.i.), supernatants were collected to determine virus yield by plaque assay and cells were processed to measure viral RNA by real time PCR (qRT-PCR).

2.4. Plaque assay

Vero cells grown as monolayers were infected with serial dilutions of the supernatants of Z-siRNA-transfected and JUNV-infected Vero cells. After 1 h adsorption, cells were washed, covered with MM containing 0.7% methylcellulose, and incubated at 37 °C under 5% CO_2. At day 7 p.i. virus plaques were counted and % inhibition with respect to the viral control was calculated. Each value was the mean of two independent experiments performed in duplicate ± standard deviation.

2.5. Real time PCR

RNA was isolated from siRNA transfected and JUNV-infected Vero cells using TRIzol (Invitrogen) according to the Manufacturer's instructions. To monitor RNA replication, cDNA was generated by using murine reverse transcriptase M-MLV (200 U/μl, Invitrogen) and random primers. This cDNA was amplified by real time PCR using SYBRGreen (Roche) detection. The mix reaction volume was 25 μl including 1 μl of cDNA, DNA polymerase GoTaq (5 U/μl, Promega) and specific primers to amplify gene z: Z Forward 5′-ATGGGCAACTGCAACGGGGCATC-3′ and Z Reverse 5′-AGCCAACAGCACCACCACCATAG-3′. Real time PCR was carried out with an initial incubation at 94 °C during 1 min followed by 40 cycles of 30 s at 95 °C, 45 s at 60 °C, 45 s at 72 °C, 2 s at 79 °C and a final incubation of 15 s at 60 °C. Amplification plots were expressed as C_T values to be analyzed with Opticon Monitor 3.1. software where C_T values represent the reaction cycle at which PCR products reach a threshold level of detection. C_T values were normalized by using *actin* as standard.

2.6. Indirect immunofluorescence assay for viral antigens

Vero cell monolayers grown on coverslips were transfected with 1 μM Z-siRNA and infected with JUNV at 24 h post-transfection as described in Section 2.3. At 24 h p.i., cells were fixed with methanol for 10 min at −20 °C for cytoplasmic immunofluorescence. Indirect staining was carried out by incubation with a rabbit anti-JUNV polyclonal serum for 1 h at 37 °C followed by fluorescein (FITC)-conjugated goat anti-rabbit IgG in the same conditions. After a final washing, cells were mounted in a glycerol solution containing 2.5% 1,4-diazabicyclo[2,2,2]octane (DABCO) and visualized by a fluorescence microscope.

2.7. Construction of JUNV protein expressing plasmids

A truncated Z lacking a stop codon was obtained by PCR-amplification using plasmid pGEM containing a full-length cDNA copy of the Z gene as template DNA together with oligonucleotides Z-F (5′-GGATCCATGGGCAACTGCAACGGGGCATC-3′) and Z-trunc-R (5′-AAAAGCGGCCGCCTGGTGGTGGTGCTGTTGGCT-3′) containing a BamHI and NotI site, respectively (underlined). The insert DNA was subsequently subcloned into the BamHI and NotI sites of the mammalian expression vector pcDNA3.1 to generate pcDNA3.1-Z trunc. Oligonucleotides EGFP-F (5′-AAAAGCGGCCGCATGGTGAGCAAGGGCGAGGAG-3′) and EGFP-R (5′-

AAAATCTAGATTACTTGTACAGCTCGTCCATGCC-3′), which contain a NotI and XbaI site, respectively (underlined), were used with plasmid pEGFP C as template DNA to PCR amplify the sequence encoding the enhanced green fluorescent protein (EGFP). The amplicon was cloned into the NotI–XbaI digested pcDNA3.1-Z trunc vector to generate the reporter plasmid pcDNA3.1-Z-EGFP, which resulted in the expression of a fusion protein Z containing EGFP in its C-terminus. The same strategy was used to generate the reporter plasmid pcDNA3.1-Z-Flag (DYKDDDDK).

In order to generate the N and GPC proteins encoding plasmids, the pcDNAHisMAX A vector was modified and AgeI and ClaI restriction sites respectively (underlined) were added into the HindIII and ApaI vector sites using the following designated primers: 5′-CTGGCTAACTAGAGAACCCACTGC-3′ and 5′-TATGGGCCCCCCGGCGCCCCCATCGATCCCACCGGTCCCCATGGTTTC-GGAGGCCG-3′. The N and GPC genes were obtained by PCR-amplification from JUNV-infected Vero cells using the following primers: N AgeI sense (5′-AAAACCGGTATGGCACACTCCAAAGAGG-3′), N ClaI antisense (5′-AAAATCGATCAGTGCATAGGCTGCC-3′), GPC AgeI sense (5′-GGGACCGGTATGGGGCAATTCATCAGC) and GPC ClaI antisense (5′-TTTATCGATGTGTCCTCTTGCGCC-3′). Then, PCR amplification fragments were cloned into modified pcDNAHisMAX A vector to obtain the pcDNAHisMAX A-N and pcDNAHisMAX A-GPC constructs, respectively.

2.8. Cell co-transfection with siRNA and JUNV protein expressing plasmids

Vero monolayers grown to 80% confluence on 12 mm diameter glass coverslips were co-transfected with 1 μg plasmid pcDNA3.1-EGFP, pcDNA3.1-Z-EGFP, pcDNA3.1-Z-Flag, pcDNAHisMAX A-N or pcDNAHisMAX A-GPC and 1 μM Z-siRNAs using Lipofectamine 2000 (Invitrogen) as described previously. As control, cells were transfected with the respective plasmid without Z-siRNA. To record EGFP expression, at 48 h post-transfection cells were fixed with methanol for 10 min at −20 °C, mounted in glycerol with DABCO and observed in a fluorescence microscope. For Z-Flag detection, cells fixed at 48 h post-transfection were incubated with rabbit anti-Flag polyclonal serum (Cell Signaling Technology) overnight at 4 °C, followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (Sigma) for 1 h at room temperature. Then, cells were mounted and visualized. For N or GPC detection, cells fixed as above were incubated first with monoclonal antibodies SA02BG12 or QC03-BF11 (Sánchez et al., 1989), respectively, then with FITC-labeled goat anti-mouse IgG.

2.9. Western blot

Vero cells co-transfected with 1 μg pcDNA 3.1-Z-Flag with or without 1 μM Z-siRNA as described above were harvested at 48 h post-transfection in sample buffer (5% SDS, 2% 2-mercaptoetanol, 10% glycerol and 0.005% bromophenol blue in 0.0625 M Tris–HCl, pH 6.8) (Promega). After boiling the lysates during 7 min, proteins were separated by 15% SDS-PAGE and blotted onto a PDVF membrane (Millipore). Membranes were incubated with a rabbit anti-Flag serum overnight at 4 °C. After washing with Tris-buffered saline (TBS, 20 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 0.1% Tween 20, a second incubation was performed with horseradish peroxidase-conjugated anti-rabbit IgG during 1 h at room temperature. As control, the presence of actin was revealed by incubation with mouse monoclonal anti-actin antibody (JLA20, Calbiochem) followed by incubation with peroxidase-conjugated anti-mouse IgG. For protein detection blots were treated with the Western Lightning Luminescence system (PerkinElmer).

3. Results and discussion

3.1. Effect of Z-siRNAs on infectious virus production

To evaluate the effect of Z-siRNAs on infectious JUNV production, Vero cells were transfected with the respective siRNA (Z1-siRNA, Z2-siRNA, Z3-siRNA, Z4-siRNA or X-siRNA), and 24 h later cultures were infected with JUNV at a MOI of 0.1. As a control, non-transfected JUNV-infected cells were also included. Supernatants were collected at 24 h p.i. and titrated by plaque assay. Cells transfected with the non-specific X-siRNAs did not exhibit a reduction in virus yield (1.26×10^6 PFU/ml), showing similar levels to non-transfected JUNV-infected Vero cells (1.17×10^6 PFU/ml), indicating that transfection of the cells with the control X-siRNA did not induce a non-specific interference with virus replication during the 24 h incubation period. By contrast, reduction in virus titer was observed in cells treated with the siRNAs corresponding to the Z transcript (Fig. 1A). Whereas Z1-siRNA reduced the virus titer by 85.3% compared to the viral control, Z2-siRNA induced a 92.8% yield reduction. In Vero cells treated with both Z1-siRNA and Z2-siRNA, the virus yield was reduced by 83.7%, showing that the combination of different silencing agents did not present any advantage (data not shown). On the other hand, Z3-siRNA and Z4-siRNA were less effective, with only 49.5 and 56.5% of virus yield inhibition, respectively.

Interestingly, the silencing effect of Z2-siRNA, the most effective inhibitor, was observed also when the virus yield inhibition was determined at longer period of incubation (48 h p.i.) with 70.5% of virus yield inhibition (Fig. 1B), and even when JUNV infection was performed at higher MOIs of 1 and 10, with 77.3 and 75.6% of virus yield inhibition, respectively (Fig. 1C).

Since the gene regions targeted for Z-siRNA design were conserved among arenaviruses (Fig. 2), we looked for a possible interference of the selected Z-siRNAs on XJCl3, another attenuated strain of JUNV, and also on the close antigenically related TCRV and the non-antigenically related LCMV. When Z-siRNAs originally designated against JUNV-IV_{4454} were evaluated against JUNV-XJCl3, a moderate virus yield inhibition was observed with Z1- to Z4-siRNAs, with 43.5, 54.7, 47.7 and 36.6% of virus yield inhibition, respectively. However, when JUNV Z-siRNAs were evaluated against the other two arenaviruses species, no inhibition was observed (Fig. 1A for TCRV and not shown for LCMV), confirming the specificity of the RNA interference mechanism.

3.2. Effect of Z-siRNA on viral RNA in JUNV-infected cells

Since siRNAs function by identifying and degrading mRNA with its complementary sequence, we next examined the effect of Z1-siRNA and Z2-siRNA on the abundance of viral RNA in JUNV-infected cells by qRT-PCR using Z-specific primers. In contrast to Vero cells transfected with the control non-silencing X-siRNA, viral RNA amplification was reduced in Vero cells transfected with both Z-specific siRNAs (Fig. 1D). Again, Z2-siRNA was the most effective inhibitor with a 12-fold reduction in viral RNA whereas Z1-siRNA induced approximately a 3-fold RNA inhibition with respect to control X-siRNA (Fig. 1D).

3.3. Effect of Z-siRNA on the expression of viral antigens in JUNV-infected cells

Vero cells treated with Z2-siRNA and infected with JUNV were also examined for viral antigen expression at 24 h p.i. by an indirect immunofluorescence assay with a rabbit anti-JUNV polyclonal serum. Hyperimmune JUNV antiserum allows the detection of the main viral proteins, the nucleoprotein N and the precursor and mature glycoproteins GPC and G1. In accordance with the reduction in virus yield produced by Z2-siRNA shown in Fig. 1A, a concomitant

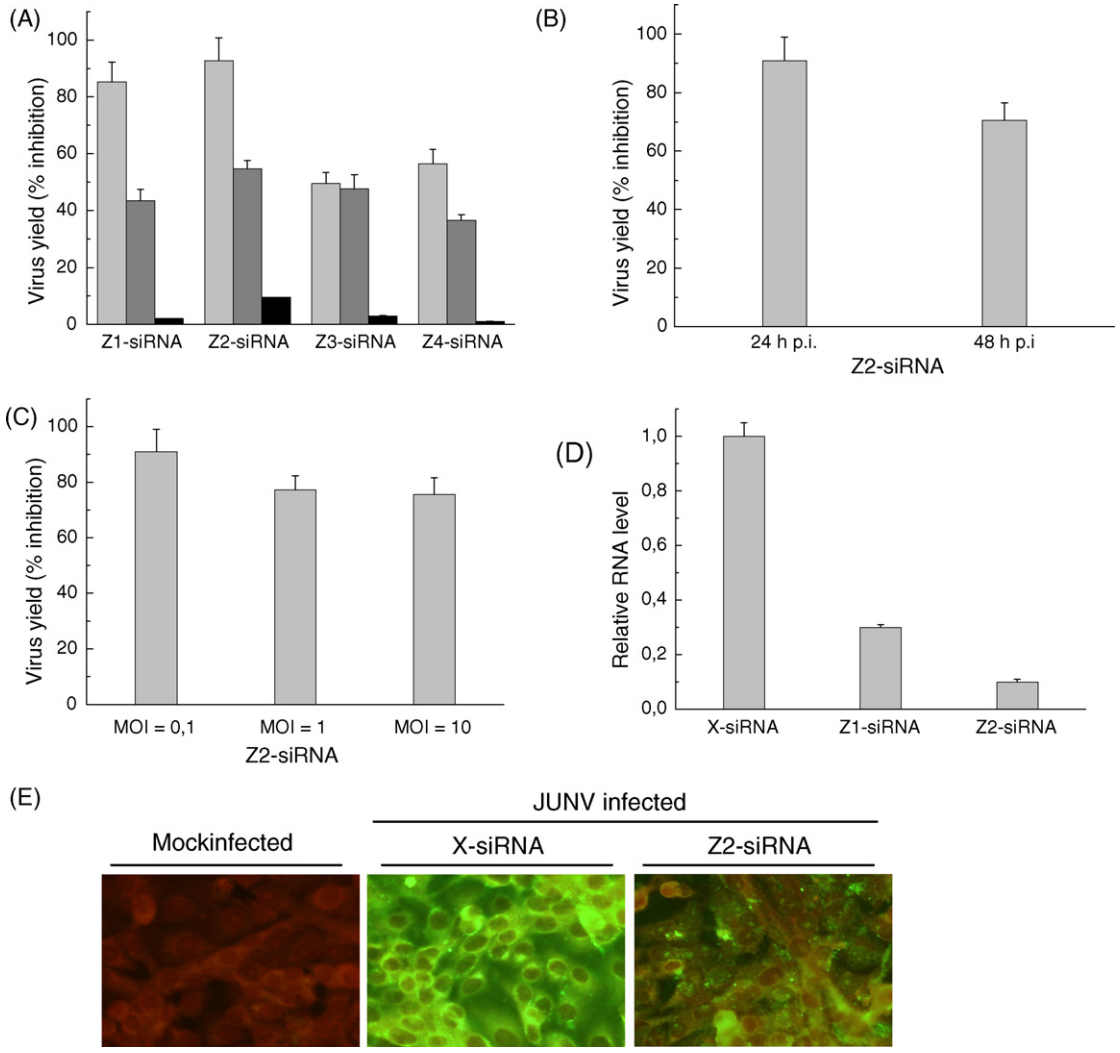

Fig. 1. Effect of Z-siRNAs on virus infection. (A) Vero cells were transfected with different Z-siRNA (Z1–Z4) and infected at a MOI of 0.1 with the IV_{4454} (light grey bars) and the XJCl3 (dark grey bars) strains of JUNV, or TCRV (black bars). Supernatants collected at 24 h p.i. were titrated by plaque assay. Inhibition of virus yield was calculated comparatively to the titer obtained in cells transfected with X-siRNA. Each value represents the mean of two independent experiments performed in duplicate ± standard deviation (S.D.). (B) Vero cells were transfected with Z2-siRNA and infected with JUNV, MOI = 0.1. Supernatants were collected at 24 or 48 h p.i. and titrated by plaque assay. Inhibition of virus yield was calculated as above. (C) Vero cells were transfected with Z2-siRNA and infected with JUNV at different MOIs. Supernatants were collected at 24 h p.i., and titrated by plaque assay. Inhibition of virus yield was calculated as above. (D) Amount of viral RNA in Vero cells transfected with Z1-siRNA or Z2-siRNA and infected with JUNV was quantified by real time RT-PCR. Values, standardized to those of actin mRNAs, were expressed as relative RNA levels comparatively to the amount obtained in cells transfected with X-siRNA and represented as mean of two independent experiments performed in triplicate ± S.D. (E) Expression of viral antigens in Vero cells transfected with Z2-siRNA or X-siRNA and infected with JUNV was detected by immunofluorescence assay using a rabbit anti-JUNV polyclonal serum. Magnification = 400×.

decrease in cytoplasmic JUNV antigen expression was observed (Fig. 1E).

3.4. *Effect of Z-siRNAs on the expression of Z-recombinant fusion protein*

To confirm that the Z-siRNAs were effective as specific inhibitors of the expression of Z protein, the effect of Z-siRNAs on the expression of recombinant Z fusion proteins was analyzed. First, the inhibitory action of Z-siRNA on the expression of the fusion protein Z-EGFP was determined by co-transfection of Vero cells with the plasmid pcDNA3.1-Z-EGFP and either the Z-siRNA or the non-silencing X-siRNA. A series of co-transfections of the reporter plasmid pcDNA3.1-EGFP and the siRNAs were assayed in parallel. At 48 h post-transfection, EGFP fluorescence was analyzed. The EGFP protein was highly expressed in cells transfected with the reporter plasmid in the presence of co-transfected Z-siRNA or X-siRNA (Fig. 3A, panels A and B). The control non-silencing X-siRNAs had no apparent effect on Z-EGFP expression (Fig. 3A, panel C), whereas the four specific Z-siRNAs produced a marked reduction in fluorescence (Fig. 3A, panels D–G). As observed in virus yield assay, Z2-siRNA appeared to silence Z-EGFP gene expression more efficiently than the other Z-siRNAs. In fact, the quantification of fluorescent cells showed that Z1- to Z4-siRNA reduced expression of the Z-EGFP fusion protein in comparison to X-siRNA by 67.4, 91.0, 46.9 and 45.2%, respectively (Fig. 3B).

The specificity of Z2-siRNA to block expression of Z protein was further assessed by testing the expression of other recombinant protein, the Z-Flag fusion protein expressed by the plasmid pcDNA 3.1-Z-Flag, by immunofluorescence and Western blot assays performed with rabbit anti-Flag antibodies. It was evident from the results shown in Fig. 3C and D that the Z-specific siRNA reduced the amount of detectable Z-Flag fusion protein in both assays whereas the co-transfection of Vero cells with the control non-silencing X-siRNA failed to block the synthesis of Z-Flag fusion protein. It is also worth to note that the expression in the same cell samples of a non-targeted host protein, actin (ca. 45 kDa), remained almost unaffected (Fig. 3D).

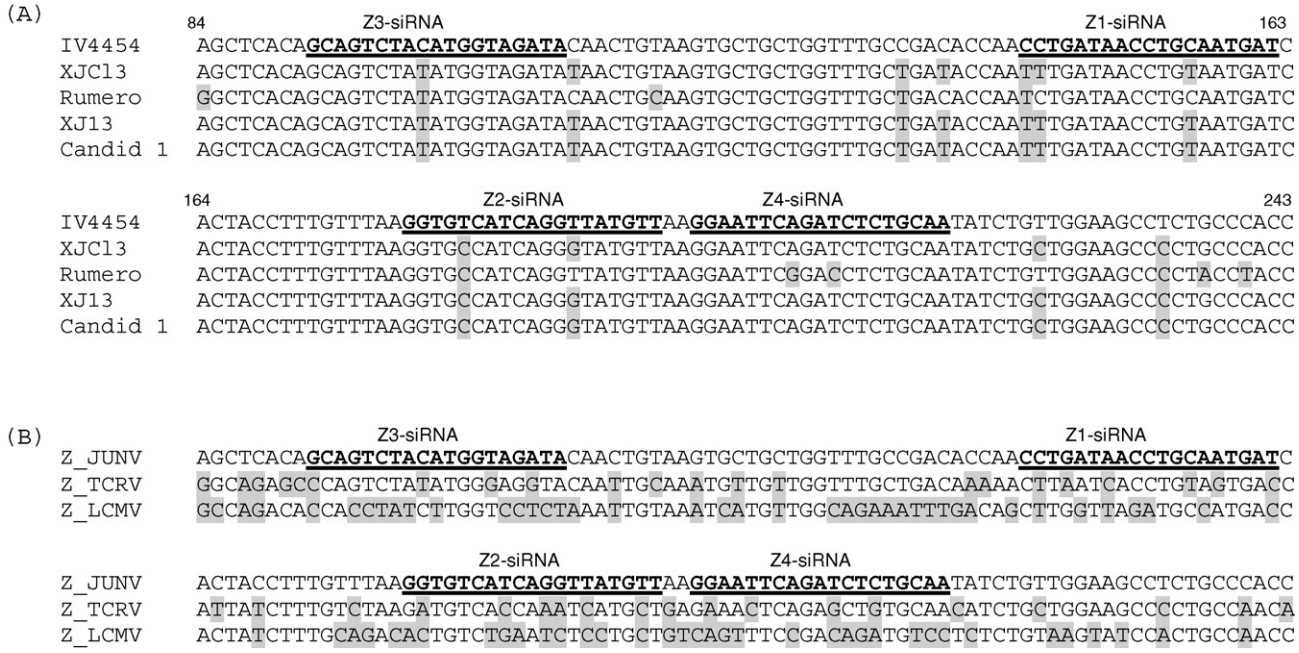

Fig. 2. Gene regions targeted for Z-siRNA design. The sequence data for different strains of JUNV (A) and the other arenaviruses TCRV and LCMV (B) for the multiple alignments of the matrix protein Z were obtained from GenBank: IV_{4454} (DQ538136), XJCl3 (unpublished), Rumero (AY619640), XJ13 (NC_005080), Candid #1 (AY819707), TCRV (NC_004292) and LCMV (AF004519).

To gain further evidence for the specificity of Z-siRNAs, we investigated whether the presence of Z2-siRNA could decrease other transiently expressed JUNV proteins. To this end, cells were co-transfected with the Z2-siRNA or X-siRNA and plasmids pcDNAHisMAX A-N or pcDNAHisMAX A-GPC encoding N and GPC JUNV proteins, respectively. As shown in Fig. 4, no effect on N and GPC expression could be detected by indirect immunofluorescence using specific monoclonal antibodies against these proteins, respectively, suggesting that off-target effects are not the cause of the JUNV yield reduction observed in Fig. 1.

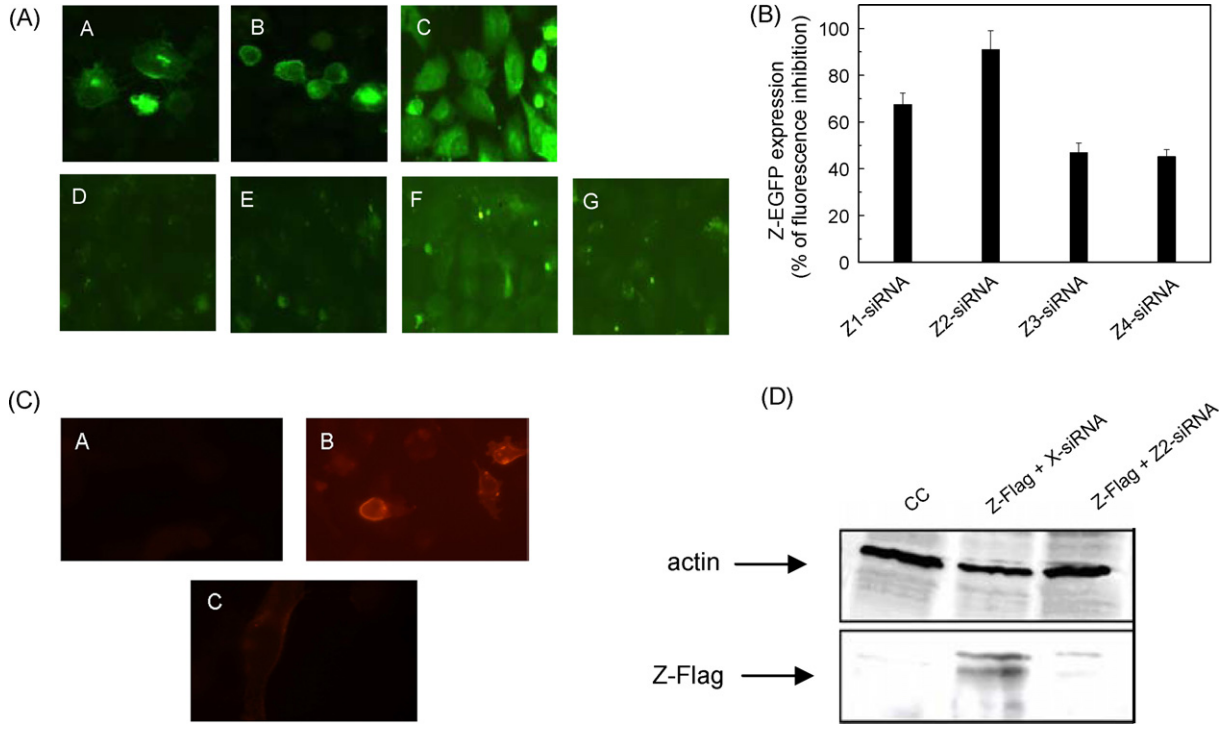

Fig. 3. Effect of Z-siRNAs on the expression of Z-recombinant fusion protein. (A) Vero cells grown on glass coverslips were co-transfected with pcDNA3.1-EGFP and X-siRNA (panel A), pcDNA3.1-EGFP and Z2-siRNA (panel B), pcDNA3.1-Z-EGFP and X-siRNA (panel C) or pcDNA3.1-Z-EGFP and the corresponding Z-siRNA (Z1–Z4) (panels D–G, respectively). At 48 h post-transfection, cells were fixed, mounted and observed in a fluorescence microscope (magnification = 400×). (C) Vero cells grown on glass coverslips were untransfected (panel A), co-transfected with pcDNA3.1-Z-Flag and X-siRNA (panel B) or pcDNA3.1-Z-Flag and Z2-siRNA (panel C). After 48 h, cells were fixed and Z-Flag expression was detected using a rabbit anti-Flag serum, followed by rhodamine-conjugated goat anti-rabbit IgG (magnification = 400×). (D) The same samples as (B) were analyzed by Western blot using a rabbit anti-Flag serum or a mouse monoclonal anti-actin antibody.

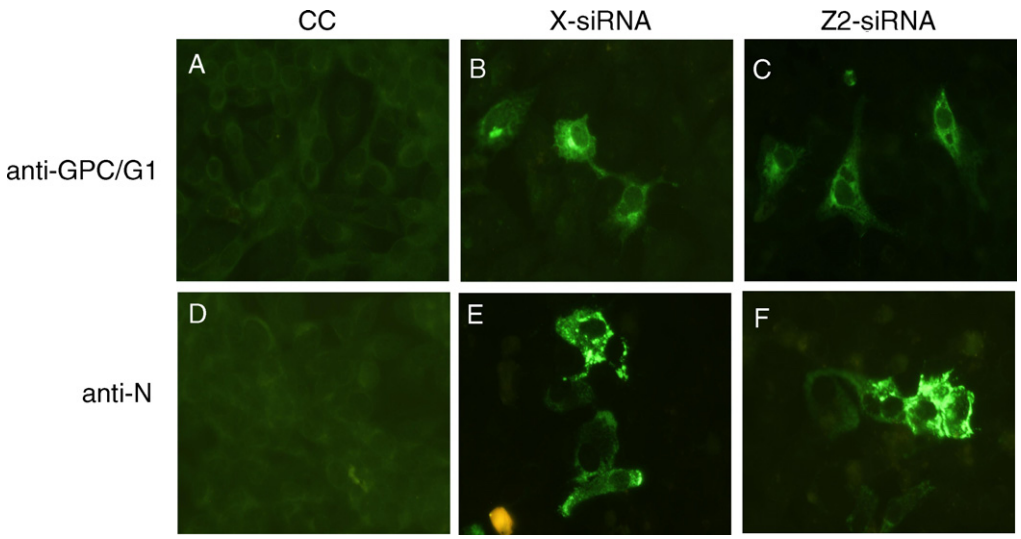

Fig. 4. Effect of Z-siRNA on JUNV nucleocapsid and glycoprotein expression. Vero cells grown on glass coverslips were untransfected (panels A and D), co-transfected with pcDNAHisMAX C-GPC or pcDNAHisMAX C-N and X-siRNA (panels B and E) or Z2-siRNA (panels C and F). At 48 h post-transfection, cells were fixed, and protein expression was detected using specific monoclonal antibodies against GPC/G1 and N, followed by FITC-conjugated goat anti-mouse IgG (magnification = 400×).

Altogether, these data showed that the siRNA targeting the Z transcript exerts its inhibitory action exclusively through a significant and specific reduction in the expression of the Z protein.

In conclusion, results presented here have shown the effective inhibitory action against the arenavirus JUNV by siRNAs directed against the sequence of the Z transcript. Four Z-specific siRNAs were tested showing variable efficacy. The most effective inhibitor was Z2-siRNA, targeted to the region encompassed by nt 179–197 of Z gene. The efficacy of this agent against JUNV was also demonstrated in virus-infected cells by testing infectious virus plaque formation, viral RNA or antigen expression, as well as in cells transfected with Z-specific reporter plasmids. Furthermore, the lack of effect of this siRNA on the expression of other JUNV proteins, such as N and GPC, confirmed the specificity of action exerted by Z2-siRNA on Z transcript. Interestingly, the inhibitory effect observed in the release of JUNV infectious progeny by Z2-siRNA (>90% inhibition at 24 h p.i.) is similar to the effect described in transient transfection of siRNAs for other viruses, but higher than the activity recently reported for siRNAs targeted to the termini of N and L genes in the arenavirus LASV (Müller and Günther, 2007). It remains to be investigated if the antiviral potential of RNA interference for JUNV infections here shown after transient transfection of synthetic siRNA targeted to Z gene can be improved by using a viral vector of a short hairpin RNA that resembles siRNAs precursors. However, siRNAs elicit potent effects at relatively low doses; so as to minimize the interference with naturally occurring processes, current therapeutic efforts seem to be focused towards the use of a pool of synthetic unmodified naked siRNAs (Davis, 2009).

Likewise, our results provide a clear evidence of the central role of Z protein in the JUNV life cycle and assess the good perspectives of this protein as antiviral target in *Arenaviridae*, in accordance with previous studies reporting the efficacy against JUNV and LCMV infections of compounds reactive with the RING finger motif of the Z protein (García et al., 2003, 2006).

The present study represents the first report of virus inhibition mediated by RNA interference for a New World arenavirus, a group in the family including four agents of severe viral hemorrhagic fevers in South America (JUNV, Machupo, Sabiá and Guanarito viruses). It would be an ideal situation to get an antiviral approach effective against all these pathogens. Regarding infectious diseases, ongoing clinical trials in the field of siRNA are developed by different companies and most of them are in the preclinical stage, with the exception of HBV and HIV studies that are in phase I, and the most advanced program concerning the treatment of infection by RSV using siRNA developed by Alnylam Pharmaceuticals that has just finished phase II (López-Fraga et al., 2008). Given the diversity of arenaviruses, it will be difficult to design a siRNA treatment able to cross-inhibit several species in the family, even using a highly conserved sequence as antiviral target. In contrast with the cross-reactivity among the Old World arenaviruses LASV, LCMV and Mopeia virus reported with siRNAs targeted to LASV (Müller and Günther, 2007), in our study we observed no efficacy when JUNV designed Z-siRNAs were used to inhibit the close antigenically related New World arenavirus TCRV. Even, the effectiveness against other JUNV strain was reduced when there is more than one nucleotide change in the target sequence, as shown for Z1- and Z2-siRNA against IV_{4454} and XJCl3 JUNV strains (Figs. 1A and 2). Thus, given the variability observed among natural and experimentally isolated JUNV strains (as shown in Fig. 2A for the Z gene of IV_{4454}, Rumero, XJCl3, XJ13 and Candid 1 strains), more detailed studies will be necessary in the design of RNAi-based therapeutics for successful clinical intervention of human pathogenic arenaviruses.

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