Inhibition of coxsackievirus B3 and related enteroviruses by antiviral short interfering RNA pools produced using φ6 RNA-dependent RNA polymerase

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Coxsackievirus B3 (CBV3) is a member of the human enterovirus B species and a common human pathogen. Even though much is known about the enteroviral life cycle, no specific drugs are available to treat enterovirus infections. RNA interference (RNAi) has evolved to be an important tool for antiviral experimental therapies and gene function studies. We describe here a novel approach for RNAi against CBVs by using a short interfering (siRNA) pool covering 3.5 kb of CBV3 genomic sequence. The RNA-dependent RNA polymerase (RdRP) of bacteriophage φ6 was used to synthesize long double-stranded RNA (dsRNA) from a cloned region (nt 3837–7399) of the CBV3 genome. The dsRNA was cleaved using Dicer, purified and introduced to cells by transfection. The siRNA pool synthesized using the φ6 RdRP (φ6–siRNAs) was considerably more effective than single-site siRNAs. The φ6–siRNA pool also inhibited replication of other enterovirus B species, such as coxsackievirus B4 and coxsackievirus A9.

A supplementary table is available with the online version of this paper.
Enterovirus infections, such as poliovirus (Gitlin et al., 2002), enterovirus 70 and 71 (Lu et al., 2004; Sim et al., 2005; Tan et al., 2007a, b, 2008), coxsackievirus A16 (CAV16) (Wu et al., 2008) and CBV3 (Merl et al., 2005; Merl & Wessely, 2007; Schubert et al., 2005; Werk et al., 2005; Yuan et al., 2005) infections have been successfully inhibited using RNAi in cell culture and in animal models (Fechner et al., 2008; Kim et al., 2008; Merl et al., 2005; Tan et al., 2007b). These studies have employed sequence-specific siRNAs or hairpin RNAs, synthesized chemically or transcribed by DNA-dependent RNA polymerases. Since identifying target sequence candidates for RNAi can be difficult and expensive, it would be beneficial to find an alternative way of producing siRNAs. One mechanism would be to digest long dsRNA into siRNA pools representing multiple sequences of the viral gene or genome. EndoRNase-produced siRNAs have been used previously against hepatitis C virus (Könke et al., 2004) and other targets (Buchholz et al., 2006). In the present study, dsRNA covering a wide area of the CBV3 genome was synthesized with the φ6 RdRP and then Dicer-digested into siRNAs (Fig. 1). Their efficiency in silencing the virus propagation was then compared with that of previously published siRNAs targeting single sites in the essential CBV3 RNA polymerase gene. In addition, we studied the possible effects of the φ6–CBV3 siRNA pool on other enteroviruses. As controls, we used unrelated siRNAs against green fluorescent protein (GFP), both as a single-site siRNA molecule as well as a φ6 siRNA pool.

For construction of the CBV3-specific siRNA pool, a fragment spanning nt 3837–7399 plus the polyA sequence was excised, using EcoRI and SpeI from a plasmid coding for the full-length mRNA of the Nanc strain of CBV3 (Harvala, 2003). This 3562 bp fragment includes the P2b, P2c, P3A, 3B, 3C and 3D genes (Fig. 1). This sequence was cloned into pBluescript II KS+ (Stratagene), the plasmid was linearized with ClaI and ssRNA was synthesized using T7 RNA polymerase. dsRNA was synthesized from this template with the φ6 RdRP and the resulting dsRNA was digested using recombinant Dicer enzyme (Stratagene). The siRNAs were purified from undigested long dsRNA using Waters Gen-Pak FAX HPLC column. The φ6–GFP siRNA pool was synthesized in a similar manner from a plasmid containing the entire enhanced GFP (eGFP) gene (Aalto et al., 2007).

Studies using single 21 nt siRNAs against CBV3 have previously targeted the RNA polymerase 3D (Schubert et al., 2005; Werk et al., 2005; Yuan et al., 2005), the viral protease 2A (Merl et al., 2005; Yuan et al., 2005) and VP1 (Ahn et al., 2005; Yuan et al., 2005). For comparison, we used two previously studied siRNAs specific to the 3D region: Sirev3, 5′-AATAGGACACTAATGTA-3′ and siRdRP2, 5′-CTAAGGACCTAAACAGGT3′ (Schubert et al., 2007), targeting the CBV3 sequence at nt 6825–6843 and 6315–6333, respectively (Qiagen). GFP-22 (Qiagen library item no. 1022064) siRNA and φ6–GFP siRNA pool, both reactive against GFP, were used as non-specific controls. We also used a transfection control which contained water instead of siRNAs.

To test the inhibition of CBV3-, CBV4- and CAV9-infections with the φ6–CBV3 siRNAs, LLC-MK2 cells (ATCC) were grown to a confluence of ~90 % and infected with 100 ng siRNA targeting CBV3 (φ6–CBV3 siRNAs, Sirev3 or siRdRP2) or with control siRNAs (φ6–GFP siRNAs or GFP-22) per well, or with plain water, using Lipofectin reagent (Invitrogen). After a 24 h incubation at 37 °C, the cell culture medium was replaced with fresh growth medium (2 % fetal calf serum in Dulbecco’s modified Eagle’s medium+gentamycin). At 20–24 h after transfection, the cells were infected with 30 p.f.u. per well (m.o.i. of ~ 0.0003) of CBV3 (Nancy strain), CBV4 (JVB strain) or CAV9 (Griggs strain) in a total volume of 100 μl and incubated for 1 h at 37 °C. Cells were washed four times with medium and incubated for 24, 48 and 72 h. The appropriate m.o.i. for the infections were determined in preliminary experiments (data not shown). Supernatants were collected and the cells were fixed with methanol for immunoperoxidase (IPS) detection and stained with a polyclonal antibody known to recognize the virus strains used in this study (Vuorinen et al., 1999).

IPS detection of the fixed cells (Fig. 2) treated with φ6–CBV3 siRNAs showed no infected cells for CBV3, CBV4 or CAV9 as late as 72 h post-infection (p.i.). φ6–GFP siRNAs also had a effect on the infection efficiency, but foci of infected cells were seen for all three viruses at 72 h p.i. No difference in numbers of infected cells was found between φ6–GFP siRNA-treated and untreated cells in CAV9 infection. CBV3- and CBV4-infected cells transfected with the φ6–GFP siRNAs showed a non-significant reduction in numbers of infected cells in comparison with untreated cells. No clear reduction was seen after treatment with Sirev3 in either CBV3-, CBV4- or CAV9-infected cells at
72 h p.i. (Fig. 2b, d, f). Treatment with siRdRP2 reduced the number of CBV3-infected cells but not CBV4- or CAV9-infected cells. CBV3 grows well in LLC cells and therefore cells were already detaching by 72 h p.i. This was seen also for Sirev3- and GFP-22-treated, infected cells. Sirev3 appeared to be somewhat toxic to the cells, as the cells detached sooner from the wells than did the untreated cells. All treated and untreated cells infected with CBV4 and CAV9 showed high numbers of infected cells by 72 h p.i. and no detectable difference could be seen between the different single-site siRNA treatments.

To study the replication and release of virus into culture medium, plaque titrations were performed on African green monkey kidney (GMK-22) cells in 12-well plates. Virus yields are shown in Fig. 3. 6-CBV3 siRNAs inhibited the CBV3 infection almost completely, with only very low virus titres detected in the supernatants even at 72 h p.i. The CBV3 siRNA pool made using the 6 RdRP also had an inhibitory effect on two related coxsackieviruses, CBV4 and CAV9, both belonging to the enterovirus B species. The reduction in virus titre was significant at all three time points (P<0.05 compared with untreated

Fig. 2. Immunoperoxidase staining of cell cultures using anti-enterovirus antibody after siRNA transfection and CBV3 (a, b), CBV4 (c, d) or CAV9 (e, f) infection (72 h p.i.). The images in (a), (c) and (e) show transfection with 6-siRNAs (CBV3 or GFP) or water, no transfection (Untreated-infected), and the cells alone (Blank). The images in (b), (d) and (f) show transfection with single-site siRNAs (Sirev3, siRdRP2 or GFP22) or water, no transfection (Untreated-infected), and the cells alone (Blank). Bar, shown in (a) 100 μm.
cells) for all three viruses (CBV3, CBV4 and CAV9) treated with the $\phi 6$–CBV3 siRNAs. Cells transfected with $\phi 6$–GFP siRNAs showed a significant reduction in virus titre for CAV9-infected cells at the earlier time points, but not at 72 h p.i. These effects were not significant in the CBV3- and CBV4-infected cells. Reduction was also seen for GFP-22-transfected cells infected with CBV4. However, there is no detectable sequence similarity between the GFP-22–siRNA sequence and the CBV3, CBV4 or CAV9 genome. siRdRP2-treated cells infected with CBV3 showed a significant decrease in virus titre at all three time points (Fig. 3d–f). At 72 h p.i., there was a 10-fold reduction compared with non-transfected cells. For the other viruses, the reduction was less pronounced but was significant for CBV4. No significant decrease in virus titre was seen following CAV9 infection. Sirev3 reduced the amount of virus significantly only at 24 h p.i. in CBV3- and CBV4-

infections. The $\phi 6$–siRNA pools had a stronger effect than the single-site siRNAs, because at 72 h p.i., the reduction caused by siRdRP2 in CBV3-infected cells was 1:10 compared with the reduction of 1:10$^4$ observed for $\phi 6$–CBV3 siRNA-treated cells.

The broad effect of the $\phi 6$–CBV3 siRNA treatment on the different human enterovirus B species could be explained by the fact that the siRNAs span a large CBV3 genome segment, and this part of the genome is 80% conserved between these related viruses. The IPS data support the findings obtained by titrating the supernatants. The results of a quantitative (q)PCR for the presence of enteroviral genome (modified from the method described by Lönnrot et al., 1999) in the cells were in accordance with the IPS and plaque titration (data not shown). The statistical analyses were performed with StatView Mann–Whitney U-

Fig. 3. Virus titres in treated cell culture supernatants collected at 24, 48 and 72 h after infection with CBV3 (a, d), CBV4 (b, e) or CAV9 (c, f). LLC cells in 96-well plates were transfected with 100 ng siRNAs 20–24 h before infection. *P<0.05, indicates that the results are statistically significantly different when compared with infected cells. (a–c) Titres in cell cultures transfected with $\phi 6$–siRNA pools. Bars: black, $\phi 6$–CBV3 siRNA; white, $\phi 6$–GFP siRNA; dark grey, infected cells only. (d–f) Titres in cell cultures transfected with single-site siRNAs. Bars: white, Sirev3; black, siRdRP2; light grey, GFP-22; dark grey, infected cells only.
test and ANOVA. siRNA treated samples were analysed in comparison with untreated infected cells. P values <0.05 were considered statistically significant.

It has been shown that some siRNAs activate the interferon (IFN) system (Kim et al., 2004; Rossi, 2009; Sleutz et al., 2003) and it was long believed that an induction of the innate immune system would be an obstacle for possible therapeutic applications of RNAi. However, it has also been shown that siRNAs do not always trigger an IFN response (Judge & MacLachlan, 2008). We performed qRT-PCR for IFNs in our cell cultures and no induction of type 1 IFNs was observed, which could be attributed to the IFNs in our cell cultures and no induction of type 1 IFNs (Judge & MacLachlan, 2008). We performed qRT-PCR for IFNs in our cell cultures and no induction of type 1 IFNs was observed, which could be attributed to the IFNs in our cell cultures and no induction of type 1 IFNs (Judge & MacLachlan, 2008).

We also observed that some single-site siRNAs and the 6–GFP siRNAs may upregulate MxA expression. The IFN response may explain why the GFP–single-site siRNA or 6–GFP siRNA pool occasionally inhibited CBV4 infection. RNA viruses use dsRNA intermediates during transcription and therefore also trigger IFN responses. Using siRNAs spanning a wider genome area may have negative consequences for normal cell machinery. Such siRNAs may also target transcription of the cellular genes. However, we did not detect an effect of the 6–siRNA pools on transcripts of housekeeping genes such as GAPDH, β-actin or 18S rRNA (Supplementary Table S1, available in JGV Online).

We observed that 6–siRNA pools against CBV3 were three orders of magnitude more efficient at reducing viral replication compared with single-site siRNAs. In addition, they also had a profound effect on CBV4 and CV9 replication. Such an effect was not observed using the single-site siRNAs. The target sites of these single-site siRNAs are not found in the CBV4 and CV9 genomes, but are present in the CBV3 genome. The 6–siRNA pools are also more likely to inhibit viruses, which may escape single-site siRNA silencing by point mutations. It might also be possible to prevent human enterovirus B infections by targeting siRNA to highly conserved ‘Cre elements’ (Lee et al., 2007). No off-target effects were detected that could be attributed to the 6–siRNA pools, probably due to the low concentration of each individual siRNA within the pool. It seems that the use of pooled siRNAs is a favourable means to target viral infections and may offer a viable alternative for single-site siRNAs.

Acknowledgements

This study was supported by the Finnish Centre of Excellence Program of the Academy of Finland (2006–2011) grant 1129648 (D.H.B) and by the Academy of Finland #211035 and #118366 (V.H.). Further support was obtained from Paulon Säätiö, Svenska Kulturfonden, Varsinais-Suomen Kulttuurirahasto, Turku Graduate School of Biomedical Sciences and Turun Yliopistosäätiö. Dr Albie van Dijk is acknowledged for her input at the early stages of this work. We also thank Terhi Helander and Camilla Aspelin for help with PCR runs and Riitta Tarkkainen for producing the 6–siRNAs.

References


