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Inhibition of red seabream iridovirus (RSIV) replication by small interfering RNA (siRNA) in a cell culture system

Lua T. Dang, Hidehiro Kondo, Ikuo Hirono*, Takashi Aoki

Laboratory of Genome Science, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato, Tokyo 108-8477, Japan

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Abstract

Small interfering RNAs (siRNAs), mediators of a process of sequence-specific gene silencing called RNA interference, have been shown to have activity against a wide range of viruses and are considered to be potential antiviral tools. Here, we describe an antiviral activity of a siRNA that targets the major capsid protein (MCP) gene of red seabream iridovirus (RSIV), a marine fish-pathogenic virus, in a cell culture system. Inhibition of RSIV replication was demonstrated by reduced MCP expression level and reduced RSIV titer. MCP-targeted siRNA (siR-MCP) dose-dependently inhibited the expression of MCP gene in cells that either transiently expressed or stably expressed the MCP gene. At 84 and 96 h after viral infection, siR-MCP reduced the expression of MCP gene by 55.2% and 97.1%, respectively. Transfection with siR-MCP reduced the production of RSIV particles in supernatants of samples infected with RSIV, while the corresponding mismatched siR-MCP (MsiR-MCP) and nsRNA controls did not exhibit this effect. These results show that MCP-targeted siRNA can effectively and specifically inhibit the expression of the target gene and hinder RSIV replication during an *in vitro* infection, providing a potential approach for the control of viral diseases in aquaculture.

Keywords: RNA interference (RNAi); Small interfering RNA (siRNA); Major capsid protein; MCP; Red seabream iridovirus; RSIV

1. Introduction

RNA interference (RNAi), mediated by double-stranded small interfering RNAs (siRNAs), has been shown to have activity against a wide range of viruses and is promising a new antiviral therapy (Andino, 2003; Coburn and Cullen, 2002; O'Brien, 2007). It is a process of sequence-specific gene silencing in the cytoplasm of eukaryotic cell, in which siRNAs of 21–23 nucleotides (nts) are associated with a multiprotein complex known as the RNA-induced silencing complex (RISC) to target homologous mRNA for degradation based on complementary base pairing. siRNAs can be processed in cells from longer double-stranded RNAs produced by viral infection, by transposons or can also be chemically introduced into cells from the outside (Agami, 2002; Carmichael, 2002). Therefore, introduction of 21–23 nts siRNA duplexes specific for viruses into cells could lead to viral mRNA degradation and inhibition of viral gene expression and viral replication. Recent studies have proven that siRNAs can inhibit replication of many kinds of viruses at several stages of infection in various cells (Ferreira et al., 2007; Haasnoot et al., 2003). siRNAs can be employed to suppress the expression of viral genes in plant cells (Yelina et al., 2002), insect cells (Adelman et al., 2002; Caplen et al., 2002), mammalian cells (Capodici et al., 2002; Novina et al., 2002; Surabhi and Gaynor, 2002), as well as aquatic animal cells (Tirasophon et al., 2005; Xie et al., 2005).

Efficient inhibition has been demonstrated for DNA viruses both in cell culture and animal models. These include hepatitis B virus (HBV) (Giladi et al., 2003; Morrissey et al., 2005; Wu et al., 2007), herpes simplex virus 1 (HSV-1) (Bhuyan et al., 2004), herpes simplex virus 2 (HSV-2) (Palliser et al., 2006), human papillomavirus type 18 (HPV-18) (Hall and Alexander, 2003), human cytomegalovirus (HCMV) (Wiebusch et al., 2004), human herpes virus 6B (HHV-6B) (Yoon et al., 2004), JC virus (JCV) (Orba et al., 2004), murine herpesvirus 68 (MHV-68) (Jia and Sun, 2003), anatid herpes virus 1 (AHV-1) (Mallanna et al., 2006), and tiger frog iridovirus (TFV) (Xie et al., 2005). The results of these studies suggest the possibility of using siRNAs as an antiviral tool against DNA viruses.

* Corresponding author. Tel.: +81 3 5463 0689; fax: +81 3 5463 0689.
E-mail address: hirono@kaiyodai.ac.jp (I. Hirono).

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Red seabream iridovirus (RSIV), a fish-pathogenic virus, causes a systemic infectious iridoviral disease known as red seabream iridoviral disease (RSIVD) in cultured marine fish in some parts of the world (Do et al., 2004; Inouye et al., 1992; Kawakami and Nakajima, 2002; Wang et al., 2003). Therefore, it is necessary to develop novel therapeutic approaches that effectively inhibit RSIV replication. RSIV is a double-stranded DNA virus with the genome encoding a major capsid protein (MCP) gene and 92 other putative open reading frames (ORFs) (Kurita et al., 2002). The MCP gene accounts for about 45% of the total protein of the virus and is needed for the cleavage and packaging of viral DNA to form viable virions (Williams, 1996). This gene has been selected to analyze the phylogenetic relationships of iridoviruses (Go et al., 2006; Imajoh et al., 2007; Lu et al., 2005) and has been confirmed to be the most suitable gene for detection and measurement of RSIV infection (Caipang et al., 2003; Dang Thi et al., 2007).

In this paper, we report the inhibition of RSIV replication using a siRNA targeting the MCP gene of the virus in a cell culture system. siRNA is a sequence-specific gene silencing mechanism, and inhibits the target gene in a dose-dependent manner (Colbere-Garapin et al., 2005; Elbashir et al., 2001; Huelsmann et al., 2006; Zheng et al., 2005). Therefore, siRNA was initially assessed for inhibitory effects on the MCP gene silencing in cells either transiently or stably expressing the MCP gene by using a plasmid expressing the target gene, and it was then tested for its inhibitory effect on RSIV replication in terms of MCP gene expression during viral infection and in terms of reduction in viral production. Our data provide evidence that siRNA can be used to selectively block viral gene expression and hence viral replication in fish cell lines.

2. Materials and methods

2.1. Cell culture and virus

Grunt Fin (GF) cells (Clem et al., 1961) and Hirame Natural Embryo (HINAE) cells (Kasai and Yoshimizu, 2001) were maintained following Lua et al. (2005). GF cells were used for propagation of virus stock while HINAE cells were used for transfection experiments.

RSIV was obtained from a spleen homogenate of RSIV-infected red seabream, and was propagated in GF cells following Lua et al. (2005). The virus titer was determined using the 50% tissue culture infective dose ($TCID_{50}$) method (Reed and Muench, 1938), and the virus stock was stored in 1 ml aliquots at −80 °C until use.

2.2. Design and synthesis of siRNAs

Three (3) duplex siRNAs were chemically synthesized for use in this study (Table 1). Among them, a siRNA (referred to as siR-MCP) targeting the MCP gene of RSIV was designed using the siRNA target finder programme of Ambion (http://www.ambion.com/sirnatargetfinder). Two other siRNAs (referred to as MsiR-MCP and nsRNA) were designed for use as controls of siRNA sequence specificity. MsiR-MCP was the corresponding mismatched siRNA of siR-MCP and was designed in accordance with previously published rules (Schyth et al., 2006). nsRNA was identical to the sequence of a siRNA (Si1) specific for MCP of TFV (Xie et al., 2005).

2.3. Construction of MCP-expressing plasmid (pCMV-MCP) and selection of stably MCP-expressing HINAE transformant

The full-length of MCP was amplified from the RSIV genome with primers containing EcoRI and XbaI sites (Table 2) by PCR. The PCR products were purified, and cloned into EcoRI and XbaI sites of pCI-neo mammalian expression vector (Promega, USA) (Fig. 1A). The MCP-expressing plasmid (pCMV-MCP) was extracted and purified using a NucleoSpin plasmid quickpure kit (Macherey-Nagel, USA) according to the manufacturer's protocol.

To generate cells stably expressing the MCP gene, HINAE cells were transfected with pCMV-MCP and cultured with selective medium (Leibovitz's L-15 medium supplemented with geneticin) (Gibco-BRL, USA) following the manufacturer's protocol. The presence of a selectable marker, the neomycin phosphotransferase gene, allowed selecting HINAE transformants that harbored pCMV-MCP under selective conditions. Normal HINAE cells were sensitive while stably MCP-expressing HINAE transformants were stable with geneticin. One month post-transfection, the transformants were checked for the expression of the MCP gene (Fig. 1B) and used to assess the inhibitory effect of siR-MCP on the MCP gene in the case of stable expression.

2.4. Transfection of plasmid DNA and siRNA

HINAE cells were seeded into 24-well or 96-well cell culture plates for about 24 h (90–95% confluent monolayer) using L-15 medium containing 15% of fetal bovine serum (FBS) (JRH Biosciences, USA) without phenol red or antibiotics prior to transfection. Cells were transfected following the manufac-

Table 1
siRNA sequences (sense strand) used in this study

siRNA name	Target sequence	Position in gene sequence	Source
siR-MCP	5′-AACAGACUGGCCAUGCUAAUU-3′	164–182	RSIV
MsiR-MCP[a]	5′-A**G**CAGACUG**A**C**U**A**C**GCUA**G**UU-3′		
nsRNA[b]	5′-CGCCUGGUUGGUACUCAAGUU-3′	237–255	TFV

[a] The corresponding mismatched siRNA of siR-MCP. Mismatched nucleotides are bold and underlined.
[b] The identical sequence of siRNA (Si1) targeting MCP of tiger frog iridovirus (TFV) (Xie et al., 2005).