Virology 411 (2011) 25–31

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

A microRNA of infectious laryngotracheitis virus can downregulate and direct cleavage of ICP4 mRNA

Lisa A. Waidner [a], Joan Burnside [b], Amy S. Anderson [b], Erin L. Bernberg [b], Marcelo A. German [c], Blake C. Meyers [d], Pamela J. Green [d], Robin W. Morgan [b,*]

[a] Elcriton, Inc. 15 Innovation Way, Suite 288, Newark, DE 19711, USA
[b] Department of Animal and Food Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, DE 19711, USA
[c] Dow AgroSciences, LLC, 16160 SW Upper Boones Ferry Road, Portland, OR 97224, USA
[d] Department of Plant and Soil Sciences, Delaware Biotechnology Institute, University of Delaware, Newark, DE 19711, USA

ARTICLE INFO

Article history:
Received 15 April 2010
Returned to author for revision
2 December 2010
Accepted 13 December 2010
Available online 12 January 2011

Keywords:
Infectious laryngotracheitis virus
Herpesvirus
microRNA
siRNA
Transcription factor
ICP4

ABSTRACT

Viral microRNAs regulate gene expression using either translational repression or mRNA cleavage and decay. Two microRNAs from infectious laryngotracheitis virus (ILTV), iltv-miR-I5 and iltv-miR-I6, map antisense to the ICP4 gene. Post-transcriptional repression by these microRNAs was tested against a portion of the ICP4 coding sequence cloned downstream of firefly luciferase. Luciferase activity was downregulated by approximately 60% with the iltv-miR-I5 mimic. Addition of an iltv-miR-I5 antagomiR or mutagenesis of the target seed sequence alleviated this effect. The iltv-miR-I5 mimic, when co-transfected with a plasmid expressing ICP4, reduced ICP4 transcript levels by approximately 50%, and inhibition was relieved by an iltv-miR-I5 antagomiR. In infected cells, iltv-miR-I5 mediated cleavage at the canonical site, as indicated by modified RACE analysis. Thus, in this system, iltv-miR-I5 decreased ILTV ICP4 mRNA levels via transcript cleavage and degradation. Downregulation of ICP4 could impact the balance between the lytic and latent states of the virus *in vivo*.

Introduction

MicroRNAs are small, non-coding RNAs found in plant, animal, and viral genomes. These molecules are considered important contributors to regulation of cellular processes because of their widespread and diverse tissue- and condition-specific expression profiles (He and Hannon, 2004) and because they can regulate at least 60% of vertebrate transcripts (Friedman et al., 2009). MicroRNAs are generally thought to function in animal cells by base-pairing to their target mRNAs, which leads to translational inhibition or mRNA degradation (Bartel, 2004; Filipowicz et al., 2008; Nilsen, 2007). Complementarity between nucleotides 2 and 7/8 of the microRNA (known as the "seed" sequence) is crucial for target recognition in mRNA destabilization, translational repression, or both, and 100% complementarity is important for RNA-induced silencing complex (RISC)-mediated cleavage (Bartel, 2009). Some microRNAs act as small interfering RNAs (siRNAs) through an RNAi mechanism via 100% complementarity to their targets (Wang et al., 2004). A number of viruses, herpesviruses in particular, encode microRNAs (Cullen, 2006; Pfeffer et al., 2005). Because of the compact nature of viral genomes, many viral microRNAs are derived from non-coding transcripts antisense to functional genes. However, regardless of genomic address, viral microRNAs can act via an siRNA mechanism (Barth et al., 2008; Seo et al., 2008, 2009; Sullivan et al., 2005; Tang et al., 2008, 2009) or via imperfect base pairing to targets, as with animal microRNAs (Grey et al., 2007; Murphy et al., 2008; Umbach et al., 2008).

Herpesviruses, which are characterized by latent infections, are likely to use microRNAs to enhance viral effects on host cells, and this can be accomplished by regulating either host or viral genes (Cullen, 2009). Epstein-Barr virus (EBV) (Pfeffer et al., 2004), Kaposi's sarcoma-associated herpesvirus (KSHV) (Cai et al., 2005), human herpes simplex virus 1 (HSV1) (Umbach et al., 2008), murine herpesvirus 68 (MHV68) (Pfeffer et al., 2005), human cytomegalovirus (HCMV) (Grey et al., 2005), Marek's disease virus 1 (MDV1) (Burnside et al., 2006; Yao et al., 2008), MDV2 (Waidner et al., 2009; Yao et al., 2007), herpesvirus of turkeys (HVT) (Waidner et al., 2009), and infectious laryngotracheitis virus (ILTV) (Waidner et al., 2009) are among the herpesviruses known to encode microRNAs. Some viral microRNAs are expressed during latency and therefore may be important in regulating the balance between productive and latent viral states. For instance, microRNAs of MDV1 (Burnside et al., 2006), MDV2 (Waidner et al., 2009; Yao et al., 2007), EBV (Grundhoff et al., 2006; Pfeffer et al., 2004), KSHV (Cai et al., 2005; Grundhoff et al.,

* Corresponding author. University of Delaware, Delaware Biotechnology Institute, Room 245, 15 Innovation Way, Newark, DE 19711, USA. Fax: +1 302 831 4054.
E-mail address: morgan@udel.edu (R.W. Morgan).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.12.023

2006; Pfeffer et al., 2005; Samols et al., 2005), HSV1 (Umbach et al., 2008), Rhesus monkey rhadinovirus (RRV) (Schafer et al., 2007), and murine herpesvirus 68 (MHV68) (Pfeffer et al., 2005) are predominantly clustered at one or two sites in the genome, and one of the clusters lies in a region active during latency. In some cases, microRNAs are antisense to sequences within (Waidner et al., 2009; Yao et al., 2007) or in the vicinity of (Burnside et al., 2006; Yao et al., 2008) the immediate early gene, ICP4.

Many viral microRNAs map antisense to viral lytic genes, but the mechanism of downregulation of the antisense targets varies among and within viruses. Viral microRNAs that are highly complementary to their targets may regulate them at the mRNA level, at the protein level, or not at all. MicroRNA-mediated cleavage is used in regulation of the large T-antigen mRNA in two human polyomaviruses (JC and BK) (Seo et al., 2008) and murine polyoma virus (Sullivan et al., 2009). Additionally, HSV2-miR-H3 is 100% complementary to the neurovirulence factor gene ICP34.5 and downregulates it in an siRNA-like manner (Tang et al., 2008). In contrast, translation inhibition is used by HSV1-miR-H2, which maps antisense to the immediate-early transactivator ICP0 gene (Umbach et al., 2008). In HCMV, miR-112-1 has high complementarity (15/22 nucleotide match) to the immediate early gene IE1 but does not affect mRNA levels even though it does decrease IE1 protein expression (Grey et al., 2007; Murphy et al., 2008). HSV miRs H3 and H4 are 100% complementary to ICP34.5, but regulation of this transcript by these microRNAs has not been addressed (Umbach et al., 2008). However, HSV miR-H6 shows extensive seed complementarity to the HSV ICP4 and does downregulate expression of ICP4 (Umbach et al., 2008).

ILTV is an alphaherpesvirus belonging to the genus *Iltoviridae*. It has a general genome structure that resembles that of HSV, but unlike most alpha-herpesviruses, it does not contain the repeat regions flanking the unique long genome segment (I/TR_L). ILTV causes a respiratory disease in chickens and pheasants, with the severity of the disease varying from decreased egg production to conjunctivitis and severe tracheal infections resulting in bloody expectoration (Bagust et al., 2000; Garcia and Riblet, 2001; Kirkpatrick et al., 2006). In addition, after a short acute infection phase of 2 weeks or less, the virus becomes latent in the central nervous system (Williams et al., 1992) and can subsequently reactivate, resulting in re-infection of exposed flocks (Hughes et al., 1989; Hughes et al., 1991). The most abundant microRNAs from ILTV, iltv-miR-I5 and iltv-miR-I6, map antisense to the ICP4 gene (Rachamadugu et al., 2009; Waidner et al., 2009), which is located in the internal and terminal repeats flanking the unique short genome segment (I/TR_S). ICP4 is a major transcriptional regulator that is essential for viral growth; it is an immediate-early gene that is repressed during latency. In the study presented here, we investigate whether iltv-miR-I5 could downregulate the immediate early gene ICP4.

Results

iltv-miR-I5 reduced expression from reporter plasmids containing complementary sites

The action of iltv-miR-I5 and iltv-miR-I6 on the expression of ILTV ICP4 was tested using luciferase reporter assays. A 409-bp fragment from the coding region of ICP4, corresponding to nucleotides 115,017–115,425 in the IR_S (146,290–146,708 in the TR_S), was cloned downstream of the luciferase gene in the pMIR-REPORT™ vector (pMiR-Report-I5-I6-target, Fig. 1, inset). This portion of the IPC4 open reading frame contains sequences that are 100% complementary to iltv-miR-I5 and iltv-miR-I6. Plasmid pMiR-Report-I5-I6-target was co-transfected into COS7 cells along with mimics and antagomiRs. Transfections included the pMIR-REPORT™ β-galactosidase plasmid to evaluate transfection efficiency, and relative luciferase activity was determined (Fig. 2). The addition of the iltv-miR-I5 mimic resulted in significant repression ($p<0.05$), with approximately 60% reduction in luciferase activity compared to control samples (Fig. 2A). Repression of luciferase activity was observed for the iltv-miR-I6 mimic but was not significant (23% reduction, $p=0.072$). Samples co-transfected with a combination of mimics for iltv-miR-I5 and iltv-miR-I6 showed a level of luciferase repression similar to that seen with iltv-miR-I5 alone (Fig. 2A), and this was also significantly lower than the negative control ($p<0.05$).

To confirm the specificity of the negative regulatory effect of iltv-miR-I5, we mutated three nucleotides in the seed target region for iltv-miR-I5 in plasmid pMiR-Report-I5-I6-target (Fig. 2B). The iltv-miR-I5 mimic significantly reduced expression of the wild-type target construct by approximately 60% and was significantly different from the control ($p<0.05$, Fig. 2C), and the addition of an antagomiR of miR-I5 alleviated this effect ($p=0.49$). Co-transfection of the iltv-miR-I5 mimic with the mutated construct did not result in a significant reduction (15% reduction, $p=0.21$, Fig. 2C). As expected, the co-transfection with the mutant construct along with the iltv-miR-I5 and its antagomiR did not differ significantly from the control transfection ($p=0.11$).

In order to assess whether physiological levels of ILTV-derived miR-I5 could downregulate ICP4, iltv-miR-I5 target luciferase constructs were transfected into LMH cells infected with ILTV. The timing of ILTV growth and microRNA expression was first tested in this cell culture system with an ILTV multiplicity of infection (MOI) of 2.2 (Supplemental Fig. 1). A qPCR based on quantification of the gC gene was used to measure total viral genomes per well. In this system, the number of ILTV genomes per well was maximal at 35–50 h post-infection (hpi) (Supplemental Fig. 1A). In addition, iltv-miR-I5 was measured by Northern analysis, and microRNA accumulation was detected as early as 19 hpi (Supplemental Fig. 1B) but was higher at later times (28 and 50 hpi). A 331-bp fragment containing the iltv-miR-I5 target region in the coding region of ICP4 (nucleotides 146,391–146,708 in the TR_S) was cloned downstream of the luciferase gene in the pMIR-REPORT™ vector (pMiR-Report-I5-target, Fig. 1, inset). Luciferase constructs WT pMiR-Report I5-target, its mutated derivative, or the empty vector pMIR-REPORT™ were transfected at 36.5 to 49.5 hpi. At 4.5 to 9 h post-transfection, cells were assayed for normalized luciferase values. In numerous experiments, both wild-type and mutated target constructs were expressed at lower levels compared to expression in cells transfected with empty vector pMIR-REPORT™ (data not shown). This finding was unexpected but reproducible, and it could indicate that a cellular microRNA or even another regulatory mechanism can target ILTV ICP4 independent of iltv-miR-I5.

The experimental protocol was modified such that the cell culture medium was not removed during the transfection process in order to allow maximum accumulation of virus and virus-encoded microRNAs. Data from the two experiments done with this modified protocol are shown in Fig. 3. Both wild-type and mutated targets were expressed at lower levels compared to expression in cells transfected with empty vector. In mock-infected cells, there was no significant difference between the wild-type and mutant reporter constructs. In the ILTV-infected cells, there was a slightly lower expression (16–17% difference) of the wild-type compared to the mutant reporter (Fig. 3), and the value obtained from the wild-type transfection was significantly lower than that of the mutated construct in both experiments ($p<0.05$). However, the effect was not large, the experimental protocol was delicate to optimize, and the assay was complicated by the background reduction of reporter expression in mock-infected cells.

iltv-miR-I5 reduced ICP4 mRNA levels by transcript cleavage

Since iltv-miR-I5 is 100% complementary to the coding region of ICP4, we hypothesized that this microRNA could act as an siRNA and