Minireview

Virus-host interactions: new insights from the small RNA world

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Abstract

RNA silencing has a known role in the antiviral responses of plants and insects. Recent evidence, including the finding that the Tat protein of human immunodeficiency virus (HIV) can suppress the host's RNA-silencing pathway and may thus counteract host antiviral RNAs, suggests that RNA-silencing pathways could also have key roles in mammalian virus-host interactions.

Over the course of evolution viruses have developed highly sophisticated mechanisms for interacting with host cells. Such interactions may involve parasitizing the cellular machinery to enhance the production of progeny viruses; budding of virions of the human immunodeficiency virus (HIV), for example, makes use of the endosomal sorting complexes (ESCRT complexes) that normally regulate the formation of the multivesicular bodies of the endosomal pathway [1]. In addition, many viruses have mechanisms for disrupting the immune response to viral infection. An example is the inhibition by the human cytomegalovirus proteins US2 and US11 of the presentation of antigens by host MHC class I molecules; this prevents the recognition and destruction of virus-infected cells by the host immune system [2]. New research is now beginning to show that the complex interaction between viruses and host cells also involves RNA-silencing pathways.

RNA silencing in animal cells is carried out by microRNAs (miRNAs) and small interfering RNAs (siRNAs) of around 22 nucleotides, which specifically hybridize with target RNAs to inhibit their expression. Perfect sequence complementarity between siRNAs and their target sequences results in the cleavage of target mRNAs by the RNA-induced silencing complex (RISC), whereas imperfect matches, as typically observed between miRNAs and their targets, result in repression of translation [3]. siRNAs are generated from long double-stranded (ds) RNAs by the Dicer RNase III enzyme [4]. Maturation of miRNAs first requires the nuclear processing of their precursor transcripts by the Microprocessor complex, which contains Drosha RNase III and a dsRNA-binding protein, Pasha [3-5]. Dicer and another dsRNA-binding protein, TRBP, are then required for the final maturation of miRNAs [3-7].

RNA silencing is critical in plant and animal development [8,9], and is important for protection against viruses in plants and insects [9,10], where it is induced by the recognition of viral dsRNA. It has been unclear whether RNA silencing has a role in immunity in vertebrates, however, even though vertebrates do have other sophisticated innate mechanisms for responding to viral dsRNA, such as the protein-kinase-R-dependent antiviral response and the Toll-like receptor system [11]. Recent studies now show that vertebrate viruses encode products that interfere with the RNA-silencing machinery [10], suggesting that RNA silencing may indeed be important for antiviral responses in vertebrates.

RNA silencing in response to virus infection could be due to miRNAs encoded by either the virus or the host. Several virus-encoded miRNAs have now been found, but their relevance to infection is in most cases unclear. In the first successful search for virus-derived small RNAs, Pfeffer and co-workers [12] identified five miRNAs encoded by the herpesvirus

Epstein-Barr virus (EBV), one of which - miR-BART2 - targets for cleavage the mRNA for EBV DNA polymerase (BALF5). More recently, computational prediction combined with cloning has identified additional miRNAs from other herpesviruses, although their functions remain unknown [13-15]. Interestingly, an miRNA has been identified in the papovavirus simian virus 40 (SV40); it is derived from the late transcript and targets the transcript of the large T antigen for cleavage [16]. This does not affect viral replication, at least *in vitro*, but may function to limit the expression of large T antigen. Abrogating this miRNA-mediated suppression of T antigen increased the recognition of SV40-infected cells by antigen-specific cytotoxic T cells [16]. The viral miRNA may thus reduce the susceptibility of the virus to the host immune system.

Viral small RNAs

Bennasser and co-workers [17] now report that HIV triggers the RNA-silencing system to produce a potentially suppressive small RNA, and that the HIV Tat protein interferes directly with the silencing system to produce a general inhibition of silencing function. During the course of its replication cycle, HIV generates multiple different spliced RNA transcripts, many with dsRNA elements that might trigger an RNA-silencing response within the infected cell. Such transcripts include the transactivation response (*TAR*) element, which is crucial for viral transcription, and the Rev-response element (*RRE*), which promotes the expression of genes from unspliced or partially spliced transcripts [18]. To determine whether any of these elements are processed by the host cell's RNA-silencing machinery so as to limit HIV infection, Bennasser *et al.* [17] used an algorithm to scan the HIV genome for perfect 19-bp hairpin RNA sequences that could potentially be processed by Dicer. They found that one such region within the *env* gene, which encodes the viral envelope glycoprotein, is indeed expressed during HIV infection as a 21-nucleotide RNA, a hallmark of Dicer-processed products. Heterologous overexpression of this small RNA, which Bennasser *et al.* call vsiRNA1, was able to knock down expression of the *env* gene in cells transfected with HIV DNA, but only modestly inhibited the production of infectious HIV particles.

Key questions regarding the function of HIV vsiRNA1 remain to be addressed. How does its natural expression level during HIV infection compare with the level required to inhibit the virus in the overexpression experiments? Is it generated from a larger precursor in a Drosha-dependent fashion, as are endogenous miRNAs, or by some other pathway? As described earlier, some viruses utilize the host's RNA-silencing machinery to generate viral miRNAs that regulate host and viral gene expression during the course of viral replication. Could the HIV vsiRNA1 provide a similar benefit? One clear way to address its function might be to mutate residues that affect the hairpin formation but do not affect

the coding sequence of the *env* gene, and then determine the effect of these mutations on the virus. It is indeed possible that this small viral RNA plays no role in infection at all, but is simply expressed as a by-product of the conservation of palindromic sequence in the *env* gene for other reasons. As such a palindrome is also found in the simian immunodeficiency virus (SIV), it may be informative to examine the effect of mutations to the hairpin structure on SIV replication and pathogenesis in the rhesus macaque. As we discuss below, Bennasser *et al.* [17] also found that HIV could suppress the host cell's RNA-silencing machinery. Given this and the other findings, it seems unlikely that the small RNA provides significant benefit to the virus.

Viral interference with host RNA silencing

HIV is not alone in affecting RNA silencing: a number of plant and animal viruses have recently been shown to suppress RNA-silencing pathways. The protein HC-Pro, encoded by the tobacco etch potyvirus, was the first viral protein identified as suppressing RNA silencing, but the mechanism of suppression remains unclear [9]. The tomato bushy stunt virus (TBSV) protein p19 binds both siRNA and miRNA duplexes and thus presumably inhibits the assembly of the RISC effector complex [10]. The vaccinia virus protein E3L, influenza protein NS1 and the Nodamura virus B2 protein are proposed to interfere with RNA silencing by sequestering dsRNAs [19,20]. Viral RNA molecules may also interfere with the RNA-silencing machinery. The adenoviral noncoding RNA VA1 inhibits RNA silencing, possibly by blocking the nuclear export of miRNA precursors by exportin-5 and/or the processing of miRNAs/siRNAs by Dicer [21].

In the case of HIV, Bennasser *et al.* [17] noticed that short hairpin RNAs designed to target the *TAR* RNA element in the 5' end of nascent HIV transcripts were ineffective when the experiments were carried out in the presence of the HIV Tat protein. Tat has long been known to play an important role in HIV replication, by recruiting to *TAR* the cyclin-dependent protein kinase Cdk9 and cyclin T1, cellular factors essential for processive transcription [22]. Strikingly, expression of Tat was able to inhibit RNA silencing of several genes, indicating that it acts as a general suppressor of RNA silencing, rather than being specific for *TAR*. By mutating the Tat protein at different sites, the authors [17] were also able to separate the ability of Tat to inhibit RNA silencing from its ability to promote HIV transcription, indicating that these are distinct activities. An HIV mutant encoding a Tat variant that lacks the ability to suppress RNA silencing replicates only marginally less well than wild-type HIV, but was significantly more sensitive to inhibition by short hairpin RNAs targeting the HIV genome.

How does Tat function to inhibit the host RNA-silencing machinery? RNA silencing mediated by synthetic siRNAs was unaffected by Tat, suggesting that Tat acts at a step upstream

of RISC assembly and function, possibly by directly suppressing Dicer-mediated dsRNA processing (Figure 1). This idea is supported by the finding that Tat was able to inhibit Dicer cleavage of substrate RNA *in vitro*. Perhaps Tat interferes with the reaction by sequestering the dsRNA substrate or by interacting with Dicer to inhibit its activity.

As Tat acts as a general suppressor of RNA silencing and inhibits Dicer activity, which is used by both the siRNA and miRNA pathways, it is conceivable that the host miRNA pathway might also be inhibited by Tat. The significance of such inhibition is highlighted by Lecellier *et al.* [23], who have shown that an miRNA expressed in human cells restricts the replication of primate foamy virus (PFV). They first found that expression of the TBSV silencing suppressor p19 enhanced PFV replication fivefold. They then mapped the region of PFV that was being targeted by RNA silencing and found that it contained a potential target sequence for the human miRNA miR-32. A locked nucleic acid antisense oligonucleotide specifically designed to inhibit miR-32 enhanced PFV replication in HeLa cells, indicating that miR-32 is indeed limiting PFV replication in human cells. Like HIV, PFV counteracts the RNA-silencing machinery with a virus-coded protein, Tas, which acts as a broad suppressor of RNA silencing. The specific mechanism by which Tas suppresses silencing is unknown. Interestingly, Lecellier *et al.* [23] propose that HIV may be targeted by several host miRNAs (miR-29b, miR-129, and miR-188). It is possible that these miRNAs play roles in inhibiting HIV gene expression and need to be suppressed by Tat. Further work is needed to dissect the function of host miRNAs in the anti-HIV response and to determine whether Tat suppresses the biogenesis and/or function of host miRNAs.

In summary, it now appears that the RNA-silencing pathway may indeed be important in vertebrate antiviral responses. In turn, some viruses may employ this pathway for their own advantage. It will be interesting to understand how viruses balance the employment (in the case of SV40) and suppression (in the case of PFV and HIV) of the host RNA-silencing pathway. Do other viruses that produce dsRNA molecules avoid triggering the antiviral effects of the host RNA-silencing system? Some viruses might express general RNA-silencing suppressors, as HIV does, to counteract host defense. For others, the dsRNA molecules are perhaps well protected by other means to avoid exposure to the host RNA-silencing system. Understanding this may assist in the identification of drugs that target viral suppressors of RNA silencing. In the case of HIV, for example, a gene therapy approach using endogenously generated siRNAs, such as short hairpin RNAs, to target HIV transcripts might have only limited effects as a result of suppression by Tat. Indeed, some experiments using short hairpin RNAs to target HIV have indicated that the virus is able to escape their antiviral activity [5]. Could small drugs be designed to target the ability of Tat to suppress RNA silencing? Disruption of this activity of Tat
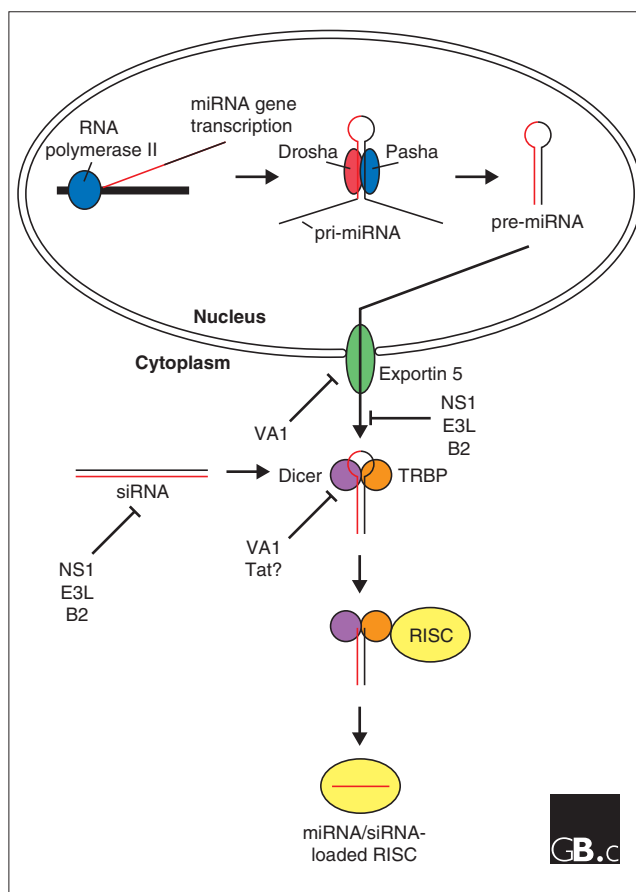

Figure 1
Viral suppressors of the mammalian RNA silencing pathway. The diagram shows the pathway of RNA silencing mediated by miRNAs and siRNAs. miRNA genes are transcribed as long transcripts in the nucleus, usually by RNA polymerase II. These long transcripts (pri-mRNAs) with local stem-loop structure are recognized and processed into miRNA precursors (pre-miRNAs) of approximately 70 nucleotides by the Microprocessor complex containing Drosha and Pasha. The highly structured pre-miRNAs are then exported into the cytoplasm by exportin-5. In the cytoplasm, pre-miRNAs are recognized and further processed into approximately 22-nucleotide mature miRNA duplexes by the Dicer-TRBP complex. Dicer also generates approximately 22-nucleotide siRNA duplexes from long dsRNAs. The miRNA or siRNA duplex is unwound during the assembly of the RNA-induced silencing complex (RISC) and only one strand is loaded while the other is degraded. The miRNA or siRNA in the RISC finds and silences its target mRNAs through sequence-specific recognition. The viral products that interfere with the pathway are shown, and the points at which they possibly act on the pathway to inhibit RNA silencing are indicated by barred lines. NS1, E3L, B2, and Tat are viral proteins (see text); VA1 is a noncoding adenoviral RNA.

in the HIV genome caused only a mild phenotype in tissue culture infection, but it will be interesting to see how an SIV strain with an equivalent mutation fares in the rhesus macaque model.

Are there host miRNAs that are used by viruses to enhance their replication? This possibility is raised by the recent discovery that miR-122 plays an important role in facilitating

hepatitis C virus replication [24]. Drugs targeting cellular miRNAs might be less likely to promote the evolution of viral 'escape' mutants. Time will tell whether a better understanding of the RNA-silencing system will have practical benefits in antiviral therapy.

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