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Review

Studying the RNA silencing pathway with the p19 protein

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ABSTRACT

The origins of the RNA silencing pathway are in defense against invading viruses and in response, viruses have evolved counter-measures to interfere with the host pathway. The p19 protein is expressed by tombusviruses as a suppressor of RNA silencing and functions to sequester small RNA duplexes, thereby preventing induction of the pathway. p19 exhibits size-specific and sequence-independent binding of its small RNA ligands, binding with high affinity to duplexes 20–22 nucleotides long. p19's binding specificity and its ability to sequester small RNAs has made it a unique protein-based tool for probing the molecular mechanisms of the highly complex RNA silencing pathway in a variety of systems. Furthermore, protein engineering of this 'molecular caliper' promises novel applications in biotechnology and medicine where small RNA molecules are of remarkable interest given their potent gene regulatory abilities.

1. Introduction

1.1. RNA silencing in antiviral defense

The discovery that double-stranded RNA (dsRNA) in the cell elicits sequence-specific inhibition of gene expression through the RNA silencing pathway has revolutionized our understanding of endogenous gene regulation and host–pathogen interactions (reviewed in [1]). In this system, endogenous and exogenous dsRNA precursors are the substrates of RNase III enzymes in the Dicer family, which generate small RNA duplexes (sRNAs) 21–24 nucleotides (nt) in length. microRNAs (miRNAs) are endogenous sRNAs with nuclear precursors that are processed in the cytoplasm [1]. miRNAs are critical in eukaryotic post-transcriptional gene regulation of nearly all cellular processes and their aberrant expression is linked to several disease states in humans, including cancer [2]. In plants and invertebrates, this pathway serves as a critical defense against invading viruses, and there is increasing evidence that this pathway has antiviral roles in vertebrates as well [3,12] (Fig. 1). Plants express several Dicer-Like (DCL) enzymes with specialized functions and DCL4 is the primary enzyme responsible for cleaving dsRNA intermediates or fold-back structures in the viral genome, generating viral-derived short-interfering RNAs (siRNAs) 21-nt long with 3′ 2-nt overhangs and 5′ phosphates [4–6]. sRNAs are the effector molecules of the pathway and are incorporated into a RNA-induced silencing complex (RISC) through interactions with members of the Argonaute protein family. Plants express a diversity of Argonaute proteins, but viral-derived siRNAs are predominantly incorporated into AGO1 or AGO2 to form the anti-viral RISC effector complex [7–9]. The sRNA-loaded RISC allows sequence specific gene silencing of the target mRNA, primarily through mRNA degradation or inhibition of translation [3,10].

1.2. Viral suppressors of RNA silencing

To counter the RNA silencing pathway, all plant viruses and several insect and mammalian viruses have evolved viral suppressors of RNA silencing (VSRS) [3,11,12]. VSRS from different viral families have extraordinary diversity in sequence and structure and employ distinct mechanisms to interfere with the host RNA silencing pathway, pointing to their independent evolution [3,13]. Functional annotation of viral genes as VSRS during infection has lent substantial insight into the mechanisms and significance of the RNA silencing pathway in viral defense [3].

Mechanisms of suppression of RNA silencing vary greatly among VSRS. Some VSRS interfere directly with the silencing machinery of the host plant [14], such as the p38 protein of *Turnip Crinkle Virus* and the 2b protein of *Cucumber mosaic virus*, which both function by interfering with AGO1-mediated gene silencing, p38 through GW motifs that serve as an Ago 'hook' [15,16] and

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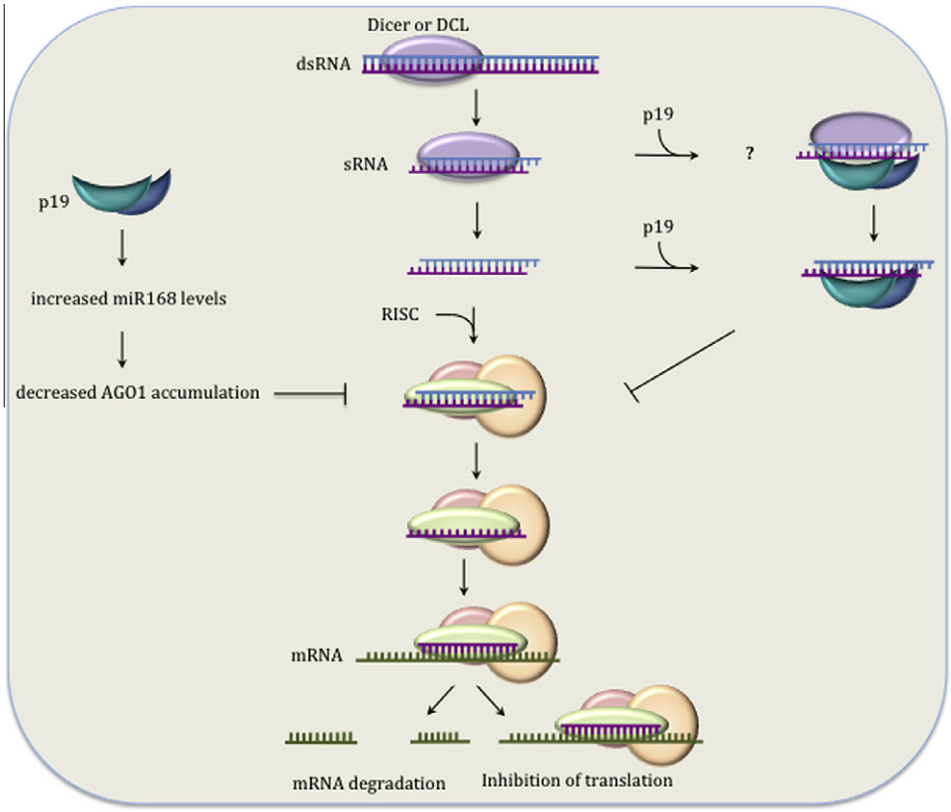

Fig. 1. The RNA silencing pathway and its suppression by the p19 protein of tombusviruses. Dicer or Dicer-like (DCL) enzymes cleave double-stranded RNA (dsRNA) into small RNA (sRNA) duplexes 21–24 nucleotides long, which are then incorporated into the RNA-induced silencing complex (RISC), which uses one strand of the duplex to elicit sequence-specific gene knockdown through mRNA degradation or inhibition of translation [10]. p19 is a viral suppressor of RNA silencing, which functions by sequestering viral-derived sRNAs (right) and preventing their incorporation into the RISC complex [30]. Kinetics studies suggest a mechanism of suppression involving a ternary complex between Dicer, sRNA and p19 [31]. p19 also functions to suppress the pathway by preventing the accumulation of AGO1, a component of anti-viral RISC, by inducing the accumulation of a host miRNA, miR168, which then downregulates AGO1 expression (left) [59].

2b through interactions with the PAZ domain [9]. Through interactions with AGO1, they prevent loading of an active RISC complex. Several VSRS interact with dsRNA, either binding long dsRNA to prevent Dicer-mediated cleavage (e.g. *Flockhouse Virus* B2 protein [17]) or binding siRNAs to prevent active-RISC formation (e.g. p19 protein of tombusviruses [18,19]). Structural analyses have demonstrated a fascinating diversity in dsRNA binding strategies by these proteins, three of which have been determined by X-ray crystallography in complex with dsRNA, lending insight into the biochemical basis for their ligand specificities and affinities (Fig. 2).

It has been suggested that sequestering sRNAs is a general strategy of VSRS to suppress the pathway [20,21], however it is becoming increasingly evident that in vitro sRNA binding activity does not always contribute to suppressor activity and may be important for other activities of the protein unrelated to VSRS activity. p19's high affinity for sRNA is critical to its function in viral pathogenesis, since point mutations abrogating binding eliminates its silencing activity in planta [22–24]. On the other hand, *Turnip Crinkle Virus* p38 binds dsRNAs of variable length [20], but its suppressor activity was completely abrogated by mutating two GW residues responsible for its interaction with AGO1 [15]. Furthermore, p38 is unlikely to function by sRNA sequestration since its transgenic expression does not prevent the mobility of siRNAs into neighbouring cells, in contrast to what is observed for *Tombusvirus* p19 and *Beet Yellow Virus* p21 proteins [25,26].

1.3. p19: molecular caliper for small RNA duplexes

The p19 protein is expressed by tombusviruses, positive-sense single-stranded RNA viruses, at high levels during infection to allow systemic invasion of the host plant [27]. It is encoded in the 3′ proximal open reading frame (ORF) *p19*, which is embedded in another ORF that encodes a 22-kDa cell-to-cell movement protein. [28,29]. The characterization of p19's role in *Tombusvirus* pathogenesis has been previously well reviewed by Scholthof [30]. p19 is the most well studied VSRS and its mechanism of suppression is quite unique; it sequesters sRNAs and prevents their incorporation into RISC (Fig. 1) [18,19]. p19 is in competition with Argonaute proteins for sRNAs but it can not de-program an already loaded RISC complex [19]. It appears from kinetics studies that p19 efficiently competes for sRNAs through a multiple turnover mechanism, as it exhibits highly reversible binding with a very high dissociation rate constant (k_{off} = 0.062 ± 0.002 s^{-1}) and may possibly intercept RISC formation by forming a ternary complex with Dicer [31].

Delineating p19's biochemical properties in vitro have been instrumental to its application as a tool for probing the RNA silencing pathway. The reported crystal structures of p19 from both *Tomato Bushy Stunt Virus* (*TBSV*) and *Carnation Italian Ringspot Virus* (*CIRV*) in complex with a 21-nt sRNA (Fig. 2A) [22,32] demonstrate how p19 functions as a 'molecular caliper' to bind the sRNA according to the length of the duplex. p19 binds the sRNA as a dimer with N-terminal helices that clasp the edges of the duplex and provide stacking interactions between tryptophan residues and the terminal nucleotides, thereby measuring the length of the duplex. p19 exhibits sequence-independent binding, as the binding surface consists of a continuous 8-stranded β-sheet that forms a concave binding surface, interacting with the sRNA backbone through hydrogen bonding and electrostatic interactions.

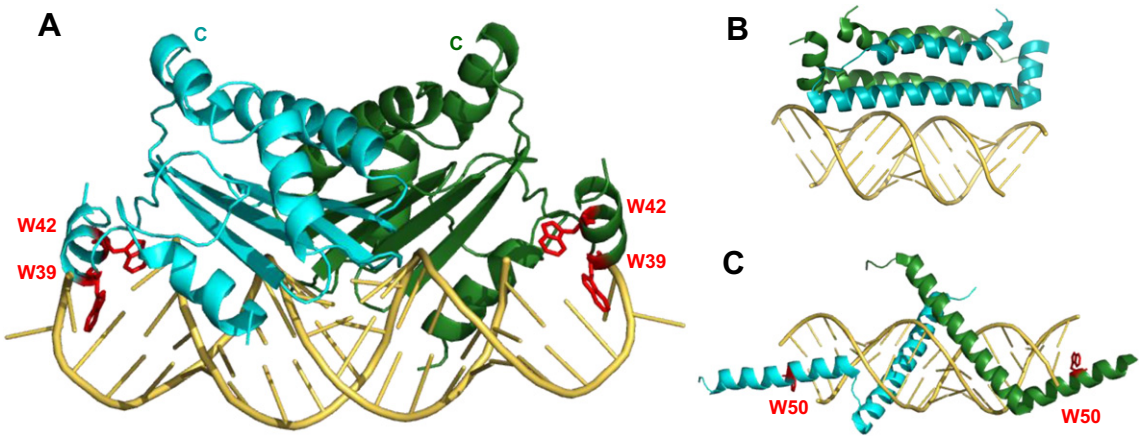

Fig. 2. Diversity in structures and mechanism of dsRNA binding among viral suppressors of RNA silencing (VSRS) as determined by X-ray crystallography. (A) *Carnation Italian Ringspot Virus* (*CIRV*) p19 in complex with a 21-nt siRNA demonstrates its sequence-independent and size-specific binding of sRNAs, where it functions as a dimer to form a 8-stranded β-sheet binding surface that interacts with one face of the sRNA backbone and N-terminal helices that clasp the ends of the sRNA duplex, with end-capping interactions provided by conserved tryptophan residues (W39 and W42, highlighted in red) (PDB: 1RPU) [78]. *Tomato Bushy Stunt Virus* (*TBSV*) p19 in complex with siRNA shows a nearly identical binding strategy (PDB: 19RF)[32]. (B) *Flockhouse virus* (*FHV*) B2 protein in complex with an 18-bp dsRNA demonstrates its sequence and length-independent binding of dsRNA, where it functions as a dimer to form a four-helix bundle that interacts with one-face of the sugar-phosphate backbone (PDB: 2AZ70) [17]. (C) *Tomato aspermy virus* (*TAV*) 2b protein (1–69) in complex with a 19 bp siRNA demonstrates its sequence-independent binding of dsRNA, where it functions as a dimer to fit its α-helical protein backbones into the major groove of the RNA duplex (PDB: 2ZI0) [78]. *TAV 2b* preferentially binds dsRNA, with some preference for duplex length conferred by tryptophan stacking interactions with siRNAs (W50, highlighted in red) but also binds long dsRNA with high affinity and dsDNA with weak affinity.

p19 evolved to have the highest affinity for products of the DCL4 enzyme, as this is the main enzyme involved in plant antiviral defense [6,4]. p19 exhibits the highest affinity reported to date of a protein for siRNAs, with a dissociation constant of approximately 0.2 nM [22,31,33]. It exhibits drastically reduced affinity for siRNAs longer or shorter than 20–22 nt long, does not bind single-stranded RNA or DNA, and requires 5′ phosphates for high affinity binding but does not require 3′-2 nt overhangs [22]. p19 also binds miRNAs (~23 nt) with high affinity both in vivo and in vitro [24,33–37]. Interestingly, p19 also suppresses RNA silencing by another mechanism unrelated to sequestration of sRNAs; in plants p19 expression induces the expression of a host miRNA, miR168, which reduces levels of AGO1, thereby inhibiting anti-viral RISC formation [59].

p19 has been broadly used as an effective suppresser of RNA silencing through expression in heterologous systems, including non-endogenous host plants [24], *Drosophila* [38], *Drosophila* embryo extracts [19], as well as human cells [24]. Furthermore, it's ability to sequester small RNAs in a variety of systems make it an ideal probe for examining the role these molecules play in diverse biological processes, not limited to host-pathogen interactions. Herein we discuss its application as a probe for small RNAs in diverse systems as well as the applications of purified, recombinant p19 proteins in small RNA detection. Furthermore, we discuss how p19 is amenable to modifications including protein fusions and altered binding specificity, which provide novel properties and invite further applications of p19 in biotechnology and therapy.

2. p19 as a tool to probe small RNA pathways

2.1. Probing small RNA biology in plants

Transgenic expression of p19 has allowed interrogation of sRNA biology in the model plant *Arabidopsis thaliana,* representing the largest impact of p19's use as a probe in heterologous systems. Examining sRNA accumulation in plants transgenically expressing cytoplasmic and nuclear variants of p19 gave initial evidence for nuclear processing of sRNA precursors, as nuclear p19 expression repressed the accumulation of 21-nt siRNAs and a 21-nt miRNA [39]. Expressing p19 also aided in a landmark study that confirmed the mobility of 21-nt siRNAs, where the level of suppression of the mobile interference signal generated from an inverted repeat construct (SUC:*SUL*) was directly correlated to the extent which p19 had sequestered 21-nt siRNAs [26]. p19's binding specificity was crucial to this experiment; distinguishing between DCL4-generated 21-nt siRNAs and the long dsRNA precursors and identifying that the siRNAs are the mobile element.

The details of the siRNA and miRNA pathways in *A. thaliana* have been teased apart by applying a 'molecular toolbox' approach, where the diverse properties of VSRS allow interrogation of the RNA silencing pathway at different points. Transgenic expression of five unrelated VSRS in *A. thaliana* all exhibit silencing activity but only *Turnip mosaic virus* (*TuMV*) P1-HcPro, *TBSV* p19, and *Peanut clump virus* (*PCV*) p15 interfere with the endogenous miRNA pathway, whereas *TCV* p38 and *Potato virus x* p25 are specific for the siRNA pathway [24]. The differential effect of these VSRS gave insight that the siRNA and miRNA pathways in plants are only partially overlapping and that developmental abnormalities associated with p19 expression are likely due to perturbation of endogenous miRNA systems [24]. Furthermore, directly comparing the effect of HcPro, p19, p15 and P38 on siRNA- and miRNA-loading of AGO1 demonstrated that although all VSRS prevented siRNA loading, only p19 prevented miRNA loading into AGO1, demonstrating its unique ability to sequester both siRNAs and miRNAs in vivo. Interestingly, even though *TCV* p38 interferes directly with AGO1, it appears to only prevent siRNA loading without affecting miRNA loading, suggesting that there are two distinct cellular pools of AGO1 that are loaded with siRNA or miRNA and are differentially targeted by VSRS. By employing VSRS, this study uncovers another layer of complexity to these intertwined but functionally distinct pathways [25].

2.2. Probing small RNA biology in Drosophila

p19 has been an effective tool in interrogating the RNA silencing pathway in *Drosophila.* It is observed that constitutively expressing cytoplasmic p19 in living flies impairs hairpin-induced RNAi without altering the endogenous miRNA pathway [40]. Furthermore, this study effectively employs the 'molecular toolbox' approach

to investigate the contribution of RNA silencing via endogenous siRNAs to heterochromatin formation in *Drosophila*; abrogating the pathway through transiently expressing p19 and *FHV* B2 in S2 cells. It was observed that expressing p19 in the nucleus via a fused nuclear localization signal (NLS-p19) suppressed silencing of heterochromatin gene markers in adult flies, implicating nuclear sRNAs in regulating heterochromatin silencing. Examining the sRNAs bound by p19 through immunoprecipitation determined that there are abundant endogenous siRNAs (endo-siRNAs) in S2 cells derived from transposable elements. Comparing the sRNA immunoprecipitates of cytoplasmic p19 versus NLS-p19 demonstrates the existence of an abundance of cytoplasmic endo-siRNAs in S2 cells as well as moderately abundant nuclear endo-siRNAs, and further suggests that a portion of the cytoplasmic endo-siRNAs are translocated back into the nucleus to elicit chromatin silencing [40].

2.3. Probing small RNA biology in mammalian cells

p19 has been used to explore the role of RNA silencing in the defense against the retrovirus *Primate Foamy Virus type 1* (*PFV*). Expressing p19 in human 293T immortalized kidney cells resulted in a marked increase in viral accumulation, suggesting that sRNAs were repressing viral replication. It was subsequently shown that an endogenous miRNA mediates the antiviral defense [41].

p19 was used to probe the sRNA milieu of mouse embryonic stem cells through immunoprecipitations of transiently expressed cytoplasmic and nuclear p19 with subsequent small RNA cloning [42]. This study demonstrated that mouse ES cells do not have abundant endogenous siRNAs. It was observed that the short RNAs immunoprecipitated with p19 had an overall higher GC content than the control RNA, indicated that p19 exhibits a preference for GC-rich RNA. Additionally, p19 expression in these cells did not lead to an accumulation of miRNA* strands in cell extracts, nor were they detected in the p19 immunoprecipitates. This is in contrast to what has been observed in plants [24,34,43], suggesting that p19 did not have access to mature miRNAs in ES cells. Interestingly, it was found that in the absence of siRNAs, p19 is capable of binding non-canonical ligands in which a short RNA species annealed to a longer species (32–35 nt) such that the dsRNA region is 19 bp long. The affinity of p19 for these species was reduced 5–10-fold as compared to canonical siRNA ligands, but still exhibits nanomolar affinity. Notably, this study also observes that expressing p19 in HEK293T cells does not prevent exogenous siRNA-mediated gene knockdown.

It appears that p19's ability to suppress RNA silencing in human cells is not universal. The results in HEK293T cells [42] are consistent with another study where transient expression of p19 in HEK393T cells did not show an increase in HIV-1 production, although stable expression of p19 resulted in a modest increase [44]. Also, we have observed that p19 does not appear to suppress RNA silencing in human hepatoma cells (Huh7), possibly due to high levels of endogenous miR-122 that also acts as a ligand for p19 (unpublished results). These findings are in contrast to the effective silencing suppression observed in HeLa cells [24], and the increased *PFV* viral accumulation upon p19 expression in HEK293T cells [41]. This inconsistency in human cells is likely due to cell-type specific factors that alter p19's function, which have yet to be fully understood.

3. p19 for small RNA detection

Recombinant, purified p19 proteins invite several applications in sRNA detection. Fortuitously, p19 is amenable to overexpression in bacteria with high soluble yields and protein fusions on both the N- and C-termini retain high affinity for sRNA ligands [19,42,45]. p19's binding properties allow detection of small RNAs for quantitative analysis, providing sensitive detection without the need for PCR-based amplification. There is widespread interest in increasing the sensitivity of miRNA detection, given that small changes in their expression patterns have drastic biological outcomes and are linked to several disease states [46]. Given p19's high affinity for both siRNAs and miRNAs, it is a unique protein-based tool for sensing sRNAs.

Recombinant CIRV p19 has been used as part of an surface-plasmon resonance (SPR) based approach for quantifying miRNA-122 from small RNA isolates of human hepatoma cells, where RNA probes are immobilized on gold surfaces and p19 detects the miRNA-bound probes [36]. This represents a unique strategy that provides a simple alternative to previous SPR-miRNA detection methods that require enzymatic surface modification and gold nanoparticle labels for signal amplification [47]. p19 has also aided miRNA detection as part of a rapid enzyme-linked immunoassay approach which exhibits femtomole sensitivity [36] (Fig. 3A). This method uses RNA probes conjugated to magnetic microbeads to capture the target miRNA, which is then bound by p19 allowing rapid detection through horseradish-peroxidase linked antibody detection of p19. This method achieves high sensitivity of miRNA detection without the inherent biases that exist in PCR-based amplification [48].

The p19 protein has been used to enhance miRNA detection by capillary electrophoresis (CE). CE is a rapid and sensitive method for miRNA detection, however previous research has shown that a lack of separation between the excess single-stranded probe and the probe:miRNA duplex precludes accurate quantitative analysis [49]. p19's binding specificity allows enhanced separation between the probe and probe:miRNA duplex, in a technique termed protein-facilitated affinity capillary electrophoresis (proFACE) [37]. Furthermore, this study employed p19 as a tool to improve miRNA detection in human serum. miRNAs are present in stable forms in human serum and are being investigated as cancer biomarkers [50,51]. Commercially available p19-coated beads [52] were applied to plasma to pre-concentrate probe:RNA duplexes allowing very sensitive detection (0.5 fM or 30 000 miRNA molecules per ml) without requiring PCR amplification [37] (Fig. 3B).

p19 has also been applied to fluorescent detection of sRNAs, where a fusion of a linked p19 dimer and a cyan fluorescent protein (CFP) allows reporting on binding of fluorescently labeled siRNA through Förster resonance energy transfer (FRET) [45] (Fig. 3C). These results suggest the possibility of developing p19 as an intramolecular FRET probe to allow sensing of unlabeled siRNA, which would allow label-free reporting on p19 binding sRNA. This is a strategy that our lab has been exploring.

4. Modifying p19 for novel properties

Mutational analysis of *Tombusvirus* genomes has allowed characterization of p19's role in viral pathogenesis and specific targeting of siRNA binding sites has allowed correlating its siRNA binding activity to its activity as a VSRS [22,23,53,54]. Mutational analysis of p19 has produced loss-of-function point mutations that are useful as negative control constructs. Other mutations confer altered binding specificity and suppressor activity, attracting novel applications in biotechnology and human disease research. p19 has been shown to be amenable to mutation and modifications, although it is clear that it awaits much broader experimentation that will likely produce distinct properties useful for dissecting the RNA silencing pathway in diverse organisms.

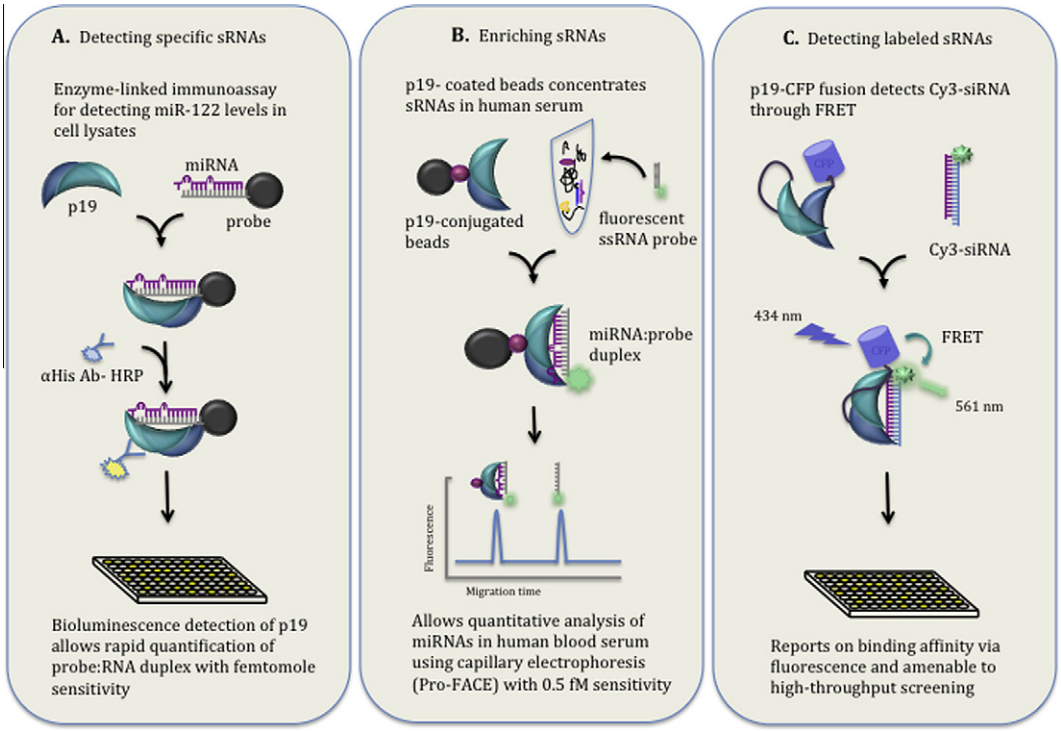

Fig. 3. Applications of p19 in small RNA detection. (A) p19 binds probe:miRNA duplexes for quantifying levels of specific miRNAs in biological samples using bioluminescence detection of p19 [36]. (B) p19 fusion protein with N-terminal maltose-binding protein and C-terminal chitin binding domain (MBP-p19-CBD) allows enrichment of sRNAs using chitin magnetic beads (New England Biolabs). Sensitive and quantitative detection of specific miRNAs in human serum by capillary electrophoresis (CE) is attained through pre-concentrating miRNA:probe duplexes using p19-coated beads as well as CE stacking, resulting in ~1000-fold enrichment of miRNAs and detection as low as 0.5 fM miRNA without PCR-based amplification [37]. p19 further aids the analysis by enhancing separation of the probe:miRNA duplexes from the excess, unbound probe, a technique termed ProFACE (protein-facilitated CE). (C) A C-terminal cyan-fluorescent protein (CFP)-p19 fusion protein detects fluorophore (DY547) labeled siRNA through Forster resonance energy transfer (FRET), allowing spectrophotometric detection of binding and determination of binding affinity which is amenable to high-throughput screening applications [45].

4.1. Loss of function mutations

Loss-of-function point mutations abrogating specific properties of p19 are of great use as control proteins to compare the effect of wild-type p19 in various heterologous systems. p19's interface between the N- and C-terminal sub-domains appears particularly sensitive to mutation, where disrupting key salt bridges that stabilize the N-terminal reading heads abrogate siRNA binding and suppressor activity. Two of these mutants, TBSV R72G and RR75–78GG are incapable of binding siRNAs in planta and do not function as suppressors, although it has been suggested that these mutations likely cause structural perturbations that may affect protein stability [22,23,53,54]. Recombinant CIRV Y73S has been shown in vitro to completely abrogate siRNA and miRNA binding and displays a conformational change from wild-type, as determined by an altered circular dichroism spectrum below 210 nm. Thermal melt analysis demonstrated that this mutant exhibits a higher Tm, possibly reflecting a more rigid conformation that is not conducive to ligand binding [33]. All of the mutants from this interface region, however, still maintain the ability to form dimers [33,54].

Another loss-of-function mutant is generated by directly targeting the end-capping tryptophans required for measuring the length of the sRNA duplex. Mutating the highly conserved Trp39 to glycine in CIRV p19 abrogated its suppressor activity in planta but was not analyzed for ligand binding activity [22]. We have observed that this mutant's ability to bind siRNAs in vitro is substantially reduced, but still binds with ~100 nM affinity (unpublished results) and molecular dynamics studies predict a loss in affinity of ~7 kcal/mol [55]. These mutants reflect that p19's binding activity and thus its suppressor activity can be tailored through modifications to the binding site, and can be used during experimentation in parallel with wild-type p19 to examine the effect of sRNA sequestration on cellular processes.

4.2. Altering p19's effect on endogenous processes

During viral infection, p19's main role is in binding vsRNAs and inhibiting the formation of anti-viral RISC, however p19 is also interacting with and effecting host cell endogenous processes. Developing p19 as a tool requires that we also understand its effect on host cellular processes that are not linked to its ability to sequester sRNAs. p19 interacts with host ALY proteins in planta through their RNA-binding domain, altering their localization from the nucleus to the cytoplasm [56,57]. p19 also alters HEN-1 mediated miRNA methylation and therefore affects miRNA stability [58]. In plants, p19 appears to specifically enhance the level of miR168 which downregulates the levels of endogenous AGO1, but this activity may be distinct from its ability to bind sRNAs [59]. Little is known regarding p19's interactions with host factors in other organisms, and will need to be examined to broaden p19's application, but will also highlight differences between RNA silencing pathways in different species.

During heterologous expression of p19, it is evident that its effect on endogenous processes is often detrimental to the host. Exploring the biochemical basis for its symptomatic effects, including viral-like symptoms and developmental defects, have implicated its interactions with the host miRNA pathways [60,61]. p19 expression in *A. thaliana* stabilizes miRNA/miRNA* duplexes thereby decreasing the activity of the template strand [24,34,43] and has been demonstrated in planta to prevent miRNA loading into

AGO1 [25]. Developmental defects in VSRS transgenic *A. thaliana* has been attributed to misregulation of miR167 target AUXIN RESPONSE FACTOR 8 (ARF8) in *Arabidopsis* [61]. Interestingly, transgenically expressing p19 and other VSRS in *Drosophila* does not cause the developmental delays and does not alter endogenous miRNA pathways, possibly inviting more applications in this organism [38].

By dissecting p19's effect in these systems we can begin to hypothesize how to overcome detrimental effects to the host while still allowing p19 to function as a suppressor protein. Intriguingly, mutational analysis of p19 suggests that its ability to bind sRNA and its other biological effects may be functionally separated. Specific targeting of the siRNA-binding sites of TBSV p19 generated several mutants unable to bind siRNA, and it was observed that targeting different regions of the protein gave distinct, host-specific biological outcomes not attributable to siRNA binding [23]. It was suggested that these mutations might alter p19's effect on other host factors, including miRNAs, although this remains to be explored experimentally.

One particular mutant of TBSV p19, R43W, displays attenuated symptomatic effects during viral infection but still displays activity as a suppressor of RNA silencing and binds siRNAs in planta, albeit reduced from wild-type levels [53,54]. Interestingly, this mutant binds longer dsRNA molecules in planta and its altered binding specificity is possibly due to an inability to form a hydrogen bond with the 5′ phosphate of a sRNA [54]. Due to its lowered symptomatic effects, R43W p19 was applied in a heterologous expression system to increase foreign gene expression in *A. thaliana* [62]. Wild-type p19 can be transiently co-expressed with a foreign gene of interest and substantially increase foreign gene expression (~50-fold) [63] and has been applied to increase the expression of several foreign genes of interest for vaccine development, such as HIV and SARS antigens [64,65], as well as therapeutic antibodies [66,67]. Generating p19-expressing stably transformed plants, however, is hampered by its toxicity [68]. Applying the less symptomatic mutant p19, R43W, results in 7-fold increase in GFP expression when transiently expressed, although stable expression results in a modest 1.7-fold increase [62]. It is possible that this mutant is impaired in its normal ability to target host processes, such as the miRNA pathway, although these important questions remain to be experimentally validated. This study provides proof-of-concept that p19 can be modified towards effective silencing activity with lowered symptomatic effects. R43W may serve as a platform from which further modifications can be made to further uncouple the unwanted symptomatic effects while retaining suppressor activity.

It is important to note that the majority of the mutational analysis performed thus far on p19 sequences has been during the context of viral infection, which has limited scope since the entire p19 gene is embedded within another ORF. We suggest that to optimize p19 as a tool in heterologous systems, more comprehensive mutational analyses performed with recombinant sequences may result in distinct outcomes desirable in different systems. For example, using recombinant CIRV p19 we observe that p19's affinity for a human miRNA can be increased ~50-fold through a single point mutation without altering its affinity for siRNA in vitro (discussed below). Through engineering efforts it may be possible to functionally separate its activity as a suppressor from its interference with endogenous processes that preclude its broader application.

4.3. Enhancing function

Recombinant CIRV p19 has been modified for enhanced function through protein engineering for applications in biotechnology and human therapy. Linking the two monomers through a semi-rigid linker $(GGGGS)_2$ between the N-terminus of one monomer and the C-terminus of the other monomer enhances the thermal stability of the construct and it's affinity for siRNA 3.5-fold over wild type p19 [69] (Fig. 4A). Having a linked p19 dimer construct facilitates its use in biotechnology and has been used for quantitative miRNA detection using ProFACE [37]. Mutagenesis studies exploring the importance of cysteine residues in the p19 protein led to the observation that mutating three cysteine residues (C110, C134 and C160) to isoleucine results in improved solubility properties, precluding the need for reducing agents during purification and storage, as is required for purification of wild type p19 proteins to prevent aggregation (unpublished results) [70].

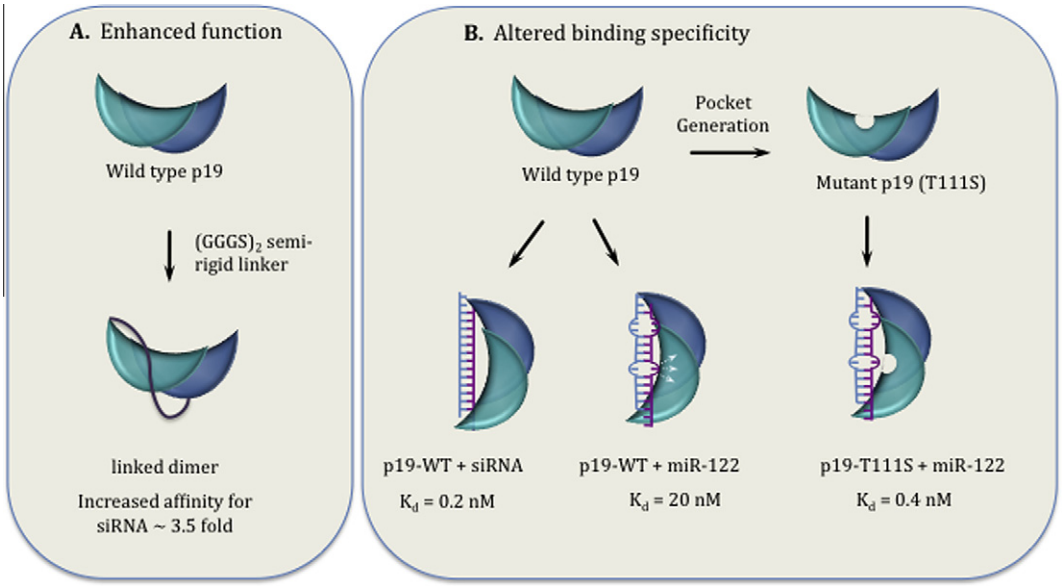

Fig. 4. Engineering the p19 protein for enhanced function and altered binding specificity. (A) Recombinant CIRV p19's properties are enhanced by linking the monomers using a semi-rigid linker (GGGS)2 between the flexible N-terminus of one monomer and the C-terminus of the other monomer. The linked dimer exhibits a ~3.5-fold enhanced binding affinity for siRNA and enhanced thermal stability [69]. (B) Site-directed mutagenesis of a single residue of the p19-binding site generates unique high-affinity interactions with the human microRNA, miR-122, resulting in a 50-fold enhancement in binding affinity. By mutating threonine 111 to serine, a potential 'pocket' in the binding site better accommodates the bulges in the miR-122 molecule and generates new hydrogen bonding interactions [33].

4.4. Altering binding specificity

Novel applications of mutant p19 proteins are forthcoming, as there is evidence that mutations in its binding site can alter its binding specificity for certain molecules. p19 exhibits highest affinity for perfectly base paired sRNA duplexes. Wild-type p19's affinity for a human miRNA, miR-122, is ~100 fold less than its affinity for a perfectly base paired duplex [33]. miR-122 is a liver-specific miRNA [71] which is used by the hepatitis C virus to aid in the viral life cycle [72–74] and whose sequestration is being investigated for human therapy [75].

Mutating a single residue in the binding site of recombinant CIRV p19, Thr111 to serine (T111S) increases its affinity for the human miRNA, miR-122, 50-fold and allows sequestering the miRNA in human hepatoma cells [33] (Fig. 4B). Interestingly, the T111S mutation did not alter the protein's affinity for siRNA, suggesting that there are unique interactions between p19's binding site and miR-122 versus the siRNA, which are likely due to structural deviations in the miRNA from base-pair mismatches. By mutating Thr111 to serine, there appears to be novel hydrogen-bond interactions with the miR-122 molecule, allowing high affinity binding that may be specific to its areas of base pair mismatches. Interestingly, another study notes that p19's affinity for another miRNA, let-7, was the same as its affinity for siRNA [31]. It is possible that certain positions of mismatches cause more potent disruptions in p19's binding.

These studies suggest that it may be possible to tailor the p19 binding site to bind specific types of molecules with high affinity, perhaps through enhancing interactions with 'bulges' in miRNAs. The positioning of mismatches in miRNAs is increasingly recognized for specific biological effects. Certain Argonaute proteins exhibit specificity based on mismatch placement [76] and transitivity in plants also depends on miRNA mismatches [77]. This is a burgeoning field in protein:RNA interactions, and the unique features of the p19 binding site among RNA binding proteins make it an excellent platform for further development as a tool in biotechnology.

5. Perspectives

Understanding the mechanisms of the RNA silencing pathway is essential for research into human disease states, among a multitude of other areas, and is prerequisite to its manipulation for desirable outcomes in therapy and biotechnology. The evolutionary arms race between hosts and pathogens has given us a unique set of proteins that potently abrogate RNA silencing and whose characterization has produced fundamental knowledge of this pathway in diverse organisms. The greatest benefit will come now from expanding this repertoire through protein engineering of these VSRS. The tombusviral p19 protein has been demonstrated to be a unique tool for small RNA sequestration in living systems and protein engineering efforts will likely lead to fine-tuning of its ligand specificity and biological effects, thus inviting broader application.

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