Published in final edited form as:

J Mol Biol. 2011 April 29; 408(2): 262–276. doi:10.1016/j.jmb.2011.02.038.

Viral RNAi suppressor reversibly binds siRNA to outcompete Dicer and RISC via multiple-turnover

Renata A. Rawlings^{1,2}, Vishalakshi Krishnan², and Nils G. Walter^{2,*}

¹ Department of Biophysics, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109-1055, USA

² Department of Chemistry, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109-1055, USA

Abstract

RNA interference (RNAi) is a conserved gene regulatory mechanism employed by most eukaryotes as a key component of their innate immune response against viruses and retrotransposons. During viral infection, the RNase III-type endonuclease Dicer cleaves viral double-stranded RNA into small interfering RNAs (siRNAs), 21–24 nucleotides in length, and helps load them into the RNA-induced silencing complex (RISC) to guide cleavage of complementary viral RNA. As a countermeasure, many viruses have evolved viral RNA silencing suppressor (RSS) proteins that tightly, and presumably quantitatively, bind siRNAs to thwart RNAi-mediated degradation. Viral RSS proteins also act across kingdoms as potential immunosuppressors in gene therapeutic applications. Here we report fluorescence quenching and electrophoretic mobility shift assays that probe siRNA binding by the dimeric RSS p19 from Carnation Italian Ringspot Virus (CIRV), as well as by human Dicer and RISC assembly complexes. We find that the siRNA:p19 interaction is readily reversible, characterized by rapid binding ($(1.69 \pm 0.07) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and marked dissociation ($k_{\text{off}} = 0.062 \pm 0.002 \text{ s}^{-1}$). We also observe that p19 efficiently competes with recombinant Dicer and inhibits formation of RISC-related assembly complexes found in human cell extract. Computational modeling based on these results provides evidence for the transient formation of a ternary complex between siRNA, human Dicer, and p19. An expanded model of RNA silencing indicates that multiple-turnover by reversible binding of siRNAs potentiates the efficiency of the suppressor protein. Our predictive model is expected to be applicable to the dosing of p19 as a silencing suppressor in viral gene therapy.

*Corresponding author. Department of Chemistry, 930 North University Avenue, University of Michigan, Ann Arbor, MI 48109-1055, USA. Phone: (734) 615-2060; Fax: (734) 647-4865; nwalter@umich.edu.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at...

Author Contributions: R.A.R. and N.G.W. designed research; R.A.R. and V.K. performed research, R.A.R. and V.K. analyzed data; and R.A.R. and N.G.W. wrote the paper.

This article contains supporting information.

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Keywords

fluorescence quenching; p19; RNA interference; systems biology modeling; viral silencing suppressor protein

Introduction

Over the past decade, RNA interference (RNAi) has become recognized as a benchmark laboratory and potential clinical tool to control gene expression, as well as a highly conserved eukaryotic immune response against viral pathogens or transposons.^{1–4} During viral infection, plant and invertebrate cells are known to activate RNAi where viral RNA is specifically recognized by the cytoplasmic RNase III-type endonuclease Dicer and degraded by an RNA-induced silencing complex (RISC). Dicer cleaves exposed stretches of double-stranded RNA into small interfering RNAs (siRNAs) 21–24 nucleotides in length (depending on species). Dicer then delivers the double-stranded siRNA via the RISC-loading complex⁵ to RISC,^{6,7} where the passenger strand of the duplex is cleaved and released while the guide strand is retained to guide specific cleavage of complementary viral RNA genomes or transcripts.^{8,9} Dicer is thus thought to play a critical dual role in RNAi by both generating virus-specific siRNA duplexes and assembling them with the necessary protein components into active RISC.¹⁰ In mammalian including human cells, artificial siRNAs have been shown to confer antiviral immunity,¹¹ but only very recently evidence has emerged to suggest that small RNAs including siRNAs are also directly generated from infecting viruses.¹²

To subvert the host RNAi response many viruses have evolved small proteins that act as RNA silencing suppressors (RSSs).^{13,14} One of the best characterized RSSs is exemplified in the plant pathogen Carnation Italian Ringspot Virus (CIRV), which encodes a 19 kDa protein termed p19, an important pathogenicity factor that is highly conserved among all tombusviruses.¹⁴ Crystal structures^{15,16} and recent molecular dynamics simulations¹⁷ show that homodimers of p19 bind a single siRNA duplex in a positively charged surface cleft (Fig. 1a). Pairs of tryptophan residues stack on both terminal base pairs to establish a sequence-independent, caliper-like size selection for siRNA duplexes with 19 base pairs and 2-nucleotide long 3'-overhangs. Sequestration and binding of Dicer-generated siRNAs by p19 has been observed to correlate with reduced viral RNA degradation, systemic symptom spread, and sustainability of the viral phenotype after infection.^{18–21} Previous electrophoretic mobility shift assays (EMSAs) have suggested that the affinity of dimeric p19 for siRNA is high with an apparent equilibrium dissociation constant of $K_p = 0.17 \pm 0.02$ nM.¹⁵ Such tight binding is consistent with a widely accepted sequestration model wherein the p19 dimer near-quantitatively binds siRNA duplexes to suppress RNAi.^{14,18} Binding of double-stranded RNA is considered to be the most common RSS mechanism,^{22,23} yet measurements of siRNA binding and dissociation kinetics to better understand sequestration have not been reported to date for any RSS protein.

Here we develop solution-based fluorescence quenching assays that observe siRNA:p19 (SP) binding to be readily reversible with not only a rapid binding rate constant ($(1.69 \pm 0.07) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$), but also a dissociation rate constant ($k_{\text{off}} = 0.062 \pm 0.002 \text{ s}^{-1}$) markedly faster than those of slow-release nucleic acid binding proteins.^{24,25} Our results are consistent, within error, with the high apparent affinity measured in EMSAs, yet expand on the sequestration model. If binding were completely irreversible, it would require the virus to generate sufficient quantities of p19 dimer to quantitatively bind all small RNAs produced enzymatically by either Dicer cleavage or the amplification effected in a plant cell through RNA-dependent RNA polymerase.² By contrast, facile reversibility of SP complex

formation opens the possibility that multiple-turnover may potentiate p19's efficacy as RSS. In particular, if p19 were to disrupt the siRNA:Dicer (SD) interaction via multiple-turnover, it would potentially interfere with the role of Dicer as obligatory RISC assembly factor.

Consistent with such a mechanism of p19 action, we observe the efficient competition of p19 for siRNA binding with active recombinant human Dicer in the absence of other known RISC components, as well as the prominent disruption of RISC assembly complexes observed in human HeLa cell extract containing the three core RISC-loading complex components Dicer, TRBP, and Ago2.⁵ Mathematical modeling demonstrates that the Dicer competition data are only accurately reproduced assuming the formation of a transient ternary complex, siRNA:Dicer:p19 (SDP). Taken together, a multiple-turnover "catch and release" mechanism emerges wherein p19 repeatedly promotes dissociation of SD complexes to efficiently suppress Dicer-mediated RISC assembly.

Results

Fluorescence assays to observe siRNA:p19 complex formation

We site-specifically labeled the antisense (guide) strand of an established luciferase-targeting siRNA duplex^{26,27} with either fluorescein or tetramethylrhodamine as fluorescence quenching probes in positions not expected to interfere with p19 binding (Fig. 1a). We found that only the tetramethylrhodamine label was efficiently quenched upon p19 addition (Fig. 1b). Addition of buffer or other proteins such as recombinant Dicer caused no such quenching (Supplementary Fig. S2). To observe a robust quenching signal upon p19 addition, we proceeded to label with both fluorophores together, allowing us to monitor a normalized tetramethylrhodamine:fluorescein (T/F) fluorescence ratio that is insensitive to dilution effects and other perturbations of an otherwise relative fluorescence signal. Next, we tested by EMSA p19 binding of this doubly-labeled siRNA against that of the unlabeled siRNA and detected no interference from the fluorophores ($K_p = 0.17 \pm 0.03$ nM in both cases, consistent with earlier EMSA studies¹⁵ (Fig. 1c). Time-resolved fluorescence resonance energy transfer (trFRET) between the two fluorophores revealed only a small increase in fluorophore distance distribution upon p19 addition (with the mean distance increasing from 43.4 to 44.5 Å; Fig. 1d), indicating that formation of the SP complex leads to only a minor deformation of the siRNA duplex, as expected from X-ray crystallography.¹⁵ We conclude that the doubly-labeled siRNA duplex is a suitable reporter for formation of the siRNA:p19 interaction in solution.

p19 binds siRNA rapidly and reversibly

Addition of increasing excess concentrations of p19 to the doubly-labeled siRNA led to an increase in the pseudo-first order rate constant of tetramethylrhodamine quenching as monitored by the T/F ratio, yielding a bimolecular binding rate constant of $k_{on} = (1.69 \pm 0.07) \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ (Fig. 2a,b). Next, dissociation of the preformed SP complex was monitored upon addition of a large excess of unlabeled siRNA (chase), yielding a dissociation rate constant of $k_{off} = 0.062 \pm 0.002 \text{ s}^{-1}$ (Fig. 2c and Supplementary Fig. S2). Together with the rapid k_{on} , a solution-based dissociation equilibrium constant of $k_{off}/k_{on} = K_{p,sol} = 0.37 \pm 0.08$ nM is obtained, within 2-fold of the EMSA predicted value (0.17 nM), attesting to a high-affinity, yet also notably reversible siRNA:p19 binding mode.

p19 efficiently competes with recombinant human Dicer for siRNA binding

The critical dual role of Dicer in RNAi as both the enzyme that produces siRNAs and as a RISC assembly factor¹⁰ suggests the siRNA:Dicer (SD) interaction as a particularly vulnerable step during RNA silencing. Previous studies in embryo extracts of the fruitfly *Drosophila* have shown that p19 suppresses RNA silencing by competing with the Dcr2-

R2D2 complex.^{18,22} To determine whether p19 also competes with human Dicer for siRNA, we employed EMSA using radio-labeled siRNA duplexes and an active recombinant human Dicer preparation, devoid of other known RISC proteins (Supplementary Fig. S1) (available as a relatively crude purification of heterologously overexpressed Dicer from Genlantis, see Materials and Methods). We measured the apparent equilibrium dissociation constant of the human SD complex as $K_D = 3.7 \pm 0.4$ nM (Fig. 3a and Supplementary Fig. S3a). The presence of a low concentration of p19 (0.17 nM), equivalent to the K_D of the SP complex, weakened this interaction by >25-fold to ~100 nM (Fig. 3a and Supplementary Fig. S3b). The presence of a saturating concentration of p19 (2.5 nM) weakened the interaction still further by >270-fold to an estimated lower limit of ~1.0 μ M (Fig. 3a and Supplementary Fig. S3c). These findings led us to hypothesize that p19 can function to inhibit RNAi in humans by interrupting the SD complex before Dicer can hand off siRNA to RISC. Reciprocal competition experiments showed that the presence of even saturating concentrations (140 nM) of Dicer lowered the apparent affinity of p19 for siRNA only marginally (~4-fold from 0.17 ± 0.03 nM to 0.81 ± 0.06 nM; Fig. 3b and Supplementary Fig. S3d,e), further supporting the hypothesis that strong competition with the SD complex is an important mode of action for p19 inhibition of RNAi in human cells.

Human siRNA-containing complexes formed in cytosolic cell extract are vulnerable to p19 challenge

Previous studies with *Drosophila* extracts have shown that p19 inhibits RISC assembly while disrupting preassembled RISC to a lesser extent.²² Our result that p19 disrupts the complex of recombinant human Dicer with siRNA suggests that p19 may also be effective in suppressing RISC assembly in mammals. To test this hypothesis and probe p19's effect on the formation of human RISC-related complexes, we used a modified *in vitro* RISC assembly assay.^{28,29} Specifically, we incubated radio-labeled siRNA duplex with 15% (v/v) cytosolic HeLa cell extract, which we showed to contain the three core RISC-loading complex components Dicer, TRBP, and Ago2 (Supplementary Figs. S1 and S5), and analyzed the products by EMSA and autoradiography (Fig. 4a,b). Three distinct siRNA-containing complexes were observed that correspond to those previously observed, including a slowly-migrating complex D that is thought to contain Dicer, as well as two faster migrating bands termed C1 and C2^{18,30} (Fig. 4b). The normalized fraction of complex C2 decreases over a 2-h period, whereas the complex C1 and D fractions increase (with estimated rate constants of $2 \times 10^{-3} \text{ s}^{-1}$, $0.8 \times 10^{-3} \text{ s}^{-1}$, and $0.4 \times 10^{-3} \text{ s}^{-1}$, respectively, Fig. 4a). To test for the presence of Dicer we performed supershift assays with anti-Dicer polyclonal antibody and found that only complex D specifically supershifts with this antibody (Fig. 4c). Similarly, Dicer is detected by Western blot in complex D only (Supplementary Fig. S5). Notably, adding increasing p19 concentrations to the cell extract prior to siRNA addition increasingly impairs the formation of all three siRNA-containing complexes, concomitant with an accumulation of the competing SP complex (Figs. 4d,e). When p19 is added to the RISC assembly assay after 2 h of pre-incubation with siRNA, the formation of particularly the Dicer-containing complex D is inhibited to a lesser extent (Figs. 4f,g). Similar results were obtained at higher HeLa cell extract concentrations and temperatures (Supplementary Fig S4).

Modeling supports formation of a transient ternary siRNA:Dicer:p19 complex

The mechanism of competition between p19 and human Dicer can be determined from mathematically modeling the two possible modes of competition for a two-protein-single-substrate system. First, competition may occur through a dissociative binding mode, where the higher affinity protein shifts the relative equilibrium of the system by binding all available substrate as it dissociates from the competitor, thus establishing a new equilibrium position (Fig. 3c). In the case of p19 and Dicer, siRNAs are pre-associated with Dicer at the

onset, due to their cleavage from longer RNAs, making such dissociative binding by p19 relatively fast only if spontaneous dissociation from Dicer is rapid. Second, competition may occur through formation of a ternary complex intermediate involving siRNA, Dicer, and p19 (Fig. 3c). Using the previously determined gel-based apparent dissociation equilibrium constants for p19 ($K_P = 0.17 \pm 0.02$ nM) and Dicer ($K_D = 3.7 \pm 0.4$ nM), we can discriminate between these two possible modes of competition by calculating the resulting expected siRNA:Dicer and siRNA:p19 complex fractions and comparing them to experiments for a given set of equilibrium conditions.³¹

Assumption of the dissociative binding mechanism shows poor correlation with experiment, as evident from the facts that changes upon adding Dicer are consistently overestimated (Fig. 3a), while changes upon adding p19 are consistently underestimated (Fig. 3b). This poor correlation suggests that ternary complex formation is needed to explain the observed efficient disruption of the siRNA:Dicer complex in the presence of p19. The inclusion of ternary complex formation into the mechanism creates a thermodynamic cycle that requires the ratio of K_D/K_{SP} to equal that of K_P/K_{SD} .³² Rearranging and using the values of K_D and K_P we find that $K_{SP}/K_{SD} = K_D/K_P = 21.6$ and, therefore, $K_{SP} = 21.6 K_{SD}$. This result suggests that the dissociation of the ternary complex into the binary siRNA:p19 complex is >20-fold more likely than dissociation into the siRNA:Dicer complex, indicating that siRNAs can indeed be effectively shuttled toward the p19 bound state by assuming transient formation of a ternary siRNA:Dicer:p19 complex. The resulting model correlates well with experiment and explains both the weak effect that Dicer addition has on the siRNA:p19 complex and the large effect that p19 addition has on the siRNA:Dicer complex (Figs. 3a,b). A similar result is observed when p19 is placed in the context of the RNAi pathway, as follows.

Modeling illustrates the benefits of reversible siRNA binding by p19

To further interpret and unify our results, we expanded a previously published, simplified steady-state kinetic model that encapsulates key features of the RNAi pathway, including RNA cleavage by Dicer, siRNA incorporation into RISC, and the resulting RISC-mediated RNA degradation.³³ We included the experimentally observed competition of p19 with the siRNA:Dicer interaction and p19's reversible interaction with siRNA. From this model the possibility of a multiple-turnover "catch and release" mechanism emerges wherein p19 is effectively recycled after successfully competing with Dicer and dissociating again from the siRNA (Fig. 5a). We next sought to establish what impact, if any, such a mechanism has on the ability of p19 to suppress RNAi (for details of the model and its analysis see Supplementary Information).

Our model suggests that the intracellular steady-state load of viral proteins (and thus viral viability) is specified by three global parameters (Fig. 5a); the efficiencies of Dicer (A), RNAi in the specific host cell type (y), and p19 (v). These three global parameters are each composed of detailed mechanistic parameters (Supplementary Table S1 and Supplementary Information). In the case of irreversible binding of p19 to an siRNA (single turnover), the relative enhancement of the viral protein load in the presence over the absence of p19, ϕ_{Irr} , is then described by:

$$\phi_{Irr} = \frac{2Av(1+y)}{(-1 - v + \sqrt{1+2(1+A)v+(-1+A)^2v^2})y + Av(2+y)}$$

leading to a negligible dependence on the p19 suppression efficiency v (Fig. 5b) when using a realistic parameter space (Table 1 and Materials and Methods).

In the case of a reversibly formed SP complex, as observed in our experimental work, two additional model parameters come into play (Fig. 5a), the fraction of siRNAs that successfully reenters the RNAi pathway after release from p19 (n) and the fraction of the total p19 that is bound to siRNA (a). Values of n close to zero lead to recycling of p19 protein without reincorporation of the released siRNA into RISC, whereas values of n close to unity effectively reduce the free concentration of p19 without changing the fate of the bound siRNA as an eventual guide for RISC. Previous studies have shown that Dicer substrates at sub-nanomolar concentrations are up to 100-fold more effective in gene knockdown than mature siRNAs,^{6,34} suggesting a low probability of $n = 0.01$ for reincorporation of free siRNA into the RNAi pathway especially at the low siRNA concentrations expected during early stages of viral infection. Previous experimental studies in *Drosophila* embryo lysate have shown that p19 is effective in preventing low nanomolar concentrations of siRNAs from incorporation into RISC but less effective in disrupting mature RISC,^{18,22} a feature also evident in our data (compare Fig. 4e,g), suggesting that indeed early stages of viral infection, where n is small, may be most effective for RNAi suppression by p19. The relative enhancement of viral protein load in the presence over the absence of p19 in our reversible binding model, ϕ_{Rev} , is then described by:

$$\phi_{Rev} = \frac{2Av(1+y)}{2Av+y(1 - aA \frac{k_{off}}{k_{catD}} + v - Av - \sqrt{(-4A(-1+a \frac{k_{off}}{k_{catD}}(A-n)) \frac{v}{\beta^2} + (-1+aA \frac{k_{off}}{k_{catD}} + (-1+A)v)^2}}}$$

where k_{off} is the dissociation constant of the SP complex and k_{catD} is the cleavage rate constant of Dicer. This model predicts a hyperbolic dependence of ϕ_{Rev} on the p19 efficiency v , with little dependence on a (Fig. 5b), which can be estimated from our experimental data in Fig. 3a,b to range between 0.1–200. Reversible siRNA binding by p19 is thus found to enhance viral protein production by up to ~2.2-fold over that observed for the irreversible binding model (Fig. 5b). Notably, even a value of $n = 0.1$ does not diminish this effect much due to a close-to-linear decrease of ϕ_{Rev} toward unity with an increase of n toward unity (data not shown).

Discussion

The small, positive-sense, single-stranded RNA genome of tombusviruses encodes p19, an RSS thought critical for symptom spread and cytotoxicity of this class of plant pathogens^{20,21,35,36} through its ability to bind and thus sequester Dicer-generated siRNAs.^{14,18} The proposed use of p19 in therapeutic and research applications including mammalian gene therapy,³⁷ vaccine production,^{38,39} and as a human retroviral protein homolog⁴⁰ motivates the study of p19 outside its natural host environment, across kingdom boundaries. We here have investigated binding of a generic siRNA by CIRV p19 through fluorescence quenching assays in combination with radioactive EMSAs. Noticeable dissociation of an siRNA from p19 ($k_{off} = 0.062 \pm 0.002 \text{ s}^{-1}$) is counterbalanced by fast association to yield a solution based dissociation equilibrium constant ($K_D = 0.37 \pm 0.08 \text{ nM}$; Fig. 2), within 2.2-fold of the high affinity previously deduced in EMSAs.¹⁵ The dissociation of siRNA from p19 is nearly three orders-of-magnitude faster than that observed for many high-affinity nucleic acid-protein complexes with dissociation rate constants on the order of 10^{-4} s^{-1} .^{24,25,41,42} Strikingly, the rate and equilibrium constants for the siRNA:p19 complex resemble those of HIV-1 protein Rev for interaction with Rev Response Element (RRE) RNA ($k_{on} = 5.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 0.14 \text{ s}^{-1}$), perhaps due to the similarly (primarily) non-sequence specific binding of a small protein to a (partially) base paired RNA duplex.⁴³

The presence of even low concentrations of p19 dramatically weakens the interaction of recombinant human Dicer with its siRNA product (25- to >270-fold, depending on the p19 concentration, Fig. 3a), in the absence of other detectable RISC components. Conversely, the presence of saturating concentrations of Dicer only slightly lowers (~4-fold) the affinity of p19 for siRNA (Fig. 3b), supporting the hypothesis that strong competition with the SD complex is an important mode of p19 action *in vivo*. Accordingly, assembly assays show that p19 also strongly disrupts RISC-related human siRNA-protein complexes observed in HeLa cell extract that we show to contain all core RISC-loading complex components (Dicer, Ago2, and TRBP;⁵ Supplementary Fig. S1d,e), suggesting that p19 is able to disrupt the SD complex even in the context of the full complement of RISC components (Fig. 4). Our experimental results inform mathematical modeling of the competition between Dicer and p19 for siRNA, as well as of the steady-state interactions between siRNA, Dicer, p19, RISC, and viral (m)RNA. We determine that the minimal model necessary to achieve the experimentally observed level of competition of p19 with Dicer for siRNA requires the postulation of a transient ternary complex that favors dissociation into the siRNA:p19 complex and free Dicer (Fig. 3). In addition, an expanded steady-state kinetic model predicts a significant enhancement in viral protein load when the siRNA:p19 interaction is assumed to be reversible, as observed experimentally. Our results thus invoke a multiple-turnover “catch and release” mechanism wherein p19, unlike the released siRNA,^{6,34} is effectively recycled after successfully competing with Dicer (Fig. 5). Notably, if the dissociation rate constant of the siRNA:p19 complex were lower and thus closer to that of a slow-release protein, p19 would be more efficient in competing with Dicer for siRNA, but also less efficient in releasing the siRNA to achieve multiple-turnover. It is tempting to speculate that evolution in this way has yielded a p19 with siRNA binding properties optimized for RNAi suppression.

As any mathematical model, ours relies on some assumptions, including an experimentally derived estimate for a low probability n of 1% for reincorporation of siRNA released from p19 into the RNAi pathway.^{6,34} The dependence of the predicted RNAi suppression has a relatively shallow dependence on n , and for any $n < 1$ predicts an enhancement of suppression through the experimentally observed reversibility of the siRNA:p19 interaction. Strikingly, the estimated 2.2-fold enhancement in RNAi suppression through reversible siRNA:p19 complex formation is similar in magnitude to the typical RNAi effect that microRNAs have on the expression of cellular genes,^{9,44} suggesting that even a modest change in gene expression level as predicted here can have profound biological consequences, in our case on the likelihood of survival of a virus in its host.

Several implications for the struggle between the virus and the cellular RNAi machinery arise from our work. First, in gene therapeutic applications p19 will be required to be effective outside of its natural host environment, where a study of the protein's potential interaction partners so far has been lacking. Here, we observe p19 in the context of human cellular components and find evidence for ternary complex formation between p19, an siRNA and human Dicer. Our experimental data suggest that the observed efficient competition of p19 with the siRNA:Dicer complex (Fig. 3) and the resulting Dicer-containing RISC assembly complexes (Fig. 4) targets a particularly critical step on the path to an activated RISC. Recent 3D reconstructions from negatively-stained electron microscopy images of human Dicer complexes depict it as a fist-like shape with a central flat surface that is thought to bind the siRNA.^{4,45,46} This architecture along with the known binding mode of p19 raises the possibility that p19 could bind the opposite face of the siRNA in a ternary complex with Dicer. Notably, crystal structures of several other viral suppressors also appear to bind only one face of the siRNA, leaving the opposite face open to potentially accommodate proteins such as Dicer.⁴⁷ While we did not observe formation of a super-shifted band as indicative of such a ternary complex in our EMSAs with siRNA,

p19, and human Dicer (Figs. 4d–g), the complex may simply be too short-lived or our EMSA too low in resolution for the complex to be detected.

Second, the previously proposed simple sequestration model would require stoichiometric p19 concentrations relative to all siRNA duplexes, native to CIRV or not, that are generated by Dicer, as well as all other small duplex RNAs bound by p19, including defective interfering RNAs,²⁰ the siRNA amplification products generated in plant and other cells by RNA-dependent RNA polymerase activity,^{2,48} and perhaps miRNA duplexes.^{19,49,50} We independently confirmed the ability of p19 to bind a human miRNA using the conserved, ubiquitous, and well-studied let-7a miRNA, which has been implicated as a tumor suppressant.⁵¹ Remarkably, we observed that p19 binds the partially mismatched, double-stranded 22-nucleotide let-7a miRNA and our 21-nucleotide siRNA with equal affinities of 0.16 ± 0.02 nM and 0.17 ± 0.03 nM, respectively (Supplementary Fig. S8). Similar to our observations, a recent study used an affinity-capture approach to show that p19 from CIRV retains high affinity for let-7 miRNA, as well as a fluorophore labeled siRNA.⁵⁰ These observations further underscore that p19 may face a large background of competitor RNAs while targeting its siRNA substrate, although the specific base pairing patterns of some plant miRNAs may disfavor their binding to p19.⁵² Our finding that formation of the high-affinity siRNA:p19 complex is readily reversible (Fig. 2) while still interfering with siRNA binding by Dicer and RISC-related assembly complexes *in vitro* (Figs. 3 and 4) raises the possibility that sub-stoichiometric p19 concentrations may suffice *in vivo* to prevent siRNAs by multiple-turnover from assembling into RISC. In this fashion, the virus could keep pace with the amplified host response during systemic RNAi self-defense of the plant host against CIRV.⁵³

Third, while our results have an immediate impact on our understanding of the physicochemical properties and biological function of p19, a more practical consideration also arises from our work. The future of RNAi based strategies may well be dependent on the ability to control and predict the relative intracellular expression levels of RISC components and potential therapeutics.^{37,54} Short hairpin RNAs (shRNA), for example, while shown *in vivo* to efficiently induce gene silencing, in long-term investigations have been found to cause dose-dependent liver injury and ultimately death in adult mice when sustained at high levels,⁵⁴ and to cause dysregulation of microRNAs and increased tumorigenesis when marginally dosed.⁵⁵ Co-expression with p19 could help ameliorate such toxic effects of shRNAs. In addition, CIRV p19 and other RSS's have been shown to aid in the transfection and difficult over-expression of alpha-, adeno-, and lentiviruses used in gene therapy and in plant based vaccines, through their ability to suppress the intracellular RNAi immune response.³⁷ For such immunosuppression to be effective, dosing of the RSS is important, analogous to the use of immunosuppressants after organ transplant surgery. In fact, our observation that at least some miRNAs tightly bind to p19 cautions that adverse developmental effects due to a sequestration of essential cellular RNAs has to be carefully avoided. Understanding and modeling relevant biophysical properties of RNAi processes in the presence of RSSs, as advanced here, is thus anticipated to become critical for a broader and safer use of RNAi therapeutics in human disease control.

Materials and Methods

Sources of p19, Dicer, HeLa cell extract, and RNA

Purified p19 protein was prepared as previously described,¹⁵ and its dimer concentration was measured by Bradford assay. Recombinant human Dicer was prepared by Genlantis using the Baculovirus system with a commercially available protocol from Invitrogen. The His-tagged Dicer was purified on a Ni²⁺ column and the His tag removed by Tev protease cleavage, which should remove any contaminating factors that do not remain bound to the

recombinant Dicer. S100 cytosolic HeLa cell extract was obtained from Jena Biosciences and the integrity of Dicer in both samples was tested by Western blot analysis (Supplementary Fig. S1a). Additionally, we demonstrated biological activity of the recombinant Dicer through cleavage assays using long double-stranded RNA substrates (Supplementary Fig. S1b). An estimate of Dicer concentration in the partially purified preparation was derived from Bradford assays to measure the total protein concentration in combination with the 5% purity quoted by Genlantis and verified by Coomassie staining of a denaturing SDS-PAGE gel (Supplementary Fig. S1c). This estimate is conservative as higher relative purities would only imply that even higher concentrations of Dicer are needed to efficiently compete with p19 for siRNA binding. All RNAs were synthesized by the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory at the Yale University School of Medicine, then deprotected and purified as previously described.^{27,56} siRNA sequences were derived from the firefly luciferase gene²⁶ and were as follows: Sense strand 5'-P-CGU ACG CGG AAU ACU UCG AAA-3'; antisense strand 5'-P-UCG AAG (Fluorescein-dT)AU UCC GCG (Amino C6-dT)AC GUG-3', where P denotes a 5'-phosphate, and Fluorescein-dT and Amino C6-dT denote the phosphoramidites of the same name from Glen Research; the antisense strand was also synthesized with only one or no modification wherein U replaced the modified dT. Where needed, tetramethylrhodamine was coupled to the Amino C6 linker and the RNA purified as described.⁵⁶ miRNA sequences were derived from the human wild-type let-7a miRNA and were as follows: Let-7a antisense strand 5'-P-UGA GGU AGU AGG UUG UAU AGU U-3'; Let-7a sense strand 5'-P-CUA UAC AAU CUA CUG UCU UUC C-3'.

Steady-state fluorescence spectroscopy

Standard fluorescence measurements were performed on an Aminco Bowman spectrofluorometer (Thermo Scientific) at 37 °C. siRNA duplexes were formed by mixing an excess of 100 nM of unlabeled sense strand with 50 nM labeled sense strand in near-physiologic standard buffer (50 mM Tris-Acetate, pH 7.4, 80 mM KCl, 20 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 0.02% (v/v) Tween 20), heat annealing for 2 min at 70 °C, and cooling to room temperature over 5 min. Fluorescein/tetramethylrhodamine doubly-labeled siRNAs were excited at 490 nm (4 nm slit width) and emission alternately detected at 520 nm (for fluorescein, F) and 585 nm (8 nm slit width, for tetramethylrhodamine, T) to calculate the T/F ratio; F or T singly-labeled siRNAs were excited/detected at 490/520 nm or 565/585nm, respectively. In a typical experiment, p19 was added to 50 nM labeled siRNA and mixed manually to a final concentration of 500 nM unless otherwise indicated. For a chase experiment, unmodified siRNA duplex (formed through annealing the unlabeled composite sense and antisense RNA strands in a 2:1 ratio in standard buffer) was added to a final concentration of 3 μM. Saturation by the chase was ensured by triplicate trials using 500 nM, 750 nM, 1.5 μM, and 3 μM (Supplementary Fig. S2).

To observe fast SP complex formation, siRNA samples were prepared as above in standard buffer to a final concentration of 40 nM. In standard buffer p19 was added to varying (at least 8-fold) final excess concentrations (0.32–1.76 μM) at 37 °C in a KinTek PMA23B stopped-flow spectrofluorometer while exciting at 520 nm and detecting emission at 585 nm. All time course were fit with single-exponential increase or decrease functions in Origin (OriginLab) as described.⁵⁷

Electrophoretic mobility shift assays

For radioactive EMSAs the non-phosphorylated antisense strand was first 5'-³²P labeled with T4 polynucleotide kinase (PNK) and γ-³²P-ATP at an RNA concentration of 800 nM. PNK was inactivated by heating to 90 °C for 10 min, then the 5' phosphorylated complementary strand was added in 2-fold molar excess and the reaction slowly cooled to

room temperature to anneal the two strands. The siRNA duplex was further purified by nondenaturing, 20% (w/v) polyacrylamide gel electrophoresis. The duplex siRNA was cut out, eluted overnight into 1 mM EDTA at 4 °C, ethanol precipitated, and dissolved in RNase-free water. Scintillation counting was done on a Beckman LS6500 Multipurpose Scintillation Counter. EMSAs were performed as described previously¹⁵ on 10-cm non-denaturing 12% (w/v) polyacrylamide gels in 0.5x TBE (44.5 mM Tris-Borate, 1 mM EDTA) and run at 500 V and 4 °C for 2 h. Dicer and p19 at varying final concentrations were added to radiolabeled siRNA duplex (50,000 cpm, < 400 pM of 5'-³²P labeled duplex) in 10 µL standard buffer and incubated for 0.5 hrs. Next, 10 µL non-denaturing loading buffer (10% glycerol, 0.5x TBE, 0.025% bromophenol blue, and 0.025% xylene cyanol) was added and each sample loaded into a well of the EMSA gel. Competition experiments were performed by preincubating < 400 pM siRNA duplex (prepared as above) in standard buffer either with 140 nM Dicer and increasing concentrations of p19, or with 0.17 nM or 2.5 nM p19 and increasing concentrations of Dicer. Gel chase experiments were done in standard buffer with 70 and 10 nM of Dicer or p19 respectively to < 400pM 5'-³²P labeled siRNA and addition of 750 nM unlabeled siRNA chase for 0, 10, 30, 60 and 120 min. Gels were wrapped in plastic wrap and exposed to phosphor screens, which were scanned on a Storm 840 PhosphorImager and quantified using Image Quant software (Molecular Dynamics). The relative fraction f of relevant protein complexes was fit with the following hyperbolic binding isotherm to extract saturation point f_{max} and half-titration point $K_{D,app}$:

$$f = f_{max} \frac{[protein]}{[protein] + K_{D,app}}$$

RISC assembly complex formation

To observe RISC assembly complexes 15, 50, or 75% (v/v) cytosolic HeLa cell extract was incubated with < 400 pM siRNA duplex (prepared as above) in a final volume of 10 µl HeLa buffer [8% (v/v) Lysis Buffer (23.7 mM HEPES, pH 7.5, 79 mM potassium acetate, 1.58 mM magnesium acetate, 5 mM DTT, and 1 mg/ml Perabloc SC (Roche)), supplemented with 20% (v/v) 5x RNAi mix (125 mM creatine phosphate, 5 mM ATP, 25 mM DTT), 2% (w/v) creatine kinase, and 20% (v/v) RNA guard RNase Inhibitor (GE Bioscience)] for 2 h at 4 °C, unless otherwise specified. For competition experiments, p19 was added in increasing concentrations to the HeLa cell extract/siRNA mixture either immediately or after a 2 h preincubation. Time courses were stopped by direct loading onto a running nondenaturing 4% (w/v) polyacrylamide gel in 0.5x TBE (44.5 mM Tris-Borate, 1 mM EDTA), run at ~50 mW/cm² and 4 °C. For comparison with a previous assay, 50 and 75% (v/v) HeLa cell extract was incubated with siRNA as described in²⁸ for 1 h at 4 °C to 37 °C and analyzed by EMSA, yielding similar results (Supplementary Fig. S4). 15% (v/v) extract was preferred for most of our work due to the better pH control afforded by this lower concentration and to conserve material. For antibody supershift EMSA, cytosolic HeLa cell extract (24 µg total protein) was pre-incubated with 0.8 µg or 1.6 µg of anti-Dicer rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) and 0.8 µg or 1.6 µg of unrelated control antibodies, either anti-DsRed rabbit polyclonal antibody (Clontech Laboratories, Inc.) or anti-β actin mouse monoclonal antibody (Sigma-Aldrich, Inc.), for 2 h at 4 °C prior to the RISC assembly assay. The final volume was 60 µl per lane. Nondenaturing 4% (w/v) polyacrylamide gel electrophoresis was used to resolve these complexes as described above. Time courses and titration curves were fit as above.

Time-resolved FRET

Time-resolved donor (fluorescein, F) fluorescence decays were collected in the presence and absence of acceptor (tetramethylrhodamine, T) using time-correlated single-photon

counting, as previously described.^{58,59} As before, the donor decay in the presence of acceptor was modeled as a weighted average of the decays for each donor-acceptor distance in the ensemble of molecules assuming a three-dimensional Gaussian distance distribution with a Förster distance of 54 Å between F and T.^{58,59}

Western Blots for Dicer Detection

For denaturing Western blots, the cytosolic HeLa cell extract, the recombinant Dicer enzyme preparation, and recombinant TRBP (Abcam) and Ago2 (Abnova) proteins were run on a NuPAGE Novex 4–12% gradient Bis-Tris Gel in the presence of a molecular weight marker (ECL Plex Rainbow marker). The gel was then electroblotted in Tris-Glycine buffer (25 mM Tris, 192 mM Glycine, and 20% (v/v) Methanol) onto a Polyvinylidene Fluoride (PVDF) membrane (Immobilon-P Membrane, Millipore) over 75 min at 300 mA, using a Bio-Rad Trans-Blot SD semi-dry transfer cell per the manufacturer's instructions. After transfer the membrane was probed with either rabbit anti-Dicer (Santa Cruz Biotechnology), mouse anti-TRBP or mouse anti-Ago2 (both Abnova) followed by goat anti-Rabbit-Cy5 and goat anti-mouse-Cy3 (both Amersham), respectively. The probed blots in Supplementary Fig. S1a,d,e were visualized on a Typhoon 9410 Variable Mode Imager (GE Healthcare Life Sciences).

For non-denaturing Westerns blots, RISC assembly complexes were formed as described above but reactions were scaled up by 6-fold. Gels were soaked in 0.1% (w/v) SDS for 15 min and then electroblotted in Tris-Glycine transfer buffer (25 mM Tris, 25 mM Glycine) onto a PVDF membrane (Immobilon-P Membrane, Millipore) over 75 min at 300 mA, using a Bio-Rad Trans-Blot SD semi-dry transfer cell per the manufacturer's instructions. After transfer, the proteins were fixed to the membrane by incubating in 5–10% (v/v) acetic acid, rinsing with deionized water, and air drying. The membrane was probed with a rabbit primary antibody against Dicer (Santa Cruz Biotechnology), followed by a goat anti-rabbit secondary antibody, conjugated with horseradish peroxidase (Zymed, Invitrogen). The blot was developed using a peroxide/enhancer solution (ECL-Plus detection, Amersham), and visualized on a Typhoon 9410 Variable Mode Imager (Supplementary Fig. S5).

Modeling p19 in the context of RNAi

For the model described schematically in (Fig. 5a), changes in viral protein expression levels were monitored at steady-state in the absence and presence of the RSS p19. Changes in free p19 protein over time depend on terms representing the natural rates of production (r_x r_t / r_{dm}), degradation (r_{dp} [p19]), the extent of siRNA binding (k_{on} [siRNA] [P]), and the siRNA:p19 complex dissociation (k_{off} [SP]). Production of p19 is governed by its transcription rate (r_x), its messenger RNA degradation rate (r_{dm}), and its translation rate (r_t), which are assumed to have reached equilibrium on a timescale faster than that of repression. The p19 protein is degraded by the first-order rate constant (r_{dp}), leading to:

$$p19'[\tau] = \frac{r_x r_t}{r_{dm}} - r_{dp}[p19] - k_{on}[siRNA][p19] + k_{off}[SP] \quad [1]$$

The amount of usable siRNA in the system, i.e., the fraction able to associate with RISC, is reduced by the amount of siRNA bound to p19. Production of siRNA is dependent on the catalytic rate constant of Dicer (k_{catD}), and siRNA is degraded with a first order rate constant (r_{ds}). The parameter n is the fraction of siRNA that reenters the RNAi pathway after p19 dissociation, yielding:

$$siRNA'[\tau] = k_{catD}[Dicer] - r_{ds}[siRNA] - k_{on}[siRNA][p19] + n * k_{off}[SP] \quad [2]$$

The time evolution of the viral protein of interest that serves as a reporter of RNAi suppression and p19 enhancement is described as:

$$viral'[\tau] = \frac{r_x r_t}{\frac{k_{catR}}{K_{DR}}[RISC][siRNA] + r_{dm}} - r_{dp}[viral] \quad [3]$$

where (k_{catR}) is the catalytic rate constant for RISC-mediated cleavage of mRNA. The additional term in the denominator of Equation 3 is a quasi-steady state assumption used in previous work^{33,60} to describe RISC mediated degradation of viral messenger RNA (mRNA). It assumes that the active concentration of siRNA-loaded RISC is approaching its steady-state value with rate constant k_{catR} . This buildup of siRNA-loaded RISC contributes to the rate of degradation of the viral mRNA, as described by the concentrations of RISC and siRNA, as well as the dissociation constant K_{DR} . The proteins not silenced by RNAi are represented at steady-state by unitless constants:

$$[Dicer] = \frac{r_x r_t}{r_{dp} r_{dm}} \quad [4]$$

$$[RISC] = \frac{r_x r_t}{r_{dp} r_{dm}} \quad [5]$$

Equations 1–3 are rescaled as described by Marshall³³ and solved at steady-state to yield:

$$P = \frac{1}{1 - Aa \frac{k_{off}}{k_{catD}} + (Av\beta)S} \quad [6]$$

$$S = \frac{1 + \frac{k_{off}}{k_{catD}} naP}{\beta + \beta vP} \quad [7]$$

$$V = \frac{1}{1 + \beta y S} \quad [8]$$

where y and β are the parameters previously described as the efficiency of the RNAi pathway and the RNAi settling time, respectively.³³ The parameters a , v , and A are the fraction of siRNA-bound relative to total p19, the efficiency of p19, and the efficiency of Dicer in a particular cell line, respectively. The level of the viral protein at steady-state is dependent mostly on S . For example, in the absence of RNAi (i.e., in the absence of Dicer) $S = 0$, so that $V = 1$, that is, the viral protein level is not suppressed. Substituting Equation 6 into Equation 7 yields two solutions for S , where we choose S_2 as the more biologically relevant one:

$$S_1 = \frac{1 - aA \frac{k_{off}}{k_{cat}D} + v - Av + \sqrt{\left(-4A(-1 + a \frac{k_{off}}{k_{cat}D}(A-n)) \frac{v}{\beta^2} + (-1 + aA \frac{k_{off}}{k_{cat}D} + (-1+A)v)^2\right)}}{2Av\beta}$$

$$S_2 = \frac{1 - aA \frac{k_{off}}{k_{cat}D} + v - Av - \sqrt{\left(-4A(-1 + a \frac{k_{off}}{k_{cat}D}(A-n)) \frac{v}{\beta^2} + (-1 + aA \frac{k_{off}}{k_{cat}D} + (-1+A)v)^2\right)}}{2Av\beta} \quad [9]$$

Substituting S_2 into Equation 8 yields:

$$V_{Rev} = \frac{1}{1 + \frac{y(1 - aA \frac{k_{off}}{k_{cat}D} + v - Av - \sqrt{\left(-4A(-1 + a \frac{k_{off}}{k_{cat}D}(A-n)) \frac{v}{\beta^2} + (-1 + aA \frac{k_{off}}{k_{cat}D} + (-1+A)v)^2\right)}}{2Av}} \quad [10]$$

If p19 binding is assumed to be irreversible (i.e., $k_{off} = 0$), then V solves to:

$$V_{Irr} = \frac{2Av}{(-1 - v + \sqrt{1 + 2(1+A)v + (-1+A)^2v^2})y + Av(2+y)} \quad [11]$$

In the absence of p19 (i.e., $P = 0$), based on Equations 7 and 8, V reduces to the fully suppressed protein level:

$$V_o = \frac{1}{1+y} \quad [12]$$

The enhancement of viral protein production due to either irreversible (ϕ_{Irr}) or reversible p19 binding (ϕ_{Rev}) is then found to be (see also Fig. 5b):

$$\phi_{Irr} = \frac{V_{Irr}}{V_o} \quad [13]$$

$$\phi_{Rev} = \frac{V_{Rev}}{V_o} \quad [14]$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jeffrey Vargason and Traci Hall for the generous provision of purified p19 protein as well as Patrick Nelson for helpful discussions on mathematical modeling. Also we thank Jennifer Doudna for providing analytical purified Dicer and Ago protein. This work was funded by an NIH grant (to N.G.W.) and by the National Academy of Science Ford fellowship (to R.A.R.).

Abbreviations used

EMSA	electrophoretic mobility shift assay
RNAi	RNA interference
RISC	RNA-induced silencing complex
siRNA	small interfering RNA
CIRV	Carnation Italian Ringspot Virus
RSS	RNA silencing suppressor

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391:806–881. [PubMed: 9486653]
2. Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. *Cell*. 2009; 136:642–655. [PubMed: 19239886]
3. Castanotto D, Rossi JJ. The promises and pitfalls of RNA-interference-based therapeutics. *Nature*. 2009; 457:426–433. [PubMed: 19158789]
4. Sashital DG, Doudna JA. Structural insights into RNA interference. *Curr Opin Struct Biol*. 2010; 20:90–97. [PubMed: 20053548]
5. MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA. In vitro reconstitution of the human RISC-loading complex. *Proc Natl Acad Sci U S A*. 2008; 105:512–517. [PubMed: 18178619]
6. Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol*. 2005; 23:222–226. [PubMed: 15619617]
7. Collingwood MA, Rose SD, Huang L, Hillier C, Amarzguioui M, Wiiger MT, et al. Chemical modification patterns compatible with high potency dicer-substrate small interfering RNAs. *Oligonucleotides*. 2008; 18:187–200. [PubMed: 18637735]
8. Ji X. The mechanism of RNase III action: how dicer dices. *Curr Top Microbiol Immunol*. 2008; 320:99–116. [PubMed: 18268841]
9. Siomi H, Siomi MC. On the road to reading the RNA-interference code. *Nature*. 2009; 457:396–404. [PubMed: 19158785]
10. Jaskiewicz L, Filipowicz W. Role of Dicer in posttranscriptional RNA silencing. *Curr Top Microbiol Immunol*. 2008; 320:77–97. [PubMed: 18268840]
11. Gitlin L, Andino R. Nucleic acid-based immune system: the antiviral potential of mammalian RNA silencing. *J Virol*. 2003; 77:7159–7165. [PubMed: 12805414]
12. Parameswaran P, Sklan E, Wilkins C, Burgon T, Samuel MA, Lu R, et al. Six RNA viruses and forty-one hosts: viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS Pathog*. 2010; 6:e1000764. [PubMed: 20169186]
13. Qu F, Morris TJ. Suppressors of RNA silencing encoded by plant viruses and their role in viral infections. *FEBS Lett*. 2005; 579:5958–5964. [PubMed: 16162340]
14. Scholthof HB. The Tombusvirus-encoded P19: from irrelevance to elegance. *Nat Rev Microbiol*. 2006; 4:405–411. [PubMed: 16518419]
15. Vargason JM, Szittyá G, Burgyan J, Hall TM. Size selective recognition of siRNA by an RNA silencing suppressor. *Cell*. 2003; 115:799–811. [PubMed: 14697199]
16. Ye K, Malinina L, Patel DJ. Recognition of siRNA by a viral suppressor of RNA silencing. *Nature*. 2003; 426:874–878. [PubMed: 14661029]
17. Xia Z, Zhu Z, Zhu J, Zhou R. Recognition mechanism of siRNA by viral p19 suppressor of RNA silencing: a molecular dynamics study. *Biophys J*. 2009; 96:1761–1769. [PubMed: 19254536]
18. Lakatos L, Szittyá G, Silhavy D, Burgyan J. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO J*. 2004; 23:876–884. [PubMed: 14976549]

19. Dunoyer P, Lecellier CH, Parizotto EA, Himber C, Voinnet O. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell*. 2004; 16:1235–1250. [PubMed: 15084715]
20. Havelda Z, Hornyik C, Valoczi A, Burgyan J. Defective Interfering RNA Hinders the Activity of a Tombusvirus-Encoded Posttranscriptional Gene Silencing Suppressor. *J Virol*. 2005; 79:450–457. [PubMed: 15596838]
21. Omarov R, Sparks K, Smith L, Zindovic J, Scholthof HB. Biological relevance of a stable biochemical interaction between the tombusvirus-encoded P19 and short interfering RNAs. *J Virol*. 2006; 80:3000–3008. [PubMed: 16501109]
22. Lakatos L, Csorba T, Pantaleo V, Chapman EJ, Carrington JC, Liu YP, et al. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO J*. 2006; 25:2768–2780. [PubMed: 16724105]
23. Meraï Z, Kerenyi Z, Kertesz S, Magna M, Lakatos L, Silhavy D. Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *J Virol*. 2006; 80:5747–5756. [PubMed: 16731914]
24. Mitsis PG, Wensink PC. Identification of yolk protein factor 1, a sequence-specific DNA-binding protein from *Drosophila melanogaster*. *J Biol Chem*. 1989; 264:5188–5194. [PubMed: 2494181]
25. Ramsden JJ, Dreier J. Kinetics of the interaction between DNA and the type IC restriction enzyme EcoR124II. *Biochemistry*. 1996; 35:3746–3753. [PubMed: 8619995]
26. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell*. 2002; 110:563–574. [PubMed: 12230974]
27. Hoerter JA, Walter NG. Chemical modification resolves the asymmetry of siRNA strand degradation in human blood serum. *RNA*. 2007; 13:1887–1893. [PubMed: 17804643]
28. Pellino JL, Jaskiewicz L, Filipowicz W, Sontheimer EJ. ATP modulates siRNA interactions with an endogenous human Dicer complex. *RNA*. 2005; 11:1719–1724. [PubMed: 16177131]
29. Pham JW, Sontheimer EJ. Separation of *Drosophila* RNA silencing complexes by native gel electrophoresis. *Methods Mol Biol*. 2005; 309:11–16. [PubMed: 15990394]
30. Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ. A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. *Cell*. 2004; 117:83–94. [PubMed: 15066284]
31. Sigurskjold BW. Exact analysis of competition ligand binding by displacement isothermal titration calorimetry. *Anal Biochem*. 2000; 277:260–266. [PubMed: 10625516]
32. Wreggett KA, De Lean A. The ternary complex model. Its properties and application to ligand interactions with the D2-dopamine receptor of the anterior pituitary gland. *Mol Pharmacol*. 1984; 26:214–227. [PubMed: 6237254]
33. Marshall WF. Modeling recursive RNA interference. *PLoS Comput Biol*. 2008; 4:e1000183. [PubMed: 18802453]
34. Hefner E, Clark K, Whitman C, Behlke MA, Rose SD, Peek AS, et al. Increased potency and longevity of gene silencing using validated Dicer substrates. *J Biomol Tech*. 2008; 19:231–237. [PubMed: 19137112]
35. Chu M, Desvoyes B, Turina M, Noad R, Scholthof HB. Genetic dissection of tomato bushy stunt virus p19-protein mediated host-dependent symptom induction and systemic invasion. *Virology*. 2000; 266:79–87. [PubMed: 10612662]
36. Silhavy D, Molnar A, Lucioli A, Szittya G, Hornyik C, Tavazza M, et al. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *EMBO J*. 2002; 21:3070–3080. [PubMed: 12065420]
37. de Vries W, Haasnoot J, van der Velden J, van Montfort T, Zorgdrager F, Paxton W, et al. Increased virus replication in mammalian cells by blocking intracellular innate defense responses. *Gene Ther*. 2008; 15:545–552. [PubMed: 18273055]
38. Lombardi R, Circelli P, Villani ME, Buriani G, Nardi L, Coppola V, et al. High-level HIV-1 Nef transient expression in *Nicotiana benthamiana* using the P19 gene silencing suppressor protein of Artichoke Mottled Crinkle Virus. *BMC Biotechnol*. 2009; 9:96. [PubMed: 19930574]

39. Zheng N, Xia R, Yang C, Yin B, Li Y, Duan C, et al. Boosted expression of the SARS-CoV nucleocapsid protein in tobacco and its immunogenicity in mice. *Vaccine*. 2009; 27:5001–5007. [PubMed: 19523911]
40. Qian S, Zhong X, Yu L, Ding B, de Haan P, Boris-Lawrie K. HIV-1 Tat RNA silencing suppressor activity is conserved across kingdoms and counteracts translational repression of HIV-1. *Proc Natl Acad Sci U S A*. 2009; 106:605–610. [PubMed: 19122141]
41. Mougél M, Ehresmann B, Ehresmann C. Binding of *Escherichia coli* ribosomal protein S8 to 16S rRNA: kinetic and thermodynamic characterization. *Biochemistry*. 1986; 25:2756–2765. [PubMed: 3521721]
42. Seimiya M, Kurosawa Y. Kinetics of binding of Antp homeodomain to DNA analyzed by measurements of surface plasmon resonance. *FEBS Lett*. 1996; 398:279–284. [PubMed: 8977123]
43. Pond SJ, Ridgeway WK, Robertson R, Wang J, Millar DP. HIV-1 Rev protein assembles on viral RNA one molecule at a time. *Proc Natl Acad Sci USA*. 2009; 106:1404–1408. [PubMed: 19164515]
44. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet*. 2010; 11:597–610. [PubMed: 20661255]
45. Lau PW, Potter CS, Carragher B, MacRae IJ. Structure of the human Dicer-TRBP complex by electron microscopy. *Structure*. 2009; 17:1326–1332. [PubMed: 19836333]
46. Wang HW, Noland C, Siridechadilok B, Taylor DW, Ma E, Felderer K, et al. Structural insights into RNA processing by the human RISC-loading complex. *Nat Struct Mol Biol*. 2009; 16:1148–1153. [PubMed: 19820710]
47. Yang J, Yuan YA. A structural perspective of the protein-RNA interactions involved in virus-induced RNA silencing and its suppression. *Biochim Biophys Acta*. 2009; 1789:642–652. [PubMed: 19501679]
48. Lipardi C, Paterson BM. Identification of an RNA-dependent RNA polymerase in *Drosophila* involved in RNAi and transposon suppression. *Proc Natl Acad Sci USA*. 2009; 106:15645–15650. [PubMed: 19805217]
49. Chapman EJ, Prokhnovsky AI, Gopinath K, Dolja VV, Carrington JC. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev*. 2004; 18:1179–1186. [PubMed: 15131083]
50. Jin J, Cid M, Poole CB, McReynolds LA. Protein mediated miRNA detection and siRNA enrichment using p19. *Biotechniques*. 2010; 48:xvii–xxiii. [PubMed: 20569217]
51. Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME. The role of let-7 in cell differentiation and cancer. *Endocr Relat Cancer*. 2011; 17:F19–36. [PubMed: 19779035]
52. Varallyay E, Valoczi A, Agyi A, Burgyan J, Havelda Z. Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation. *EMBO J*. 2010; 29:3507–3519. [PubMed: 20823831]
53. Baulcombe D. RNA silencing in plants. *Nature*. 2004; 431:356–363. [PubMed: 15372043]
54. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*. 2006; 441:537–541. [PubMed: 16724069]
55. Beer S, Bellovin DI, Lee JS, Komatsubara K, Wang LS, Koh H, et al. Low-level shRNA Cytotoxicity Can Contribute to MYC-induced Hepatocellular Carcinoma in Adult Mice. *Mol Ther*. 2010; 18:161–170. [PubMed: 19844192]
56. Walter NG. Probing RNA structural dynamics and function by fluorescence resonance energy transfer. *Curr Protocols Nucleic Acid Chem*. 2002; 11.10.11–11.10.23.
57. Gondert ME, Tinsley RA, Rueda D, Walter NG. The catalytic core structure of the trans-acting HDV ribozyme is subtly influenced by sequence variation outside the core. *Biochemistry*. 2006; 45:7563–7573. [PubMed: 16768452]
58. Rueda D, Wick K, McDowell SE, Walter NG. Diffusely bound Mg²⁺ ions slightly reorient stems I and II of the hammerhead ribozyme to increase the probability of formation of the catalytic core. *Biochemistry*. 2003; 42:9924–9936. [PubMed: 12924941]

59. Gerczei T, Shah BN, Manzo AJ, Walter NG, Correll CC. RNA chaperones stimulate formation and yield of the U3 snoRNA-Pre-rRNA duplexes needed for eukaryotic ribosome biogenesis. *J Mol Biol.* 2009; 390:991–1006. [PubMed: 19482034]
60. Groenenboom MA, Maree AF, Hogeweg P. The RNA silencing pathway: the bits and pieces that matter. *PLoS Comput Biol.* 2005; 1:155–165. [PubMed: 16110335]
61. Ma E, MacRae IJ, Kirsch JF, Doudna JA. Autoinhibition of human dicer by its internal helicase domain. *J Mol Biol.* 2008; 380:237–243. [PubMed: 18508075]
62. Bartlett DW, Davis ME. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res.* 2006; 34:322–333. [PubMed: 16410612]
63. Rose SD, Kim DH, Amarzguioui M, Heidel JD, Collingwood MA, Davis ME, et al. Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res.* 2005; 33:4140–4156. [PubMed: 16049023]

Fig. 1. Fluorescence assays to detect siRNA:p19 complex formation. (a) Crystal structure of p19 (blue) bound to an siRNA (grey). The nucleotides to which the fluorophores fluorescein (F) and tetramethylrhodamine (T) are attached are highlighted in green and red, respectively. (b) The T-only labeled siRNA (red) is quenched upon p19 addition, while the F-only labeled siRNA (green) is unaffected by p19. (c) EMSA analysis shows that the doubly-labeled ft-siRNA (red) binds to p19 with the same affinity as the unlabeled siRNA (black). (d) Time-resolved FRET measures the F:distances for the single-stranded, protein-free double-stranded, and p19-bound double-stranded siRNA 35 Å, 43.4 Å, and 44.5 Å, respectively.

Fig. 2.

Kinetics of formation and dissociation of the siRNA:p19 complex. (a) Pseudo-first order kinetics of the binding of doubly-labeled siRNA (50 nM) to excess p19, measured through the T/F ratio during stopped-flow mixing. Scatter points denote raw data; lines denote single-exponential fits to extract rate constants. (b) Plot of the observed pseudo-first rate constants from panel A over the p19 concentration. The linear fit yields the indicated bimolecular binding rate constant, k_{on} . (c) Fluorescence detection of the binding of doubly-labeled siRNA (50 nM) to p19 (500 nM) and of complex dissociation upon addition of an unlabeled siRNA chase (3 μM). A single-exponential fit (red line) yields the indicated dissociation rate constant, k_{off} .

Fig. 3.

Competition of p19 with human Dicer for siRNA binding as detected by EMSA. (a) The fraction of siRNA bound to Dicer over increasing Dicer concentrations in the absence of p19 (black squares), and presence of 0.17 nM (open red circles) or 2.5 nM p19 (blue dots). Solid lines, corresponding fits of hyperbolic binding isotherms. Dashed lines indicate the predicted curves based on a dissociative binding mechanism (siRNA has to first dissociate from Dicer to bind p19). (b) Fraction of siRNA bound to p19 over increasing p19 concentrations in the absence (black squares) or presence of a saturating Dicer concentration of 140 nM (open green circles). Solid lines, corresponding fits of hyperbolic binding isotherms. The dashed-dotted line indicates the predicted curve based on a dissociative binding mechanism. (c) Schematic of the two competition models between Dicer and p19 for siRNA; the dashed box highlights the part unique to the ternary complex formation model.

Fig. 4.

Competition of p19 with human siRNA-containing complexes found in cytosolic HeLa cell extract. (a) Normalized fraction of siRNA bound to complexes D (green), C2 (red), and C1 (black), where the highest point is normalized to unity in each complex over time, as derived from the gel in panel B. The relative abundances of C1 and complex D increase over time with estimated rate constants of $8 \times 10^{-4} \text{ s}^{-1}$ and $4 \times 10^{-4} \text{ s}^{-1}$, respectively, whereas C2 decreases in relative abundance with a rate constant of $2 \times 10^{-3} \text{ s}^{-1}$. (b) Formation of complexes D, C2, and C1 after 10, 30, 60 and 120 min incubation of siRNA in cytosolic HeLa cell extract. Samples were loaded onto a running 4% native polyacrylamide gel, leading to the indicated differences in migration. (c) Anti-Dicer antibody-based supershift of siRNA-containing complexes. Dicer containing complex D shifts in the presence of anti-Dicer antibody (lane 3: 0.8 μg ; lane 4: 1.6 μg). Lanes 1, 5 and 6 indicate the mobility of the complexes in the absence of any antibody. Lanes 9–12 show negative controls where 0.8 μg or 1.6 μg of anti-actin (lanes 9, 10) or anti-DsRed (lanes 11, 12) were added. (d) Quantification of the normalized fraction of siRNA bound in each complex, as derived from gels as in panel E. Fractions were normalized to the zero time point. (e) Formation of

complexes after 2.5 h incubation of siRNA in cytosolic HeLa cell extract, and increasing concentrations of p19. (f) Quantification of the normalized fraction of siRNA bound in each complex, as derived from gels as in panel E, normalized as in D. (g) Formation of complexes D, C1, and C2 after 2 h incubation of siRNA in cytosolic HeLa cell extract, followed by the addition of increasing concentrations of p19 and further incubation for 30 min.

Fig. 5.

Steady-state kinetic modeling of p19 action in RNAi suppression. (a) Schematic of our expanded “catch and release” mathematical model. A , y , and v describe the efficiencies of Dicer, RNAi in the cell, and p19, respectively. n is the fraction of siRNAs that successfully reenters the RNAi pathway after release from p19. The mature human RISC (with single stranded siRNA bound) may or may not contain Dicer (dashed outline). (b) Enhancement of viral protein load over unsuppressed protein level, assuming reversible and irreversible p19 binding (ϕ_{Rev} and ϕ_{Irr} , respectively). The fraction of siRNA:p19 complex over free p19, a , will increase as the efficiency of p19 (v) increases. The enhancement in the level of viral protein due to reversible p19 binding will therefore fall within the range (grey area) determined for a ranging from 0.1 (upper bound) to 200 (lower bound).

Table 1**Model Parameters**

Name	Description (units)	Determined From	Value
kcatD	Catalytic Rate of Dicer (nM/h)	Literature ⁶¹	8.143×10^6
rdp	Protein Degradation Rate Constant (h^{-1})	Literature ⁶²	0.35
rds	siRNA Degradation Rate Constant (h^{-1})	Literature ⁶²	0.029
koff	p19 Dissociation Rate Constant (h^{-1})	Determined	223.2
kon	p19 Association Rate Constant ($\text{nM}^{-1} \text{h}^{-1}$)	Experimentally Determined	608.4
n	Fraction of siRNA that reenter the RNAi pathway	Experimentally Literature ⁶³	0.01
a	Fraction of bound verses unbound p19	Estimated from experimental data	1–100
Cellular Volume	Intracellular volume (L)	Literature ⁶²	1.4×10^{-12}
Grouped Parameters			
y	RNAi Efficacy Parameter specific to HEK293 cells	Literature ³³	1.22
A	Dicer Efficacy Parameter	kcatD/rdp	2.33×10^7
β	RNAi Settling Time	rds/rdp ³³	8.29×10^{-2}
v	p19 Efficacy Parameter	Range	0–1