Targeting of Dicer-2 and RNA by a Viral RNA Silencing Suppressor in *Drosophila* Cells

Nan Qi,^a Lei Zhang,^a Yang Qiu,^a Zhaowei Wang,^a Jie Si,^a Yongxiang Liu,^a Xue Xiang,^a Jiazheng Xie,^a Cheng-Feng Qin,^b Xi Zhou,^a and Yuanyang Hu^a

State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, Hubei, China,^a and State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China^b

RNA interference (RNAi) is a eukaryotic gene-silencing mechanism that functions in antiviral immunity in diverse organisms. To combat RNAi-mediated immunity, viruses encode viral suppressors of RNA silencing (VSRs) that target RNA and protein components in the RNAi machinery. Although the endonuclease Dicer plays key roles in RNAi immunity, little is known about how VSRs target Dicer. Here, we show that the B2 protein from Wuhan nodavirus (WhNV), the counterpart of Flock House virus (FHV), suppresses *Drosophila melanogaster* RNAi by directly interacting with Dicer-2 (Dcr-2) and sequestering double-stranded RNA (dsRNA) and small interfering RNA (siRNA). Further investigations reveal that WhNV B2 binds to the RNase III and Piwi-Argonaut-Zwille (PAZ) domains of Dcr-2 via its C-terminal region, thereby blocking the activities of Dcr-2 in processing dsRNA and incorporating siRNA into the RNA-induced silencing complex (RISC). Moreover, we uncover an interrelationship among diverse activities of WhNV B2, showing that RNA binding enhances the B2–Dcr-2 interaction by promoting B2 homodimerization. Taken together, our findings establish a model of suppression of *Drosophila* RNAi by WhNV B2 targeting both Dcr-2 and RNA and provide evidence that an interrelationship exists among diverse activities of VSRs to antagonize RNAi.

As a form of nucleic acid-based silencing, RNA interference (RNAi) plays essential roles in the cellular response to viral infection in plants and invertebrates (3, 13, 34, 50). In virus-infected cells, aberrant accumulation of viral single-stranded RNA (ssRNA) triggers its conversion into viral replicative intermediate double-stranded RNA (vRI-dsRNA), which is processed by the dsRNA-specific endonuclease Dicer into 21- to 23-nucleotide (nt) small interfering RNAs (siRNAs). Next, the virus-derived siRNAs (viRNAs) are transferred from Dicer to Argonaute (AGO) proteins in the RNA-induced silencing complex (RISC), which then guides the specific degradation of homologous viral ssRNAs (1, 8, 11, 50). In mammals, RNAi directed by viral and cellular microRNAs (miRNAs) also contributes to host innate immunity against viral infection (18, 27, 35).

To combat RNAi-mediated immunity, many plant and animal viruses encode viral suppressors of RNA silencing (VSRs) that target different components in the RNAi machinery. By sequestering dsRNA and siRNA, plant VSRs like turnip crinkle virus (TCV) capsid protein and tombusvirus P19 protein inhibit the production of siRNAs and hinder the incorporation of siRNAs into RISC (25, 41, 52). Additionally, direct interaction with AGO protein is known as a common approach of many plant and insect VSRs, such as cucumber mosaic virus (CMV) 2b protein, TCV P38 protein, sweet potato mild mottle virus (SPMMV) P1 protein, and cricket paralysis virus (CrPV) 1A protein, for suppressing RISC-mediated mRNA cleavage (2, 17, 36, 54).

Although Dicer plays essential roles in RNAi immunity, the mechanism by which Dicer can be directly targeted by VSRs is still poorly understood. The core protein of hepatitis C virus (HCV) was previously reported to interact with Dicer; however, whether this interaction is required for the RNA silencing suppression activity of HCV core protein has not been determined (7, 51). Furthermore, several VSRs have been reported to be able to target both RNA and protein components in the RNAi machinery. For

example, TCV P38 was shown to target both RNA duplexes and AGO1 (2, 33). A question that remains to be answered, however, is whether an interrelationship exists between diverse activities of VSRs that mediate RNA binding and interaction with RNAi protein components (51).

The ideal model for studying viral pathogenesis and RNAi immunity is the persistent infection of *Drosophila melanogaster* cells with Flock House virus (FHV), the most extensively studied member of the *Nodaviridae* family, which encodes a well-defined VSR designated B2 (1, 6, 10, 16, 29). During the course of FHV infection, 5'-terminal vRI-dsRNA initiated by viral RNA-dependent RNA polymerase (RdRP) triggers RNAi immunity, which is suppressed by B2 protein because B2 associates with RdRP and binds to vRI-dsRNA, thereby leading to the inhibition of the production of siRNAs by Dicer-2 (Dcr-2) (1). Recently, an interaction of FHV B2 with the Piwi-Argonaut-Zwille (PAZ) domain of Dcr-2 was detected *in vitro* (45). Although whether this interaction plays essential roles in the suppression of *Drosophila* RNAi by FHV B2 remains unclear, it appears that the nodaviral B2 protein might be a multifunctional VSR that targets both RNA and protein components in the RNAi machinery (49).

Wuhan nodavirus (WhNV), the counterpart of FHV, has been characterized in considerable detail by our group and shown to be distinct from FHV in some aspects (5, 31, 32, 39, 40). In addition to elucidating the special strategy of subgenomic RNA3 synthesis (40), we have previously shown that WhNV B2 employs a novel

Received 28 December 2011 Accepted 6 March 2012

Published ahead of print 21 March 2012

Address correspondence to Xi Zhou, zhouxi@whu.edu.cn.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.07229-11

TABLE 1 Oligonucleotides and primers used in this work

Primers ^a	Sequence (5' to 3')	Purpose
B2-For	<u>GAATTC</u> ACC ATGGCGAACGACAACCAGAAAC (EcoR I)	Construction of plasmids for constitutive expression of B2 WT and mutants in S2 cells and prokaryotic expression of GST fusion proteins
B2-Rev	TCTAGACTGAGTTTCGATGGGTCTGCC (XbaI)	
B2R24A-For	<u>GAATTC</u> ACC ATGGCGAACGACAACCAGAAACAAGCATTGACA GCGTACCAAGCACTGGTCCGCCCAAGTAATGGCGACCgc cACG (EcoR I)	Chemically synthesized siRNA that induces RNA silencing of GFP in S2 cells
B2R24A-Rev	TCTAGACTGAGTTTCGATGGGTCTGCC (XbaI)	
B2ΔN20-For	<u>GAATTC</u> ACC ATGGCGACCCGAACGCTGGT (EcoR I)	Construction of transcription templates for synthesis of WhNV RNA1, dsRNA, siRNA, and probes by T7 RNA polymerase
B2ΔN20-Rev	TCTAGACTGAGTTTCGATGGGTCTGCC (XbaI)	
B2ΔC21-For	<u>GAATTC</u> ACC ATGGCGAACGACAACCAGAAAC (EcoR I)	
B2ΔC21-Rev	TCTAGACTGTCTGGCTGCCTCCAGCATCCGT (XbaI)	
GST-RNase-For	<u>GAATTC</u> CGATATTTGTTGCAGGCCCTCAC (EcoR I)	
GST-RNase-Rev	CTCGAGTTAGAGCTTTGCGTTCTCCGCTAG (XhoI)	
GST-PAZ-For	<u>GAATTC</u> GAAAGATTTTGAGGGCAAAATT (EcoR I)	
GST-PAZ-Rev	CTCGAGTTAAATGCGGTTGCCGTACTTTGA (XhoI)	
siRNA-GFP	Homologous sequence GGTTATGTACAGGAACGCA Sense strand p-GGUUAUGUACAGGAACGCAU Antisense strand UUCCAAUACAUGUCCUUGCGU-p TAATACGACTCACTATAGATGGCTAGCAAAGGAGAAGAAGCTT	
ds-GFP-For	TAATACGACTCACTATAGATGGCTAGCAAAGGAGAAGAAGCTT	
ds-GFP-Rev	TAATACGACTCACTATAGTTGAAGTTAGCTTTGATTCCATTTC	
si-GFP-For	TAATACGACTCACTATAGTTATGTACAGGAACGCA	
si-GFP-Rev	TAATACGACTCACTATAGAACCAATACATGTCCTTGCGT	
ds-RNA1-For	TAATACGACTCACTATAGATAAAACGTAACGTGACAAATAT	
ds-RNA1-Rev	TAATACGACTCACTATAGGGTCTCCATGATTCTCGAGAACTGT	
RNA1-For	TAATACGACTCACTATAGATAAAACGTAACGTGACAAATAT	
RNA1-Rev	AATACCTGTTTATTGGTGGTGAGAT	
sense probe-For	TTTCTCTTATGGTGTTCATGCTT	
sense probe-Rev	TAATACGACTCACTATAGAAAGATATAGTGCCTTCCTGTACA	
anti probe-For	TAATACGACTCACTATAGTTTCTCTTATGGTGTTCATGCTT	
anti probe-Rev	AAAGATATAGTGCCTTCCTGTACA	
RNA1 probe-For	TAATACGACTCACTATAGACAGTATGGGTTACTACGCAGGCAT	
RNA1 probe-Rev	ATTTTCGACCCGGTAGACTGCGGCGT	

^a Sequence-specific primers are designed according to the sequences with GenBank accession numbers AY962576 (WhNV RNA1), NC 011521.1 (GFP), and NP 523778.2 (Dcr-2). The WhNV RNA1, B2 protein, GFP, and Dcr-2 ORF region were amplified from pWh1 (G,0) constructed in our previous work (40) using pIZT-V5/His (Invitrogen) and Dcr-2 cDNA clone (GenScript) vectors. Underlined characters indicate restriction endonuclease sites, and the endonuclease types are shown in parentheses. Nucleotides replaced for mutagenesis are shown with lowercase characters. The T7 RNA polymerase promoter is shown in italics. The Kozak translation initiation sequence is indicated with bold characters.

RNA-binding mode different from that of FHV B2 to sequester dsRNA and siRNA (39). Moreover, we revealed that the homodimerization of WhNV B2 is required for its RNA-binding activity, while RNA binding in turn promotes B2 homodimerization (39). This positive feedback effect indicates that diverse activities of VSRs might interact with each other.

Here, we report that in addition to sequestering dsRNA and siRNA, WhNV B2 suppresses RNA silencing by directly interacting with Dcr-2 in *Drosophila* Schneider 2 (S2) cells. Further investigations show that WhNV B2 binds to the RNase III and PAZ domains of Dcr-2. Through these interactions, WhNV B2 blocks the activities of Dcr-2 in processing dsRNA and incorporating siRNAs into RISC. Moreover, we uncover an interrelationship among diverse activities of WhNV B2, showing that RNA binding to B2 enhances the B2–Dcr-2 interaction by promoting B2 homodimerization. Thus, our findings establish a model of suppression of *Drosophila* RNAi by WhNV B2 targeting Dcr-2 and RNA that not only extends our understanding about how VSRs interact with Dicer but also sheds light on the synergistic effects among diverse activities of VSRs to antagonize RNAi.

MATERIALS AND METHODS

Plasmids. The commercial InsectSelect glow system vector, pIZT/V5-His (Invitrogen, Carlsbad, CA), was used to construct plasmids that generate stable *Drosophila* S2 cell lines with constitutive expression of green fluorescent protein (GFP) and WhNV B2 wild-type (WT) or mutated proteins. pRISE-ds-GFP, a hairpin-shaped dsRNA expression plasmid that targets the GFP open reading frame (ORF) region of nucleotides 1 to 500, was constructed as described previously (23). Plasmids for the prokaryotic expression and purification of maltose-binding protein (MBP)-tagged WT or mutant B2 proteins were constructed in our previous work (39). Mutations were introduced into the B2 coding region by PCR-mediated mutagenesis according to our standard protocol (39). To construct plasmids that express glutathione S-transferase (GST)-tagged PAZ (corresponding to nucleotides 2623 to 2769) and the RNase (nucleotides 4346 to 4596) domain of Dcr-2, PCR products amplified from Dcr-2 cDNA (GenScript, Piscataway, NJ) were cloned into the pGEX-6P-1 vector (Amersham Biosciences, Piscataway, NJ). The oligonucleotides are shown in Table 1.

Cell culture and extract preparation. We cultured *Drosophila* S2 cells in semisuspension at 27°C in Schneider's insect medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco). The gen-

eration of stable S2 cell lines expressing GFP and WhNV B2 proteins was performed by transfecting S2 cells with pIZT/V5-His vectors that contained different transgenes, followed by a series of antibiotic selection steps according to the manufacturer's protocol (Invitrogen). To prepare cell extracts, cells were collected for extract preparation at 1×10^7 cells per ml, washed in ice-cold phosphate-buffered saline (PBS), and resuspended in a hypotonic buffer (10 mM HEPES [pH 7.4], 2 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF]). Lysates were centrifuged at $20,000 \times g$ at $4^\circ C$ for 20 min and stored at $-70^\circ C$ as previously reported (4).

Protein-protein interaction. Coimmunoprecipitation assays were conducted according to our standard protocol (39). After incubation with antibodies (anti-His monoclonal antibody or preimmune mouse antisera) and pretreated protein G-agarose beads (Roche, Basel, Switzerland) at $4^\circ C$ for 1 h, the antibody-bound complexes were captured and washed. Proteins and RNAs were extracted from the captured complexes by boiling in 12% SDS–polyacrylamide gel sample buffer or using a Poly-Gel RNA extraction kit (Omega Biotek, Norcross, GA), with subsequent preparation for Western blotting and Northern blotting analyses as previously described (39). The monoclonal antibodies recognizing Dcr-1 and Dcr-2 were purchased from Abcam (Oxford, United Kingdom). The methods for generating T7-transcribed digoxigenin (DIG)-labeled RNA probes were described previously (39); the oligonucleotides are shown in Table 1.

GST pulldown assays were performed with GST-tagged proteins and recombinant MBP fusion proteins, which were expressed and purified as previously described (39). After a washing step and resuspension in TENN buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.5% NP-40, and 50 mM NaCl), 200 μ l glutathione Sepharose (GE Healthcare, Piscataway, NJ) was added to the reaction mixtures containing 2 μ g GST-tagged and 2 μ g MBP fusion proteins and the mixtures were incubated at $4^\circ C$ for 1 h. Following washing and collection, proteins that bound to the glutathione Sepharose were subjected to 12% SDS–PAGE and Western blotting.

RNAse III-mediated cleavage assays. RNAse III-mediated cleavage assays were conducted according to our standard protocol (39), using T7-transcribed 500-bp dsRNA derived from GFP as precursor. Reactions were performed in 20- μ l reaction mixture volumes containing 0.1 μ M dsRNA, $1 \times$ RNAse III buffer (20 mM Tris-HCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT [Sigma, Saint Louis, MO], 140 mM NaCl, 2.7 mM KCl [pH 7.9]), and 1 U recombinant RNAse III (a generous gift from Yi Zhang). After incubation at $37^\circ C$ for 30 min, reaction products were resolved by Tris-borate-EDTA (TBE)–agarose gel electrophoresis on 1.2% gels, and the RNA was stained with ethidium bromide at 1 μ g/ml.

In vitro RISC assembly and mRNA cleavage assays. *In vitro* RISC assembly and subsequent mRNA cleavage assays were performed using standard RNAi reactions as previously described (37, 48), with minor modifications. For the RISC assembly assay, each 20- μ l reaction mixture contained 50% (vol/vol) S2 cell extracts, 500 μ M ATP (New England BioLabs, Ipswich, MA), 5 mM DTT, 1 U RNasin (Promega, Madison, WI). The mixtures were incubated with 0.3 μ M DIG-labeled siRNA transcribed by T7 RNA polymerase at $25^\circ C$ for 30 min as previously reported (14). For the mRNA cleavage assay, RISC preassembled with 0.3 μ M chemically synthesized anti-GFP siRNA (RiboBio, Guangzhou, China) was incubated with 50 nM mRNA derived from nucleotides 1 to 500 of the GFP ORF and labeled with DIG at $25^\circ C$ for 30 min. S2 cell extracts were chromatographed on a Superdex-200 gel filtration column (GE Healthcare), and the fractions were incubated with DIG-labeled siRNA to identify the RISC and RISC-loading complex (RLC) as described previously (37).

Far-Western blotting assays. Far-Western blotting assays were conducted as described in our previous studies (39). After being separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membrane, 0.5 μ g GST, GST-PAZ, and GST-RNase was gradually renatured at $4^\circ C$ in HEPES buffer (20 mM HEPES-KOH, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 10% glycerol [pH 7.4]) containing

5% nonfat milk. Then, the membranes were washed and incubated with 50 μ g MBP-tagged WT B2 or mutated proteins in 4 ml incubation buffer (10 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 1% nonfat milk powder [pH 7.4]) supplemented or not with 50 μ g 500-bp dsRNA at room temperature for 3 h. The immunoblots were detected by using anti-MBP monoclonal antibody (New England BioLabs) (1:10,000 dilution) and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:2,000 dilution), with BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium; Promega) as a substrate.

RESULTS

WhNV B2 protein suppresses dsRNA-induced RNA silencing in *Drosophila* S2 cells. Our previous work reported that WhNV B2 forms a homodimer that binds to both dsRNA and siRNA, thereby inhibiting Dicer-mediated cleavage and the incorporation of siRNA into RISC (39). Furthermore, we have dissected the RNA-binding and homodimerization domains of B2 essential for its RNAi suppression activity (39). Helix α 1 of the N terminus mediates B2 homodimerization, whereas helices α 2 and α 3 constitute the RNA-binding domain (Fig. 1A). Thus, deleting the homodimerization domain (Δ N20) or replacing the RNA binding essential residue Arg24 with alanine (R24A) completely abolishes the RNAi suppression activity of B2 (39) (Fig. 1A). Interestingly, although the last two helices, comprised of C-terminal amino acids 1 to 21, have been shown to be dispensable for the RNA-binding or homodimerization activities (39), we found that deleting this portion of B2 still compromised its RNAi suppression activity (Fig. 1A). This unexpected observation led us to wonder whether in addition to sequestering RNA duplexes, WhNV B2 employs another strategy to block RNAi via its C-terminal region.

To uncover the detailed mechanism by which WhNV B2 functions, we generated a series of stable S2 cell lines, the control cell line that constitutively expresses GFP (S2/GFP) and the cell lines that simultaneously express GFP and wild-type B2 (S2/GFP+B2) using the InsectSelect glow system vector pIZT/V5-His (Fig. 1B). Following a series of antibiotic selections, multiple oligoclonal cell lines were screened via fluorescence and Western blotting analyses for the expression of GFP and B2, and the desired cell lines expressing high levels of GFP and/or B2 were expanded (Fig. 1C). In the same manner, we generated other stable cell lines that expressed mutated B2 proteins defective in RNA binding (B2R24A), defective in homodimerization (B2 Δ N20), or in which C-terminal amino acids 1 to 21 were deleted (B2 Δ C21).

At 72 h after transfection of pRISE-ds-GFP, a plasmid expressing dsRNA targeting GFP (anti-GFP dsRNA), the GFP mRNA transcripts and the production of siRNA in stable cells were determined via Northern blotting analyses. Compared to the results for the control cell line, anti-GFP dsRNA-induced RNAi was dramatically inhibited by WT B2 in S2/GFP+B2 cells (Fig. 1C and D, lanes 2 to 5), whereas the RNAi suppression activity of B2 was reduced or completely abolished in B2R24A and B2 Δ N20 cells, respectively (Fig. 1D, lanes 6 and 8). Notably, although B2 Δ C21 retains the RNA-binding and homodimerization activities (39), deleting C-terminal amino acids 1 to 21 of B2 resulted in a reduction of its RNAi suppression activity (Fig. 1D, lane 7). Moreover, since a previous study reported that a 400-bp dsRNA produced from the 5' end of FHV genomic RNA1 is the major substrate of Dcr-2 (1), we cotransfected stable S2 cell lines with WhNV genomic RNA1

FIG 1 WhNV B2 inhibits RNA silencing of GFP induced by dsRNA in *Drosophila* S2 cells. (A) A schematic representation of the functional domains of B2. Indicated are the five alpha helices ($\alpha 1$ to $\alpha 5$) and their locations. The dimerization domain (DMD) is located in helix $\alpha 1$, and the RNA-binding domain (RBD) is composed of helices $\alpha 2$ and $\alpha 3$. A series of mutations are indicated, with their impacts on the diverse activities of B2 shown as “+” and “-.” a.a., amino acids. R24A, the replacement of Arg24 with alanine, which abolishes the RNA-binding activity of B2, is highlighted in red and shown with an asterisk. (B) Generation of stable S2 cells expressing GFP and B2. Illustrated are the plasmids constructed for stably expressing GFP (pIZT/V5-His) and expressing both GFP and B2 (pIZT-B2) in S2 cells. OpIE1 and OpIE2, OpMNPV IE-1 gene and IE-2 gene promoters, respectively; SV40, simian virus 40; pA, polyadenylation sequence; MCS, multiple-cloning site. (C) Fluorescence analyses of the stable cell line (S2/GFP) with constitutive expression of GFP (1) and the stable cell line (S2/GFP+B2) that simultaneously expressed GFP and B2 (2) in the absence (-) or presence (+) of plasmid carrying dsRNA targeting GFP (anti-GFP dsRNA). (D) Suppression of dsRNA-induced RNAi by WT B2 and mutants in S2 cells. At 72 h after transfection of the anti-GFP dsRNA-expressing plasmid pRISE-ds-GFP, RNA was harvested from stable S2 cells that expressed WT or mutant B2 as indicated above each lane (lanes 5 to 8) and then subjected to Northern blotting to determine the effects of WT B2 or mutants on the reduction in GFP mRNA and the production of siRNA. The expression of WT B2 and mutants was detected by Western blotting using anti-B2 polyclonal antibodies. RNA harvested from mock-transfected cells and the stable cell line expressing GFP were assayed as negative controls (lanes 1, 2, and 4). 18S rRNA and β -actin were used as the loading controls. (E) WhNV B2 suppresses RNA silencing of WhNV genomic RNA1 that is induced by RNA1-derived dsRNA. As indicated above each lane (lanes 3 to 6), the stable S2 cells expressing WT B2 and mutants were cotransfected with *in vitro*-transcribed WhNV genomic RNA1 (nucleotides 1 to 3149) with dsRNA derived from 5'-end nucleotides 1 to 400 of RNA1 (dsRNA1). At 72 h posttransfection, RNA was harvested from cells and then subjected to Northern blotting to determine the effects of WT B2 and mutants on the reduction in RNA1 and the production of siRNA. RNA harvested from cells cotransfected without dsRNA1 (Neg. 1) and from cells cotransfected with dsRNA1 in the absence of WT B2 (Neg. 2) was assayed as negative controls (lanes 1 and 2). 18S rRNA was used as the loading control. (+)RNA1, plus-strand RNA1.

and the dsRNA derived from the 5'-end nucleotides 1 to 400 of RNA1 using an *in vitro* transcription system. These efforts resulted in RNA silencing of WhNV genomic RNA1 in S2 cells, which was found to be blocked by B2 (Fig. 1E). Taken together, our findings indicate that in addition to sequestering RNA duplexes, WhNV B2 suppresses *Drosophila* RNAi via a novel strategy that is mediated by its C-terminal region.

WhNV B2 interacts with Dcr-2 via its C-terminal region in S2 cells. Given that FHV B2 has been shown to bind to the PAZ domain of Dcr-2 *in vitro* (45), we sought to determine whether WhNV B2 could interact directly with Dcr-2 via its C-terminal region in S2 cells. For this purpose, lysates from S2/GFP or S2/GFP+B2 cells transfected with the anti-GFP dsRNA-expressing

plasmid pRISE-ds-GFP were immunoprecipitated with control antiserum or anti-His monoclonal antibody recognizing the His-tagged B2 protein. Subsequently, the B2 immunoprecipitates were immunoblotted with anti-Dcr-2 monoclonal antibody. Dcr-2 coimmunoprecipitated with B2 (Fig. 2A, lane 2); however, no Dcr-2 was detected in the control antiserum-captured complex or immunoprecipitates from control cells (Fig. 2A, lane 3, and D, lane 1). Interestingly, although Dcr-2 also coimmunoprecipitated with B2 in the absence of dsRNA, the B2-Dcr-2 interaction was stronger in the presence of dsRNA (Fig. 2A, compare lanes 2 and 2'). To further validate this observation, when dsRNA was present or absent, the relative ratios of Dcr-2 in immunoprecipitates and lysates were quantified from three independent experiments. The pres-

FIG 2 The C-terminal region of WhNV B2 mediates the B2–Dcr-2 interaction. (A) Coimmunoprecipitation (Co-IP) analyses of lysates from stable S2 cells expressing B2. Cells were transfected (lanes 1 to 3) or not (lanes 1' to 3') with plasmid expressing anti-GFP dsRNA. At 72 h after transfection, cell lysates were immunoprecipitated with control antiserum or anti-His monoclonal antibody recognizing His-tagged B2. B2-immunoprecipitates (I.P.) were subjected to Western blotting using anti-B2 polyclonal antibodies (bottom panel) and anti-Dcr-2 monoclonal antibody (top). The lysates were loaded as input control (lanes 1 and 1'). IB, immunoblot. (B) Quantification of the B2–Dcr-2 interaction enhanced by dsRNA. The relative ratios of Dcr-2 in immunoprecipitates and lysates (input) when dsRNA was present (+) or absent (–) were quantified. Error bars represent standard deviation values from three separate experiments. (C) WhNV B2 does not interact with Dcr-1 in S2 cells. The experimental conditions used were the same as described for panel A. At 72 h after transfection of pRISE-ds-GFP, cell lysates were immunoprecipitated with anti-His monoclonal antibody recognizing His-tagged B2. The proteins in immunoprecipitates were detected with anti-B2 polyclonal antibodies and anti-Dcr-1 monoclonal antibody. (D) Mapping the domain of WhNV B2 responsible for the B2–Dcr-2 interaction. As described for panel A, lysates from stable S2 cells expressing WT B2 (lane 2) or mutants (lanes 3 to 5) in the presence of anti-GFP dsRNA were analyzed via Co-IP and subjected to Western blotting with indicated antibodies. As a negative control, lysates from control cell line S2/GFP were analyzed (lane 1). β -Actin was used as the loading control.

ence of dsRNA was shown to increase the B2–Dcr-2 interaction nearly 3-fold (Fig. 2B). In addition, we found that B2 did not immunoprecipitate with Dicer-1 (Dcr-1) (Fig. 2C), which plays key roles in the *Drosophila* miRNA-mediated RNAi pathway (28). These results reveal a specific interaction of WhNV B2 with Dcr-2 in S2 cells that is stronger in the presence of dsRNA.

To determine the functional domain of B2 responsible for the B2–Dcr-2 interaction, we deleted C-terminal amino acids 1 to 21 of B2, which resulted in complete abolishment of the B2–Dcr-2 interaction (Fig. 2D, lane 4), whereas B2R24A retained its Dcr-2 interaction activity (Fig. 2D, lane 3). Interestingly, the ability of B2 Δ N20 to interact with Dcr-2 was also defective (Fig. 2D, lane 5), suggesting that the homodimerization of B2 is required for its interaction with Dcr-2. We conclude that the C-terminal domain of WhNV B2 mediates its interaction with Dcr-2.

WhNV B2 binds to the RNase III and PAZ domains of Dcr-2.

Dcr-2 is an essential component of the RNAi machinery that functions at both the initiation and effector steps of the siRNA-based RNAi pathway. In the initiation step, Dcr-2 processes dsRNA into siRNAs by the endonuclease activity of its RNase III domain. In the effector step, the PAZ domain of Dcr-2 recognizes the characteristic 3' overhangs of siRNA, thereby mediating the incorporation of siRNA into RISC (47). To define the domain(s) of Dcr-2 responsible for its interaction with B2, we performed glutathione S-transferase (GST) pulldown assays by fusing GST tags with the RNase III and PAZ domains of Dcr-2. As illustrated in Fig. 3A, the PAZ-containing module of Dcr-2 (amino acids 875 to 923) and the functional region of the RNase III domain (amino acids 1448 to 1532) (30) were constructed as N-terminal GST fusion proteins, designated GST-PAZ and GST-RNase, while WT B2 and B2 Δ C21 were fused with maltose-binding protein (MBP) tags. GST-PAZ and GST-RNase but not GST alone were able to pull down MBP-B2 but not MBP (Fig. 3B, lanes 7, 8, 10, and 12). Consistently, we found that B2 Δ C21, which lacks Dcr-2 interaction activity, could not be pulled down by GST-PAZ or GST-RNase (Fig. 3B, lanes 9 and 11). These results indicate that WhNV B2 binds to the RNase III and PAZ domains of Dcr-2.

RNA binding enhances the interaction of WhNV B2 with Dcr-2. In our previous work and the present study, we demonstrated that the homodimerization of WhNV B2 is required for its RNA binding (39) and its interaction with Dcr-2 (Fig. 2C). Moreover, RNA binding to B2 was shown to promote B2 homodimerization (39), and we found that the B2–Dcr-2 interaction was enhanced by dsRNA in S2 cells (Fig. 2A and B). These observations led us to wonder whether RNA binding to B2 could enhance the B2–Dcr-2 interaction by promoting the homodimerization of B2. To this end, we established an *in vitro* system to analyze the interaction between the B2 and Dcr-2 domains (RNase III and PAZ) by far-Western blotting, and then examined whether the protein–protein interactions were enhanced by dsRNA.

After being separated by 12% SDS–PAGE and transferred to PVDF membrane, GST-PAZ, GST-RNase, and GST proteins on the membrane were incubated with MBP-B2 or MBP and then blotted using anti-MBP monoclonal antibodies. Consistent with the results of the GST pulldown assays (Fig. 3B), RNase III and PAZ interacted with MBP-B2 (Fig. 4A, lanes 3 and 4), whereas MBP alone did not induce the protein–protein interaction (Fig. 4A, lanes 3' and 4').

Our previous work revealed that the presence of dsRNA promotes the homodimerization of WT B2 but not B2R24A, because this mutant is defective in RNA-binding activity (39). Consistent with our previous observations in cells (Fig. 2A and B), the presence of dsRNA was shown to increase the B2-PAZ and B2-RNase interactions 3-fold (Fig. 4B, compare top and bottom levels of panel 1, and C). In contrast, because B2R24A is unable to bind to

FIG 3 WhNV B2 binds to the RNase III and PAZ domains of *D. melanogaster* (*Dm*) Dcr-2 via its C-terminal region. (A) Schematic illustration of full-length and truncated forms of Dcr-2. Indicated are the locations and fragment regions of the RNase III (RIII) and PAZ domains that were tagged with GST as bait proteins. WT B2 and mutant B2ΔC21 were tagged with MBP at the N terminus as target proteins. RBF, Ras-responsive binding factor; dsRBD, dsRNA-binding domain. (B) GST pulldown assays showing the interaction of B2 with the RNase III and PAZ domains of Dcr-2. Two micrograms of each MBP-tagged target protein was incubated with the indicated bait protein (2 μg each) using glutathione resins, and the mixture was then subjected to 12% SDS-PAGE. Lanes 1 to 6, input of all bait and target proteins, with their gel mobilities detected by staining with Coomassie brilliant blue R250. Lanes 7 to 12, proteins pulled down by glutathione resins were detected by Coomassie brilliant blue staining (top panel) and Western blotting with indicated antibodies (bottom 2 panels). Lanes 7 and 12, negative controls for ruling out the possibility of any nonspecific interactions of tags (GST and MBP) with bait or target proteins.

dsRNA, the presence of dsRNA did not enhance the interaction of B2R24A with the RNase III domain or PAZ domain (Fig. 4B, compare top and bottom levels of panel 2, and C). Additionally, neither B2ΔC21 nor B2ΔN20 interacted with the RNase III domain or PAZ domain (Fig. 4B, panels 3 and 4). Altogether, our findings show that although the B2–Dcr-2 interaction is RNA independent, RNA binding to B2 enhances the B2–Dcr-2 interaction.

WhNV B2 inhibits Dcr-2-mediated cleavage by sequestering dsRNA and blocking the endonuclease activity. Previously, we reported that WhNV B2 forms a complex with dsRNA, thereby preventing Dicer-mediated cleavage (39). In this study, WhNV B2 was shown to bind to the RNase domain of Dcr-2, which prompted us to determine whether B2 could block the endonuclease activity of Dcr-2 in addition to sequestering dsRNA. Bacterial RNase III is a Dicer homolog and was usually used as the Dicer substitute for *in vitro* experiments (15, 20, 39, 53). To determine whether B2 could directly target Dcr-2 *in vitro*, we performed RNase III-mediated cleavage protection assays (Fig. 5A). This experiment was divided into two groups. For group 1, 0.1 μM T7-

transcribed 500-bp dsRNA substrate, which mimics the *in vivo* plasmid carrying anti-GFP dsRNA, was incubated with 30 μM MBP-tagged WT B2 or mutants at 25°C for 30 min and was then digested by 1 U RNase III. For group 2, RNase III was preincubated with WT B2 or mutants at 37°C for 30 min before dsRNA was added to the reaction mixture.

The production of RNase III-processed siRNAs was significantly different between group 1 and group 2. In group 1, WT B2 was shown to protect dsRNA from being cleaved by RNase III into siRNAs (Fig. 5A, lane 4). Because B2ΔC21 retains its RNA-binding activity, the deletion of C-terminal amino acids 1 to 21 did not result in a loss of the B2–RNA complex (Fig. 5A, lane 5). Interestingly, although B2R24A did not bind to dsRNA (no B2–RNA complex was detected), it still inhibited the endonuclease activity of RNase III, as a portion of dsRNA was protected from digestion (Fig. 5A, lane 6). In group 2, however, B2R24A and B2ΔC21 behaved differently than they did in group 1. When RNase III was preincubated with B2R24A, free dsRNA was detected but no siRNAs were (Fig. 5A, lane 9), indicating that the endonuclease activity of RNase III was completely blocked. In contrast, a por-

FIG 4 RNA binding enhances the interaction of WhNV B2 with Dcr-2. (A) Far-Western blotting analyses showing the interaction of B2 with the RNase III and PAZ domains of Dcr-2. After being separated by 12% SDS-PAGE and transferred to PVDF membrane, 0.5 μ g renatured GST-RNase or GST-PAZ (lanes 3, 3', 4, and 4') was overlaid with 50 μ g MBP-B2 or MBP as indicated and immunoblotted with anti-MBP monoclonal antibody. MBP was loaded as a positive control for validating the effectiveness of MBP antibody used for blotting (lanes 1 and 1'), and a GST tag was loaded as a negative control for ruling out the possibility of nonspecific interaction of B2 with GST (lanes 2 and 2'). (B) Far-Western blotting analyses showing the interaction of WT B2 and mutants with the RNase III and PAZ domains in the absence [dsRNA (–)] or presence [dsRNA (+)] of 50 μ g 500-bp dsRNA. The overlaid proteins, MBP-tagged WT B2 and mutants, are indicated above the panel. Proteins were stained with Coomassie brilliant blue as loading controls. (C) Quantification of protein-protein interactions enhanced by dsRNA. The interactions of WT B2 or B2R24A with PAZ (black) and RNase (gray) when dsRNA was absent or present were quantified, and the enhancement (fold change relative to the results when dsRNA was not present) of protein-protein interactions by dsRNA was determined. Error bars represent standard deviation values from three separate experiments.

tion of dsRNA was associated with B2ΔC21, whereas other portions were digested into siRNAs (Fig. 5A, lane 8). These findings suggest that although B2ΔC21 did not block the endonuclease activity of RNase III, it can compete with RNase III for precursor dsRNA, thus resulting in partial protection. Neither the MBP tag nor B2ΔN20 inhibited RNase III-mediated cleavage (Fig. 5A, lanes 1 and 10).

To confirm whether WhNV B2 can block the endonuclease activity of Dcr-2 in cells, we harvested RNA from the stable S2 cells transfected with pRISE-ds-GFP and subjected it to Northern blotting analyses to determine the ratio of pre-Dicer dsRNA to post-Dicer siRNAs. When WT B2 was absent, 500-bp dsRNA was completely digested into ~20-bp siRNAs by Dcr-2 (Fig. 5B, lane 1), whereas a dramatic increase in the dsRNA/siRNA ratio was observed in the presence of WT B2 (Fig. 5B, lane 2). If the accumulation of dsRNA was due only to the

RNA-binding activity of B2, dsRNA should have been totally cleaved into siRNAs in the context of B2R24A, because this mutant is defective in binding to dsRNA. However, although B2R24A did not precipitate any dsRNA or siRNA in cells (Fig. 5B, lane 3'), we observed an obvious accumulation of dsRNA (Fig. 5B, lane 3), whereas deleting the Dcr-2 interaction domain of B2 (C-terminal amino acids 1 to 21), which did not affect its ability to precipitate RNA in cells (Fig. 5B, lane 4'), still resulted in a decrease in the dsRNA/siRNA ratio (Fig. 5B, lane 4). Taken together with the RNAi suppression results shown in Fig. 1D, our findings reveal that WhNV B2 inhibits Dcr-2-mediated dsRNA cleavage by sequestering dsRNA and directly blocking the endonuclease activity of Dcr-2, thereby suppressing the RNAi pathway in S2 cells.

WhNV B2 suppresses RISC-mediated mRNA cleavage by blocking the incorporation of siRNA into RISC. Having shown

FIG 5 WhNV B2 inhibits the production of siRNA by sequestering dsRNA and blocking the endonuclease activity of Dcr-2. (A) RNase III-mediated cleavage assays were divided into two groups using T7-transcribed 500-bp dsRNA derived from GFP as precursor. For group 1, dsRNA was incubated with WT B2 and mutants at 25°C for 30 min before being cleaved by RNase III (lanes 4 to 6). For group 2, RNase III was preincubated with WT B2 and mutants at 37°C for 30 min before being supplemented with dsRNA (lanes 7 to 9). After the cleavage was conducted at 37°C for 30 min, the reaction products were separated on 1.2% TBE-agarose gels, and RNAs were visualized by ethidium bromide staining. The locations of input dsRNA, RNase III-processed siRNA, and B2-RNA complex are indicated by arrows. (B) Suppression of the endonuclease activity of Dcr-2 by WT B2 and mutants in S2 cells. Left, RNA harvested from stable S2 cells transfected with anti-GFP dsRNA was subjected to Northern blotting to determine the ratio of pre-Dicer dsRNA to post-Dicer siRNAs in the absence (lane 1) or presence (lane 2) of WT B2 or mutant as indicated above each lane (lanes 3 to 5). Right, RNAs precipitated with WT B2 or mutants were detected via Northern blotting. Cells were lysed and immunoprecipitated with control antiserum (lane 6') or with anti-His monoclonal antibodies recognizing the His-tagged WT B2 (lane 2') and B2 mutants (lanes 3' to 5'). RNA extracted from the immunoprecipitates was subjected to Northern blotting with probes recognizing both dsRNA and siRNA, as indicated by cartoons and arrows.

that WhNV B2 binds to the PAZ domain of Dcr-2, we next sought to ask if B2 could hinder Dcr-2 from incorporating siRNA into RISC. Ordered pathways for the stepwise *in vitro* assembly of RISC have been previously established using S2 cell extracts to examine the capability of VSRs to inhibit the incorporation of siRNA into RISC (4, 19, 24). To determine whether WhNV B2 could block siRNA loading onto RISC by interacting with Dcr-2, we incubated serial dilutions of MBP-tagged WT B2 or mutated proteins with S2 cell extracts and then added digoxigenin (DIG)-labeled siRNA to the reaction mixtures. Subsequently, the formation of RISC was quantified to determine the impacts of B2 on the incorporation of siRNA into RISC.

As previously reported (37, 38), three silencing complexes (R2D2-Dcr-2 initiator [RDI], RISC-loading complex [RLC], and RISC) were assembled in an ordered pathway via incubation of S2 cell extracts with siRNA, and we observed RLC and RISC complexes resolved on native agarose gels (Fig. 6A, lane 2). As the concentration of B2 increased, the formation of RLC and RISC was compromised, followed by the accumulation of a new complex, B2-siRNA (Fig. 6A, lanes 3 to 6). When the concentration of B2 reached 80 nM, the majority of free siRNA was shifted into the

B2-siRNA complex, whereas additional B2 protein (120 nM) did not decrease the ratio of the remaining RISC (Fig. 6A, lanes 5 and 6). Notably, although the same concentration of B2R24A did not shift any siRNA, the formation of RISC was still impaired (Fig. 6A, lane 7), whereas B2ΔC21 competed with Dcr-2 for siRNA, thereby leading to incomplete inhibition of RISC assembly (Fig. 6A, lane 8). In contrast, neither B2ΔN20 nor MBP had any effect on RISC assembly (Fig. 6A, lanes 9 and 10). Moreover, by separating the silencing complexes on 4% native polyacrylamide gels, we found that WhNV B2 also affected the formation of the RDI complex that is composed of Dcr-2, R2D2, and siRNA (Fig. 6A, middle). These results suggest that WhNV B2 inhibits the RISC assembly by suppressing siRNA binding to Dcr-2.

Next, we examined whether the impacts of B2 on RISC assembly would affect the capability of RISC to cleave target mRNA. Before chemically synthesized anti-GFP siRNA was supplemented, S2 cell extracts were preincubated with 120 nM WT or mutated B2 proteins. Then, the relative activities of mRNA cleavage (ratio of cleavage product/mRNA) were quantified. We found that siRNA-loaded RISC cleaved most of the mRNA, with its cleavage products at the expected position (Fig. 6B, lane 2). Reflective of their abilities to undermine RISC assembly (Fig. 6A), WT B2, B2R24A, and B2ΔC21 dramatically inhibited the relative mRNA cleavage activity (Fig. 6B, lanes 3 to 5). Furthermore, our findings were confirmed by examining the activities of WT B2 and mutants in rescuing GFP mRNA from siRNA-induced RNA silencing in S2 cells (Fig. 6C), thereby demonstrating that WhNV B2 inhibits RISC-mediated mRNA cleavage by blocking the incorporation of siRNA into RISC.

When siRNA duplexes are incorporated into RISC, the passenger strands of siRNA are preferentially excluded from RISC and rapidly degraded, thereby liberating the guide strands (22, 44). Consistently, at 72 h posttransfection with chemically synthesized anti-GFP siRNA, no passenger strand siRNA (siRNA*) was detected in the control cells (Fig. 6D, lane 1). In contrast, the presence of WT B2 caused an aberrant accumulation of siRNA* (Fig. 6D, lane 2). Additional immunoprecipitation assays showed that WT B2 sequestered siRNA* in cells (Fig. 6D, lane 2'). Compared to the results with WT B2, we detected relatively low levels of siRNA* in the presence of B2R24A or B2ΔC21 (Fig. 6D, lanes 3 and 4). Since B2R24A and B2ΔC21 retain the capability of B2 to either block the PAZ domain or sequester siRNA (Fig. 6D, lanes 3' and 4'), their activities in suppressing the incorporation of siRNA into RISC were incomplete. Also, we found that deleting the homodimerization domain (N-terminal amino acids 1 to 20) of B2 completely abolished its activity in binding to siRNA. (Fig. 6D, lanes 5 and 5'). In summary, we conclude that WhNV B2 blocks the incorporation of siRNA into RISC by sequestering siRNA and interacting with Dcr-2, thereby inhibiting RISC-mediated mRNA cleavage.

DISCUSSION

During the course of viral infection, the host Dicers function as pattern recognition receptors that recognize vRNA-dsRNA as a pathogen-associated molecular pattern, thereby leading to the initiation of RNAi-mediated antiviral immunity (12, 21). Although the necessity of Dicers in the RNAi pathway is well recognized, little is known about how VSRs antagonize host RNAi immunity by targeting Dicers. Here, we demonstrate that in addition to sequestering dsRNA and siRNA, targeting of Dcr-2 by WhNV B2 is

FIG 6 WhNV B2 inhibits RISC-mediated mRNA cleavage by blocking the incorporation of siRNA into RISC. (A) Suppression of siRNA loading onto RISC by B2 *in vitro*. Before the incorporation of DIG-labeled siRNA, S2 cell extracts were incubated with MBP (lane 10), MBP-tagged WT B2 in multiple concentrations (lanes 3 to 6), or mutants (lanes 7 to 9) at 37°C for 30 min, and the mixtures were then applied to 1.5% native agarose gels (left) or 4% native polyacrylamide gels (middle) to determine the formation of silencing complexes by Northern blotting analysis. Locations of RISC, RLC, RDI, B2-siRNA complex, and unbound siRNA on the gels are indicated. The formation of RISC and RLC was plotted (right); as the concentration of WT B2 (black diamond) increased (from 10 to 120 nM), the formation of RISC and RLC was compromised. (B) Suppression of RISC-mediated mRNA cleavage by B2 *in vitro*. Before the incorporation of chemically synthesized anti-GFP siRNA, S2 cell extracts were incubated with 120 nM MBP, MBP-tagged WT B2, or MBP-tagged B2 mutants as indicated above each lane (lanes 3 to 7) at 37°C for 30 min. Markers at left are in nucleotides. Subsequently, reconstituted RISC was incubated with 500-nt transcripts of GFP mRNA that had been DIG labeled *in vitro* to determine the cleavage activity of RISC. RNA recovered from the reaction mixture was subjected to denaturing agarose gels to detect the DIG signals. Locations of mRNA and the cleavage products in the gels are indicated, and the relative silencing activities were quantified and are shown in a bar diagram (bottom). (C) Suppression of siRNA-induced RNA silencing by B2 in S2 cells. At 72 h after transfection of 50 nM chemically synthesized anti-GFP siRNA, RNA harvested from stable S2 cells expressing WT B2 and mutants as indicated above each lane was subjected to Northern blotting to determine the effects of WT B2 and mutants on the reduction in GFP transcripts. 18S rRNA and β -actin were used as the loading controls. (D) Passenger strand siRNA (siRNA*) immunoprecipitated with WT B2 or mutants (lanes 2' to 5') was detected by Northern blotting as described for Fig. 3B. As input controls, siRNA*s harvested from cell lysates were loaded (lanes 2 to 5). RNA extracted from cell lysates in the absence of B2 (Neg. 1) or lysates immunoprecipitated with control antiserum (Neg. 2) were loaded as negative controls (lanes 1, 1', and 6').

a novel mechanism of suppression of *Drosophila* RNAi. By binding to RNA duplexes and interacting with Dcr-2, WhNV B2 efficiently blocks the activities of Dcr-2 in processing dsRNA and incorporating siRNA into RISC. These results provide evidence that WhNV B2 is a multifunctional VSR that targets both RNA duplexes and Dcr-2 in the RNAi machinery.

The RNase III enzyme Dicers of *Drosophila*, Dcr-1 and Dcr-2, play distinct roles in miRNA- and siRNA-silencing pathways, respectively (28). Genetic studies on *Drosophila* RNAi immunity have shown that Dcr-2 but not Dcr-1 is required for the biogenesis of siRNAs from several RNA viruses during the course of infection (1, 8, 16, 50). In this study, we report that WhNV B2 interacts specifically with Dcr-2 but not with Dcr-1 in S2 cells. These observations support the idea that the primary role of nodaviral B2

proteins is to antagonize siRNA-directed RNAi immunity rather than to block the miRNA-directed RNAi pathway (1, 46).

In our previous and present studies, we have shown that RNA binding (39), self-interaction (homodimerization) (39), and Dicer interaction are mediated by different domains of WhNV B2 (Fig. 1A). Although the interaction of WhNV B2 with Dcr-2 is independent of RNA, we found that RNA binding enhanced the B2–Dcr-2 interaction that requires the homodimerization of B2. Interestingly, our previous work found that the homodimerization of B2 is also required for binding to RNA, and RNA binding in turn promotes B2 homodimerization (39). Taken together, these results suggest that B2 functions as a homodimer, and RNA binding to B2 promotes the homodimerization of B2, thereby enhancing the B2–Dcr-2 interaction. Thus, our findings reveal an

FIG 7 A schematic illustration of the interrelationship among diverse activities of WhNV B2. As indicated by the straight arrows, the homodimerization of B2 is required for both the RNA-binding and Dcr-2 interaction activities of B2. The curved arrows represent the promotion of B2 homodimerization and the B2-Dcr-2 interaction by RNA binding.

interrelationship and synergistic effects among diverse activities of WhNV B2 (Fig. 7).

Here, we propose a model of the suppression of *Drosophila* RNAi immunity by WhNV B2 in which it targets both RNA duplexes and protein components (Fig. 8). In WhNV-infected cells, a portion of B2 homodimers spontaneously associate with Dcr-2 on the surface of the RNase III and PAZ domains through its C-terminal domain, thus forming an initial complex that is in close proximity to the site where WhNV replication occurs on the outer mitochondrial membrane (Fig. 8A). When the replication of plus-strand RNA is initiated, nascent vRI-dsRNA binding to B2 promotes B2 homodimerization, thereby leading to rearrangement and conformational changes in the initial B2-Dcr-2 complex, which then recruits additional dissociative Dcr-2 and B2 homodimers. This RNA-protein complex confers more effective inhibition of the production of viRNAs at the initiation step (Fig. 8B) and the incorporation of siRNAs into RISC at the effector step (Fig. 8C), because WhNV B2 not only sequesters vRI-dsRNA and viRNAs but also blocks the activities of Dcr-2 both in processing vRI-dsRNA and incorporating viRNAs into RISC. Our future studies will determine how WhNV B2 suppresses RNAi immunity during the course of viral infection.

Notably, the innate immunity of vertebrates, unlike that of invertebrates, is a network of antiviral defenses in addition to RNAi, such as interferon and dsRNA-inducible protein kinase R pathways, which are also induced by viral dsRNA (9, 26, 42). Therefore, to ensure the survival of nodaviruses in a wide range of hosts from insects to mammals (10, 43, 46), it is plausible that nodaviral B2 proteins evolve to respond to various host innate immunity strategies by simultaneously sequestering dsRNA and interacting with Dicer. If so, targeting of Dicer and RNA might be a general RNAi suppression strategy that is employed by other nodaviruses, such as FHV, to adapt to diverse host environments.

Because of the similarity and differences between the B2 proteins from WhNV and FHV, WhNV is also a valuable model which could be supplementary in studies using FHV for elucidating the mechanisms of viral pathogenesis and RNAi immunity. Given that the domains of WhNV B2 that mediate different activities have been mapped, functional complementation with distinct WhNV B2 mutants would be an ideal strategy to identify a potential VSR and study its possible mechanism(s).

In summary, we establish a model of the suppression of *Drosophila* RNAi by WhNV B2 targeting Dcr-2 and RNA and, furthermore, reveal the interrelationship and synergistic effects among

FIG 8 Model for the suppression of *Drosophila* RNAi immunity by WhNV B2. (A) In WhNV-infected cells, a portion of B2 homodimers (blue oval) spontaneously associate with Dcr-2 (cyan square) via its C-terminal region, thereby forming an initial complex that is in close proximity to the site where WhNV replication occurs on the outer mitochondrial membrane. (+) RNA, plus-strand RNA; (–) RNA, minus-strand RNA. (B and C) When the replication of plus-strand RNA is initiated, nascent vRI-dsRNA binding to B2 promotes the homodimerization of B2, thereby leading to rearrangement and conformational changes in the initial B2-Dcr-2 complexes, which then recruit additional dissociative Dcr-2 and B2 homodimers. The RNA-protein complex confers effective inhibition of the production of viRNAs (B) and the incorporation of siRNAs into RISC at the effector step (C), thereby leading to the suppression of RISC-mediated viral RNA cleavage. TSN, VIG, and dFMR1 are the auxiliary factors of RISC; AGO2 is the core RISC protein.

diverse activities of WhNV B2. These findings deepen our understanding about how VSRs interact with Dicer and lead us to speculate that the synergistic effects might be a general strategy for multifunctional VSRs to antagonize RNAi and promote viral replication.

ACKNOWLEDGMENTS

We are grateful to Yi Zhang, Yuji Kageyama, and Yasushi Hiromi for generously providing experimental materials. We also thank Robert E. DeJournett and Jiali Si for carefully reading the manuscript and Markeda Wade for professionally editing the manuscript.

This work was supported by the Chinese 111 project (grant B06018),

the National Natural Science Foundation of China (grant 30870112), and the Natural Science Foundation of Hubei Province (grant 2011CDB444).

REFERENCES

- Aliyari R, et al. 2008. Mechanism of induction and suppression of antiviral immunity directed by virus-derived small RNAs in *Drosophila*. *Cell Host. Microbe* 4:387–397.
- Azevedo J, et al. 2010. Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. *Genes Dev.* 24:904–915.
- Baulcombe D. 2004. RNA silencing in plants. *Nature* 431:356–363.
- Bernstein E, Caudy AA, Hammond SM, Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363–366.
- Cai D, et al. 2010. Characterization of Wuhan nodavirus subgenomic RNA3 and the RNAi inhibition property of its encoded protein B2. *Virus Res.* 151:153–161.
- Chao JA, et al. 2005. Dual modes of RNA-silencing suppression by Flock House virus protein B2. *Nat. Struct. Mol. Biol.* 12:952–957.
- Chen W, et al. 2008. HCV core protein interacts with Dicer to antagonize RNA silencing. *Virus Res.* 133:250–258.
- Chotkowski HL, et al. 2008. West Nile virus infection of *Drosophila melanogaster* induces a protective RNAi response. *Virology* 377:197–206.
- Cullen BR. 2006. Is RNA interference involved in intrinsic antiviral immunity in mammals? *Nat. Immunol.* 7:563–567.
- Dasgupta R, Selling B, Rueckert R. 1994. Flock House virus: a simple model for studying persistent infection in cultured *Drosophila* cells. *Arch. Virol. Suppl.* 9:121–132.
- Deleris A, et al. 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* 313:68–71.
- Ding SW. 2010. RNA-based antiviral immunity. *Nat. Rev. Immunol.* 10:632–644.
- Ding SW, Voinnet O. 2007. Antiviral immunity directed by small RNAs. *Cell* 130:413–426.
- Donze O, Picard D. 2002. RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. *Nucleic Acids Res.* 30:e46.
- Fenner BJ, Goh W, Kwang J. 2007. Dissection of double-stranded RNA binding protein B2 from betanodavirus. *J. Virol.* 81:5449–5459.
- Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler JL. 2006. Essential function in vivo for Dicer-2 in host defense against RNA viruses in *Drosophila*. *Nat. Immunol.* 7:590–597.
- Giner A, Lakatos L, Garcia-Chapa M, Lopez-Moya JJ, Burgyan J. 2010. Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. *PLoS. Pathog.* 6:e1000996.
- Gottwein E, Cullen BR. 2008. Viral and cellular microRNAs as determinants of viral pathogenesis and immunity. *Cell Host. Microbe* 3:375–387.
- Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293:1146–1150.
- Ji X. 2008. The mechanism of RNase III action: how dicer dices. *Curr. Top. Microbiol. Immunol.* 320:99–116.
- Kemp C, Imler JL. 2009. Antiviral immunity in *Drosophila*. *Curr. Opin. Immunol.* 21:3–9.
- Khvorova A, Reynolds A, Jayasena SD. 2003. Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115:209–216.
- Kondo T, Inagaki S, Yasuda K, Kageyama Y. 2006. Rapid construction of *Drosophila* RNAi transgenes using pRISE, a P-element-mediated transformation vector exploiting an in vitro recombination system. *Genes Genet. Syst.* 81:129–134.
- Lakatos L, et al. 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO J.* 25:2768–2780.
- Lakatos L, Szittyi G, Silhavy D, Burgyan J. 2004. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO J.* 23:876–884.
- Langland JO, Cameron JM, Heck MC, Jancovich JK, Jacobs BL. 2006. Inhibition of PKR by RNA and DNA viruses. *Virus Res.* 119:100–110.
- Lecellier CH, et al. 2005. A cellular microRNA mediates antiviral defense in human cells. *Science* 308:557–560.
- Lee YS, et al. 2004. Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117:69–81.
- Li H, Li WX, Ding SW. 2002. Induction and suppression of RNA silencing by an animal virus. *Science* 296:1319–1321.
- Lim do, H, Kim J, Kim S, Carthew RW, Lee YS. 2008. Functional analysis of dicer-2 missense mutations in the siRNA pathway of *Drosophila*. *Biochem. Biophys. Res. Commun.* 371:525–530.
- Liu C, et al. 2006. Sequence analysis of coat protein gene of Wuhan nodavirus isolated from insect. *Virus Res.* 121:17–22.
- Liu C, et al. 2006. Isolation and RNA1 nucleotide sequence determination of a new insect nodavirus from *Pieris rapae* larvae in Wuhan City, China. *Virus Res.* 120:28–35.
- Meraï Z, et al. 2006. Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *J. Virol.* 80:5747–5756.
- Mueller S, et al. 2010. RNAi-mediated immunity provides strong protection against the negative-strand RNA vesicular stomatitis virus in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 107:19390–19395.
- Muller S, Imler JL. 2007. Dicing with viruses: microRNAs as antiviral factors. *Immunity* 27:1–3.
- Nayak A, et al. 2010. Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in *Drosophila*. *Nat. Struct. Mol. Biol.* 17:547–554.
- Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ. 2004. A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. *Cell* 117:83–94.
- Pham JW, Sontheimer EJ. 2005. Molecular requirements for RNA-induced silencing complex assembly in the *Drosophila* RNA interference pathway. *J. Biol. Chem.* 280:39278–39283.
- Qi N, et al. 2011. RNA binding by a novel helical fold of B2 protein from Wuhan nodavirus mediates RNAi suppression and promotes B2 dimerization. *J. Virol.* 85:9543–9554.
- Qiu Y, et al. 2011. Internal initiation is responsible for synthesis of Wuhan nodavirus subgenomic RNA. *J. Virol.* 85:4440–4451.
- Qu F, Ren T, Morris TJ. 2003. The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. *J. Virol.* 77:511–522.
- Samuel CE. 2001. Antiviral actions of interferons. *Clin. Microbiol. Rev.* 14:778–809.
- Scherer WF, Hurlbut HS. 1967. Nodamura virus from Japan: a new and unusual arbovirus resistant to diethyl ether and chloroform. *Am. J. Epidemiol.* 86:271–285.
- Schwarz DS, et al. 2003. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115:199–208.
- Singh G, et al. 2009. Suppression of RNA silencing by Flock House virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. *FASEB J.* 23:1845–1857.
- Sullivan CS, Ganem D. 2005. A virus-encoded inhibitor that blocks RNA interference in mammalian cells. *J. Virol.* 79:7371–7379.
- Tomari Y, Zamore PD. 2005. Perspective: machines for RNAi. *Genes Dev.* 19:517–529.
- Tuschl T, Zamore PD, Lehmann R, Bartel DP, Sharp PA. 1999. Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev.* 13:3191–3197.
- Venter P, Jovel J, Schneemann A. 2010. Insect nodaviruses, p 251–282. In: Asgari S, Johnson K (ed), *Insect virology*. Caister Academic Press, Norfolk, United Kingdom.
- Wang XH, et al. 2006. RNA interference directs innate immunity against viruses in adult *Drosophila*. *Science* 312:452–454.
- Wu Q, Wang X, Ding SW. 2010. Viral suppressors of RNA-based viral immunity: host targets. *Cell Host. Microbe* 8:12–15.
- Ye K, Malinina L, Patel DJ. 2003. Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* 426:874–878.
- Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. 2004. Single processing center models for human Dicer and bacterial RNase III. *Cell* 118:57–68.
- Zhang X, et al. 2006. Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. *Genes Dev.* 20:3255–3268.