Published in final edited form as:

Science. 2006 April 21; 312(5772): 452–454.

RNA Interference Directs Innate Immunity Against Viruses in Adult *Drosophila*

Xiao-Hong Wang^{1,*}, Roghiyh Aliyari^{2,*}, Wan-Xiang Li², Hong-Wei Li², Kevin Kim⁴, Richard Carthew⁴, Peter Atkinson³, and Shou-Wei Ding^{1,2,†}

¹Graduate Program for Microbiology, University of California, Riverside, California CA 92521, USA.

²Department of Plant Pathology and Center for Plant Cell Biology, Institute for Integrative Genome Biology, University of California, Riverside, California CA 92521, USA.

³Department of Entomology, University of California, Riverside, California CA 92521, USA.

⁴Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois IL 60208, USA.

Abstract

Innate immunity against bacterial and fungal pathogens is mediated by Toll and immune deficiency (Imd) pathways, but little is known about the antiviral response in *Drosophila*. Here, we demonstrate that an RNA interference pathway protects adult flies from infection by two evolutionarily diverse viruses. Our work also describes a molecular framework for the viral immunity, in which viral double-stranded RNA produced during infection acts as the pathogen trigger whereas *Drosophila* Dicer-2 and Argonaute-2 act as host sensor and effector, respectively. These findings establish a *Drosophila* model for studying the innate immunity against viruses in animals.

RNA interference (RNAi) silences gene expression through small interfering RNAs (siRNAs) and microRNAs (miRNAs). In *Drosophila melanogaster* (1) Dicer-2 (Dcr-2) produces siRNAs whereas Dicer-1 (Dcr-1) recognizes precursors of miRNAs. The small RNAs are assembled with an Argonaute (Ago) protein into related effector complexes, such as RNA-induced silencing complex (RISC), to guide specific RNA silencing (1).

RNA silencing provides an antiviral mechanism in plants and animals (2-6). Plant viruses have evolved diverse strategies for evading the RNA silencing immunity and expression of viral suppressors of RNAi (VSRs) is essential for infection and virulence (6). However, it is unknown if antiviral silencing in plants is controlled by a specific small RNA pathway targeted by plant VSRs. Bacterial and fungal infections of *D. melanogaster* induce the Toll and immune deficiency (Imd) pathways, leading to transcriptional induction of antimicrobial peptide effectors via NF- κ B-like signaling processes (7). However, it has been unclear if either pathway plays a role in *Drosophila* innate immunity against viruses (8,9). Our previous work in cell culture has indicated that RNAi might mediate the viral immunity in *D. melanogaster* (3). Here we investigated if RNAi indeed provides protection against virus infection in *Drosophila* embryos and adults.

FHV contains an RNA genome (10) divided among two plus-strand molecules, RNAs 1 and 2. RNA2 (R2) encodes the single virion structural protein whereas RNA1 (R1) encodes protein A, the viral RNA-dependent RNA polymerase (RdRP), and B2, a VSR (3,4,11) expressed after RNA1 replication from its own mRNA, RNA3 (Fig. s1). In the absence of R2, R1 replicated

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: dingsw@ucr.edu

autonomously, accumulated to high levels, and produced abundant RNA3 in wild type (wt) *D. melanogaster* embryos 30 hours after injection with R1 transcripts synthesized *in vitro* (Fig. 1, lane 2). No FHV RNAs accumulated in wt embryos injected with R1fs transcripts that contain a frame-shift mutation in the RdRP ORF (Fig. 1, lane 1). FHV RNAs were also not readily detected in wt embryos injected with the second mutant of R1, R1ΔB2, which does not express the VSR (Fig. 1, lane 3). However, abundant accumulation of R1ΔB2 (Fig. 1, lane 9), but not of R1fs (Fig. 1, lane 7), occurred in the mutant *Drosophila* embryos that carried a homozygous null mutation in *ago-2* (*ago-2⁴¹⁴*), which is essential for RNAi in *Drosophila* (1,12,13). These data indicated that viral RNA replication in *Drosophila* embryos triggers an RNAi-mediated virus clearance in an *Ago-2*-dependent manner and effective RNAi suppression by B2 is necessary to achieve normal accumulation of FHV RNAs.

In *Drosophila* *Ago-2* acts downstream of *Dicer-2* (*Dcr-2*) to recruit siRNAs, the products of *Dcr-2* activity, into the siRNA-dependent RISC (siRISC) (1,14). Thus, a genetic requirement for *ago-2* in FHV RNA clearance implicates *Dcr-2* in the RNAi antiviral effector mechanism. To test this hypothesis, we injected R1, R1fs, and R1ΔB2 transcripts into embryos carrying a homozygous *dcr-2* null mutation, *dcr-2^{L811fsX}*. Northern blot hybridizations showed that although FHV RNAs remained undetectable in *dcr-2* embryos injected with R1-fs (Fig. 1, lane 4), viral RNA accumulation was rescued in the *dcr-2* embryos injected with R1ΔB2 transcripts (Fig. 1, lane 6). This result shows that *Dcr-2* is required to initiate RNAi-mediated clearance of FHV RNAs in *Drosophila* embryos.

To investigate if the RNAi pathway protects *Drosophila* from virus infection, we injected adult flies of either wt or *dcr-2* genotype with purified FHV virions. The FHV isolate was of low virulence in wt flies since approximately 50% of infected flies survived 15 days post inoculation (dpi; Fig. 2A) despite a detectable virus load (Fig. 2B, lanes 1-6). Inoculation with the same dose of FHV resulted in 60% mortality by 6 dpi and 95% by 15 dpi in *dcr-2* flies (Fig. 2A). Mock inoculation with buffer had little effect on either wt or *dcr-2* flies for as long as the observation was made. Both Northern and Western blot analyses revealed that the virus accumulated more rapidly and to much greater levels in *dcr-2* than wt flies (Fig. 2B, C). Thus, *dcr-2* mutant exhibit enhanced disease susceptibility to FHV in comparison with wt flies, demonstrating that *Dcr-2* is also required to mount an immune response that protects adult *Drosophila* against FHV infection.

R2D2 contains tandem dsRNA-binding domains and forms a heterodimer with *Dcr-2* *in vivo* that is required for siRNA loading into RISC (1,15). We found that flies homozygous for a loss-of-function mutation in *r2d2* exhibited a similar enhanced disease susceptibility phenotype as *dcr-2* to FHV infection (Fig. 2). Thus, R2D2 also participates in the innate immunity pathway that protects adult flies from FHV infection. Notably, although FHV accumulated to extremely high levels in both *dcr-2* and *r2d2* mutant flies, abundant viral siRNAs were detected only in *r2d2* flies and viral siRNAs were below the level of detection in *dcr-2* flies (Fig. 2D). Thus, FHV infection is detected by *Dcr-2*, leading to production of FHV siRNAs. However, R2D2 is not required for the production but is essential for the function, of viral siRNAs, which is consistent with the genetic requirements for processing the artificially introduced dsRNA (1, 15).

To investigate if the RNAi pathway in *Drosophila* is specific against nodaviruses and not other classes of RNA viruses, we assessed the susceptibility of wt, *dcr-2* and *r2d2* flies to *Cricket paralysis virus* (CrPV). CrPV contains a non-segmented plus-strand RNA genome, but belongs to a group of picorna-like viruses (16). CrPV is significantly more virulent than FHV in *Drosophila* since injection of CrPV at much lower titers resulted in mortality of 70% of wt flies by 15 dpi (Fig. 3A). However, we found that CrPV induced enhanced disease susceptibility in both *dcr-2* and *r2d2* mutant flies (Fig. 3A). Approximately 60% of the infected mutant flies

were dead by 6 dpi, and more than 95% were dead by 15 dpi (Fig. 3A). In addition, Northern blots indicated that the virus accumulated more rapidly and to greater levels in the mutant flies (Fig. 3B). Thus, both *dcr-2* and *r2d2* are required for protection of *Drosophila* against CrPV.

CrPV infection of cultured S2 cells induced antiviral silencing, illustrated by detection of CrPV-specific siRNAs (Fig. 4A). Antiviral silencing against FHV in S2 cells induced by FR1gfp as described previously (11), was suppressed by CrPV superinfection, leading to de-repression of GFP (Fig. 4B, left). Two ORFs are encoded by the CrPV RNA genome (16) (Fig. s2). We did not observe suppression of antiviral silencing in S2 cells co-transfected with a plasmid expressing either the entire downstream ORF of CrPV or the individual mature virion proteins processed from the polyprotein (Fig. 4C, lanes 4-10). In contrast, RNAi suppression was detected after co-transfection with a plasmid expressing either the entire upstream ORF of CrPV or its N-terminal 140 codons (Fig. 4C, pA in lane 1). However, the suppressor activity was not detected after a frameshift mutation was introduced into pA (Fig. 4C, lane 2), thus identifying the N-terminal fragment of 140 amino acids of the CrPV non-structural polyprotein as a VSR.

In *D. melanogaster* Imd signaling is stimulated by Gram-negative (Gram⁻) bacterial infection whereas Toll signaling is triggered by Gram-positive (Gram⁺) bacterial infection (7,17). To determine if loss of the RNAi pathway initiated by Dcr-2 had an impact on the Toll and Imd signaling processes, wt, *dcr-2* and *r2d2* flies were subjected to immune challenge by inoculation with *Escherichia coli* (Gram⁻) or *Micrococcus luteus* (Gram⁺). Northern blot hybridizations detected significant transcriptional induction of the antimicrobial peptide gene *Diptericin A* six hours post immune challenge (hpi) with either *E. coli* or *M. luteus*, which declined at 24 hpi (online supplementary Fig. s3) as described (17). Similar induction patterns for *Diptericin A* were observed in *dcr-2* and *r2d2* flies inoculated with the Gram⁺ and Gram⁻ bacteria (Fig. s3). Furthermore, we found that induction of either *Attacin A* or *Drosomycin* by the Gram⁺ and Gram⁻ bacteria was also not altered in *dcr-2* and *r2d2* flies as compared to wt flies (Fig. s3). These results indicate that induction of antimicrobial peptide genes via Toll and Imd signaling pathways is not compromised in *dcr-2* and *r2d2* flies.

Nodaviruses and the polio-like CrPV belong to two different superfamilies of animal RNA viruses. We demonstrate that the same set of RNAi pathway genes is required for *Drosophila* defense against FHV and CrPV and that both viruses encode a potent VSR. These results collectively show that RNAi pathway functions as a common viral immunity mechanism in *Drosophila* and that RNAi suppression represents a general counterdefensive strategy used by insect viruses. Furthermore, a genetic requirement for Dcr-2, R2D2 and Ago-2 in antiviral silencing establishes a molecular framework for the innate immunity against viruses in *D. melanogaster*. None of Dcr-2, R2D2 and Ago-2 plays a detectable role in either the production or function of miRNAs in *D. melanogaster* (1). Thus, our work identifies the dsRNA-siRNA pathway of RNAi as providing the innate immunity against virus infection in *Drosophila*, and establishes that dsRNA produced during virus replication acts as the pathogen trigger whereas Dcr-2 and Ago-2 act as the host sensor and effector of the immunity, respectively. These results support and extend the previous findings on antiviral silencing in *C. elegans* (4,5).

Although NF- κ B-like signaling in the Toll and Imd pathways do not appear to play a role in the RNAi-directed viral immunity mechanism in *D. melanogaster*, the fly mutant defective in the Jak kinase Hpscotch exhibited a modest increase in susceptibility to infection with *Drosophila C* virus, suggesting an antiviral role of the Jak-STAT signaling (8). Nonetheless, we believe that RNAi-based immunity is independent of the Jak-STAT signaling since virus infection is not known to induce the RNAi pathway in *Drosophila* (8) and FHV induction of the Jak-STAT responsive gene *vir-1* was unaltered in the *dcr-2* and *r2d2* mutants as shown by

our recent work. Since the Toll and Imd pathways are highly conserved in vertebrates (7), the *Drosophila* model established for RNAi may also be useful for the analyses of the innate antiviral immunity in vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

1. Tomari Y, Zamore PD. *Genes Dev* 2005;19:517. [PubMed: 15741316]
2. Hamilton AJ, Baulcombe DC. *Science* 1999;286:950. [PubMed: 10542148]
3. Li HW, Li WX, Ding SW. *Science* 2002;296:1319. [PubMed: 12016316]
4. Lu R. *Nature* 2005;436:1040. [PubMed: 16107851]
5. Wilkins C. *Nature* 2005;436:1044. [PubMed: 16107852]
6. Voinnet O. *Nat Rev Genet* 2005;6:206. [PubMed: 15703763]
7. Hoffmann JA. *Nature* 2003;426:33. [PubMed: 14603309]
8. Dostert C. *Nat Immunol* 2005;6:946. [PubMed: 16086017]
9. Zambon RA, Nandakumar M, Vakharia VN, Wu LP. *Proc Natl Acad Sci U S A* 2005;102:7257. [PubMed: 15878994]
10. Ball LA, Johnson KL. *Adv Virus Res* 1999;53:229. [PubMed: 10582101]
11. Li WX. *Proc Natl Acad Sci U S A* 2004;101:1350. [PubMed: 14745017]
12. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. *Science* 2001;293:1146. [PubMed: 11498593]
13. Okamura K, Ishizuka A, Siomi H, Siomi MC. *Genes Dev* 2004;18:1655. [PubMed: 15231716]
14. Lee YS. *Cell* 2004;117:69. [PubMed: 15066283]
15. Liu Q. *Science* 2003;301:1921. [PubMed: 14512631]
16. Wilson JE, Powell MJ, Hoover SE, Sarnow P. *Mol Cell Biol* 2000;20:4990. [PubMed: 10866656]
17. Irving P. *Proc Natl Acad Sci U S A* 2001;98:15119. [PubMed: 11742098]
18. Dong XF, Natarajan P, Tihova M, Johnson JE, Schneemann A. *J Virol* 1998;72:6024. [PubMed: 9621065]
19. We thank JL Imler for a stimulating discussion, A Schneemann for providing CrPV and the anti-FHV serum, A Raikhel for the bacterial strains, and members of the Ding and Atkinson laboratories for comments and technical assistance. This project was supported by NIH grant AI052447 and USDA grant 2005-35319-15331 (to S.W.D.). Partial support for this work was provided by NIH grants (to P.A. and R.W.C.)

Fig 1. Antiviral silencing in *Drosophila* embryos requires Dcr-2 and Ago-2. Northern blot detection of FHV RNA accumulation in *wt*, *dcr-2*, and *ago-2* embryos micro-injected with *in vitro* synthesized transcripts R1, R1fs and R1ΔB2 as shown in Fig. s1.

Fig 2.

The siRNA pathway controls the innate immunity against FHV in adult flies. **(A)** Survival of *wt*, *dcr-2* and *r2d2* flies after FHV infection. Data shown represented mean of triplicates and the error bars indicate standard derivation. **(B, C)** Detection of viral RNAs by a probe specific for RNAs 1 and 3, and viral coat protein (CP) by a rabbit anti-FHV serum (18) in *wt*, *dcr-2*, and *r2d2* flies injected with FHV. **(D)** Detection of FHV siRNAs in the infected adult flies as in **C** (*d2*, *dcr-2*; *r2*, *r2d2*). The membrane was also probed for miR-8 and U6 RNA.

Fig 3. *dcr-2* and *r2d2* flies exhibit enhanced disease susceptibility to CrPV. **(A)** Survival of *wt*, *dcr-2* and *r2d2* flies after CrPV infection. Data shown represented mean of triplicates and the error bars indicate standard derivation. **(B)** Accumulation of CrPV genomic RNA in *wt*, *dcr-2*, and *r2d2* adults injected with CrPV.

Fig 4.

Induction and suppression of antiviral silencing by CrPV. **(A)** Accumulation of the genomic RNA and siRNAs of CrPV in infected S2 cells. **(B)** pFR1gfp directed transcription of a recombinant FHV RNA1 in which the coding sequence for B2 was replaced by that of GFP. S2 cells were transfected with pFR1gfp alone (middle) or pFR1gfp plus either dsRNA-targeting Ago-2 (right) or CrPV superinfection (left). **(C)** Identification of CrPV RNAi suppressor. Cells were co-transfected with pFR1gfp and a plasmid as indicated (see Fig. s2), and total RNA analyzed for the accumulation of pFR1gfp-encoded RNA1 and RNA3. P, the empty plasmid; VP, the virion protein precursor; V1, VP1; V2, VP2; V3, VP3; V4, VP4; V0, VP0 (precursor for VP3 and VP4); A, the first 140 codons of the upstream ORF; ΔA, a frameshift mutant of A; and b, the first 107 codons of the upstream ORF. NS1 is an RNAi suppressor of Influenza A virus as described previously (11).