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DIP1 plays an antiviral role against DCV infection in *Drosophila melanogaster*

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ABSTRACT

Disconnected Interacting Protein 1 (DIP1) is a dsRNA-binding protein that participates in a wide range of cellular processes. Whether DIP1 is involved in innate immunity remains unclear. Here, DIP1 was found to play an antiviral role in S2 cells. Its antiviral action is specific for DCV infection and not for DXV infection. *dip1* mutant flies are hypersensitive to DCV infection. The increased mortality in *dip1* mutant flies is associated with the accumulation of DCV positive-stranded RNAs *in vivo*. This study demonstrated that *dip1* is a novel antiviral gene that restricts DCV replication *in vitro* and *in vivo*.

1. Introduction

Innate immunity provides immediate and broad-spectrum protection against microbial infection, and innate immunity processes are often conserved across different kingdoms. Viruses are natural pathogens of fruit flies, and several antiviral mechanisms have been well studied in fruit flies [1–3]. The Toll pathway is required for the efficient inhibition of Drosophila X virus (DXV) replication and has been shown to control the survival of DXV-infected flies [4,5]. The Imd pathway has been reported to provide antiviral defenses against Sindbis virus (SINV) and Cricket paralysis virus (CrPV) infection in *Drosophila* [6,7]. The JAK-STAT pathway has been shown to contribute to the antiviral response of *Drosophila* against Drosophila C virus (DCV) and SINV in *Drosophila* [6,8]. The RNAi-mediated antiviral response in *Drosophila* has been studied with evolutionarily diverse viruses including DXV, DCV, CrPV and Flock house virus (FHV) [4,9–11].

RNAi is currently considered the major antiviral immune defense mechanism in *Drosophila*. The siRNA pathway utilizes Ago2 and Dcr-2 and is activated by dsRNA [9,12]. Fruit flies with mutations in components of the Dcr-2/R2D2/Ago2 pathway are susceptible to viral infection [10]. Dcr-2 has RNase III activity and is crucial for the cleavage of dsRNAs into siRNAs, which is essential for RNAi [13,14]. These siRNAs generated by Dcr-2 have the hallmark of Dcr-2 products, a 3′ 2-nt overhang. These siRNAs are incorporated into AGO2, assembled into RNA-induced silencing complexes (RISC) and used to perform a defense response against specific virus-derived RNAs [15,16]. Several dsRNA-binding proteins, such as Dcr-2, Adar, Loqs and R2D2, play crucial roles in RNAi pathways and the defense response to viral infection [17–19].

Disconnected Interacting Protein 1 (DIP1) is a member of the dsRNA-binding protein family [20]. It was previously shown to interact with proteins involved in transcription regulation and chromosomal silencing, such as Ultrabithorax (Ubx) and disconnected (Disco) [21,22]. DIP1 may mediate epigenetic mechanisms required for the establishment and maintenance of cell fate specification [20]. Daniel et al. reported that DIP1 binding to pre-tRNA and ADAT is involved in tRNA processing and maturation, but the function of DIP1 and the role of its dsRNA-binding domains are still unclear [22,23]. Thus, it is valuable to determine whether DIP1 has similar RNA-processing or immune-related functions to other dsRNA-binding proteins, such as Dcr-2, Adar, Loqs and R2D2. In this study, we found that DIP1 has an antiviral role against DCV in both cultured S2 cells and adult fruit flies.

2. Materials and methods

2.1. Fly strains

All flies were raised on standard cornmeal-agar medium and all experiments were performed at room temperature. *dip1* (*dip1*^{EY02625}) mutants were purchased from Bloomington drosophila stock center (Bloomington no. 15577). *ago2* (*ago2*^{414})

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and *dcr-2* (*dcr-2*^{L811fsX}) mutants containing null alleles have been described previously [24,25]. Canton-S (*csw*) flies were used as the wild type (WT) control.

2.2. Preparation of DCV virions

DCV virions were purified following a previously published protocol with minor modification [10]. Briefly, S2 cells were inoculated with DCV crude virions and were collected three days later. These cells were mixed with 0.5% Nonidet P-40 and were subjected to 3 freeze/thaw cycles. After digestion with RNase A, the sample was placed on a 30% sucrose cushion containing 0.05M HEPES (pH 7.0), 5 mM $CaCl_2$, 0.1% β-mercaptoethanol, and 0.1% Bovine Serum Albumin (BSA) and then ultracentrifugated at 25,000 rpm for 2 h. The pellet was resuspended in 500 μl HEPES buffer and the insoluble material was removed by centrifugation at 14,000 rpm at 4 °C. The virion suspensions were aliquoted and stored at −80 °C for future study. DCV was inactivated at 100 °C for 10 min.

2.3. Inoculation of adult fruit flies with DCV virions

For virion inoculation, 3–4-day-old flies of the indicated genotypes were infected *Drosophila* C virus as described [26]. Briefly, the fruit flies were transferred into vials containing fresh medium and injected with the virions into their thorax by a FemtoJET microinjector (Eppendorf, Germany). The infected flies were monitored for mortality every day or were collected at the indicated time points for other assays. The Infectivity experiments were repeated at least three times.

2.4. Cell culture and gene knockdown by dsRNAs

Drosophila S2 cells were cultured at 25 °C in Schneider's insect medium (SIGMA) supplemented with 10% heat-inactivated fetal bovine serum, 5 mM sodium bicarbonate, 5 mM calcium chloride, 100 U/mL penicillin, and 100 mg/mL streptomycin.

The dsRNAs of *dip1*, *ago2*, *dcr-2* and *β-gal* were generated by *in vitro* transcription using a T7 transcription kit (TOYOBO, Japan). DNA templates of *ago2*, *dcr-2* and *β-gal* were amplified for dsRNA synthesis using primer sets that were previously described [27]. The DNA template of the *dip1* gene was amplified using the primer set (P1: T7-GGGACAAGAAGTTGCGACAG, P2: T7-GCGTTCTTGGCTGTCATTTT). S2 cells were incubated with dsRNAs in medium free of fetal bovine serum (FBS) for 30 min and then, the medium was replaced with fresh medium containing 12% FBS. Two days after dsRNA bathing, the cells were infected with DCV and assayed at 2 days post-infection.

2.5. Quantitative RT-PCR and Northern blot

Total RNA was extracted from adult flies and *drosophila* S2 cells using Trizol (Invitrogen, USA). cDNAs were synthesized with random primers using a reverse transcription kit (Thermo Scientific, USA). SYBR Premix Ex Taq (Bio-Rad, USA) was used for quantitative real-Time PCR. The expression levels of all genes as determined by real-time PCR were normalized to the level of rp49 RNA for relative quantification. Viral RNA was detected by Northern blot using standard procedures with a DIG-labeled RNA probe (Roche, USA).

3. Results

3.1. Antiviral role of DIP1 against DCV virus in S2 cells

DCV is a single-stranded positive-sense RNA virus of the *Dicistroviridae* family and a natural viral pathogen of fruit flies. Previous studies have shown that both RNA silencing and the JAK-STAT

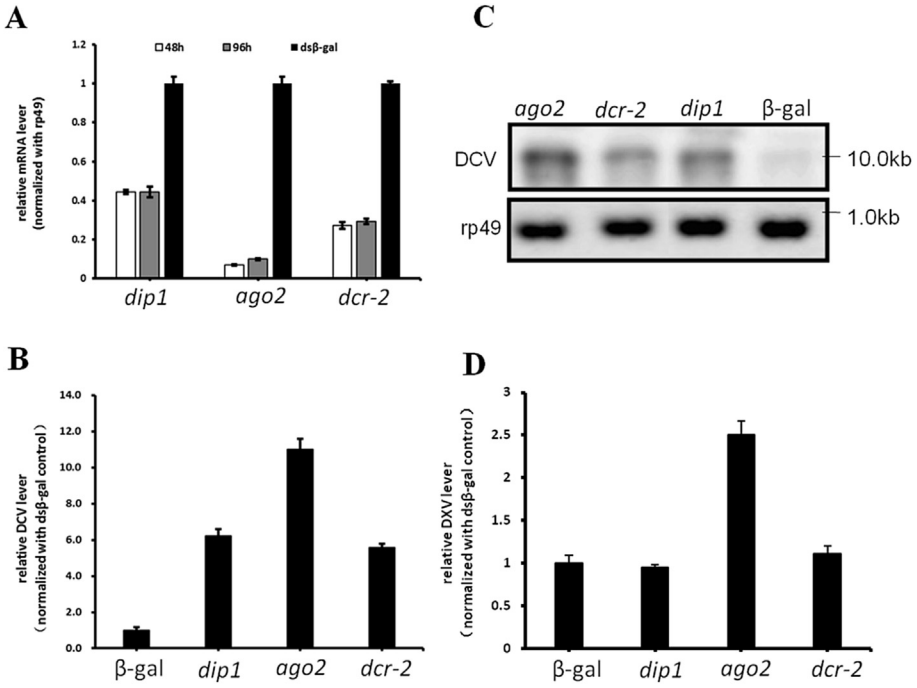

Fig. 1. DIP1 specifically inhibits DCV replication in S2 cells. (A) The knockdown efficiency of *dip1*, *ago2*, *dcr-2* genes in S2 cells treated with corresponding dsRNAs. Cells were collected at the indicated time points after treatment, and the expression levels of the genes were analyzed by qRT-PCR. The expression levels were normalized to the corresponding values expressed in the S2 cells treated with *β-gal* dsRNAs. (B & C) S2 cells were treated with dsRNAs 2 days before DCV virion inoculation, then the accumulation of DCV RNA in S2 cells was analyzed by qRT-PCR (B) and Northern blot (C) at 48 h post-inoculation. (D) S2 cells were treated with dsRNAs 2 days before DXV virion inoculation; at 48 h post-inoculation, the accumulation of DXV RNAs in S2 cells was analyzed by qRT-PCR. Each data point represents the mean value of triplicate experiments, and the error bars indicate the corresponding standard deviations.

pathway contribute to the antiviral response of *Drosophila* against DCV infection [10,8]. To determine whether DIP1, which contains two dsRNA-binding domains, is involved in that antiviral defense, cultured S2 cells were treated with dsRNAs of the *dip1* gene before DCV virion inoculation. The mRNA level of the *dip1* gene was knocked down to ~45% of that in control S2 cells treated with dsRNAs of β-galactosidase (*β-gal*), and the efficiency of the knockdown of *dip1* was stable at 48 h and 96 h after dsRNAs treatment (Fig. 1A). The knockdown of the *dip1* gene in S2 cells did not cause any noticeable changes in cell morphology or cell proliferation. As Ago2 and Dcr-2 are the core components of the RNA-induced silencing complex (RISC), which is involved in the RNAi pathway, S2 cells were treated with dsRNAs of both *ago2* and *dcr-2* genes as a positive control. Similar knockdown to that for the *dip1* gene was observed for the *dcr-2* and *ago2* genes; the mRNA levels of *dcr-2* and *ago2* were 30% and 10% of those in S2 cells treated with *β-gal* dsRNA, respectively (Fig. 1A).

Then, the pretreated S2 cells were further inoculated with DCV virus. After 48 h, the S2 cells were collected and the DCV positive-strand RNAs were quantified by qRT-PCR. Compared with control S2 cells pretreated with *β-gal* dsRNAs, the viral positive-strand RNA levels were 6.2, 11 and 5.8 times higher in S2 cells pretreated with dsRNAs of *dip1*, *ago2* and *dcr-2*, respectively (Fig. 1B). The accumulation of viral positive-strand RNAs in S2 cells was confirmed by Northern blot analysis (Fig. 1C). These results demonstrate that DIP1 has a significant antiviral role against DCV infection in S2 cells, and the antiviral action of DIP1 is comparable to that of Dcr-2 but is lower than that of Ago2.

3.2. Negligible antiviral role of DIP1 against DXV in S2 cells

To test whether DIP1 has broad antiviral function against diverse viruses, DXV of the *Birnaviridae* family was tested in S2 cells following the same protocol as used for DCV. The genome of DXV is comprised of two dsRNA segments: RNA1, 3.36 kb and RNA2, 3.24 kb. In S2 cells treated with dsRNAs of *ago2* and *dcr-2*, the DXV RNA levels increased to 2.5 and 1.2 times that in control S2 cells, respectively, and these results are consistent with those previously reported [4]. In contrast, dip1 knockdown had a negligible impact on DXV replication in S2 cells (Fig. 1D). Together, our results show that DIP1 play an antiviral role against DCV infection but not against DXV, which has a dsRNA genome.

3.3. Hypoexpression of the dip1 genes in dip1 mutants

dip1 mutant flies were purchased from the Bloomington drosophila stock center (Bloomington no. 15577) and were constructed by inserting the transposon gypsy into the 5′ end of the longest DIP1 cDNA [28]. The level of *dip1* gene expression in the mutants is approximately 50% lower than that in wild type fruit flies (Fig. 2A). In wild type fruit flies, DCV infection up-regulates a specific set of genes, including *virus-induced RNA 1* (*vir-1*) [8]. To test whether *dip1* mutants have a normal response to DCV infection, *dip1* mutant flies were inoculated with DCV virions, and the expression level of *vir-1* was measured by qRT-PCR. The *vir-1* gene was up-regulated by 2.6 and 5 times at 3 and 4 days post-inoculation compared to before inoculation (Fig. 2B), respectively, which suggests that the DCV infection in *dip1* mutants induces *vir-1* expression to the same extent as in wild type flies.

3.4. dip1 mutant flies are hypersusceptible to DCV infection

To test whether DIP1 has an antiviral role against DCV infection *in vivo*, wild type flies and *dip1*, *ago2* and *dcr-2* mutant flies were challenged with DCV virions. Wild-type fruit flies were resistant to

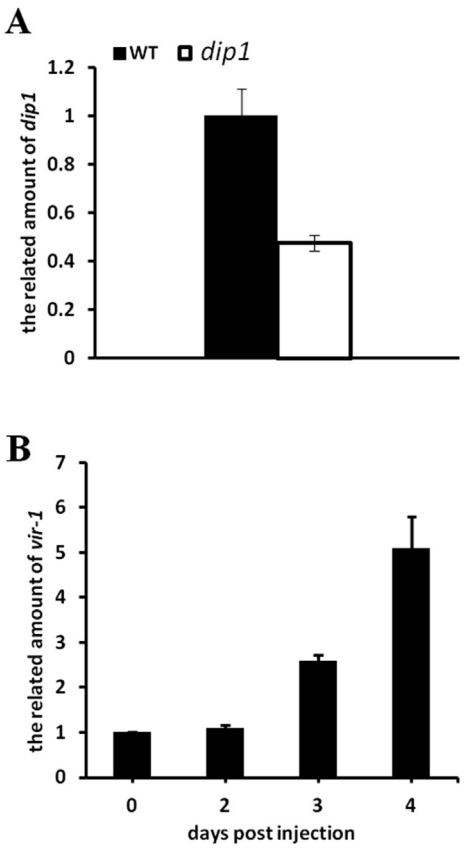

Fig. 2. (A) *dip1* mutant flies showed hypoexpression of the *dip1* gene. Total RNAs were isolated from wild type (WT) and *dip1* mutant flies, and the expression level of the *dip1* gene was measured by qRT-PCR and normalized to that in the wild type flies. (B) *dip1* mutant flies were challenged with DCV virions. Then, flies were collected, and the level of *virus-induced RNA 1* (*vir-1*) was determined at the indicated days after injection. The initial value of *vir-1* before DCV injection was used for normalization. Each data point represents the mean value of triplicate experiments, and the error bars indicate the corresponding standard deviations.

DCV infection as indicated by an 80% survive rate at 10 days post-inoculation (Fig. 3A). In contrast, the survival rate of *dcr-2*, *ago2* and *dip1* mutants were 45%, 30% and 17% at 10 days post-inoculation, respectively. These results indicated that *dip1* mutants are hypersusceptible to DCV infection, even compared to *ago2* and *dcr-2* mutant flies. *dip1* mutant flies showed the earliest onset of mortality with a median survival of ~3.5 days; this was shorter than the median survivals of the *dcr-2* and *ago2* mutants, which were both 5~6 days (Fig. 3A).

To exclude the possibility that the mortality of the *dip1* mutants was caused by non-specific effects of DCV infection, the *dip1* mutant flies were also injected with PBS buffer and heat-inactivated DCV virions as a control. The *dip1* flies injected with dead DCV virions and PBS buffer were almost all alive after 10 days (Fig. 3B), which suggests that the mortality of *dip1* mutants was due to DCV replication *in vivo* not any non-specific effect of inoculation.

3.5. Higher accumulation of DCV positive-strand RNA in dip1 mutant flies

To determine if the increased mortality of *dip1* mutants was associated with an increase in viral replication, DCV positive-strand RNAs were measured in wild type flies and *dip1* mutant flies at 4 time points over 72 h after DCV inoculation. Living flies were collected at 12, 24, 48 and 72 h post-inoculation. In the wild type flies, no obvious accumulation of DCV positive-strand RNA was

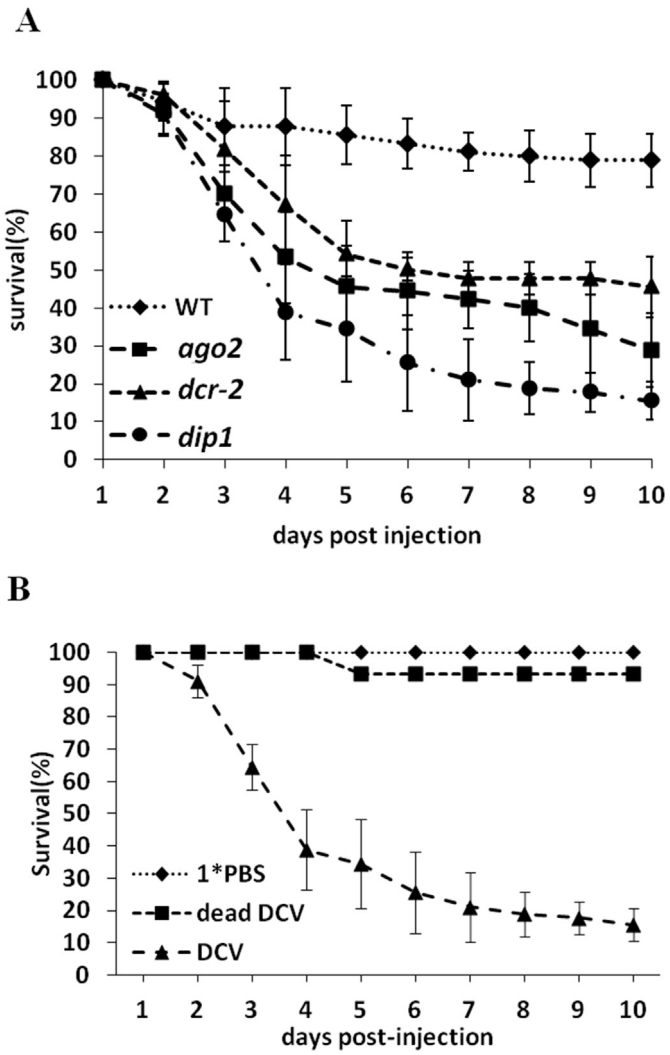

Fig. 3. *dip1* mutant flies are hypersensitive to DCV infection. (A) Survival curves of WT flies and *ago2*, *dcr-2* and *dip1* mutant flies after DCV injection. Flies were injected in the thorax with DCV and then monitored daily for mortality. (B) Survival curve of *dip1* mutant flies after inoculation with mock (PBS), head-inactivated DCV virions and DCV virions. Each data point represents the mean value of triplicate experiments, and the error bars indicate the corresponding standard deviations.

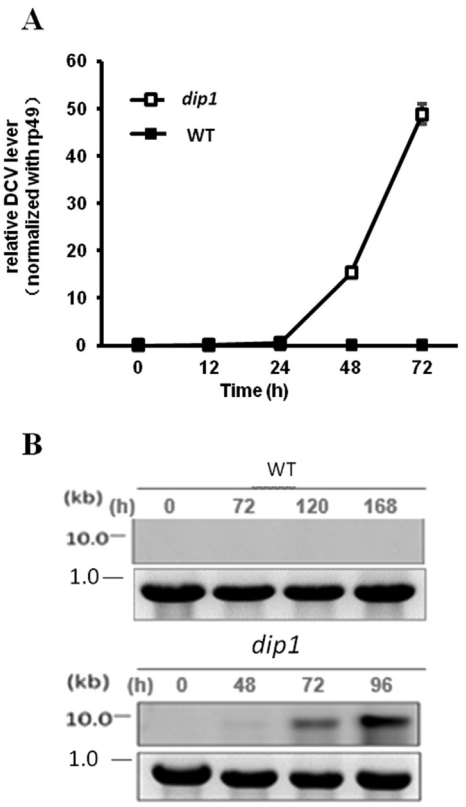

Fig. 4. Wild type and *dip1* flies were challenged with DCV, and flies were collected at the indicated time points post-infection. The level of DCV RNA was measured by qRT-PCR and normalized to the level of rp49 RNA (A). The level of DCV RNA was also analyzed by Northern blot (B) with rp49 RNA as the loading control.

observed, but in the *dip1* mutants, the amount of DCV positive-strand RNA increased sharply after 24 h (Fig. 4A). The ratios of viral positive-strand RNA to Drosophila Rp49 RNA were 17 and 48 times higher in *dip1* mutants than in the wild type flies at 48 and 72 h (Fig. 4A), respectively. The total RNA isolated from living wild type and *dip1* mutant flies at distinct time points after post-inoculation were also detected by Northern blot. DCV positive-strand RNAs were identified in wild type flies at 7 days (168 h) post-inoculation. In contrast, the bands were visible at 48 h post-inoculation and became much stronger at 96 h post-inoculation in *dip1* mutants (Fig. 4B). Both qRT-PCR and Northern blot showed that the amount of viral positive-strand RNAs was correlated with the mortality rate of wild type and *dip1* mutant flies. Thus, DIP1 has a defensive role against DCV infection and represses the replication of DCV virus *in vivo*.

4. Discussion

In fruit flies, the essential roles of many dsRNA binding protein in antiviral defense have been established. However, whether DIP1, which contains two dsRNA-binding domains, has an important immune function is not yet known. We demonstrated an antiviral function of DIP1 in both cultured S2 cells and adult flies. Knock-down of the *dip1* gene in S2 cells led to increased replication of DCV viruses. When the *dip1* knockdown mutant flies, which had ~50% *dip1* gene expression compared to wild type flies, were infected with DCV, a higher amount of viral positive-strand RNAs and greater mortality were observed. These results clearly suggest that DIP1 has an antiviral role against DCV infection that is stronger than those of Ago2 and Dcr-2, two core components of the RNA silencing pathway.

It is worth noting that DIP1 has no impact on the replication of DXV in S2 cells. Although RNA silencing is currently considered the major antiviral immune defense mechanism in Drosophila, Dcr-2, the core component of the RNAi pathway, has no obvious impact on DXV replication [4]. It is reasonable to hypothesize that DIP1 and Dcr-2 proteins have similar antiviral mechanisms. Previous studies have shown that DIP1 binds with high affinity to a subset of dsRNA ligands, including microRNA precursor stem-loops, and interacts with many proteins [22,23]. DIP1 may serve as a recruitment factor that guides the RNA processing, possibly by binding with viral dsRNAs to facilitate Dcr-2 processing.

Conflict of interest

None.

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Transparency document

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