TRBP and eIF6 Homologue in *Marsupenaeus japonicus* Play Crucial Roles in Antiviral Response

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Abstract

Plants and invertebrates can suppress viral infection through RNA silencing, mediated by RNA-induced silencing complex (RISC). Trans-activation response RNA-binding protein (TRBP), consisting of three double-stranded RNA-binding domains, is a component of the RISC. In our previous paper, a TRBP homologue in *Fenneropenaeus chinensis* (Fc-TRBP) was reported to directly bind to eukaryotic initiation factor 6 (eIF6). In this study, we further characterized the function of TRBP and the involvement of TRBP and eIF6 in antiviral RNA interference (RNAi) pathway of shrimp. The double-stranded RNA binding domains (dsRBDs) B and C of the TRBP from *Marsupenaeus japonicus* (Mj-TRBP) were found to mediate the interaction of TRBP and eIF6. Gel-shift assays revealed that the N-terminal of Mj-TRBP strongly binds to double-stranded RNA (dsRNA) and that the homodimer of the TRBP mediated by the C-terminal dsRBD increases the affinity to dsRNA. RNAi against either *Mj*-TRBP or *Mj*-eIF6 impairs the dsRNA-induced sequence-specific RNAi pathway and facilitates the proliferation of white spot syndrome virus (WSSV). These results further proved the important roles of TRBP and eIF6 in the antiviral response of shrimp.

Introduction

Viral diseases, especially white spot syndrome virus (WSSV) cause a great loss to the shrimp culture. Investigation of the antiviral mechanism of shrimp would help to control those diseases. Several studies demonstrate that the RNA interference (RNAi) play an important role in shrimp antiviral immunity [1]. Small interfering RNA (siRNA)/microRNA (miRNA)-induced RNA silencing has been demonstrated to be an important pathway regulating eukaryotic gene expression. This process depends on 20–23-nt siRNAs and miRNAs, guiding the RNA-induced silencing complex (RISC) to recognize and silence the target mRNA [2,3,4]. The core of RISC is composed of three proteins: Dicer, trans-activation response RNA-binding protein (TRBP), and Argonaute 2 (Ago2) [2,3]. TRBP is a protein partner of the human Dicer and serves as a bridge between the double-stranded RNA (dsRNA) and Dicer, requiring for RNA silencing mediated by siRNAs/miRNAs [2,3]. MacRae et al. used purified TRBP, Dicer, and Ago2 to assemble the RISC-loading complex in vitro. The complex shows dicing, slicing, guide-strand selection, and Ago2-loading activities [5,6]. Introduction of those genes to *Sacharomyces cerevisiae* could reconstitute RNAi in budding yeast [7]. Eukaryotic initiation factor 6 (eIF6) is also a component of the human RISC, depletion of eIF6 abolish the miRNA-mediated silencing [8].

There is increasing evidence suggesting the crucial role of RNAi in invertebrate antiviral immunity. Significant amounts of dsRNA are produced following productive infection with positive-strand ssRNA, dsRNA, or DNA viruses [9]. These dsRNAs are thought to be subjected to the RNAi pathway, generating an antiviral response. The first direct evidence was reported in fruit fly, in which the flock house virus is both an initiator and a target of RNA silencing [10]. DsRNA-induced sequence-specific antiviral silencing and nonspecific immunity to white spot syndrome virus (WSSV), Taura syndrome virus, and yellow-head virus have also been reported in shrimp [1,11,12,13].

TRBP belongs to the dsRNA-binding protein family, containing three dsRNA-binding domains (dsRBDs) [3,14]. The first two dsRBDs bind to dsRNA with an affinity that is independent of dsRNA length and sequence [15,16]. The second dsRBD of RDE-4, a TRBP homologue in *Caenorhabditis elegans*, plays a primary role in interaction with dsRNA [15]. The C-terminal dsRBD mediates binding to Dicer [3,4,17], protein kinase R (PKR) [18], and PKR-associated activator (PACT) [19], instead of binding to RNA. In our previous study, full-length cDNAs that encode a human TRBP2 homologue (*Fe*-TRBP) and an eIF6 protein (*Fe*-eIF6) in Chinese white shrimp (*Fenneropenaeus chinensis*) were cloned, and direct interaction between those two proteins was demonstrated. The proliferation of WSSV was reduced after injection of recombinant *Fe*-TRBP, implying a crucial role of *Fe*-TRBP in the antiviral defense response of shrimp [20]. However, the mechanism of antiviral silence in shrimp remains obscure. In this study, different dsRNA-binding domains (dsRBDs) of TRBP from
**Marsupenaeus japonicus** (Mj-TRBP) were expressed in *Escherichia coli*, and pull-down assays were performed to show which dsRBDs mediate the association with eIF6. Gel shift assays were performed to identify the dsRNA-binding activity of dsRBDs. Results would further characterize the dsRNA- and eIF6-binding activities of Mj-TRBP, and proving the important roles of Mj-TRBP and Mj-eIF6 in dsRNA-induced antiviral silencing.

**Materials and Methods**

**Biological material**

*M. japonicus* (about 10–15 g each) were bought from a shrimp market in Jinan, Shandong Province, China and kept in tanks containing aerated seawater.

**Cloning of Mj-TRBP and Mj-eIF6 gene**

Total RNA isolation and reverse transcription of the RNA were performed as described previously [20]. Two primers, MjTRBF and MjTRBFR, were designed according to the homologous gene sequences in order to obtain the complete open reading frame (ORF) of Mj-TRBP. The PCR procedure was as follows: 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s and 1 cycle at 72°C for 10 min.

Mj-eIF6 was cloned using MjIF6exF and MjIF6exR primers (Table 1), which were designed based on homologous gene sequences. The PCR procedure was as follows: 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s and 1 cycle at 72°C for 10 min.

**Recombinant expression of different dsRBDs of Mj-TRBP**

The three dsRBDs of Mj-TRBP were denoted as TRBP-DA, TRBP-DB, and TRBP-DC. TRBP-DA (369 bp), TRBP-DB (294 bp), TRBP-DC (257 bp), TRBP-DB (701 bp, containing TRBP-DA and TRBP-DB), and TRBP-DC (701 bp, containing TRBP-DB and TRBP-DC) fragments of Mj-TRBP were amplified from hemocyte cDNA using the primers: MjTRBF1 and MjTRBFR1, MjTRBF2 and MjTRBFR2, MjTRBF3 and MjTRBFR3, respectively (Table 1). *EcoR* I and *XhoI* restriction sites were inserted at the beginning and end of the DNA fragments, so that the PCR products could be cloned into the *EcoR* I and *XhoI* restriction sites of pET-30a. Recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells, which were then cultured in Luria-Bertani medium with 25 µg/ml ampicillin. When the OD600 of the culture reached 0.5, isopropyl-β-D-thiogalactoside (IPTG; 0.1 mM) was added. After 3 h of culture, cells were collected by centrifugation at 6000 rpm for 10 min. They were then resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4) containing 0.2% Triton X-100. Following cell sonication and centrifugation, proteins were purified using His-Bind resin (Novagen), according to the manufacturer’s instructions. The purified proteins were analyzed by 15% SDS-PAGE and stained with Coomassie brilliant blue G250.

**Pull-down assays**

The pull-down assay was performed as described previously [20]. Briefly, TRBP-DA with an N-terminal His tag was expressed in *E. coli*. After induction by IPTG and sonication, 10 ml lysed cells were incubated with 1 ml His-Bind Resin for 5 min and washed with 10 ml binding buffer (0.5 M NaCl, 20 mM Tris-Cl (pH 7.9), 5 mM imidazole) and then 6 ml washing buffer (0.5 M NaCl, 20 mM Tris-Cl (pH 7.9), 30 mM imidazole). About 200 µg purified eIF6 with the N-terminal His tag removed by incubation with thrombin (eIF6ΔHis-tag) was added and incubated with the His-tagged protein and His-Bind Resin for 10 min at 4°C. eIF6ΔHis-tag and eIF6 with His-tag were analyzed by SDS-PAGE, and the difference in mobility was observed, ensuring the His-tag of eIF6ΔHis-tag was completely removed. After 3 washes with 10 ml washing buffer, proteins were eluted with eluting buffer (0.5 M NaCl, 20 mM Tris-Cl (pH 7.9), 1 M imidazole). The eluted proteins were analyzed by 15% SDS-PAGE. Pull-down assays were also performed to identify the interaction between TRBP-DA and eIF6, TRBP-DC and eIF6, TRBP-DB and eIF6, TRBP-DB and eIF6, and TRBP-DB and TRBP.

**dsRNA preparation**

dsRNA was prepared as described previously [21], with slight modifications. DNA fragments were amplified using MjTRBF1

**Table 1. Oligonucleotide primers used in this study.**

| Primer Name | Nucleotide Sequence (5’-3’) |
|-------------|-----------------------------|
| MjTRBF1     | TCATGTATACATCAACCTACCAAAAA  |
| MjTRBFR1    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF2     | GGATCCCTGACTCACTATAGG       |
| MjTRBFR2    | GCGGATCCCTGACTCACTATAGG     |
| MjTRBF3     | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR3    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF4     | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR4    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF5     | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR5    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF6     | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR6    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF7     | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR7    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF8     | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR8    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF9     | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR9    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF10    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR10   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF11    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR11   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF12    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR12   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF13    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR13   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF14    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR14   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF15    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR15   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF16    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR16   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF17    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR17   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF18    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR18   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF19    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR19   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF20    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR20   | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBF21    | CCCCTTCTCCCTAACAACAGTG     |
| MjTRBFR21   | CCCCTTCTCCCTAACAACAGTG     |
and MjTRBP5R (543 bp), MjeIF6iF and MjeIF6iR (802 bp), MjPOeF and MjPOeR (810 bp), GFPiF and GFPiR (467 bp) primers, respectively (Table 1). A T7 promoter was linked to both ends of the PCR products. After extraction with phenol/ chloroform and precipitation with ethanol, DNA fragments were used as templates for dsRNA synthesis.

Transcription was carried out as follows: 8 μg DNA templates was mixed with 25 μL 5x transcription buffer, containing 80 U T7 RNA polymerase (Fermentas, USA), 3 μL 10 mM A/U/C/G/TTP each (Fermentas, USA), and 120 U RNasin (TaKaRa, Japan). RNase-free water was added to a volume of 125 μL. After incubation at 37 °C for 4 h, dsRNAs were incubated at 75 °C for 5 min and then cooled to room temperature for annealing. To remove the template, 40 U RNase-free DNase I (Fermentas, USA) was added and the solution was incubated at 37 °C for 30 min. After extraction with phenol/chloroform and precipitation with ethanol, dsRNAs were resuspended in 60 μL RNase-free water. The dsRNA purity and integrity were determined using agarose gel electrophoresis. The dsRNAs were quantified using a spectrophotometer (GeneQuant; Amersham Biosciences).

**Gel-shift assay**

Mj-eIF6 dsRNA was prepared as above, with additional 0.35 μL 10 mM digoxigenin (Dig)-labeled UTP (Roche Applied Sciences) added to the transcription buffer during preparation. The VP28 dsRNA was also prepared using the DNA template amplified with VP28F1 and VP28R1 (193 bp) primers from the genomic DNA of WSSV. All components of the reaction were added to 20 μL PBS, including: 10 mM DTT, 10 U RNasin (TaKaRa, Japan), 2.5 μg Dig-labeled Mj-eIF6 dsRNA (802 bp) or 0.5 μg Dig-labeled VP28 dsRNA (193 bp), and 0.5 μg protein (dsRBDs). The mixture was incubated at 4 °C for 30 min to allow binding and was subsequently loaded onto a 5% native polyacrylamide gel. After electrophoresis, proteins and Dig-labeled dsRNA were transferred to a nylon membrane, and an anti-Dig-phosphatase antibody (AB) was used to detect dsRNA. AB binding was visualized by incubation of the membrane with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride. Recombinant adenylyl kinase 1 (expressed in the same E. coli system, a gift of from Dr. Weiwei Zheng, paper is under preparation) from Helicoverpa armigera (Ha-AK) was used as a control.

**In vivo RNAi assay**

RNAi assay was performed according to a previously described method [21]. Briefly, 60 μg of Mj-TRBP dsRNA, Mj-eIF6 dsRNA, Mj-prophenoloxidase (Mj-PO) dsRNA or control dsRNA GFP was injected into the abdominal segment of M. japonicus. Injections were repeated 24 h after the first injection. The total RNA from hemocytes was extracted at 24 h post the second injection. Transcription of Mj-TRBP Mj-eIF6 and Mj-PO were detected by quantitative real time PCR to confirm knockdown using the MjTRBP-F3 and MjTRBP-R2, MjeIF6RTF and MjIF6RTR, MjPORTF and MjPORTTR primers (Table 1).

To investigate the role of Mj-TRBP and Mj-eIF6 in the RNAi pathway, shrimps were injected with dsRNAs against Mj-TRBP, Mj-eIF6 and GFP [control] as above. To examine whether the activity of RNAi pathway was impaired by the silencing of these protein, Mj-PO dsRNA was subsequently injected to the shrimp. Transcription of Mj-PO was analyzed by quantitative real time PCR.

**Functional analysis of Mj-TRBP and eIF6**

The preparation and quantification of WSSV inocula were done as described previously [20]. M. japonicus subjects were divided into 3 groups and injected with 60 μg Mj-TRBP dsRNAs, Mj-eIF6 dsRNAs, and Mj-GFP dsRNAs, as above. A second injection was done 24 h later. WSSV (1.5 × 10⁸ copies/shrimp) was injected into the abdominal segment of shrimps 24 h after the second injection of dsRNA. At 36 h post-WSSV-infection, genomic DNA was extracted from the gills of the shrimp, and quantitative RT-PCR was performed to quantify WSSV.

In another experiment, one group of shrimp were injected with WSSV (5.0 × 10⁷ copies/shrimp) and 8 μg of Mj-TRBP, while the other groups received the same amounts of WSSV and 8 μg of TRBP fragments (DA, DB, DC, DAB and DBC). The control group was injected with WSSV and 8 μg of Ha-AK2. At 24 h post injection, genomic DNA was extracted from shrimp gills, and quantitative real time PCR was performed to quantify WSSV.

![Figure 1. Scheme of the dsRBDs of Mj-TRBP and the SDS-PAGE analysis of recombinant dsRBDs of Mj-TRBP and Fc-eIF6 expressed in E. coli.](image-url)
Figure 2. Pull-down assays that identify interactions between dsRBDs of *Mj*-TRBP and *Fc*-eIF6. A: Recombinant His-tagged TRBP-DA was incubated with His-Bind resin, to which eIF6ΔHis-tag (the His tag was removed from recombinant eIF6 by thrombin treatment and SDS-PAGE show the different mobility of eIF6 and eIF6ΔHis-tag, ensuring the His-tag of eIF6ΔHis-tag was removed completely) was added and incubated for 10 min. Wash buffer did not elute either protein, and elution buffer only eluted TRBP-DA, indicating the incapability of TRBP-DA to bind eIF6. B, C, D, E and F: The interactions between eIF6 and TRBP-DB, eIF6 and TRBP-DC, eIF6 and TRBP-DAB, eIF6 and TRBP-DBC eIF6 and full length TRBP (positive control) were identified by Pull down assays, respectively. G: The eIF6ΔHis-tag was incubated with His-Bind resin, and no protein was eluted after wash. H: To verify those proteins, western blot analysis was performed using TRBP (left) or eIF6 (right) specific antibody. The results indicated that eIF6 could be co-eluted with those TRBP fragments containing the second and third dsRBD, which indicated that the TRBP-DB and -DC, but not TRBP-DA mediated the interaction between TRBP and eIF6.

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Results

Cloning of TRBP and eIF6 in M. japonicus

*Mj*-TRBP was cloned from *M. japonicus*, and the complete ORF is composed of 1032 bp encoding a 343-aa protein. The cDNA sequence of *Mj*-TRBP was deposited in GenBank (accession no. HM149250). Alignment revealed high sequence similarity between *Mj*-TRBP and the homologue in *F. chinensis* (99.42% identity) (Fig. S1). Two additional isoforms of *Mj*-TRBP, *Mj*-TRBP2 (GenBank accession no. HM149251) and *Mj*-TRBP 3 (GenBank no. HM149252), were also identified. These isoforms are identical except for the lack of 60 and 141 bp between the second and the third dsRBDs.

The homologue of eIF6 in *M. japonicus* was cloned (GenBank no. HM149253). The complete ORF of *Mj*-eIF6 is composed of 738 bp encoding a 245-aa protein. Alignment analysis showed that *Mj*-eIF6 is highly homologous to *Fc*-eIF6 (97.55% identity, Fig. S2).

TRBP-DB and TRBP-DC mediate the interaction between TRBP and eIF6 in shrimp

We previously reported the interaction between TRBP and eIF6 in shrimp by screening the T7 phage display library and confirming by pull-down assay [20]. Similar to other members of the TRBP family, *Mj*-TRBP contains three dsRBDs, denoted as TRBP-DA (27–92 aa), TRBP-DB (130–196 aa) and TRBP-DC (272–338 aa), and it remains unclear which domains mediate the interaction with eIF6. To address this, different domains of *Mj*-TRBP were recombinant-expressed and purified (Fig. 1). His-tag pull-down assays were performed to identify the interaction between these dsRBDs and *Fc*-eIF6. In Fig. 2A, His-tagged TRBP-DA was incubated with His bind resin, and subsequently the resin was incubated with eIF6ΔHis-tag (the His tag of eIF6 was removed by treatment with thrombin *in vitro*). SDS-PAGE show the different mobility of eIF6 and eIF6ΔHis-tag, and ensure the His-tag of eIF6ΔHis-tag was removed completely. After stringent wash, only TRBP-DA was eluted, indicating TRBP-DA could not bind eIF6. While eIF6ΔHis-tag was proved to be unable to bind His bind resin non-specifically (Fig. 2G). Pull-down assays were also performed between eIF6 and TRBP-DB/DC/DAB/DBC, and both eIF6 and TRBP-DB/DC/DAB/DBC could be eluted (Fig. 2B,C,D,E). Full length TRBP was used as positive control (Fig. 2F). All the proteins were verified by *Fc*-TRBP or *Fc*-eIF6 antibody (Fig. 2H). Results indicated that all dsRBDs could bind to eIF6, except TRBP-DA. Therefore, both TRBP-DB and TRBP-DC mediate the association of TRBP with eIF6 (Fig. 2).

TRBP-DA has a higher affinity with dsRNA, and the TRBP-DC can enhance the dsRNA-binding activity of TRBP

To investigate the dsRNA binding activity of *Mj*-TRBP, recombinant, gel-shift experiments were performed using 0.5–1.5 μg proteins (TRBP, dsRBDs or *Ha*-AK as a control) and 2.5 μg Dig-labeled *Mj*-eIF6 dsRNA (802 bp). The results show that TRBP and TRBP-DA have a high affinity for dsRNA, and TRBP-DB and BC only weakly bind to dsRNA. TRBP-DAB has a high affinity for dsRNA, and TRBP-DA has a very high affinity with dsRNA. The affinity of dsRBD A of *Mj*-TRBP could bind to dsRNA with high affinity. The affinity of dsRBD B was very weak. dsRBD C was unable to bind to dsRNA.

![Figure 3. Gel-shift assays that identify the dsRNA-binding activity of recombinant dsRBDs of *Mj*-TRBP and full length recombinant *Fc*-TRBP. A, B: 0.5 μg (A) and 1.5 μg (B) recombinant protein were incubated with 2.5 μg dsRNA (802 bp). Ha-AK served as control. C, D, E, F: Increasing amounts of recombinant protein (TRBP, TRBP-DAB, TRBP-DA, and TRBP-DB) were added and incubated with 2.5 μg dsRNA; the number under each lane represents the amount of recombinant proteins added to the dsRNA. *Fc*-TRBP and dsRBD A of *Mj*-TRBP could bind to dsRNA with high affinity. The affinity of dsRBD B was very weak. dsRBD C was unable to bind to dsRNA.

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medium affinity between TRBP-DA and B. TRBP-DC is completely unable to bind to dsRNA (Fig. 3 A, B). Different concentrations of TRBP, TRBP-DAB, TRBP-DA, and TRBP-DB were subjected to the binding affinity assay, and the results revealed that the binding activity of those proteins to dsRNA occurs in a dose dependent manner (Fig. 3C, D, E, F). Gel-shift assays were repeated using 193 bp Dig-labeled VP28 dsRNA (from WSSV), and the results confirmed that TRBP-DA has a high affinity for dsRNA, TRBP-DB has a weak affinity, and TRBP-DC cannot bind to dsRNA (Fig. 4). These results also suggest that the TRBP-binding activity is independent of dsRNA sequence.

Both full length TRBP and TRBP-DAB contain dsRBD A and dsRBD B, so they were expected to have equal affinity for dsRNA, because of the incapability of dsRBD C to bind dsRNA. However, results showed that the dsRNA affinity of full length TRBP is much higher than that of TRBP-DAB (Fig. 3A, B, C, D). One possible explanation is that the C-terminal region of TRBP assists in the association of other dsRBDs with dsRNA, although it is incapable of binding to dsRNA by itself. Pull-down assays showed that TRBP-DC can interact with full length TRBP and that there is no association observed between TRBP-DC and TRBP-DAB and between full length TRBP and TRBP-DAB (Fig. 5A).

Therefore, TRBP-DC seems to mediate the dimerization of TRBP. As a result, TRBP could form dimer via the C-terminal dsRBD and bind to dsRNA co-operatively, exhibiting a significantly higher affinity to dsRNA than TRBP-DAB, which was lack of the C-terminal region and could not form dimer (Fig. 5B). Native PAGE was performed to confirm the dimerization of TRBP-DC (Fig. S3). The dimer of TRBP-DC exhibits smear bands with a decreased migration compared to TRBP-DC monomer. The result might suggest that the TRBP-DC could form different polymers in vitro.

**TRBP and eIF6 are involved in dsRNA-induced RNA silencing**

In our previous study, TRBP was show to be involved in antiviral innate immunity of shrimp [20]. Given the fact that both TRBP and eIF6 is components of RISC, we further investigated the roles of TRBP and eIF6 in dsRNA-induced silencing in shrimp. To clarify this question, dsRNA was prepared to knockdown the MjTRBP or MjeIF6. In Fig. 6 A and B, injection of MjTRBP or MjeIF6 dsRNA (dsTRBP, dsEIF6) can significantly knock down the transcription of MjTRBP or MjeIF6 in shrimp respectively, comparing to the shrimp injected with GFP dsRNA (dsGFP).

To examine whether silencing of MjTRBP, MjeIF6 affects the RNAi pathway, the dsRNA of Mj-prophenoloxidase (dsPO) was prepared, and then injected with or without dsTRBP, dsEIF6 and dsGFP. In Fig. 6D, agarose gel electrophoresis were performed to ensure the same amount of dsRNA was injected. Injection of dsPO could significantly down regulate mRNA level of MjPO. However, the mRNA level of MjPO still remained high when the dsPO were injected with either dsTRBP or dsEIF6 (Fig. 6 C), implying dsRNA induced RNA silence was impaired by knocking down TRBP or eIF6. Therefore, our results indicated that both MjTRBP and MjeIF6 played crucial roles in the dsRNA/siRNA induced RNA silence.

In another experiment, recombinant proteins (Fc-TRBP, TRBP-DA, DB, DC, DAB, DBC, Ha-AK as control) and WSSV (8.0 ×10^7 copies/shrimp) were co-injected into the shrimp. After 24 h, the titer of WSSV decreases remarkably in shrimp injected with TRBP (about 2.33 ×10^7 copies/g tissues) compared with the control group (about 4.06 ×10^7 copies/g tissues) (Fig. 7B), whereas the replication of WSSV in shrimp injected with TRBP fragments is not significantly different from the control group, implying that the full length of TRBP is required for the antiviral function.

**Discussion**

In the RNAi pathway, dsRNA was processed into siRNA by Dicer, and then siRNA was assembled into large ribonucleoprotein complexes called RISCs, the core of which is composed of Dicer, Ago2, and TRBP [2,22,23]. The siRNA-induced gene silencing mechanism is conserved in a wide range of eukaryotic organisms from plants to mammals. Since the discovery of dsRNA-mediated RNAi in C. elegans in 1998 [24], more evidence

![Figure 4. Gel-shift assays that identify the dsRNA-binding activity of recombinant dsRBDs of MjTRBP and full length recombinant TRBP. 0.5 μg (up) and 1.5 μg (down) protein were incubated with 2.5 μg 193-bp dsRNA. Ha-AK served as the control. doi:10.1371/journal.pone.0030057.g004](image-url)
have supported the important roles of RNAi in invertebrates, plants, and fungi [25]. DsRNAs produced by viruses during their infectious cycles [9] serve as the substrate of Dicer, thus triggering antiviral RNAi response. Blocking RNAi pathway leads to higher virus susceptibility and lethality in Drosophila [26, 27, 28]. DsRNA-mediated antiviral RNAi has been reported in plants, flies, worms, and shrimps [1, 11, 25]. In this study, TRBP and eIF6 were proven to be involved in the dsRNA-induced RNAi pathway. Silence of either TRBP or eIF6 significantly impairs the RNAi pathway and increases WSSV replication. These results indicate that TRBP-DC mediates the dimerization of full length TRBP. The C-terminal of TRBP and TRBP-DC to dsRNA in shrimp. TRBP-DAB binds dsRNA uncooperatively and exhibits lower affinity. C-terminal dsRBD of TRBP mediate the dimerization of TRBP, resulting in a higher affinity of TRBP to dsRNA compared to TRBP-DAB. 

Figure 5. A: Pull-down assays showing that TRBP-DC mediates the homo-dimerization of TRBP in shrimp. Left: Recombinant His-tagged TRBP-DC was incubated with His-Bind resin, and then TRBP-DABHis-tag was added. After stringent wash, only TRBP-DC was eluted, indicating TRBP-DAB could not bind to TRBP-DC. Middle: Pull down assay was performed to identify the interaction between TRBP-DC and full length TRBP, and both proteins could be eluted, suggesting the interaction between TRBP-DC and full length TRBP. Right: pull down assay show TRBP-DAB could not bind to full length TRBP. These results indicate that TRBP-DC mediates the dimerization of full length TRBP. B: Illustration of the differences in affinity of full length TRBP and TRBP-DC to dsRNA in shrimp. TRBP-DAB binds dsRNA uncooperatively and exhibits lower affinity. C-terminal dsRBD of TRBP mediate the dimerization of TRBP, resulting in a higher affinity of TRBP to dsRNA compared to TRBP-DAB. doi:10.1371/journal.pone.0030057.g005

TRBP stabilize the Ago2–Dicer interaction, and Dicer, TRBP, and Ago2 are required for siRNA- and miRNA-mediated RNAi [2, 4, 6, 8, 29, 30]. Chendrimada et al. isolated a large TRBP-containing complex that includes Dicer, Ago2, MOV10, eIF6, and 60S ribosome proteins [8, 31, 32]. The C-terminal domain of human TRBP (referred to as the Medipal domain) mediates protein–protein interactions with PKR, Dicer, PACT etc [19, 33, 34, 35, 36, 37]. Results of this study showed that TRBP-DB and TRBP-DC mediate the association of Mj-TRBP with eIF6 in shrimp. The dsRBD C of Mj-TRBP is very similar to the Medipal domain of human TRBP and is suggested to have a similar function in protein binding (Fig. S4). The C-terminal of TRBP is reported to contain a 69aa Dicer binding site [17], mediating the association with Dicer, and is essential for antiviral RNAi. Our results are consistent with Parker’s report [38], in which the C-terminal of RDE-4 is required for Dicer activity in C.elegans, and the mutant in C-terminal of RDE-4 blocks siRNA production. Our results show that injection of the TRBP protein inhibits the proliferation of virus, but injection of TRBP-DAB not, implying a role of C-terminal dsRBD of TRBP in antiviral immunity. Our results also indicate that the full length of TRBP is required for the antiviral function of TRBP.
dsRBD2 of human TRBP is necessary to bind HIV transactivation response (TAR) RNA upper-stem/loop site, and the two Arg and Lys residues are important for the RNA-binding activity [35,36,37]. However, results of this study showed that the N-terminal dsRBD of Mj-TRBP has a high dsRNA affinity and TRBP-DB can only weakly bind to dsRNA. There are 5 different residues in the equivalent peptides of TRBP-DB (KKLAKRQAAYKMTQL) and TR13, where the replacement of an Arg (R) by Leu (L) might lead to the low dsRNA affinity of TRBP-DB (Fig. S3 boxed sequence). The equivalent peptide in TRBP-DA (KKKAKHAAAKAVL) does not contain Arg. Further study is needed to clarify the high dsRNA affinity of TRBP-DA.

The gel-shift assays showed that the dsRNA affinity of full length TRBP is much higher than the affinity of TRBP-DAB, and then pull-down assays showed that TRBP can form dimers in the C-terminal region (TRBP-DC), allowing for the cooperative binding of Mj-TRBP to dsRNA (Fig. 5B). TRBP is capable of binding to itself to form dimer [3,41,42]. Paker et al. reported that RDE-4, a TRBP homologue in C. elegans, is a homodimer in solution, and the C-terminal domain of RDE-4 is required for dimerization. RDE-4 displays higher affinity to longer dsRNA.
because multiple RDE-4 form long clusters along the dsRNA [15,38]. Results of this study showed that TRBP in shrimp is homo-dimerized by the C-terminal region and display a higher affinity to dsRNA than TRBP-DB.

In conclusion, this study found that the TRBP-DA of Mj-TRBP strongly binds to dsRNA, while TRBP-DB only weakly binds to dsRNA. The C-terminal region (TRBP-DC) increases TRBP affinity to dsRNA by mediating dimerization of TRBP and that dsRNA. The C-terminal region (TRBP-DC) increases TRBP strongly binds to dsRNA, while TRBP-DB only weakly binds to dsRNA. Both Mj-TRBP and Mj-eIF6 are necessary for dsRNA-induced silencing and play important roles in antiviral immunity in shrimp.

Supporting Information

Figure S1 Multiple alignments of Fc-TRBP1 (GenBank no. EU679001) with Mj-TRBP1-3.

Figure S2 Multiple alignments of Fc-eIF6 (GenBank no. EU679001) with Mj-eIF6.

Figure S3 Native PAGE and Western blot was performed to confirm the dimerization of TRBP-DC(left panel). After Native PAGE of TRBP-DA, DB and DC, the proteins in the PAGE gel were transferred onto a nitrocellulose membrane and were detected with TRBP antibody. TRBP-DA and DB were used as control. TRBP-DC dimer exhibits a smear bands above the band of DC monomer. SDS-PAGE and Western blot were performed to verify the identity of the bands (right panel).

Figure S4 Multiple alignments of Mj-TRBP (GenBank no. HM149251) amino acid sequences from other animals. Two TR13 sequence of human TRBP and the equivalent peptides in other animal are marked with a box. The following sequences were selected from GenBank: F. c. (EU679001), Aedes aegypti (XP_001659426.1), D. rerio (NP_956291.1), Drosophila melanogaster (NP_609646.1), Homo sapiens (AAP66731.1), Mus musculus (AAH020218.1), Rattus norvegicus (NP_001030113.1), Xenopus tropicalis (NP_001025646.1). Dark shadow: identity = 100%; Grey shadow: identity ≥ 75%; Light grey shadow: identity ≥ 50%. The alignment was performed by DNAMan 3.1.

Author Contributions

Conceived and designed the experiments: JXW SW. Performed the experiments: SW AJC LJS. Analyzed the data: SW JXW. Contributed reagents/materials/analysis tools: JXW XFZ. Wrote the paper: SW JXW. Performed the experiments and writing: SW.

References

1. Robalino J, Bartlett TC, Chapman RW, Gross PS, Browsky CL, et al. (2007) Double-stranded RNA and antiviral immunity in marine shrimp: inducible host mechanisms and evidence for the evolution of viral counter-responses. Dev Comp Immunol 31: 539–547.
2. Chendrimada TP, Gregory RI, Kamaraswamy E, Norman J, Gooch N, et al. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436: 740–744.
3. Haase AD, Jaskiewicz L, Zhang H, Laine S, Sack R, et al. (2005) TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep 6: 961–967.
4. Rossi JJ (2005) Mammalian Dicer finds a partner. EMBO Rep 6: 927–929.
5. De N, Macae J. Purification and Assembly of Human Argonaute, Dicer, and TRBP Complexes. Methods Mol Biol 725: 107–119.
6. MacRae JI, Ma E, Zhou M, Robinson CV, Doudna JA (2008) In vitro reconstitution of the human RISC-loading complex. Proc Natl Acad Sci U S A 105: 512–517.
7. Suk K, Choi J, Suzuki Y, Otsuki SB, Mello JC, et al. Reconstitution of human RNA interference in budding yeast. Nucleic Acids Res 39: e49.
8. Chendrimada TP, Fan KJ, Ji X, Baillat D, Gregory RI, et al. (2007) MicroRNA silencing through RISC recruitment of eIF6. Nature 447: 823–828.
9. Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR (2006) Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol 80: 5059–5064.
10. Roux MM, Pain A, Klippel KR, Dhar AK (2002) The lipopolysaccharide and beta-1,3-glucan binding protein gene is upregulated in white spot virus-infected shrimp (Penaeus stylirostris). J Virol 76: 7140–7149.
11. Robalino J, Bartlett T, Shepard E, Prior S, Jaramillo G, et al. (2005) Double-stranded RNA induces sequence-specific antiviral silencing in addition to non-specific immunity in a marine shrimp: convergence of RNA interference and innate immunity in the invertebrate antiviral response? J Virol 79: 13561–13571.
12. Tirasophon W, Roshom Y, Panyim S (2005) Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. Biochem Biophys Res Commun 334: 102–107.
13. Yodmuang S, Tirasophon W, Roshom Y, Chinnarutong W, Panyim S (2006) YHV-protease dsRNA inhibits YHV replication in Penaeus monodon and prevents mortality. Biochem Biophys Res Commun 341: 351–356.
14. Gatignol A, Buckler-White A, Berkhout B, Jeang KT (1991) Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. Science 251: 1597–1600.
15. Parker GS, Maity TS, Bass BL (2008) dsRNA binding properties of RDE-4 and TRBP reflect their distinct roles in RNAi. J Mol Biol 384: 967–979.
16. Saunders LR, Barber GN (2003) The dsRNA binding protein family: critical roles, diverse cellular functions. FASEB J 17: 961–983.
17. Daniels SM, Melendez-Pena CE, Scarborough RJ, Daher A, Christensen HS, et al. (2009) Characterization of the TRBP domain required for dicer interaction and function in RNA interference. BMC Mol Biol 10: 38.
18. Gupta V, Huang X, Patel RC (2003) The carboxy-terminal, M3 motifs of PACT and TRBP have opposite effects on PKR activity. Virology 313: 283–291.
19. Laraki G, Clerzius G, Daher A, Melendez-Pena C, Daniels S, et al. (2008) Interactions between the double-stranded RNA-binding proteins TRBP and PACT define the Medipal domain that mediates protein-protein interactions. RNA Biol 5: 92–103.
20. Wang S, Liu N, Chen AJ, Zhao XF, Wang JX (2009) TRBP homolog interacts with eukaryotic initiation factor 6 (eIF6) in Fenneropenaeus chinensis. J Immunol 182: 3250–3256.
21. Liu H, Jiravanichpaisal P, Soderhall I, Cerenius L, Soderhall K (2006) Antilipopolysaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish Pacifastacus leniusculus. J Virol 80: 10363–10371.
22. Bernstein E, Candy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363–366.
23. Rana TM (2007) Illuminating the silence: understanding the structure and function of small RNAs. Nat Rev Mol Cell Biol 8: 23–36.
24. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, et al. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391: 806–811.
25. Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. Cell 130: 413–426.
26. Aliyari R, Wu Q, Li HW, Wang XH, Li F, 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.
27. 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.
28. van Rij RP, Saleh MC, Berry B, Foo C, Houk A, et al. (2006) The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev 20: 2985–2995.
29. Duarte M, Graham K, Daher A, Battisti PL, Bannwarth S, et al. (2000) Characterization of TRBP1 and TRBP2. Stable stem-loop structure at the 5’ end of TRBP2 mRNA resembles HIV-1 TAR and is not found in its processed pseudogene. J Biomed Sci 7: 494–506.
30. Wang HW, Noland C, Siridechadilok B, Taylor DW, Ma E, et al. (2009) Structural insights into RNA processing by the human RISC-loading complex. Nat Struct Mol Biol 16: 1148–1153.
31. Cook HA, Koppetsch BS, Wu J, Theurkauf WE (2004) The Drosophila SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. Cell 116: 817–829.
32. Tomari Y, Da T, Haley B, Schwarz DS, Bennett R, et al. (2004) RISC assembly defects in the Drosophila RNAi mutant armitage. Cell 116: 831–841.
33. Chang KY, Ramos A (2005) The double-stranded RNA-binding motif, a versatile macromolecular docking platform. FEBS J 272: 2109–2117.
34. Daher A, Laraki G, Singh M, Melendez-Pena CE, Bannwarth S, et al. (2009) TRBP control of PACT-induced phosphorylation of protein kinase R is reversed by stress. Mol Cell Biol 29: 234–243.
35. Daviet L, Erard M, Droein D, Duarte M, Vaquero C, et al. (2000) Analysis of a binding difference between the two dsRNA-binding domains in TRBP reveals the modular function of a KR-helix motif. Eur J Biochem 267: 2419–2431.
36. Erard M, Barker DG, Amalric F, Jeang KT, Gatignol A (1998) An Arg/Lys-rich core peptide mimics TRBP binding to the HIV-1 TAR RNA upper-stem/loop. J Mol Biol 279: 1085–1099.
37. Gatignol A, Buckler C, Jeang KT (1993) Relatedness of an RNA-binding motif in human immunodeficiency virus type 1 TAR RNA-binding protein TRBP to human PI/dsI kinase and Drosophila staufen. Mol Cell Biol 13: 2195–2202.
38. Parker GS, Eckert DM, Bass BL (2006) RDE-4 preferentially binds long dsRNA and its dimerization is necessary for cleavage of dsRNA to siRNA. RNA 12: 807–818.
39. Eamens AL, Smith NA, Curtin SJ, Wang MB, Waterhouse PM (2009) The Arabidopsis dhl2 and dhl3 genes are required for accumulation of small interfering RNA and for silencing of viral RNA. Mol Plant Microbe Interact 22: 310–320.
40. Yamashita S, Nagata T, Kawae K, Takego M, Kikawa T, et al. (2001) Structures of the first and second double-stranded RNA-binding domains of human TAR RNA-binding protein. Protein Sci 10: 118–130.
41. Cosentino GP, Venkatesan S, Beresford FC, Green SR, Mathews MB, et al. (1995) Double-stranded-RNA-dependent protein kinase and TAR RNA-binding protein form homo- and heterodimers in vivo. Proc Natl Acad Sci U S A 92: 9445–9449.
42. Hinti EG, Sallaz NC, Scobey VC, Jantsch MF (2004) Oligomerization activity of a double-stranded RNA-binding domain. FEBS Lett 574: 25–30.