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The Dicer from oyster *Crassostrea gigas* functions as an intracellular recognition molecule and effector in anti-viral immunity

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ABSTRACT

Dicer, as a member of ribonuclease III family, functions in RNA interference (RNAi) pathway to direct sequence-specific degradation of cognate mRNA. It plays important roles in antiviral immunity and production of microRNAs. In the present study, a Dicer gene was identified from oyster *Crassostrea gigas*, and its open reading frame (ORF) encoded a polypeptide (designed as *Cg*Dicer) of 1873 amino acids containing two conserved ribonuclease III domains (RIBOc) and a double-stranded RNA-binding motif (DSRM). The deduced amino acid sequence of *Cg*Dicer shared identities ranging from 18.5% to 46.6% with that of other identified Dicers. The mRNA transcripts of *Cg*Dicer were detectable in all the examined tissues of adult oysters, with the highest expression in hemocytes (11.21 ± 1.64 fold of that in mantle, $p < 0.05$). The mRNA expression level of *Cg*Dicer in hemocytes was significantly up-regulated (36.70 ± 11.10 fold, $p < 0.01$) after the oysters were treated with double-stranded RNA (dsRNA). In the primarily cultured oyster hemocytes, the mRNA transcripts of *Cg*Dicer were significantly induced at 12 h after the stimulation with poly(I:C), which were 2.04-fold ($p < 0.05$) higher than that in control group. Immunocytochemistry assay revealed that *Cg*Dicer proteins were mainly distributed in the cytoplasm of hemocytes. The two most important functional domains of *Cg*Dicer, DSRM and RIBOc, were recombinant expressed in *Escherichia coli* transetta (DE3), and the recombinant DSRM protein displayed significantly binding activity to dsRNA and poly(I:C) *in vitro*, while the recombinant RIBOc protein exhibited significantly dsRNase activity to cleave dsRNA *in vitro*. These results collectively suggested that *Cg*Dicer functioned as either an intracellular recognition molecule to bind dsRNA or an effector with ribonuclease activity, which might play a crucial role in anti-viral immunity of oyster.

1. Introduction

Viruses are stealth invaders that hijack the host machinery to replicate, and cause disease. Multicellular organisms have evolved various sophisticated defense systems against diverse viruses, such as clustered regularly interspaced short palindromic repeats/Cas (CRISPR-Cas) system, RNA interference (RNAi) system, and interferon (IFN) system [1]. RNAi system is an evolutionally highly conserved mechanism for gene silencing, which plays an important role in recognizing the virus components and inducing anti-viral effectors [2–4]. In RNAi anti-viral immunity, virus-derived RNA is firstly recognized by Dicer, which could then degrade the double-stranded RNA (dsRNA) into short (21–23 oligonucleotides) small interfering RNAs (siRNAs) [5]. Subsequently, the siRNAs bind to Argonaute and form an RNA-induced silencing complex (RISC) to destroy the complementary mRNAs in an cascade amplification manner [6].

As a major component of RNAi system, Dicer is a member of the ribonuclease III (RNase III) family, which recognizes the 5′ and 3′ helical ends of dsRNA substrates and cleaves at a specific distance to produce 21–27 nt products [7]. Dicer contains conserved helicase domain, PAZ (Piwi/Argonaute/Zwille) domain, dsRNA binding domain, and RNase III domain [8]. The structures of Dicer proteins are complicated, and they have structural differentiation during the evolution [9]. There is only one Dicer protein reported in vertebrates, which produces both miRNAs and siRNAs [9]. In *Drosophila melanogaster*, there are two Dicers, of which Dicer-1 cleaves stem-loop RNA

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precursors (pre-miRNAs) to generate miRNAs and Dicer-2 cleaves longer dsRNA with perfect complementarity to produce siRNA [10]. The domain structures of Dicer or Dicer-like proteins from some lower eukaryotes are simpler than those from higher eukaryotes. For example, there are only one PAZ and two RNase III domains in the Dicer from *Giardia intestinalis* [11]. In *Caenorhabditis elegans*, Dicer contains an N-terminal DExH/DEAD-box type RNA helicase domain, two RNase III-like domains, and a C-terminal dsRNA binding motif [12]. So far, six Dicers have been reported in plants [13]. There are four Dicer homologues (Dicer-likes, DCLs) identified in *Arabidopsis thaliana*, five DCLs in poplar and six DCLs in rice [13]. The notable expansion of DCL family members in monocot and dicot plants may reflect the deployment of RNA silencing approach in antiviral defense [14].

Dicer, as a sensor of viral nucleic acid, is presumed to be involved in antiviral immune responses. It can recognize virus-derived RNA as pathogen-associated molecular patterns (PAMPs), and then initiate the RNAi pathway to clear invading virus [15]. A flexible linker of 7 aa between the RNase III and dsRBD domains in Dicers allow induced fit of the relative orientation of the two domains upon binding dsRNA. The highly conserved lysines in the RNase IIIa and IIIb domains play a critical role in the phosphodiester bond cleavage reaction [16]. In mammalian, Dicer could dramatically inhibit adenovirus (Ad) replication by cleavage Ad-encoding small RNAs (VA-RNAs) [17]. Dicer is an indispensable antiviral protein in many organisms including invertebrates and plants [15]. The disease susceptibility to cricket paralysis virus (CrPv) increased in the Dicer-1 mutant *D. melanogaster* [18]. Knockdown of Dicer-1 lead to higher loads of gill-associated virus (GAV) and increasing mortalities of shrimp *Penaeus monodon* [19]. In *Litopenaeus vannamei*, the mRNA expression level of *Lv*Dicer-1 was up-regulated in both hemocytes and gills after Taura syndrome virus (TSV) infection [20]. In plants, there may be some functional overlap among Dicer paralogs, particularly in the case of antiviral Dicers, where one Dicer may compensate for loss of a paralog's function [21]. DCL-1 from *A. thaliana* is responsible for the production of miRNAs, while DCL-2, DCL-3 and DCL-4 are involved in the processing of long dsRNA [22].

The Pacific oyster (*Crassostrea gigas*) is an important economic mollusk cultured world widely. Unfortunately, the frequent outbreaks of viral diseases have caused large-scale mortalities and substantial economic losses to oyster aquaculture [23]. Since oysters lack the lymphocyte-mediated adaptive immunity, and they only possess innate immunity which is a common character of both vertebrates and invertebrates [24]. The conserved RNAi is postulated to be an ancient and important immune mechanism to provide effective protection against viral invasion. It has been reported that dsRNA could be processed in oysters, and long dsRNAs could promote an anti-viral response in Pacific oyster and hampering ostreid herpesvirus 1 replication [25,26]. However, the cellular and molecular machinery of antiviral RNAi defense system and the involvement of dsRNA processing in antiviral immunity are still not well understood in oyster. In the present study, a Dicer (*Cg*Dicer) was identified from oyster *C. gigas* with the aims to (1) characterize its molecular features and phylogeny, (2) monitor its mRNA expressions during early developmental stages and responses after immune stimulation in adult oysters, and (3) determine its dsRNA binding and processing activities.

2. Materials and methods

2.1. Experimental animals

Adult Pacific oysters (*C. gigas*), about 180 g in weight and 12–16 cm in length, were collected from a local aquaculture farm in Dalian, Liaoning Province, China. The oysters were cultured for 1–2 weeks in tanks with continuously aerated seawater at 15 ± 2 °C prior to the experiment. Female mice (six-week old, SPF Kunming mice) used for preparing polyclonal antibody were purchased from Dalian Institute of Drug Control.

2.2. Sample collection

After temporarily acclimatized, nine blank oysters were sacrificed and their hemocytes were harvested from hemolymph by centrifugation at 800 *g*, 4 °C for 10 min. The tissues including mantle, gills, hepatopancreas, lip and adductor muscle were also collected randomly as three parallel samples (three individuals in each parallel). All samples were stored at −80 °C in 1 mL TRIzol™ reagent (Invitrogen, California, USA) for RNA extraction, gene cloning and quantitative real-time PCR (qRT-PCR).

2.3. Primary hemocytes culture and immune stimulation

The primary hemocytes were cultured *in vitro* for the stimulation with poly(I:C) (Polyinosinic–polycytidylic acid potassium salt, Sigma, USA, P9582). Hemolymphs from 20 blank oysters were collected with anticoagulant (20.8 $g{\cdot}L^{-1}$ glucose, 8.0 $g{\cdot}L^{-1}$ sodium citrate, 3.36 $g{\cdot}L^{-1}$ EDTA, 22.5 $g{\cdot}L^{-1}$ NaCl, pH 7.5) and centrifuged immediately at 800 *g*, 4 °C for 10 min to harvest the hemocytes. The isolated hemocytes were gently resuspended in Leibovitz's L-15 medium (Gibco, USA) supplemented with 1% antibiotics (10,000 $\mu g{\cdot}mL^{-1}$ streptomycin, 10,000 $U{\cdot}mL^{-1}$ penicillin, Gibco, USA) and additional salts (20.2 $g{\cdot}L^{-1}$ NaCl, 0.54 $g{\cdot}L^{-1}$ KCl, 0.6 $g{\cdot}L^{-1}$ $CaCl_2$, 3.9 $g{\cdot}L^{-1}$ $MgCl_2$, 1 $g{\cdot}L^{-1}$ $MgSO_4$, pH 7.2–7.4) [27], and subsequently seeded in 12-well cell culture plate (Corning, USA) at a density of 6×10^5 $cells{\cdot}mL^{-1}$. For poly(I:C) immune stimulation, 14 μL poly(I:C) (5 $mg{\cdot}mL^{-1}$ in PBS) was added into 1.4 mL culture medium and incubated with primary hemocytes at 16 °C. The hemocyte samples from each experimental group were collected at 3, 6, 12, 24 and 48 h after stimulation (N = 3) for subsequent RNA extraction. The untreated primary hemocytes were used as control.

For the dsRNA stimulation, six oysters were randomly divided into two groups with three individuals in each group. The oysters in the treatment group received individually an injection of 100 μL dsRNA (1000 $ng{\cdot}\mu L^{-1}$ in DEPC-treated water). The dsRNA was *in vitro* synthesized with eGFP DNA fragment (653 bp) as template by using T7 polymerase (Takara) according to the manufacture. The untreated oysters were used as control. The hemocyte samples from each group were collected at 12 h after stimulation (N = 3) for subsequent RNA extraction.

2.4. RNA extraction, cDNA synthesis

Total RNA was extracted from samples using TRIzol™ reagent (Invitrogen, USA). In brief, the samples in 1 mL TRIzol™ reagent were homogenized with lapping rod (hemocytes were broken by repeatedly pipetting up and down with 1 mL syringe), and 0.2 mL chloroform was then added into the tissue homogenate to separate total RNA. After precipitating with isopropanol, the total RNA was washed twice with 75% ethanol, vacuum dried and resuspended in RNase-free water for cDNA synthesis.

The cDNA synthesis was performed using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's instruction. The first-strand cDNA synthesis was performed with primer oligo (dT) (all the information of cloning primers used in this study was listed in Table 1). About 1 μg total RNA was added into the 20 μL reaction system. The mixture was incubated in 37 °C for 15 min, 85 °C for 15 s. The cDNA mix was diluted to 1:20 and stored at −80 °C for next processing.

2.5. Gene cloning and sequence analysis of CgDicer

A gene encoding Dicer was identified (designed as *Cg*Dicer, NCBI No. CGI_10015093) from the genome of oyster *C. gigas*. The nucleic acid sequence information of *Cg*Dicer in oyster was retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). A pair of gene specific primers, *Cg*Dicer-F and *Cg*Dicer-R

Table 1
Primers used in this study.

Primer name	Sequence (5′— 3′)
Clone primers	
Oligo (dT)	GGCCACGCGTCGACTAGTACT
*Cg*Dicer-F	ATCAGAGGTCCAAAAATAAATC
*Cg*Dicer-R	CAAATCCTTCAGTCTGTTACCCTT
RT primers	
*Cg*Dicer-RTF	ATAGATGTCCCTAAATGTAACCTCATAGTCA
*Cg*Dicer-RTR	CATGTATGGCAGTAGAACTTTAGATGTGTCC
*Cg*EF-RTF	AGTCACCAAGGCTGCACAGAAAG
*Cg*EF-RTR	TCCGACGTATTTCTTTGCGATGT
Recombination primes	
r*Cg*Dicer-RIBOcF	CGCGGATCCTGTACCATATTGCAAGCTTTAACC
r*Cg*Dicer-RIBOcR	CCGCTCGAGCAGCAGGTATTTGTCTATTTGCG
r*Cg*Dicer-DSRMF	CCGGAATTCAGCATTCCAAAGTCTCCAGTAC
r*Cg*Dicer-DSRMR	CCGCTCGAGTACAAGTCCGCCAGCTTG

(Table 1), were designed to amplify the open reading frame (ORF) sequence of *Cg*Dicer by using LaTaq DNA polymerase (Takara). The PCR products were inserted into pMD19-T simple vector (Takara), and confirmed by nucleotide sequencing. The resulting sequences were analyzed by using BLAST algorithm (http://www.ncbi.nlm.gov/blast/) and subjected to cluster analysis.

A translation tool (http://web.expasy.org/translate) was used to predict the amino acid sequence, theoretical molecular weight, and isoelectric point of *Cg*Dicer. The protein sequences of Dicers from different species were obtained from NCBI databases. The protein domains of Dicers were predicted by using online software the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). Multiple sequence alignment analysis of Dicers was performed with the ClustalW multiple alignment programs (http://www.ebi.ac.uk/clustalw/). The phylogenetic tree was constructed by the Neighbor-Joining (NJ) method with the software of MEGA 6.0. The results were tested for reliability over 1000 bootstrap replicates.

2.6. Quantitative real-time PCR (qRT-PCR) analysis

The mRNA distribution of *Cg*Dicer in different tissues and its temporal expression in hemocytes after immune stimulation were examined by SYBR Green fluorescent qRT-PCR using the ABI Quantstudio Sequence Detection System (Applied Biosystems, USA). Two specific primers, *Cg*Dicer-RTF and *Cg*Dicer-RTR (Table 1), were used to amplify a 264 bp fragment. The oyster elongation factor (CgEF) (GenBank accession No. NM_001305313) fragment, amplified with a pair of primers *Cg*EF-RTF and *Cg*EF-RTR (Table 1), was employed as internal reference. The experiment was conducted according to previous description with some modification [28]. The qRT-PCR reaction mixture (10 μL) consisted of 2 × SYBR Green PCR Master mix, 50 × ROX Reference Dye, 0.4 mM each of the forward and reverse primers, and 2 μL of cDNA template. The program of qRT-PCR was operated as follow: 95 °C for 30s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. Dissociation curve analysis was conducted to confirm that the only one product was amplified at the end of each PCR. The relative expression level of *Cg*Dicer mRNA was calculated by the comparative CT ($2^{-\Delta\Delta CT}$) method [29]. All results were given based on relative mRNA expression of mean ± SD (N = 3).

2.7. Recombinant expression and purification of recombinant CgDicer and preparation of its polyclonal antibody

The cDNA fragment encoding the RIBOc domain of *Cg*Dicer was amplified by primers r*Cg*Dicer-RIBOcF and r*Cg*Dicer-RIBOcR (Table 1), of which a *Bam*H I and an *Xho* I site sequences were added to their 5' ends, respectively. The PCR fragments were digested by restriction enzymes *Bam*H I and *Xho* I, and ligated into the same restriction enzyme sites of expression vector pET-30a. The recombinant plasmid (pET-30a-*Cg*Dicer-RIBOc) was transformed into *Escherichia coli* Transetta (DE3) (TransGen Biotech), and the null pET-32a vector without any inserting sequence was selected as a negative control, which could express the 6 × His-tag-Trx protein (designated rTrx) when transformed into *E. coli* Transetta (DE3). The positive transformants were cultured in LB medium at 37 °C with shaking at 180 rpm. When the culture medium reached OD_{600} of 0.4–0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the LB medium at a final concentration of 1 mM, and cultured for additional 6 h to induce the expression of recombinant protein. The recombinant protein of *Cg*Dicer-RIBOc with a 6 × His-tag at C-terminal was harvested from crude extract of bacteria, purified by Ni^{+} affinity chromatography, and dialyzed extensively. The purified recombinant proteins were stored at −80 °C for subsequent experiment.

In order to obtain soluble protein, the cDNA fragment encoding *Cg*Dicer-DSRM was amplified by primers r*Cg*Dicer-DSRMF and r*Cg*Dicer-DSRMR (Table 1). The PCR fragments were digested by restriction enzymes (*Eco*R I and a *Xho* I) and ligated into the same restriction enzyme digested expression vector pGEX-4T-1. The recombinant plasmid (pGEX4T-1-*Cg*Dicer-DSRM) was transformed into *E. coli* Transetta (DE3) (TransGen Biotech) to induce protein expression as mentioned above. And the null pGEX-4T-1 vector without any inserting sequence was selected as a negative control, which could express the GST-tag protein (designated rGST) when transformed into *E. coli* Transetta (DE3). The recombinant protein of *Cg*Dicer-DSRM with a GST-tag was purified by glutathione affinity chromatography and dialyzed extensively. The purified recombinant proteins were stored at −80 °C for subsequent experiment.

To prepare polyclonal antibody, six-week-old mouse were subcutaneously stimulated with r*Cg*Dicer-RIBOc protein according to the previous description [30]. Briefly, r*Cg*Dicer-RIBOc (0.3 mg·mL^{-1}) were emulsified with isometric Freund's complete adjuvant (Sigma, USA) and then used to immunize each female mouse. The second and third immunizations were executed on the 14th and 21st day with incomplete adjuvant (Sigma, USA). The fourth inoculation was executed on the 28th day using purified proteins, and finally the serum was collected on the 35th day. The anti-r*Cg*Dicer serum was stored at −80 °C for subsequent experiment.

2.8. Western blotting assay

The efficiency and specificity of polyclonal antibodies against *Cg*Dicer was verified by western blotting assay. The r*Cg*Dicer-RIBOc protein was separated by SDS-PAGE and further transferred onto the 0.22 μm nitrocellulose membrane (Sangon Biotech, China). After washing with TBS-T (TBS with 0.1% (w/v) Tween 20) for three times (5 min for each time), the membranes were blocked in 5% skim milk powder solution (skimmed milk diluted in TBS-T) at 37 °C for 2 h, followed by the incubation with polyclonal antibody against r*Cg*Dicer (diluted at 1:2000 with 5% skim milk powder solution) at 37 °C for 1 h. After washing with TBS-T for three times, the membranes were incubated with the HRP-linked goat-anti-mouse IgG (Sango Biotech, China) (diluted at 1:2000 with 5% skim milk powder solution) at 37 °C for 1 h followed by three times of washing with TBS-T. Finally, the membrane was incubated in Western lighting ECL substrate system (Thermo Scientific, USA) in dark for 2 min, and captured image by Amersham Imager 600 system (GE Healthcare, Bonston, USA).

2.9. Immunocytochemistry assay

Immunocytochemistry of hemocytes was performed to exam the subcellular localization of *Cg*Dicer. Hemolymphs were collected from three oysters and immediately centrifuged at 800 *g*, 4 °C for 10 min to harvest the hemocytes. The hemocytes were resuspended in L-15 media (with additional saline 20.2 g·L^{-1} NaCl, 0.54 g·L^{-1} KCl, 0.6 g·L^{-1} $CaCl_2$, 1.0 g·L^{-1} $MgSO_4$, 3.9 g·L^{-1} $MgCl_2$) and dropped on polysine

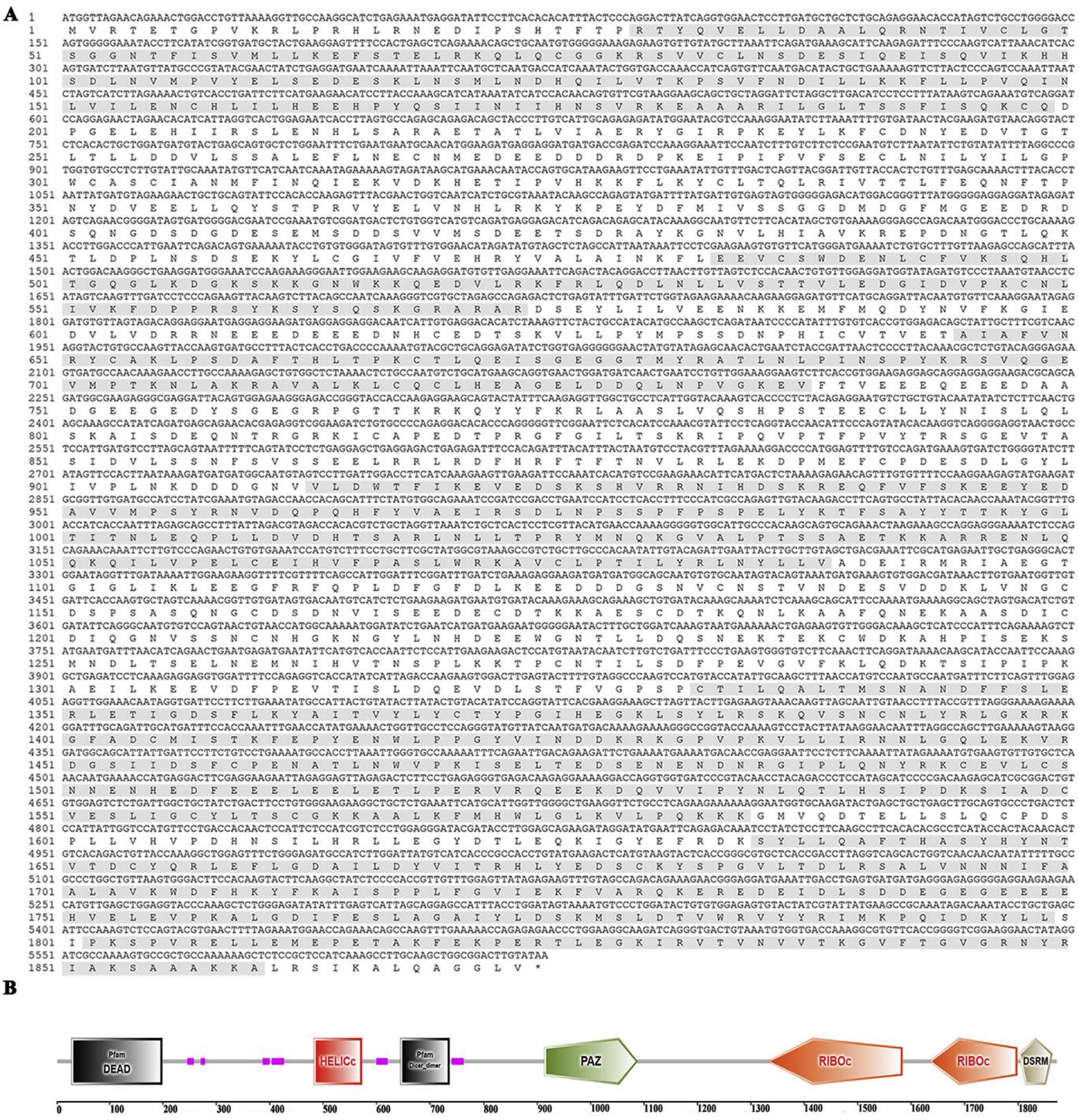

Fig. 1. Sequence features and domain architecture of *Cg*Dicer. A: Nucleotide and amino acid sequences of *Cg*Dicer. The gray lighting sequence presents the N-terminal DEAD domain, helicase domain, dicer_dimer domain, PAZ domain, two RNase III nuclease domains (RIBOc), and double-stranded RNA-binding motif (DSRM) sequentially. B: The domain architecture of *Cg*Dicer were predicted.

microscope adhesion slides (Solarbio, China) for 1 h to form monolayer of cells. After fixed with 4% paraformaldehyde (PFA) for 20 min, the hemocytes were permeabilized with 0.1% Triton X-100 at room temperature for 5 min and washed with 0.01 M PBS-T (PBS with 0.1% (w/v) Tween 20) for three times, then blocked with 3% BSA (Fetal bovine serum albumin diluted in PBS-T) at room temperature for 30 min. The hemocytes were incubated with prepared r*Cg*Dicer-RIBOc polyclonal antibody (with a dilution of 1:500 in 3% BSA) at room temperature for 1 h with the pre-immune serum as negative control. After three times washing with PBS-T, Alexa Fluor 488-conjugated anti-mouse secondary antibody (with a dilution of 1:2000 in 3% BSA) was added and incubated at room temperature in the dark for 1 h followed by washing with PBS-T. DAPI (Beyotime life, with a dilution of 1:10000 in PBS) was used to stain the nucleus. The hemocytes were observed under fluorescence microscope (Axio Imager A2 (ZEISS)).

2.10. Elisa assay

The enzyme linked immunosorbent assay (ELISA) was performed to detect the dsRNA binding activity of *Cg*Dicer according to previous

description with some modification [31]. Briefly, the 96-well microliter plates (Costar) were coated with 1 μg dsRNA or poly(I:C) dissolved in one hundred microliter of carbonate-bicarbonate buffer (0.015 mol·L^{-1} Na_2CO_3, 0.035 mol·L^{-1} $NaHCO_3$, pH 9.6) at 4 °C overnight, respectively. The plate was washed three times (5 min for each time) with TBS-T and then blocked with 3% BSA in TBS-T at 37 °C for 1 h. After three times washing with TBS-T, one hundred microliter of recombinant *Cg*Dicer-DSRM with varied concentration (5 μg·mL^{-1}, 2.5 μg·mL^{-1}, 1.25 μg·mL^{-1}, 0.625 μg·mL^{-1}, 0.3125 μg·mL^{-1}, 0.15625 μg·mL^{-1}) was added in the wells and incubated at 37 °C for 1 h rGST protein (100 μg·mL^{-1}) was added to the wells as negative group at the same time. One hundred microliter of anti-GST-tag monoclonal antibody (1:5000 dilution in 3% BSA, Sangon Biotech, China) and HRP-linked goat-anti-mouse IgG (1:5000 dilution in 3% BSA, Sangon Biotech, China) were added and incubated at 37 °C for 1 h sequentially. After an efficient wash, one hundred microliter of TMB (dihydrochloride) (Solarbio, California, USA) was added to each well and incubated at room temperature in dark for 15 min. The reaction was stopped by adding one hundred microliter of 1 mol·L^{-1} H_2SO_4 per well and the absorbance of OD_{450} was measured by Tecan Infinite M1000 PRO absorbance microplate reader (Tecan, Switzerland). All assays were carried out in triplicate, and the date were presented as mean ± SD (n = 3). The values of r*Cg*Dicer-DSRM group, rGST group and TBS group were recorded as P, N and B, respectively. Samples with P (sample)-B (blank)/N (negative)-B (blank) > 2.1 were considered as positive [32].

2.11. dsRNA cleavage assay

The dsRNA was *in vitro* synthesized with eGFP DNA fragment (653 bp) as template by using T7 polymerase (Takara) according to the manufacture, and the dsRNA cleavage assay was performed according to the previous description with modification [33]. The assay was performed at 37 °C in a total volume of 10 μL with 5 μg r*Cg*Dicer-RIBOc protein, 2 μg dsRNA, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 1 μL RNase inhibitors. rTrx protein was used as negative control at the same time. The reactions were stopped by adding 2 μL gel loading buffer (36% glycerol, 30 mM EDTA, 0.025% SDS, 0.1% xylene cyanol and 0.1% bromphenol blue) on ice. The samples were analyzed by 1% agarose gel electrophoresis.

2.12. Statistical analysis

All the data were shown as mean ± S.D, and two-sample Student's test was performed for the comparisons between groups. Multiple group comparisons were subjected to one-way ANOVA and followed to a Tukey multiple group comparison using Statistical Package for Social Sciences (SPSS) 16.0 Software. The statistically difference was considered as significant at $p < 0.05$.

3. Results

3.1. Identification and characterization of CgDicer

The full-length cDNA sequence of *Cg*Dicer gene was of 5622 bp encoding a peptide of 1873 amino acids (Fig. 1A) with a predicted molecular mass of 213.12 kDa and a theoretical isoelectric point of 5.01. SMART program analysis revealed that *Cg*Dicer contained the conserved domains of Dicer family, including an N-terminal DEAD domain, a helicase domain, a dicer_dimer domain, a PAZ domain, two RNase III nuclease domains (RIBOc), and a double-stranded RNA-binding motif (DSRM) (Fig. 1B). The deduced amino acid sequence of *Cg*Dicer shared high identity with other previously reported Dicer proteins (Table 2), such as 46.6% identity with Dicer of *Mizuhopecten yessoensis* (XP_021347347.1), 39.7% identity with Dicer of *Mus musculus* (NP_683750.2), 39.2% identity with Dicer of *Homo sapiens* (NP_085124.2), 31.0% identity with Dicer-1 of *Drosophila melanogaster* (NP_524453.1), and 18.5% identity with Dicer-2 of *Drosophila melanogaster* (NP_523778.2). Multiple sequence alignment revealed that the functional domains of *Cg*Dicer were highly conserved with that in other species (Fig. 2). The deduced amino acid sequence of PAZ domain in *Cg*Dicer shared identity ranging from 61.02% to 86.93% with that of other species. Most of the key amino acid residues (Phe981, Tyr993, Tyr994, Arg1018, Arg1023 and Glu1058) in the PAZ domain were conserved in *Cg*Dicer (Fig. 2A). The functional domains of RIBOc and DSRM also showed high identity with that from other species. The deduced amino acid sequence of RIBOc domain and DSRM domain in *Cg*Dicer shared identity ranging from 44.12% to 67.01% and 67.69%–92.19% with that of other Dicers, respectively. The catalytic residues (Asp1357, Glu1552, Asp1663 and Glu1765) were identified in the RNase III domain in *Cg*Dicer, which were highly conserved (Fig. 2B–C).

Table 2
Comparison of sequence identity between *Cg*Dicer and other species.

	Species	Percent identity (%)
Vertebrates	*H. sapiens* Dicer	39.2
	M. musculus Dicer	39.7
	B. taurus Dicer	38.8
	G. gallus Dicer	39.5
	D. rerio Dicer	39.3
Arthropods	*D. melanogaster* Dicer-1	31.0
	D. melanogaster Dicer-2	18.5
	A. aegypti Dicer-1	31.5
	A. aegypti Dicer-2	20.2
	P. vannamei Dicer-1	27.2
	P. vannamei Dicer-2	22.2
	P. monodon Dicer-1	27.2
	P. monodon Dicer-2	22.5
Mollusks	*M. yessoensis* Dicer	46.6

3.2. The phylogenetic evolution of CgDicer

Fifteen Dicers from different organisms were structurally and evolutionarily compared and analyzed. As shown in the domain schematic (Fig. 3A), all the Dicers harbored a helicase domain, a dicer_dimer domain, a PAZ domain, two RIBOc domains, and a DSRM domain. One more N-terminal domains (DEAD domain) were existed in *Cg*Dicer, vertebrate Dicers and Dicer-2s from arthropod, but not arthropod Dicer-1s. A phylogenetic tree was constructed depending on the amino acid sequences of these Dicers using neighbor-joining (NJ) method (Fig. 3B). *Cg*Dicer shared a close evolutionary relationship with Dicer from scallop (*Mizuhopecten yessoensis*), and then clustered with Dicers from vertebrates, including human (*Homo sapiens*), cow (*Bos taurus*), mouse (*Mus musculus*), chicken (*Gallus gallus*) and fish (*Danio rerio*), and finally gathered with the Dicer-1s from arthropod including shrimp (*Penaeus vannamei* and *Penaeus monodon*), mosquitoe (*Aedes aegypti*), and fruit fly (*Drosophila melanogaster*). *Cg*Dicer showed relatively far relationship with Dicer-2s from *A. aegypt*, *D. melanogaster*, *P. vannamei*, and *P. monodon*.

3.3. The mRNA expression pattern of CgDicer

The expression of *Cg*Dicer mRNA at different developmental stages was analyzed with the data deposited in the oyster genome database [34], in which the expression level was displayed as value of Reads Per Kilobase per Million mapped reads (RPKM). *Cg*Dicer was highly expressed during embryonic development with the expression levels of 22.05 RPKM in two cells (TC), 15.44 RPKM in four cells (FC), 22.15 RPKM in early morula (EM), and then declined to the basal level (less than 10 RPKM) at the larval stage (Fig. 4A).

In the adult oyster, *Cg*Dicer mRNA could be detected in hemocytes and all the tested tissues including mantle, gills, hepatopancreas, lip

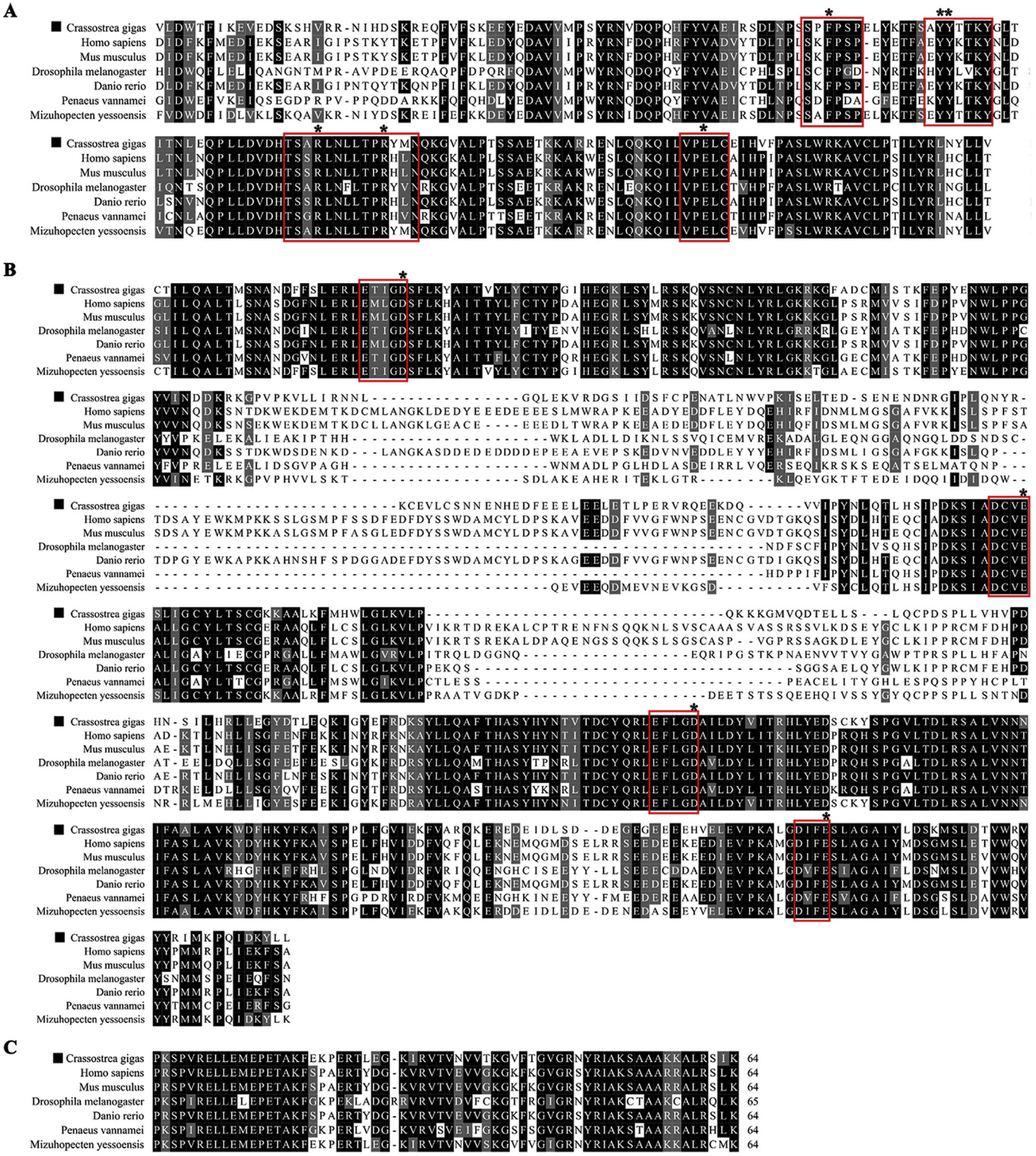

Fig. 2. Multiple sequence alignment of the conserved domains in *Cg*Dicer with that of Dicers from other organisms. A. PAZ domains; B. two Ribonuclease III domains (RIBOc); C. dsRNA binding motif (DSRM). The key amino acid residues are marked by black asterisks (Phe981, Tyr993, Tyr994, Arg1018, Arg1023, Glu1058 in the PAZ domain and Asp1357, Glu1552, Asp1663, Glu1765 in the RIBOc domain). The Dicers used for Multiple sequence alignment include *Homo sapiens* Dicer (NP_085124.2), *Mus musculus* Dicer (NP_683750.2), *Drosophila melanogaster* Dicer-1 (NP_524453.1), *Danio rerio* Dicer (NP_001154925.1), *Penaeus vannamei* Dicer-1 (ACF96960.1), *Mizuhopecten yessoensis* Dicer (XP_021347347.1).

and adductor muscle with the highest level in hemocytes, which was 11.21 ± 1.64 fold ($p < 0.05$) of that in mantle (Fig. 4B). The expression level of *Cg*Dicer mRNA in adductor muscle (6.08 ± 1.38 fold, $p < 0.05$) and lip (4.12 ± 1.65 fold, $p < 0.05$) was also significantly higher than that in mantle (Fig. 4B). There was no significant difference of *Cg*Dicer mRNA expression among the tissues of gills, hepatopancreas, and mantle.

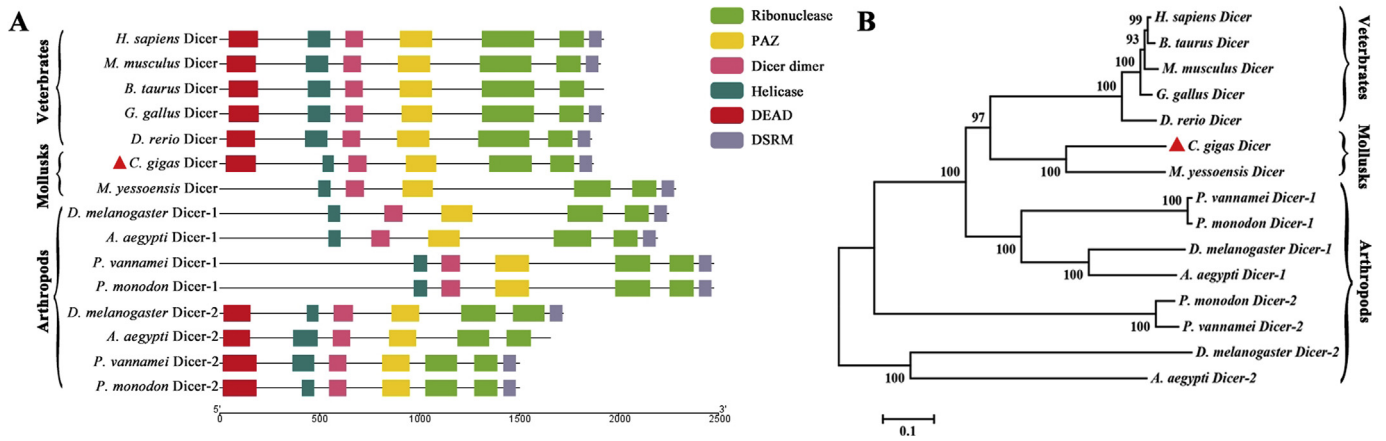

Fig. 3. Structure and evolutionary analysis of *Cg*Dicer with other Dicers. A: Schematic comparison of the structural domains of Dicers among different species. The protein domains of Dicers were predicted by using the online software the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). The Dicers used for structural analysis include: *Homo sapiens* Dicer (NP_085124.2), *Mus musculus* Dicer (NP_683750.2), *Bos taurus* Dicer (NP_976235.1), *Gallus gallus* Dicer (NP_001035555.1), *Danio rerio* Dicer (NP_001154925.1), *Mizuhopecten yessoensis* Dicer (XP_021347347.1), *Penaeus vannamei* Dicer-1 (ACF96960.1), *Penaeus vannamei* Dicer-2 (AEB54796.1), *Penaeus monodon* Dicer-1 (ABR14013.1), *Penaeus monodon* Dicer-2 (AGL08684.1), *Drosophila melanogaster* Dicer-1 (NP_524453.1), *Drosophila melanogaster* Dicer-2 (NP_523778.2), *Aedes aegypti* Dicer-1 (EAT47261.1), *Aedes aegypti* Dicer-2 (AAW48725.1). B: Phylogenetic tree of Dicers from different species. MEGA 6.0 program was used to construct the tree by neighbor-joining (NJ) algorithm based on the deduced amino acid sequences of Dicers.

3.4. The expression pattern of CgDicer mRNA after immune stimulation

To detect the involvement of *Cg*Dicer in the immune response, the expression level of *Cg*Dicer mRNA was examined in the hemocytes after poly(I:C) stimulation *in vitro* and the dsRNA injection *in vivo*. Oyster hemocytes were primarily cultured *in vitro* and stimulated with poly (I:C). The expression level of *Cg*Dicer mRNA was measured by qRT-PCR at 3, 6, 12, 24 and 48 h after stimulation. The expression level of *Cg*Dicer mRNA in oyster hemocytes was up-regulated at 12 h post poly (I:C) stimulation which was 2.04 ± 0.50 fold of that in control group ($p < 0.05$), and then recovered and decreased to 0.41 ± 0.15 fold of that in control group ($p < 0.05$) at 24 h (Fig. 5A). After the oysters received an injection of dsRNA, the mRNA expression level of *Cg*Dicer in hemocytes was up-regulated significantly (36.70 ± 11.10 fold of that in control group, $p < 0.01$) at 12 h (Fig. 5B).

3.5. Recombinant protein and polyclonal antibody of CgDicer

The recombinant plasmid pET-30a-*Cg*Dicer-RIBOc and pGEX-4T-1-*Cg*Dicer-DSRM were transformed into *E. coli* transetta (DE3), and the expression of recombinant proteins were induced by IPTG. The whole cell lysates of positive clones were analyzed by SDS-PAGE, and the distinct bands with a molecular mass of nearly 60 kDa and 35 kDa were revealed as predicted (Fig. 6A and C), respectively. The purified recombinant protein of *Cg*Dicer-RIBOc (r*Cg*Dicer-RIBOc) was employed to immunize mice to prepare polyclonal antibody. A unique and clear band about 60 kDa was revealed by western blotting (Fig. 6B). In the negative control group, no visible band was observed with mouse pre-immune serum (data not shown).

3.6. Subcellular localization of CgDicer in oyster hemocytes

Immunocytochemistry was performed to detect the subcellular localization of endogenic *Cg*Dicer in oyster hemocytes. The nucleus was stained by DAPI in blue, and the positive signals of r*Cg*Dicer-RIBOc were observed in green, which were distributed dominantly in the cytoplasm of hemocytes (Fig. 7).

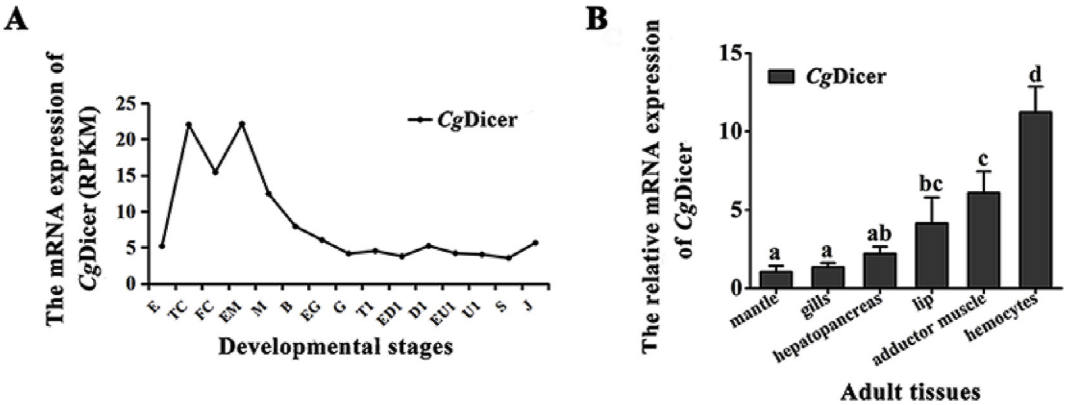

Fig. 4. The expression level of *Cg*Dicer mRNA during embryonic development and in different adult tissues. A: Expression level of *Cg*Dicer mRNA at different developmental stages (depending on oyster genome data. E, egg; TC, two cells; FC, four cells; EM, early morula; M, morula; B, blastula; EG, early gastrula stage; G, gastrula; T1, trochophore 1; ED1, early D-larva 1; D1, D-larva 1; EU1, early umbo larva 1; U1, umbo larva 1; S, spat; and J, juvenile.). The expression level was displayed as value of Reads Per Kilobase per Million mapped reads (RPKM). B: The relative expression level of *Cg*Dicer mRNA in different tissues of oyster detected by qRT-PCR. Vertical bars represent the mean ± S.D. (N = 3) for each tissue. The different letters indicated significant differences comparing with other groups ($p < 0.05$, ANOVA).

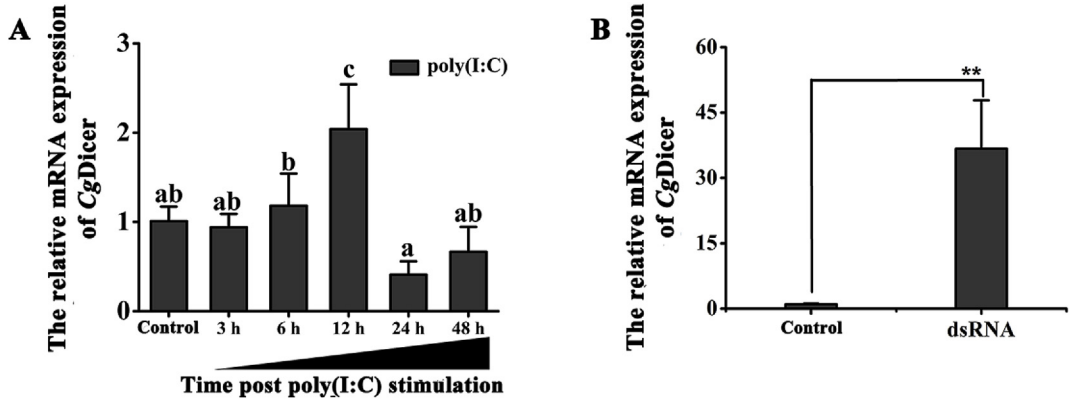

Fig. 5. The expression changes of *Cg*Dicer mRNA after immune stimulations. A: Temporal expression level of *Cg*Dicer mRNA in primary cultured oyster hemocytes after poly(I:C) immune stimulation. B: The expression level of *Cg*Dicer mRNA in oyster hemocytes at 12 h after dsRNA immune stimulation. The untreated primary hemocytes and oyster hemocytes were used as control. Vertical bars represent the mean ± S.D. (N = 3) for each group. The different letters indicated significant differences comparing with other groups ($p < 0.05$, ANOVA). Asterisks indicate significant differences (**: $p < 0.01$).

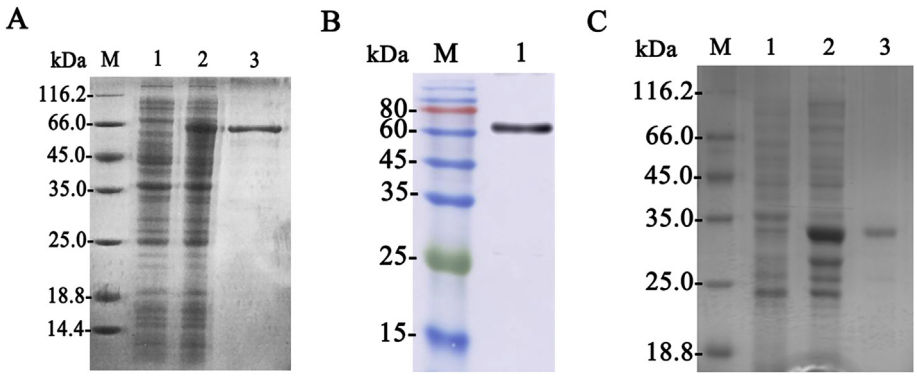

Fig. 6. SDS-PAGE and western-blotting analysis of purified r*Cg*Dicer. A: Lane M: protein molecular standard; lane 1: negative control for r*Cg*Dicer-RIBOc (without induction); lane 2: induced r*Cg*Dicer-RIBOc; lane 3: purified r*Cg*Dicer-RIBOc. B: lane M: protein molecular standard; lane 1: western blotting based on the sample of line3 to assay the specificity of the polyclonal antibody against r*Cg*Dicer-RIBOc. C: Lane M: protein molecular standard; lane 1: negative control for r*Cg*Dicer-DSRM (without induction); lane 2: induced r*Cg*Dicer-DSRM; lane 3: purified r*Cg*Dicer-DSRM.

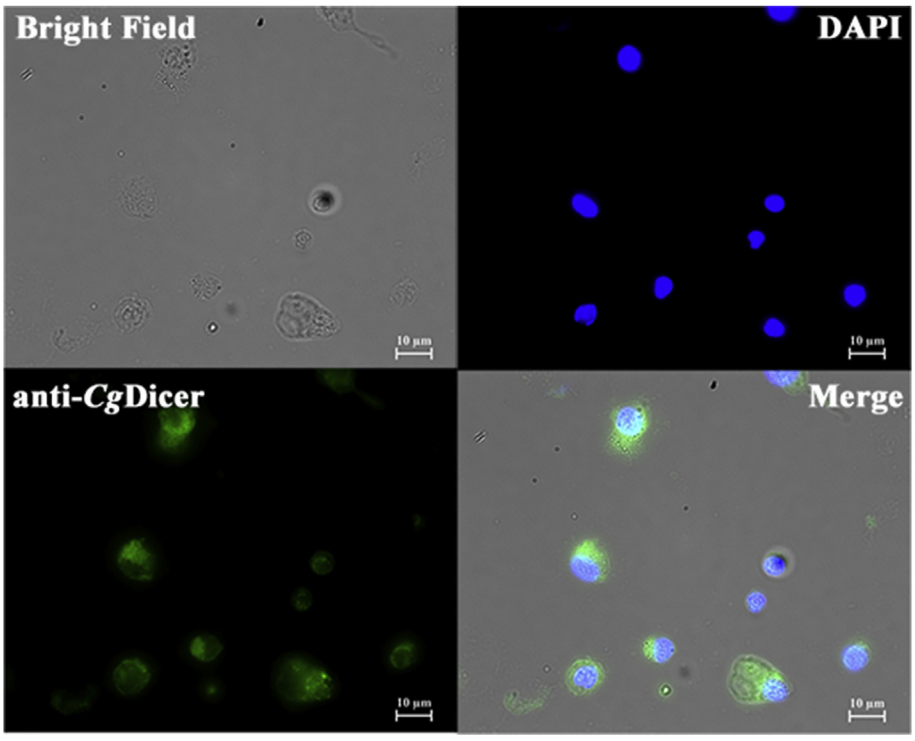

Fig. 7. Subcellular localization of *Cg*Dicer in oyster hemocytes. The morphology of hemocytes is shown in bright field. The nuclei of hemocytes were stained with DAPI shown in blue. Positive signal of *Cg*Dicer was green. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

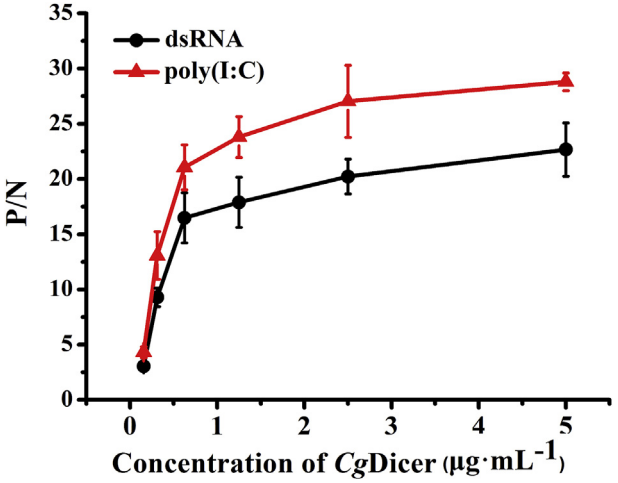

Fig. 8. The dsRNA binding activity of DSRM domain in *Cg*Dicer. The DSRM domain of *Cg*Dicer was prokaryotically expressed and purified, incubated with dsRNA and poly(I:C), respectively. The binding activity of r*Cg*Dicer was examined by ELISA assay. The values of r*Cg*Dicer-DSRM group, rGST group and TBS group were recorded P, N and B, respectively. Samples with P (sample)-B (blank)/N (negative)-B (blank) > 2.1 were considered as positive. Results are representative of average three such experiments.

3.7. The activity of CgDicer to bind dsRNA

To examine the binding activity of *Cg*Dicer toward dsRNA, the recombinant protein of *Cg*Dicer-DSRM (r*Cg*Dicer-DSRM) with GST-tag (Fig. 6C) was incubated with dsRNA and poly(I:C), and the activity of *Cg*Dicer to bind dsRNA was examined by ELISA assays. r*Cg*Dicer-DSRM exhibited strong affinity to poly(I:C) and dsRNA with a dose-dependent manner *in vitro*. With the protein concentration increased from 0.15625 to 5 μg·mL^{-1}, the P/N value for r*Cg*Dicer-DSRM to bind poly(I:C) and dsRNA increased significantly from 4.32 to 28.80 and 3.05 to 22.66, respectively (Fig. 8). No significant binding activity of rGST protein was observed (data not shown).

3.8. The activity of CgDicer to cleave dsRNA

To investigate the dsRNA cleavage activity of *Cg*Dicer, 5 μg r*Cg*Dicer-RIBOc was incubated with 2 μg dsRNA substrate, and the degradation of dsRNA was analyzed by 1% agarose gel electrophoresis. After 90 min incubation with r*Cg*Dicer-RIBOc, the intact dsRNA was gradually reduced over time, while no significant cleavage of dsRNA was observed in the rTRX control group (Fig. 9).

4. Discussion

RNAi is an important and evolutionally conserved anti-viral immune process in which dsRNA triggers the degradation of cognate mRNA [35]. The initiation of RNAi requires the recognition and cleavage of exogenous or endogenous dsRNA by an RNase III family enzyme Dicer [4]. Dicer or Dicer-like enzyme has been identified in a wide-ranging of species from plants to humans. However, the involvement of RNAi in the antiviral immunity of *C. gigas* are still not well understood.

The number of Dicer gene locus varies greatly in the genome of different organisms. It was reported that only one Dicer was identified in vertebrates, while two members were reported in arthropods and even more in plants. For example, there is one Dicer identified in human (*Hm*Dicer), two Dicer genes in fruit fly (*Dm*Dicer-1 and *Dm*Dicer-2) and shrimp (*Pm*Dicer-1 and *Pm*Dicer-2). In the present study, only one intact Dicer homologue (*Cg*Dicer) was identified in *C. gigas* with 1873 amino acids, which was predicted to contain the conserved N-terminal DEAD domain, a helicase domain, a dicer_dimer domain, a PAZ domain, two RIBOc domains, and a C-terminal DSRM domain. Though another truncated Dicer-like gene (Dicer-like protein 2, NCBI No. CGI_10020752) was annotated in *C. giga* genome, it contained only the N-terminal DEAD domain and helicase domain of intact Dicer homologue, which was more likely a member of DExD/H-box helicases family. These six domains have all been identified in the mere Dicer of vertebrates and Dicer-2 of arthropods. But the N-terminal DEAD domain was absent in Dicer of scallop and Dicer-1 of arthropods. Structurally, *Cg*Dicer was closer associated with Dicer in vertebrates, or Dicer-2 but not Dicer-1 sub-family in arthropods. Functionally, the N-terminal DEAD domain was inferred to be important for the processing of different RNA substrates. In *D. melanogaster*, the *Dm*Dicer-2 containing a functional DExD/H helicase domain was reported to process dsRNA substrates and then trigger antiviral RNAi, while *Dm*Dicer-1 without this domain was responsible for processing hairpin-structured pre-miRNAs [10]. Therefore, *Cg*Dicer was inferred to be able to induce antiviral RNAi based on the same N-terminal domains of *Dm*Dicer-2.

The Dicers from metazoans are highly conserved in structure [15]. The multiple sequence alignment analysis of the full-length Dicers showed that *Cg*Dicer shared 18.5%–46.6% identity with that of other Dicers, with the highest identity with that of scallop. *Cg*Dicer also showed higher sequence identity with vertebrate Dicers and arthropod Dicer-1s than that with arthropod Dicer-2s. Correspondingly, *Cg*Dicer shared closer evolutionary relationship with the Dicers from scallop and vertebrates, and finally gathered with arthropod Dicer-1s in the phylogenetic tree. Though there was no N-terminal DEAD domain, the six C-terminal domains in Dicer-1s shared a higher sequence identity with that of *Cg*Dicer and vertebrate Dicers. The C-terminal domains of *Cg*Dicer and vertebrate Dicers were suspected to function in a manner more like arthropod Dicer-1s responsible for the maturation of miRNA. Multiple sequence alignment of the C-terminal functional domains

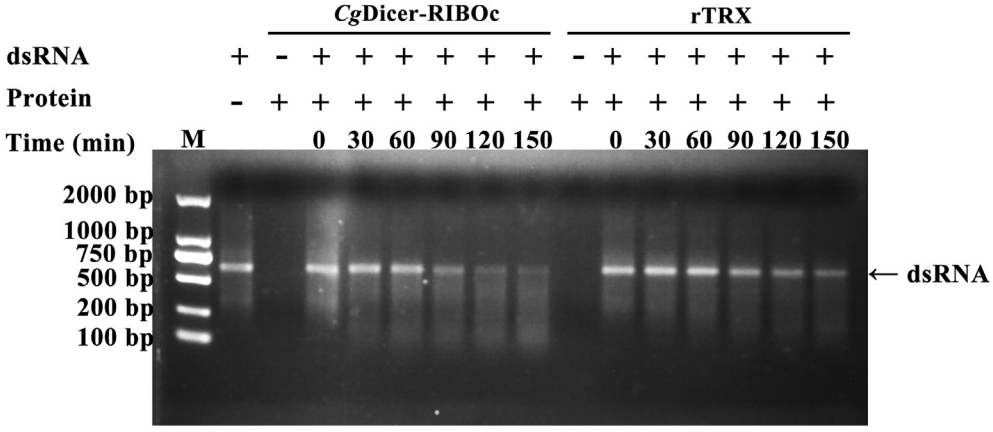

Fig. 9. The dsRNA cleavage activity of RIBOc domain in *Cg*Dicer. The RIBOc domain of *Cg*Dicer was prokaryotically expressed and purified, incubated with dsRNA, and its dsRNA cleavage activity of r*Cg*Dicer was examined by dsRNA cleavage assay. rTrx protein was used as negative control. The samples were analyzed by 1% agarose gel.

(PAZ, RIBOc and DSRM) revealed that *Cg*Dicer was highly conservative with other Dicers. The PAZ domain was responsible for protein-protein interaction, and it could bind to the 2-nucleotide 3' overhang of dsRNA with its binding pocket [16,36]. Some key amino acid residues (Phe981, Tyr993, Tyr994, Arg1018, Arg1023 and Glu1058) were also identified in the PAZ domain of *Cg*Dicer. The functional domains of RIBOc and DSRM, which were responsible for dsRNase activity and dsRNA binding activity, respectively, were also found to be conserved in *Cg*Dicer. The highly conserved catalytic residues (Asp1357, Glu1552, Asp1663 and Glu1765) were also identified in RIBOc domain of *Cg*Dicer. The PAZ and RIBOc domains of Dicer could act as a molecular ruler to precisely cleavage appropriate size siRNAs [36]. The DSRM, together with PAZ and RIBOc domains, were thought to be essential for dsRNA recognition and spatial cleavage [36]. Structurally, the conservations of *Cg*Dicer C-terminal sequence with Dicer-1 and N-terminal of *Cg*Dicer with Dicer-2 suggested that *Cg*Dicer might possess functions as Dicer-1 and Dicer-2 simultaneously.

To explore the potential physiological function of *Cg*Dicer, its expression pattern was monitored in both developmental stages of larvae and different tissues of adult oysters. *Cg*Dicer was found to be constitutively expressed at all the examined development stages with a higher expression level during the embryonic period and a moderate expression level during the larval stage. Previous studies have documented that Dicer is involved in the regulation of development. *Lv*Dicer-1 from *L. vannamei* was reported to function as a maternal factor during fertilized eggs stage [20]. In *C. elegans*, Dicer was found to be related to developmental timing by regulating the maturation of small RNA molecules [37]. In *Drosophila* ovary, Dicer-1 was reported to be responsible for egg cell maturation, fertilization or embryogenesis [38]. In bovine, the Dicer was observed to be highly expressed during embryonic development, which was suggested to be involved in degradation of different maternal mRNAs during oogenesis [39]. The high expression levels of *Cg*Dicer in two cells, four cells and early morula indicated that *Cg*Dicer might played a role in embryonic development of oysters. Moreover, the transcripts of *Cg*Dicer were constitutively expressed in hemocytes and all the tested tissues, including mantle, gills, hepatopancreas, lip, and adductor muscle. The highest expression level of *Cg*Dicer mRNA was detected in hemocytes, which was significantly higher than that in other test tissues. *Cg*Dicer protein was mainly located in cytoplasm of oyster hemocytes, which was consistent with the reports in transfected cells of human and mouse that *Hm*Dicer and *Mm*Dicer were both distributed in the cytoplasm [33,40]. Similarly, *Pm*Dicer-1 and *Lv*Dicer-1 were also highly expressed in hemocytes of the shrimps *P. monodon* and *L. vannamei*, respectively [19,20]. As the hemocytes were regarded as important defensive components of innate immunity in invertebrates [41–43], the highly expression in hemocytes suggested that *Cg*Dicer might play important roles in immune defense. The expression level of *Cg*Dicer mRNA was further investigated in the cultured hemocytes of oyster after immune stimulation. It was significantly up-regulated at 12 h post poly(I:C) stimulation, which was consistent with the report in shrimp that *Lv*Dicer-1 was significantly up-regulated after TSV infection [20]. Since the mRNA expression of *Cg*Dicer in the primary cultured hemocytes reached the highest level at 12 h after poly(I:C) stimulation, the mRNA expression of *Cg*Dicer in oyster hemocytes was examined at 12 h post dsRNA stimulation *in vivo*. The mRNA expression of *Cg*Dicer was also found to be up-regulated significantly in hemocytes after dsRNA stimulation *in vivo*. The expression level of *Manduca sexta* Dicer-2 mRNA was also up-regulated in response to injection with eGFP-dsRNA [44]. It has been reported that long dsRNAs could promote an anti-viral response in Pacific oyster to hamper ostreid herpesvirus 1 replication [25]. Dicer could act as a sensor to directly bind and cleave foreign dsRNA, and induce a specific and direct antiviral RNAi response [15]. The highly up-regulation of *Cg*Dicer was suspected to participate in the activation of anti-viral response. Collectively, these results implied that *Cg*Dicer was responsive to poly(I:C) and dsRNA stimulations and might be engaged in antiviral immune defense in oysters.

Pathogen recognition is the first step in triggering effective immune response [43]. Since invertebrates possess only innate immunity, they must rely on various pattern recognition receptors (PRRs) to recognize and bind the signature and conserved microbial structures called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan (PGN), poly(I:C), and dsRNA [45]. Antiviral activity mediated by Dicer in lower organisms occurs via small interfering RNA production following cytoplasmic sensing of viral dsRNA [15]. The recognition and degradation capacities of Dicers are accomplished by different protein domains, among which RNase III domain and dsRNA binding domain are found to display dsRNase activity and dsRNA binding activity, respectively [9]. In *C. elegans*, embryos extracts containing Dicer could produce siRNAs in a time-dependent fashion from the dsRNA but not from the ssRNA [37]. In the present study, r*Cg*Dicer-DSRM protein exhibited affinity to poly(I:C) and dsRNA with a dose-dependent manner *in vitro*, which was similar as human Dicer that the putative dsRNA-binding domain located at the C-terminus could bind dsRNA *in vitro* with a dose-dependently affinity [33]. The dsRBD of mouse Dicer also could bind to dsRNA *in vitro* [16]. Some other proteins containing dsRNA binding domain, such as PKR (double-stranded RNA-dependent protein kinase) and Rnt1p (a major RNase III protein in *Saccharomyces cerevisiae*), also utilize this domain to bind dsRNA to modulates its own function activation [46,47]. The significant binding activity of *Cg*Dicer-DSRM indicated that *Cg*Dicer might possess the capacity of recognizing virus nucleoside analogue. The dsRNase activity of *Cg*Dicer was also examined by incubation dsRNA with r*Cg*Dicer-RIBOc protein, and a gradual degradation of dsRNA was witnessed, indicating obvious cleavage activity of r*Cg*Dicer-RIBOc. Similar results have been reported in human, in which human recombinant Dicer cleavge dsRNA dose-dependently [11,33]. The RNase III of mouse Dicer also could cleavge dsRNA *in vitro* [16]. Meanwhile, a lysine residue, highly conserved in Dicer RNase IIIa and RNase IIIb domains was also found in *Cg*Dicer, which had the potential to participate in the phosphodiester bond cleavage reaction by stabilizing the transition state and leaving the scissile bond group [16]. RNase IIIa and RNase IIIb in a Dicer molecule form two catalytic sites through intramolecular dimerization, and DSRM and PAZ assist the cleavage reaction [16]. These results implied that *Cg*Dicer might be involved in antiviral immunity through recognition and cleavage of dsRNA to reduce the impaction of viruses.

To sum up, a novel *Cg*Dicer was identified from Pacific oyster *C. gigas*. The mRNA of *Cg*Dicer was constitutively expressed in all the tested tissues with the highest expression level in hemocytes, and its expression could be induced by dsRNA and poly(I:C). *Cg*Dicer was mainly distributed in cytoplasm of hemocytes. The two most important functional domains of *Cg*Dicer, DSRM and RIBOc, displayed nucleic acid-binding and ribonuclease activities, respectively. These result collectively suggested that *Cg*Dicer could function as an intracellular recognition molecule and effector, and possibly confer anti-viral immune protection in oysters.

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