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Short communication

Characterization of a gC1qR homolog from sea cucumber *Apostichopus japonicus*

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ABSTRACT

gC1qR is a multifunctional and multiligand binding protein that plays important roles in inflammation and infection. In this study, a novel gC1qR homolog called AjgC1qR from the invertebrate sea cucumber *Apostichopus japonicus* was cloned and characterized. The open reading frame of AjgC1qR encoded 292 amino acid residues with a conserved mitochondrial targeting sequence and MAM33 domain. Multiple sequence alignment and phylogenetic analyses proved that AjgC1qR is a homolog of the gC1qR family. Spatial mRNA transcription in five tissues revealed the ubiquitous expression of *AjgC1qR*. The highest and lowest levels of expression were found in the tentacle and muscle, respectively, and *AjgC1qR* expression was remarkably up-regulated in coelomocytes after *Vibrio splendidus* challenge. Moreover, the recombinant rAjgC1qR protein exhibited high binding activity toward pathogen-associated molecules, such as lipopolysaccharides, peptidoglycan, and mannan. These findings demonstrate that AjgC1qR may play important roles in innate immunity and function as a pathogen recognition receptor.

1. Introduction

Complement component 1q (C1q) is the first subcomponent of the classical pathway of the complement system that acts as an efficient pathogen recognition molecule linking innate and acquired immunity [1–3]. C1q comprises two major structural and functional regions, namely, a collagen-like “stalk” (cC1q) and a globular “head” (gC1q) [4,5]. gC1qR, a receptor for C1q, is a multifunctional protein that plays a crucial role in host defense by binding to gC1q [6]. The gC1qR molecule was originally isolated from Raji cells and is located on the cell surface of the plasma membrane [7]. gC1qR, as a highly anionic cellular and multicompartmental protein, is also known as receptor for the globular head domains of C1q (C1qBP), hyaluronan-binding protein 1 (HABP1), splicing factor 2-associated protein (p32), or p33 [7–9]. gC1qR is a mitochondrial protein found in various cellular compartments but not in erythrocytes [10,11]; the receptor is a multiligand protein that binds to a broad range of other proteins [12], including plasma (e.g., fibrinogen) [13], microbial (e.g., *Staphylococcus aureus*, herpesvirus) [14,15], and cellular (e.g., α_{1B}-adrenergic receptor) proteins [11]. What's more, gC1qRs modulate the activation of immune responses under inflammation and infection in vertebrates [12,16,17]. Hu et al. [18] revealed that p32 serves as a key host factor for respiratory syncytial virus (RSV) production, thereby indicating that p32 plays important roles in RSV infection. Chen et al. [1] reported that Nile tilapia gC1qR may be involved in the host defense against bacterial infection.

Several studies of gC1qR in invertebrate species, including giant freshwater prawn *Macrobrachium rosenbergii* [19], *Exopalaemon carinicauda* [20], and swimming crab *Portunus trituberculatus* [21], have focused on the functions of pathogen recognition receptors (PRRs) in innate immunity. These molecules are dramatically induced by pathogenic bacteria and viruses, thus suggesting that gC1qR regulates innate immune responses and host defenses. The EsgC1qR protein from Chinese mitten crab could potently bind to ligands and direct inhibit bacterial activity [22]. Zhang et al. [20] reported that the mRNA expression of gC1qR in the hepatopancreas of *E. carinicauda* is markedly induced after pathogen infection, and the recombinant EcgC1qR protein could inhibit the growth of *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. Numerous studies have demonstrated that gC1qR plays important roles in innate immunity and functions as a docking receptor for various extracellular or intracellular and structural or functional molecules [23,24]. However, the physiological and biological roles of

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this protein in marine echinoderm species have yet to be elucidated.

The sea cucumber *Apostichopus japonicus* (Echinodermata, Holothuroidea), a commercially important species in China aquaculture, is severely threatened by infectious diseases [25,26]. Skin ulceration syndrome, which is the most contagious and lethal disease in the sea cucumber culture industry, results in the mass mortality of sea cucumbers and huge economic losses. Therefore, the host defense mechanism of sea cucumbers should be determined, and new strategies should be explored to control the incidence of such infectious diseases. In the present study, AjgC1qR in *A. japonicus* was cloned and characterized, and the functions of this protein were investigated to reveal its role in innate immunity against pathogen infection.

2. Materials and methods

2.1. Rearing, challenge, and sample collection of experimental animals

Healthy adult sea cucumbers (average weight, 125 ± 15 g) were collected from Dalian Pacific Aquaculture Company (Dalian, China) and acclimatized in 30 L of aerated natural seawater (salinity, 28 ± 1‰; temperature, 16 ± 1 °C) for 3 days before viral challenge.

For spatial expression analysis, five tissue samples, including coelomocytes, muscle, tentacle, respiratory trees, and intestine, were collected from five healthy sea cucumbers using sterilized scissors and tweezers. Coelomic fluids were collected by syringe, passed through a 160 μm sterile nylon mesh, and then centrifuged at 800 × *g* for 10 min to harvest coelomocytes. Other tissues (wet weight, ~100 mg) were ground into powder in liquid nitrogen using a mortar and a pestle. We performed five biological replicates for tissue distribution analysis and stored samples at −80 °C for subsequent RNA extraction.

Vibrio splendidus was isolated from sea cucumbers suffering from skin ulceration syndrome at the indoor farms of Jinzhou Hatchery on May 2013, and its identity was determined by 16S rDNA sequencing analysis. The bacterium was preserved in glycerol, stored at −80 °C, and then used for the infection experiment. First, the bacterium was cultured overnight in liquid 2216E broth (Tryptone 5 g L^{-1}, yeast extract 1 g L^{-1}, pH 7.6) at 28 °C and 150 rpm. Bacterial cells were collected by centrifugation at 6000 × *g* for 10 min and resuspended in filtered seawater for the infection experiment. The sea cucumbers were randomly divided into six tanks, each containing 10 individuals. Five of the tanks were exposed to resuspended *V. splendidus* at a final concentration of 10^7 CFU mL^{-1}. The same number of individuals in another tank served as the control group. After exposure for 0, 6, 12, 24, 48, and 96 h, coelomocytes were collected as described above for further study. No sea cucumber died over the course of the experiment.

2.2. Cloning of the full-length cDNA of AjgC1qR

The partial sequence of the *AjgC1qR* gene was generated by screening the *A. japonicus* transcriptome database [27]. BLASTx analysis of the fragment showed that the sequence lacks complete 5′ and 3′ ends compared with those of other similar species. Therefore, gene-specific primers for *AjgC1qR* (Table 1) were designed on the basis of the acquired unigenes. The full-length cDNA sequence of the gene was subsequently cloned using the SMARTer® RACE 5′/3′ Kit (TaKaRa) following the manufacturer's instructions. The desired PCR products were purified and cloned into the pMD19-T vector (TaKaRa). Positive clones for each product were sequenced at Sangon (Shanghai, China), and the complete *AjgC1qR* cDNA sequence was obtained by overlapping the correct fragments. The full-length cDNA sequence of *AjgC1qR* was analyzed using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed using Expert Protein Analysis System (http://www.expasy.org/). Protein domain features and mitochondrial targeting sequences were predicted using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) and MITOPROT (https://ihg.gsf.de/ihg/mitoprot.html) [28], respectively. N-Glycosylation sites were predicted by using online software (http://www.cbs.dtu.dk/services/NetNGlyc/). The molecular mass (MM) and theoretical isoelectric point (*pI*) of the proteins were calculated on the basis of the deduced amino acids by using the ProtParam tool (http://www.expasy.ch/tools/protparam.html). Multiple alignment analysis of AjgC1qR was performed by using ClustalW2 multiple alignment software (http://www.ebi.ac.uk/clustalw/), and phylogenetic and molecular evolutionary analyses were conducted using MEGA 7.0.

Table 1
Primers used in this study.

Primer Name	Primer Sequence (5′–3′)	Used for
AjgC1qR 5-1	GTCCAATGATGAGCCTGTCCTAT	5′ RACE
AjgC1qR 5-2	GCTGGTGCGACTTGAAACATTAG	
AjgC1qR 3-1	CCAGCAATAAAGATAGGACAGGC	3′ RACE
AjgC1qR 3-2	TCCAACGATAGCAGGATTCTCAG	
AjgC1qR F	ATGGCATTTTCTAATGTTTCAAG	Code mature
AjgC1qR R	CTATTCTTTGATGGTGTCCTTCA	peptide
*AjgC1qR Bam*H I F	GGATCCATGGCATTTTCTAATGTTTCAAG	Vector
AjgC1qR Xho I R	CTCGAGCTATTCTTTGATGGTGTCCTTCA	construction
AjgC1qR qF	CCAGCAATAAAGATAGGACAGGC	Real-time PCR
AjgC1qR qR	ACTGAGAATCCTGCTATCGTTGG	
Ajβ-actin qF	CCATTCAACCCTAAAGCCAACA	Real-time PCR
Ajβ-actin qR	ACACACCGTCTCCTGAGTCCAT	
M13-RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	

2.3. Tissue distribution and expression pattern of AjgC1qR

The distribution of *AjgC1qR* in coelomocytes, muscle, tentacle, respiratory trees, and intestine was determined by PCR (Applied Biosystem 7500 Real-time PCR System). The expression level of *AjgC1qR* in coelomocytes after pathogen challenge was also analyzed by qRT-PCR. Total RNA was isolated from each sample by using Trizol (TaKaRa) reagent, and cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa). The specific primers used for qRT-PCR analysis are listed in Table 1; here, *Ajβ-actin* served as the reference. Amplification was carried out in 20 μL reaction volumes containing 8 μL of the cDNA (1:100 dilution), 0.8 μL of each of the primers, 0.4 μL of ROX Reference Dye II, and 10 μL of SYBR Green Mix (TaKaRa). Reaction mixtures were incubated for 5 min at 95 °C and subjected to 40 amplification cycles of 15 s at 95 °C, 20 s at 60 °C, 30 s at 72 °C and then a melting curve stage after the cycling stage. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression level of *AjgC1qR*; here, values obtained denote n-fold differences relative to the calibrator [29]. The expression level of *AjgC1qR* in muscle tissues was used as a calibrator to determine the expression levels of the gene in other tissues. The expression level of *AjgC1qR* in coelomocytes at 0 h served as the calibrator in the bacterial challenge experiment. The data are presented as relative expression levels (means ± SD, n = 5), and one-way ANOVA was applied to discern significant differences between the control and experimental groups. Differences were considered significant at $p < 0.05$ (one asterisk) and $p < 0.01$ (two asterisks).

2.4. Recombinant protein expression and purification

The full-length cDNA sequence of *AjgC1qR* was cloned with gene-specific primers (Table 1), double digested with *Bam*H I and *Xho* I, and ligated into the pET28a vector (Novagen). The recombinant vector (pET-28a-AjgC1qR) was transformed into *Transetta* (DE3) Chemically Competent Cell (TransGen, China) and sequenced to ensure correct construction. The positive transformant was subsequently incubated in LB medium containing 50 μg mL^{-1} kanamycin at 37 °C and 180 rpm.

The expression of the His-tagged fusion protein was induced with 1 mM isopropyl-b-D-thiogalactopyranoside at 37 °C and collected after another 3 h of induction. Bacterial pellets were harvested by centrifugation at 8000 × *g* for 5 min at 4 °C and resuspended in inclusion body washing solution with 2 M urea containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5% Triton × 100 (pH 7.9). The pellets were sonicated at 4 °C for 10 min in cycles of 5 s of sonication at 10 s intervals under 300 W power. The cell lysates were centrifuged at 10,000 × *g* for 10 min at 4 °C to collect inclusion bodies, which were then solubilized in 8 M urea containing 20 mM Tris-HCl, 150 mM NaCl, 0.1% β-mercaptoethanol, 0.2% Triton × 100, and 30 mM imidazole (pH 7.9). The solubilized protein was partially purified using Ni-NTA Seflnose™ resin following the manufacturer's instructions (Sangon, China). The purified recombinant protein was refolded in gradient urea (6, 4, 2, 0 M, pH 7.9) to ensure the removal of urea and other contaminants. Each operation was conducted for 12 h at 4 °C. The refolded protein was subjected to 12% SDS-PAGE, and protein concentrations were measured using the BCA Protein Assay Kit (Sangon, China).

2.5. Pathogen-associated molecular pattern (PAMP) binding analysis

For PAMP binding analysis, enzyme-linked immunosorbent assay (ELISA) was performed to detect the direct binding of rAjgC1qR to lipopolysaccharides (LPS, *Escherichia coli* 055:B5) (Sigma), peptidoglycan (PGN, *Staphylococcus aureus*) (Sigma), and mannan (MAN, *Saccharomyces cerevisiae*) (Sigma) by using previously described methods [30]. Three types of PAMPs were dissolved in carbonate–bicarbonate buffer (50 mmol L^{-1}, pH 9.6) at a final concentration of 1 mg mL^{-1}. After addition of a total of 20 μL of each PAMP solution to the wells of 96-well plates, the plates were incubated overnight at 4 °C and washed three times with PBS containing 0.05% Tween-20 (PBST; pH 7.2). The wells were blocked with 200 μL of 5% BSA in PBS for 1 h at 37 °C and washed three times with PBST. Afterward, 100 μL of rAjgC1qR protein of different doses (0, 20, 40, 60, 80, and 100 μg) was added to the wells, and the plates were incubated once more for 1 h at 37 °C. Each well was washed three times and incubated with 100 μL of mouse anti-His-tagged monoclonal anti-body (1:1000 dilutions). The samples were incubated for 1 h at 37 °C. Another 100 μL of AP-labeled goat anti-mouse IgG (1:3000 dilutions) was added to the wells, and the plates were incubated once more for 1 h at 37 °C. The plates were then washed four times with PBST. Finally, 100 μL of the PNPP chromogenic substrate reagent (Beijing Seitz Biotechnology Company) was added to each well. Samples were incubated at room temperature in the dark for 30 min, and the chromogenic reaction was terminated by adding 50 μL of 3 M NaOH per well. Absorbance was recorded at 450 nm by using a microplate reader (Thermo Scientific). Recombinant extracellular signal-regulated protein kinases (ERK) from the same prokaryotic expression system were treated under the same conditions and used as the negative control group, as reported in our previous study without any PAMP binding activities [31]. BSA (0, 20, 40, 60, 80, and 100 μg) was set as the control, and TBS without any exogenetic protein supply was set as the blank control. Five replicates were performed for each sample, and the data are presented as mean ± SD (n = 5).

3. Results and discussion

3.1. Characterization of A. japonicus gC1qR sequence

The complete cDNA of the *gC1qR* gene of *A. japonicus* was obtained by overlapping the original EST and each RACE product. The sequence was submitted to the NCBI database and denoted as *AjgC1qR* (MK309404). The full-length cDNA of *AjgC1qR* comprised 1665 base pairs consisting of a 5′-UTR of 126 bp and a 3′-UTR of 660 bp with two RNA instability signals (Fig. 1). The AjgC1qR protein was predicted to have an MM of 32.4 kDa and consist of 292 amino acids with an estimated theoretical *pI* of 4.51. In general, mammalian gC1qR contains the

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1    GCCGATAACGTGACTGTAGTGTGTAAACCTATTCCGCAAATTTTGCAAAATAGGCATAGA
61   CTAGTTTATGTGTGTCGAATGAACAGTTGAACCGTACTGCCCTTAGAAGAAGAAAAAATA
1          M  A  F  S  N  V  S  S  R  T  S  R  I  I  S  R  A  A
121  CATACAATGGCATTTTCTAATGTTTCAAGTCGCACCAGCCGAATTATAAGCCGAGCGGCA
19   Q  F  T  R  S  I  E  A  T  S  S  C  V  N  Q  I  N  R  S  L
181  CAATTTACAAGATCCATTGAGGCTACAAGTTCATGTGTTAACCAAATCAACAGAAGTCTG
39   A  R  S  V  W  H  L  S  S  N  K  D  R  T  G  S  S  L  D  V
241  GCTCGCTCTGTATGGCATTTGTCCAGCAATAAAGATAGGACAGGCTCATCATTGGACGTG
59   V  G  S  V  Q  K  S  V  T  W  P  G  K  P  C  T  C  G  C  A
301  GTAGGCAGTGTACAAAAGTCTGTGACATGGCCCGGCAAACCCTGCACATGTGGATGTGCT
79   G  L  H  T  E  A  D  K  Q  L  S  N  F  L  A  E  E  I  Q  I
361  GGTTTGCATACAGAGGCTGACAAGCAGTTGTCCAACTTCCTAGCAGAAGAAATCCAGATC
99   E  K  E  S  Q  K  N  P  K  I  P  T  I  A  G  F  S  V  T  T
421  GAAAAAGAAAGCCAGAAGAACCCCAAAATTCCAACGATAGCAGGATTCTCAGTTACCACT
119  D  G  A  E  V  T  L  T  K  E  G  S  S  D  E  R  V  Q  V  I
481  GATGGCGCAGAAGTGACCCTTACCAAAGAAGGAAGCTCAGATGAAAGGGTGCAAGTCATT
139  F  N  V  N  H  S  V  E  F  E  E  N  V  E  A  E  P  P  A  D
541  TTCAATGTCAATCACTCAGTTGAGTTTGAGGAGAACGTTGAGGCAGAACCACCAGCTGAC
159  S  K  D  T  D  T  P  D  G  Q  M  R  S  Y  P  D  F  R  V  E
601  AGCAAAGATACCGATACACCAGATGGGCAGATGAGGTCATACCCCGACTTCCGTGTGGAA
179  L  T  K  S  G  K  S  T  I  A  V  Q  C  S  Y  T  T  D  D  D
661  CTGACGAAGTCAGGAAAGTCTACGATTGCAGTCCAGTGTAGCTACACGACCGATGATGAC
199  F  N  A  E  E  E  V  E  E  G  E  A  D  D  P  Q  D  D  L  F
721  TTTAATGCAGAGGAGGAGGTGGAGGAGGGAGAGGCAGATGATCCTCAAGATGATCTCTTT
219  L  I  D  E  V  Y  M  F  K  D  Q  A  N  E  K  T  Y  R  V  G
781  CTGATTGATGAGGTGTACATGTTCAAAGACCAAGCCAATGAAAAGACCTACAGGGTTGGG
239  S  E  V  M  Q  G  E  M  Y  D  H  L  L  A  L  L  E  S  R  G
841  TCAGAGGTCATGCAAGGGGAGATGTACGACCATTTATTGGCCCTGCTGGAGAGTCGAGGT
259  V  D  N  D  F  A  D  K  L  S  D  F  A  S  S  F  E  H  S  S
901  GTCGACAACGATTTTGCAGATAAGCTATCGGACTTCGCCAGCAGTTTCGAACACTCATCC
279  F  I  K  F  L  E  S  L  K  D  T  I  K  E  *
961  TTCATCAAGTTTCTTGAGTCCCTGAAGGACACCATCAAAGAATAGACAAGTCCTGTATCT
1021 GATAATACAACAACCACCACCAATCTGTCACGAGTATAATCACCCACCGTCAAGAGTAGG
1081 TTACAAATCACATGTCGGCATAACCGTGTGCATCTTTGGTCTGGTATTCAATAATGACAA
1141 CTACTGAATATTTATGAATAAGTGATCCCCCATTCATATCCCTGAGATCAGTAAATGTTA
1201 TCTAGGAAATTGGTCTCAGGCAAAAGTAGTCACGCCCAGGGATTTGAGACTTCCTTGCTT
1261 GTGTTCTTTTGCCTTCACCGATGCCACCTTAACGAAGACTCAACATTGTTAATGACGCAT
1321 GAATTTGCATGTTTGACGGTTACTCACCTTACACATACCTACCTCCTTCCATGTTATCCA
1381 CATTACCAACAGAAAACTGCATCCATTTGTGGATTCTTTAGAAAGGATAATCAAGTACTA
1441 ACAACCTGCTGCAGTTACTTTAAAATTAAATGAAGCTTCAGTGACAATTGCGATCGTTGT
1501 ATTCTCCAGTAAGTGGATATTCAAATATTAGCTGATGAATTATCAAGTGGAAATTTATTC
1561 ATGTTCACCCCTCTTGGACTTGTTTGATGTTACGGCAGCTTGTTACTTTGTCTCTTGATA
1621 ATGCCTCTACACTAATCGATGCTAGAATCTCATAAAAAAAAAAAA
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Fig. 1. cDNA and deduced amino acid sequence of *A. japonicus gC1qR*. The start codon is indicated in black. An asterisk indicates the stop codon. The mitochondrial acidic matrix protein domain (MAM33) is underlined, and the mitochondrial cleavage site is shadowed in gray. N-Glycosylation sites are boxed in red. RNA instability sequences (ATTTA) are indicated by bold italics. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

MAM33 (mitochondrial acidic matrix protein of 33 kDa) domain, which binds to the globular head of C1q and inhibits the activation of C1 [7,16]. SMART analysis indicated that the predicted AjgC1qR protein also contains a MAM33 domain (residues 92–290) at its C terminal. In addition, mitochondrial targeting sequences with lengths of 45 amino acids were found at the N terminal of the deduced protein sequence, which is usually synthesized as a per-protein and can be directed to the mitochondrial matrix [7,32]. Moreover, three N-glycosylation sites of Asn^{5}, Asn^{35}, and, Asn^{142} were found; these sites suggest that glycosylation may occur in eukaryotic cells [33]. The site in which glycosylation occurs in sea cucumber will be verified in our future work.

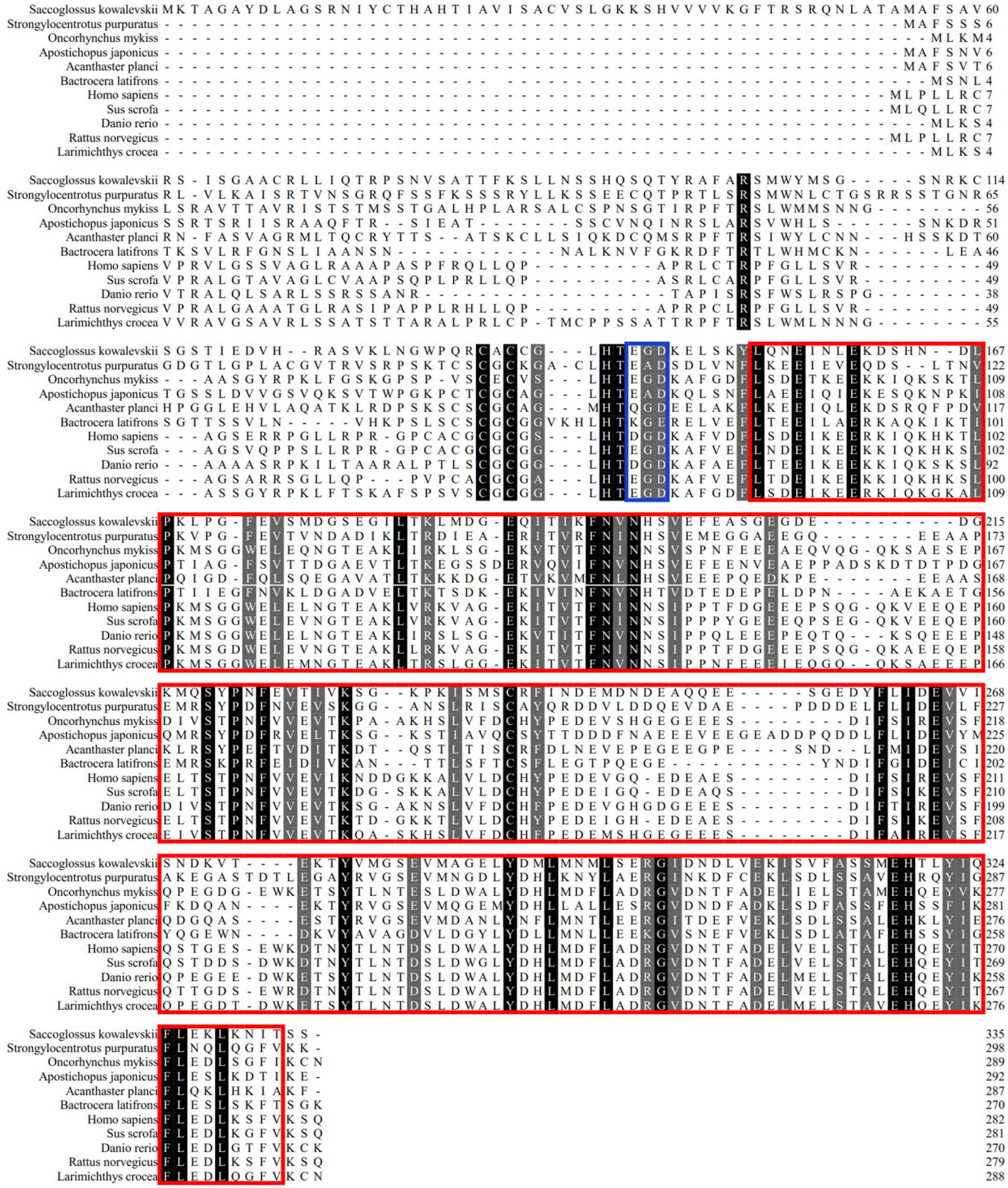

Fig. 2. Alignment of the predicted amino acid sequence of AjgC1qR with other gC1qRs by using ClustalW2 multiple alignment software. Consensus residues are with a threshold of over 80% identity are shaded by using Multiple Align Show. Identical residues are indicated in black, and similar residues are in light gray. MAM33 domains are marked with a red box, while EGD motifs are marked with a blue box. The accession numbers of relevant protein sequences are as follows: *Saccoglossus kowalevskii* (XP_002737600.2), *Latimeria chalumnae* (XP_014349317.1), *Strongylocentrotus purpuratus* (XP_789452.2), *Oncorhynchus mykiss* (XP_021473073.1), *Acanthaster planci* (XP_022105090.1), *Bactrocera latifrons* (XP_018788294.1), *Homo sapiens* (AAH00435.1), *Sus scrofa* (XP_020923404.1), *Danio rerio* (NP_001017858.2), *Rattus norvegicus* (NP_062132.2), *Larimichthys crocea* (XP_010741426.2), and *Apostichopus japonicus* (MK309404). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

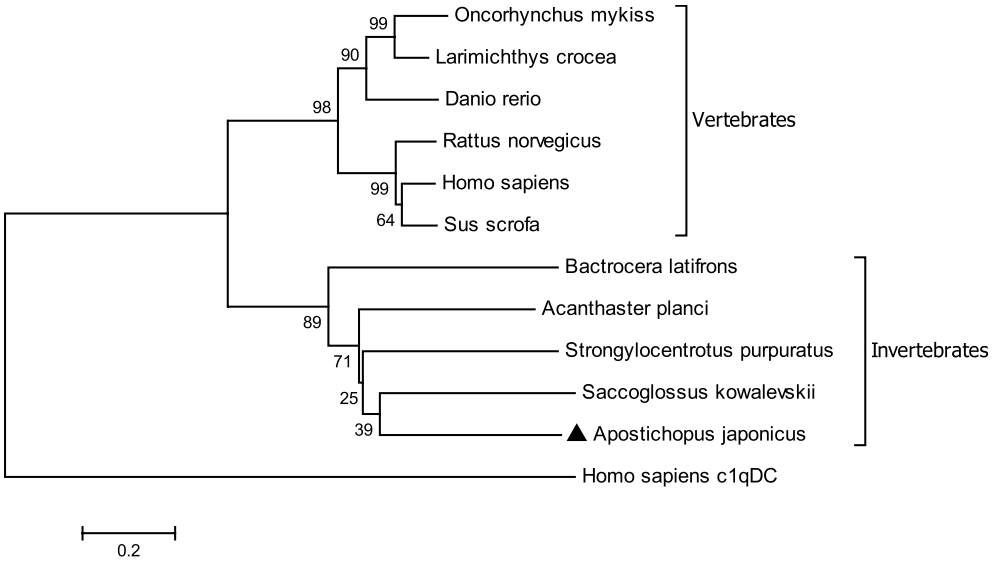

Fig. 3. Phylogenetic tree based on the amino acid sequences of gC1qR. The tree was obtained by bootstrap analysis via the neighbor-joining method, and numbers on branches represent the bootstrap values for 1000 replications. The family of c1qDC (c1q domain-containing protein) was used as the out-group. The accession number of the *Homo sapiens* c1qDC protein sequence is NP_001107573.1.

3.2. Comparative similarity and phylogenetic analysis

Multiple sequence alignment of AjgC1qR with other gC1qRs showed highly conserved MAM33 domains, and the N-glycosylation site of Asn^{142} was conserved from invertebrates to humans (Fig. 2). The traditional RGD motif (Arg–Gly–Asp) has been demonstrated to function in cell attachment via integrin on the cell surface and prevent bacterial infection [22,33]. However, the RGD motif was not observed in the AjgC1qR sequence, similar to *Strongylocentrotus purpuratus* gC1qR (AAH00435.1). In the present case, whether the EAD domain can bind to integrin requires further verification. Pairwise sequence alignment showed that AjgC1qR is most sequence-similar (61.0%) to *Bactrocera latifrons* (XP_018788294.1). Moreover, AjgC1qR shared 41.6%, 32.1%, and 28.3% identity and 60.3%, 52.3%, and 48.2% similarity with *S. purpuratus*, *Danio rerio* (NP_001017858.2), and *Homo sapiens* (AAH00435.1), respectively.

A phylogenetic tree was constructed via the neighbor-joining method by using the c1qDC family (c1q domain-containing proteins) as an out-group to analyze the evolutionary position of gC1qR members. The phylogenetic tree showed that gC1qRs from vertebrates and invertebrates are clustered separately into two different branches (Fig. 3). One main cluster was formed by vertebrates, and the other was formed by invertebrates. gC1qR proteins from mammals and fishes were clustered into a large branch of vertebrates. The AjgC1qR protein was initially clustered with *Saccoglossus kowalevskii* (XP_002737600.2), *Acanthaster planci* (XP_022105090.1), *S. purpuratus*, and *Bactrocera latifrons* (XP_018788294.1). Hence, AjgC1qR has a close evolutionary relationship with invertebrate species.

3.3. Tissue-specific expression of AjgC1qR

The mRNA transcript levels of *AjgC1qR* in five tissues were investigated by qPCR to gain better understanding on the biological roles of the gene in sea cucumber; here, *Ajβ-actin* was used as the internal control (Fig. 4). While *AjgC1qR* was ubiquitously and highly expressed in the tentacle (20.0-fold, $p < 0.01$) and coelomocytes (15.9-fold, $p < 0.05$), its expression was relatively lower in the intestine (3.15-fold, $p < 0.05$) and respiratory trees (2.90-fold, $p < 0.05$) compared with that in muscle tissues. Recent studies show that the *gC1qR* gene is widely expressed in nearly all organizations and mainly expressed in immune tissues, such as the liver [1], hepatopancreas [19], and intestine [34], thereby suggesting that it participates in various physiological processes, particularly in immune responses. Zhang et al. [20] found that *EcgC1qR* mRNA from *E. carinicauda* is predominately

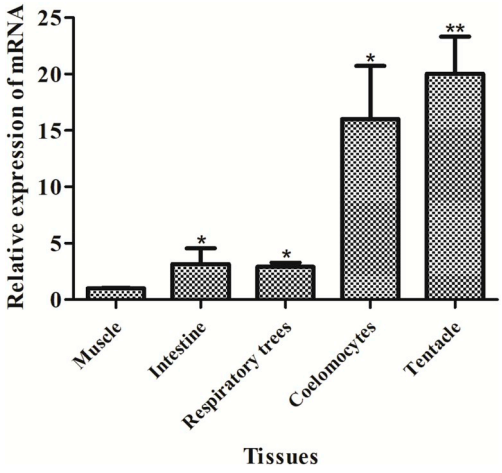

Fig. 4. Tissue distribution of *AjgC1qR* in normal *A. japonicus* detected by quantitative PCR. Transcript levels in the intestine, coelomocytes, tentacle, and respiratory trees were normalized to that in the muscle. Values are given as mean ± SD, n = 5.

expressed in hemocytes and the hepatopancreas. However, Ning et al. [21] reported minimal expression of *PtgC1qR* from *Portunus trituberculatus* in the normal hemocytes of male and female crabs. Similar tissue distributions have been observed in *EsgC1qR* [22], which is highly expressed in hemocytes and lowly expressed in nerve and muscle. In invertebrates, hemocytes are important immune organs that play crucial roles in immune responses [35,36]. In echinoderms, coelomocytes play key roles in recognizing and eliminating invasive pathogens [37]. In the present work, the expression level of *AjgC1qR* in coelomocytes was high, which indicates that the gene may play positive regulatory roles in innate immunity. Sea cucumbers possess branching tentacles that are mainly used to filter seawater and serve an important site of entry of microorganisms [38]. The high expression of *AjgC1qR* in tentacle indicates the important role of the gene against pathogen invasion.

3.4. Expression analysis of AjgC1qR after pathogen challenge

The expression pattern of *AjgC1qR* in coelomocytes upon *V. splendidus* infection was investigated to further understand its potential function in the innate immunity of sea cucumber (Fig. 5). In contrast to the control, the *AjgC1qR* transcript was slowly up-regulated within the first 6 h after pathogen challenge. The expression level of *AjgC1qR* then

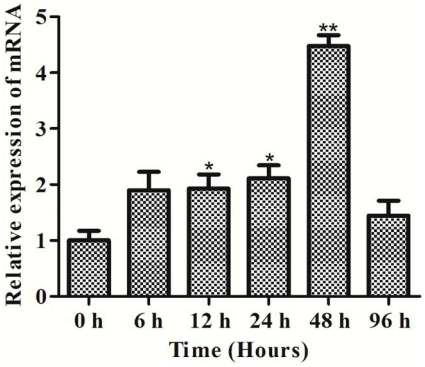

Fig. 5. Expression levels of *AjgC1qR* in *A. japonicus* coelomocytes infected with *V. splendidus*. Values are given as mean ± SD, n = 5. Asterisks indicate significant differences: *$p < 0.05$, **$p < 0.01$.

significantly increased at 12 h (1.93-fold, $p < 0.05$) and peaked at 48 h (4.48-fold; $p < 0.01$). While the expression of the *AjgC1qR* transcript gradually decreased thereafter, it remained higher compared with the control after 96 h. Li et al. [34] found that the mRNA transcript of *Fenneropenaeus chinensis* FcgC1qR in hemocytes is significantly elevated by *Vibrio anguillarum* and *Staphylococcus aureus* challenge. Ye et al. [19] showed that the mRNA expression of *MrgC1qR* is markedly enhanced after white spot syndrome virus infection or *V. anguillarum* challenge. The transcript level of *MrgC1qR* in the liver, spleen, and head kidney increases after *Streptococcus agalactiae* challenge [18]. These results strongly support our hypothesis that the *gC1qR* gene plays an important role in innate immunity against bacterial infection.

3.5. PAMP binding activity of AjgC1qR

gC1qR is a multiligand binding protein that can bind to pathogens or various ligands [39]. ELISA was performed to determine whether recombinant rAjgC1qR could bind to PAMPs. The recombinant rAjgC1qR was purified and refolded, and SDS-PAGE presented a unique band with a molecular size of approximately 40 kDa (Fig. 6). The MM of rAjgC1qR is larger than that of AjgC1qR because the recombinant rAjgC1qR contains a His-tag. rAjgC1qR could bind to different PAMPs, including LPS, PGN, and MAN, in a dose-dependent manner (Fig. 7). The binding activity was sharply increased by supplying 20–100 μg of the recombinant rAjgC1qR. No apparent binding activities were found in the negative control group (ERK) despite the use of different PAMPs and doses. Moreover, all of the OD_{450} values of BSA were approximately 0.3–04, thereby indicating that the protein could not bind to PAMPs. Most studies indicate that gC1qR proteins could bind to various PAMPs, such as LPS and PGN [19,20,22]. LPS is a complex glycolipid located in the outer membrane of Gram-negative bacteria [40], PGN forms in the

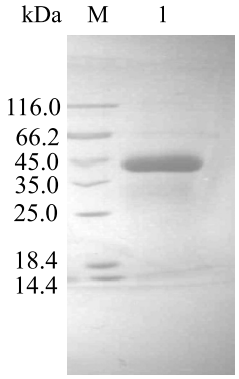

Fig. 6. SDS-PAGE analysis of the recombinant rAjgC1qR. M: protein molecular standard; 1: purified rAjgC1qR protein.

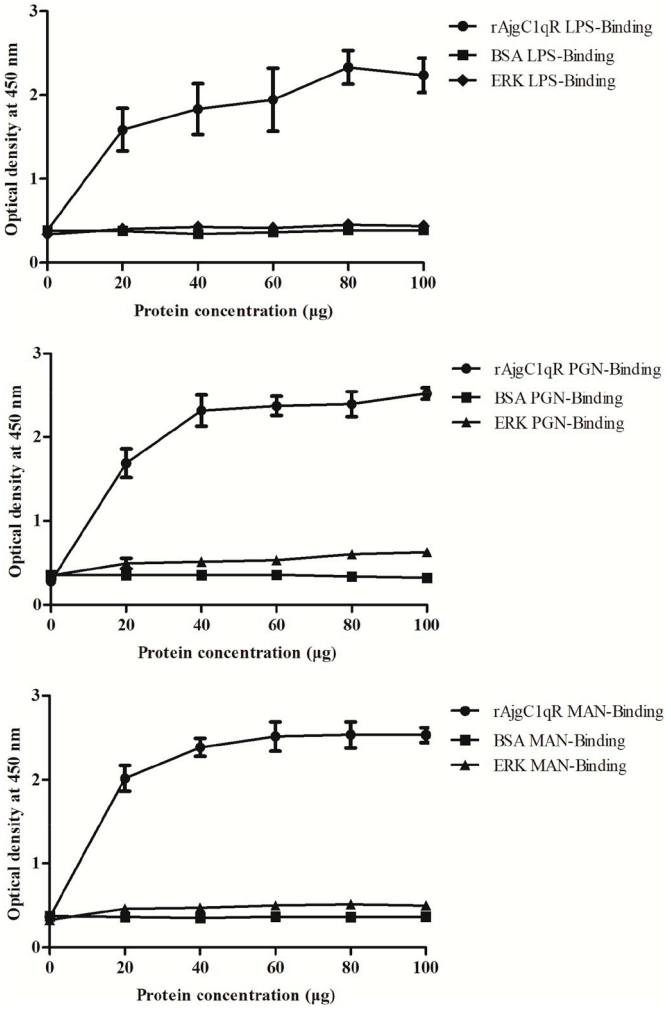

Fig. 7. Binding activity of rAjgC1qR toward different PAMPs. Values are given as mean ± SD, n = 5.

outer wall of Gram-positive bacteria [41], and MAN is a component of fungal cell surfaces [42]. PAMPs are primarily recognized via highly conserved and high-affinity receptors called PRRs. Thus, our results suggest that the rAjgC1qR protein could bind to PAMPs and may function as a PRR in innate immunity.

4. Conclusion

We cloned and characterized the full-length cDNA of the *AjgC1qR* gene in *A. japonicus* and investigated its roles in the innate immunity of *A. japonicus*. Our findings, although inconclusive, suggest that *AjgC1qR* is activated following bacterial infection and functions as a PRR in the innate immunity of sea cucumber.

Notes

The authors declare no competing financial interest.

Acknowledgments

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