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# The involvement of cysteine-rich intestinal protein in early development and innate immunity of Asiatic hard clam, *Meretrix meretrix*

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ABSTRACT

Cysteine-rich intestinal protein (CRIP), a $Zn^{2+}$-binding protein, contains a single copy of the highly conserved double-zinc-finger structure known as the LIM (lin-11-isl-1-mec-3) motif. In this paper, a cDNA encoding *MmCRIP* was isolated from the Asiatic hard clam *Meretrix meretrix*. The full-length cDNA of *MmCRIP* consists of a 237-bp open reading frame that encodes a polypeptide of 78 amino acids with a predicted molecular weight (MW) of 8635.8 Da and theoretical isoelectric point (pI) of 9.01. Bioinformatics analysis showed that it belonged to a new member of the CRIP subfamily. Relationship analysis revealed that MmCRIP has high-levels of sequence similarity to many CRIPs reported in other animals, particularly in invertebrates. Real-time PCR analysis showed that the highest level of *MmCRIP* expression was in hemocyte tissue and at pediveligers stage. To investigate immune function, mature clams were challenged with *Aeromonas hydrophila*. During *A. hydrophila* infection, up-regulation of *MmCRIP* transcript in clam's hemocyte, gill and hepatopancreas was detected. DsRNAi (double-strand RNA interference) approach was employed to study the function of MmCRIP and the data showed that inactivation of the *MmCRIP* gene blocked larvae development and caused mass mortalities. The probable roles of MmCRIP in clam early development and innate immunity are presented for the first time.



## 1. Introduction

The Asiatic hard clam *Meretrix meretrix* is an important marine bivalve for commercial fisheries along coastal and estuarine areas in China, Korea, Japan, Vietnam and India [1]. Like other marine bivalves, the larvae and adults of *M. meretrix* possess different morphology and behavior patterns. The fertilized egg hatches for a free-swimming, plankton-like larva (trochophore), which then experience two veliger stages (D-veliger and pediveliger) and become a benthonic postlarva through metamorphosis [2]. Metamorphosis was also proved to be the critical phase in terms of mortality in the life cycle for bivalves [3]. The developmental biology of marine bivalves is essential for both scientific research and aquaculture.

Cysteine-rich intestinal protein (CRIP) was first identified in rat intestine through its developmental regulation during the neonatal period [4]. It belongs to the LIM/double-zinc-finger protein family, members of which includes cysteine- and glycine-rich protein-1 (CSRP1), rhombotin-1 (RBTN1), rhombotin-2 (RBTN2), and rhombotin-3 (RBTN3) [5]. LIM domain is a highly conserved double-zinc-finger structure that comprises $Cys_2HisCys$ and $Cys_4$ sequences [6]. Because the zinc-binding domain has been shown to be present in three gene transcription factors, lin-11 (*Caenorhabditis elegans* cell lineage gene), rat isl-1 (rat insulin I gene enhancer region-binding protein), and mec-3 (*C. elegans* gene required for differentiation of mechanosensory neurons) it was termed as LIM motif [7–9]. CRIP contains unique LIM domain and a short additional conserved motif. This LIM domain has proposed to have a function of facilitating protein–protein or protein-DNA interactions [6,9].

Most functional studies of CRIPs were focused on vertebrates, especially human and rat. For rat, is has high expression level in small intestine; lower amounts in colon, lung, spleen, adrenal,

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testicle, skin, heart, skeletal muscle, and stomach; no expression in brain, kidney, or day 19 placenta [4,9,10]. CRIP has been suggested to play an indispensable role in the host defense system. The differential expression of CRIP can alter cytokine patterns and the immune response in transgenic mice [11]. A study pointed out that CRIP might also play a role in Th1/Th2 cytokines balance [6]. In human, CRIP has been observed to be overexpressed in several tumor types [12–16], and it was identified as a novel marker for early detection of cancers [17].

Though well documented in vertebrates, CRIPs have been rarely characterized in invertebrates to date. In the present study, a clam homolog of the mammalian CRIP, named MmCRIP, was identified as a candidate development related gene from *M. meretrix* based on the transcriptomic data on early development stages. The functions of MmCRIP in clam development and immunity were analyzed via quantitative real-time PCR (qRT-PCR) and RNAi methods.

## 2. Materials and methods

### 2.1. Animal

The Asiatic hard clams *M. meretrix* (2–3 years old), used as parent clams or for bacterial challenge were bought from a market in Qingdao, China and acclimated for about 2 weeks (approximate 25 °C, 30‰ salinity and under continuous aeration) in the laboratory. Parent clam culture, spawning, and larvae rearing were conducted as described by Wang et al. [18].

Larvae of four developmental stages including trochophore, D-veliger, pediveliger and postlarva were collected from four individuals after fertilization, respectively. To determine the tissue-specific expression of MmCRIP, four tissues including intestine, muscle, hepatopancreas and gills, were collected from five healthy adult individuals. Haemolymph from these five hard clams was also collected and then immediately centrifuged at 800 g, 4 °C for 10 min to harvest the hemocytes. For bacterial infection experiments, 50 adult clams were challenged according to the procedure reported by Yue et al. [19]. Briefly, the acclimated clams were injected with 50 μl of *Aeromonas hydrophila* suspension at the concentration of ~$5 \times 10^7$ CFU $ml^{-1}$ per clam. Five individuals were randomly sampled at 0, 6, 12, 24, 36 and 48 h post injection. The hemocyte, gill and hepatopancreas samples were collected for real-time PCR analysis. All samples were reserved in liquid nitrogen before processing for RNA extraction.

### 2.2. RNA extraction and reverse transcription

Total RNA was isolated from larvae and tissues using an RNAiso Plus kit (TaKaRa, Japan). The cDNA was synthesized with TransScript Reverse Transcriptase (TransGen Biotech, China) using total RNA as template and AOLP as primer. Table 1 demonstrated the sequences of primers used in experiments.

### 2.3. Cloning of MmCRIP

A transcript of gene encoding for CRIP-like protein was found in the *M. meretrix* transcriptome database. The 3′-end of *MmCRIP* was obtained by 3′ RACE with gene-specific primer CR-F1 and adapter primer AP. The PCR parameters were as follows: 1 cycle of 94 °C for 4 min, 35 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 60 s, followed by the final extension at 72 °C for 10 min. PCR products were gel-purified and cloned into the pMD19-T simple vector (TaKaRa, Japan) and then sequenced at Shanghai Sangon Company (Shanghai, China).

**Table 1**
Primers used for MmCRIP.

| Name | Sequence (5′→3′) |
|---|---|
| AOLP | GGCCACGCGTCGACTAGTAC$(T)_{16}$(A/C/G) |
| AP | GGCCACGCGTCGACTAGTAC |
| CR-F1 | GACACGACACCAGCATTGCC |
| CR-F2 | AACATTGTCTGCTGGTTCTCA |
| CR-R2 | TGTTATTAGTGCTGCGGGTT |
| ACT-F | TTGTCTGGTGGTTCAACTATG |
| ACT-R | TCCACATCTGCTGGAAGGTG |
| CR-F3 | TAGGATCCATGCCTAAGTGTCCGAACTGT |
| CR-R3 | GCCTCGAGTTACTTTTTGTACGTATAAC |
| EGFP-F | CGGAATTCATGGTGAGCAAGGGCGAGGA |
| EGFP-R | CGCTCGAGCTTGTACAGCTCGTCCATGC |

### 2.4. Bioinformatics analysis for MmCRIP

Firstly, the coding sequence of *MmCRIP* was identified based upon alignments with known *CRIP* sequences, which derived from NCBI's GenBank database. BioEdit software (version: 7.0.9.1) was used to deduced the amino acid sequence of *MmCRIP*. Then, molecular weight (Mw) and isoelectric point (pI) of the protein was calculated based upon its amino acid composition using the Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). The 3D structure was predicted using SWISS MODEL in ExPASy database (http://www.expasy.ch/). SignalP 4.1 program was utilized to predict the presence of signal peptide in amino acid sequence (http://www.cbs.dtu.dk/services/SignalP/) [20].

For identification of putative protein motifs analysis, the following programs were performed: Motif-Scan software (Pfam HMMs global models database) available at SIB (Swiss Institute of Bioinformatics; http://hits.isb-sib.ch), the Proscan (http://pbil.ibcp.fr) and the PESTfind software available at the EMBOSS (European Molecular Biology Open Software Suite): epestfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind) [21].

Finally, MmCRIP was aligned with known CRIP amino acid sequences from other species using ClustalW multiple alignment. Neighbor-joining tree was generated from multiple sequence alignments using MEGA software (version 4.0). Bootstrap analysis of 1000 replicates was carried out to determine the confidence of tree branch positions [22].

### 2.5. qRT-PCR analysis of MmCRIP mRNA expression

*MmCRIP* mRNA expression was detected by SYBR Green fluorescent quantitative real-time PCR (qRT-PCR) in a BioRad CFX96 detection system with forward primer CR-F2 and reverse primer CR-R2. The amplification conditions were as follows: 1 cycle of denaturation at 95 °C for 3 min and 40 cycles of 95 °C for 20 s, anneal and extension at 60 °C for 30 s. In order to confirm whether only one specific PCR product was amplified, a melt cycle, in which PCR product was denatured from 55 to 95 °C, was added to each thermal profile to produce melting-curve. All reactions were run in four replicates with *β-actin* as an internal reference gene (forward primer ACT-F and reversed ACT-R). The relative expression of *MmCRIP* was calculated using the comparative Ct method with the formula $2^{-\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct$ target $- \Delta Ct$ reference) [23].

Data obtained from qRT-PCR analysis were subjected to one-way analysis of variance (one-way ANOVA). Moreover, the *P*-values less than 0.05 were considered statistically significant.

### 2.6. Preparation of dsRNA of MmCRIP

Procedures for the preparation of dsRNA and detection of silencing efficiency were similar as described previously [24]. Briefly, the recombinant plasmid pL4440-MmCRIP expressing



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