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A novel junctional adhesion molecule A (CgJAM-A-L) from oyster (*Crassostrea gigas*) functions as pattern recognition receptor and opsonin

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ABSTRACT

Junctional adhesion molecule (JAM), a subfamily of immunoglobulin superfamily (IgSF) with a couple of immunoglobulin domains, can act as regulator in homeostasis and inflammation of vertebrates. In the present study, a structural homolog of JAM-A (designated CgJAM-A-L) was screened out from oyster, *Crassostrea gigas*, through a search of JAM-A D1 domain (N-terminal Ig domain in JAM-A). The cDNA of CgJAM-A-L was of 1188 bp encoding a predicted polypeptide of 395 amino acids. The immunoreactive area of CgJAM-A-L mainly distributed over the plasma membrane of hemocytes. After *Vibro splendidus* or tumor necrosis factor (CgTNF-1) stimulation, the mRNA transcripts of CgJAM-A-L in hemocytes increased significantly by 4.46-fold and 9.00-fold ($p < 0.01$) of those in control group, respectively. The recombinant CgJAM-A-L protein (rCgJAM-A-L) could bind multiple PAMPs including lipopolysaccharides (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA), mannose (MAN), β-glucan (GLU) and poly(I:C), and various microorganisms including *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *Vibro anguillarum*, *V. splendidus*, *Pastoris pastoris* and *Yarrowia lipolytica*. The phagocytic rates of oyster hemocytes towards Gram-negative bacteria *V. anguillarum* and yeast *P. pastoris* were significantly enhanced after the incubation of rCgJAM-A-L, and even increased more significantly after the pre-incubation of rCgJAM-A-L with microbes ($p < 0.01$). The results collectively indicated that CgJAM-A-L functioned as an important pattern recognition receptor (PRR) and opsonin in the immune defense against invading pathogen in oyster. Moreover, as the most primitive specie with homolog of JAMs, the information of CgJAM-A-L in oyster would provide useful clues for the evolutionary study of JAMs and immunoglobulins.

1. Introduction

Immunoglobulin superfamily (IgSF) is a diversed collection of proteins containing one or more immunoglobulin (Ig) domains, which performs a wide range of functions in various fundamental processes, such as immune response, neural cell development, anti-tumoral action, and simply in cell adhesion (Brümmendorf and Lemmon, 2001; Halaby and Mornon, 1998; Rougon and Hobert, 2003). The Ig domain was characterized by 7–10 β-strands, distributed between two sheets with typical topology and connectivity (Halaby et al., 1999; Harpaz and Chothia, 1994), and they have diverged in structure as V-set, C-set and I-set. Based on the binding character of atomic mechanisms, Ig domains are often found to provide an ideal structural platform for IgSF to generate a large spectrum of potential protein-protein interaction surfaces (Vaughn and Bjorkman, 1996). Among them, Ig domain containing cell adhesion molecules (IgSF CAMs) mainly contribute to innate immunity through cell-mediated defense reactions, such as phagocytosis and cell spreading (Schmidt et al., 2010).

As canonical representatives of IgSF CAMs, junction adhesion molecules (JAMs) are found to be located within tight junctions initially, and they are usually composed with two immunoglobulin domains (Ig domains), one transmembrane spanning segment and a cytoplasmic tail (Luissint et al., 2014). Based on the distinctive

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adhesion properties and cellular localizations (Aurrand-Lions et al., 2001a, 2001b; Liang et al., 2002; Moog-Lutz et al., 2003), JAMs were given the names of JAM-A (Malergue et al., 1998), JAM-B (Cunningham et al., 2000), and JAM-C (Arrate et al., 2001), which shared 32–38% sequence identity. JAM-A is differentiated from JAM-B and C in the absence of extra pair of cysteines within their membrane-proximal Ig domain (Palmeri et al., 2000). No general pattern of canonical Ig domain subtypes was evidenced among the JAM proteins (Mandell and Parkos, 2005). For example, murine JAM-A contains two V-set domains (Kostrewa et al., 2001), while human JAM-A has been reported to contain a V-type and I-type domain by crystal structure analysis (Prota et al., 2003). In accordance with structure and location, JAMs regulate leukocyte/platelet/endothelial cell interactions for the tight junction formation and maintenance in the immune system (Bazzoni, 2011; Bradfield et al., 2007; Coyne, 2009; Naik et al., 2008). Besides epithelial tight junction formation and maintenance, JAM-A, with D1 domain (N-terminal Ig domain) strikingly resembling the variable domain of immunoglobulin, IgAk, also took roles in defective polarization and cell motility to enhance the activation of adaptive immunity (Cera et al., 2009; Dermody et al., 2009). JAM-B was reported in regulation of rolling and firming adhesion of T lymphocytes under shear stress (Ludwig et al., 2009). For JAM-C, it promoted leukocyte migration by controlling cell polarization (Woodfin et al., 2011). Beyond those, JAMs also took roles as virus receptors, under the activation of NF-kB signaling (Coyne, 2009).

In invertebrate, although the tight junction has been evidenced in arthropods (Lane, 1981) and ascidian (Martinucci et al., 1988), the homologs of JAMs are not well documented to date. For instance, JAM-B has been revealed in the genome of red beetle (Kim et al., 2010), but its function is still obscure. Intriguingly, a structural homolog search of JAM-A D1 domain from invertebrate screened out a collection of precursors of adaptive immune factors with high haplotype variations (Dermody et al., 2009), such as Dscam and variable region containing chitin-binding protein (VCBP). In fruit fly, tens of thousands of isoforms can be generated for Dscam by alternative splicing for acquired immunity (Rimer et al., 2014). In amphioxus, a large number of VCBP alleles and haplotypes have been approved to promote bacterial recognition and phagocytosis (Dishaw et al., 2010). It is favored that the identical IgSF proteins in invertebrates could serve as precursors of soluble adaptive immune effectors. Ig or Ig-like domains seem to be more abundant in mollusc than that in other invertebrates. For instance, a total of 284 Ig-like domain contained proteins were identified in oyster *Crassostrea gigas* genome, and a clear expansion was revealed when comparing with honey bee *Apis mellifera* (127) and fruit fly *Drosophila melanogaster* (137) (Zhang et al., 2012). However, the knowledge about homolog of JAMs and their roles in invertebrates are still extremely limited.

The oyster *C. gigas* is a bivalve native to Asia, and contributes weightily to the aquaculture industry of northern China, Korea and Japan. The recent released genome sequences provided valuable information to investigate the invertebrate immune system. The information about the immunological roles and evolutional trace of JAMs will pave a new way to further understand the heterogeneous immune system of mollusc and the precursors to adaptive immune effectors. In the present study, the genome of oyster *C. gigas* was screened with JAM-A D1 domain as a query, and a structural homolog protein containing three Ig domains was identified (designated as CgJAM-A-L) with the main objectives (1) to identify the structural homolog and trace the precursor of JAMs from *C. gigas*, (2) to investigate its temporal expression after the treatments of *Vibro splendidus* and cytokine, (3) to survey its binding activity to pathogen-associated molecular pattern (PAMP) and microorganism, (4) to examine its effect on the enhancement of hemocyte phagocytosis to understand its opsonic role in oyster.

2. Materials and methods

2.1. Oysters and microbes

Adult oysters *C. gigas* (average shell length of 13.0 cm) were collected from a local farm in Qingdao, Shandong Province, China, and maintained in aerated freshwater at 15 ± 2 °C for a week before processing.

Bacteria *Micrococcus luteus* (Microbial Culture Collection Center, Beijing, China) and *Escherichia coli* TOP10 F' (Transgen) were suspended in LB medium at 37 °C. *Staphylococcus aureus* (Microbial Culture Collection Center, Beijing, China) was grown in LB medium at 28 °C. *Vibrio anguillarum* (provided by Dr. Mo) and *V. splendidus* (Liu et al., 2013) were grown in 2116E medium at 28 °C. Fungi *Pichia pastoris GS115* (Invitrogen) and *Yarrowia lipolytica* (provided by Dr. Chi) were grown in YPD medium at 28 °C.

2.2. Immune stimulation and hemolymph collection

Ninety oysters were employed and randomly divided into 3 groups for the stimulation experiment. The oysters in two treatment groups received an injection of recombinant protein of CgTNF-1 (1 ng) (Sun et al., 2014) and *V. splendidus* (1×10^7 CFU mL^{-1}) in 100 μL phosphate buffered saline (PBS, 0.14 M NaCl, 3 mM KCl, 8 mM $NaH_2PO_4 \cdot 12H_2O$, 1.5 mM K_2HPO_4, pH 7.4), respectively. The rest oysters received an injection of equivalent PBS, and were employed as blank group. After treatment, the oysters were returned to water tanks and 6 individuals were randomly sampled at 3, 6, 12 and 24 h post-injection in *V. splendidus*-challenged and PBS-treated group, and at 6 h in CgTNF-1-treated group. The hemolymph were collected and centrifuged at 800 × g, 4 °C for 10 min to harvest the hemocytes. All these hemocyte samples were stored at −80 °C after addition of 1 mL RNAiso plus reagent (Takara) for subsequent RNA extraction.

2.3. Sequence analysis of CgJAM-A-L

The cDNA sequence was cloned by PCR with primers CgJAM-A-L-Fw and CgJAM-A-L-Rv (Table 1). The deduced amino acid sequence of CgJAM-A-L was analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org). The signal peptide was predicted by the SignalP 4.1 server (http://www.cbs.dtu.dk/service/SignalP), and the protein domains were revealed by the Pfam

Table 1
Primers used in this study.

Primer name	Sequence (5′ − 3′)
Clone primers	
Oligo(dT)-adapter	GGCCACGCGTCGACTAGTACT$_{17}$
T7	GTAATACGACTCACTATAGGGC
CgJAM-A-L-Fw	ATGATAATGCTCGGATTACTC
CgJAM-A-L-Rv	TTTTATTATCACAGAAAGAGTTC
Sequence primers	
M13-Fw	CGCCAGGGTTTTCCCAGTCACGAC
M13-Rv	GAGCGGATAACAATTTCACACAGG
RT-PCR primers	
CgEF1-α-rtFw	AGTCACCAAGGCTGCACAGAAAG
CgEF1-α-rtRv	TCCGACGTATTTCTTTGCGATGT
CgJAM-A-L-rtFw	ACAACTACGCCATCACCTTCC
CgJAM-A-L-rtRv	AGGCTGTCCGTTCCTACTGC
Recombination primes	
CgJAM-A-L-exFw	GGAATTCCATATGATGATAATGCTCGGATTACTC
CgJAM-A-L-exRv	CGCGGATCCTTTTATTATCACAGAAAGAGTTC