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Molecular characterization of a novel tetraspanin from the oyster, *Crassostrea ariakensis*: Variation, localization and relationship to oyster host defense

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ABSTRACT

We identified a tetraspanin family member gene, named Ca-TSP, in the oyster *Crassostrea ariakensis* and found that the transcription profiles of Ca-TSP were variable in the oyster hemocytes. Three distinct patterns of variation of Ca-TSP were observed. Using immunofluorescence and immunoelectron microscopy, we show that Ca-TSP was present in granules and in vesicular structures of the oyster hemocyte. Sequence analysis, structural features and immunogold electron microscopy showed that Ca-TSP is an integral membrane glycoprotein of granules of hemocyte and may be a novel CD63-like gene of the tetraspanin family of molluscs. The gene expression analysis of Ca-TSP using isolated oyster hemocytes, was done following challenge of the oysters with LPS and Poly I:C. The Ca-TSP mRNA levels increased in hemocytes in the first 12 h after LPS and Poly I:C stimulation, and decreased after the addition of H_2O_2. Western blot analysis using anti-Ca-TSP antibody indicated that gene expression and protein levels were similar. The recombinant Ca-TSP was found to significantly inhibit hemocytes aggregation. Our results suggested that Ca-TSP participates in the innate immunity of the oyster.

1. Introduction

Tetraspanins (also referred to as tetraspans or TM4SF proteins) are a family of four-transmembrane-domain proteins, found in mammals, insects, worms and fungi [1–3]. Tetraspanin proteins regulate cell morphology, motility, invasion, fusion and signaling as organizers of multi molecular membrane complexes. The first tetraspanin protein, ME491/CD63, was characterized in 1988 and the hallmark family sequence motifs were reported in 1990 [4–7]. In addition to four transmembrane domains these proteins feature several conserved amino acid residues including a highly conserved CCG motif, where two cysteine residues are required for formation of essential disulphide bonds of the second extracellular loop [8,9].

There is a mounting body of evidence that suggests that invertebrates have complex immune responses and significant immune diversity [10–13]. The oysters have been used as a model for the examination of the interactions between the host immune system and pathogens in molluscs [14–18]. Recent studies have used sequence tags (ESTs) of oyster obtained from cDNA libraries [19–21] to identify genes involved in physiological response of oysters to both environmental stressors and pathogens [22,23].

The biological role of tetraspanins in blood cell functions has been well established, but there are no reports about their functional role in the molluscs. In this study, we examined the possible roles of a novel tetraspanin family member, named Ca-TSP, in the oyster *Crassostrea ariakensis*. Here we report the sequence variation from a tetraspanin family gene and biological activity of the recombinant CA-TSP. We also examined the localization of Ca-TSP in the hemocyte of *C. ariakensis* and analyzed gene expression after stimulation of the oyster with LPS, Poly I:C and H_2O_2.

2. Material and methods

2.1. Ethic statement

The oysters used in the present study were marine cultured animals, and all the experiments were conducted according to the regulations of Chinese local and central governments.

2.2. Animals, hemolymph collection, hemocyte plating and immune challenge

Oysters, *C. ariakensis*, were maintained in tanks containing aerated artificial seawater at room temperature (22 ± 1 °C).

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Hemolymph was collected from the pericardial cavity using a sterile 1 ml syringes pre-loaded with 0.5 ml ice-cold simple salt solution (SSS, 102.4 g/l NaCl, 1.8 g/l KCl, 5.1 g/l $CaCl_2$, 11.8 g/l $MgCl_2$, and 16.7 g/l $MgSO_4$) [24] containing 9 mM EDTA. The hemolymph was centrifuged for 10 min at 300× *g* to pellet the cells. Hemocyte monolayers were prepared for as described previously [18]. Hemocytes were incubated with stimulants (Lipopolysaccharide, 10 mg LPS; 150 μM H_2O_2; 10 mg polyinosinic:polycytidylic acid, poly I:C). These stimulants were all dissolved in SSS. Hemocytes were collected after 12–16 h after treatment.

2.3. RNA extraction and cloning of oyster Ca-TSP and sequence analysis

Total RNA extraction from hemocytes of oyster was carried out as described [25]. The full-length sequence of Ca-TSP was obtained from the oyster hemocyte cDNA library developed in our laboratory. cDNA library was constructed with the SMART™ cDNA library Construction Kit (Clontech, USA) according to the manufacturer's instruction. Original RNA samples were treated to remove residual DNA and MMLV Reverse Transcriptase and Advantage 2 PCR Kit were used for first and 2nd strand synthesis. The primers used in this study are shown in Table 1. RT-PCR was performed using the following thermocycler conditions: 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s and a final elongation step at 72 °C for 5 min.

For sequence variation of Ca-TSP, RT-PCR and molecular cloning were done using hemocytes cDNA prepared from individual oyster. Ten clones of a single hemocyte cDNA library were randomly picked up and sequenced. The cDNA clones of library from 20 oysters were sequenced. *Pfu* polymerase (Takara, Japan) was used in the detection of sequence variation, and a low cycle number re-amplification of a 10-fold diluted mixed-template PCR product was used to reduce artificial genetic diversity [26]. Actin and elongation factor were used as control genes. *C. ariakensis* species was identified by multiplex species-specific PCR [27].

Sequence was processed by SMS (Sequence Manipulation Suite). Sequence similarity was determined using BLAST program (NCBI). Sequence alignment of the LEL (large extracellular loop) region of tetraspanins was performed using DNAman. Neighbor-Joining analysis of distances with aligned sequences was used to create a phylogenetic tree with MEGA (version 4.0).

2.4. Quantitative PCR analysis of Ca-TSP expression

Extraction of total RNAs from challenged hemocytes was conducted as described above, and the RNA reverse transcribed into cDNA, using M-MLV reverse transcriptase (Invitrogen, USA). Real-time PCR was performed using the SYBR *Premic Ex Tag*™ KIT (Takara, Japan). Real-time RT-PCR was carried out to measure the expression level of Ca-TSP, following the Real-time PCR protocol described by manufacturers. The primers used in Real-time PCR are shown in Table 1. PCR reaction was performed in a 25 μl volume, 10 μM of each specific primer, 2 μl of cDNA and 12.5 μl SYBR*Premix Ex Taq*™ in an iCycler iQ™ thermocycler (Bio-Rad) using the following procedure: 30 s at 95 °C, followed by 40 cycles of 94 °C for 10 s, 58 °C for 20 s. The relative expression level of Ca-TSP was calculated according to the $2^{-\Delta\Delta CT}$ method [28]. And 28S rDNA gene (GenBank accession no. AF137052) was used as a housekeeping gene. All data were given in terms of relative mRNA expression as mean ± S.E. ($N = 6$) and analyzed by Student's *t*-test, and $P < 0.05$ was considered to be statistically different.

Table 1
Specific primers used in this study.

Ca-TSP ORF	
Forward primer	5′-ATGGGGTTGGACTGTGGA-3′
Reverse primer	5′-TTAAGCCAAGTTTCCTTCGCC-3′
Ca-TSP Real-time	
Forward primer	5′-CAAAACCAGCTAAACTCAGTC-3′
Reverse primer	5′-GCTGCTGTACTTTGAGGTATC-3′
28S rDNA	
Forward primer	5′-GAATCCCTCATCCTAGCGA-3′
Reverse primer	5′-CACGTACTCTTGAACTCTCTC-3′

2.5. Production and purification of recombinant Ca-TSP and anti-Ca-TSP antibodies

PCR products for LEL (Large Excellular Loop) region (120–240 amino acids) and total open reading frame (ORF) of Ca-TSP were digested with restriction enzymes (*Bam* HIand *Xho*I), then ligated to pET28a vector (Novagen, USA). The resulting recombinant plasmids pET28a-Ca-TSP was transformed to *Escherichia coli* BL21 (DE3) and induced with 1 mM isopropyl β-D-thiogalactoside (IPTG). The recombinant fusion proteins were purified by affinity chromatography through the nickel–nitrilotriacetic acid agarose resins (Novagen, USA). The denatured recombinant protein was refolded by dialysis against phosphate-buffered saline (PBS; 10 mM phosphate buffer pH 7.4) and 5% glycerol at 4 °C overnight. Anti-Ca-TSP rabbit polyclonal antibodies were raised against recombinant His-tagged Ca-TSP. Anti-Ca-TSP antibodies were affinity-purified with the antigen.

2.6. Western blotting

The Ca-TSP samples were re-suspended in 2 × SDS sample buffer (1:1), resolved under reducing condition using 12% SDS-PAGE, and transferred by electroblotting onto polyvinylidene fluoride membrane. Membranes were blocked for 1 h at room temperature using 5% skim milk in phosphate-buffered saline contain 0.1% Tween 20 (PBST) and probed with anti-Ca-TSP antisera (1:15,000) at 4 °C overnight. Subsequently, membranes were washed with PBST and incubated with goat anti-rabbit Ig G antibody, conjugated with horseradish peroxidase (1:30,000, Pierce) for 1 h at room temperature. The membranes were washed with PBST and then exposed to SuperSignal West Pico Chemiluminescent Substrate System (Pierce) before exposure to X-ray film. Quantification for total protein was performed using Bradford method [29].

2.7. Immunofluorescence and immunoelectron microscopy of the tetraspanin

Immunofluorescence microscopy was performed according to the method described in Ref. [30] and modified by Yang [15]. Briefly, hemocytes suspensions were settle onto slides and incubated in a humid chamber at room temperature for 15 min. Slides were washed by simple salt solution (SSS). The hemocytes were fixed using three consecutive fixing solutions: prefixed using 0.002% glutaraldehyde in SSS for 10 min; fixed in 1% formaldehyde, 0.25% Triton X-100 in phosphate-buffered saline (PBS, 0.15 M phosphate buffer pH 7.4) for 10 min; postfixed in cold (−20 °C) methanol for 15 min. Slides were washed three times in PBS and incubated in blocking solution (2% v/v normal goat serum) for 30 min in a humid chamber. Slides were washed and the slides containing fixed hemocytes were probed using anti-Ca-TSP antibody for 45 min at room temperature in a humid chamber. Cells were washed as described above, and incubated with goat anti-rabbit immunoglobulins conjugated to Dye Light 549 (Pierce, 1:500). Negative controls included pre-immune sera as well as