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Blocking the large extracellular loop (LEL) domain of *Fc*Tetraspanin-3 could inhibit the infection of white spot syndrome virus (WSSV) in Chinese shrimp, *Fenneropenaeus chinensis*

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ABSTRACT

Tetraspanins belong to the transmembrane 4 superfamily (TM_4SF), which span the cell membrane 4 times and act as bridges or connectors. Increasing evidences have shown that tetraspanins play important role in virus infection. The large extracellular loop (LEL) of a tetraspanin is considered as a possible target of some virus. Tetraspanins are widely found in invertebrates, but the functional roles of most invertebrate tetraspanins have remained unknown. Recently, a tetraspanin, called *Fc*Tetraspanin-3, was cloned from the cDNA library of Chinese shrimp, *Fenneropenaeus chinensis*. The *Fc*Tetraspanin-3 constitutive expression in all examined tissues and the expression of the gene were highly induced in hepatopancreas, lymphoid organ and intestine by white spot syndrome virus (WSSV) challenge. In this study, we expressed and purified the recombinant peptide containing the LEL domain of *Fc*Tetraspanin-3, and produced the anti-LEL polyclone antibody. The expression of *Fc*Tetraspanin-3 was observed by real-time PCR and Western blot. Also, the localization of *Fc*Tetraspanin-3-positive cells in intestine and hepatopancreas were revealed by immunofluorescence. The results of anti-LEL antibody blocking experiments shown that the antibody can significantly reduce the mortality of shrimp challenged by WSSV. Additionally, dsRNA interference was utilized to examine the functional role of *Fc*Tetraspanin-3 in response to WSSV infection, and a sensible decrease of the viral copy number in the tetraspanin knockdown shrimp. These results suggested the blocking of LEL domain of *Fc*Tetraspanin-3 could inhibit the infection of WSSV. *Fc*Tetraspanin-3 might play an important role in response to WSSV infection, and the LEL domain of *Fc*Tetraspanin-3 might mediate the entry of WSSV.

1. Introduction

Tetraspanins belong to the transmembrane 4 superfamily (TM4SF), a large family of evolutionarily conserved cell-surface proteins that include at least 33 members in mammals [1]. They are widely distributed in many cell types of eukaryotic organisms [2]. As transmembrane proteins, tetraspanins can act as a bridge to connect the proteins outside or inside the cell membrane. A tetraspanins web is formed by the tetraspanins–proteins complex, and the web is believed to involve in fundamental functions of immunity system, and consequently, signaling between cells and inside cells, regulating cell activation and adhesion, participating in recognition and infection of some viruses [2–4].

The common structure of tetraspanin protein consists of four transmembrane (TM) domains, a small extracellular loop (SEL) and a large extracellular loop (LEL) [5]. The four highly conserved cysteine residues maintained in LEL can form a mushroom-like shape [6], which defines an important structure in their specific functions [7,8]. At least 20 tetraspanin proteins express on the surface of leukocytes in human, which belong to CD molecules, and are highlighted by the important functions observed in the immune system [9]. Specific tetraspanin family members are selectively associated with specific viruses and affect multiple stages of infection, including initial cellular attachment to syncytium formation and viral particle release [7]. CD9 is considered to be involved in feline immunodeficiency virus (FIV), canine distemper virus and human immunodeficiency virus-1 (HIV-1) infections [10–12]. The antibody of CD9 could inhibit FIV [13]. CD9 and CD81 can modulate HIV-1-induced membrane fusion [14], and the specific antibody which recognized the LEL of CD9 could suppress

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the release of HIV-1 [15]. CD81 is the receptor protein identified by hepatitis C virus (HCV) [6,16,17]. LEL blocking by antibody could prevent the invasion of HCV, the LEL of CD81 was proved to interact specifically with HCV [17]. CD63 relates to the entrance and egress of HIV-1 [18]. HIV-1 infection can be inhibited by antibodies which bind to the LEL of CD63 [19]. CD82 participates in the infection of human T-cell leukemia/lymphoma virus type 1 (HTLV-1) [7,20,21]. Research confirms that the association of HTLV-1 with tetraspanin-enriched microdomain is mediated by the inner loop of CD82 [22]. CD151 could cooperate in porcine reproductive and respiratory syndrome virus (PRRSV) infection, and antibody treatment of CD151 completely blocked PRRSV infection [23]. There were compelling evidences proved by using antibody that tetraspanins and especially the extracellular structure of LEL play key role in the route of pathogens infection.

Our previous study cloned and identified the full length cDNA of one tetraspanin (*Fc*Tetraspanin-3) from Chinese shrimp *Fenneropenaeus chinensis*, and observed that it was markedly up-regulated in the live WSSV-challenged shrimp tissues [24]. In order to understand the function of *Fc*Tetraspanin-3 during the WSSV infection, the LEL motif sequence of *Fc*Tetraspanin-3 was cloned into pGEX-4T-1 vector and the recombinant peptide was expressed in *E*scherichia *coli* BL21 (DE3). Subsequently, the anti-LEL polyclone antibody was prepared and used to analyze the expression profiles in different tissues and to perform antibody blocking experiments for WSSV infection. Additionally, dsRNA interference was utilized to examine the functional role of *Fc*Tetraspanin-3 in response of WSSV infection.

2. Materials and methods

2.1. Experimental animals and virus

Chinese shrimp *F. chinensis* with a body length of 6.5 cm ± 1 cm (weighing 4–5 g) were fed with clam meat at our lab. Before doing experiments, the shrimp were tested by a WSSV-specific PCR reaction as described previously [25], and only WSSV-free shrimp were used for the experiments.

WSSV virus was originally isolated from the infected *F. chinensis*. Intact WSSV virions were purified as described previously [25]. The purified WSSV used for infection experiments was calculated to be 6×10^5 copies/μl in the viral genomic copies by quantitative real-time PCR.

2.2. Expression and isolation of recombinant peptide

*Fc*Tetraspanin-3 (EF032649) contains a 720 bp open reading frame (ORF), which encodes 239 amino acids [24]. The position of LEL motif in *Fc*Tetraspanin-3 is from 104 to 205, containing 101 amino acids, and the molecular weight (MW) of LEL is about 11 kDa. Based on the LEL motif nucleotide sequence, a pair of primers, LEL-f and LEL-r (shown in Table 1) were designed by Primer Premier 5.0 program. The few sequences located on the both sides of LEL motif were either cloned by the primers. Restriction enzyme cutting site sequence of *Bam*HI and *Sal*I were introduced separately to the 5′ end of LEL-f and LEL-r, and obtained LEL-F and LEL-R (shown in Table 1). PCR was performed using the previously cloned shrimp cDNA [24] and primers LEL-F and LEL-R, under the following conditions: one cycle of 94 °C for 5 min; 35 cycles including denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s; followed by one cycle of 72 °C for 10 min. Then the amplified fragment was ligated into pMD-19T vector (Invitrogen). The positive transformant pMD-LEL was amplified and gel-purified. Plasmid pMD-LEL was restriction double digested and the gene portion of LEL was ligated into the pGEX-4T-1 vector (Invitrogen) at the *Bam*HI and *Sal*I sites. LEL sequence was expressed in *E. coli* BL21 (DE3) by induction with 1 mM IPTG (Promega). Cells were harvested by centrifugation 10000 rpm for 5 min and resuspended in phosphate-buffered saline (PBS, pH 7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4), with Triton X-100 added to a final concentration of 1%. The cell suspension was disrupted by sonication in 400 w for 30 min. The cell lysate was centrifuged at 12,000 rpm for 20 min at 4 °C to collect the inclusion bodies, which were then solubilized in denaturing solution (0.1 M PBS, pH 7.4, 8 M Urea, and 10 mM DTT), Under the condition of 4 °C, the denaturing proteins were dialyzed against renaturing solution (0.1 M PBS, pH 7.4, 4 M Urea, and 10 mM DTT) for 24 h, and then dialyzed against 0.1 M PBS, pH 7.4 for 24 h. The vacuum filter was performed through a 0.45 μm nitrocellulose membrane. The recombinant GST-LEL peptide was purified using glutathione-agarose column (GSTrap HP 1 ml, GE Healthcare) according to the recommended protocol (GE). A denaturing SDS-12%(w/v) PAGE gel was used to analyze all the samples, and the band at 38 KDa (presumed to be GST-LEL) stained strongly by Coomassie Blue was excised from the gel and confirmed by Mass Spectrometry after tryptic digest using the reported method [26].

Table 1
The primers list.

Primer	Sequences (5′–3′)	NCBI accession number	Annealing temperature (°C)
LEL-F	CGAGGATCCGCCATC CTTATCTTCGTCTA	EF032649.1	58
LEL-R	CGAGTCGACCTAAGCA ACCACAACATTCTG		
LEL-f	GCCATCCTTATCTTCGTCTA		55
LEL-r	CTAAGCAACCACAACATTCTG		
EGFP-f	CAGTGCTTCAGCCGCTACCC	EU716633.1	58
EGFP-r	AGTTCACCTTGATGCCGTTCTT		
vp28-f	ATGGATCTTTCTTTCACTCTTTC	EU414753.1	55
vp28-r	GGTCTCAGTGCCAG		
18s rRNA-f	TATACGCTAGTGGAGCTGGAA	AY438005.1	58
18s rRNA-r	GGGGAGGTAGTGACGAAAAAT		

2.3. Antiserum preparation

Antiserum against the recombinant LEL peptide was made separately by immunizing white rabbit according to the reported method [27,28]. And, the anti-LEL antibody was purified with protein A-Agarose Kit (Beyotime, Shanghai, China) as described [29], and quantified by Bradford Method according to the Kit (Sangon, Shanghai, China). Final concentration of the purified anti-LEL was 2 μg/μl and stored at −80 °C.

2.4. Western blot detection and immunofluorescence localization

To analyses the expression level of *Fc*Tetraspanin-3 in shrimp tissues, nine different shrimp tissues, including stomach, epidermis, heart, ventral nerve cord, hemocyte, gill, intestine, lymphoid organ and hepatopancreas were collected from 9 untreated individuals and each tissue from three individuals was pooled together and grinded in liquid nitrogen. Tissue debris was lysed by Ripa lysis buffer (Solarbio, Beijing, China), and about 50 μg extracted proteins were separated by SDS-PAGE using a 12% acrylamide tris/tricine gel. Proteins were then electro-transferred onto 0.45 μm PVDF membranes (Millipore) in an electrophoretic transfer (Bio-Rad) using a 50-mM Tris–HCl buffer, pH8.0 with constant voltage at 100 V for 60 min. Following blocked with 5%BSA and 0.1%Tween20 (v/v) in PBS at 4 °C overnight, the membrane was