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Syntenin is involved in the bacteria clearance response of kuruma shrimp (Marsupenaeus japonicus)

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ABSTRACT

Syntenin is a multifunctional cytosolic adaptor protein that contributes to cell migration, proliferation, attachment, and apoptosis, as well as immune response to virus, in vertebrates. However, the functions of syntenin in the antibacterial response of invertebrates remain unclear. In this study, we identified a syntenin-like gene (MjSyn) from the kuruma shrimp (Marsupenaeus japonicus) and detected its function in the antibacterial immunity of shrimp. The full-length MjSyn was 1223 bp with a 963 bp open reading frame that encodes 320 amino acids. The deduced MjSyn proteins contained two atypical PDZ domains (sequence repeat that was first reported in the postsynaptic density protein or PSD-95, DlgA, and ZO-1 protein), an N-terminal domain, and a C-terminal domain. Reverse transcription (RT)-PCR results showed that MjSyn was expressed in all tested tissues. Quantitative real-time PCR analysis revealed that MjSyn transcripts in the hemocyte, gill, and intestine were significantly induced at various time points after infection with Staphylococcus aureus and Vibrio anguillarum. The knockdown of the expression of MjSyn by RNA interference resulted in a significant decrease in the phagocytic ability and increased bacteria number in vivo of shrimp. Moreover, the expression of MjCnx, a cytoplasmic and membrane location lectin chaperone protein, was inhibited in the MjSyn-knocked down shrimp, which indicated a possible calnexin-related way. Thus, the MjSyn participates in the bacterial clearance response of kuruma shrimp, thereby providing new insight into the function of this kind of important adaptor protein.

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1. Introduction

Adaptor proteins are physiologically pertinent molecules that are responsible for the regulation and integrity of signaling pathways by interacting with relevant proteins via specific conserved domains [1]. The PDZ (previously known as Discs-Large homology repeats [DHRs] or GLGF repeats)-domain-containing protein is an important and well-known family of adaptor proteins. These proteins are abundant (more than 400 copies in the human genome) in metazoan genomes and have also been identified in bacteria, plants, and yeast [2]. The PDZ domain is an 80–100 amino acid sequence homolog that was named by the three proteins in which these domains have been identified: the brain synaptic protein PSD-95, the Drosophila seapate junction protein Discs-Large, and the epithelial tight junction protein ZO1 [3–5]. Crystallographic and/or nuclear magnetic resonance (NMR) results show that the PDZ domains consist of six β-strands and two α-helices, which are folded into a compact globular with an extended groove between the β-strand B and α-helix B for peptide ligand binding [6–8]. Functionally, the PDZ domain recognizes and binds the cytoplasmic tails of the transmembrane receptors and channels or mediates the interaction with internal motif of other proteins. This behavior contributes to the two main functions of PDZ-containing proteins, namely, the organization of multi-protein signaling complexes and the establishment, as well as maintenance, of cell polarity [9,10].

Syntenin is a PDZ-domain-containing protein that binds to the cytoplasmic domains of the syndecans [11]. This protein is also known as an interferon γ-induced potential melanoma differentiation-associated gene (mda-9) [12]. Structurally, syntenin contains an N-terminal domain (NTD), a C-terminal domain (CTD), and two PDZ domains [13]. Syntenin binds with the PDZ binding motif (PBM) of more than 20 proteins through its PDZ domains [14]. This protein also participates in various important physiological processes, such as cancer metastasis [15], early development [16,17], axonal outgrowth [18], synaptic transmission, intracellular trafficking, and signaling transduction [19], through the formation of macromolecular complexes with other proteins.

Syntenin has been recently implicated in several viral infections. For example, syntenin regulates actin polymerization,
phosphatidylinositol 4,5-bisphosphate (PIP2) production, and human immunodeficiency virus type 1 (HIV-1) contact to target CD4+ T-cells [20]. During severe acute respiratory syndrome coronavirus (SARS-CoV) infection, syntenin binds to the E protein PDZ-binding motif (PBM) of SARS-CoV, activates the p38 MAPK, and leads to the over-expression of inflammatory cytokinesis [21]. The involvement of syntenin in the response to white spot syndrome virus (WSSV) infection in shrimp has also been reported [22]. However, whether syntenin functions in the bacterial infection process of animals remains unclear.

In this study, a syntenin-like gene (MjSyn) was cloned from Marsupenaeus japonicus, and its expression profile to the bacterial infection was investigated. Furthermore, RNA interference (RNAi), combined with bacterial clearance and phagocytic activity assays, was conducted to disclose the function of MjSyn in the antibacterial immunity of shrimp.

### 2. Materials and methods

#### 2.1. Animal challenge and tissue collection

Kuruma shrimp (M. japonicus, 10 g in average) were purchased from an aquaculture market in Jinan City, Shandong Province,
China. The shrimp were cultured in an aerated seawater (24‰ salinity) tank at about 22°C and kept for 24 h prior to use. *Staphylococcus aureus* (AS1.89) and *Vibrio anguillarum* [American Type Culture Collection (ATCC) 43305] were obtained from the Organism Culture Collection (SDMCC) of State Key Laboratory of Microbial Technology in Shandong University. For the immune challenge, the overnight-cultured *S. aureus* (2 × 10⁷ CFU, 15 μL per individual) or *V. anguillarum* (2 × 10⁷ CFU, 15 μL per individual) were injected into the abdominal segment of each shrimp. The control groups were injected the same volume of phosphate-buffered saline (PBS, 140 mM of NaCl, 2.7 mM of KCl, 10 mM of Na₂HPO₄, 1.8 mM of KH₂PO₄, and pH 7.4). Tissue samples, which included the hemolymph, hemocytes, heart, hepatopancreas, gills, stomach, and intestines, were collected using a previously described method [23].

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from the aforementioned samples using TRIzon Reagent (KWBio, Beijing, China). The first-strand cDNA was synthesized following the manufacturer’s protocol of SMART cDNA kit (Clontech, California, USA) by using 4 μg of RNA. The primer used was Smart F and Oligo anchor R (Table 1).

2.3. cDNA cloning and sequence analysis

The full-length syntenin sequence (*MjSyn* for abbreviation) was obtained by random sequencing of the shrimp ovary cDNA library. The online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and SMART program (http://smart.embl-heidelberg.de/) were used to analyze the similarity of *MjSyn* with other syntenins and predict the putative domain of *MjSyn* protein. The phylogenetic tree of selected syntenin proteins was constructed using the MEGA 4.0 program [24]. Tertiary structure prediction was performed using the SWISS-MODEL prediction algorithm (http://swissmodel.expasy.org/) and displayed in PyMOL software.

2.4. RT-PCR and quantitative real-time PCR

Reverse transcription PCR (RT-PCR) was used to analyze the *MjSyn* expression in various tissues, which included the hemocyte, gill, hepatopancreas, stomach, heart, intestine, sperm, and ovaries. The primers used in the RT-PCR assay were *MjSynRTF* and *MjSynRTR* (Table 1). The PCR process was as follows: 94°C for 3 min, 28 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and the last step of 72°C for 5 min *β-actin* was amplified with *Mjβ-actinF*, as well as *Mjβ-actinR*, and used as internal control (primer sequences are shown in Table 1).

Quantitative real-time PCR (qPCR) was performed to investigate the expression patterns of *MjSyn* in the tissues (hemocyte, gills, and intestine) of *V. anguillarum*- or *S. aureus*-challenged shrimp and the expression patterns of phagocytosis-related genes (*Rab5*, *Arp2-3*, *Ran*, *Calnexin*, and *Myosin*) in normal or *MjSyn*-knocked down shrimp tissue. The qPCRs were performed in a real-time thermal cycler (Bio-Rad, USA), with the gene specific primers (for phagocytosis-related genes) and primers that were used in the RT-PCR analysis (for *MjSyn*)(Table 1), following the instruction of SYBR...
qPCR pre-mixture protocol (BioTeke Corporation, Beijing, China) and the methods in our previous report [23]. All samples were repeated in triplicate. The data for qRT-PCR were calculated by the $2^{-\Delta\Delta CT}$ method and shown by GraphPad Prism software (GraphPad, San Diego, USA). Significant difference was analyzed by two-tailed unpaired t-test and accepted at $P < 0.05$.

2.5. RNAi in vivo

A 500 bp MjSyn fragment was amplified with two specific primers, namely, MjSynF and MjSynR (Table 1), and used as template for dsRNA synthesis. DsRNA was prepared as previously described [25]. The final concentration of dsRNA was 4 μg/μL. The GFP dsRNA was synthesized simultaneously as the control.

For RNAi, each shrimp was injected with 4 μg/μL dsRNA (dsMjSyn or dsGFP) into the abdominal segment. Injection was repeated after 24 h. The RNAi efficiency was assessed by RT-PCR or qPCR on the total RNAs from the gills of three randomly selected shrimp.

2.6. Western blot

After the RNAi, the gill and intestine tissues of shrimp in the experimental (dsMjSyn injection) and control (dsGFP injection) groups were dissected and homogenized in a lysis buffer (150 mM of NaCl, 3 mM of EDTA, 50 mM of Tris-HCl, 1 mM of phenylmethanesulfonyl fluoride, and pH 7.5) and centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was then collected. The samples were analyzed by 12.5% SDS-PAGE according to the Laemmli method [26]. Immunoblot analysis was conducted with polyclonal antibodies against MjCnx (gift from Jin-xing Wang lab) [27] (1:200) or β-Actin (1:200) as the primary antibodies. The signal was visualized with staining catalyzed by peroxidase.

2.7. Bacteria clearance assay after RNAi in vivo

The shrimp were randomly divided into 7 groups with 15 animals in each group. The shrimp in the first group were cultured with no treatment and considered as the normal control. The shrimp in the other groups were injected separately with 15 μL of PBS (second and fifth group), dsMjSyn (third and sixth group), and dsGFP (fourth and seventh group). Then, 24 h after the second dsRNA injection, each shrimp in the second to fourth groups was challenged with 15 μL of S. aureus bacteria (2 × 10⁶ CFU/g), whereas the shrimp in the fifth to seventh groups were challenged with 15 μL of V. anguillarum bacteria (2 × 10⁶ CFU/g).

The hemolymph was drawn from the ventral sinus of each shrimp at 1 and 6 h post bacterial challenge (at least three shrimp at each time point) by using a sterile 26-gauge syringe preloaded with 1 mL of anticoagulant (450 mM of NaCl, 10 mM of KCl, 10 mM of EDTA, 10 mM of HEPES, and pH 7.45) [28]. The hemolymph was centrifuged at 800 × g for 10 min to remove the hemocyte and then diluted with PBS (1:1000) for use. Then, 50 μL of each sample was plated onto a modified 2216E agar plate (0.5% tryptone, 0.1% yeast extract, 1.5% agar, 0.01% FeCl₃, and 2.4% sea salt). The plates were placed at 37 °C for 12 h. The clones on the plates were counted and recorded. The assay was repeated in triplicate. The data were

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Fig. 3. Phylogenetic analysis of syntenins from multiple species. The GenBank accession numbers are shown in the neighbor-joining tree. MjSyn is marked with a black triangle. Neighbor-joining tree was constructed using MEGA 4.0 with 1000 bootstrap replications to determine the reproducibility of the results.
subjected to statistical analysis via unpaired Student’s t-test. Significant differences were accepted at \( P < 0.05 \).

The gills of the selected shrimp at each time point from each group were dissected at the same time for RNA extraction and cDNA transcription. The first-strand cDNA was diluted 100-fold as the template for qPCR.

2.8. Phagocytosis assay

FITC-labeled bacteria (S. aureus and V. anguillarum) were prepared according to a published method [28]. The bacteria were heated in 75 °C water for 30 min and incubated with fluorescein isothiocyanate (FITC, 0.1 mg/mL, dissolved in 0.1 M NaHCO₃) for 1 h at 4 °C. After washing six times with 0.1 M NaHCO₃, the bacteria were resuspended in PBS. The shrimp in the experimental and control (20 shrimp per group) groups were injected into dsMjSyn or dsGFP separately for RNAi in vivo as described in Section 2.6. Then, FITC-labeled S. aureus or V. anguillarum (10⁷ CFU per individual, 10 shrimp per group) were injected into the shrimp. Hemocytes were isolated after 30 min, washed twice by PBS, and fixed in 4% paraformaldehyde for 20 min at room temperature. After washing twice with PBS, the cells were resuspended using 100 μL of PBS, and 50 μL of the cells was dropped onto poly-lysine-coated glass slides. The hemocytes were laid for 30 min at room temperature and stained with DAPI (1 mg/mL in PBS) for 10 min. After the final washing step with PBS (six times, each for 5 min), hemocyte phagocytosis was observed under a fluorescence microscope (Olympus BX51, Japan) at 400 × magnification. Assays were repeated thrice. Statistical analysis was performed using the following formula: Phagocytosis percentage = Number of cells with phagocytized bacteria/Number of cells in vision.

3. Results

3.1. Cloning and sequence analysis of MjSyn

The full length of cDNA of MjSyn was 1223 bp, including a 116 bp 5′ UTR, a 144 bp 3′ UTR, and a 963 bp ORF encoding a 320 amino acid peptide (Accession number: KP241935). Blastp and multiple sequence alignment analysis showed that MjSyn had low sequence similarity with syntenins from other animals (from 31% to 51%), except syntenin from the penaeid species, Penaeus monodon (93% identity) (Fig. 1). However, the PDZ domains of all the syntenins were highly conserved, including that of MjSyn (Fig. 1).

The tertiary structure of PDZ domains of MjSyn (MjPDZ) was further predicted using the SWISS-MODEL prediction algorithm and compared with that from human syntenin 1 (HsPDZ). The result showed that each of the PDZ domains of MjSyn consisted of two α-helixes (α1 and α2) and five β-sheets (β1 to β5) (Fig. 2B). The two PDZ domains of the Homo sapiens syntenin were composed of two α-helixes and six β-sheets (Fig. 2A). Amino acid alignment indicated that the amino acid performed the α2-β6 of HsPDZ1 and...
β4–α2 of HsPDZ2, which are considerably different from the α2–β5 of MjPDZ1 and β4–α2 of MjPDZ2, respectively (Fig. 2C).

The syntenins were classified into four clades by using phylogenetic analysis. The syntenin clades were as follows: I (largest of the clades), vertebrates; II, arthropods (including MjSyn and syntenin from P. monodon); III, platyhelminthes; IV, with only one syntenin from Brugia malayi (Fig. 3).

3.2. MjSyn expression was upregulated by bacterial challenge

Semi-quantitative RT-PCR was performed to analyze the tissue distribution of MjSyn at the transcriptional level. The result showed that MjSyn was expressed in all the tissues selected (hemocytes, gill, hepatopancreas, stomach, heart, intestine, testes, and ovary), with relatively high level in the gill, stomach, heart, and intestine tissues (Fig. 4A). qPCR was performed to investigate the expression profiles of MjSyn in the hemocyte, gill, and intestine after S. aureus (Gram-positive bacteria) or V. anguillarum (Gram-negative bacteria) challenge. Upon V. anguillarum challenge, the transcription of MjSyn expression was upregulated above fivefold both in the hemocyte (Fig. 4B) and gill (Fig. 4C) over the PBS-control at 6 h post-injection (hpi) and then decreased to normal level in the hemocyte at 48 hpi and in the intestine at 12 hpi but kept upregulated in the gill until 48 hpi (Fig. 4C and D). For S. aureus challenge, the transcription of MjSyn was upregulated in the hemocyte (Fig. 4B), gill, and intestine at 24 hpi and kept to 48 h in the gill and intestine tissues (Fig. 4C and D). These results indicated that MjSyn may have been involved in the antibacterial immunity of shrimp.

3.3. Silencing of MjSyn expression decreased the bacterial clearance ability in vivo

The bacterial clearance assay combined with the RNAi to further investigate the function of MjSyn in shrimp. The bacteria in the hemolymph of shrimp at 1 and 6 h post S. aureus or V. anguillarum injection with or without RNAi were cultured and counted. The efficiency of RNAi was detected by qPCR at the same time (Fig. 5 and Fig. S1). The result showed that the expression of MjSyn was knocked down by 20% or 10% at 6 h post S. aureus (Fig. 5A) or V. anguillarum challenge (Fig. 5A and C), but no significant changes were found in the control groups (dsGFP + S. aureus or V. anguillarum). At the corresponding time, bacteria numbers in the hemolymph of MjSyn knockdown shrimp were significantly increased compared with that in the control groups (Fig. 5B and D). These results showed that MjSyn had important roles in the clearance of both S. aureus and V. anguillarum bacteria in shrimp.
3.4. RNAi of MjSyn impaired the phagocytosis efficiency in shrimp

Considering that phagocytosis is a main cellular response in the bacterial clearance reaction, phagocytosis assay together with the RNAi was further conducted. The expression of MjSyn was knocked down by approximately 60% at 30 min post the FITC-labeled bacteria injection (Fig. 6A). At this time, the phagocytic activity of shrimp decreased for V. anguillarum (from 23% to 3%) and S. aureus (from 7% to 4%), which indicated that knocking down MjSyn impaired the phagocytosis of hemocytes (Fig. 6B).

3.5. Knockdown of MjSyn reduced the expression of calnexin

The expression of some phagocytosis-related genes, which included Rab5, Arp2-3, Ran, calnexin, and myosin, was detected by qPCR to investigate the possible molecules related to MjSyn (Fig. 7). The result showed that the expression of calnexin (MjCnx) was inhibited, whereas the expression of other genes was unchanged or upregulated (Rab5) when MjSyn was knocked down (Fig. 7A). Western blot was conducted to confirm the change in the expression of MjCnx in the protein level. The MjCnx protein expression was considerably downregulated in the gill and intestine tissues after MjSyn knockdown (Fig. 7B). Bacterial challenge was performed after MjSyn or GFP knockdown to further confirm the relationship between calnexin and MjSyn. The expression of MjCnx and Rab5 gene was investigated by qPCR at 6 hpi (Fig. 7C and D). The results showed that the expression levels of MjCnx and Rab5 were both upregulated by bacterial challenge in the experimental and control groups. However, the upregulation of the expression of calnexin (MjCnx) was restrained in the MjSyn knockdown group compared with that in the GFP knockdown group, but the expression of Rab5 was unchanged (Fig. 7C and D).

4. Discussion

In this study, we identified and characterized an adaptor protein, MjSyn, from M. japonicus. We also examined its expression and antibacterial functions. The syntenin protein of shrimp showed remarkable sequence differences with those from vertebrates and other invertebrates, such as mosquito [29] and filarial [30], except for the PDZ domains. The syntenin protein has four domains: an NTD, two PDZ domains, and a CTD. The PDZ domains of HsSyn have been well illustrated both in function and structure. Generally, they bind to the C-terminus of membrane-located target proteins and mediate the assembly of dynamic multi-protein complexes at the membrane. Although only 26% sequence identities in amino acids have been reported, the two PDZ domains of MjSyn have similar globular structures, which usually comprise six β-strands (β1–β6) and two α-helices (α1 and α2) [13]. The two PDZ domains of MjSyn showed relatively higher sequence identities with that of HsSyn. Although the predicted tertiary structure of MjSyn PDZ domains express reduced β-strands (β1–β5), the peptide binding surface of these domains are almost identical to those of human’s, such as the positively charged residues in β2 and α2 of PDZ1 (Fig. 2). These results indicated that a conserved peptide binding character probably exists in the shrimp. Compared with the PDZ domains, the functions of NTD and CTD are not well documented. Some functions of the NTD in HsSyn, such as the recruitment of the transcription factor SOX4 and eukaryotic translation initiation factor 4A (EIF4A) into signaling complexes and interaction with Alix and ubiquitin, have been reported [31,32]. However, the NTD of syntenins from various species have low sequence identities, except for some identified sequence motif (such as LYPML motif and the tyrosine phosphorylation site) for specific peptide binding or regulation. In HsSyn, the LYPML motif function in Alix binding and the tyrosine phosphorylation sites regulate the interaction of HsSyn with the receptor type protein tyrosine phosphates (Fig. 1). Whether the NTD of MjSyn has the same binding function should be elucidated.

MjSyn is broadly distributed in shrimp tissues with higher transcription located in the gill, heart, and intestine tissues. In mammals, syntenins are also widely expressed, with higher transcripts in the heart and placental tissues in adult human and strongest expression in the brain, testes, lung, and heart tissues of rat [33,34]. To date, the regulation of mdr-9/syntenin has not been extensively studied. It was only reported to be an interferon-inducible gene [12], which was induced by TNFα or fibrocinet in mammals [35]. In aquatilia, the upregulation of the mRNA of syntenin homologs was reported in marine teleost olive flounder Paralichthys olivaceus by hypoxia and in shrimp P. monodon by WSSV infection [22,36]. In our study, both the Gram-positive and Gram-negative bacteria induced significant upregulation of syntenin, which indicated a potential role of syntenin in the antibacterial response of shrimp. This function was then investigated by bacteria clearance assay and phagocytic activity following the RNAi. Knockdown of the expression of MjSyn in vivo of shrimp resulted in
significant increase in bacteria numbers, and the impaired phagocytosis ability confirmed the important role of MjSyn in bacteria clearance.

In invertebrates, bacteria challenge stimulates innate immune responses, which include phagocytosis or encapsulation, prophenoloxidase cascade, and humoral response [37]. Phagocytosis is one of the most powerful and immediate ways to eliminate microbe infection and comprises a series of complex events and proteins [38]. In the present study, the transcripts of some phagocytosis-related proteins were detected after knockdown MjSyn. Unexpectedly, the expression of traditional phagocytosis-related proteins (myosin, Ran, Arp2/3, and Rab5) was unaffected, contrary to the expression of calnexin (MjCnx), which was inhibited in the mRNA and protein levels. Calnexin is a lectin chaperone that is usually localized to the endoplasmic reticulum membrane and comprises a dedicated maturation system known as Cnx/calreticulin (Crt) cycle with another lectin chaperone Crt [39]. However, although Calnexin was reported to be involved in the phagocytosis since year 2001, no further studies have been reported until this year. Zhang et al. showed that calnexin in M. japonicus (MjCnx) has a cytoplasmic and membrane location in hemocytes, and it facilitates the phagocytosis efficiency of hemocytes to V. anguillarum by its binding ability to bacteria and carbohydrate [27]. In our study, knockdown of MjSyn decreased the phagocytosis ability of hemocytes to V. anguillarum and S. aureus as well as the expression of MjCnx, which indicates a tight relationship of two proteins. The MjCnx was supposed to be one of the pathogen recognition receptors located in the cytomembrane in shrimp. The most characteristic function of syntenin is its binding capacity to the cytosolic tail of various transmembrane receptors or cytoplasmic partners for signaling complexes assemble or signal transmission. At the same time, syntenin has been reported to be vital for the regulation of F-actin polymerization in HIV entry and CXCR4-induced chemotaxis of T-cells [20,40]. Actin polymerization is also an important step during phagocytosis. Thus, MjSyn may function in the phagocytosis anti-bacteria immunity of shrimp through two mechanisms: participate the MjCnx anti-bacteria response and (or) influence phagosome formation through regulate actin polymerization process. Further studies are needed to confirm these mechanisms.

In conclusion, a syntenin homolog gene, MjSyn, was cloned from M. japonicus. This gene was widely distributed in the tissues and upregulated upon bacteria stimulation. Knockdown of MjSyn in shrimp resulted in weak phagocytosis to bacteria and increased bacteria number in vivo of shrimp, which revealed an important role of syntenin in the antibacterial immunity of shrimp. To our knowledge, this study is the first report of this multiple function adaptor protein involved in the bacteria clearance response of crustacean.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.02.016.
