Developmental and Comparative Immunology 46 (2014) 356–363

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Calnexin functions in antibacterial immunity of *Marsupenaeus japonicus*

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ARTICLE INFO

Article history:
Received 22 January 2014
Revised 4 May 2014
Accepted 4 May 2014
Available online 21 May 2014

Keywords:
Calnexin
Marsupenaeus japonicus
Phagocytosis
Pattern recognition receptor
Innate immunity

ABSTRACT

Calnexin (Cnx) is an endoplasmic reticulum membrane–bound lectin chaperone that comprises a dedicated maturation system with another lectin chaperone calreticulin (Crt). This maturation system is known as the Cnx/Crt cycle. The main functions of Cnx are Ca^{2+} storage, glycoprotein folding, and quality control of synthesis. Recent studies have shown that Cnx is important in phagocytosis and in optimizing dendritic cell immunity. However, the functions of Cnx in invertebrate innate immunity remain unclear. In this research, we characterized Cnx in the kuruma shrimp *Marsupenaeus japonicus* (designated as *Mj*Cnx) and detected its function in shrimp immunity. The expression of *MjCnx* was upregulated in several tissues challenged with *Vibrio anguillarum*. Recombinant *Mj*Cnx could bind to bacteria by binding polysaccharides. *Mj*Cnx protein existed in the cytoplasm and on the membrane of hemocytes and was upregulated by bacterial challenge. The recombinant *Mj*Cnx enhanced the clearance of *V. anguillarum* in vivo, and the clearance effects were impaired after silencing *MjCnx* with RNA interference assay. Recombinant *Mj*Cnx promoted phagocytosis efficiency of hemocytes. These results suggest that *Mj*Cnx functions as one of the pattern recognition receptors and has crucial functions in shrimp antibacterial immunity.

1. Introduction

Chaperones comprising a large family of proteins have important functions in various physiological processes, such as cell survival promotion, protein aggregation prevention, stress response, signaling, transcription and differentiation (Frydman, 2001; Young et al., 2004). Recently, attention has turned to the roles of molecular chaperones in modulating immune functions, because they ensure the proper folding of immunological proteins and assist with antigen presentation and activation of immune cells (Wang et al., 2009a). Calnexin (Cnx) and calreticulin (Crt) are the most extensively studied glycoprotein-specific chaperones because of their functions in translocation, protein folding, and the quality control of newly synthesized polypeptides (Williams, 2006). Cnx is a lectin chaperone that is localized to the endoplasmic reticulum (ER) lumen; Cnx has a large lumenal domain, a transmembrane domain (TM), and a short cytosolic tail (Wada et al., 1991). Cnx and another lectin chaperone, Crt, are components of a dedicated maturation system known as Cnx/Crt cycle, which mainly functions in Ca^{2+} storage, promotion of correct folding, and oligomerization of many glycoproteins in the ER (Ellgaard and Frickel, 2003; Rutkevich and Williams, 2010). Previous reports discussed the nonchaperone functions of Cnx and Crt. For example, Cnx and Crt purportedly have important functions in phagocytosis (Muller-Taubenberger et al., 2001). Phagocytosis is a phylogenetically conserved process that is critical not only for the removal of apoptotic bodies and dying tumor cells but also for the disposal of infected pathogens (Greenberg and Grinstein, 2002). Some studies reported that Crt has functions in plant antibacterial immunity (Qiu et al., 2012). Recent studies have revealed that Cnx is involved in apoptotic processes induced by ER stresses (Guerin et al., 2009). Cnx-deficient cells are resistant to apoptosis in mammalian cells (Zuppini et al., 2002). In fission yeast, Cnx overexpression causes apoptosis; Cnx regulates apoptosis induced by inositol starvation (Guerin et al., 2008, 2009). Studies on crustaceans found that Crt prevents apoptosis in crayfish after viral infection with gC1qR (Watthanasurorot et al., 2013) and that Crt may be related to the antiviral response in shrimp (Luana et al., 2007; Wang et al., 2007). Information on the function of Cnx in crustacean immunity is lacking (Wang and Wang, 2013).

In this research, we initially identified a Cnx in the kuruma shrimp *Marsupenaeus japonicus* and analyzed its function in shrimp immunity. Results indicated that *Mj*Cnx may be a sensor of infected bacteria and may promote phagocytosis of hemocytes in shrimp. Our report contributes to the knowledge on Cnx function in invertebrate innate immunity.

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http://dx.doi.org/10.1016/j.dci.2014.05.009
0145-305X/

2. Materials and methods

2.1. Biological materials, chemicals, and microorganisms

Shrimp weighing approximately 12–15 g each were purchased from a seafood market in Jinan (Shandong, China) and cultured in tanks filled with aerated seawater. Lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5, lipoteichoic acid (LTA) from *Staphylococcus aureus*, peptidoglycan (PGN) from *Micrococcus luteus* (designated as PGN (M)), and peptidoglycan (PGN) from *S. aureus* (designated as PGN (S)) were purchased from Sigma (St. Louis, MO, USA). *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus*, and *E. coli* were maintained in our laboratory. *Vibrio anguillarum* was provided by Professor Jianhai Xiang of the Institute of Oceanology, Chinese Academy of Sciences.

2.2. Immune challenge of shrimp, RNA extraction, and cDNA synthesis

In immune challenge experiments, we injected *V. anguillarum* (approximately 3×10^7 CFU/shrimp) into the abdomen of each shrimp using a previously described method (Wang et al., 2009c). For hemocyte isolation, the hemolymph was obtained with a syringe containing 1 mL of anticoagulant buffer [0.45 M NaCl, 10 mM KCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.45] from the ventral sinus of the shrimp. Total RNA from the hemocytes, heart, hepatopancreas, gills, stomach, and intestine of the shrimp were extracted using Unizol (Biostar, Shanghai, China) at 2, 6, 12, and 24 h postinfection. RNA from the tissues of phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) challenged shrimp was isolated as control. RNA at 5 μg was used to synthesize first-strand cDNA with the RevertAid First-strand cDNA synthesis kit (Fermentas, Burlington, Canada) using a pair of primers SMART F and oligo anchor R (Table 1).

2.3. Cloning of MjCnx gene

A specific primer *Mj*Cnx F (Table 1) was designed using a nucleotide sequence obtained by transcriptome sequencing of intestine in our laboratory and pairing with 3′ anchor R primer (Table 1) to amplify the 3′ end of *Mj*Cnx cDNA. Polymerase chain reaction (PCR) was performed as follows: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 1 min; and 72 °C for 10 min.

Table 1
Sequences of primers used in this research.

Primer name	Sequence (5′–3′)
SMART F	TACGGCTGCGAGAAGACGACAGAAGGG
Oligo anchor R	GACCACGCGTATCGATGTCGACT$_{16}$(A/C/G)
*Mj*Cnx F	CTGGAAGTGGGAATGAAGCT
3′ anchor R	GACCACGCGTATCGATGTCGAC
RT F	TTCAAGGGCAAGTGGCG
RT R	AACGCCCGCCTGTCC
Actin F	AGTAGCCGCCCTGGTTGTAGAC
Actin R	TTCTCCATGTCGTCCCAGT
EX F	TACTCAGAATTC (*Eco*RI) GCATATCTGATGGAAACA
EX R	TGAGTACTCGAG (*Xho*I) GACAACTATATTGTCGAA
RNAi F	TAATACGACTCACTATAGGGATAATGTTGCTGAAGCTTAC
RNAi R	TAATACGACTCACTATAGGGCTTTGTGTTGTTCTG GTTG
GFP RNAi F	TAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
GFP RNAi R	TAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC

The *Eco*RI, *Xho*I restriction sites and T7 promoters are underlined.

2.4. Sequence analyses

*Mj*Cnx was compared with other Cnx proteins by similarity analysis, which was performed using online BLASTX (http://www.ncbi.nlm.nih.gov/). The signal peptide and putative domain were forecasted using online SMART program (http://smart.embl-heidelberg.de/). GeneDoc software and MEGA 4.0 were used to perform the sequence alignment and to construct a phylogenetic tree, respectively. The phylogenetic tree was developed using the neighbor-joining method (Tamura et al., 2007).

2.5. Semi-quantitative reverse transcription PCR (RT-PCR) and relative quantitative real-time PCR (qRT-PCR)

The tissue distribution of *Mj*Cnx was determined by RT-PCR using specific primers RT F and RT R (Table 1). Actin F and Actin R (Table 1) were used to amplify *β-actin* as the internal control. PCR was performed as follows: 94 °C for 3 min; 28 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 30 s; and 72 °C for 10 min. qRT-PCR was performed to analyze the expression patterns of *Mj*Cnx after *V. anguillarum* challenge using the same primers used in RT-PCR. The amplification procedure was as follows: 95 °C for 10 min; 39 cycles of 95 °C for 20 s, 62 °C for 1 min, and 75 °C for 2 s; and a subsequent melt from 70 to 95 °C. Each experiment was repeated in triplicate. qRT-PCR data were calculated by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The unpaired Student's *t*-test was used to analyze the significant difference, and significant difference at $P < 0.05$ was accepted.

2.6. Recombinant expression and purification of MjCnx and preparation of antiserum

Using the full-length cDNA sequence of *Mj*Cnx as basis, we designed a pair of primers EX F and EX R (Table 1) to amplify the fragment encoding for the Crt domain of *Mj*Cnx. The amplified fragment and the PGEX-4T-1 vector were digested with *Eco*RI and *Xho*I, and then ligated at 16 °C overnight. Subsequently, the recombinant plasmids were transformed into competent *E. coli* BL21 (DE3) cells. The expression of the recombinant *Mj*Cnx (r*Mj*Cnx) was induced by isopropyl β-D-1-thiogalactopyranoside. The soluble r*Mj*Cnx was purified using glutathione Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions and analyzed by 12.5% SDS–PAGE. The glutathione S-transferase (GST) tag was also purified from the competent *E. coli* BL21 (DE3) cells with parent PGEX-4T-1 vectors as control protein. The purified r*Mj*Cnx was used to raise antiserum in New Zealand rabbits following a previously described method using (Shi et al., 2008).

2.7. Western blot

V. anguillarum (approximately 3×10^7 CFU/shrimp) was injected into the abdomen of each shrimp. After 24 h, heart, hepatopancreatic, gills, stomach, and intestine from the challenged shrimp were collected and homogenized in a lysis buffer (150 mM NaCl, 1 mM Phenylmethanesulfonyl fluoride (PMSF), 3 mM EDTA, 50 mM Tris–HCl, pH 7.5) and then centrifuged at 12,000*g* for 10 min at 4 °C to collect the supernatant. Shrimp hemocytes isolated from the hemolymph were also homogenized. The proteins of hemocytes and other tissues from PBS-challenged and unchallenged shrimp were collected as the controls. Protein concentrations were measured using the Bradford method (Bradford, 1976). Approximately 200 μg of proteins from each sample from the three groups was analyzed by 12.5% SDS–PAGE according to the Laemmli method (Laemmli, 1970). Immunoblot analysis was conducted as previously described (Wang et al., 2009b). The