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CsCTL1, a teleost C-type lectin that promotes antibacterial and antiviral immune defense in a manner that depends on the conserved EPN motif

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ABSTRACT

Many C-type lectins (CTLs) have been identified in teleost, however, the *in vivo* function of fish CTLs is essentially unknown. In this study, we examined the function of a CTL (CsCTL1) from tongue sole. CsCTL1 possesses the conserved EPN motif required for mannose binding in mammals but unknown in function in fish. Recombinant CsCTL1 (rCsCTL1), but not the mutant rCsCTL1M bearing substitutions at EPN, interacted with and agglutinated a limited range of bacteria. The agglutinating ability of rCsCTL1 was abolished in the absence of calcium or presence of mannose. Binding of rCsCTL1 to bacteria promoted phagocytosis and antimicrobial activity of head kidney monocytes. Fish administered with rCsCTL1 exhibited enhanced resistance against bacterial and viral infections. These results provide the first evidence that the EPN site is essential to a fish CTL and that, in addition to antibacterial properties, a fish CTL promotes the immune defense against viral infection as well.

1. Introduction

Lectins are a group of carbohydrate-binding proteins. They were first discovered in plants, but subsequently they are found to exist ubiquitously in bacteria, invertebrates, and vertebrates. In animals, lectins are divided into several subgroups, one of which is the C-type lectin (CTL) (Klipatrick, 2000). CTL was originally defined as a group of secreted or transmembrane proteins that require Ca^{2+} for carbohydrate binding (Cummings and McEver, 2009). CTL is a large family with diverse members, which include collectins, selectins, and proteoglycans (Cummings and McEver, 2009; Tanne and Neyrolles, 2011). All CTLs have a carbohydrate-recognition domain (CRD) that mediates specific recognition and binding to oligosaccharides in the extracellular matrix and on solid surfaces such as microbes (Drummond and Brown, 2013). Ca^{2+} is thought to be involved in ligand binding and maintenance of the CRD structure (Drickamer, 1999; Zelensky and Gready, 2005). In addition to the classical CTLs, many proteins with C-type lectin-like domain (CTLD) have been identified, some of which, however, are Ca^{2+}-independent and do not bind carbohydrates (Geijtenbeek et al., 2004).

Functionally, C-type lectins are known to participate in many fundamental cellular processes including cell–cell interaction, adhesion, endocytosis, and cellular signaling. Since CTLs are able to bind sugar molecules on various microorganisms such as bacterial pathogens, they belong to a group of proteins called pattern recognition receptors (PRRs) (Akira, 2009; Cummings and McEver, 2009; Geijtenbeek and Gringhuis, 2009). PRRs are components of the innate immune system that recognize pathogen-associated molecular patterns (PAMPs), which are molecules commonly associated with microbial pathogens (Medzhitov, 2007). Binding of lectins to pathogens can trigger a series of immune responses, such as enhanced phagocytosis and complement activation, which lead to clearance of the targeted pathogens (Cambi et al., 2005; Endo et al., 2006; Garred, 2008; van den Berg et al., 2012).

In teleost, CTLs of various types have been identified and characterized in secondary structure (Fujiki et al., 2001; Ji et al., 2014; Kondo et al., 2007; Liu et al., 2011; Nakao et al., 2006; Nikolakopoulou and Zarkadis, 2006; Savan et al., 2004; Tasumi et al., 2002; Tsutsui et al., 2007; Vitved et al., 2000; Wei et al., 2010; Yu et al., 2013b; Zhang et al., 2010). In general, fish CTLs possess the fundamental structures highly conserved in higher vertebrates, such as the CRD domain and the mannose-type carbohydrate-binding motif EPN. However, the functional importance of these conserved structures in fish CTLs is not known. In addition, the *in vivo* effect of fish CTLs on microbial, in particular viral, infection is unclear. In the present work, we reported the first characterization of a CTL (named CsCTL1) from the teleost fish tongue sole (*Cynoglossus semilaevis*). We investigated the *in vitro* as well as the *in vivo* immune effects of CsCTL1 against bacterial and viral pathogens and examined the essentialness of the EPN motif to the function of CsCTL1.

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2. Materials and methods

2.1. Fish

Clinically healthy tongue soles (average 13.6 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Before the experiment, fish were randomly sampled and verified to be absent of bacterial pathogens in liver, kidney, and spleen as reported previously (Zhou et al., 2014). For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA).

2.2. Sequence analysis

The cDNA sequence of CsCTL1 has been reported previously (GenBank accession no. XP_008329461.1). The cDNA and amino acid sequences of CsCTL1 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. Multiple sequence alignment was created with ClustalX.

2.3. Quantitative real time reverse transcription–PCR (qRT-PCR)

2.3.1. qRT-PCR analysis of CsCTL1 expression in fish tissues under normal physiological conditions

Kidney, blood, intestine, gill, spleen, brain, muscle, heart, spleen, and liver were taken aseptically from five tongue soles (as described above) and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously (Long et al., 2014). The expression level of *CsCTL1* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin (ACTB) as an internal reference (Long et al., 2014). The experiment was performed three times, each time with five fish.

2.3.2. qRT-PCR analysis of CsCTL1 expression in fish tissues during bacterial and viral infections

Vibrio anguillarum C312, a bacterial pathogen isolated from diseased fish, was cultured in Luria–Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.8; the cells were washed with PBS and resuspended in PBS to 1×10^6 CFU/ml. The fish viral pathogen megalocytivirus RBIV-C1 (Zhang et al., 2013) was resuspended in PBS to 5×10^5 copies/ml. Tongue soles (as described above) were divided randomly into three groups and injected intraperitoneally with 50 μl *V. anguillarum*, megalocytivirus, or PBS. Kidney, spleen, and liver were taken from the fish (five at each time point) at 1 h, 4 h, 12 h, 24 h, and 48 h post-bacterial infection and at 1 d, 3 d, 5 d, and 7 d post-viral infection. Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above. For bacterial infection, the internal reference genes for kidney, spleen, and liver were ACTB, ribosomal protein L18 (RPL18), and 18S rRNA respectively (Long et al., 2014); for viral infection, the internal reference genes for kidney and spleen were ACTB (Long et al., 2014). The experiment was performed three times.

2.4. Plasmid construction

The protein expression vector pET259 was constructed by inserting linker L811 (5′-TATGGCATTTAAATCTC-3′) into pET258 (Zhang and Sun, 2007) between NdeI/XhoI sites. To construct pEtCsCTL1, which expresses His-tagged recombinant CsCTL1 (rCsCTL1), the coding sequence of CsCTL1 was amplified by PCR with primers CTL1F1 (5′-GATATCATGTTATCTTTAAGCGAACCTGAGCC-3′, underlined sequence, EcoRV site) and CTL1R1 (5′-GATATC AAGACAGCTCGAGGGACGGGTTGCAC-3′, underlined sequence, EcoRV site); the PCR products were ligated with the T–A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the *CsCTL1*-containing fragment, which was inserted into pET259 at the SwaI site, resulting in pEtCsCTL1. pEtCsCTL1M, which expresses His-tagged recombinant CsCTL1M (rCsCTL1M) that bears three alanine substitutions at the EPN site, was constructed by overlap extension PCR as follows: the first overlap PCR was performed with the primers CTL1F1 (as above) and MR1 (5′-GCTAGCTGCCGCTCCTACGGC CCAGTCCAGAAAT-3′), the second overlap PCR was performed with the primers MF2 (5′-GTAGGAGCGGCAGCTAGCCAAGGCAACGAA GACT-3′) and CTL1R1 (as above), and the fusion PCR was performed with the primer pair CTL1F1/CTL1R1. The PCR products were inserted into pET259 as above.

2.5. Purification of recombinant proteins

Escherichia coli BL21(DE3) (purchased from TransGen Biotech Beijing, China) was transformed with pEtCsCTL1 and pEtCsCTL1M; the transformants were cultured in LB medium at 37 °C to mid-logarithmic phase, and isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growing at 37 °C for an additional 4 h, the cells were harvested by centrifugation, and His-tagged proteins were purified using Ni-NTA Agarose (QIAGEN, Valencia, USA) as recommended by the manufacturer. The proteins were treated with Triton X-114 to remove endotoxin as reported previously (Chen et al., 2013). The proteins were then concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, USA). The concentrated proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of the proteins was determined using the Bradford method with bovine serum albumin as the standard. Native gel electrophoresis was carried out using Amersham High Molecular Weight Calibration Kit for native electrophoresis (GE Healthcare, Piscataway, NJ, USA).

2.6. Agglutination assay

The Gram-negative bacteria *Edwardsiella tarda* TX1, *Pseudomonas fluorescens* TSS, and *Vibrio harveyi* T4D have been reported previously (Sun et al., 2009; Yu et al., 2013a); *Aeromonas hydrophila* was purchased from China General Microbiological Culture Collection Center (CGMCC), Beijing, China. *E. coli* DH5α was from Tiangen (Beijing, China). The Gram-positive bacteria *Micrococcus luteus* and *Staphylococcus aureus* were purchased from CGMCC. For agglutination assay, *A. hydrophila*, *E. tarda*, *P. fluorescens*, *V. anguillarum*, and *V. harveyi* were cultured in Luria–Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.9; *E. coli*, *M. luteus*, and *S. aureus* were cultured in LB medium at 37 °C to an OD_{600} of 0.9. The cells were washed with TBS buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.5) and resuspended in TBS or TBS containing 10 mM $CaCl_2$ (TBS–Ca^{2+} buffer) to 2×10^9 CFU/ml. rCsCTL1 and rCsCTL1M were added to the bacterial cells to the final concentration of 50 μM. After incubation at 25 °C for 1 h, bacterial cells were stained with 4,6-diamino-2-phenyl indole (DAPI) (Invitrogen, USA) according to manufacturer's instructions, and agglutination was observed with a fluorescence microscope (Nikon E800, Japan). To examine the effect of ethylene diamine tetraacetic acid (EDTA), bacteria were incubated with rCsCTL1 as described above in TBS–Ca^{2+} buffer containing 4 mM EDTA. To determine agglutination titer, rCsCTL1 was subjected to 2-fold dilution; each dilute was incubated with *V. anguillarum* or *M. luteus* and examined for agglutination as above. The greatest dilution that could still cause agglutination was defined as agglutination titer. To