A Novel C-Type Lectin from the Shrimp *Litopenaeus vannamei* Possesses Anti-White Spot Syndrome Virus Activity∗†

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C-type lectins play key roles in pathogen recognition, innate immunity, and cell-cell interactions. Here, we report a new C-type lectin (C-type lectin 1) from the shrimp *Litopenaeus vannamei* (LvCTL1), which has activity against the white spot syndrome virus (WSSV). LvCTL1 is a 156-residue polypeptide containing a C-type carbohydrate recognition domain with an EPN (Glu99-Pro100-Asn101) motif that has a predicted ligand binding specificity for mannose. Reverse transcription-PCR analysis revealed that LvCTL1 mRNA was specifically expressed in the hepatopancreas of *L. vannamei*. Recombinant LvCTL1 (rLvCTL1) had hemagglutinating activity and ligand binding specificity for mannose and glucose. rLvCTL1 also had a strong affinity for WSSV and interacted with several envelope proteins of WSSV. Furthermore, we showed that the binding of rLvCTL1 to WSSV could protect shrimps from viral infection and prolong the survival of shrimps against WSSV infection. Our results suggest that LvCTL1 is a mannose-binding C-type lectin that binds to envelope proteins of WSSV to exert its antiviral activity. To our knowledge, this is the first report of a shrimp C-type lectin that has direct anti-WSSV activity.

Lectins are a group of nonimmunogenic proteins possessing at least one noncatalytic domain that binds reversibly to specific carbohydrates and usually agglutinates cells (16, 35). They exist ubiquitously in animals, plants, and bacteria. Due to their ability to bind to specific carbohydrates on the surfaces of microorganisms, lectins have been regarded as primary candidates for pattern recognition receptors (PRRs) in animal innate immunity. Calcium-dependent (C-type) lectins, which contain a characteristic carbohydrate recognition domain (CRD) with two or three pairs of disulfide bonds, are believed to mediate pathogen recognition and play an important role in the clearance of pathogens in innate immunity (6, 15, 42, 47). C-type lectins can be further classified into a number of subgroups based on their functions and structures (5, 7). Mammalian mannose-binding lectin (MBL), a liver-derived pluri-}

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- MBL has also been shown to link the innate immune system to the acquired immune system (2, 10).
- C-type lectins in invertebrates are also involved in innate immune responses, including the promotion of phagocytosis (18, 28), nodule formation, encapsulation, melanization (20, 48), and the activation of prophenoloxidase (48, 49). A number of C-type lectins in invertebrates, particularly in insects and shrimps, have been isolated and characterized (20, 25, 28, 29, 31, 36, 46–49). In the shrimp *Penaeus monodon*, two C-type lectins containing a single CRD have been characterized (28, 29). In *P. japonicus*, an *N*-acetylglucosamine (GlcNAc)-specific lectin and another lectin have also been reported (21, 46). A C-type lectin in *L. vannamei* with two CRDs (LvCTL) is expressed, but only in the hepatopancreas (31). In *Femmeropeneaus chinensis*, two C-type lectins (FcCLeC-1 and Fc-hsL) have been reported (25, 36). FcCLeC-1 contains dual CRDs and is expressed in hemoocytes, while Fc-hsL contains only one CRD and is expressed specifically in the hepatopancreas. These shrimp C-type lectins have ligand binding specificities for carbohydrates such as *N*-acetylglucosamine and lipopolysaccharide, and they can agglutinate erythrocytes and exhibit antimicrobial activity against some bacteria and fungi. Moreover, many expressed sequence tags (ESTs) encoding lectins have been identified in cDNA libraries from healthy shrimps (*P. japonicus, L. vannamei, and L. setiferus*). In addition, MBL has been used to link the innate immune system to the acquired immune system (2, 10).
The Pacific white shrimp, *L. vannamei*, is a commercially important species of cultured penaeid shrimps in China, as well as worldwide. With the rapid production and development of *L. vannamei* worldwide, shrimp diseases have become widespread and, in recent years, have threatened the shrimp industry. WSS, which is caused by WSSV, is the most severely damaging disease of shrimps and other crustaceans around the world (13, 24, 26). The outbreak and spread of WSS have resulted in the high mortality of farmed penaeid shrimps and huge economic losses in many regions of the world, especially in Southeast Asia (13, 24, 26, 39). The viral pathogen, WSSV, is a member of a new genus, *Whisppovirus*, in the *Nimaviridae* family (41), with double-stranded DNA (about 300 kb) and envelope proteins (39, 45). Since the first report of WSSV in 1993, major concerns in the world aquaculture industry have been raised, and great effort has been made in preventing and controlling the disease in recent years (24, 26, 39, 45). Even so, shrimp defense mechanisms, particularly those against viruses, are poorly understood.

The shrimp defense system is believed to rely largely on innate immunity. The innate immune systems of shrimps consist of PRRs that recognize and bind to specific patterns on the surfaces of pathogens (3, 22). Previously, we employed suppression subtractive hybridization technology to differentially screen cDNA libraries from normal and WSSV-infected *L. vannamei* shrimps by using WSSV-resistant tester and WSSV-susceptible *L. vannamei* as the driver (51). More than 1,000 qualified ESTs were obtained from the positive clones of the cDNA libraries. Many ESTs encoding C-type lectins are upregulated in virus-resistant shrimps compared to their levels of regulation in susceptible shrimps, particularly in virus-resistant shrimps at 48 h postinfection (51). Subsequently, several of these lectins have been cloned and characterized.

In this study, we report a novel anti-WSSV C-type lectin, CTL1, from the shrimp *L. vannamei* (LvCTL1). LvCTL1 contains a single CRD. It exhibits hemagglutinating and sugar binding activities as well as a strong affinity for WSSV. LvCTL1 binds to several envelope proteins from WSSV virions. More importantly, LvCTL1 exhibits high antiviral activity against WSSV both in vitro and in vivo.

**MATERIALS AND METHODS**

**Cloning of full-length LvCTL1 cDNA.** A partial cDNA sequence of LvCTL1 was obtained from the clone RS4E82, which was isolated from the subtraction cDNA library described previously (51); the sequence was homologous to a C-type lectin from *P. monodon* (PmAV; GenBank accession no. AAQ75589) (29). Based on the EST sequences, gene-specific primers (GSP1 and GSP2) were designed for the rapid amplification of 3' and 5' cDNA ends (RACE) to amplify the full-length cDNA of LvCTL1. Then, gene-specific primers GSP3 and GSP4 were designed to confirm the full-length cDNA of LvCTL1 from 3'- and 5'-end-overlapping fragments (Table 1). RACE was carried out using a BD SMART RACE cDNA amplification kit (Clontech, CA), using the adapter-ligated cDNA, which was synthesized from total RNA of the hepatopancreas according to the manufacturer's instructions. Total RNA was prepared from the hepatopancreas, hemocytes, gills, eyestalks, muscles, brains, hearts, pyloric ceca, nerve cords, intestines (midgut), spermatids, and stomachs of healthy WSSV-susceptible *L. vannamei* shrimps. All of the RNA samples were treated with DNase (Promega) to remove contaminating DNA, and the single-stranded cDNAs were synthesized using Moloney murine leukemia virus reverse transcriptase (RT; Promega) with an oligo(dT)18 primer by following the manufacturer's instructions. Equal amounts of cDNAs from the shrimp tissues were used as templates for PCR. The LvCTL1 cDNA fragment was amplified with primers LvCTL1-F and LvCTL1-R (Table 1), using the following conditions: initial denaturation at 94°C for 3 min and then 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 8 min. The constitutively expressed β-actin gene was amplified with the specific primers β-actin-F and β-actin-R (Table 1) and used to normalize PCR products. The PCR products were analyzed on a 1% agarose gel and sequenced to confirm the identity of the LvCTL1 cDNA.

**Expression and purification of recombinant LvCTL1.** A cDNA fragment encoding a mature LvCTL1 protein (residues 21 to 156) (Fig. 1A) was amplified by PCR using *Taq* polymerase (Promega) with the specific primers Re-F and Re-R (Table 1). BamHI and XmaI restriction sites were added to the 5' ends of Re-F and Re-R (after the stop codon), respectively (Table 1). The PCR fragment was cloned into the pGem-T easy vector (Promega), completely digested with BamHI and XmaI (NEB, United Kingdom), and then cloned into the BamHI/XmaI sites of the expression vector pQE-30 (Qiagen, Germany). The recombinant plasmid (pQE-30-LvCTL1) was transformed into competent *Escherichia coli* M15(pREP4) (Qiagen, Germany) cells for expression of recombinant proteins. Bacterial cells expressing recombinant proteins were then harvested and sonicated, and the inclusion bodies were resuspended in 15 mM phosphate-buffered saline (PBS) containing 8 M urea. Recombinant LvCTL1 protein was purified by affinity chromatography with Ni-nitrilotriacetic acid-agarose (Qiagen, Germany) under denaturing (8 M urea) conditions according to the manufacturer's instructions (Qiagen, Germany). Purified recombinant protein was dialyzed against 1× PBS-glycerol buffer (PBS with 5% glycerol, pH 7.0) at 4°C overnight. The resulting protein was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R-250.

**Production of antibodies.** The polyclonal antibody against recombinant LvCTL1 was produced by immunizing BALB/c mice according to the conventional method (34). The titer of the antiserum was then determined by an enzyme-linked immunosorbent assay (ELISA) to determine the optimal working concentration.

**Table 1. Primers used for this experiment**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>LvCTL1 cDNA cloning</td>
<td></td>
</tr>
<tr>
<td>GSP1 ..........................</td>
<td>CTGTCCTCTACCCCTACGGGCC</td>
</tr>
<tr>
<td>GSP2 ..........................</td>
<td>GTGCTACCCGATGGTGTGTC</td>
</tr>
<tr>
<td>GSP3 ..........................</td>
<td>GTGCTACCTGACGGCAAGACCC</td>
</tr>
<tr>
<td>GSP4 ..........................</td>
<td>CAGTACATTCAATATTCTTTG</td>
</tr>
<tr>
<td>RT-PCR</td>
<td></td>
</tr>
<tr>
<td>LvCTL1-F ......................</td>
<td>TCGTGGCCAAGTAGGGTTGGTC</td>
</tr>
<tr>
<td>LvCTL1-R ......................</td>
<td>CGTATCCACTTATAGGGC</td>
</tr>
<tr>
<td>β-actin-F .....................</td>
<td>AGATGCTGTGACGGCAAGAATGA</td>
</tr>
<tr>
<td>β-actin-R .....................</td>
<td>TCAGGCCAGTTCTCGGTCTGG</td>
</tr>
</tbody>
</table>

* a The BamHI restriction site is underlined.  
* b The XmaI restriction site is underlined.

**AB197373.** PmLec from *Penaeus semisulcatus* (accession no. AB197373).  
**LvLec** (accession no. AB197374).  
**FecLec-1** from *F. chinensis* (accession no. AAX63905).  
**FcLec-2** (accession no. ABA54612).  
**PmAV** (accession no. AAQ75589).  
**PmLec-2** (accession no. AAZ29608).  
**Multiple-sequence alignment** of the LvCTL1 CRD with CRDs from other shrimp C-type lectins is performed using the ClustalW multiple-alignment software.

**Expression of LvCTL1 in tissues.** Total RNA was prepared from the hepatopancreas, hemocytes, gills, eyestalks, muscles, brains, hearts, pyloric ceca, nerve cords, intestines (midgut), spermatids, and stomachs of healthy WSSV-susceptible *L. vannamei* shrimps. All of the RNA samples were treated with DNase (Promega) to remove contaminating DNA, and the single-stranded cDNAs were synthesized using Moloney murine leukemia virus reverse transcriptase (RT; Promega) with an oligo(dT)18 primer by following the manufacturer's instructions. Equal amounts of cDNAs from the shrimp tissues were used as templates for PCR. The LvCTL1 cDNA fragment was amplified with primers LvCTL1-F and LvCTL1-R (Table 1), using the following conditions: initial denaturation at 94°C for 3 min and then 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 8 min. The constitutively expressed β-actin gene was amplified with the specific primers β-actin-F and β-actin-R (Table 1) and used to normalize PCR products. The PCR products were analyzed on a 1% agarose gel and sequenced to confirm the identity of the LvCTL1 cDNA.

**Sequence analysis.** The similarity analysis of the nucleotide and amino acid sequences of LvCTL1 was carried out using the BLAST programs at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Translation of the cDNA was performed using the Expert Protein Analysis System (http://au.expasy.org/). Signal sequence and CRD domain predictions were conducted using SMART software. Shrimp C-type lectin uses for comparisons include PmLec-1 (GenBank accession no. AB197373). PmLec from *Penaeus semisulcatus* (accession no. AB197373). LvLec (accession no. AB197374). FecLec-1 from *F. chinensis* (accession no. AAX63905). FcLec-2 (accession no. ABA54612). PmAV from *P. monodon* (accession no. AAQ75589). PmLec-2 (accession no. AAZ29608). Multiple-sequence alignment of the LvCTL1 CRD with CRDs from other shrimp C-type lectins is performed using the ClustalW multiple-alignment software.
FIG. 1. *L. vannamei* LvCTL1 cDNA and amino acid sequences. (A) Full-length cDNA and deduced amino acid sequences. The amino acid sequence is represented by a single capital letter below the nucleotide sequence (GenBank accession no. DQ858900). The putative signal peptide sequence is underlined; four conserved cysteine residues that define the C-type lectin domain are shaded, and four additional cysteine residues are shaded and underlined. The EPN motif for ligand binding specificity is boxed. The polyadenylation signal sequence AATAAA is double underlined. (B) Multiple-sequence alignment of the CRD of LvCTL1 with CRDs of other shrimp C-type lectins. The CRDs of LvCTL1 and four other shrimp C-type lectins were aligned using ClustalX. Conserved cysteine residues that define the C-type lectin domain are shaded and indicated by asterisks. The EPN or QPD motif is also shaded. Shown are LvCLec-1 (accession no. DQ858899, lec5D), PmCLec-1-CRD1 (accession no. ABJ97373), PmCLec-1-CRD2 (accession no. ABJ97374), PmCLec-CRD1 (accession no. ABJ97372), PmCLec-CRD2 (accession no. ABJ97373), LvCLT-CRD1 (accession no. ABJ97374), LvCLT-CRD2 (accession no. ABJ97374), FeCLec-1-CRD1 (accession no. AAX63905), FeCLec-1-CRD2 (accession no. AAX63905), FeCLec-2 (accession no. AB45612), PmAV (accession no. AAQ75589), and PmCLec-2 (accession no. AAZ29608).
concentration. Recombinant LvCTL1 protein samples were separated using 15% SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane (Roche Biosciences, Germany). The membrane was blocked with blocking buffer containing 5% nonfat milk powder in PBS with 0.1% Tween 20 (PBS-T) for 2 h and then incubated with mouse anti-LvCTL1 antibody (or monoclonal antibody against the His tag; Novagen) for 2 h, followed by incubation with peroxidase-conjugated secondary antibody (1:10,000 dilution in PBS-T; Promega) for 1.5 h. LvCTL1-specific signals were visualized after the addition of diaminobenzidine colorimetric chromogen (Boster, China).

Expression of LvCTL1 protein in the hemolymph of shrimps after WSSV infection. To detect LvCTL1 protein in the hemolymph of shrimps after WSSV infection, healthy WSSV-susceptible L. vannamei shrimps were challenged with WSSV as described previously (51), and hemolymph was collected from the pericardial sinus located at the first abdominal segment of each shrimp at 3, 6, 12, 24, 36, 48, and 72 h postinjection, using a 1-mL sterile syringe. The hemolymph was then immediately centrifuged for 10 min at 5,000 × g at 4°C and the cell-free plasma was collected. Plasma from unchallenged shrimps was also prepared by the same method. The plasma samples were first diluted with distilled water (1:5, vol/vol) and then subjected to 15% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane (Roche Biosciences, Germany). The membrane was blocked with 5% nonfat milk powder in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.2, 150 mM NaCl) for 4 h. After being washed three times with TBS with 0.1% Tween 20 (TBS-T), the membrane was then incubated with the mouse antiserum against LvCTL1 for 2 h. Antibody binding was visualized by a colorimetric reaction catalyzed by peroxidase-conjugated goat anti-mouse antibody (1:10,000 dilution in TBS; Promega).

ELISA-based assay of LvCTL1 expression in the hemolymph of WSSV-resistant and -susceptible L. vannamei shrimps were obtained by means of a selective-breeding program (51). Two families were selected to perform an ELISA-based assay for LvCTL1 expression. Family A has a survival rate of up to 70% after WSSV infection, while family B has a mortality rate of 100% after WSSV infection. Hemolymph samples were collected in tubes containing 10% saline solution (0.9%, vol/vol, NaCl) from both WSSV-resistant and -susceptible L. vannamei shrimps at 3, 6, 12, 24, 36, 48, and 72 h postinjection. LvCTL1 concentration. Recombinant LvCTL1 protein samples were separated using 15% SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane (Roche Biosciences, Germany). The membrane was blocked with blocking buffer containing 5% nonfat milk powder in PBS with 0.1% Tween 20 (PBS-T) for 2 h and then incubated with mouse anti-LvCTL1 antibody (or monoclonal antibody against the His tag; Novagen) for 2 h, followed by incubation with peroxidase-conjugated secondary antibody (1:10,000 dilution in TBS; Promega). The membrane was washed with PBS-T, and Western blotting was performed with monoclonal antibody against the His tag to confirm the expression of the polypeptide in TBS-T containing 0.1% BSA for 2 h at room temperature. The binding of recombinant proteins to WSSV was detected by immunoblotting, using mouse monoclonal antibody to the His tag (Novagen) or mouse polyclonal antibody specific for LvCTL1 for 2 h, followed by incubation with goat anti-mouse immunoglobulin G conjugated to peroxidase as a secondary antibody (1:10,000 dilution in PBS-T; Promega) for 1.5 h at room temperature. Then, a 3,3-diaminobenzidine solution (Boster, China) was added to develop the brown product for 5 min, and the reaction was stopped with water.

Interaction of recombinant LvCTL1 with WSSV proteins by protein pull-down assay. The cDNA fragment encoding mature LvCTL1 (residues 21 to 156) (Fig. 1A) was cloned into the pMAL-c2X plasmid (NEB) for the expression of recombinant LvCTL1 in E. coli BL21 cells. The MbP or MbP-lectin fusion protein was fixed to amylose resins (NEB, United Kingdom) to express mLbP or mLbP-lectin fusion protein in E. coli BL21 cells. The MBP or MBP-lectin fusion protein was fixed to amyllose resins, and then used for pull-down assay with recombinant LvCTL1. After 6 days of culture in a 48-well microplate, the plates were washed three times and blocked with blocking buffer containing 5% milk powder in TBS-T overnight. The membrane was washed with PBS-T and then incubated with mouse monoclonal antibody to the His tag (Novagen) or mouse polyclonal antibody specific for LvCTL1 for 2 h, followed by incubation with goat anti-mouse immunoglobulin G conjugated to peroxidase as a secondary antibody (1:10,000 dilution in PBS-T; Promega) for 1.5 h at room temperature. Then, a 3,3-diaminobenzidine solution (Boster, China) was added to develop the brown product for 5 min, and the reaction was stopped with water.

Identification of recombinant LvCTL1 by mass spectrometry. The in-gel enzymatic digestion and mass spectrometry analysis were performed by the Shanghai Institutes for Biological Sciences, Research Center for Proteome Analysis. The digested protein was separated and identified with a Finnigan LTQ mass spectrometer (ThermoQuest, San Jose, CA), coupled with a Surveyor high-performance liquid chromatography system (ThermoQuest). First, a Microsorve reverse-phase column (C18, 0.15 mm by 120 mm; Thermo Hypersil, San Jose, CA) was used to separate the protein digests. Solvent A was 0.1% (vol/vol) formic acid, and solvent B was 0.1% (vol/vol) formic acid in 100% (vol/vol) acetonitrile. The solvent B gradient was held at 2% for 15 min and increased linearly to 98% in 90 min. The peptides were eluted from the C18 microcapillary column at a flow rate of 0.3 μl/min, and the column was operated directly in a microcapillary MSQ spectrometer with the application of a spray voltage of 3.2 kV and with a capillary temperature of 170°C. The full scan ranges from mass 400 to 2,000. Protein identification using raw data from tandem mass spectrometry was performed with SEQUEST software (University of Washington, licensed to Thermo Finnigan) based on se-
quences in the Swiss-Prot database. The species is WSSV. A relative molecular mass of 57 Da was added to the average molecular mass of cysteines in tandem-mass spectrometry data searching. Both b ions and y ions were included in the database search.

In vitro anti-WSSV activity of recombinant LvCTL1. To test whether recombinant LvCTL1 has anti-WSSV activity, a coculture assay was performed. This assay is based on the percentages of cytopathic effects (CPE) and dead cells induced by WSSV infection relative to those of normal cells. Hemocytes from healthy L. vannamei shrimps were collected as the primary cells and challenged with WSSV according to the method described by Jiang et al. (17). The L. vannamei hemocytes were cultured in vitro at 24 ± 0.5°C with 5% ± 0.2% CO₂ in a 96-well microplate in the modified Leibovitz’s L-15 medium (80% 1× Leibovitz’s L-15; Gibco) and supplemented with 15% fetal bovine serum (HyClone), 1.0 g/liter glucose, 0.3 g/liter glutamine, 0.1 mg/liter vitamin C, 12.0 g/liter NaCl, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate (pH 7.2). The cells were observed daily with an inverted phase-contrast microscope (Nikon, Japan), and half of the culture medium was replaced with fresh culture medium every day. The number of dead cells was counted before inoculating them into 96-well microplates at about 3 × 10⁶ cells per well for WSSV challenge experiments. The WSSV inoculum was prepared according to the method described in our previous work (51). WSSV dilutions (50 μl for each well) were preincubated with the same volume of serial dilutions of LvCTL1 (the ultimate LvCTL1 concentrations were 10 μg/ml, 20 μg/ml, 40 μg/ml, and 60 μg/ml) or BSA (as a control) in serum-free culture medium for 20 min at 4°C and then added to the confluent hemocytes, which had been cultured in a 96-well microplate for 2 weeks. This procedure gave a final WSSV dilution of 10⁻³ virion after the addition of the hemocyte suspension. After 2 h of incubation, the viral solutions were removed, and completely fresh medium was added. The cells were incubated at 25°C and monitored for 5 days. CPE and the number of death cells induced by WSSV were recorded. Assays were performed in triplicate.

In vivo anti-WSSV activity of recombinant LvCTL1. The anti-WSSV activity of recombinant LvCTL1 was also tested in healthy L. vannamei shrimps challenged with WSSV. Healthy Pacific white shrimps with an average weight of 15 g were kept in separate 500-liter tanks (each tank contained 30 shrimps) of well-aerated seawater (at 26 ± 1°C) and acclimatized to laboratory conditions for 1 week before experiments. Shrimps were fed a commercial diet three times daily, and approximately 50% of the seawater was changed per day. Twelve tanks of the healthy shrimps were divided into four groups (three tanks for each group). Shrimps were injected intramuscularly with sterile 1× PBS solution (as a negative control; group 1), WSSV inoculum (as a positive control; group 2), or WSSV inoculum preincubated with LvCTL1 (60 μg/ml; group 3) or pET-32a-His-tag (group 4) for 20 min at 4°C (50 μl per shrimp). During shrimp culture, shrimp mortality was monitored daily for 9 days, and the dead shrimps were examined by PCR to confirm infection with WSSV.

Statistical analysis. Student’s t test was used for statistical comparison of the mean cumulative mortality rates across groups in the antiviral activity experiment in vivo. P values of <0.05 were considered statistically significant.

RESULTS

Cloning of the LvCTL1 cDNA from L. vannamei. Based on the initial partial cDNA sequence of LvCTL1, gene-specific primers (GSP1 and GSP2) (Table 1) were then designed and used for RACE reactions to obtain both the 5’ and 3’ ends of the cDNA, and the full-length cDNA was assembled from multiple overlapping clones. Then, the sequence was verified by reamplifying the ends of the cDNA, and the full-length cDNA was assembled from multiple overlapping clones. The sequence was then verified by reamplifying the ends of the cDNA, and the full-length cDNA was assembled from multiple overlapping clones. The sequence was verified by reamplifying the ends of the cDNA, and the full-length cDNA was assembled from multiple overlapping clones.

The CRD of LvCTL1 contains an EPN motif (Glu99-Pro100-Asp101), which has a predicted ligand binding specificity for mannose in mammalian C-type CRDs (8). This result suggests that LvCTL1 may bind to mannose. The calculated molecular mass of the mature LvCTL1 protein (residues 21 to 156) is 15.86 kDa, with an estimated pI of 5.20.

Comparison of LvCTL1 with other shrimp C-type lectins. Database searches with the deduced amino acid sequence of LvCTL1 showed that LvCTL1 is most similar to the C-type lectin FcCllec-2 (GenBank accession no. ABAS54612) (77% identity). The CRD of LvCTL1 was also highly similar to that of FcCllec-2 (accession no. ABAS54612) (79% identity), and it is also similar to CRDs of PsClec (accession no. ABI97373) (34% identity), LvCt (accession no. ABI97374) (34%), PmClec-1 (accession no. ABI97373) (32%), and PmAv (accession no. AAQ75589) (28%). Based on the BLASTP analysis, several shrimp C-type lectins were selected and aligned with LvCt (Fig. 1B). These shrimp lectins contain at least one C-type CRD. The alignment showed that the CRD of LvCTL1 contains all 14 invariant amino acid residues and 16 of 18 highly conserved amino acid residues that define a C-type CRD (6), including the conserved cysteine residues that stabilize the C-type lectin domain and the EPN motif for the ligand binding specificity of mannose (Fig. 1B).

LvCTL1 mRNA is specifically expressed in the hepatopancreas. Reverse transcription-PCR (RT-PCR) was performed to determine tissue-specific expression of LvCTL1 mRNA. The LvCTL1 transcript was highly expressed in the hepatopancreas, and a much lower expression of LvCTL1 was detected in the stomach. However, the LvCTL1 transcript was not detected in the hemocytes, eyestalks, muscles, brains, gills, intestines (midgut), pyloric ceca, nerve cords, spermataries, or hearts of healthy L. vannamei shrimps (Fig. 2A and B). These results suggest that the LvCTL1 transcript is specifically expressed in the hepatopancreases of shrimps.

Expression and purification of recombinant LvCTL1. Competent E. coli M15(pREP4) cells were transformed with the recombinant expression vector pQE30-LvCTL1, and the recombinant protein, which has a six-His-tag addition to the N terminus, was expressed abundantly after IPTG (isopropyl-β-D-thiogalactopyranoside) induction. The recombinant LvCTL1 protein was expressed as inclusion bodies and purified with Ni-nitrotriacetic acid agarose under denaturing conditions. After being refolded by gradient dialysis against PBS buffer, almost all of the purified LvCTL1 protein became soluble, and the purity was estimated to be higher than 95% (Fig. 2C, lane 3). Recombinant LvCTL1 has an apparent mass of ~16 kDa as determined by SDS-PAGE, which matched the predicted molecular mass calculated from the deduced amino acid sequence. Recombinant LvCTL1 was specifically recognized by both monoclonal antibody against the His tag and polyclonal antibody against LvCTL1 (Fig. 2C, lanes 4 and 5).

Expression of LvCTL1 protein in shrimp hemolymph is induced after WSSV infection. The hemolymph was collected from healthy shrimps or shrimps infected with WSSV at different times (3, 6, 12, 24, 36, 48, and 72 h) postinfection, and LvCTL1 was detected by immunoblotting, using polyclonal antibody against recombinant LvCTL1 (Fig. 2D). LvCTL1 protein was not detected in the hemolymph of healthy (unchallenged) shrimps. After WSSV infection, a 24-kDa protein was detected in the hemolymph at 12 h postinfection, which was assumed to be the natural glycoprotein of LvCTL1, just like other shrimp lectin glycoproteins from Fenneropenaeus merguiensis, Penaeus monodon, and Macrobrachium rosenbergii (32, 33, 50). The concentration of LvCTL1 in hemolymph increased steadily up to 48 h postinfection (Fig. 2D, lanes 4, 5,
However, LvCTL1 in hemolymph decreased dramatically at 72 h postinjection (Fig. 2D, lane 8). These results suggest that LvCTL1 protein in hemolymph was induced after WSSV infection.

**Expression of LvCTL1 in the hemolymph of WSSV-resistant and -susceptible shrimps.** To compare the expression levels of LvCTL1 in the hemolymph of WSSV-resistant and -susceptible shrimps, an ELISA-based assay was performed. Cell-free plasma samples were prepared from both the WSSV-resistant and -susceptible L. vannamei shrimps at 0, 3, 6, 12, 24, 36, 48, and 72 h after WSSV injection and used for the ELISA. Total proteins extracted from hemolymph samples from both the WSSV-resistant and -susceptible L. vannamei shrimps at 0, 3, 6, 12, 24, 36, 48, and 72 h after WSSV injection were assayed by the ELISA procedure. The 96-well microtiter plates were coated overnight at 4°C with 20.0 μg total protein per well in about 100 μl of protein extracts. The bound peroxidase activity was determined by a reaction with TMB (3,3′,5,5′-tetramethylbenzidine), and the OD value was measured by spectrophotometry, using 450-nm filters. Negative-control plates included plates coated with BSA. For each sample, the OD of the control well was subtracted from the OD of the hemolymph sample well. All ELISAs were performed in triplicate, with the data given in mean values.

After WSSV injection were gradually increased and obviously higher than those of the hemolymph samples from the healthy control shrimps. The mean OD values from the hemolymph samples of the WSSV-resistant shrimps after WSSV injection were significantly higher than those of the hemolymph samples from the WSSV-susceptible shrimps (Fig. 2E). These results indicated that the expression levels of LvCTL1 in the hemolymph of the WSSV-resistant shrimps were significantly higher than those in the hemolymph of the WSSV-susceptible shrimps after WSSV injection, suggesting that induced expression of LvCTL1 is a response against the WSSV infection.

**Hemagglutinating activity and carbohydrate binding specificity of recombinant LvCTL1.** Recolded recombinant LvCTL1 protein was used to perform a hemagglutination assay with erythrocytes from different animals. Among the animal eryth-
TABLE 2. Sugar specificities of LvCTL1

<table>
<thead>
<tr>
<th>Sugar name</th>
<th>MIC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Mannose</td>
<td>5</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>15</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>35</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>95</td>
</tr>
<tr>
<td>Lactose</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Rockeyes tested, LvCTL1 agglutinated mouse and chicken erythrocytes more effectively than rabbit erythrocytes with a minimal agglutination concentration of 6.25 μg/ml, and it agglutinated fish and rabbit erythrocytes at a concentration higher than 12.5 μg/ml. Recombinant LvCTL1 also agglutinated shrimp hemocytes at a concentration higher than 80.0 μg/ml. However, no agglutination could be observed in the BSA control under the same conditions. When mouse hemocytes were agglutinated in the presence of EDTA, agglutination was inhibited when the EDTA concentration was higher than 2.0 mM (agglutination occurred at EDTA concentrations of 0, 0.5, 1.0, and 1.5 mM), indicating that the agglutination of animal erythrocytes by recombinant LvCTL1 is calcium dependent.

To test the carbohydrate binding specificity of LvCTL1, an inhibitory hemagglutination assay was performed. Carbohydrates were preincubated with recombinant LvCTL1, and the mixtures were then added to mouse erythrocytes. Among the carbohydrates tested, D-mannose and D-glucose inhibited the hemagglutinating activity of recombinant LvCTL1 most effectively, with minimal concentrations of 5 and 15 mM, respectively (Table 2). D-Fructose inhibited the hemagglutinating activity of LvCTL1 at a higher concentration (35 mM), while D-galactose, lactose, and sucrose had little inhibitory activity (Table 2). These results suggest that LvCTL1 has ligand binding specificity for mannose/glucose, which is consistent with the predicted ligand specificity of mannose.

**Recombinant LvCTL1 binds to WSSVs.** To test whether LvCTL1 can directly bind to WSSVs, immunostaining was performed. WSSV virions were spotted onto a nitrocellulose membrane and then incubated with recombinant LvCTL1, Lypn (a His-tagged control protein from fish [unpublished]), or pET-32a-His-tag. Binding of recombinant proteins to WSSV virions was detected by monoclonal antibody against the His tag or polyclonal antibody specific for LvCTL1. As shown in Fig. 3a, binding of recombinant LvCTL1 to WSSV was detected by both anti-His tag and LvCTL1-specific antibodies (Fig. 3aA and B). No Lypn or pET-32a-His-tag bound to WSSV (Fig. 3aC and D). These results suggest that LvCTL1 may bind to proteins on the surfaces of WSSV.

**Recombinant LvCTL1 interacts with WSSV envelope proteins.** To further confirm that recombinant LvCTL1 can interact with proteins from WSSVs, a pull-down assay was performed. WSSV proteins pulled down by MBP-lectin but not by MBP were analyzed by mass spectrometry and identified as VP95, VP28, VP26, VP24, VP19, and VP14 (Fig. 3b), with sequence coverage of 21%, 28%, 62%, 53%, 41%, and 46%, respectively. These results suggest that LvCTL1 may bind to these WSSV envelope proteins.

Recombinant LvCTL1 has anti-WSSV activity in vitro and in vivo. To test whether recombinant LvCTL1 has direct anti-WSSV activity, an in vitro assay was first performed with a primary culture of *L. vannamei* hemocytes. Hemocytes from healthy shrimps were collected and cultured in 96-well microplates. Next, the primary hemocytes were infected with WSSV that had been preincubated with recombinant LvCTL1 or BSA (as a control), and the cells were monitored for 5 days. These hemocytes were viable after 2 days, but after 3 days, clumps of infected cells and lysed cells were found in wells that were inoculated with BSA-treated WSSV (data not shown). There were no significant changes, however, in the hemocytes inoculated with recombinant LvCTL1-treated WSSV (data not shown). At 5 days postinfection, CPE were apparent in the hemocytes inoculated with BSA-treated WSSV; most cells became detached from the wells, and cell debris was observed (Fig. 4aB). On the other hand, only small clumps of infected cells and very little cell debris were found in the hemocytes inoculated with LvCTL1 (40 μg/ml)-treated WSSV (Fig. 4aC). When the concentration of recombinant LvCTL1 was 20 μg/ml or 60 μg/ml, the appearance of infected cell clumps was also delayed, and the amounts of cell clumps and CPE induced by WSSV were decreased (data not shown). The hemocytes with-

![FIG. 3. Interaction of recombinant LvCTL1 with WSSV.](http://jvi.asm.org/)

(a) Binding of recombinant LvCTL1 to the WSSV virions. Purified WSSV was dotted onto a nitrocellulose membrane and probed with recombinant LvCTL1, Lypn (a fish protein that was also expressed with the pET-32a vector), or pET-32a-His-tag. Binding of recombinant proteins to the viruses was detected by mouse monoclonal antibody against the His tag or mouse polyclonal antibody specific for LvCTL1. (A) The membrane was probed with recombinant LvCTL1 and detected with anti-His tag antibody. (B) The membrane was probed with recombinant LvCTL1 and detected with anti-His tag antibody. (C) The membrane was probed with pET-32a-His-tag and detected with anti-His tag antibody. (D) The membrane was probed with Lypn and detected with anti-His tag antibody. (b) Protein pull-down assay for WSSV proteins that interact with the lectin LvCTL1. The cDNA fragment encoding mature LvCTL1 (Fig. 1A, residues 21 to 156) was cloned into the pMAL-C2X plasmid (NEB, United Kingdom) to express an MBP-lectin fusion protein in *E. coli* BL21 cells. The MBP or MBP-lectin fusion protein was fixed to amylase resins. The purified WSSV proteins were lysed and centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was diluted five times with TBS containing 1% Triton X-100, 10 mM EDTA, and protease inhibitor. Then, the viral protein extract was added to the MBP or MBP-lectin fusion protein-fixed amylase resins, and the mixture was incubated for 1 h at 4°C. The amylase resins were then washed with TBS buffer containing 1% Triton X-100, and the pull-down proteins were analyzed by SDS-PAGE. Lane 1, proteins pulled down by MBP; lane 2, proteins pulled down by MBP-lectin; lane 3, protein molecular standard.
out WSSV inoculation grew well throughout the experimental period (Fig. 4aA). However, the anti-WSSV activity of LvCTL1 was not significant when the concentration of LvCTL1 was 10 μg/ml (data not shown). These results suggest that LvCTL1 can protect shrimp hemocytes from WSSV infection.

To determine whether LvCTL1 also has anti-WSSV activity in shrimps in vivo, healthy L. vannamei shrimps were infected with untreated WSSV (positive control), recombinant pET-32a-His-tag-treated WSSV, or PBS (negative control), and the mortality of shrimps was monitored daily for 9 days. Initial mortality was observed after the first or second day postinjection in all three groups of shrimps infected with WSSV as well as in the negative-control group (first day). On the fifth day postinfection, the mortality of the shrimps infected with recombinant LvCTL1-treated WSSV (15.6%) was significantly lower \((P < 0.05)\) than that of the shrimps infected with pET-32a-His-tag-treated WSSV (43.3%) or untreated WSSV (58.9%), and no mortality was observed in the negative-control shrimps (Fig. 4b and Table 3). On the ninth day postinfection, the mortality of shrimps infected with LvCTL1- and pET-32a-His-tag-treated WSSV reached 47.8% and 100%, respectively, and no mortality was observed in the negative-control shrimps. These results suggest that recombinant LvCTL1 can protect shrimps from WSSV infection.

**DISCUSSION**

C-type lectins exist in almost all animals. They are capable of binding to specific carbohydrates in a Ca\(^{2+}\)-dependent manner and play an important role in nonself-recognition and the clearance of invading microorganisms (6, 8, 42, 47). In this study, we isolated a cDNA for a novel C-type lectin (named LvCTL1) from the Pacific white shrimp, L. vannamei, by using ESTs and RACE. LvCTL1 contains a single CRD that is similar to PmAV (black tiger shrimp P. monodon) (29) and Fe-hsL.

**TABLE 3. Cumulative mortalities of the shrimp L. vannamei after the injection of PBS, WSSV, or WSSV preincubated with recombinant LvCTL1 or pET-32a-His-tag protein**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial no. of shrimps ((n = 360))</th>
<th>Mortality postinfection (%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 5</td>
</tr>
<tr>
<td>PBS</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>WSSV</td>
<td>90</td>
<td>13.3 ± 1.00</td>
</tr>
<tr>
<td>WSSV +</td>
<td>90</td>
<td>11.1 ± 0.57</td>
</tr>
<tr>
<td>PET-32a</td>
<td>90</td>
<td>11.1 ± 0.57</td>
</tr>
<tr>
<td>WSSV +</td>
<td>90</td>
<td>6.7 ± 1.00</td>
</tr>
<tr>
<td>LvCTL1</td>
<td>90</td>
<td>11.1 ± 0.57</td>
</tr>
</tbody>
</table>

\(^a\) Results are means from three independent experiments with standard deviations. \(P\) values were determined using analysis of variance, and the significance of differences was determined by the \(t\) test \((P < 0.05)\). Values in the same column with different letters (A and B) are significantly different.
C-type lectins, such as MBL, have been shown to be able to bind envelope glycoproteins of human immunodeficiency virus (4) and envelope proteins of the influenza A virus (19), and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrins can interact with envelope glycoproteins of Ebola virus (1) and bind to Dengue (37) and hepatitis C (27) viruses. Thus, it is likely that LvCTL1 may bind to the surface glycoproteins of WSSV to inhibit the infection.

So far, approximately 58 structural proteins, including 9 nucleocapsid proteins and 32 envelope proteins, have been identified in the WSSV virions (23), and they may play key roles in the initial infection of WSSV in shrimps (40). These data facilitate us further in studying antiviral activity. It was shown that even a single WSSV envelope protein, such as VP28, can prolong the survival of shrimps following WSSV challenge when it is injected intramuscularly or administered orally, possibly by competitive saturation of receptor sites of the host cells relevant for viral adhesion and viral penetration (43). Likewise, antibodies raised against VP28 can also neutralize and inhibit WSSV infection (40).

We showed that recombinant LvCTL1 protein, when bound to WSSV, significantly increased the survival of shrimps against WSSV challenge and also protected shrimp hemocytes from WSSV infection (Table 3; Fig. 4). LvCTL1 may serve as a PRR to recognize and bind envelope proteins of WSSV, to block the virus from entering cells, or to neutralize the virus. Our future goal is to investigate how the binding of LvCTL1 to WSSVs inhibits the infection.

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