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A Short-Form C-Type Lectin from Amphioxus Acts as a Direct Microbial Killing Protein via Interaction with Peptidoglycan and Glucan[1]

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To investigate the evolution and immune function of C-type lectin in amphioxus, the primitive representative of the chordate phylum, we identified three C-type lectins consisting solely of a carbohydrate recognition domain and N-terminal signal peptide and found that they had distinct express patterns in special tissues and immune response to stimulations analyzed by quantitative real-time PCR. We characterized the biochemical and biological properties of AmphiCTL1, which was dramatically up-regulated in amphioxus challenged with *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and zymosan. Immunohistochemistry demonstrated that the localization of AmphiCTL1 protein was exclusively detected in the inner folding tissues of the hepatic diverticulum. Recombinant AmphiCTL1 was characterized as a typical Ca^{2+}-dependent carbohydrate-binding protein possessing hemagglutinating activity, preferentially bound to all examined four Gram-positive bacteria and two yeast strains, but had little binding activity toward four Gram-negative bacteria we tested. It aggregated *S. aureus* and *S. cerevisiae* in a Ca^{2+}-dependent manner and specifically bound to insoluble peptidoglycan and glucan, but not to LPS, lipoteichoic acid, and mannan. Calcium increased the intensity of the interaction between AmphiCTL1 and those components, but was not essential. This lectin directly killed *S. aureus* and *S. cerevisiae* in a Ca^{2+}-independent fashion, and its binding to microorganism cell wall polysaccharides such as peptidoglycan and glucan preceded microbial killing activity. These findings suggested that AmphiCTL1 acted as a direct microbial killing C-type lectin through binding microbial targets via interaction with peptidoglycan and glucan. Thus, Amphi CTL1 may be an evolutionarily primitive form of antimicrobial protein involved in lectin-mediated innate immunity. *The Journal of Immunology,* 2007, 179: 8425–8434.

Innate immunity constitutes the first line of defense against microbial invasion in the animal kingdom based on pattern recognition. The ability to recognize the exposed glycans on the cell surface of potential pathogens by host humoral or cell-associated lectins is considered a primary role for pattern recognition molecules in innate immunity (1–4). Lectins are generally organized as oligomers of noncovalently bound subunits, each displaying a carbohydrate recognition domain (CRD)[3] that binds to the sugar ligands, usually a nonreducing terminal monosaccharide or oligosaccharides. C-type lectins are proteins that contain CRDs and bind carbohydrate structures in a Ca^{2+}-dependent manner. Calcium ions are directly involved in ligand binding, as well as in maintaining the structural integrity of the CRD that is necessary for the lectin activity (5). C-type lectin domains (CTLDs) refer to protein domains that are homologous to the CRDs of the C-type lectins, or which have structure resembling the structure of the prototypic C-type lectin CRD (6). In vertebrates, CTLD represents a very large family that encompasses up to 17 subgroups (6), most of which are able to bind pathogen-associated molecular patterns (PAMPs) and microorganisms themselves through sugar moieties such as *N*-acetyl-glucosamine (GlcNAc), mannose, *N*-acetyl-mannosamine, fucose, and glucose (7). Recently, a new form of C-type lectin-mediated innate immunity is described in which mouse Reg|||γ, along with its human counterpart hepatocarcinoma-intestine pancreas/pancreatic-associated protein (HIP/PAP), has direct bactericidal activity as a secreted C-type lectin (8). Reg|||γ can bind to bacterial peptidoglycan on the surface of commensal Gram-positive bacteria and regulates crypt colonization in the mouse intestine.

In the absence of the vertebrate adaptive immunity, the whole burden of the antipathogenic defense lies in the innate immune system for invertebrates. To protect themselves from the invading microorganisms, invertebrates must rely on the effective and sophisticated innate immune system. The recognition of the

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[3] Abbreviations used in this paper: CRD, carbohydrate recognition domain; CTLD, C-type lectin domain; AmphiCTL, amphioxus C-type lectin; PAMP, pathogen-associated molecular pattern; DAB, diaminobenzidine; LB, Luria-Bertani; TRX, thioredoxin; LTA, lipoteichoic acid; PGN, peptidoglycan; EST, expressed sequence tag; HA, hemagglutinating activity; Q-PCR, quantitative real-time PCR; HIP, hepatocarcinomanin-intestine pancreas; PAP, pancreatitis-associated protein.

characteristic carbohydrate structures is considered as a universal and effective method of distinguishing self from nonself in invertebrates. The abundance of C-type lectins in invertebrates (such as *Drosophila melanogaster* and *Caenorhabditis elegans*) is a very effective repertoire for defending pathogens. C-type lectins from invertebrates such as the insect, starfish, and shrimp have been found to be involved in various biological responses, for instance, promotion of phagocytosis (9), activation of the prophenol oxidase system (10, 11), nodule formation (12), and antibacterial activity (13, 14).

CTLDs are found almost exclusively in Metazoa and are highly conserved in vertebrates but with considerable diversity among invertebrates. For example, the C-type lectin repertoire of *C. elegans* and *D. melanogaster* is drastically different from each other, and from the known vertebrate groups (6). The studies of CTLDs on those organisms that occupy crucial positions in the animal tree will give important implications for understanding the origin and evolution of the functional systems in which CTLDs are involved. Amphioxus is the most primitive representative of the chordate phylum, hence constitutes an important reference to the immunity evolution of CTLDs. There are >1000 C-type lectin gene models in the amphioxus genome based on our analysis, compared with 100~200 genes in other species such as human, fugu, and *C. elegans* (6, 15, 16) and most of them derive from lineages specific to amphioxus. Half of amphioxus CTLDs consist solely of a CRD domain (S. Huang, S. Yuan, Y. Yu, L. Gua, unpublished observation). In this study, we characterized three C-type lectins with a single CRD domain from the Chinese amphioxus, one of which possesses direct microbial killing activity by binding their microbial targets via interaction with peptidoglycan (PGN) and glucan carbohydrates.

Materials and Methods

Reagents

LPS from *Escherichia coli* 0111:B4, lipoteichoic acid (LTA) from *Staphylococcus aureus*, mannan from *Saccharomyces cerevisiae*, glucan from *S. cerevisiae*, zymosan from *S. cerevisiae*, maltose, mannose, GlcNAc, BSA, DMSO, 3,3′,5,5′-tetramethylbenzidine, and lactose Sepharose 4B were purchased from Sigma-Aldrich. PGN from *S. aureus* and *Bacillus subtilis* were purchased from Fluka Chemical. FITC (isomer I) and PHA were purchased from Amersco. Recombinant protein G agarose was purchased from Invitrogen Life Technologies. Sucrose, lactose, galactose, fructose, and glucose were obtained from Guangzhou Pure Chemical. Diaminobenzidine (DAB) and HRP-labeled IgG were purchased from Boster Chemical.

Preparation of microbial cells

Staphylococcus epidermidis and *Enterococcus faecium* were clinical strains isolated from the patients of the Third Affiliated Hospital of Sun Yat-Sen University (China). *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Aeromonas sobria*, and *B. subtilis* were isolated from amphioxus. They were characterized by using standard microbiological and biochemical procedures in the Medical Diagnostic Laboratory of the Third Affiliated Hospital, Sun Yat-Sen University. In addition to these strains, *S. cerevisiae* (American Type Culture Collection (ATCC) 9763), *S. aureus* (ATCC 12598), *Pichia pastoris* and *E. coli* DH5α were used. *V. parahaemolyticus* was cultured with a TCBS agar plate at 26°C while other bacteria separated from amphioxus were cultured at 26°C with aeration in Luria-Bertani (LB) medium prepared with fresh seawater. Other bacterial strains were cultured at 37°C in LB prepared with the distilled water. *S. cerevisiae* and *P. pastoris* were cultured with 2× YPD medium with 3% glucose (4% bactotryptone, 2% bacto-yeast extract (pH 5.8)) at 30°C. All microbial strains were harvested by centrifugation at 3500 × *g* for 10 min and resuspended in the buffer for an appropriate concentration. Quantification was performed by plating various bacteria dilutions on agar plates.

Cultivation and immune stimulation of amphioxus and preparation of tissue and embryo samples

Matured adults of Chinese amphioxus, *Branchiostoma belcheri* (genus *Branchiostoma*, family *Branchiostomidae*) were obtained from Xiamen (Fujian Province, China), and cultured at 25°C in a tank filled with air-pumped circulating sterilized seawater. Spawning females swimming up to lay eggs from the sand were caught with a net and immediately put into a large petri dish containing naturally inseminated seawater. The embryos at each period were collected and quickly frozen in liquid nitrogen. Adult amphioxus was not fed for 10 days before separating tissues under an optical microscope. For immune stimulations, a live microbial PBS suspension (15 μl/animal, 10^9 CFU/μl), component PBS suspension (15 μl/animal, 1 mg/ml), or PBS was injected into the coelom of amphioxus. Twenty random samples of each treatment were collected after injection and frozen by liquid nitrogen as experimental samples.

Full-length cDNA cloning and sequencing of amphioxus C-type lectins

Expressed sequence tags (EST) with sequence similarity to C-type lectin were identified from a series of EST libraries constructed by our laboratory. The 5′-RACE was performed using a GeneRACE kit (Invitrogen Life Technologies) according to the manufacturer's instructions. Gene-specific primers were designed for amplifying the 5′ end sequence and the full-length sequence of amphioxus C-type lectins. In all PCR, total RNA of whole amphioxus was used as templates. And the amplified fragments were cloned into the pGEX-T easy vector (Promega) and sequenced by the PerkinElmer ABI Prism 3730 DNA sequencer.

Quantitative real-time PCR (Q-PCR) analysis of gene expression patterns for amphioxus C-type lectins

Q-PCRs were performed and analyzed as described (17). Total 40 cycles were performed with the primers 5′-GTGGCTACGAATACTGGGTG AACG-3′ (forward) and 5′-ATTGCGATTGCCGAGAAATGTGTC-3′ (reverse) for AmphiCTL1, 5′-AACTGCTACCGCTTCTCCACCGAC CA-3 (forward) and 5′-TGTGTCTCGTTGTTCTT-CGCCGTTGC-3′ (reverse) for AmphiCTL2, and 5′-GGGAACAACGGATACAAGGGATG-3′ (forward) and 5′-TGGAGGACGCTGACAGATGAAC-3′ (reverse) for AmphiCTL3. All samples were analyzed in three duplications and the results were expressed as relative fold of one sample in each experiment as mean ± SD.

Preparation of recombinant AmphiCTL1 proteins

Recombinant AmphiCTL1 proteins were expressed in two different systems. The nonfusion protein is expressed with pET21b system (Novagen) to prepare for the antiserum as the Ag. The coding region for mature AmphiCTL1 (Val^{24} to Ala^{151}) is amplified by primers (forward primer: 5′-GGAATTCCATATGGTTACCCGATATGAACGTCGT-3′; reverse primer: 5′-CCGCTCGAGTTAGGCTGATGCATTGTTGCCTCT-3′) from the pGEM-T Easy vector (Promega) carrying AmphiCTL1 full-length cDNA. *Nde*I and *Xho*I endonuclease sites (underlined) were included at the 5′ end of the forward and reverse primer, respectively, while one strong stop codon was incorporated at the 5′ end of the reverse primer to produce native protein AmphiCTL1. The PCR products were digested with *Nde*I and *Xho*I, gel-purified, ligated to pET21b plasmid prepared in the same way, and confirmed by DNA sequencing to construct the expression vector pET-AmphiCTL1. The expression plasmids were introduced into *E. coli* BL21 (DE3) to express recombinant protein. The recombinant protein was denatured in 8 M urea and renatured by three dialysis steps as described (18), and each dialysis step was performed for at least 12 h at 4°C. Then, the soluble renatured protein was purified with lactose Sepharose 4B.

To obtain the nature soluble protein, we expressed recombinant Amphi CTL1 using a thioredoxin (TRX) fusion system containing a 6× His tag to facilitate the purification of fusion proteins on a Ni^{2+}-chelating Sepharose column and partner TRX to help the proteins fold correctly. Specific cDNA was amplified with the primers 5′-GGGGTACCGTTACCCGATATG AACGTCGT-3′ (forward) and 5′-ATAAGAATGCGGCCGCTCAGGCT GATGCATTGTTGCC-3′ (reverse). *Kpn*I and *Not*I endonuclease sites (underlined) were included at the 5′ end of the forward and reverse primer, respectively. The amplified fragments were inserted into pTRX between the *Kpn*I and *Not*I site, and the expression plasmid was introduced into *E. coli* BL21 (DE3). The supernatant of the recombinant protein was applied to a Ni^{2+}-chelating Sepharose column, pooled by elution with 150 mM imidazole.

The purified recombinant proteins were desalted to TBS (50 mM Tris · Cl (pH 7.5), 150 mM NaCl) with G-25 column, and concentrated by filtration through an Ulterafree centrifugal filter device (Millipore). Protein concentration was determined using a Bio-Rad Protein Assay dye reagent and BSA as a standard.

Preparation of AmphiCTL1 Ab

The inclusion body of AmphiCTL1 expressed by pET21b system was washed with 2 M urea, and the pellets were dissolved with the sample buffer and subjected to the SDS-PAGE. The specific band of AmphiCTL1 was injected to the New Zealand white rabbit over a period of 2 mo to prepare the anti-AmphiCTL1 serum. The cross-reaction Ab of the antiserum was eliminated following the methods of Braunagel (19) with slight modification. The 10 ml of *E. coli* BL21 (DE3) incubated overnight was collected at 4000 × *g* for 10 min and washed with TBS, then resuspended with 500 μl of TBS and frozen-thawed for four times. Then, the mixture was quickly boiled at 100°C for 5 min to destroy the activity of the proteases. The cells were lysed by sonication for 10 min, incubated at 56°C for 30 min, and then mixed at 4°C with the 100 μl of serum for 12 h. Cell debris was removed by centrifugation. The IgG fraction was purified from bacteria-preabsorbed serum using recombinant protein G agarose (Invitrogen Life Technologies), and the preimmune serum was treated with the same methods.

Localization of AmphiCTL1 protein by immunohistochemistry

Amphioxus was severed into three to four pieces, and fixed in freshly prepared 4% (w/v) paraformaldehyde in 100 mM PBS (pH 7.3) at 4°C for 24 h. After dehydration, they were embedded in paraffin, and sectioned at 5 μm. The sections were mounted on slides, and dried at 42°C for 4 h. They were dewaxed in xylene for 10 min (two changes for 5 min each) followed by immersion in absolute ethanol for 10 min (two changes for 5 min each), and then rehydrated in 95, 90, 80, and 70% ethanol (one change for 5 min) and brought to 100 mM PBS. After rinsing with distilled water for 5 min, the endogenous peroxidase activity in the sections was quenched with incubation in 3% (v/v) H_2O_2 at room temperature for 15 min, which was followed by a 5-min wash in redistilled water. Subsequently, the sections were preincubated with 5% BSA in 20 mM PBS (pH 7.3) at room temperature for 30 min, washed in 20 mM PBS for 5 min, and then incubated for 1 h with anti-AmphiCTL1 IgG diluted 1/1000 with 20 mM PBS containing 5% BSA in a humidified chamber at 37°C. The control sections were similarly incubated with preimmune rabbit Ab. Both experimental and control sections were washed three times for 3 min each in 20 mM PBS, and incubated further with HRP-labeled anti-rabbit IgG diluted 1/200 with PBS containing 5% BSA at room temperature for 1 h. The chromomeric reaction was achieved by addition of 0.015% (w/v) DAB containing 0.1% $NiCl_2$ and 0.02% (v/v) H_2O_2 in 50 mM Tris · Cl buffer (pH 7.6) and maintaining in the dark for 5 min. Then, the sections were restained with the hematoxylin for 2 min. In the last two steps, all the sections were washed three times for 10 min in redistilled water. Then, the sections dehydrated in 70, 80, 90, 95%, and absolute ethanol (one change for 5 min) were brought to dimethyl benzene. The sections were mounted in Canada balsam, observed, and photographed under a Nikon microscope.

Hemagglutination inhibition assays

Hemagglutinating activity (HA) of AmphiCTL1 was tested on trypsin-treated glutaraldehyde-fixed rabbit erythrocytes as described (20). Rabbit erythrocytes (2%, v/v) were prepared in TBS containing 10 mM $CaCl_2$. The sugar solution was dissolved with the same buffer as erythrocytes. HA was measured by a series of 2-fold dilutions of purified recombinant proteins with equal protein concentration in 96-well microtiter plate with V-shaped well bottoms. The lowest dilution that caused distinct hemagglutination was determined after 1 h at room temperature. To analyze the calcium-dependent activity, an excess of EDTA was added. To analyze the inhibitory effect of sugars on hemagglutinating activity, the buffer was replaced by different sugar solutions. The reciprocal of the highest dilution of the lectin showing visible hemagglutination was recorded as the titer and the specificity was determined as the lowest dilution of sugars that inhibited HA of the lectin solution. TRX as negative control and PHA as positive control were tested the same as AmphiCTL1.

Microbial-binding assays

Approximately 5 × 10^7 microbes were incubated with 5 μg of targeted proteins in TBS containing 10 mM $CaCl_2$ by gentle orbital rotation overnight at 4°C. Microbes were pelleted and washed five times with 1 ml of binding buffer. The washed pellets were then suspended with reducing sample buffer quickly and denatured by heating at 100°C for 15 min. Protein binding was analyzed by Western blot, as follows. Proteins were fractionated by electrophoresis through 15% SDS-PAGE and electrophoretically transferred onto a nitrocellulose blot membrane (Pall Corporation). Membranes were blocked with 5% nonfat milk and PBST at room temperature for 2 h and washed three times with PBST. Anti-AmphiCTL1 Ab was diluted 1/5000 in PBST and incubated with the membranes overnight at 4°C. After washing three times with PBST, membranes were incubated for 1 h with HRP-labeled anti-rabbit IgG Ab diluted 1/1000 in PBS. The membranes were washed three times with PBS and detected with DAB. In this experiment, Sepharose 4B was treated with the same method as negative control. In some experiment, EDTA was added to eliminate calcium activity.

Microbial aggregation assays

For FITC labeling of the microbes, microbes collected from plate or liquid cultures were suspended in 1 ml of buffer at pH 9.0 containing 50 mM Na_2CO_3 and 10 mM NaCl, and were mixed with 50 μl of FITC solution (10 mg/ml in DMSO). The reaction was incubated at room temperature in the dark for 1 h with gentle agitation and washed four times with TBS. FITC-labeled microbes and 10 μg of recombinant proteins were mixed and incubated 1 h at room temperature in the presence of 10 mM $CaCl_2$. The agglutinating reaction was examined under fluorescence microscopy.

Binding assay of AmphiCTL1 with the components of microorganisms

A total of 20 μg of LPS, LTA, PGN, glucan (PGN and glucan are ultrasonically solubilized), and mannan were used to coat a 96-well microtiter plate (TPP) in 100 mM PBS (pH 7.3) overnight at 4°C. Nonspecific binding to the wells was prevented by the addition of PBS containing 3% BSA overnight at 4°C. Several concentrations of AmphiCTL1 protein were then added to the wells and incubated for 1 h at room temperature. Bound AmphiCTL1 was detected with anti-AmphiCTL1 Ab diluted 50,000-fold for 1 h at room temperature, followed by a 30-min incubation with a 1/1000 dilution of HRP-labeled IgG. Between each incubation step, unbound protein, Ab, or HRP-labeled anti-mouse IgG was washed off five times. Color was developed by adding 3,3′,5,5′-tetramethylbenzidine liquid substrate, and the absorbance was read at 450 nm. The assay was repeated five more times with similar procedures.

The PGN and glucan-binding activities of AmphiCTL1 were also detected by pull-down assays. Insoluble PGN or glucan was incubated at 4°C for 1 h with 5 μg of AmphiCTL1 in TBS in the presence or absence of 10 mM $CaCl_2$. The samples were centrifuged at 15,000 × *g* for 10 min and PGN or glucan pellets fractions were washed with the same buffer, centrifuged, and dissolved in reducing sample buffer. Pellets were analyzed on 15% SDS-PAGE and then transferred to the nitrocellulose membrane and detected by the anti-AmphiCTL1 Ab.

Antimicrobial activity assays

Antimicrobial activities against *S. aureus* and *S. cerevisiae* were performed on a petri dish. Thirty milliliters of warm nutrient agar (1.0%) mixed with microbes were poured into a 90-mm plate. The pores are 0.5 cm in diameter perforated with perforex. Then, the targeted protein or antibiotic in 100 μl of TBS was added to the pores at the finial concentration of 1 μg/μl. The plates were incubated at suitable temperature for 16 or 40 h. A transparent ring around the pores indicated antibacterial activity.

The growth curves of *S. aureus* and *S. cerevisiae* cultured with Amphi CTL1 were tested as follows. Two single colonies were picked up and transferred into 1 ml of LB or 2× YPD broth. A volume of 50 μl of cell suspension was added to an equal volume of TBS or purified various concentrations of AmphiCTL1 with or without 10 mM $CaCl_2$ in TBS. Each sample was incubated with aeration at 200 rpm and the OD at 600 nm (OD_{600}) was measured every 1 h. An inhibition assay was performed by preincubating protein for 10 min with soluble PGN or glucan before the addition of microbes.

Electron microscopy

For electron microscopy, mid-log phase cultures of *S. aureus* were washed and resuspended in 1 ml of TBS. A total of 500 μl of resuspended bacteria was added to each of two reactions containing either buffer with 100 μg of TRX, or 100 μg of TRX-AmphiCTL1. Reactions were incubated for 2 h at 37°C. The bacterial pellets were washed three times with TBS, and then fixed overnight at 4°C in 4% paraformaldehyde, 0.1% glutaraldehyde in 100 mM PBS. Tissues were dehydrated in an ethanol gradient at decreasing temperatures (0 to −35°C), then embedded in KM4 under UV illumination for 2 days. Embedded tissues were then sectioned using a microtome,

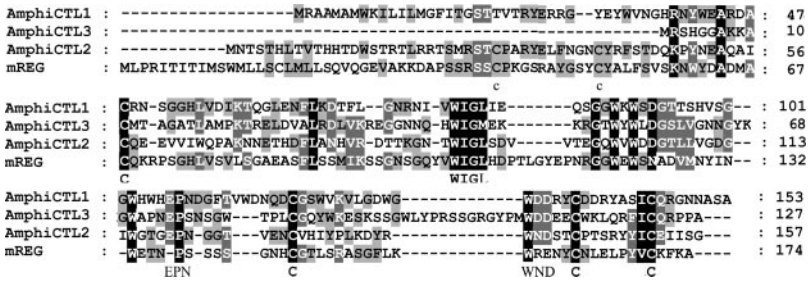

FIGURE 1. Multiple alignment of amphioxus C-type lectins with mice reg|||γ (NP_035390). Residues identical with the threshold of 60% in all sequences were shaded. Residues in black background indicate higher levels of amino acid similarity. The conserved cysteine residues and motif in character were listed in the last line.

floated onto grids, visualized using the JEOL electron cryomicroscope, and imaged on Kodak SO163 films.

Results

Cloning and sequence analysis of amphioxus C-type lectins

ESTs with sequence similarity to C-type lectin were identified from a series of EST libraries established in our laboratory. The full-length cDNA sequences of these ESTs were obtained by 5′-RACE and three of them encoded a sole CRD domain and N-terminal secreted signal. They encoded 153-, 157-, and 127-aa putative proteins composed of a 23-, 28-, and 17-aa signal peptides and CRD domains in C-terminal, named as AmphiCTL1, Amphi CTL2, and AmphiCTL3, respectively. These amphioxus C-type lectins shared 30∼33% identity with each other, also with other species C-type lectins, such as mice reg|||γ. Alignment analysis was conducted for these amphioxus C-type lectins and mice reg|||γ, and several signature sequences of the C-type lectin family were found to be conserved (Fig. 1). The WIGL and WND motifs conserved in the classical C-type lectins also existed in amphioxus C-type lectins with a little variation, such as the WIGM for AmphiCTL3 and WDD for AmphiCTL2/3. The four cysteine residues, which are important to form the CRD internal disulfide bridges, were completely conserved. But two unconserved cysteine residues existing in some CRDs were not found in AmphiCTL1 and AmphiCTL3. The EPN, the key motif in forming coordinates with mannose/glucose in C-type lectins (21), was also found in AmphiCTL1 and AmphiCTL2, but was replaced by EPS in AmphiCTL3.

Expression patterns of amphioxus C-type lectins

Q-PCR analysis of amphioxus C-type lectins transcripts revealed that each had a distinct temporal- and spatial-specific expression pattern (Fig. 2, *A* and *B*). Amphioxus C-type lectins were expressed during the entire embryonic development, with a high expression level at 4 h (blastula stage) and 5.5 h (gastrulae stage), consistent with our previous results that most development and immune-related genes were increased in gastrulae stage because of the changing from multiple cell organization into organ formation in this period (17). These lectins had a relatively high expression at 16 h stage, especially for AmphiCTL3 (Fig. 2*A*). At the adult stage, AmphiCTL1 and AmphiCTL2 transcripts were detected in all tissues with the highest levels in the hepatic diverticulum for AmphiCTL1 and intestine for AmphiCTL2. The AmphiCTL3 transcript was abundant in the skin, ovary, intestine, and muscle, weak in the gill, notochord, and hepatic diverticulum (Fig. 2*B*).

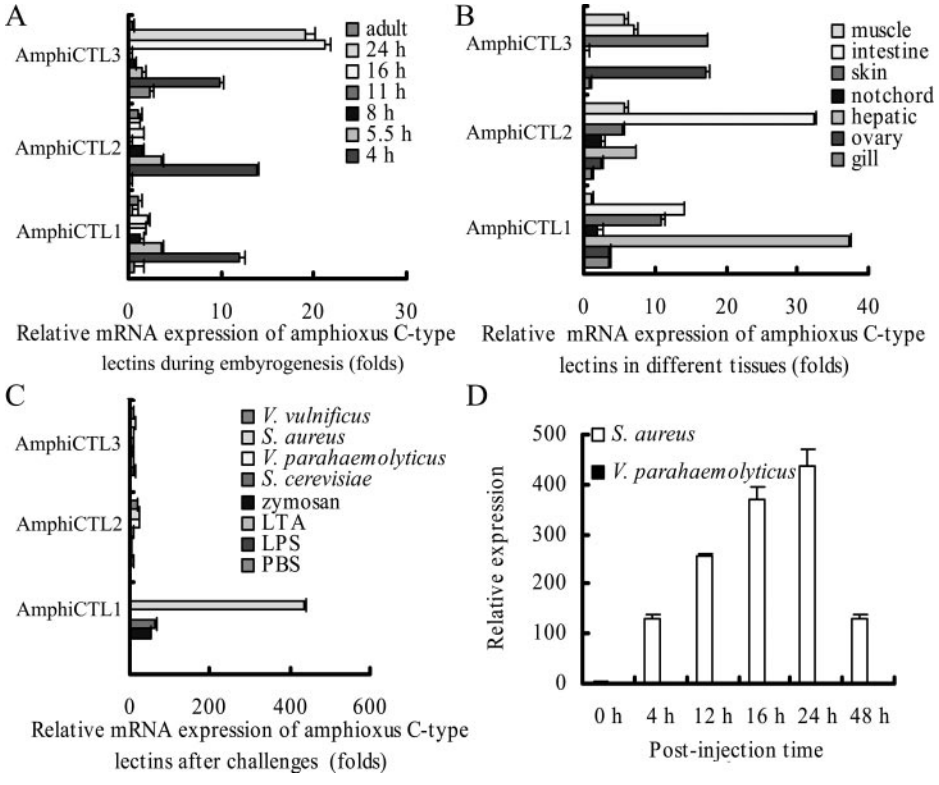

FIGURE 2. Q-PCR analysis of the expression patterns of amphioxus C-type lectins. *A*, The relative level of amphioxus C-type lectin mRNA during the embryogenesis. Data were expressed as a ratio to *AmphiCTL1* mRNA expression in adult. *B*, The relative level of amphioxus C-type lectins mRNA in different tissues. Data were expressed as a ratio to *AmphiCTL1* mRNA expression in muscle. *C*, The relative level of amphioxus C-type lectin mRNA at 24 h after different immune stimulation. Data are expressed as a ratio to *AmphiCTL1* mRNA expression in samples injecting with PBS. *D*, The time course of *AmphiCTL1* mRNA expression at 4, 8, 12, 24, and 48 h after *S. aureus* challenge (□) compared with injection with *V. parahaemolyticus* (■). Data were expressed as an increasing ratio of *AmphiCTL1* mRNA expression to unchallenged naive animals. In each experiment, values were normalized to the cytoplasmic actin expression, and mean ± SD is plotted.

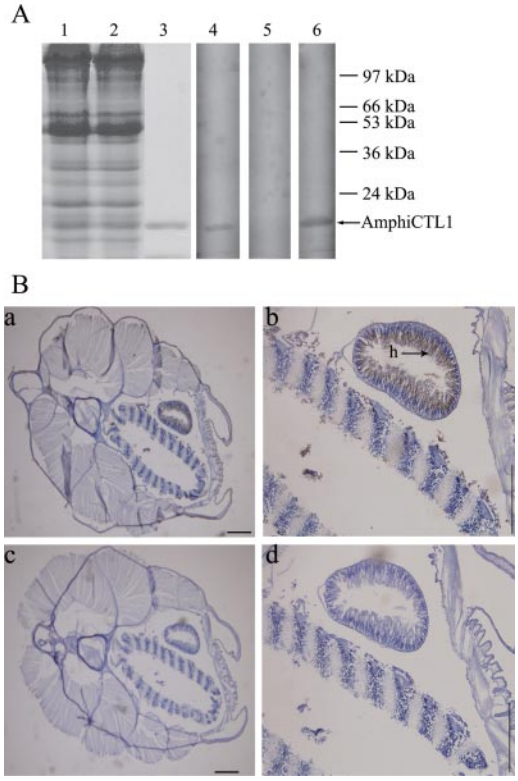

FIGURE 3. Immunohistochemical staining of AmphiCTL1 in amphioxus. *A*, Detection of AmphiCTL1 proteins in the total amphioxus extracts with affinity purified serum. Total proteins were extracted from amphioxus with the lysed buffer (10 mM Tris · Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA) and subjected to the 12% SDS-PAGE and staining with Coomassie brilliant blue (*lanes 1* and *2*), and detected by anti-AmphiCTL1 Ab (*lane 4*) or preimmune rabbit Ab (*lane 5*) by Western blot. Purified AmphiCTL1 recombinant protein was also subjected to the 12% SDS-PAGE and staining with Coomassie brilliant blue (*lane 3*) and detected by anti-AmphiCTL1 Ab (*lane 6*) as positive control. *B*, Serial sections of amphioxus were stained with affinity purified Ab. A polyclonal Ab raised against recombinant AmphiCTL1 was able to recognize AmphiCTL1 in the hepatic delivercilum (h) of amphioxus (*a* and *b*). Preimmune rabbit Ab (*c* and *d*) could not stain those tissues. The bar: 200 μm.

To determine whether these lectins were differentially regulated upon infection, Q-PCR analysis was also used to examine the relative expression of these lectins in amphioxus after challenged with different microbes and their cell wall components 24 h later (Fig. 2*C*). The results revealed 5~20-fold increased expression of AmphiCTL2 and AmphiCTL3 in all stimulated amphioxus, while the increased expression of AmphiCTL1 was specific for the stimulation of zymosan, *S. cerevisiae* and *S. aureus*, and up to 70~400-fold compared with the injection with PBS. Additional experiments of the infection time-course analysis revealed 130.2-fold induction of AmphiCTL1 transcript after injection of *S. aureus* 4 h compared with unchallenged naive amphioxus; the transcript increased up to 24 h postinjection (437.1-fold at most) then decreased to 129.5-fold at 48 h, while the AmphiCTL1 transcript had little change after challenge with *V. parahaemolyticus* compared with naive animals.

Localization of AmphiCTL1 protein by immunohistochemistry

The specific expression patterns of AmphiCTL1 in the tissues and immune response to stimulations prompted us to further study it. To identify the biological properties of AmphiCTL1, we used the pET21b system to express it, but recombinant protein was insoluble. The inclusion bodies washed with the urea and separated by SDS-PAGE were injected into the rabbit to prepare for the immune serum. The specificity of anti-AmphiCTL1 polyclonal Ab purified with protein G agarose was examined by Western blot of amphioxus total protein. A single band, which corresponded well with the deduced mature AmphiCTL1 protein, was detected by the anti-AmphiCTL1 Ab, but not by the preimmune Ab (Fig. 3*A*), indicating that the anti-AmphiCTL1 Ab specifically recognized the native protein in amphioxus. The localization of AmphiCTL1 in amphioxus was then examined by immunohistochemistry using this Ab (Fig. 3*B*). The anti-AmphiCTL1 Ab specifically stained in the inner folding tissues of the hepatic diverticulum, consistent with the tissue expression patterns analyzed by Q-PCR. No specific staining was observed with the preimmune serum.

Table I. *HA of AmphiCTL1 on erythrocytes*

Samples	Minimal Agglutinating Concentration (nM)
TRX plus $CaCl_2$	ND[a]
PHA	2.5
TRX-AmphiCTL1 plus $CaCl_2$	20
TRX-AmphiCTL1 plus $CaCl_2$ plus EDTA	ND[b]

[a] ND, Not detected.
[b] Not detected at 1 mM.

Determination of sugar-binding specificity by HA

To obtain the soluble protein, TRX fusion system was used to express soluble fusion AmphiCTL1 protein, which can be purified with a Ni^{2+}-chelating Sepharose column. The HA was tested with AmphiCTL1 fusion protein on rabbit erythrocytes. AmphiCTL1 proteins at 20 nM with 10 mM $CaCl_2$ could induce the hemagglutination of the erythrocytes, while the addition of EDTA inhibited the HA (Table I), indicating that the lectin activity of AmphiCTL1 was in a Ca^{2+}-dependent manner. The competition inhibition of HA was seen in the presence of at least 125 mM lactose, 200 mM maltose, sucrose, galactose, mannose, and GlcNAc, 250 mM glucose (Table II). Up to 250 mM fructose and 1 mg/ml LPS, LTA, and mannan did not inhibit the HA induced by AmphiCTL1.

Binding and aggregating of AmphiCTL1 to microbes

The above-mentioned experiments demonstrated that AmphiCTL1 was mainly synthesized in the hepatic diverticulum, which had long been considered as the precursor of vertebrate liver (22), and the high expression of AmphiCTL1 in this tissue was induced by

Table II. *Effects of saccharides on HA of AmphiCTL1*

Saccharides	Minimal Inhibitory Concentration
Maltose	200 mM
D-glucose	250 mM
Lactose	125 mM
Galactose	250 mM
Sucrose	200 mM
D-mannose	200 mM
Fructose	>250 mM
N-acetyl-D-glucosamine	200 mM
LPS from *E. coli* 0111:B4	>1 mg/ml
LTA from *S. aureus*	>1 mg/ml
Mannan from *S. cerevisiae*	>1 mg/ml

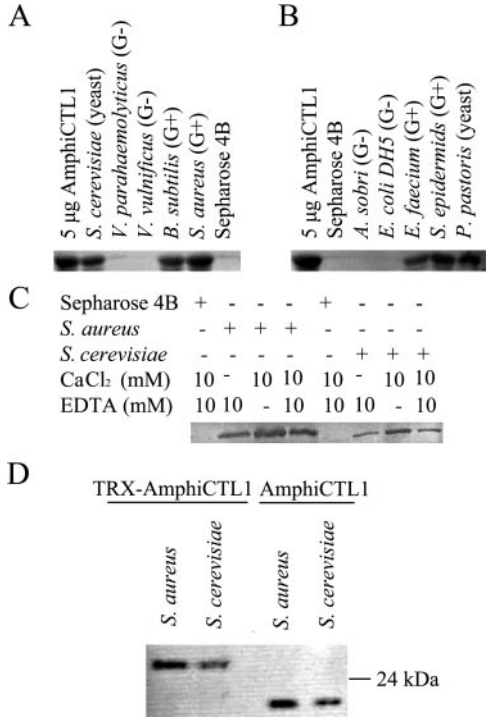

FIGURE 4. Binding of the microorganisms by AmphiCTL1 protein. *A*, Living microbial strains were incubated with TRX-AmphiCTL1 fusion protein in the presence of 10 mM $CaCl_2$ and the stirringly washed pellets were subjected to the SDS-PAGE and detected by Western blot with anti-AmphiCTL1 Ab. *B*, TRX-AmphiCTL1 could also bind with other Gram-positive bacteria and yeast. *C*, The effect of calcium on the binding activity of TRX-AmphiCTL1 with *S. aureus* and *S. cerevisiae*. *D*, The AmphiCTL1 protein without an extra part could also bind with *S. aureus* and *S. cerevisiae*.

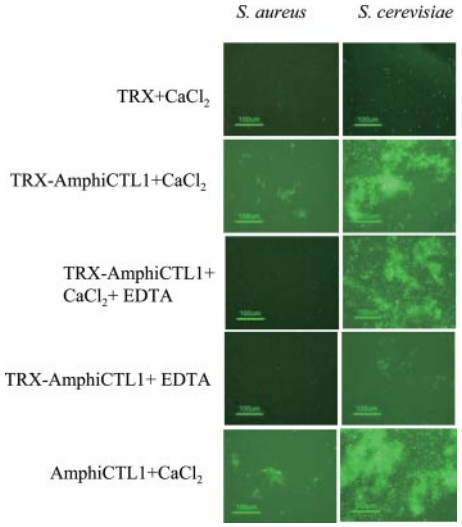

FIGURE 5. Aggregation of the microbes by AmphiCTL1. The targeted proteins were incubated with the FITC-labeled *S. aureus* (1×10^7 CFU/ml) or *S. cerevisiae* (1×10^6 CFU/ml) in the absence or presence of 10 mM Ca^{2+} and mixed 1 h at room temperature and examined under fluorescence microscopy.

the specific stimulation, indicating that AmphiCTL1 played an important role in defending against the infection in this region. Other members of C-type lectin, such as mannose-binding lectin and Reg|||γ, bound to microbial surface carbohydrates and triggered innate immune responses based on previous studies (8, 23). Therefore, we hypothesized that AmphiCTL1 might similarly bind to those microbes. First, we incubated those microbes with the targeted protein, and the microbial pellets were assessed by Western blot using anti-AmphiCTL1 Ab. As shown in Fig. 4*A*, Amphi CTL1 was found to bind to Gram-positive bacteria and yeast, but not to the Gram-negative bacteria, and up to 80% protein were bound to the microbes estimated by band intensity compared with the equal amount of unincubated AmphiCTL1. To test that its microbial killing activity was broad-spectrum to other Gram-positive bacteria and yeast, we incubated AmphiCTL1 with other microbes and obtained the same result (Fig. 4*B*). In the absence of calcium, however, AmphiCTL1 could still bind to the *S. aureus* and *S. cerevisiae*, indicating that calcium was not essential to the microbial-binding activity of AmphiCTL1. In the presence of calcium, the band intensity was thicker than that in the absence of it, suggesting that calcium could increase the intensity of interaction between AmphiCTL1 and microbes (Fig. 4*C*). The above experiment was processed with the recombinant fusion protein, which was composed of a TRX-His-tag part plus the normal AmphiCTL1 protein. To determine whether the extra part could affect the microbial-binding activity of AmphiCTL1, we obtained the soluble nonfusion AmphiCTL1 protein by denaturing-renaturing the inclusion body expressed by pET21b and incubated them with *S. aureus* and *S. cerevisiae*. In the absence of calcium, the nonfusion AmphiCTL1 could also bind with these microorganisms, suggesting that the extra part had no effect on interaction between AmphiCTL1 and microbes (Fig. 4*D*).

To determine whether the binding activity of AmphiCTL1 could induce the aggregation of those microbial pathogens, we incubated FITC-labeled *S. aureus* and *S. cerevisiae* with TRX-AmphiCTL1 fusion protein and assessed microorganism aggregation by fluorescence microscopy. The addition of AmphiCTL1 caused a strong aggregation of *S. cerevisiae*, and a weak aggregation of *S. aureus*. The addition of 10 mM EDTA inhibited the *S. aureus* aggregation induced by AmphiCTL1, but did not completely inhibit the aggregation of *S. cerevisiae* (Fig. 5). However, the addition of 10 mM EDTA inhibited absolutely the aggregation of *S. cerevisiae* in calcium free in the buffer solution. We also tested the effect of the TRX-His-tag part on the aggregation activity, and found that AmphiCTL1 itself could induce the aggregation of *S. aureus* and *S. cerevisiae* in the presence of calcium. These data indicated that the TRX-His-tag part had no extra effect on Amphi CTL1 function. Because we used the denaturing/renaturing method to obtain the nonfusion AmphiCTL1 protein from the inclusion body, AmphiCTL1 may not fold precisely to the natural form which may cause subtle functional change for the recombinant protein. Thus, we chose the fusion protein for conducting following the functional studies.

Binding of AmphiCTL1 to the microbial components

AmphiCTL1 binding directly to microorganisms may be dependent on recognizing the components of the microbial cell wall, especially the carbohydrate groups on these molecules. Then, we performed a plate ELISA to test the interaction between AmphiCTL1 and those microorganism components. Microtiter plates were coated with the microbial cell wall components and incubated with AmphiCTL1 followed by detecting with anti-AmphiCTL1 Ab. Notably, AmphiCTL1 protein interacted with soluble PGN from *S. aureus* and *B. subtilis*, glucan from *S. cerevisiae*, but not with LPS, LTA, and mannan (Fig. 6*A*).

The binding activities of AmphiCTL1 to the insoluble PGN and glucan were also tested by the co-pull down assay. PGN and glucan were incubated with AmphiCTL1 and the unbounded protein

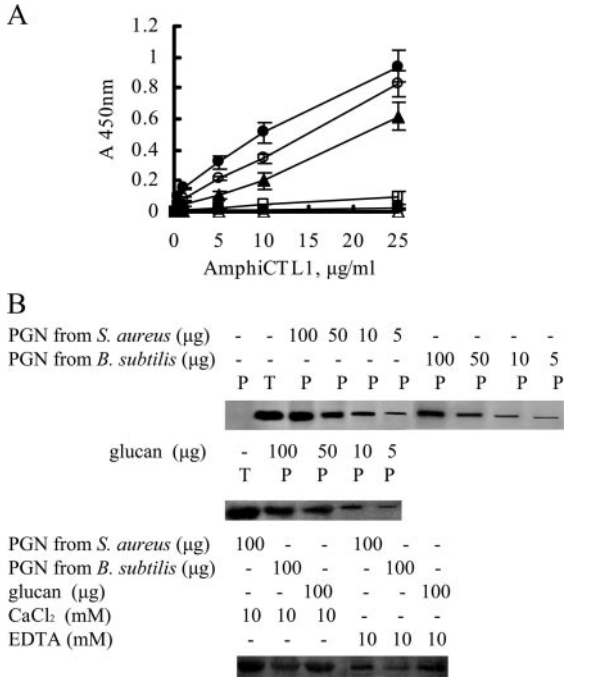

FIGURE 6. AmphiCTL1 directly interacted with the components of the microorganism cell walls. *A*, ELISA analysis of the interaction between recombinant fusion TRX-AmphiCTL1 and the components. Plates were coated with 20-μg components, which were washed and incubated with AmphiCTL1 (concentrations, horizontal) at 4°C overnight and detected with anti-AmphiCTL1 Ab. Results are representative of average six such experiments. Background absorbance without protein was subtracted. ■, LPS from *E. coli* 0111:B4; □, LTA from *S. aureus*; ▲, glucan from *S. cerevisiae*; △, mannan from *S. cerevisiae*; ●, PGN from *S. aureus*; ○, PGN from *B. subtilis*. Soluble glucan and PGN were generated by sonication of insoluble components. *B*, Co-pull down assay analysis of TRX-AmphiCTL1 binding with the PGN and glucan. The interaction between AmphiCTL1 and the PGN/glucan was concentration dependent and calcium was not essential for its binding activity. T, total protein; P, pellet protein.

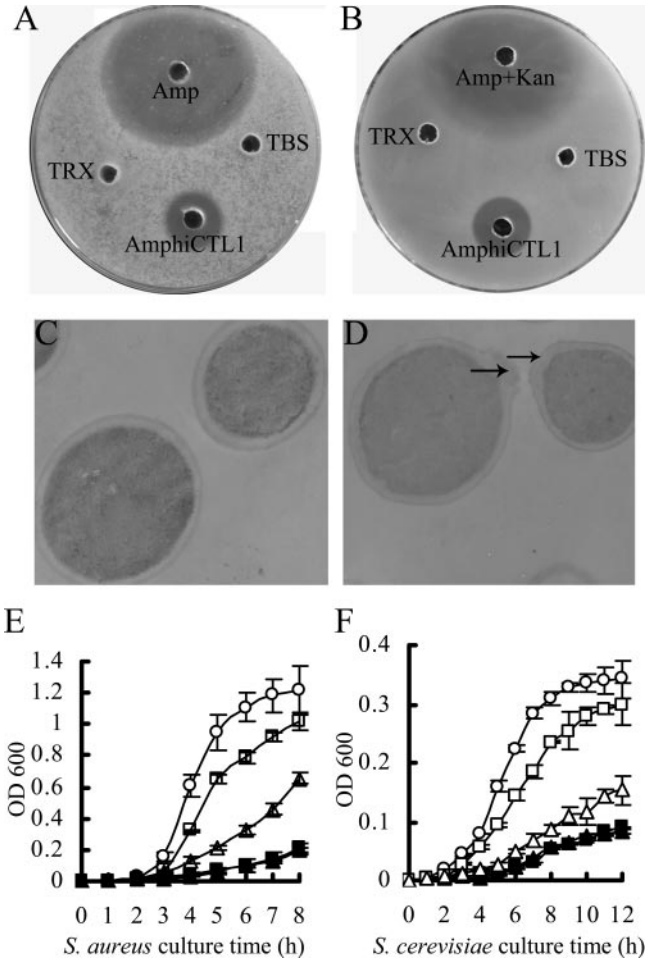

FIGURE 7. AmphiCTL1 possessed microbial killing activity. *A* and *B*, Antibacterial activity against *S. aureus* (*A*) and *S. cerevisiae* (*B*) were performed on petri dish. The finial concentration of 1 μg/μl TRX-Amphi CTL1 fusion protein, TRX, ampicillin (Amp), or kanamycin (Kan) in 100 μl of TBS was introduced to pores of the agar plates mixed with microbes. Then, the plates were incubated at 37°C for 16 h (*S. aureus*) or 30°C for 40 h (*S. cerevisiae*). A transparent ring around the pores signifies antibacterial activity. *C* and *D*, Transmission electron microscopy of *S. aureus* after incubation with TRX (*C*) or TRX-AmphiCTL1 (*D*) 2 h later. Arrows indicate the damaged cell surface and the cytoplasmic leakage. Magnification: 140,000. *E* and *F*, Growth suppressive tests of TRX-AmphiCTL1 against *S. aureus* (*E*) and *S. cerevisiae* (*F*). *S. aureus* or *S. cerevisiae* mixed with targeted proteins and OD_{600} were measured every 1 h after starting the cultures. In inhibition assay, soluble PGN from *S. aureus* and soluble glucan from *S. cerevisiae* were preincubated with its corresponding microbes. The data are the average ± SD of three independent cultures. ○, TRX; ■, 200 μg/ml AmphiCTL1 + 10 mM $CaCl_2$; ▲, 200 μg/ml AmphiCTL1; △, 20 μg/ml AmphiCTL1 + 10 mM $CaCl_2$; □, 200 μg/ml AmphiCTL1 + 10 mM $CaCl_2$ + PGN/glucan.

was separated from pellets by centrifugation. From our results, AmphiCTL1 directly interacted with the PGN from *S. aureus* and *B. subtilis*, glucan from *S. cerevisiae*. The interacting intensity of the PGN/glucan with AmphiCTL1 was dependent on the dose of the saccharides. AmphiCTL1 had a higher affinity with the PGN of *S. aureus* than those of *B. subtilis*, because the relative thick bands were detected in those lanes in which AmphiCTL1 was incubated with PGN of *S. aureus*. In the absence of calcium, AmphiCTL1 still interacted with the PGN/glucan, though the interaction intensity between PGN/glucan and AmphiCTL1 was reduced compared with that in the presence of calcium (Fig. 6*B*). These results demonstrated that AmphiCTL1 recognized microorganisms and directly interacted with the cell wall components on those microorganisms.

Antibacterial activity of AmphiCTL1 against S. aureus *and* S. cerevisiae

To determine the antimicrobial activity of AmphiCTL1, its inhibition effect on the growth of microbes was examined. Compared with the TBS and TRX, a transparent ring was found to be around the pores with AmphiCTL1 like the pores with ampicillin or kanamycin in the plates of *S. aureus* and *S. cerevisiae* (Fig. 7, *A* and *B*). We used transmission electron microscopy to visualize morphological changes in *S. aureus* cell after exposure to AmphiCTL1 (Fig. 7, *C* and *D*). The images revealed evidence of cell damage and cytoplasmic leakage remarkably similar to those obtained with the mouse Reg|||γ and human HIP/PAP (8). These indicated that AmphiCTL1 directly killed the microorganisms by cell wall permeabilization. We also tested the effect of AmphiCTL1 concentration on the antimicrobial activity. The antimicrobial activity of AmphiCTL1 was dose-dependent with 200 μg/ml AmphiCTL1 strongly suppressing the microbial growth, but 20 μg/ml AmphiCTL1 suppressing it weakly. To test the effect of polysaccharides on the antimicrobial activity of AmphiCTL1, PGN or glucan was preincubated with the protein. AmphiCTL1 antimicrobial activity against *S. aureus* was inhibited with PGN from *S. aureus*, and

antimicrobial activity against *S. cerevisiae* was also inhibited with glucan from *S. cerevisiae*, indicating that lectin binding to the polysaccharide carbohydrate preceded killing microbes. We also tested the calcium-dependent property on its antimicrobial activity, and found that the calcium had no significant effect on the antibacterial activity of AmphiCTL1 (Fig. 7, *E* and *F*).

Discussion

In invertebrates such as amphioxus, the innate immune system is particularly important, because they lack the vertebrate adaptive immune system. The recognition of infectious pathogens is an important process for animals to mount an immune response. C-type lectins are a main kind of pattern recognition molecule in the innate immune system (3). They encompass a large family of proteins found almost exclusively in Metazoa. More than 100~200 human or *C. elegans* proteins containing CTLD have been identified (15, 16). But an enormous expansion of CTLD-containing proteins is found in the amphioxus genome with >1000 gene models encoding the CTLD domain (S. Huang, S. Yuan, Y. Yu, Y. Guo, and A. Xu, unpublished observation). It is generally hypothesized that a relatively small number of these C-type lectins function as opsonin or activate the complement pathway in the innate immune system (3, 6). Consequently, the many-fold amplification of this gene family in the amphioxus genome suggests that it is likely to function as a part of a fundamentally different immune mechanism. Moreover, studies on the C-type lectins from cephalochordate amphioxus, which occupy a crucial position in the animal evolution tree, may find the important functional link between the vertebrate and invertebrate C-type lectin families, especially in the innate immune system. In this study, three C-type lectins consisting of a C-terminal CRD and an N-terminal secreted signal were identified from Chinese amphioxus. This is the first report of C-type lectins in protochordate to date. These amphioxus C-type lectins have distinct express patterns in special tissues and responding to stimulations, and AmphiCTL1 may be an evolutionarily primitive form of lectin-mediated immunity through direct microbial killing activity. This study may help us understand the defense mechanisms to the pathogens in the invertebrate such as amphioxus and the immune evolution of the C-type lectin families from invertebrates to vertebrates.

C-type lectins are divided further into 17 subgroups in vertebrates and 7 subgroups in invertebrates (6, 15). Both group VII C-type lectins consist of only one CRD and a short N-terminal secreted signal. Approximately 500 C-type lectin gene models possessing the same structure as the known group VII C-type lectins are found in the amphioxus genome, most of them cluster into a group outside the vertebrate clade (S. Huang, S. Yuan, Y. Yu, Y. Guo, and A. Xu, our unpublished data), indicating that the group VII C-type lectins may have specific function in amphioxus. In this study, three of them were cloned from the Chinese amphioxus. Although all CRDs have sequence similarity, including conserved hydrophobic residues and four invariant cysteine residues, they can be divided into two types: a "short form" ~115 residues long with four invariant cysteine residues and a "long form" ~130 residues long, which includes two additional disulfide-bonded cysteine residues at the N terminus (24, 25). AmphiCTL1 and AmphiCTL3 were short form because they had a CRD with four cysteine residues, while AmphiCTL2 was regarded as long form because it had a CRD with two additional cysteines. In our experiments, we found that the transcripts of these amphioxus C-type lectins had a distinct temporal- and spatial-specific expression pattern, and were elevated in amphioxus stimulated by different microorganisms and corresponding cell wall components (Fig. 2). These results indicated that they were related to the defense mechanisms. However, these C-type lectins consist of secreted CRDs that lack collagenous domains required for complement recruitment, so they might function as fundamentally different immune mechanisms such as possessing the direct antibacterial activity the same as the tunicate *Polyandrocarpa* lectin (13). This hypothesis was proven by the biological properties of recombinant AmphiCTL1 protein.

Calcium was required for carbohydrate binding in mammalian C-type lectins, such as mannose-binding protein, which was a direct ligand for sugars (21). In this study, we found that calcium was essential to the hemagglutinating and microbial aggregation activities of AmphiCTL1, but not to the microbial binding and growth suppression activities of this lectin. The microbial aggregation activity of AmphiCTL1 was also in Ca^{2+}-dependent manner, though 10 mM EDTA did not completely inhibit the aggregation against *S. cerevisiae*. This phenomenon could be explained by the fact that *S. cerevisiae* is a eukaryote that can release calcium flux from its cells when the cell wall is damaged, which is consistent with the microbial killing activity of AmphiCTL1. This presumption was confirmed because the aggregation against *S. cerevisiae* was completely inhibited when the buffer solutions were absolutely absent of calcium (Fig. 5). Although the C-type lectin family includes members that bind their ligands in a calcium-dependent manner, many other C-type lectins show the same Ca^{2+}-dependent or -independent activity as AmphiCTL1. A C-type lectin of Conger eel, conCL-s, with binding to sugar in a Ca^{2+}-dependent manner, showed Ca^{2+}-independent activity in its yeast-binding (26). Immulectin-2, the insect immulectin family with sequence similarities to C-type lectins, did not require calcium for its binding activity (27). Mouse Reg|||γ and its human counterpart HIP/PAP did not require calcium for binding peptidoglycan and chitin (8). It seems that C-type lectins may bind to ligands using an alternative mechanism different from the classical one used by mammalian C-type lectins. From our experimental results, we postulate that calcium is not a direct ligand or essential factor for binding, but it may affect the formation of dimers or oligomers, which are required for agglutinating activity.

Most C-type lectins are able to bind PAMPs and microorganisms themselves through recognizing carbohydrate, so as to directly be involved in innate defense mechanisms as part of the acute-phase response to infection. The expression of Amphi CTL1 was quickly up-regulated after challenged with zymosan, *S. cerevisiae*, and *S. aureus*, indicating that AmphiCTL1 was also an inducible acute-phase protein to play a crucial role in host defense, which was consistent with its binding and aggregating activity to the invading pathogens. AmphiCTL1 could bind with all examined Gram-positive bacteria and yeast, but not with the Gram-negative bacteria studied. The microbial-binding activity of AmphiCTL1 was exerted by the interaction with PGN and glucan, but not with LPS, LTA, and mannan, which are the PAMPs existing in the microbial cell wall. The component binding results were consistent with transcript levels of AmphiCTL1 in amphioxus after challenge with cell wall components. These results suggested that AmphiCTL1 was a pattern recognition protein that recognized the PAMPs by the extended glycan chains of PGN and glucan. Though Amphi CTL1 did not bind to the examined Gram-negative bacteria, it interacted with the PGN from *B. subtilis*, which has the same

structure as the PGN of Gram-negative bacteria. This phenomenon could be explained by three differences between Gram-positive and -negative bacterial PGN. First, the affinity of AmphiCTL1 to Gram-negative PGN (*B. subtilis*) was lower than the Gram-positive PGN (*S. aureus*) (Fig. 6*B*). Second, PGN existing in the Gram-negative bacteria is only 10~20% of cell wall components, compared with up to 50~80% in Gram-positive bacteria. Third, the PGN of Gram-negative bacteria is buried in periplasmic space, while that of the Gram-positive bacteria is exposed on the surface. The capacity of C-type lectins to sense microorganisms is highly dependent on the density of the PAMP present on the microbial surface (28). Therefore, AmphiCTL1 cannot interact and respond to the equal dose of Gram-negative bacteria compared with Gram-positive bacteria.

An important part of innate immunity is that a group of protein has antimicrobial activity in addition to immune recognition. These proteins all play essential roles in nonspecific host defenses by preventing or limiting infections by their ability to selectively recognize potential pathogens. Most proteins exert their antifungal or antibacterial effects by interacting with and destabilizing the microbial membrane, leading to cell death. Except the traditional antimicrobial protein, such as defensin (29) and lysozyme (30), several C-type lectins have been reported to have antibacterial activity. In invertebrates, the C-type lectin purified from tunicate *Polyandrocarpa misakiensis* displayed a strong antibacterial activity even at the concentration of 1 μg/ml (13). The recombinant protein of scallop CFLec-1 displayed a remarkable inhibiting effect on Gram-positive bacteria *Micrococcus lutens* and relatively weak lytic activity against Gram-negative bacteria *E. coli* JM109 (14). In vertebrates, the conglutinin from rat demonstrated antibacterial activity against *E. coli* and *Salmonella typhimurium* in vitro (31). Mouse Reg|||γ and its human counterpart HIP/PAP have direct bactericidal activity on Gram-positive bacteria (8). In these reports, only Reg|||γ and HIP/PAP were demonstrated to kill the bacteria by cell wall permeabilization. In our study, we found that AmphiCTL1 could directly kill the microorganisms such as *S. aureus* and lead its cell wall damage the same as Reg|||γ and HIP/PAP, and had wide-range microorganism killing activity. AmphiCTL1 had the same effect on the yeast as *S. cerevisiae* by interaction with glucan, while either Reg|||γ or HIP/PAP reduced the viability of fungal microorganisms. These indicated that the primitive C-type lectin in amphioxus possessed the more wide-range microorganism recognition than its vertebrate homologs. It is an inducible microbial killing protein that seeks out its microbial targets via interaction with the bacterial PGN or yeast glucan and represents a new function for invertebrate lectin-mediated immunity.

Although AmphiCTL1 has the ability to kill the Gram-positive bacteria and fungi, it is plausible that those C-type lectins with the same structure as AmphiCTL1 may also interact with the microbes in a similar way. AmphiCTL1, which is abundant in the hepatic diverticulum, may have suppressing effect on the restricted Gram-positive bacterial and yeast strains in the fluid flow, where the resident microflora consists mostly of Gram-positive bacteria in amphioxus (Y. Yu, M. Pen, and A. Xu, unpublished data). The other amphioxus C-type lectins, AmphiCTL2 and AmphiCTL3, consisted of the same structure as AmphiCTL1, but had different expression patterns analyzed by Q-PCR. Though the Q-PCR may have possible pitfalls for measuring gene expression, this technique can still tell us the relative expression of this targeted gene. From the Q-PCR, we found that AmphiCTL2 possessed the highest levels in the intestine and was up-regulated by the all stimulations, so it was deduced that AmphiCTL2 might mainly defend against those pathogens invading the intestine. AmphiCTL3 might be a skin mucosal lectin because its transcript was abundant in the skin and it has high expression in the larva stage of amphioxus. At 16 h stage, the ovum theca of amphioxus embryo functioning as the first defense line was shucked off and the embryo was exposed to the culture environment. Up-regulation of AmphiCTL3 in this stage was consistent with the highest transcript level in the skin. AmphiCTL3 might defend against those microorganisms exposed to the body surface, so AmphiCTL3 was up-regulated by all the stimulations. Thus, the C-type lectin family in amphioxus may recognize and defend many strains of pathogens collectively.

Taken together, the results from this work indicate that the primitive form of lectin-mediated immunity also exists in amphioxus, as determined here by AmphiCTL1 as an example. This finding contributes not only to understanding the complexity of immunity in amphioxus in general, but also to the interesting facts on the evolution of the lectin-mediated immune system in particular. Our studies on AmphiCTL1 in amphioxus indicates that these animals can defend themselves against microbial invaders by a secreted microbial killing C-type lectin. Furthermore, we find that C-type lectins with a similar structure to AmphiCTL1 are abundant in the amphioxus genome, which may represent the primitive immune recognition strategy and be a main immune defense mechanism against different microorganisms in different tissues. They may constitute a defense network against almost all possible invading microorganisms. In view of the fact that the bactericidal protein Reg|||γ and HIP/PAP exist in high vertebrate, it may be predicted that such a diversity of C-type lectins also occurs in other vertebrate and invertebrate species. In short, this finding sheds new light on the lectin-mediated immune system, particularly its functional evolution from invertebrates to vertebrates.

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Disclosures

The authors have no financial conflict of interest.

References

1. McGreal, E. P., L. Martinez-Pomares, and S. Gordon. 2004. Divergent roles for C-type lectins expressed by cells of the innate immune system. *Mol. Immunol.* 41: 1109–1121.
2. Holmskov, U., S. Thiel, and J. C. Jensenius. 2003. Collections and ficolins: humoral lectins of the innate immune defense. *Annu. Rev. Immunol.* 21: 547–587.
3. Vasta, G. R., H. Ahmed, and E. W. Odom. 2004. Structural and functional diversity of lectin repertoires in invertebrates, protochordates and ectothermic vertebrates. *Curr. Opin. Struct. Biol.* 14: 617–630.
4. Fujita, T., M. Matsushita, and Y. Endo. 2004. The lectin-complement pathway–its role in innate immunity and evolution. *Immunol. Rev.* 198: 185–202.
5. Drickamer, K. 1999. C-type lectin-like domains. *Curr. Opin. Struct. Biol.* 9: 585–590.
6. Zelensky, A. N., and J. E. Gready. 2005. The C-type lectin-like domain superfamily. *FEBS J.* 272: 6179–6217.
7. Geijtenbeek, T. B., S. J. van Vliet, A. Engering, B. A. 't Hart, and Y. van Kooyk. 2004. Self and non-self recognition by C-type lectins on dendritic cells. *Annu. Rev. Immunol.* 22: 33–54.
8. Cash, H. L., C. V. Whitham, C. L. Behrendt, and L. V. Hooper. 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313: 1126–1130.
9. Luo, T., H. Yang, F. Li, X. Zhang, and X. Xu. 2006. Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*. *Dev. Comp. Immunol.* 30: 607–617.
10. Yu, X. Q., H. Gan, and M. R. Kanost. 1999. Immulectin, an inducible C-type lectin from an insect. *Manduca sexta*, stimulates activation of plasma prophenol oxidase. *Insect Biochem. Mol. Biol.* 29: 585–597.
11. Yu, X. Q., and M. R. Kanost. 2000. Immulectin-2, a lipopolysaccharide-specific lectin from an insect. *Manduca sexta*, is induced in response to Gram-negative bacteria. *J. Biol. Chem.* 275: 37373–37381.

12. Koizumi, N., M. Imamura, T. Kadotani, K. Yaoi, H. Iwahana, and R. Sato. 1999. The lipopolysaccharide-binding protein participating in hemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with two different tandem carbohydrate-recognition domains. *FEBS Lett.* 443: 139–143.
13. Suzuki, T., T. Takagi, T. Furukohri, K. Kawamura, and M. Nakauchi. 1990. A calcium-dependent galactose-binding lectin from the tunicate *Polyandrocarpa misakiensis*: isolation, characterization, and amino acid sequence. *J. Biol. Chem.* 265: 1274–1281.
14. Wang, H., L. Song, C. Li, J. Zhao, H. Zhang, D. Ni, and W. Xu. 2007. Cloning and characterization of a novel C-type lectin from Zhikong scallop *Chlamys farreri. Mol. Immunol.* 44: 722–731.
15. Drickamer, K., and R. B. Dodd. 1999. C-type lectin-like domains in *Caenorhabditis elegans*: predictions from the complete genome sequence. *Glycobiology* 9: 1357–1369.
16. Drickamer, K., and A. J. Fadden. 2002. Genomic analysis of C-type lectins. *Biochem. Soc. Symp.* 69: 59–72.
17. Yuan, S., Y. Yu, S. Huang, T. Liu, T. Wu, M. Dong, S. Chen, Y. Yu, and A. Xu. 2007. Bbt-TNFR1 and Bbt-TNFR2, two tumor necrosis factor receptors from Chinese amphioxus involve in host defense. *Mol. Immunol.* 44: 756–762.
18. Yu, X. Q., M. E. Tracy, E. Ling, F. R. Scholz, and T. Trenczek. 2005. A novel C-type immulectin-3 from *Manduca sexta* is translocated from the hemolymph into the cytoplasm of hemocytes. *Insect Biochem. Mol. Biol.* 35: 285–295.
19. Braunagel, S. C., D. M. Elton, H. Ma, and M. D. Summers. 1996. Identification and analysis of an *Autographa californica* nuclear polyhedrosis virus structural protein of the occlusion-derived virus envelope: ODV-E56. *Virology* 217: 97–110.
20. Nowak, T. P., P. L. Haywood, and S. H. Barondes. 1976. Development regulated lectin in embryonic chick muscle and a myogenic cell line. *Biochem. Biophys. Res. Commun.* 68: 650–657.
21. Weis, W. I., K. Drickamer, and W. A. Hendrickson. 1992. Structure of a C-type mannose-binding protein complexed with an oligosaccharide. *Nature* 360: 127–134.
22. Müller, J. 1844. Ueber den Bau und die Lebenserscheinungen des *Branchiostoma lubricum Costa*, *Amphioxus lanceolatus*. *Yarrell. Abh. K. Preuss Akad. Wiss. Berl.* 1844: 79–116.
23. Ezekowitz, R. A. 2003. Role of the mannose-binding lectin in innate immunity. *J. Infect. Dis.* 187(Suppl. 2): S335–S339.
24. Day, A. J. 1994. The C-type carbohydrate recognition domain (CRD) superfamily. *Biochem. Soc. Trans.* 22: 83–88.
25. Drickamer, K. 1995. Increasing diversity of animal lectin structures. *Curr. Opin. Struct. Biol.* 5: 612–616.
26. Tsutsui, S., K. Iwamoto, O. Nakamura, and T. Watanabe. 2007. Yeast-binding C-type lectin with opsonic activity from conger eel (*Conger myriaster*) skin mucus. *Mol. Immunol.* 44: 691–702.
27. Yu, X. Q., and Y. Ma. 2006. Calcium is not required for immulectin-2 binding, but protects the protein from proteinase digestion. *Insect Biochem. Mol. Biol.* 36: 505–516.
28. Cambi, A., M. Koopman, and C. G. Figdor. 2005. How C-type lectins detect pathogens. *Cell. Microbiol.* 7: 481–488.
29. Bulet, P., C. Hetru, J. L. Dimarcq, and D. Hoffmann. 1999. Antimicrobial peptides in insects; structure and function. *Dev. Comp. Immunol.* 23: 329–344.
30. Hancock, R. E., and M. G. Scott. 2000. The role of antimicrobial peptides in animal defenses. *Proc. Natl. Acad. Sci. USA* 97: 8856–8861.
31. Friis-Christiansen, P., S. Thiel, S. E. Svehag, R. Dessau, P. Svendsen, O. Andersen, S. B. Laursen, and J. C. Jensenius. 1990. In vivo and in vitro antibacterial activity of conglutinin, a mammalian plasma lectin. *Scand. J. Immunol.* 31: 453–460.