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A Novel C1q Family Member of Amphioxus Was Revealed to Have a Partial Function of Vertebrate C1q Molecule[1]

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C1q is the target recognition protein of the classical complement pathway and a major connecting link between innate and adaptive immunities. Its globular signature domain is also found in a variety of noncomplement protein that can be grouped together as a C1q family. In this study, we have cloned and identified a novel C1q family member in cephalochordate amphioxus and named it as AmphiC1q1. The high transcriptional levels of this gene were detected during all stages of embryonic development, and the section in situ hybridization demonstrated that AmphiC1q1 was mainly expressed in the ovary, intestine, and nerve system of mature individuals. The transcript of AmphiC1q1 was up-regulated by LPS and Gram-negative bacteria, but hardly by lipoteichoic acid and *Staphylococcus aureus*. The recombinant AmphiC1q1 protein could not bind with *N*-acetyl-glucosamine and did not possess hemagglutinating activity, indicating that AmphiC1q1 could not act as its lamprey homologue. But both the full-length protein and its truncated globular domain of C1q protein could interact with LPS. Moreover, recombinant AmphiC1q1 protein could inhibit collagen-induced platelet aggregation, but the truncated globular C1q domain protein would not, indicating that the blocking activity of AmphiC1q1 protein was via the collagen region of the protein. Our study on the primitive form of C1q family in protochordate will shed a light on understanding the gradual functional evolution of C1q family and eventual formation of mammalian homologues. *The Journal of Immunology,* **2008, 181: 7024–7032.**

The C1q domain was first identified in the A, B, and C chains of the C1q subcomponent of the complement C1 complex and named as globular domain of C1q (gC1q)[4] for its globular structure (1). In addition to being the key component of classical complement pathway, C1q is involved in several other immunological processes, including maintenance of immune tolerance via clearance of apoptotic cells, phagocytosis of bacteria, neutralization of retrovirus, cell adhesion, and modulation of dendritic cells, B cells, and fibroblasts. Its ability to carry out such diverse functions is aided by its capacity to engage a broad range of ligands, such as envelope proteins of certain retroviruses, β-amyloid peptide, LPS, porins from Gram-negative bacteria, phospholipids, apoptotic cells, and some acute-phase reactants via its gC1q domain and/or collagen-like region (CLR) (2).

gC1q domain was also found in many noncomplement proteins that have similar crystal structure to that of the multifunctional TNF ligand family, and also have diverse functions (2). C1qTNF-related protein 1 (CTRP1) was a vascular wall protein that inhibited collagen-induced platelet aggregation by blocking von Willebrand factor binding to collagen (3). CaOC1q-like factor, a novel member of C1q family from color crucian carp *Carassius auratus*, was identified to be specifically expressed in the ovary (4). The other members of this family, such as adiponectin, G protein couples receptor-interacting protein, also play important roles in energy homeostasis and signal transduction (5–7).

However, agnathans lamprey C1q emerged as a lectin and functioned as an initial recognition molecule, which led the C1q be related with the lectin pathway and the innate immunity (8). This finding prompted us to investigate its homologue in a more primitive species. Cephalochordate amphioxus represent the most basal extant chordate lineage (9). Based on analyzing the genome of this species, we have found that there are 50 C1q domain-containing gene models in its genome (10), in contrast to 29 in human and 5 in sea urchin (11). In the present work, we have cloned and identified one member of C1q family, and named it as AmphiC1q1, from amphioxus, which occupies crucial positions in the animal evolution tree. Moreover, AmphiC1q1 is the most primitive form of this family reported to date. This study can help us understand the evolution of the C1q family and eventual formation of mammalian homologue.

Materials and Methods

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

Adults of Chinese amphioxus (*Branchiostoma belcheri tsingtauense*) were obtained from Qingdao, China. Animals were kept in large vessels with

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[4] Abbreviations used in this paper: gC1q, globular domain of C1q; CLR, collagen-like region; CRF, C1q-related factor; CTRP or cqt, C1q and TNF-related protein; GlcNAc, *N*-acetyl-glucosamine; ME, minimum-evolution; PRP, platelet-rich plasma; TRX, thioredoxin.

aeration and supplied with fresh seawater and plankton daily. In breeding seasons, blastula, gastrula, neurula, and larva were collected. Tissues were separated under optical microscope. Live *Vibrio vulnificus* (G^-), *Vibrio parahaemolyticus* (G^-), and *Staphylococcus aureus* (G^+) PBS suspension (15 μl/animal, 10^9 CFU/μl); LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich); and lipoteichoic acid from *S. aureus* (Sigma-Aldrich) PBS suspension (15 μl/animal, 1 mg/ml) were injected into the coelom of amphioxus. PBS was injected as the negative control. Twenty random samples of each treatment were collected after 48-h injection and frozen by liquid nitrogen as experimental samples.

Isolation of full-length AmphiC1q1 cDNA and genome

A partial cDNA fragment of AmphiC1q1 was isolated by degenerate oligonucleotide-based PCR cloning with primers 5′-GG(A/G)GAACG(C/G/A)GGGT T(C/T)CCT-3′ (forward) and 5′-AAC(T/C)AAATGICCATT(G/A)AAGGTG-3′ (reverse), and total RNA of adult animal was extracted as templates. To obtain a complete cDNA sequence, 3′ and 5′ RACE were performed using the GeneRACE Kit (Invitrogen), according to the manufacturer's instructions, with gene-specific primers (5′-GSP_1, 5′-GCTGCG AATG-ACAGAGAAGGCTGAT-3′; 5′-GSP_2, 5′-CTTGGGTCCAGGAG GTCCAGCAACA-3′; 3′-GSP_1, 5′-CGCAGCATCATGCAGTCCCAGA-3′; and 3′-GSP_2, 5′-GACCAGGTATGGATGAGGATGGACTA-3′). The amplified fragments were cloned into the pGEX-T easy vector (Promega) and sequenced by the PerkinElmer ABI Prism 3730 DNA sequencer. Primers 5′-TCAGCAGCAGTCCCACCAT-3′ (forward) and 5′GATTTGTAG TCACAACAGAGG-3′ (reverse) were used to amplify the full-length cDNA and genomic sequences. The PCR products were cloned into pGEM-T easy vector and sequenced.

Southern blot hybridization of genomic DNA

For each lane, 20 μg of digested genomic DNA from adult animals was fractionated by 0.8% agarose gel electrophoresis and transferred to Hybond N^+ nylon membrane (Amersham Biosciences). A 417-bp cDNA probe of gC1q domain was amplified by primers 5′-CCACCAGAGAAACTC CAGGC-3′ (forward) and 5′-CGTGGGGAAAACTAAATGTCCA-3′ (reverse), and then labeled with the digoxigenin DNA Labeling System Kit (Roche). After overnight hybridization and stringency washing, the nylon membranes were immunodetected according to the protocol.

Alignment and phylogenetic analysis

Clustalw 1.83 was used in multiple sequence alignments. Alignments were edited and refined with GeneDoc software. Phylogenetic trees were built with Mega v3.1. Minimum-evolution (ME) method was used to calculate trees with 1000 bootstrap tests. The draft genome sequence of amphioxus *Branchiostoma floridae* and the corresponding gene predictions, annotations, searching, and downloading services can be accessed on the website http://genome.jgi-psf.org/Brafl1/Brafl1.home.html. Other sequences used in the alignment and phylogenetic analyses were extracted from GenBank and Ensemble.

PCR analysis of the AmphiC1q1 gene expression patterns

RT-PCR and real-time PCR were performed as the method described (12). A total of 40 cycles was performed with the primers 5′-CCTGGCGGT CAGCATCTATGG-3′ and 5′-CCTGGTGGGTTGTCTTGTGGG-3′. All samples were analyzed in three duplications, and the results were expressed as relative fold of one sample in each experiment and as mean ± SD.

Section in situ hybridization

Adult amphioxus was cut into three to four pieces, and fixed in 4% paraformaldehyde in MOPS buffer at room temperature for 2 h or at 4°C overnight, dehydrated in gradated ethanol, and stored in 70% ethanol at –20°C for embedding in paraffin. Then the embedded tissue blocks were cut into 8 μm. Following deparaffinization, the sections were rehydrated, absterged, and proteinase K digested, respectively. Digoxigenin-labeled sense and antisense probes synthesized with full-length AmphiC1q1 cDNA were added respectively at a concentration of 1 μg/ml, and the section was hybridized overnight at 42°C. After high stringency washing, sections were immunodetected by NBT/5-bromo-4-chloro-3-indolyl phosphate stock solution (Roche).

Construction of expression vectors and preparation of recombinant proteins

Full-length AmphiC1q1 molecule without signal peptide and its gC1q domain protein were expressed as fusion proteins with the thioredoxin (TRX) fusion system containing a 6× His tag to facilitate the purification of fusion

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  1 TTC AGC AGC AGT CCC ACC ATG ACG TCC CTC CTG TAC ACT GTC CTG ATC ATA CCA GCC TGC   60
  1                         M  T  S  L  L  Y  T  V  L  I  I  P  A  C                 14
 61 CTG GCG GTC AGC ATC TAT GGC CAG GGG TCA TAT TCT GAC CAG GGG AAA CAT TCT GGC CGA  120
 15 L  A  V  S  I  Y  G  Q  G  S  Y  S  D  Q  G  K  H  S  G  R                    34
121 GGG AAA CAG TCT GAG GAG GCG CCT GAG AGT GGC CAG TGT GTG AGG TGC TGC CCA CAG GAC  180
 35 G  K  Q  S  E  E  A  P  E  S  G  Q  C  V  R  C  C  P  Q  D                    54
181 AAC CCA CCA GGG CCT CAG ATC ATC GCT CTG CCT CAA CAC ATG GCA TCA GAG AAG GGA GAT  240
 55 N  P  P  G  P  Q  I  I  A  L  P  Q  H  M  A  S  E  K  G  D                    74
241 AAA GGA GAA CGT GGG TTC CCT GGA AGA ACA GGG AAG ATG GGA CCA AAA GGA CAC AAG GGT  300
 75 K  G  E  R  G  F  P  G  R  T  G  K  M  G  P  K  G  H  K  G                    94
301 GTT GCT GGA CCT CCA GGA CCT GAG GGA GTA GCC GGC CCT CCT GGC CCA CCA GAG AAA CTC  360
 95 V  A  G  P  P  G  P  E  G  V  A  G  P  P  G  P  P  E  K  L                   114
361 CAG GCA TCA GCC TTC TCT GTC ATC CGC AGC AAA CCC ATG GAG GGG ACC AAG TAT TAC CAG  420
115 Q  A  S  A  F  S  V  I  R  S  K  P  M  E  G  T  K  Y  Y  Q                   134
421 GTG GTC ATG TAC GAC AAA GTG TTG GTT AAC ACT GGA GAA CAC TTC AAC TTG TTC ACA GGG  480
135 V  V  M  Y  D  K  V  L  V  N  T  G  E  H  F  N  L  F  T  G                   154
481 AAG TTC TAC TGC GTC ATC CCT GGC ATC TAC TAC TTC TCT GCC ACA GTC CAC AGC TAC AAC  540
155 K  F  Y  C  V  I  P  G  I  Y  Y  F  S  A  T  V  H  S  Y  N                   174
541 AGC CGG ACG ACC TAC CTA CAT CTG ATG AAG AAC AAC CAA CCA CAG GTC ATC CTG TTT GCC  600
175 S  R  T  T  Y  L  H  L  M  K  N  N  Q  P  Q  V  I  L  F  A                   194
601 CAG GAG GGG GAC CGC AGC ATC ATG CAG TCC CAG ACG GTC ATG TTG GAG CTG ATA GTG GGG  660
195 Q  E  G  D  R  S  I  M  Q  S  Q  T  V  M  L  E  L  I  V  G                   214
661 GAC CAG GTG TGG ATG AGG ATG GAC TAC GGA GAC CAT CTC GCC ATA TTT GGT GAT GAG GAA  720
215 D  Q  V  W  M  R  M  D  Y  G  D  H  L  A  I  F  G  D  E  E                   234
721 GAT GCC TAC ATC ACC TTC AGT GGA CAT TTA GTT TTC CCC ACG TAT AAC ATG TGA TTT ATC  780
235 D  A  Y  I  T  F  S  G  H  L  V  F  P  T  Y  N  M  *                         251
781 AGA CGT ATA TTG TAA GAA TGA ATA TCC TGA AAA TTT TTG ATA CTA AGA GTC CTG ATT TTT  840
841 TTT GTC CTT TAC ACA TAT GTC AGC CAC ATG GTA TAC CAG TAC CTC TGT TGT GAC TAC AAA  900
901 TCA                                                                            903
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FIGURE 1. Nucleotide and deduced amino acid sequences of AmphiC1q1 (accession FJ154078). The signal peptide was in italics, and the collagen region was marked with underlining.

proteins on a Ni^{2+} chelating Sepharose column and partner TRX to help the proteins fold correctly. Specific cDNA sequence encoding the mature protein encompassing amino acids Val^{17} to Met^{251} was amplified with the primers 5′-*GG*GGTACCG*ACGACGACGACAAA*GTCAGCATCTATGG CCAGGG-3′ (forward) and 5′-*ATAAGAAT*GCGGCCGCTCACATGTT ATACGTGGGGAAAA-3′ (reverse), whereas gC1q domain encompassing amino acids Pro^{110} to Thr^{248} was amplified with the primers 5′-*GG*GGT ACC*GACGACGACGACAAA*CCACCAGAGAAACTCCAGGC-3′ (forward) and 5′-*ATAAGAAT*GCGGCCGCTTATTACGTGGGGAAAA CTAAATGTCCA-3′ (reverse). *Kpn*I and *Not*I endonuclease sites (underlined) were included at the 5′ ends of the forward and reverse primer, and the enterokinase site (*italics*) was followed with the *Kpn*I endonuclease site, respectively. Two stop codons were added following the *Not*I endonuclease site in the reverse primer of the gC1q. The amplified fragments were inserted into pTRX between the two sites, and the expression plasmids were introduced into *E. coli* Rosetta-gami with additions of 50 mg/L ampicillin, 15 mg/L kanamycin, 35 mg/L chloramphenicol, and 12.5 mg/L tetracycline. The 2×YT with identical antibiotics was inoculated overnight with a transformed positive clone at 37°C. When the absorbance at 600 nm reached a value of 0.6, ispropylthiogalactoside was added to a final concentration of 0.2 mM and the cultures were allowed to rock for 36 h at 12°C. The bacteria were harvested by centrifugation at 5000 × g for 10 min. Cell pellets were resuspended and disrupted using a sonicator in TBS (pH 8.5 for AmphiC1q1-FL, and pH 7.5 for AmphiC1q1-gC1q) containing 150 mM NaCl. After addition of Triton X-100 to a final concentration of 1%, the suspensions were mixed gently for 30 min to solubilize the fusion protein. 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 their corresponding TBS with G-25 column concentrated by filtration through an ULTRAFREE centrifugal filter device (Millipore). The concentrated proteins were also analyzed by gel filtration on Sephacryl S300 (Amersham Biosciences). Protein concentration was determined using a Bio-Rad protein assay dye reagent and BSA as a standard. The TRX protein was purified with the same method.

Identification of the possible ligands for lectin of AmphiC1q1

Excess AmphiC1q1-FL was incubated overnight with 10 μl of *N*-acetylglucosamine (GlcNAc) Sepharose 4B at 4°C by gentle rocking in the presence or absence of 10 mM $CaCl_2$. The beads washed stringently with TBS (with or without 10 mM $CaCl_2$) were then suspended with 10 μl of reducing sample buffer quickly and denatured by heating at 100°C for 15 min. Then the supernatant was subjected to 15% SDS-PAGE and analyzed by immunoblotting. Briefly, proteins separated by SDS-PAGE were transferred to nitrocellulose membrane and detected with a mouse anti-His mAb and HRP-labeled anti-mouse IgG Ab.

Hemagglutinating activity of AmphiC1q1-FL was tested on trypsin-treated glutaraldehyde-fixed rabbit erythrocytes, as described (12). Rabbit erythrocytes (2%, v/v) were prepared in TBS. Hemagglutination activity was measured in 96-well microtiter plate with V-shaped well bottoms with

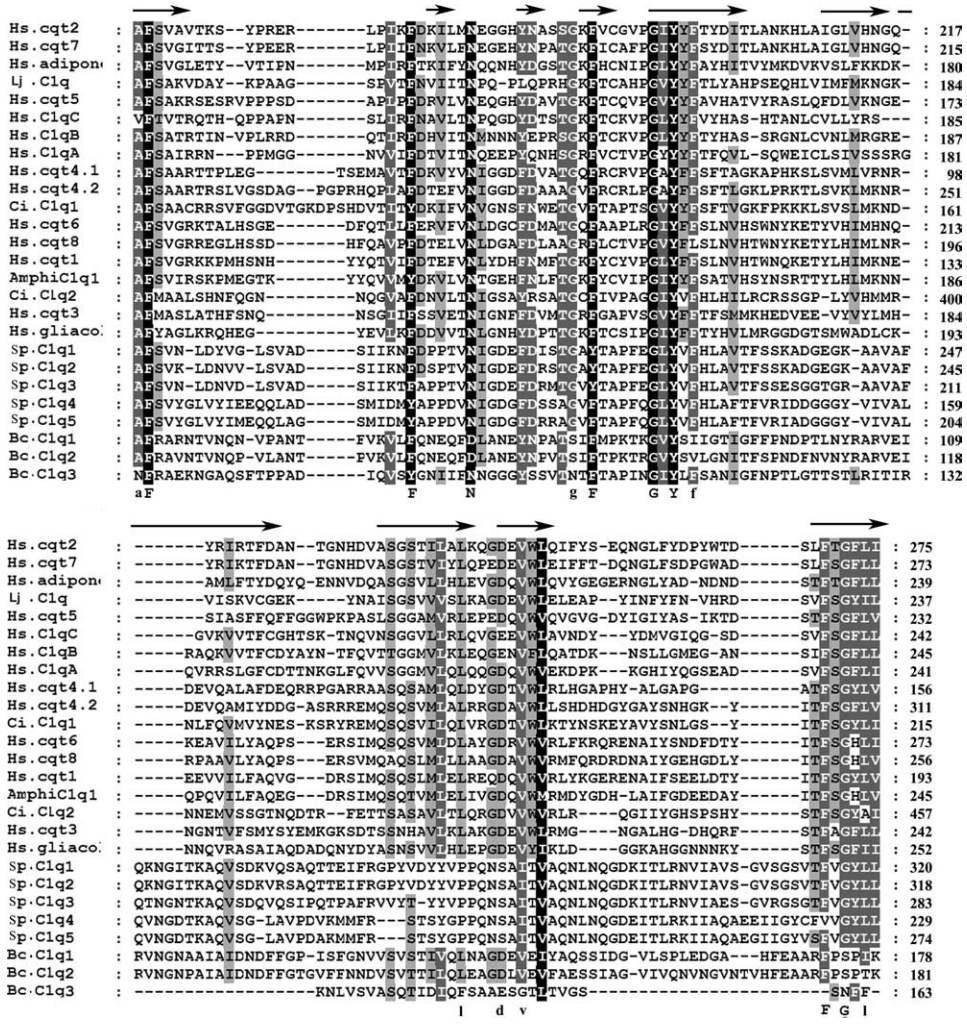

FIGURE 2. Sequence alignment of gC1q domain from various species. Arrows above the alignment represent β-strand positions found in the crystal structure of adiponectin C1q domain. The eight invariant and seven highly conserved residues of human gC1q collection were marked with majuscule and lowercase letters underneath the alignment, separately. Bc, *Bacillus cereus*; Ci, *Ciona intestinalis*; Hs, *Homo Sapiens*; Lj, *Lampetra japonicus*; Mm, *Mus mouse*; Sp, *Strongylocentrotus purpuratus*. The sequence used in alignment and phylogenetic analysis is indicated, as follows: AmphiC1q1 (FJ154078); Bc.C1q1 (AAP09230); Bc.C1q2 (AAP09231); Bc.C1q3 (AAP09378); Sp.C1q1 (AAK-11302); Sp.C1q2 (AAK11303); Sp. C1q3 (AAG16425); Sp.C1q4 (AAK-11309); Sp.C1q5 (AAK11305); Ci. C1q1 (ENSCINP00000006605); Ci. C1q2 (ENSCINP00000005965); Lj.C1q (BAD22833); Hs.cqt1 (AAQ88790); Hs.cqt2 (AAH11699); Hs.cqt3 (AAQ-88754); Hs.cqt4 (AAH35628); Hs.cqt5 (AAH29485); Hs.cqt6 (AAH20551); Hs. cqt7 (AAH24015); Hs.cqt8 (P60827); Hs. gliacolin (CAH73145); Hs.adiponectin (NP_004788); Mm.cqt1 (NP_064343); Mm.cqt2 (AAH30324); Mm.cqt4 (AAY-21929); Mm.cqt5 (AAH25174); Mm. cqt6 (AAH71187); and Mm.cqt7 (AAH90967).

excess purified recombinant proteins in the presence or absence of 10 mM $CaCl_2$. Protein that induced visible hemagglutination was recorded as possessing hemagglutinating activity.

Binding of AmphiC1q1 and its gC1q domain protein to the microbial components was performed by ELISA and pull-down assay, as described (12). Briefly, 20 μg of LPS, lipoteichoic acid, peptidoglycan from *S. aureus* (Fluka), glucan from *Saccharomyces cerevisiae* (Sigma-Aldrich), and mannan from *S. cerevisiae* (Sigma-Aldrich) were used to coat 96-well microtiter plate (Corning Glass) in PBS (pH 7.3) overnight at 4°C. Peptidoglycan and glucan are ultrasonically solubilized. Nonspecific binding to the wells was prevented by the addition of PBS containing 3% BSA overnight at 4°C. Several concentrations of targeted proteins were then added to the wells in the presence of 10 mM $CaCl_2$ and incubated for 1 h at room temperature. Bound proteins were detected with His mAb and HRP-labeled IgG. Between each incubation step, unbound protein, His mAb, 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.

Platelet aggregation assay

Collagen-induced platelet aggregation was performed using human platelet-rich plasma (PRP). AmphiC1q1 proteins were mixed with PRP by gentle rocking in a 96-well flat-bottom plate. Collagen I and thrombin were added at a final concentration of 1.25 μg/ml, the plate was agitated at 37°C on the microplate reader, and turbidity was monitored as percentage of light transmitted at 632 nm after 21.5 min.

Results

Identification and characterization of AmphiC1q1

To identify the C1q in Chinese amphioxus, *B. b. tsingtaunese*, we designed the degenerate primers according to the C1q sequences of lamprey, which was the most relative species to amphioxus with mRNA sequence available, to amplify the homologous gene. After amplification, we cloned and sequenced the full-length cDNA of AmphiC1q1 with 903 bp long (accession FJ154078), which contained an open reading frame of 756 bp encoding a 251-aa protein (Fig. 1). The deduced protein sequence contained a 16-aa signal peptide, a collagen-like sequence with 13 Gly-Xaa-Yaa repeats, and a C-terminal 137-aa gC1q domain. AmphiC1q1 shared the highest identity (51%) at the amino acid level with human C1q and TNF-related protein 1 (CTRP1 or cqt1), which was a novel member of the C1qTNF-related protein family containing characteristic

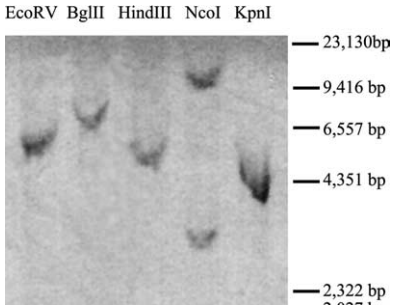

FIGURE 3. Southern blot analysis of AmphiC1q1. Genomic DNA isolated from adult animals was transferred to a Hybond N^+ nylon membrane after digestion by indicated enzymes, and detected with digoxigenin-labeled probes.

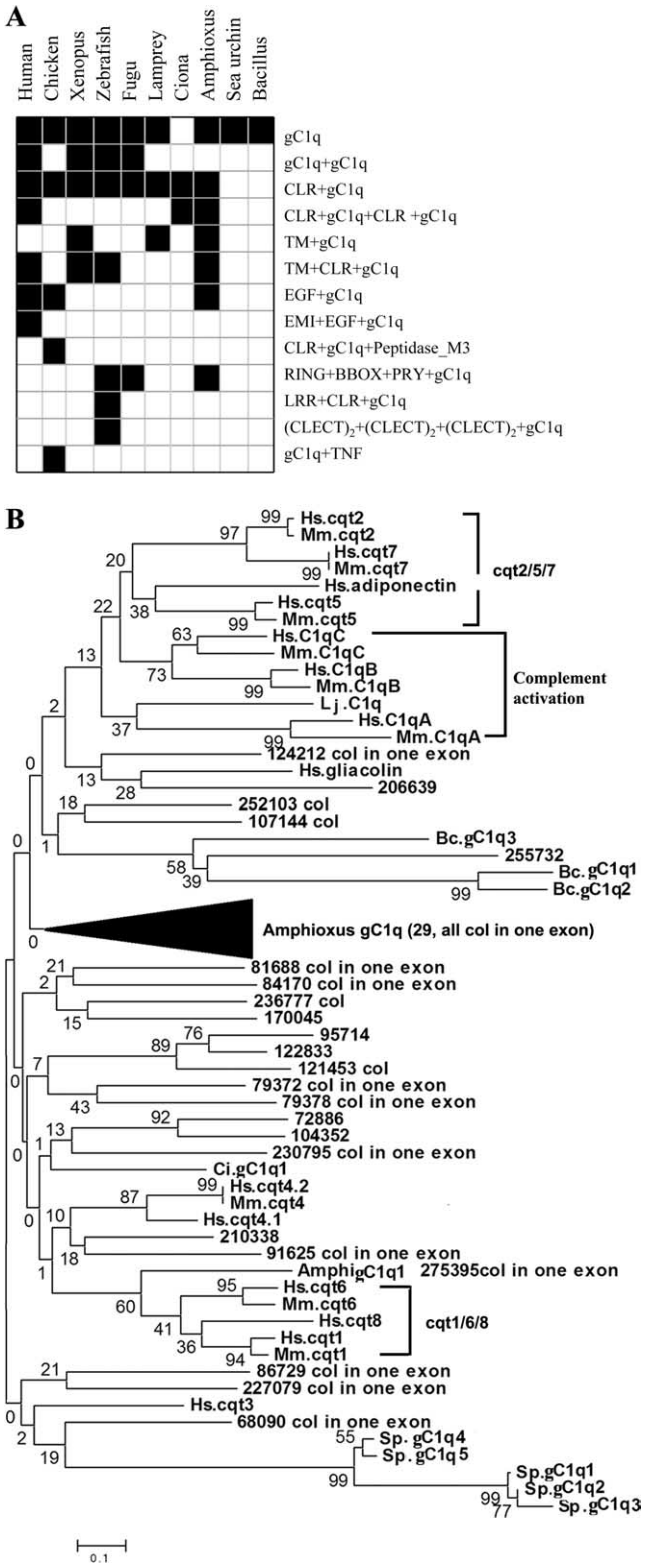

FIGURE 4. Domain architecture and evolutionary analysis of gC1q domains. *A*, The distribution and representative domain architectures of the gC1q domains across various evolutionary taxa have been compared via presence (black) or absence (white) of the domain architecture. Abbreviations are indicated, as follows: TM, transmembrane region; EGF, epidermal growth factor domain; EMI, EMILIN domain; RING, really interesting new gene; BBOX, Bbox type zinc finger domain; PRY, associated with SPRY (SPla/RYanodine receptor) domain; LRR, leucine-rich repeat domain; CLECT, C-type lectin domain; CLR, collagen-like region; gC1q, the globular domain of C1q-related protein; $(CLECT)_2$, exactly two CLECT domains are present adjacent to each other; and Pkinase, serine threonine-type protein kinase. *B*, The ME tree of 3 *Bacillus*, 5 sea urchin, 2 ascidian, 1 lamprey, 50 amphioxus, and mammalian representative C1q domain-containing proteins. The gene ID of amphioxus C1q-related molecule is referred from the website http://genome.jgi-psf.org/Brafl1/Brafl1.home.html.

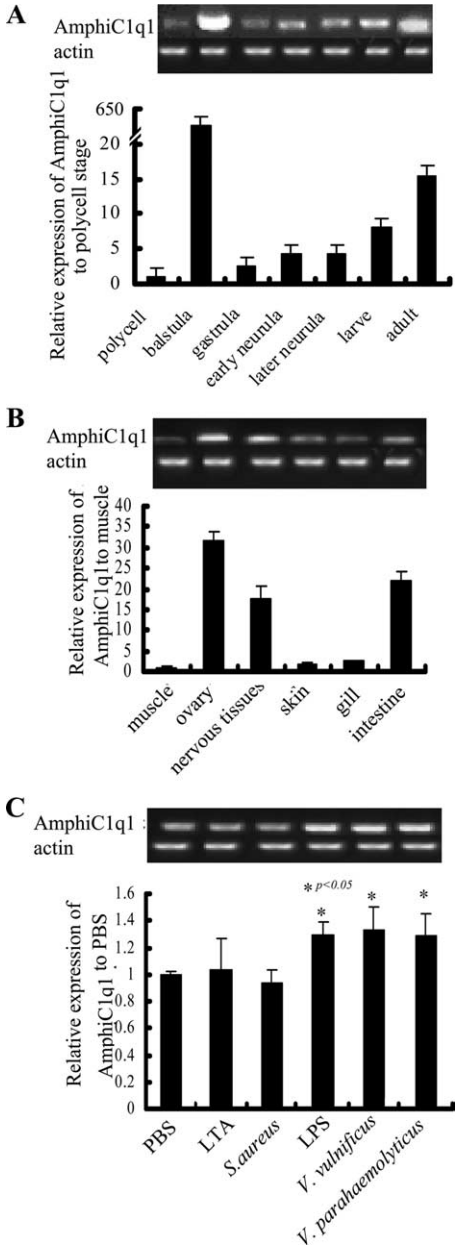

FIGURE 5. Expression patterns of AmphiC1q1 analyzed by real-time PCR and RT-PCR. *A*, The temporal expression patterns of AmphiC1q1 mRNA during embryogenesis. Data were shown as a ratio to AmphiC1q1 expression level in polycell stage. *B*, The tissue expression patterns of AmphiC1q1. Data were indicated as a ratio to AmphiC1q1 expression level in muscle. *C*, AmphiC1q1 expression after challenge with different stimulations. Data were shown as a ratio to AmphiC1q1 expression level after injection of PBS 48 h later. Asterisks indicated significant up-regulation to PBS treatment at $p < 0.05$.

collagen and TNFα-like gC1q domains with ~135 aa, and showed marked expression in vascular wall tissues. The gC1q domain in the deduced protein was more similar to the corresponding gC1q domain of G protein couples receptor-interacting protein and cqt1 (57% identity) from *Homo sapiens*, whereas other similar gC1q

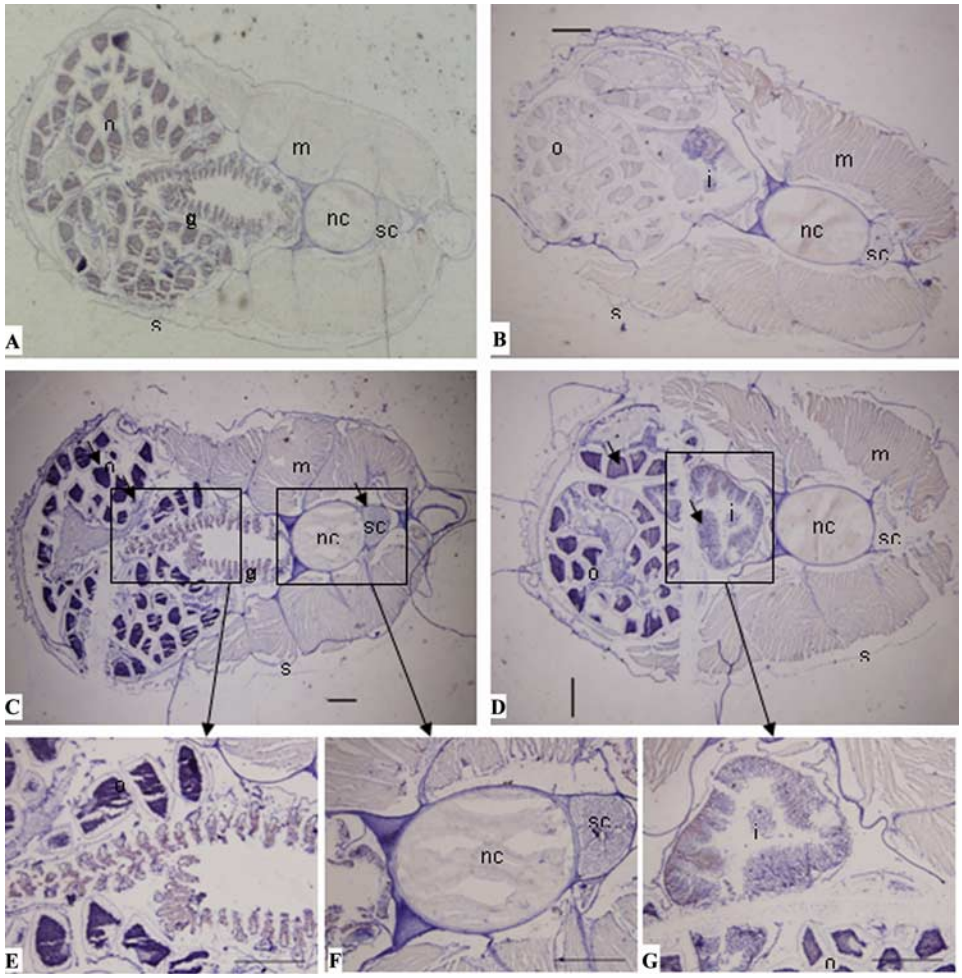

FIGURE 6. Section in situ hybridization of AmphiC1q1 in adult female. g, gill; i, intestine; s, skin; m, muscle; nc, notochord; sc, spinal cord; o, ovary. *A* and *B*, Sense probe; *C–G*, antisense probe. The bar indicated 200 μm.

domain had lower identities to the molecules such as C1q and TNF-related protein 4 (cqt4) (38% identity), complement component 1 chain A (34% identity), adiponectin (33% identity), and cerebellin 1 (33% identity).

Within the alignment profile, several well-conserved regions interspersed by less-conserved regions were identified, and they tended to correspond to conserved β-strands and connecting loops, respectively. The C1q family proteins from the sea urchin shared the highest homology with each other compared with those in other species, and this holds true for *Bacillus* Bc.C1q1,2 proteins as well, but not to AmphiC1q1. The 15 highly conserved residues in human gC1q domains were also conserved in AmphiC1q1. Among these, eight residues, invariant for all human C1q domains, were also invariable in AmphiC1q1 (positions F119, N144, F156, G162, Y164, F240, and G242), except F132, which was Y in human gC1q domains (Fig. 2). The seven highly conserved residues (positions A118, G154, F166, L211, D215, V217, and L244) were also conserved in AmphiC1q1 and two gC1qs from ascidian, but some of them were not conserved in sea urchin. These conserved residues might play important roles in target binding and formation or stabilization of the hydrophobic core of the gC1q structure (13–16).

We also cloned the genomic sequence of AmphiC1q1 (accession FJ154079) and found that AmphiC1q1 was a single intron gene. The first exon encoded the leader sequence, a short N-terminal region, and nearly half of the collagen region. The second exon encoded the remaining part of the collagen region and the gC1q domain. There was only one copy of AmphiC1q1 gene in amphioxus draft genome, which was confirmed by Southern blot hybridization (Fig. 3). Except two bands generated by the digestion of *Nco*I, only single band was detected in lanes corresponding to digestions with *Eco*RV, *Bgl*II, *Hind*III, or *Kpn*I. Considering one *Nco*I restriction enzyme cleavage site in the probe sequence, we could believe that AmphiC1q1 had a single copy of gene in the whole genome.

Domain architecture and evolution of amphioxus C1q family protein

During the course of evolution, C1q family proteins had entrenched themselves into various functional roles by variation of critical amino acids as well as associating with other taxonomic domains. Amphioxus genome encoded 50 gC1q gene models (10), in contrast to 29 in human and 5 in sea urchin. In addition, 42 of 50 amphioxus C1q-like genes adopted the typical CLR + gC1q architecture, and 33 of them encoded half of collagen and the entire gC1q domains in the same exon, just like AmphiC1q1. In amphioxus, the gC1q domain was also paired to a transmembrane region, which could be seen in higher vertebrate except aves. The pairing of PRY, BBOX, and RING domains with the gC1q domain seemed to be restricted to the ray-finned fishes and amphioxus only (Fig. 4*A*). PRY, BBOX, and RING domains were frequently associated with the pyrin domain (an Ig-like domain), which was a protein-protein interaction motif, and belonged to a family comprising death, death effector, and caspase recruitment domains. These results indicated that this domain architecture of C1q family proteins played an important role in innate immunity.

By using the ME method, we constructed a phylogenetic tree based on the gC1q domains of all C1q-domain-containing proteins from the genome of *B. florida*, *Bacillus cereus*, *Ciona intestinalis*, and *Stronglocentrotus purpuratus*, the representative proteins of *H. sapiens, Mus mouse*, and *Lampetra japonicus* (Fig. 4*B*). The phylogenetic analysis indicated that amphioxus gC1qs were highly diverged from the known gC1q proteins. Most members of amphioxus gC1qs were clustered into eight branches; the biggest clade included 29 gC1qs-containing collagen region and gC1q domain, which was encoded by a single exon. No orthologous C1q gene involved in complement activation was identified in amphioxus draft genome. Also, no orthologue of mammalian cqt2/5/7 subfamily was identified. However, AmphiC1q1 was the only orthologue of mammalian cqt1/6/8 subfamily with strong statistical support, indicating that AmphiC1q1 might be the primitive form of the cqt1/6/8 subfamily.

Expression patterns of AmphiC1q1

The expression of AmphiC1q1 during the embryonic development was investigated by real-time PCR. The mRNA expressions of AmphiC1q1 gradually arise along with the embryonic development, except at blastula stage (Fig. 5*A*). The abundant AmphiC1q1 expression in mid-blastula stage was consistent with the zygotic gene expression initiated at mid-blastula stage. After activation in mid-blastula stage, the AmphiC1q1 mRNA expression decreased to a low level in early gastrula stage. From early gastrula stage to adult, the gradual increase of the mRNA expression suggested that the AmphiC1q1 might play important roles in the embryogenesis.

We also used real-time PCR to analyze the distribution of AmphiC1q1 mRNA in adult tissues. AmphiC1q1 had a broad tissues distribution, mainly in nerve tissues, ovary, and gut (Fig. 5*B*). To detect the distribution of AmphiC1q1 mRNA in different tissues, we performed section in situ hybridization of female adult amphioxus with sense and antisense probes of gC1q region. AmphiC1q1 was detected strongly in the cytomembrane of mature oocytes, dorsal nerve cord, villi of epithelial cells of gut, the connective tissues, and coelomic cells. Relative weak expression was also detected in the epidermis of metapleura fold, the epithelial cells lining the wall of coelom, hepatic diverticulus, and pharyngeal slits (Fig. 6, *C–G*).

To investigate whether AmphiC1q1 was involved in the acute response of immune system or not, we challenged the adult amphioxus with different stimulations and detected the changed expression of AmphiC1q1 compared with PBS treatment by real-time PCR. The transcripts of AmphiC1q1 were weakly changed after amphioxus challenged with *S. aureus* and lipoteichoic acid, whereas LPS and two kinds of Gram-negative bacteria, *V. vulnificus* and *V. parahaemolyticus*, could induce a high expression of AmphiC1q1 compared with PBS injection (Fig. 5*C*). The results suggested the expression patterns of AmphiC1q1 could be changed only by challenge with specific microorganisms such as Gram-negative bacteria.

AmphiC1q1 does not function as lamprey LC1q

It has been reported that the C1q of lamprey was emerged as a GlcNAc-binding lectin and functioned as an initial recognition molecule of the complement system in innate immunity (8). To identify whether AmphiC1q1 could function as lamprey LC1q or not, we obtained the recombinant proteins with pTRX fusion expression system in *E. coli* Rosetta-gami (Fig. 7, *A* and *B*) and the molecular mass of fusion recombinant proteins were 38 kDa for AmphiC1q1-FL and 28.8 kDa for its gC1q domain under reducing conditions. Under nonreducing conditions, the 38-kDa AmphiC1q1-FL fusion protein migrated to three fractions, mainly at ≈76 kDa, slightly at ≈152 kDa, and >175 kDa (Fig. 7*C*). To further establish its multimeric nature, this protein was subjected to gel filtration by using Sephacryl S300. This 38-kDa protein was separated to three peaks: the first and second peak were low in quantity, corresponding to fractions ~at 660 and 152 kDa, and the fraction of the biggest peak was at 76 kDa (Fig. 7, *D* and *E*). The gel filtration and nonreducing PAGE gave the same result, indicating that AmphiC1q1-FL fusion protein formed dimer (76 kDa), tetramer (152 kDa), and multimer (≈660 kDa) under the nondenaturing conditions, and the dimer pattern was the major type of this protein.

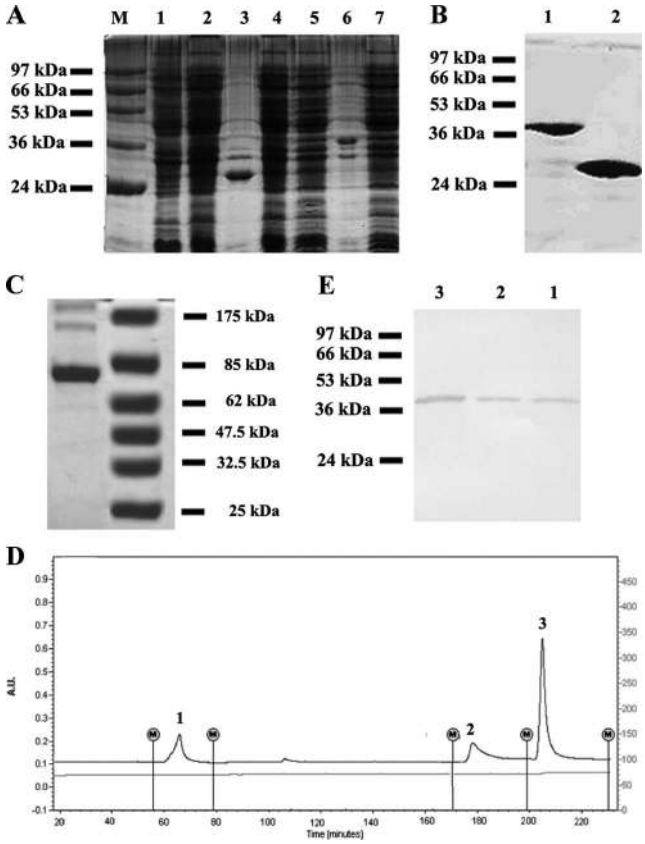

FIGURE 7. Expression and purification of AmphiC1q1 protein. *A*, The expression of recombinant AmphiC1q1 in Rosetta-gami. *Lane 1*, Total proteins of uninduced pETTRX-AmphiC1q1-FL; *lane 2*, total proteins of induced pETTRX-AmphiC1q1-FL; *lane 3*, pellets from induced pETTRX-AmphiC1q1-FL after sonication; *lane 4*, supernatant from induced pETTRX-AmphiC1q1-FL after sonication; *lanes 5–7*, total proteins, pellets, and supernatant of induced pETTRX-AmphiC1q1-gC1q. *B*, Purified AmphiC1q1 proteins by Ni^{2+}-chelating affinity chromatography run under reducing condition. *Lane 1*, TRX-AmphiC1q1-FL; *lane 2*, TRX-AmphiC1q1-gC1q. *C*, AmphiC1q1-FL protein was subjected to PAGE under nonreducing conditions. *D*, Gel filtration analysis of the AmphiC1q1-FL protein on Sephacryl S300. Numbers indicated those proteins in *E* were collected at this peak. *E*, SDS-PAGE profile of the proteins at peak under reducing conditions.

To investigate the GlcNAc-binding activity of AmphiC1q1, we incubated the GlcNAc Sepharose 4B beads with the purified recombinant AmphiC1q1 protein and examined the binding activity by Western blot. However, GlcNAc Sepharose 4B beads could not co-pull down with AmphiC1q1 in the presence or absence of calcium, indicating that AmphiC1q1 could not function like LC1q to bind with GlcNAc.

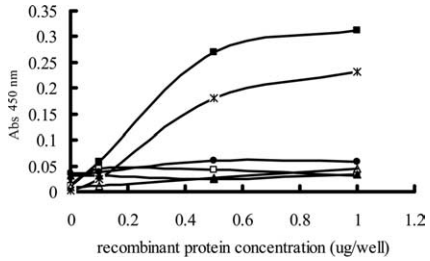

FIGURE 8. ELISA analysis of the interaction between recombinant fusion proteins and the components. Plates were coated with 20 μg of components, which were washed and incubated with AmphiC1q1-FL and its truncated gC1q domain protein (concentrations, horizontal) in the presence of 10 mM $CaCl_2$ at 4°C overnight and detected with mouse His mAb. Results are representative of average six such experiments. Background absorbance without protein was subtracted. ■, TRX-AmphiC1q1-FL+ LPS from *E. coli* 0111:B4; ∗, TRX-AmphiC1q1-gC1q + LPS from *E. coli* 0111:B4; □, TRX-AmphiC1q1-FL+ lipoteichoic acid from *S. aureus*; ▲, TRX-AmphiC1q1-FL+ glucan from *S. cerevisiae*; △, TRX-AmphiC1q1-FL+ mannan from *S. cerevisiae*; ●, TRX-AmphiC1q1-FL+ peptidoglycan from *S. aureus*.

AmphiC1q1 can bind LPS, but do not possess hemagglutinating activity

To investigate whether AmphiC1q1 could interact with other ligands for lectin except GlcNAc, we examined the hemagglutinating activity of the recombinant AmphiC1q1 protein with rabbit erythrocytes. As the positive control, AmphiCTL1 could induce strong hemagglutination in the presence of calcium (12), but AmphiC1q1 could not even with addition of excess targeted protein in the presence or absence of calcium. This result indicated that AmphiC1q1 did not possess hemagglutinating activity, nor did it recognize those glycan exposed on the surface of rabbit erythrocytes.

Interestingly, mannose-binding lectin, one of the principal lectins in humans, possesses no hemagglutinating properties, but can bind with the components of the microorganism to activate the complement pathway (17). Mammalian C1q binds not only to Igs, but also to other substances, such as C-reactive protein and endotoxins, resulting in complement activation (1). To examine whether AmphiC1q1 could bind with other components existing in the microbes, we used the ELISA to analyze the interaction between AmphiC1q1 protein and several microbial components such as LPS, lipoteichoic acid, peptidoglycan, mannan, and glucan. As shown in Fig. 8, in the presence of calcium, AmphiC1q1-FL and its gC1q domain proteins could interact with LPS, whereas both proteins had no binding activities with other components. And the pull-down assay also demonstrated that those targeted proteins did not bind with peptidoglycan and glucan (data not shown).

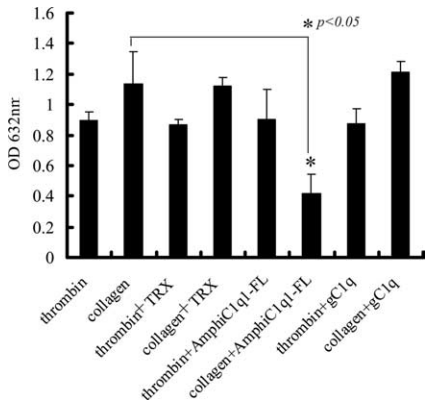

FIGURE 9. Inhibition of collagen-induced platelet aggregation by AmphiC1q1. Turbidity was monitored as percentage of light transmitted at 632 nm. Asterisk indicates significant decrease at $p < 0.05$.

AmphiC1q1 possesses the ability to inhibit collagen-induced platelet aggregation

Considering the possibility that CTRP1, which shared the highest homology with AmphiC1q1, could inhibit collagen-induced platelet aggregation (3), we performed a similar test for AmphiC1q1. Incubation of PRP with fibrillar collagen type I and thrombin produced a rapid aggregation of platelets, as described (3). AmphiC1q1 virtually completely inhibited the aggregation of platelets induced by fibrillar collagen type I, but not to thrombin. However, the truncated gC1q domain protein had no significant effects on aggregation of platelets by collagen type I and thrombin (Fig. 9).

Discussion

The complement system mediates a chain reaction of proteolysis and assembly of protein complexes that result in the elimination of invading microorganisms (18, 19). From an evolutionary perspective, the classical and lytic pathways seem to have emerged at the cartilaginous fish stage, when vertebrate adaptive immunity was established (20, 21). However, C1q, the Ig recognition molecule of the classical pathway of the vertebrate, has recently been shown to emerge as a lectin that interacted with mannose-binding lectin-associated serine proteases and activated the C3/C4-like thioester protein in the lamprey, a jawless fish that lacks conventional vertebrate adaptive immune system (8). The finding raises the possibility that some of C1q-related proteins possess lectin activity and the C1q-mediated complement activation occurs in even more primitive species.

Amphioxus represents the most basal extant chordate lineage (9), and hence, constitutes an important reference to study the immunity evolution. To date, there is little functional information for the complement system of amphioxus, except for a few reports on the presence of C3, C6 (22), mannose-binding lectin-associated serine protease 1/3 (23), Bf/C2 (24), and complement-like activity in the humoral fluid (25). From the phylogenomic perspective, amphioxus appeared to have a sophisticated complement system full of amphioxus-specific novelties (10). Although it has been demonstrated that the lectin pathway and alternative pathway were present in the amphioxus (23), there was no evidence as yet of the presence of C1q-mediated complement system.

We have identified 50 C1q-domain-containing molecules from the draft genome of *B. florida*, in contrast to 29 in human (10). However, no orthologous C1q gene involved in complement activation was identified in amphioxus draft genome. Before the genome data of *B. florida* was released, we have amplified an amphioxus C1q molecule, AmphiC1q1, by degenerate primers designed according to lamprey LC1q from *B. b. tsingtauense*. The phylogenetic analysis indicated that AmphiC1q1 was a primitive representative of subfamily cqt1/6/8, outside of the clade of the C1q involved in complement activation. Functional experiments of the recombinant AmphiC1q1 protein demonstrated that it did not possess the same function as lamprey LC1q, such as recognizing GlcNAc, but this protein could specifically bind LPS, corresponding well with high expression of this gene after challenge with LPS and two kinds of Gram-negative bacteria. These data indicated that AmphiC1q1 might also be involved in the innate immune system of amphioxus in a manner different from the lamprey LC1q, though whether it mediated complement activity or not was still unknown. However, there were many reports that C1q interacted

with LPS derived from a number of Gram-negative bacteria via its CLR and/or gC1q domain to activate complement system (26, 27). The interaction between AmphiC1q1 and LPS existed in the most primitive chordate amphioxus, like its mammalian homologue, indicating the functional conservation during the evolution of C1q-related proteins.

As mentioned above, AmphiC1q1 did not possess the same function as lamprey LC1q to mediate complement activation, but had the LPS-binding activity as a new member of gC1q family with a conserved C1q-like domain. To characterize this novel molecule, we used real-time PCR to analyze its expression pattern. Although the methods we used in this study may have possible pitfalls because the total amount of cells is different in different stages and tissues, it can still tell us the relative expression of this targeted gene to some extent. AmphiC1q1 was detected in all stages of embryo development, indicating AmphiC1q1 might play important roles in the embryogenesis, which adds a new dimension to the versatility of the family. From the in situ hybridization, AmphiC1q1 was also expressed in the central nerver system of amphioxus, such as dorsal nerve cord, suggesting that this gene could be responsible for a more basic housekeeping function for neurons, just like the C1q subunits and C1q-related factor (CRF). C1q protein had been found to localize in Alzheimer's plaques, facilitating the nucleation phase of β-amyloid aggregation (28). CRF might play a role in neuronal differentiation, and cells regulating motor control required a higher level expression of this gene (29). Because AmphiC1q1 displayed a homology to C1q and CRF, it would lead us to speculate that AmphiC1q1 might have the similar function as C1q and CRF in the nervous system. AmphiC1q1 was most detected in the cytomembrane of mature oocytes, similar to CaOC1q-like proteins localizing in egg envelope of the oocytes at cortical granule stage and vitellogenesis stage, indicating that AmphiC1q1 might have the unique function in oogenesis, oocyte maturation, and egg fertilization, the same as CaOC1q-like factor (4). Moreover, AmphiC1q1 was particularly detected in the structures that were exposed to invading microorganisms, such as coelomic cells and villi of epithelial cells of gut, suggesting that AmphiC1q1 might be involved in the defense against those pathogens, corresponding well with interaction between LPS and AmphiC1q1 protein, as mentioned above.

Because AmphiC1q1 displayed the highest homology to CTRP1, which was markedly expressed in vascular wall tissue, and played a role in collagen-induced platelet activation and aggregation (3), just like C1q (30), we investigated whether AmphiC1q1 might exhibit similar functions. Interestingly, initial studies indicated that AmphiC1q1 could block collagen-induced platelet aggregation, and the truncated gC1q domain did not possess this function (Fig. 9), indicating that CLR of AmphiC1q1 played a very important role in blocking platelet aggregation. Although platelet has not been found to exist in the amphioxus, AmphiC1q1 might block the platelet-like aggregation related to the biochemical progress of amphioxus immune defense. In contrast, there may be other ligands of AmphiC1q1 that exist in the mammalian platelet that are not known to us, so we need to further study these ligands in the host cell. Early work on the evolution of vertebrate fibrinogen suggested a common origin of the arthropod hemolymph coagulation and the vertebrate blood coagulation systems, and a common ancestor for the innate immunity and the blood coagulation systems (31). The role of AmphiC1q1 on platelet-like aggregation may also provide some evidence for understanding the evolution of the C1q-mediated innate immunity and the blood coagulation systems.

In summary, the newly identified function of this new member of the C1q family in this study may shed light on further exploration of this complicated molecule. Although whether AmphiC1q1 was involved in the complement activity or others in the immune system of amphioxus was still unknown, it was revealed to have a partial function of vertebrate C1q family, such as the interaction between C1q and LPS, and blocking collagen-induced platelet aggregation the same as CTRP1. The functional versatility of this molecule may be resulted in the great expansion of its gC1q family repertoire in amphioxus. Amphioxus had more than 50 C1q-related genes that had been found in the amphioxus draft genome, compared with 5 in sea urchin and 2 in ascidian (10). The sudden expansion of C1q-related genes in amphioxus may lead to that the function of many molecules, such as 29 amphioxus gC1qs clustered into a clade, were not well differentiated, compared with mammalian ones. Some molecules, such as AmphiC1q1, may even possess other functions beyond its mammalian homologue because the functional differentiation of this primitive form was not fully evolved as that of high species. In short, the study of the primitive form of mammalian C1q family in the most basal extant chordate lineage will provide insight into the evolution of C1q family and C1q-mediated complement system, and especially shed light on the functional evolution of its vertebrate homologues.

Disclosures

The authors have no financial conflict of interest.

References

1. Kishore, U., and K. B. M. Reid. 2000. C1q: structure, function, and receptors. *Immunopharmacology* 49: 159–170.
2. Kishore, U., C. Gaboriaud, P. Waters, A. K. Shrive, T. J. Greenhough, K. B. Reid, R. B. Sim, and G. J. Arlaud. 2004. C1q and tumor necrosis factor superfamily: modularity and versatility. *Trends Immunol.* 25: 551–561.
3. Lasser, G., P. Guchhait, J. L. Ellsworth, P. Sheppard, K. Lewis, P. Bishop, M. A. Cruz, J. A. Lopez, and J. Fruebis. 2006. C1qTNF-related protein-1 (CTRP-1): a vascular wall protein that inhibits collagen-induced platelet aggregation by blocking VWF binding to collagen. *Blood* 107: 423–430.
4. Chen, B., and J. Gui. 2004. Identification of a novel C1q family member in color crucian carp (*Carassius auratus*) ovary. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 138: 285–293.
5. Scherer, P. E., S. Williams, M. Fogliano, G. Baldini, and H. F. Lodish. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.* 270: 26746–26749.
6. Hu, E., P. Liang, and B. M. Spiegelman. 1996. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J. Biol. Chem.* 271: 10697–10703.
7. Innamorati, G., E. Bianchi, and M. I. Whang. 2006. An intracellular role for the C1q-globular domain. *Cell. Signal.* 18: 761–770.
8. Matsushita, M., A. Matsushita, Y. Endo, M. Nakata, N. Kojima, T. Mizuochi, and T. Fujita. 2004. Origin of the classical complement pathway: lamprey orthologue of mammalian C1q acts as a lectin. *Proc. Natl. Acad. Sci. USA* 101: 10127–10131.
9. Putnam, N. H., T. Butts, D. E. K. Ferrier, R. F. Furlong, U. Hellsten, T. Kawashima, M. Robinson-Rechavi, E. Shoguchi, A. Terry, J.-K. Yu, et al. 2008. The amphioxus genome and the evolution of the chordate karyotype. *Nature* 453: 1064–1071.
10. Huang, S., S. Yuan, L. Guo, Y. Yu, J. Li, T. Wu, T. Liu, M. Yang, K. Wu, H. Liu, et al. 2008. Genomic analysis of the immune gene repertoire of amphioxus reveals extraordinary innate complexity and diversity. *Genome Res.* 18: 1112–1126.
11. Tang, Y. T., T. H. Hu, M. Arterburn, B. Boyle, J. M. Bright, S. Palencia, P. C. Emtage, and W. D. Funk. 2005. The complete complement of C1q-domain-containing proteins in *Homo sapiens*. *Genomics* 86: 100–111.
12. Yu, Y., Y. Yu, H. Huang, K. Feng, M. Pan, S. Yuan, S. Huang, T. Wu, L. Guo, M. Dong, et al. 2007. A short-form C-type lectin from amphioxus acts as a direct microbial killing protein via interaction with peptidoglycan and glucan. *J. Immunol.* 179: 8425–8434.
13. Kojouharova, M. S., M. G. Gadjeva, I. G. Tsacheva, A. Zlatarova, L. T. Roumenina, M. I. Tchorbadjieva, B. P. Atanasov, P. Waters, B. C. Urban, R. B. Sim, et al. 2004. Mutational analyses of the recombinant globular regions of human C1q A, B, and C chains suggest an essential role for arginine and histidine residues in the C1q-IgG interaction. *J. Immunol.* 172: 4351–4358.
14. Roumenina, L. T., M. M. Ruseva, A. Zlatarova, R. Ghai, M. Kolev, N. Olova, M. Gadjeva, A. Agrawal, B. Bottazzi, A. Mantovani, et al. 2006. Interaction of C1q with IgG1, C-reactive protein and pentraxin 3: mutational studies using recombinant globular head modules of human C1q A, B, and C chains. *Biochemistry* 45: 4093–4104.
15. Roumenina, L. T., A. A. Kantardjiev, B. P. Atanasov, P. Waters, M. Gadjeva, K. B. Reid, A. Mantovani, U. Kishore, and M. S. Kojouharova. 2005. Role of Ca^{2+} in the electrostatic stability and the functional activity of the globular domain of human C1q. *Biochemistry* 44: 14097–14109.

16. Zlatarova, A. S., M. Rouseva, L. T. Roumenina, M. Gadjeva, M. Kolev, I. Dobrev, N. Olova, R. Ghai, J. C. Jensenius, K. B. Reid, et al. 2006. Existence of different but overlapping IgG- and IgM-binding sites on the globular domain of human C1q. *Biochemistry* 45: 9979–9988.
17. Wallis, R. 2002. Structural and functional aspects of complement activation by mannose-binding protein. *Immunobiology* 205: 433–445.
18. Walport, M. J. 2001. Complement: first of two parts. *N. Engl. J. Med.* 344: 1058–1066.
19. Walport, M. J. 2001. Complement: second of two parts. *N. Engl. J. Med.* 344: 1140–1144.
20. Fujita, T. 2002. Evolution of the lectin-complement pathway and its role in innate immunity. *Nat. Rev. Immunol.* 2: 346–353.
21. Fujita, T., M. Matsushita, and Y. Endo. 2004. The lectin-complement pathway: its role in innate immunity and evolution. *Immunol. Rev.* 198: 185–202.
22. Suzuki, M. M., N. Satoh, and M. Nonaka. 2002. C6-like and C3-like molecules from the cephalochordate, amphioxus, suggest a cytolytic complement system in invertebrates. *J. Mol. Evol.* 54: 671–679.
23. Endo, Y., M. Nonaka, H. Saiga, Y. Kakinuma, A. Matsushita, M. Takahashi, M. Matsushita, and T. Fujita. 2003. Origin of mannose-binding lectin-associated serine protease (MASP)-1 and MASP-3 involved in the lectin complement pathway traced back to the invertebrate, amphioxus. *J. Immunol.* 170: 4701–4707.
24. He, Y., B. Tang, S. Zhang, Z. Liu, B. Zhao, and L. Chen. 2008. Molecular and immunochemical demonstration of a novel member of Bf/C2 homolog in amphioxus *Branchiostoma belcheri*: implications for involvement of hepatic cecum in acute phase response. *Fish Shellfish Immunol.* 24: 768–778.
25. Zhang, S., C. Wang, Y. Wang, R. Wei, G. Jiang, and H. Ju. 2003. Presence and characterization of complement-like activity in the amphioxus *Branchiostoma belcheri tsingtauense*. *Zool. Sci.* 20: 1207–1214.
26. Zohair, A., S. Chesne, R. H. Wade, and M. G. Colomb. 1989. Interaction between complement subcomponent C1q and bacterial lipopolysaccharides. *Biochem. J.* 257: 865–873.
27. Roumenina, L. T., K. T. Popov, S. V. Bureeva, M. Kojouharova, M. Gadjeva, S. Rabheru, R. Thakrar, A. Kaplun, and U. Kishore. 2008. Interaction of the globular domain of human C1q with *Salmonella typhimurium* lipopolysaccharide. *Biochim. Biophys. Acta* 1784: 1271–1276.
28. Afagh, A., B. J. Cummings, D. H. Cribbs, C. W. Cotman, and A. J. Tenner. 1996. Localization and cell association of C1q in Alzheimer's disease brain. *Exp. Neurol.* 138: 22–32.
29. Berube, N. G., X. H. Swanson, M. J. Bertram, J. D. Kittle, V. Didenko, D. S. Baskin, J. R. Smith, and O. M. Pereira-Smith. 1999. Cloning and characterization of CRF, a novel C1q-related factor, expressed in areas of the brain involved in motor function. *Brain Res. Mol. Brain Res.* 63: 233–240.
30. Peerschke, E. I., and B. Ghebrehiwet. 1990. Platelet C1q receptor interactions with collagen- and C1q-coated surfaces. *J. Immunol.* 145: 2984–2988.
31. Kairies, N., H. G. Beisel, P. Fuentes-Prior, R. Tsuda, T. Muta, S. Iwanaga, W. Bode, R. Huber, and S. Kawabata. 2001. The 2.0-A crystal structure of tachylectin 5A provides evidence for the common origin of the innate immunity and the blood coagulation systems. *Proc. Natl. Acad. Sci. USA* 98: 13519–13524.