Functional Characterization of a Ficolin-mediated Complement Pathway in Amphioxus*‡§

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The ficolin-mediated complement pathway plays an important role in vertebrate immunity, but it is not clear whether this pathway exists in invertebrates. Here we identified homologs of ficolin pathway components from the cephalochordate amphioxus and investigated whether they had been co-opted into a functional ficolin pathway. Four of these homologs, ficolin FCN1, serine protease MASP1 and MASP3, and complement component C3, were highly expressed in mucosal tissues and gonads, and were significantly up-regulated following bacterial infection. Recombinant FCN1 could induce hemagglutination, discriminate among sugar components, and specifically recognize and aggregate several bacteria (especially Gram-positive strains) without showing bactericidal activity. This suggested that FCN1 is a dedicated pattern-recognition receptor. Recombinant serine protease MASP1/3 formed complexes with recombinant FCN1 and facilitated the activation of native C3 protein in amphioxus hemolymph fluid, in which C3 acted as an immune effector. We conclude that amphioxus have developed a functional ficolin-complement pathway. Because ficolin pathway components have not been reported in non-chordate species, our findings supported the idea that this pathway may represent a chordate-specific innovation in the evolution of the complement system.

The jawed vertebrate complement system is not only a major humoral effector, but possibly represents a fundamental shift from the antimicrobial peptide-based humoral immunity which is prevalent in most invertebrates (6). Hence, the origin of the vertebrate complement system is not trivial. Previous efforts have dated the origin of some complement pathways to before the radiation of jawed vertebrates. For example, it has been suggested that a functional alternative pathway is present in a basal deuterostome, the purple sea urchin (7). The jawless vertebrate lamprey, which lacks Ig-based antibodies, possesses a prototype of the classical pathway, in which the C1q protein recognizes pathogen-associated carbohydrates instead of antibody-antigen complexes (8). In addition, functional lectin pathways mediated by GBL (a C-type lectin without collagen) and MBL (a collectin), which can trigger the complement cascade, have been found in both urochordates and lampreys (9, 10). However, thus far no functional ficolin pathways have been found outside the jawed vertebrate lineage (11–13).

Ficolins contain a middle collagen (COL) region and a C-terminal fibrinogen-like (FBG) domain (14). In comparison, collectins contain a middle COL region and a C-terminal C-type lectin (CTL) domain (15), while C1q proteins contain a middle COL region and a C-terminal C1q-like domain (16). All three types of lectins use COL regions for serine protease binding and C-terminal domains for target recognition (either pathogens or antibody-antigen complexes). The ficolin pathway is with a more recent discovery than the collectin and C1q pathways.

The abbreviations used are: PRR, pathogen-recognition receptors; FCN, ficolin protein; FBG domain, fibrinogen-like domain; MBL, mannose-binding lectin; GBL, glucose-binding lectin; MASP, MBL-associated serine protease; GlcNAc, N-acetylgalactosamine; ORF, open-reading frame; CCP, complement control protein; CUB, complement C1r/C1s; Uegf, Bmp1 module; HA, hemagglutinin activity.

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Ficolin was first documented as a transforming growth factor (TGF)-β1-binding protein on pig uterus membranes in 1991 before their opsonic functions had been recognized (17). There are three human ficolins. Human ficolin1 (HmFCN1) recognizes Staphylococcus aureus and smooth Salmonella typhimurium (LT2), and recruits MASPs1 and 2 to cleave C4 possibly through interaction with N-acetylglycosamine (GlcNAc). It also displays a binding preference for sialyl-N-acetylglycosamine (SiaLacNAc) (18). HmFCN2 interacts with several strains of bacteria, including Escherichia coli, S. typhimurium TV119, and S. aureus (19–22), possibly through interaction with LTA, 1,3-β-d-glucan and glucose/galactose rings (23–26). HmFCN2 activates the complement cascade through a Ca²⁺-dependent association with MASPs (22). HmFCN3 binds to Aerococcus viridans (27) and a few types of ligands, including d-fucose, galactose, and acetylated albumin (27–29). HmFCN3 activates the complement system by the cleavage of C4 based on binding to A. viridans (27). Many studies have reported that mutations or ectopic expression of ficolins are associated with human diseases, including systemic lupus erythematosus (30), chronic rheumatic heart disease (31), rheumatoid arthritis (32), fever, and neutropenia (33).

Amphioxus is the oldest extant chordate lineage and is invaluable for the understanding of vertebrate origins and chordate biology. Early studies showed that amphioxus share several categories of complement components with vertebrates (6, 12). However, despite thousands of vertebrate homologs in invertebrates, not many of them (especially non-house-keeping genes) organize and function in ways similar to their vertebrate counterparts. In this work, we cloned and expressed BjFCN1, a canonical ficolin gene from amphioxus Branchiostoma japonicum (Bj), as well as three other relevant genes including BjMASP1, BjMASP3, and BjC3. We demonstrated that BjFCN1 is a dedicated PRR for the amphioxus complement system, which can interact with BjMASP1/3 and therefore lead to the activation of BjC3. Our findings not only suggest that a functional ficolin pathway have been established in the last common ancestor of chordates, but help to piece together the evolutionary story of the chordate complement system.

EXPERIMENTAL PROCEDURES

Amphioxus Collection, cDNA Cloning, and Sequence Analysis—Adult Chinese amphioxus (B. japonicum) were collected from Qingdao, China and reared in aerated sea water with algae. Partial cDNAs of BjFCN1, BjMASP1, BjMASP3, and BjC3 were cloned with gene-specific primer pairs (supplemental Table SI). Full-length cDNAs were isolated by using RACE technology (Invitrogen). These cDNA sequences were cloned into pGEX-T vector (Promega). These cDNAs were then purified from the cell medium and concentrated by using Dig RNA synthesis kit (Roche). Hybridization was carried out at 42 °C overnight, with probe concentration of 1 μg/ml. After extensive washes, sections were subjected to signal detection by using NBT/5-bromo-4-chloro-3-indolyl phosphate stock solution (Roche).

Immune Stimulation and Real-time Quantitative PCR Analysis—Several strains of bacteria including S. saprophyticus, S. hemeolyticus, S. aureus, E. faecalis, Vibrio anguillarum, Bacillus subtilis, Acinetobacter calcoaceticus, Klebsiella pneumoniae, and E. coli were obtained from the Third Affiliated Hospital of Sun Yat-Sen University (China), and verified by sequencing their 16SrRNAs. LPS (from E. coli 0111:B4) and LTA (from S. aureus) were purchased from Sigma. In the immune stimulation, live S. aureus (10⁶ cells/ml), live V. anguillarum (10⁵ cells/ml), LPS (1 mg/ml), and LTA (1 mg/ml) were suspended/dissolved in PBS buffer (137 mM NaCl, 3 mM KC1, 1 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and injected into the gut and coelom of amphioxus (15 μl animal⁻¹), respectively. The injection of PBS was used as control. Twenty animals of each treatment were collected at 1, 2, 4, 8, 12, and 24 h post-injection and frozen until use. Total RNA was purified by using RNeasy Plus Mini kit (Qiagen) and then treated with DNaseI (Promega). Double-stranded cDNAs were synthesized with SYBR perfect real-time series kit (Takara) and treated through an ULTRAFREE centrifugal filter device (Millipore). The PCR program was set for 40 cycles, with annealing temperature at 60 °C and extending temperature at 70 °C. Experiments for each sample were repeated for three times. Expression levels of each gene were determined by 2−ΔΔCt method, using GAPDH as endogenous control.

Vector Construction and Recombinant Protein Preparation—The recombinant BjFCN1 protein (without signal peptide) fused with TRX-His tag was expressed using pET32a vector (Novagen). GST-tagged recombinant BjMASP1/3-N protein (common N-terminal portion of BjMASP1 and 3 without signal peptide, CUB1-EGF-CUB2 domain) was expressed using pGEX 4T-2 vector (Promega). His-tagged recombinant BjMASP1-C protein (C-terminal portion of BjMASP1, CCP2-SP domain) and BjC3 protein (amino acid residues 579–755) were expressed using pET21b and pET28a vectors (Novagen), respectively. The inclusion recombinant BjC3, BjMASP1/3-N, and BjMASP1-C proteins were denatured and renatured as described above. Protein samples were purified with Ni²⁺-chelating Sepharose column (BjFCN1, BjC3, and BjMASP1-C) or Glutathione Sepharose™-4B column (BjMASP1/3-N) in TBS buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.5). Purified proteins were desalted with a G-25 column, and then concentrated by filtration through an ULTRAFREE centrifugal filter device (Millipore). TRX protein used as a negative control was purified by the same method. Purified recombinant BjC3 was sent to the Fourth Military Medical University, China for the preparation of monoclonal antibody. Flag-tagged full-length BjFCN1 with signal peptide was inserted into pcDNA3.0 vector and transfected into HEK293T cells. The protein was purified from the cell medium and concentrated as described above. All proteins were examined by Bio-
Rad protein assay with BSA (bovine serum albumin) as a standard.

**Hemagglutination and Sugar Binding Assays**—Hemagglutination activity (HA) of His-tagged recombinant BjFCN1 was examined by using mouse erythrocytes as previously described (38). Calcium and EDTA were added to the working buffer (TBS buffer) to investigate the effect of calcium on BjFCN1 HA. HA-based assays for BjFCN1 sugar binding activity were also conducted. Assessed sugar components included maltose, β-glucose, lactose, galactose, sucrose, α-mannose, fructose, GlcNAc, LPS, and LTA. The lowest dilution of each sugar component that inhibited HA of BjFCN1 was considered the minimal inhibitory concentration of that sugar. Purified TRX protein was used as control.

The binding activity of His-tagged recombinant BJFCN1 to GlcNAc and α-lactose was also performed by co-pull-down assays. After being equilibrated with TBS buffer, GlcNAc agarose beads were incubated with rough BjFCN1 protein (150 μg) containing CaCl₂ or EDTA (10 mM each) for 2 h at 6°C. After extensive washes, the bound proteins were eluted with 30 μl of SDS-PAGE loading buffer (100 mM Tris-HCl, 0.01% bromphenol blue, 36% glycerol, 4% SDS, pH 6.5) and heated for 15 min at 100°C before subjecting to SDS-PAGE. The binding activity with lactose agarose was tested by the same method. Rough BJFCN1 protein (30 μg) alone was used as control.

**Bacteria Binding and Aggregation Analysis**—Bacterial cells were suspended in TBS buffer (approximately A₆₀₀ = 2). His-tagged recombinant BJFCN1 protein (100 μg) was mixed with bacteria suspension in the presence of CaCl₂ or EDTA (10 mM each) and incubated at 4°C overnight. After extensive washes, the bound proteins were dissociated from the pellets by loading buffer, and analyzed by SDS-PAGE and Western blotting (with anti-BjC3 mAb) and supplemental Fig. S1). BjFCN1 has a long linker sequence between the signal peptide and the COL region compared with HmFCN2 (Fig. 1B). All conserved cysteines that are important for stabilizing the FBG domain (Fig. 1A) are conserved. These conserved cysteines might play a role in stabilizing the collagen domain. In addition to BjFCN1, we identified three other complement genes, designated BjMASP1 (supplemental Fig. S3), BjMASP3 (supplemental Fig. S4), and BjC3 (supplemental Fig. S5), which are orthologs to human MASP1, MASP3, and C3, respectively.

**Expression Regulation of Four Amphioxus Putative Ficolin Pathway Components**—The tissue distribution of BJFCN1, BjMASP1, BjMASP3, and BjC3 mRNAs in adult amphioxus was analyzed by performing section in situ hybridization (Fig. 2). While only slight or no expression was detected in skin, spinal cord, muscle, and notochord, all four genes were strongly expressed in ovary, gut, gill, and hepatic cecum (the equivalent of mammalian liver). These four tissues are known to be major sites involved in amphioxus immunity. The co-expression of these genes suggested that their encoding proteins might physically interact with each other during immune responses. Indeed, an early study reported that there was a high concentration of C3 protein in amphioxus ovary (40); thus our findings implied that ficolin and MASPs might play a role in the activation of this C3 arsenal in ovary.

The time course expression of these genes during immune challenge was also monitored using qRT-PCR. Both BJFCN1 and BjC3 were quickly and highly up-regulated in response to bacterial components LPS and LTA, whereas BjMASP1/3 was...
mildly up-regulated (the probe detected the expression of BjMASP1 and 3 simultaneously because it targets the common N-terminal region of two genes) (Fig. 3). We reason that MASPs may not require high expression levels because of their powerful catalytic activity. Also, once C3 is activated, the alternative pathway will step in to sustain and amplify the reaction. We also observed that BjFCN1 responded to whole bacteria more slowly and less dramatically, suggesting that purified bacterial components are more potent immune stimulants than the whole bacteria (Fig. 3A).

**Hemagglutinin and Sugar Binding Activity of BjFCN1**—Flag-tagged recombinant BjFCN1 was transiently transfected into HEK293T cells. The abundance of this protein was detected in cell culture medium after transfection, suggesting that BjFCN1 is a secreted protein. The molecular mass of this secreted protein is about 50 kDa, slightly larger than the predicted molecular mass of the hemagglutinin and sugar binding domain. Homo sapiens, Hm; Mus musculus, Mm; Xenopus laevis, Xe; Asciidiacea, Halocynthia roretzi, As. The sequences used in the alignment are indicated as follows: HmFCN2 (NP_004099); HmFCN3 (NP_003656); MmFCN1 (NP_032021); XeFCN3 (NP_001079138); AsFCN1 (BAB60704); AsFCN3 (BAB60706).

**FIGURE 1.** A, sequence alignment of ficolins from various species. B, architecture of cephalochordate amphioxus BjFCN1 and human HmFCN2. The deduced protein of BJFCN1 is a canonical ficolin, which consists of an N-terminal signal peptide, a middle collagen (COL) region, and a C-terminal fibrinogen-like (FBG) domain.
lar weight of 46 kDa, possibly due to post-translated glycosylation. Electrophoresis also showed that this protein was separated into three bands, suggesting that, under natural conditions, BjFCN1 forms a dimer and a tetramer through disulfide bonds (Fig. 4A).

We also expressed recombinant TRX-His-BjFCN1 protein in E. coli BL21 (DE3) and obtained purified soluble fusion protein with a Ni²⁺-chelating Sepharose column. The recombinant protein was ~64 kDa, corresponding to the predicted molecular mass (Fig. 4B). TRX-His-tagged BjFCN1 protein induced hemagglutination of mouse erythrocytes in the presence of CaCl₂. This activity was completely inhibited by the addition of EDTA (Fig. 4C), suggesting that the lectin activity of BjFCN1 is Ca²⁺-dependent. Further competition inhibition assays showed that 10 mM galactose, 10 mM d-glucose, 50 mM lactose, 150 mM d-mannose, 180 mM GlcNAc, 200 mM fructose, 200 mM sucrose, and 0.005 mg/ml LTA could sufficiently inhibit the hemagglutinating activity of BjFCN1 (Table 1), suggesting that BjFCN1 has different binding specificities to these sugars. However, as much as 500 mM maltose and 2 mg/ml LPS did not affect BjFCN1’s hemagglutinating activity, suggesting that they are not ligands for BjFCN1. Soluble TRX protein was purified and used as control, but showed no hemagglutinating activity.

Because LTA is abundant in Gram-positive bacteria, whereas LPS is present in Gram-negative bacteria, BjFCN1 may preferentially target Gram-positive bacteria.

GlcNAc is a part of the components of microbial cell wall and anthropoid cuticle, such as peptidoglycans and chitins. So far, all tested vertebrate ficolins have affinity for GlcNAc, and this affinity is critical for human ficolins. To verify whether amphioxus ficolins have this critical function, affinity chromatography technology was used to assess the interaction between BjFCN1 and GlcNAc-agarose. Simultaneously, the interaction between BjFCN1 and α-lactose agarose was evaluated. The bound BjFCN1 proteins could be concentrated by more than 100 fold with GlcNAc and α-lactose. Notably, the effect for BjFCN1-GlcNAc interaction was consistent in both calcium and calcium-free (EDTA-added) conditions, suggesting that calcium cations affect BjFCN1’s hemagglutinin activity but do not completely block its interaction with ligands.

Differential Binding Affinities of BjFCN1 to Bacteria—From the above assays, we inferred that BjFCN1 may serve as a PRR for bacteria through their surface carbohydrates. Hence, we incubated His-tagged recombinant BjFCN1 protein with bacteria and examined its binding activity by using Western blotting. We found that BjFCN1 protein could bind to all examined bacteria with different affinities in the presence of 10 mM CaCl₂ (Fig. 5A). We also noted that the binding affinity was significantly stronger for Gram-positive bacteria than for Gram-negative bacteria, except for V. anguillarum. This binding activity could be completely inhibited by adding 10 mM EDTA.

BjFCN1 protein was incubated with FITC-labeled bacteria to analyze its bacterial aggregating activity. As shown in Fig. 5B, fluorescence microscopy showed that, in the presence of cal-
BjFCN1 protein induced strong aggregation of *S. aureus* and weak aggregation of *B. subtilis*, *S. hemeolyticus*, and *E. faecalis*. The parallel experiments with purified TRX protein as control generated no such effects (Fig. 5). BjFCN1 may be a dual function receptor, not only recognizing bacteria but also aggregating them. This function is largely observed with Gram-positive bacteria.

We performed a series of experiments evaluating bactericidal and inhibitive activities, but failed to detect any such action for BjFCN1. This is in contrast with early reports (see “Discussion”), suggesting that unlike other invertebrate FBG-containing proteins, BjFCN1 could mainly function as a dedicated PRR.

The N-terminal Portion of BjMASP1/3 Forms a Complex with BjFCN1—In mammals, MASP1, 2, and 3 are major activating serine proteases for the complement lectin pathway. These MASP proteins are normally pre-coupled with collectins or ficolins (collectin/ficolin-MASP complexes) in humoral fluid. When collectins or ficolins are engaged with pathogens, the coupled MASPs are activated to cleave C4 (a paralog of C3). Amphioxus has an ortholog of vertebrate MASP1/3 gene (41). As stated previously, the mRNA of BjMASP1/3, BjFCN1, and BjC3
were co-localized and concentrated in the gut and the gonads, and were significantly up-regulated following bacterial infection. As described, BjFCN1 recognized and aggregated bacteria without showing bactericidal activity, which suggested that BjFCN1 relies on other downstream pathways to clear bacteria. The presence of a COL region suggests that BjFCN1 may complete its function by recruiting humoral MASPs. Prompted by this, we designed a GST pull-down assay to determine if there was physical interaction between BjMASP1/3 and BjFCN1.

Similar to mammalian MASPs (42, 43), full-length recombinant BjMASP1 and 3 are large and insoluble, so we expressed the N-terminal portion and the C-terminal domain of BjMASP1/3 separately (Fig. 6A). The GST-tagged N-terminal portion of BjMASP1/3 (designated BjMASP1/3-N) was purified and had a molecular mass of ~55 kDa, corresponding to the predicted molecular mass (Fig. 6B). Recombinant BjMASP1/3-N protein was mixed with His-tagged recombi-
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**A**

![Architecture of amphioxus BjC3 and human HmC3.](image)

**B**

![Binding activity of humoral BjC3 to bacteria.](image)

**C**

![Bacterial binding analysis of humoral BjC3.](image)

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A nont BjFCN1 protein and incubated with glutathione–Sepharose™ 4B beads. The bound BjFCN1 protein co-pulled with BjMASP1/3-N using the beads was analyzed by Western blotting. It showed that BjFCN1 can clearly form a complex with BjMASP1/3-N (Fig. 6C, GST-tagged human rhinovirus 3C protease protein (GST-3C) did not bind with BjFCN1.

The C-terminal Protease Domain of BjMASP1 Cleaves Humoral BjC3—The His-tagged C-terminal protease domain of BjMASP1 (designated BjMASP1-C) was expressed and purified and found to be ~37 kDa, corresponding to the predicted molecular mass (Fig. 6D). As does HmC3, BjC3 contains a β chain and an α chain, which join together via disulfide bond. The α chain starts with the ANA domain, which can be cleaved off to form the C3a and C3b fragments (the BjC3 structure shown in Fig. 7A). To assess whether BjMASP1-C catalyzes the cleavage of humoral C3 in amphioxus, we expressed a portion of C3 protein (containing the C-terminal of the β chain and the N-terminal of the α chain) and raised a monoclonal Ab (anti-BjC3 mAb) against it (Fig. 7A). The binding site for this mAb was located in the C-terminal of the β chain (Fig. 7A). Subsequently, we extracted fresh humoral fluid, which is known to contain a high concentration of C3 proteins, from adult amphioxus. The humoral fluid and the BjMASP1-C protein were mixed and incubated for 2 h in the presence of calcium or EDTA. The mixture was analyzed by Native PAGE and SDS-PAGE under non-reducing conditions, and anti-BjC3 mAb was used in Western blotting as a negative control. The incubation of humoral fluid alone, humoral fluid with GST-tagged BjMASP1/3-N protein, and humoral fluid with BSA were conducted in parallel. These experiments demonstrated that in the presence of BjMASP1-C proteins, but not in other controlled conditions, the molecular size of BjC3 was altered, and the cleavage of humoral BjC3 was accelerated (Fig. 7B). As indicated by Native PAGE, the migration of BjC3b did not correspond to its molecular mass and lagged behind intact BjC3 (Fig. 7B, left panel). Amphioxus MASP1/3 have acquired the function that all mammalian MASP1/3 share, that is, to bridge ficolins and C3 during complement activation.

DISCUSSION

In vertebrates, the ficolin pathway is a combination of ficolins and over ten other proteins, including MASPs (MASP1, 2, 3), C3-like proteins (C3, C4, C5), and several regulatory components (45). Only two unambiguous orthologs of these vertebrate genes have been found in amphioxus, including MASP1/3 and C3 (12). However, the structural and functional resemblance between BjFCN1 and vertebrate ficolins raises the possibility that amphioxus could be a PRR mainly for Gram-positive bacteria, and is capable of activating the complement pathway to destroy bacteria. Hence, BjFCN1 structurally and functionally resembles vertebrate ficolins.
sibility that there exists a functional ficolin pathway in amphioxus. We have expressed BjMASP1/3 recombinant protein and successfully demonstrated that it is able to interact with both BjFCN1 and BjC3, suggesting a BjFCN1-BjMASP1/3-BjC3 pathway in amphioxus (Fig. 8). Although this amphioxus counterpart is simplified and rudimentary compared with the full-fledged vertebrate ficolin pathway, it suggested that the basic framework of the ficolin pathway had been established since the radiation of chordate phylum. Furthermore, because of the apparent lack of ficolins in basal deuterostomes (12, 46), this pathway may represent a prototype of vertebrate ficolin-mediated complement pathway in the evolution of the complement system.

The formation of the sophisticated human complement system appears to be a slow and gradual process with intermittent leaps. The core complement component, C3-like proteins, are ancient immune effectors present in most metazoans (47–49), whereas other key components seem to have arisen with deuterostomes. Factor B is a symbol of the alternative pathway, whereas other key components seem to have arisen with deuterostomes (12, 46), this pathway may represent a prototype of vertebrate ficolin-mediated complement pathway in the evolution of the complement system.

Our works based on amphioxus have helped to piece together this story and provide more detail. In a previous genomic survey, we demonstrated that amphioxus possesses all major complement components, including collectins, ficolins, C1q proteins, MASPLike serine proteases, factors B, C3, C6, etc (12). In a subsequent transcriptomic analysis, we suggested that these genes work together through their co-regulation patterns (6). In the current study, we demonstrated that some of these genes have been organized into a functional ficolin pathway. Hence, evidence leads to the hypothesis that the framework of the modern vertebrate complement system has been established in the most recent common chordate ancestor. Following this hypothesis, we envisage that with further evolution, two rounds of whole genome duplication at the origin of vertebrates increased the gene number and made this system more intricate; and, in the jawed vertebrate lineage, the system was further involved in adaptive immunity by contributing one type of lectin receptor (i.e. C1q) to form the classical pathway.

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