Cloning and Characterization of Novel Ficolins from the Solitary Ascidian, *Halocynthia roretzi*  

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Ficolins are animal lectins with collagen-like and fibrinogen-like domains. They are involved in the first line of host defense against pathogens. Human ficolin/P35 as well as mannose-binding lectin (MBL) activates the complement lectin pathway in association with MBL-associated serine proteases. To elucidate the origin and evolution of ficolins, we separated ~40 kDa (p40) and ~50 kDa (p50) N-acetylglucosamine-binding lectins from hemolymph plasma of the solitary ascidian. Binding assays revealed that p40 recognizes N-acetyl groups in association with a pyranose ring and that p50 recognizes N-acetylglucosamine alone. Based on the amino acid sequences of the proteins, we isolated two clones each of p40 and p50 from the ascidian hepatopancreas cDNA and determined the entire coding sequences of these clones. Because all of the clones contained both collagen-like and fibrinogen-like domains, we concluded that these were homologs of the mammalian ficolin family and designated ascidian ficolins (AsFCNs). The fibrinogen-like domain of the AsFCNs shows 45.4–52.4% amino acid sequence identity with the mammalian ficolin family. A phylogenetic tree of the fibrinogen-like sequences shows that all the fibrinogen-like domains may have evolved from a common ancestor that branched off an authentic fibrinogen. These results suggest that AsFCNs play an important role with respect to ascidian hemolymph lectin activity and the correlation of different functions with binding specificity.

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Lectins play an important role in innate immunity in animals. Among them, collectins and ficolins have structural and functional similarities. Collectins are characterized as lectins with a collagen-like domain and a carbohydrate-recognition domain and include mannose-binding lectin (MBL)$(1)$, SP-A, SP-D$(2)$, conglutinin, and CL-43$(3)$. These binding specificities for carbohydrates vary, and calcium ions are required for binding. MBL in serum has an opsonic activity$(4)$ and the capacity to activate complement$(5)$. MBL is associated with serine proteases, termed MBL-associated serine proteases (MASPs), and upon binding of MBL through its carbohydrate-recognition domain to carbohydrates such as mannose or N-acetylgalactosamine (GlcNAc) of pathogens, MASPs cleave complement components$(6–9)$. Complement activation by MBL in association with MASPs is called the lectin pathway$(7,10)$.  

Ficolins are a group of proteins that consist of a collagen-like domain and a fibrinogen-like domain. They were originally identified as transforming growth factor-$\beta$-binding proteins on porcine uterus membrane$(11)$. Ficolins have been identified in mammals including human$(12–15)$, rodents$(16,17)$, pigs$(11)$, and hedgehog$(18)$ and have tissue-specific distributions. Serum ficolins from human, mouse, and pig are lectins with a common binding specificity for GlcNAc$(12,15–17)$. It is likely that the fibrinogen-like domain of serum ficolins is responsible for carbohydrate binding$(19)$. In human serum, two types of ficolin, named ficolin/P35 (ficolin L)$(12,14)$ and ficolin H (Hakata antigen)$(15)$, have been identified and both of them have lectin activity. Another ficolin, termed ficolin M$(14)$, or P35-related protein$(13)$, whose mRNA is found in leukocytes and lung is not considered to be a serum protein. Recently it has been reported that ficolin M might act as a phagocytic receptor or adaptor on circulating monocytes for microorganism recognition$(12)$. Ficolin/P35 acts as an opsonin and enhances phagocytosis of *Salmonella typhimurium* by neutrophil$(20)$. It also activates the lectin complement pathway in association with MASPs$(21)$. These findings suggest that ficolins are involved in host defense through innate immunity in the vertebrate.

The molecular details of invertebrate innate immune systems and their possible connection to vertebrate innate immunity have not been fully elucidated. Ascidians occupy a pivotal intermediary position between invertebrates and vertebrates. Therefore, studies on the host defense mechanisms of ascidian could provide us with important information about the evolution of a primitive innate immune system of vertebrates. The recent identification of cDNA encoding the third component of complement (AsC3)$(22)$, two types of MASP (AsMASPa and AsMASPb)$(23)$, and glucose-binding lectin (GBL)$(24)$ suggests that the lectin complement pathway is present in ascidian, *Halocynthia roretzi*. This implies that lectins homologous to vertebrate MBL or ficolins might exist in ascidian. Here we describe the isolation and cloning of GlcNAc-binding lectins with a collagen-like domain and a fibrinogen-like domain, designated as ascidian ficolins (AsFCNs), from hemolymph plasma of the solitary ascidian, *H. roretzi*.  

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The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AB049619, AB049620, AB049621, and AB049622.

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†The abbreviations used are: MBL, mannose-binding lectin; BSA, bovine serum albumin; AsFCN, ascidian ficolin; MASP, MBL-associated serine protease; PAGF, polyacrylamide gel electrophoresis; PSIL, photostimulated luminescence; PDDP, polyvinylidene difluoride; DPPE, dipalmitoyl phosphatidylethanolamine; GlcNAc, N-acetylgalactosamine; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; ORF, open-reading frame.
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EXPERIMENTAL PROCEDURES

Materials and Reagents—The solitary ascidian, *H. roretzi*, was obtained from local dealers in Fukushima, Japan. Sepharose 4B was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Acetylated Sepharose 4B was prepared by treating Sepharose 4B with 0.1 M HCl at 50 °C for 3 h. GlcNAc-agarose, fetuin, asialofetuin, bovine serum albumin (BSA) conjugated with lactose (Lac-BSA), and cellulose (Cel-BSA) were purchased from Sigma (St Louis, MO); BSA conjugated with N-acetylgalactosamine (GalNAc-BSA), N-acetyllactosamine (LacNAc-BSA), and NeuAc2-3-N-acetyllactosamine (Sia-LacNAc-BSA) were purchased from Dextra Laboratories (UK); neoglycoproteins of Man-BSA, Glc-BSA, and Gal-BSA; and hybrid-type oligosaccharides (27); human fetuin and human asialofetuin, both conjugated to BSA with lactose (Lac-BSA), and cellobiose (25, 26); RNase B, high mannose-type oligosaccharides, and asialofetuin and the neoglycoproteins lactose-conjugated BSA (Lac-BSA) were purchased from Sigma (St Louis, MO); BSA conjugated with lactose (Lac-BSA), and cellobiose (25, 26); RNase B, high mannose-type oligosaccharides, and RNase B, high mannose-type oligosaccharides; RNase B, high mannose-type oligosaccharides; GlcNAc-agarose, fetuin, asialofetuin, bovine pancreatic RNase B, human fetuin, and human asialofetuin, both conjugated to BSA with lactose (Lac-BSA), and the neoglycoproteins lactose-conjugated BSA (Lac-BSA) were used for binding assays. In each neoglycoprotein, ~19–42 mol of carbohydrate were conjugated to 1 mol of BSA. Ovulamin contains high mannose-type and hybrid-type oligosaccharides (25, 26); RNase B, high mannose-type oligosaccharides (27); human fetuin and human asialofetuin, both complex-type oligosaccharides and O-linked oligosaccharides (28, 29). Glycoproteins and neoglycoproteins were dissolved in phosphate-buffered saline at the concentrations indicated in the figures. 100 µl of each solution was dot-blotted onto a PVDF membrane (Millipore, Bedford, MA) using an Immunodot filter apparatus (ATTO, Tokyo, Japan). The membranes were blocked for 1 h at 4 °C without or with 1% (w/v) BSA, 0.1% SDS, and 200 mM NaCl at pH 7.8, containing 50 mM NaCl, 5 mM CaCl2, and then chromatographed on a Mono Q column. After eluting the column with starting buffer, two major proteins designated p40 and p50 were eluted with starting buffer containing 150 mM GlcNAc. The eluted fractions were dialyzed against 25 mM Tris-HCl, pH 7.8 containing, 50 mM NaCl, 5 mM CaCl2, and then chromatographed on a Mono Q column, followed by elution with a linear NaCl gradient to 0.5 M. The preparation was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using the Laemmli protocol.

Binding Assay of p40 and p50 with Glycoproteins and Neoglycoproteins—The ascidian ficolin-like proteins (p40 and p50) were labeled with Na125I and IODO-GEN from Pierce, Rockford, IL. The glycoproteins and neoglycoproteins were preincubated for 1 h at 4 °C without or with 1, 10, or 100 mM 125I-labeled p40 (400,000 cpm/ml) or 125I-labeled p50 (350,000 cpm/ml) in Buffer A containing 0.1% (w/v) BSA. After the incubation, the membrane was washed with Buffer A and air-dried. Binding of the radioactive ascidian lectin was measured using a Bio-imaging analyzer BAS-1800II (Fujifilm, Tokyo, Japan) and is indicated as the intensity of the photostimulated luminescence (PSL).

For inhibition assays, 100 µl of each GlcNAc-BSA solution (0.0002–5.0 µg/100 µl) was dot-blotted onto a PVDF membrane. The membranes were blocked and rinsed as described above. Labeled p40 and p50 were incubated for 1 h at 4 °C without or with 1, 10, or 100 mM GlcNAc, 10 mM sodium acetate, or 10 mM N-acetylglycine in Buffer A containing 0.1% (w/v) BSA. The mixture was then overlaid on the membrane and binding of the radioactive lectin to the GlcNAc-BSA was determined as described above.

Binding Assay of p40 with Neoglycolipids—Neoglycolipids were prepared by reductive amination from dipalmitoyl phosphatidylethanolamine (EPC) preincubated for 1 h at 4 °C without or with 1, 10, or 100 mM GlcNAc, 10 mM sodium acetate, or 10 mM N-acetylglycine in Buffer A containing 0.1% (w/v) BSA. The mixture was then overlaid on the membrane and binding of the radioactive lectin to the GlcNAc-BSA was determined as described above.
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RESULTS

Purification of Ascidian Ficolin-like Lectins, p40 and p50—The hemolymph plasma was subjected to affinity chromatography on a GlcNAc-agarose column, and ascidian ficolin-like lectins were eluted with GlcNAc like human ficolin/P35. Two major proteins with molecular masses of ~40 and ~50 kDa by SDS-PAGE under reducing conditions were found in the eluate from GlcNAc-agarose column (Fig. 1A) and were tentatively designated p40 and p50, respectively. Further purification was achieved by ion-exchange chromatography on a Mono Q column. As shown in Fig. 1B, each fraction of purified p40 and p50 showed a single band by SDS-PAGE under reducing conditions. Under non-reducing conditions, p40 and p50 are resolved mainly into 180 and 240 kDa components and 150, 300, and 450 components, respectively. Two-dimensional electrophoresis indicated that these proteins could be reduced to ~40 kDa and ~50 kDa, respectively (data not shown). These results suggest that p40 and p50 are composed of subunits linked to form homopolymers via disulfide bonds, as is the case with human ficolin/P35.

Binding Specificity of p40 for Glycoproteins, Neoglycoproteins—To investigate the lectin activity of p40, its binding specificity for various glycoproteins and neoglycoproteins was evaluated. In a binding assay with neoglycoprotein, p40 bound GlcNAc-BSA, GalNAc-BSA, and Sia-LacNAc-BSA but not to the other neoglycoproteins (Fig. 2A). In particular, p40 recognized GlcNAc with the most sensitivity (GlcNAc > GalNAc > Sia-LacNAc). Furthermore, it was notable that p40 did not bind to LacNAc-BSA with carbohydrate structure of Galβ1–4GlcNAc, suggesting that this lectin can recognize non-reducing terminal carbohydrate residues carrying N-acetyl group such as GlcNAc, GalNAc, and NeuAc. Another binding assay was performed with natural glycoproteins, i.e. ovalbumin (OVA) with high mannose-type and hybrid-type oligosaccharides, bovine RNase B (RN) with only high mannose-type oligosaccharids, human fetuin and human asialofetuin, both with complex-type oligosaccharides and O-linked oligosaccharides. The results showed that p40 bound to fetuin, which has oligosaccharides with N-acetylneuraminic acid at their non-reducing termini (Fig. 2B). Furthermore, one of the simplest chemicals containing an N-acetyl group, N-acetylglycine, inhibited p40 binding to GlcNAc-BSA (Fig. 2C).

We next performed similar binding assays using neoglycolipids prepared by reductive amination from dipalmitoyl phosphatidylethanolamine (DPPE) and the following carbohydrates as described previously (30): GlcNAc, GlcNAc2, GlcNAc3, GlcNAc4, GlcNAc5, GlcNAc6, and GlcNAc3/2Man0–1/6-GlcNAc91–2Man0–1/3Man (GlcNAc2Man3). These conjugates were chromatographed on a TLC plate, which was then used for a binding assay with 125I-labeled p40. As shown in Fig. 2D, radiolabeled p40 bound to all the neoglycolipids with the exception of GlcNAc-DPPE. The failure of p40 to bind to GlcNAc-DPPE may be caused by a structural change in the pyranose ring by reductive amination from DPPE. These results suggest that carbohydrate recognition by p40 may require not only an N-acetyl group but also an intact pyranose ring structure.

Amino Acid Sequence Analyses of p40 and p50—The NH2-terminal amino acid sequences of p40 (27 amino acids) and p50 (24 amino acids) were determined. These sequences showed no significant similarity to other known proteins. In p40, the amino acid sequence, HNEDLXTGLPNQLQEHXSLEPSGVIE, was obtained and an additional weak arginine (R) and threonine (T) appeared at positions 1 and 22 (shown in bold) of the mature protein, respectively. After fragmentation of p40 and p50 with S. aureus V8 protease, partial internal amino acid sequences were also determined. The NH2-terminal sequence (VYCDLTSDGGWTVFQRMDGSVDF) of one of the peptides from p40 was the same as that of a peptide from p50. This sequence has significant similarity to a portion of the fibrogen-like sequences in mammalian ficolin, tenasin and fibrinogen.

cDNA Cloning of p40 and p50—Using degenerated primers prepared based on the NH2-terminal amino acid sequences of p40 and p50, and those of the fragmented peptides, we performed nested PCR. The cDNA sequence completed by 5’- and 3’-RACE showed that p40 cDNA contains a 972-base pair open-reading frame (ORF) encoding 324 amino acids preceded by a leader peptide of 17 amino acids, which we designated AsFCN1 (Fig. 4). The predicted molecular size of mature AsFCN1 was calculated to be 34,799 Da. In the cDNA cloning of AsFCN1, another cDNA clone was identified with hepatopancreas cDNA. This cDNA clone, termed AsFCN2, encodes 324 amino acids, which had 92.6% identity with AsFCN1 and had the same domain structure (Fig. 4). The p50 cDNA contains a 1,068-base pair ORF encoding 356 amino acids preceded by a leader peptide of 21 amino acids. The predicted molecular size of mature p50, designated AsFCN3, was calculated to be 38,229 Da.
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Fig. 2. Carbohydrate recognition by p40. A, binding of p40 to neoglycoproteins. Nine neoglycoproteins, BSA conjugated with N-acetylglucosamine (GlcNAc-BSA), N-acetylgalactosamine (GalNAc-BSA), N-acetyllactosamine (LacNAc-BSA), NeuAc α2–3 N-acetyllactosamine (Sia-LacNAc-BSA), mannose (Man-BSA), glucose (Glc-BSA), galactose (Gal-BSA), lactose (Lac-BSA), and cellobiose (Cel-BSA) were dot-blotted onto a PVDF membrane in a volume of 100 μl each at the concentration indicated. The membrane was incubated with 125I-labeled p40 and then analyzed for p40 binding. The level of binding to each dot is indicated as intensity of the photostimulated luminescence (PSL). B, inhibition of p40 binding by GlcNAc. The neoglycoprotein, GlcNAc-BSA in a volume of 100 μl was dot-blotted onto a PVDF membrane at the concentration indicated. 125I-labeled p50 was preincubated without (Control) or with 1, 10, or 100 mM GlcNAc and then subjected to the binding assay.

Fig. 3. Carbohydrate recognition by p50. A, binding of p50 to neoglycoproteins. Nine neoglycoproteins were dot-blotted onto a PVDF membrane in a volume of 100 μl at the concentration indicated, as described in the legend to Fig. 2. The membrane was incubated with 125I-labeled p50 and then analyzed for p50 binding. The level of binding to each dot is indicated as intensity of the photostimulated luminescence (PSL). B, inhibition of p50 binding by GlcNAc. The neoglycoprotein, GlcNAc-BSA in a volume of 100 μl was dot-blotted onto a PVDF membrane at the concentration indicated. 125I-labeled p50 was preincubated without (Control) or with 1, 10, or 100 mM GlcNAc and then subjected to the binding assay.

overall amino acid sequence identity between AsFCN1 and AsFCN3 was 69.3%. In the cDNA cloning of AsFCN3, we also identified another clone, termed AsFCN4. AsFCN4 cDNA contains a 1023-base pair ORF encoding 341 amino acids, which has 76.2%, 69.2, and 70.9% identities to AsFCN1, 2, and 3, respectively.

Because AsFCN1 and AsFCN3 contained three and four potential N-linked glycosylation sites, respectively, the molecular sizes observed on SDS-PAGE seem to result from glycosylation. Both AsFCN1 and AsFCN3 have a short collagenous sequence, five Gly-X-Y triplet repeats (where X and Y represent any amino acid) and a COOH-terminal fibrinogen-like domain, suggesting ficolin-like lectins. As shown in Fig. 4, alignment of the amino acid sequences of these four AsFCNs indicated that they are closely similar to each other, especially in their fibrinogen-like domains. The fibrinogen-like domain sequences of the AsFCNs showed from 45.4 to 52.4% identity with those of human ficolins.

Northern Blot Hybridization of AsFCNs—Because the fibrinogen-like domains of the AsFCNs are highly homologous, the 5′-UT sequences of AsFCN1, 3, and 4 were used as probes in Northern blots. As shown in Fig. 5, the major transcripts of AsFCN1, 3, and 4 expressed in the hepatopancreas are about 1.3, 1.4, and 1.3 kilobases long, respectively. From a comparison with the signal intensities, it appears that AsFCN3 and 4 are the major products in ascidian hepatopancreas. The faint signal observed in the AsFCN1 blot could result from both AsFCN1 and 2 transcripts because the sequences of AsFCN1 and 2 are very similar. No detectable signals of AsFCNs were observed in Northern blots with the poly(A) fraction from ascidian hemocytes (data not shown).

Phylogenetic Tree of Ficolins, Fibrinogen-like Domain-bearing Proteins and Fibrinogens—The phylogenetic relationships between AsFCNs and other fibrinogen-like proteins were analyzed by neighbor-joining trees using the regional amino acid sequences of their fibrinogen-like domains. As shown in Fig. 6, all of the sequences in fibrinogen-like proteins examined such
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**DISCUSSION**

In this study, we isolated two novel ascidian lectins present in hemolymph from a solitary ascidian, *H. roretzi* by affinity chromatography using GlcNAc-agarose followed by chromatography on Mono Q, and cloned four lectins from cDNA libraries using GlcNAc-agarose followed by chromatography on Mono Q, and cloned four lectins from cDNA libraries.

As FcNs, mammalian ficolins, tenascins, angiopoietins, and a horseshoe crab tachylectins roughly formed a single large branch. In this tree, four AsFCNs formed a tight cluster. The branch of the AsFCNs did not originate directly from that of the mammalian ficolins, which also formed independently a tight branch, making it difficult to define their origin. Members of the tenascin family also formed a unique branch between the branches of the AsFCNs and the mammalian ficolins. The phylogenetic relationship of these three groups is not convincing, because the relevant bootstrap values are not high enough to support the branching definitely. The fibrinogen-like sequences of AsFCNs were slightly more similar to those of tenascins, angiopoietins, and mammalian ficolins, which also formed independently a tight cluster (data not shown). These results suggest that all the fibrinogen-like domains may have evolved from a common ancestor that branched off an authentic fibrinogen. In this tree, four AsFCNs form a group that is independent of the mammalian ficolin family. Another phylogenetic tree, which was constructed based on the entire amino acid sequences of AsFCNs and the mammalian ficolin family, indicated that the AsFCNs again branched from a common ancestor. The fibrinogen-like domains indicated that the fibrinogen-like proteins formed a cluster independent of the primary fibrinogen sequences (Fig. 6), suggesting that all the fibrinogen-like domains have evolved from a common ancestor that branched off an authentic fibrinogen.

Fig. 3. Northern blotting analysis of AsFCNs. A membrane filter containing 2 μg of poly(A)^+ fraction from hepatopancreas was hybridized with ^32P-labeled cDNA fragments (a, AsFCN1 nucleotides 7–326; b, AsFCN3 nucleotides 14–326; c, AsFCN4 nucleotides 69–384).
the same except for two amino acids, histidine (H) and serine (S) of AsFCN1, and arginine (R) and threonine (T) of AsFCN2, respectively (Fig. 4). The latter two amino acids were also recognized as an additional signal in the amino acid sequence analysis of p40. Therefore, it is possible that p40 contains both AsFCN1 and AsFCN2. With Northern blot analysis, we showed major expression of AsFCN1, 3 and 4 transcripts in ascidian hepatopancreas (Fig. 5). In this experiment, we could not design a specific probe for AsFCN2 because of its high similarity with AsFCN1. The message obtained with the AsFCN1 probe contained that of AsFCN2. Fig. 5 shows lower expression of AsFCN1/2 in hepatopancreas, which corresponds to the quantity of p40 in ascidian hemolymph (Fig. 1A). On the other hand, the p50 transcript seems identical to that of the AsFCN3 transcript since a different size of AsFCN4 transcript is observed in the p50 transcript seems identical to that of the AsFCN3 transcript. On the other hand, the binding specificity of p50 (AsFCN3) involves recognition of GlcNAc alone (Fig. 3). Thus, each fibrinogen-like domain of AsFCNs has a lectin activity associated with a different sugar specificity. Although the AsFCNs have much shorter collagen-like sequences than the mammalian ficolins, the present study elucidated the structural and functional features common to the ascidian and mammalian ficolins, including domain structure, multimeric property, and lectin activity.

In the primitive complement system of ascidian, the activation pathway consisting of at least of C3 (21), factor B (36), and MASPs (22) has been demonstrated and corresponds to the mammalian alternative and lectin pathways. Although an ascidian MBL homolog has not been fully identified, we have identified a similar lectin, termed glucose-binding lectin (GBL), which lacks a collagen-like domain in its NH2-terminal region and which specifically recognizes glucose residues (23). GBL is associated with ascidian MASPs that activate ascidian C3 (21). Recently, we showed that human ficolin/P35, associated with ascidian MASPs (22) has been demonstrated and corresponds to the ascidian and mammalian ficolins, including domain structure, multimeric property, and lectin activity.
Thus, the identification of AsFCNs described in this report may contribute to the clarification of the primitive complement system.

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