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Cloning and Characterization of Mannose-Binding Lectin from Lamprey (Agnathans)\(^1,2\)

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The recognition of pathogens is mediated by a set of pattern recognition molecules that recognize conserved pathogen-associated molecular patterns shared by broad classes of microorganisms. Mannose-binding lectin (MBL) is one of the pattern recognition molecules and activates complement in association with MBL-associated serine protease (MASP) via the lectin pathway. Recently, an MBL-like lectin was isolated from the plasma of a urochordate, the solitary ascidian. This ascidian lectin has a carbohydrate recognition domain, but the collagen-like domain was replaced by another sequence. To elucidate the origin of MBLs, the aim of this study is to determine the structure and function of the MBL homolog in lamprey, the most primitive vertebrate. Using an N-acetylgalcosamine (GlcNAc)-agarose column, MBL-like lectin (p25) was isolated from lamprey serum and cDNA cloning was conducted. From the deduced amino acid sequence this lectin has a collagenous region and a typical carbohydrate recognition domain. This lectin also binds mannose, glucose, and GlcNAc, but not galactose, indicating that it is structurally and functionally similar to the mammalian MBLs. Furthermore, it associated with lamprey MASP.s, and the MBL-MASP activated lamprey C3 in fluid-phase and on the surface of pathogens. In conjunction with the phylogenetic analysis, it seems likely that the lamprey MBL is an ortholog of the mammalian MBL. Because acquired immunity seems to have been established only from jawed vertebrates onward, the lectin complement pathway in lamprey, as one of the major contributors to innate immunity, plays a pivotal role in defending the body against microorganisms. The Journal of Immunology, 2006, 176: 4861–4868.

Immunity to infection is mediated by two general systems, acquired (or adaptive) and innate (or natural). Innate immunity was formerly thought to be a nonspecific immune response characterized by phagocytosis. However, innate immunity has considerable specificity and is capable of discriminating between pathogens and self (1). The complement system that consists of three activation pathways is engaged in both acquired and innate immunity to infection (2). MBL belongs to the collectin family of proteins (3). Human MBL has an apparent molecular mass of \(300 - 650\) kDa and consists of 32–18 monomeric subunits of \(30 - 65\) kDa each. Each subunit contains an N-terminal region rich in cysteine, a collagen-like domain, and a carbohydrate recognition domain (CRD). Through its CRD, MBL binds carbohydrates with 3- and 4-hydroxyl groups in the pyranose ring in the presence of Ca\(^{2+}\) (4, 5). Prominent ligands for MBL are mannose and N-acetylgalcosamine (GlcNAc), whereas carbohydrates that do not fit this steric requirement have undetectable affinity for MBL. This steric selectivity of MBL, along with differences in the spatial organization of the ligands, allows for the specific recognition of carbohydrates on pathogenic microorganisms including bacteria, fungi, parasitic protozoans, and viruses and avoids recognition of self (5).

Accumulating evidence indicates that adaptive immunity was established at an early stage in the evolution of the jawed vertebrates. The complement system has a more ancient origin, and all major invertebrate deuterostome groups so far studied, sea urchin, ascidians and amphioxus, as well as jawless vertebrates such as lamprey and hagfish have this system (6). Because these animals are believed to have diverged before the emergence of jawed vertebrates, their complement systems are expected to be simpler than those of higher vertebrates. Recent biochemical identification of several components of the lectin pathway from solitary

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2 The nucleotide sequence reported in this paper has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number AB195797.

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4 Abbreviations used in this paper: MBL, mannose-binding lectin; MASP, MBL-associated serine protease; GBL, glucose-binding lectin; CRD, carbohydrate recognition domain; GlcNAc, N-acetylgalcosamine.
ascidian, *Halocynthia roretzi*, revealed that the primitive complement system consisting of lectin-MASP complex, C3 and C3 receptor, functions in an opsonic manner (14–18). An MBL-like lectin and ficolins were identified as the recognition molecules of the lectin pathway in ascidians. The purified MBL-like lectin binds specifically to glucose and was designated glucose-binding lectin (GBL). Sequence analysis of GBL reveals that the C-terminal half contains a CRD that is homologous to C-type lectins, but the collagen-like domain was replaced by another sequence (17). These results raise the possibility that GBL evolved early as a prototype of MBL. To test this hypothesis, we focused on lamprey (agnathans), one of the most primitive vertebrates lacking an adaptive immune system. In this study, we describe an ortholog of mammalian MBL with a collagens region and a typical CRD in lamprey. This lamprey MBL is associated with serine proteases of the MASP family. When lamprey MBL recognizes yeast as the pathogens, the MBL-MASPs activate C3, a key component of the complement system.

**Materials and Methods**

**Purification of lamprey MBL and C3**

Lampreys, *Lampetra japonica*, were obtained from local dealers in Fuku-shima, Japan. Serum from lampreys was applied to GlcNAc-agarose (Sigma-Aldrich) equilibrated with Tris buffer (50 mM Tris, 150 mM NaCl, and 20 mM CaCl₂). After the column had been washed with starting buffer, elution was conducted with 0.3 M mannose-containing buffer. The eluted fractions were dialyzed against 25 mM Tris-HCl (pH 7.8), containing 50 mM NaCl and 5 mM CaCl₂, and then chromatographed on a Mono Q column (Amersham Biosciences), before being eluted with a linear NaCl gradient to 0.5 M. The preparation was analyzed by SDS-PAGE using the Laemmli system and proteins were stained with Coomassie brilliant blue R-250. Collagenase digestion was conducted by incubating 10 mg of lamprey C3 in 50 mM Tris, 150 mM NaCl and 10 mM CaCl₂ (pH 7.5) at 37°C for 3 h. Lamprey C3 was purified according to published methods (19) with a modification. Briefly, lamprey serum was precipitated with poly-ethylene glycol and subjected to ion-exchange chromatography, followed by gel filtration. To examine C3 activation, 8 mg of lamprey C3 was incubated with various amounts of MBL-MASPs in 50 mM Tris, 150 mM NaCl, and 10 mM CaCl₂ (pH 7.5) at 37°C for 30 min and the reaction mixture was then subjected to SDS-PAGE.

**Preparation of Abs**

Monospecific antiserum to lamprey p25 (MBL) or lamprey C3 was raised by immunizing rabbits with purified proteins in CFA. IgG of anti-C3 Ab was absorbed with A-Sepharose. To remove the natural Abs to yeast for FACS analysis, both Abs were absorbed with an excess amount of yeast. The Abs to MASP(s) were also prepared by immunizing rabbits with synthetic peptides of MASP-A H chain, and recombinant L chain peptides of MASP-B and MASP-1.

**Amino acid sequence analysis**

The N-terminal amino acid of lamprey p25 was blocked. Therefore, its internal amino acid sequence was determined. After lamprey p25 was digested with collagenase as described, digested products were subjected to SDS-PAGE under reducing conditions and then electroblotted onto polyvinylidene difluoride membranes (Millipore). The protein bands were stained, excised, and then analyzed using a protein sequencer (model 476A; Applied Biosystems). Lamprey p25 was also digested with *Staphylococcus aureus* V8 protease (Sigma-Aldrich) according to the method in the Cleveland study (20). Digested peptides were electroblotted and analyzed as described.

**Cloning of p25 cDNA**

The liver and various tissues were removed immediately before use. RNA was isolated from these tissues using the acid guanidine thiocyanate method, and the poly(A)⁺ fraction was purified by passage through an oligo(dT) cellulose column (Clontech Laboratories). cDNAs were prepared from liver RNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies). Four degenerated primers were synthesized based on the amino acid sequence that had been determined for the products of lamprey MBL, digested with collagenase and on the conserved sequences of the MBL family: AKGEKGE (5′-YYMDGGGRSMRAARGGRGA-3′), WNDVPCS (5′-KDRCAKRNYRTCRITCCA-3′), KGDKGDA (5′-AARGGNAYAARGNGAYGC-3′), and NWNDGEPN (5′-TTKG GYTCYYHYKNTCCARTT-3′). A part of the p25 cDNA was amplified by a nested RT-PCR using lamprey liver cDNA as a template and two primer sets (5′-YYMDGGGRSMRAARGGRGA-3′ and 5′-KDRCAKRNYRTCRITCCA-3′) for the first; (5′-AARGGNAYAARGNGAYGC-3′ and 5′-TTKG GYTCYYHYKNTCCARTT-3′) for the second. PCR products of the expected size were cloned into pGEM-T easy vector (Promega) and sequenced by the dideoxy method using an autosequencer (model 4000; LI-COR). The subcloned 321-bp DNA was 32P-labeled and used as a probe for screening a ZAP cDNA library. A total of 2 × 10⁶ plaques of a liver ZAP II cDNA library were screened. Positive clones were subcloned in pBluescript II (SK⁺) by in vivo excision (Stratagen) and sequenced by the method previously described.

**Northern blot hybridization and RT-PCR**

A membrane filter blotted with 0.4 µg of poly(A)⁺ RNA from various tissues of lamprey was hybridized with a 32P-labeled specific cDNA fragment of the lamprey MBL (nucleotides 49–666) at 42°C overnight in 50% formamide, 5× Denhardt’s solution, 5× SSPE (1× SSPE is 9 mM sodium phosphate, 150 mM NaCl, and 1 mM EDTA (pH 7.4)), 0.5% SDS, and 200
to prepare liposomes containing the neoglycolipid. The liposomes were then adsorbed to HPA sensors (Fig. 3A) at a surface plasmon resonance (SPR) level of 20,000 RU on a HPA sensor chip with a BIAcore 3000 instrument. Liposome without neoglycolipids was also adsorbed as a reference. Binding of lamprey MBL to carbohydrate moiety on the surface of the sensor chip was analyzed. All sensorgrams were recorded at a flow rate of 20 μl/min at 25°C in running buffer (10 mM Tris-HCl (pH 7.3), containing 150 mM NaCl and 10 mM CaCl₂).

**Immunoblotting**

After SDS-PAGE (10%) gel, proteins were transferred from the gel to a polyvinylidene difluoride membrane and the blot was probed with rabbit Abs against lamprey MBL, MASP-A, MASP-B, and MASP-1. Peroxidase-conjugated anti-rabbit IgG was used as a secondary Ab and the blot was developed with tetramethylbenzidine solution (Wako Chemical).

**Flow cytometry**

The binding of lamprey MBL and C3 to yeast was analyzed by flow cytometry. Yeast (AH109) was obtained from Clontech Laboratories and the yeast cells (2 × 10⁷) in GVB (veronal buffered saline supplemented with 0.1% gelatin, 2 mM CaCl₂ and 0.5 mM MgCl₂) were incubated with lamprey serum, GlcNAc-agarose-treated serum, or/and partially purified MBL-MASPs complex as described below, and then washed three times with GVB to detect MBL binding, and with GVB supplemented with 10 mM EDTA and 0.1% gelatin (EDTA-GVB) to detect C3 binding, respectively. The washed cells were then reacted on ice for 30 min with 20 μl of anti-C3 Ab IgG (0.4 mg/ml) or 15 μl of anti-MBL antiserum and stained on ice for 30 min with 20 μl of FITC-conjugated swine anti-rabbit IgG (DakoCytomation Japan). The yeast was washed three times with PBS between each reaction. Reactivities were evaluated on a FACScan flow

*FIGURE 2.* Alignment of the entire amino acid sequence of lamprey MBL with mammalian MBL. The entire amino acid sequences of lamprey, human, rat, and mouse MBLs were aligned using Clustal W software with reference to the invariant residues of C-type lectins (31). Gaps inserted during alignment are indicated by dashes. The double-thick line shows the collagene-like sequences. Asterisks and dots below the sequences indicate the residues are conserved and similar among these six sequences, respectively. The underlined sequences were directly determined by amino acid sequence and arrows indicate the sites of the designed primers.
cytometry (BD Biosciences) and compared with controls consisting of bacteria treated with primary Abs and FITC-conjugated swine anti-rabbit IgG. Preparation of GlcNAc-agarose-treated serum and partially purified MBL-MASPs complex

Lamprey serum (400 µl) was incubated with 80 µl of GlcNAc-agarose beads on ice for 1 h in 50 mM Tris, 150 mM NaCl, and 10 mM CaCl₂ (pH 7.5) in a total of 800 µl. After washing four times with the same buffer, the beads were treated four times with 50 ml of the described buffer containing 0.3 M mannose (total 200 µl), and the eluted materials consisting of crude MBL-MASPs complex were massively dialyzed against PBS.

Results

Purification of lamprey MBL-like lectins, p25

Serum from the Lampetra japonica lamprey was subjected to GlcNAc-agarose column chromatography in the presence of Ca²⁺. The column was sequentially eluted with mannose and with GlcNAc, as described in Materials and Methods, because it is predicted that human MBL can be eluted with mannose, and L-ficolin, one of human serum ficolins, can be subsequently eluted with GlcNAc. Both of these lectins are found to complex with human serum ficolins, can be subsequently eluted with GlcNAc (23). The predicted molecular mass of the mature protein was 23,217 Da, and there is no N-linked glycosylation site. Sequence analysis revealed that it is a homolog of mammalian MBL, as it contains a collagen-like sequence in the N-terminal half and a CRD in the C-terminal half (Fig. 2). Lamprey MBL shared ∼30% identity at the amino acid level with human MBL. Northern blotting

Northern blot analysis was performed using several samples of liver, heart, gill, intestine, blood, and brain. As shown in Fig. 3A, the major transcript of lamprey MBL expressed in the liver is ∼2.6-kb long and several faint bands were observed. To clarify the nature of these faint signals, we performed RT-PCR using the same tissue samples as a template. A single band 230-bp long, which corresponds to the collagenous portion, was observed in liver, and no band was detected in the other tissues (Fig. 3B).

Phylogenetic tree of MBL and collectins

The phylogenetic tree was constructed based on the amino acid sequences of 15 members of the MBL family including lamprey MBL. As shown in Fig. 4A, lamprey MBL branches first at the root together with the ancestors for mammalian, chicken, and bony fish MBLs, suggesting an ancient origin. Interestingly, four members of bony fish MBLs form a tight cluster, although binding specificity is different among these MBLs. The mammalian MBLs form a cluster that is divided into two subgroups, MBL-A and MBL-C. To further examine the evolutionary origin of MBL, we constructed another tree including the other members of the collectin family. As shown in Fig. 4B, this tree shows clearly distinct groups for MBL, surfactant proteins SP-D, SP-A, and collectins CL-L1, CL-K1, and CL-P1, although the ascidian collectins (Grails) (28) and ascidian GBL form a subgroup separated from the MBL family. In this tree, lamprey MBL forms again a cluster together with the other MBL members, although the bootstrap percentage for its branching is not so high. Based on these results, it is likely that the lamprey MBL is an ortholog of mammalian MBL.

Binding specificity of lamprey MBL for carbohydrates

To determine the specificity of lamprey MBL binding to various carbohydrates, we used surface plasmon resonance. As shown in Fig. 5A, MBL bound to oligosaccharides composed of mannose (Man5) and GlcNAc (GN5), but not lactose (Lac), suggesting that MBL does not bind to terminal galactose. To confirm these results and to determine whether lamprey MBL binds to glucose or N-acetylgalectosamine, we performed additional experiments. The lamprey serum was applied to several carbohydrate-conjugated agarose beads, and eluted materials were analyzed by immunoblotting. The lamprey MBL binds to glucose, in addition to mannose and GlcNAc, but not to N-acetylgalectosamine (Fig. 5B).
Complex formation of MBL with MASPs, and activation of C3 by MBL-MASPs

Next, we determined whether the purified MBL fraction contained the lamprey serine proteases MASPs, which had been reported as homologs of mammalian MASPs (29). To identify MASPs in the MBL preparation, Western blotting was performed using Abs against MASP-A, MASP-B, and MASP-1. In the case of MASP-A, the H chain band (65 kDa) was observed, and in the case of MASP-B and MASP-1, the L chain bands (34 and 35 kDa) were observed (Fig. 6A). To confirm that the complex formed, the MBL preparation was subjected to molecular sieve chromatography using Sepharose 6 (Fig. 6B). In the presence of Ca\textsuperscript{2+}, the main peak appeared in the fraction around 700 kDa (10.67 ml), whereas in the presence of EDTA the main peak (10.67 ml) disappeared, and the two peaks were separately recovered in the peaks of 12.85 and 14.47 ml. By SDS-PAGE and Western blotting analyses, the main peak contained both lamprey MBL (p25) and MASPs, and in the presence of EDTA the peaks of 12.85 and 14.47 ml contained MBL and MASPs, respectively (data not shown). These results indicate that lamprey MBL associates with MASPs in the presence of Ca\textsuperscript{2+}.

In the experiments shown in Fig. 7, we examined whether the MBL-MASP complexes activated lamprey C3. The C3 \textalpha-chain was cleaved by MBL-MASPs in a dose-dependent manner, yielding an \textalpha'-chain. As previously reported (27), purified MASP-A cleaved the C3. Although the precise function of each MASP is not known, it is possible that the binding of lamprey MBL-MASPs to carbohydrates on pathogens results in C3 activation. This possibility is examined in the following experiments.

Next, we asked whether lamprey MBL bound to yeast using lamprey serum by flow cytometry. As shown in Fig. 8A, the MBL bound to yeast and its binding was inhibited by mannose. Previously, C3 in
Discussion

In this study, we isolated a novel lectin present in the serum of a lamprey, *Lampetra japonicus*, by affinity chromatography using GlcNAc-agarose followed by chromatography on Mono Q, and cloned its cDNA from cDNA libraries of the lamprey liver. The deduced amino acid sequence of the lamprey lectin has a major feature of the mammalian MBL family: it contained a collagen-like domain and a CRD. In comparison with the mammalian MBL family, its N-terminal half has 15 Gly-X-Y triplets without gaps, whereas the mammalian MBLs have 18–20 Gly-X-Y triplets that are interrupted to form a bend in the triple helix (8). Its CRD is a C-type lectin, containing 16 of the 18 highly conserved amino acid residues including four cysteine residues that are involved in disulfide bonds within the domain (30, 31). Five residues (Glu^{184}, Asn^{186}, Glu^{191}, Asn^{205}, and Asp^{206}), which have been reported to bind directly to mannose, GlcNAc, and glucose in the presence of Ca^{2+}, a considerable amount of C3 bound to yeast in the reconstitution experiments, indicating that MBL recognizes mannan of yeast and the associated MASPs cleavage C3 into C3b that is deposited on their surfaces and acts as an opsonin. From these results, it is concluded that MBL is one of the lectins that act as a recognition molecule in the lectin pathway of the lamprey complement system.

(lamprey serum was reported to binds to zymozan, a cell wall of yeast, and to act as an opsonin (19), although the recognition molecule involved has not been identified. Because MBL bound to yeast as shown, to clarify the lamprey complement system, C3 binding to yeast was analyzed by flow cytometry. As shown in Fig. 8B, a considerable amount of C3 bound to yeast in the reconstitution experiments, indicating that MBL recognizes mannan of yeast and the associated MASPs cleavage C3 into C3b that is deposited on their surfaces and acts as an opsonin. From these results, it is concluded that MBL is one of the lectins that act as a recognition molecule in the lectin pathway of the lamprey complement system.

As mentioned, sequence analysis of CRDs revealed that Glu^{185} and Asn^{187} (EPN) sequences are highly conserved among mammalian and bird MBLs. Galactose-binding CRDs have Gln^{185} and Asp^{187} (QPD) sequences at these critical positions (10), and site-directed mutagenesis has shown that mannose-specificity can be changed to galactose-specificity by replacing Glu^{185} and Asn^{187} (EPN) with Gln^{185} and Asp^{187} (QPD) (11). In bony fish (carp, zebrafish and goldfish) several lectins have been characterized and their deduced primary structure indicates selectivity for galactose (QPD). Recently, another carp MBL with specificity for mannose (EPN) was purified (32). Previously, we purified and cloned an MBL-like lectin (GBL) from a urochordate, the solitary ascidian *Halocynthia roretzi* (17). Sequence analysis of GBL reveals that the C-terminal half of the ascidian lectin contains a CRD that is homologous to a C-type lectin (EPN), but a collagen-like domain was replaced by another sequence that has an α-helix structure similar to the configuration of Gly-X-Y repeats. In the present study, we purified the lamprey lectin that has a collagenous region and a typical (EPN) CRD. When lamprey MBL was compared with ascidian GBL in carbohydrate binding specificity, ascidian GBL only binds to glucose, but lamprey MBL binds to mannose, GlcNAc, and glucose-like mammalians MBLs. Therefore, it is possible that the ascidian GBL evolved early as a prototype of MBL, and during evolution GBL may have acquired the broad binding specificity for carbohydrates and the collagen structure characteristic of MBL. Thus, considering the phylogenetic analysis, it seems likely that the lamprey lectin is ancestor of the vertebrate MBL.

Another important finding is that lamprey MBL associates with three types of MASP (MASP-1, MASP-A, and MASP-B). These MASPs have been identified as cDNA sequences, but recently, MASP-A was purified at the protein level (27), whereas MASP-2,
C1r, and C1s have not been identified (29). MASPs are classified into three types (MASP-1, MASP-2 and MASP-3) based on the codon encoding the serine residue at the active center of the serine protease domain and the gene organization (4). Lamprey MASP-A and MASP-B were classified into the MASP-3 group in a phylogenetic tree (29), although their gene structure did not support their orthology as MASP-3. In human and mouse, MASP-2 is involved in the activation of C4 and C2, similar to what is observed with C1s, whereas we showed that MASP-1 directly cleaves C3 (33). The functions of MASP-3 have not yet been clarified. Among lamprey MASPs expressed with MBL, we demonstrated that MASP-1 is an ortholog of mammalian MASP-1. Like human MASP-1, lamprey MASP-1 may cleave C3 directly. Also, we demonstrated that MASP-B associated with lamprey MBL. As reported previously, lamprey MASP-A that associated with lamprey C1q, activated C3 (27). Although their precise functions are not known, MASP-1 and MASP-B have been identified in this study at the protein level. Thus, MBL forms complexes with three MASPs, which activate C3, showing the presence of the primordial lectin pathway in lamprey.

Previously, we proposed that the primitive complement system consists of a lectin, an associated protease and C3, the key components of the complement system. It appears in an ascidian, our closest invertebrate relative (4, 5, 13), although the origin of the complement system can be traced back to echinoderms and further back to arthropods because C3 and C2/factor B-like sequence have been identified in sea urchin (12, 34) and horseshoe crab (35). In the ascidian complement system, ficolins and MBL-like lectin (GBL) function as the recognition molecules. In lamprey, by contrast, C1q, the recognition molecule of the classical pathway in mammals, has been identified, but it has lectin-like activity and is associated with MASPs that activate C3 (27). In the present study, we cloned and characterized lamprey MBL that is also associated with MASPs. Interestingly, lamprey C1q bound only to GlcNAc, whereas MBL bound to mannose, GlcNAc, and glucose, showing that lamprey MBL has overlapping and distinct specificities for carbohydrates. In addition, we show that the lamprey complement system consists of at least the lectin-MASP complex and C3, similar to the ascidian system. However, the recent identification of soluble regulatory proteins of the complement system such as lamprey C4-bp (36) and factor H (A. Matsushita and T. Fujita, unpublished observation) leads to the prediction that the lamprey complement system may be more sophisticated than the ascidian system. Furthermore, C2/factor B-like sequences have been cloned in horseshoe crab, sea urchin, ascidians, and lamprey, though their functions are yet to be clarified. As the alternative pathway was thought to be an ancient mechanism, it is of particular interest to solve the entire molecular architecture of complement system in lamprey and this is currently under investigation.

**Disclosures**

The authors have no financial conflict of interest.

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