Mannose-binding lectin (MBL) is a C-type lectin involved in the first line of host defense against pathogens and it requires MBL-associated serine protease (MASP) for activation of the complement lectin pathway. To elucidate the origin and evolution of MBL, MBL-like lectin was isolated from the plasma of a urochordate, the solitary ascidian Halocynthia roretzi, using affinity chromatography on a yeast mannan-Sepharose. SDS-PAGE of the eluted proteins revealed a major band of ~36 kDa (p36). p36 cDNA was cloned from an ascidian hepatopancreas cDNA library. Sequence analysis revealed that the carboxy-terminal half of the ascidian lectin contains a carbohydrate recognition domain (CRD) that is homologous to C-type lectin, but it lacks a collagen-like domain that is present in mammalian MBLs. Purified p36 binds specifically to glucose but not to mannose or N-acetylgalcosamine, and it was designated glucose-binding lectin (GBL). The two ascidian MASPs associated with GBL activate ascidian C3, which had been reported to act as an opsonin. The removal of GBL-MASPs complex from ascidian plasma using Ab against GBL inhibits C3-dependent phagocytosis. These observations strongly suggest that GBL acts as a recognition molecule and that the primitive complement system, consisting of the lectin-proteases complex and C3, played a major role in innate immunity before the evolution of an adaptive immune system in vertebrates. The Journal of Immunology, 2001, 167: 4504–4510.
Materials and Methods

Materials

Solitary ascidians, H. roretzi, were obtained from a local dealer (Ishimiya) in Fukushima, Japan. Hemolymph was collected from the solitary ascidian by cutting the tunic matrix without injuring internal organs, followed by centrifugation. Acsidian hemocytes were collected as described previously (14).

Purification of ascidian lectin, lectin-proteases complex, and C3

To prepare MBL-like lectin for the initial experiment, ~1 liter of the plasma was concentrated 4-fold using a PVDF Cassettes (Millipore, Bedford, MA). The concentrated material was treated with polyethylene glycol 4000 at a final concentration of 7%. The precipitate was dissolved in 20 mM Tris-HCl buffer containing 1 mM NaCl and 50 mM CaCl2, pH 7.8 (starting buffer), and applied to a yeast mannan-Sepharose 4B column (10). After washing the column, MBL-like lectin was eluted with the starting buffer containing 0.3 M mannose. The eluted preparation was analyzed by SDS-PAGE, dialyzed against 25 mM Tris, 25 mM NaCl, 5 mM CaCl2, pH 7.8, and then applied to a Mono Q column (Amersham Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with the same buffer. Elution was conducted with a linear NaCl gradient to 0.6 M. Under reducing conditions, a band of 36 KDa (p36) was observed. This preparation was used for amino acid sequence analysis and for assaying binding to carbohydrates.

To prepare the lectin-protease complexes, hemolymph of the solitary ascidians was collected in the presence of protease inhibitors, 10 mM 6-amino-n-capric acid, 10 mM benzanilide, 100 µM (p-aminophenyl) methanesulfonylfluoride (p-APMSF; Wako Pure Chemical Industries, Osaka, Japan), 20 µM p-nitrophenyl-p-guanidino-benzoate (Merck, Darmstadt, Germany), and 100 µM Pefabloc (Pentapharm, Basel, Switzerland). After centrifugation, the plasma was applied to a mannan-Sepharose 4B column equilibrated with starting buffer. After washing the column with starting buffer, p36 with minor bands was eluted with starting buffer containing 300 mM glucose. It was purified further on a Mono Q column, using the buffer described above. AsC3 was purified according to the method described (14).

Preparation of Abs

Polyclonal anti-ascidian MASPa Ab was obtained from rabbits immunized with synthetic peptides corresponding to the last 18 amino acid residues of ascidian MASPa (13) that had been coupled to keyhole limpet hemocyanin. The specific anti-MASPa Ab was affinity-purified using peptide-coupled Sepharose. Monospecific antiserum to the ascidian MBL-like proteins (p36) was raised by immunizing rabbits with purified carbohydrates for the initial experiment, 10 µg/ml. The ascidian MBL-like protein (p36) was labeled with Na125I (New England Nuclear, Boston, MA) and used as a probe for screening a ZAP cDNA library. A subcloned 506-bp DNA was labeled with 32P and used as a probe for screening a ZAP cDNA library. The concentrated material was treated with polyethylene glycol 4000 at a final concentration of 7%. The precipitate was dissolved in 20 mM Tris-HCl buffer containing 1 mM NaCl and 50 mM CaCl2, pH 7.8 (starting buffer), and applied to a yeast mannan-Sepharose 4B column (10). After washing the column, MBL-like lectin was eluted with the starting buffer containing 0.3 M mannose. The eluted preparation was analyzed by SDS-PAGE, dialyzed against 25 mM Tris, 25 mM NaCl, 5 mM CaCl2, pH 7.8, and then applied to a Mono Q column (Amersham Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with the same buffer. Elution was conducted with a linear NaCl gradient to 0.6 M. Under reducing conditions, a band of 36 KDa (p36) was observed. This preparation was used for amino acid sequence analysis and for assaying binding to carbohydrates.

The lectin p36 was subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and stained at 4 °C for 2 h. After centrifugation at 15,000 rpm for 10 min, the precipitates were dried up and dissolved in SDS-PAGE buffer, and subjected to SDS-PAGE under reducing or nonreducing conditions, followed by autoradiography.

Immunoblotting

After SDS-PAGE (10% gel), proteins were transferred from the gel to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and stained at 4 °C for 2 h. After centrifugation at 15,000 rpm for 10 min, the precipitates were dried up and dissolved in SDS-PAGE buffer, and subjected to SDS-PAGE under reducing or nonreducing conditions, followed by autoradiography.

The binding of AsC3 to yeast was assayed by flow cytometry. Yeast cells (W303D; 5 × 10^5) in 50 µl of normal Herbst's artificial seawater (450 mM NaCl, 9.4 mM KCl, 48 mM MgSO4, 48 mM CaCl2, 32 mM Na2SO4, and 3.2 mM NaHCO3, pH 7.6) were incubated at 20 °C for 10 min with 0.1% gelatin. The labeled cells were then washed at 4 °C for 4 times with 10 ml of anti-AsC3 Ab (1 mg/ml) and stained at 4 °C for 30 min with 20 µl of 100 µg/ml FITC-conjugated swine anti-rabbit Iggs (DAKO Japan, Tokyo, Japan). The yeast was washed twice with 10 mM EDTA and 0.1% gelatin.
between each reaction. Reactivities were evaluated by FACScan flow cytometry (BD Biosciences, Mountain View, CA).

**Phagocytosis assay**

Hemolymph was collected from an ascidian, and fresh plasma was prepared. To prepare glucose-binding lectin (GBL)- or C3-depleted plasma, the fresh plasma was treated with each Ab, and the resulting Ag-Ab complexes were removed with protein A-Sepharose. Rabbit normal IgG was used as a control. Each plasma was incubated with yeast (W303D) and after washing, the coated yeast was reacted with the ascidian hemocytes. Hemocytes that ingested at least one yeast were considered positive. The degree of phagocytosis was expressed as the ratio of the number of positive hemocytes to the number of total hemocytes (21).

**Results**

**Purification of the ascidian lectin**

To purify the ascidian lectin homologous to mammalian MBL, plasma from *H. roretzi* was concentrated and precipitated with polyethylene glycol 4000. The precipitate was dissolved in buffer, applied to a yeast mannose-Sepharose column and eluted with mannose. SDS-PAGE of the eluted proteins revealed a major band of ~36 kDa under reducing conditions (Fig. 1). The 36-kDa protein (p36) was further purified by Mono Q chromatography. Under nonreducing conditions, p36 appeared as three bands with apparent molecular mass of ~70, 140, and 210 kDa. These results indicate that the ascidian lectin is composed of homodimers consisting of ~36-kDa subunits bound by means of intermolecular disulfide bonding as is the case with mammalian MBL.

For cDNA cloning of p36, its amino-terminal amino acid sequence (15 amino acids; EDEQTLACRRVTKAES) was determined. The amino-terminal amino acid sequences of 19- and 8-kDa fragments (IQVAGFEEELNEEFQEMKRR and NTAWGPNEPNDGNE), which were obtained by digesting p36 with *S. aureus* V8 protease, were also determined.

**cDNA cloning of p36**

Based on the amino-terminal amino acid sequences of p36, and the 8-kDa fragment produced by protease digestion, we designed degenerated primers and performed nested PCR. A single band of ~500 bp, which was amplified, was cloned and then sequenced by the dideoxy method. The deduced amino acid sequence revealed that it contained the complete amino acid sequence of the 19-kDa fragment of p36, which was not used for PCR. An ascidian hepatopancreas cDNA library was screened using this PCR product as a probe and a 1-kb-long cDNA clone with an open reading frame of 672 bp, which is predicted to encode a 224 aa protein including a 17-residue leader peptide, was isolated (Fig. 2). The predicted molecular mass of the protein was 23,716 Da, indicating that the mature 36-kDa protein may be glycosylated, because there are three N-linked glycosylation sites. Sequence analysis revealed that the carboxy-terminal half of the ascidian protein contains a CRD. Unlike mammalian MBL, the N-terminal portion of the lectin lacks a collagenous region consisting of Gly-X-Y triplet repeats (where X and Y are any amino acid) (8, 9). The nonlectin part of the molecule has no significant similarity to any known sequences.

The sequence of the ascidian p36 was aligned with sequences of mammalian MBLs (Fig. 2). A short stretch of the nonlectin region of the protein shows limited similarity to the portion of the MBL collagen region where the Gly-X-Y triplets are interrupted to form a bend in the triple helix (9). The p36 CRD has a sequence identity of 22.4% to the human MBL. The CRD is of the C-type, containing 13 of the 18 highly conserved amino acid residues, including four cysteines that are involved in disulfide bonds within the domain (22). Five residues (Glu184, Asn186, Glu191, Asn205, and Asp206), which have been reported to bind directly to mannos, GlcNAc, and glucose in the presence of Ca2+, (23), are completely conserved among these proteins, indicating that the ascidian lectin belongs to the mannose-binding family.

**Northern blotting**

Northern blot analysis was performed using two potential sources of plasma proteins, ascidian hepatopancreas and hemocytes. As shown in Fig. 3, the p36 probe detected a single transcript band from hepatopancreas. The p36 mRNA is 1.1 kb in length, which corresponds to the full size of the cDNA. A positive signal was not detected in blood cells.

**Binding specificity of p36 for carbohydrates**

We evaluated the binding specificity of the ascidian lectin, p36, for various neoglycolipids. As shown in Fig. 4, p36 bound to oligosaccharides composed of glucose, but not to those containing mannos, GlcNAc, or galactose. We performed additional experiments to confirm these results. Purified p36 was applied to a mannose- or a GlcNAc-Sepharose column, but it did not bind (data not shown). There are no previous reports of lectins specific for glucose residues although several lectins that recognize β-1,3-glucan have been described (24, 25). Therefore, we designated the ascidian lectin GBL. We also performed similar assays of GBL binding to oligosaccharides derived from six mammalian glycoproteins (20). It did not bind to the high mannose, complex, or hybrid-type oligosaccharides of mammalian glycoproteins (data not shown), suggesting that GBL recognizes only those glucose residues in the polysaccharides of microorganisms. By flow cytometric analysis, GBL was shown to bind to yeast and to its cell wall, zymosan, in the presence of Ca2+, probably through β-1,3-glucan (data not shown). During the preparation of GBL on the yeast mannose-Sepharose column, GBL probably bound, not to the mannose residue of mannan, as does mammalian MBL, but to glucose-containing polysaccharides, probably β-1,3-glucan, which was a contaminant in the yeast mannan used to prepare the column. Also, the purified GBL was applied to mannan-Sepharose and eluted with glucose. The same results were obtained with mannanose. The
reason why the mannose disrupted the binding of GBL to the man-nan-Sepharose is not clear. It is possible that the mannose used in
elution contains the epimer form.

**GBL associates with MASPs**

Next, we determined whether the lectin fraction contained the as-
cidian serine proteases MASPa and MASPb, which had been re-
ported as homologues of mammalian MASP-1 (13, 26). Ascidian
hemolymph was collected in the presence of a range of protease
inhibitors. After centrifugation, the plasma was applied to a man-
nan-Sepharose column, eluted with glucose, and further puri-

![C-type lectins](image)

Figure 2. Amino acid sequence alignment of the ascidian lectin. The entire amino acid sequences of the ascidian p36 and human (Hu), rat (Ra), and
mouse (Mo) MBLs were aligned using ClustalW software with reference to the invariant residues of C-type lectins (21). The signal peptide cleavage site
is indicated by an arrowhead. Residues identical in all proteins are indicated by asterisks, and the five residues directly involved in sugar binding (22) are
marked by diamonds. Gaps inserted during alignment are indicated by dashes.

![C-type lectins](image)

Figure 3. Northern blotting analysis of GBL. A membrane filter con-
taining total RNA (10 µg) from hepatopancreas (lane 1) and hemocytes
(lane 2) was hybridized with [32P]-cDNA corresponding to nucleotides
1–911. The 18S rRNA bands are presented to show the amount and quality
of RNA loaded in each lane. Size markers are indicated on the left.

![C-type lectins](image)

Figure 4. Binding specificity of the ascidian lectin for glycoconju-
gates. Neoglycolipids prepared from 10 oligosaccharides were chromatographed on a TLC plate. The plate was overlaid with 125I-p36, subjected to
autoradiography (lower panel), and then stained with orcinol (upper pan-
el). Lane 6, where GlcNAc-containing neoglycolipid was chromatographed,
was not stained with orcinol but with primulin, which stains the
lipid portion. The 10 neoglycolipids were maltopentaose (Mal5; lane 1),
isomaltopentaose (Isomal5; lane 2), cellopentaose (Cello5; lane 3), lam-
inaripentaose (Lami5; lane 4), mannopentaose (Man5; lane 5), chitopen-
taose (GN5; lane 6), oligosaccharide having two N-acetylglucosamines and
three mannose residues (GN2M3; lane 7), 3'Gal1-11032-sialyllactose (3'Gal1-11032 SL; lane 8), 6'Gal1-11032-sialyllactose (6'Gal1-11032 SL; lane 9), and lactose (Lac; lane 10).
Activation of C3 by GBL-MASPs complex

Because human MASP-1 is reported to activate C3 directly (27), we examined whether the ascidian MASPs associated with GBL could cleave AsC3. Purified AsC3 was incubated with the GBL-MASPa-MASPb complex, and proteolytic activation was monitored by SDS-PAGE. As shown in Fig. 6, the C3 α-chain was cleaved in a dose-dependent manner, yielding an α'-chain. Because the primary structures of the proteases suggested that only MASPa has trypsin-like specificity (13), AsC3 is probably cleaved by MASPa rather than by MASPb, although this point needs to be confirmed at the biochemical level.

We reported previously that C3 in ascidian plasma binds to yeast and acts as an opsonin (14), although the recognition molecule involved has not been identified. Because GBL binds to yeast, to clarify the ascidian complement system we reacted the isolated GBL-MASPs complex and C3 with yeast, and C3 binding to yeast was analyzed by flow cytometry. As shown in Fig. 7, a considerable amount of C3 bound to yeast in the reconstitution experiments, indicating that GBL recognizes carbohydrates of yeast, probably β-1,3-glucan, and that the associated MASPs activate C3. From these results, it appears that GBL is one of the lectins that acts as a recognition molecule in the lectin pathway of the ascidian complement system.
Phagocytosis assays

To confirm the above results, we performed phagocytosis assays. Usually, 20–30% of ascidian leukocytes (hemocytes) ingested at least one noncoated yeast cell, and in the case of yeast treated with ascidian plasma, 40–60% of hemocytes ingested one or more yeast cells. As reported previously, this opsonic activity is derived from C3 (14). In the experiments shown in Fig. 8, we also found that the opsonic effect of ascidian plasma was eliminated in GBL-depleted plasma in a dose-related manner. In the presence of EDTA or EGTA, the opsonic activity could not be detected, suggesting that Ca$^{2+}$ is required for the activation of C3 in this system. In addition, we showed that the purified GBL-MASPs and C3 enhanced phagocytosis. Therefore, it is clear that GBL acts as the recognition molecule, and as the result, the activated C3 is fixed on yeast which is phagocytosed through the recently identified ascidian complement receptor (28).

Discussion

In the present study, we identified a novel ascidian lectin from hemolymph of a solitary ascidian, *H. roretzi*, and cloned it from a cDNA library of the ascidian hepatopancreas. The deduced amino acid sequence of this lectin predicts that it has a typical CRD and lacks a collagen region, which is conserved in vertebrate MBLs. From an analysis of the binding specificity to neoglycolipids, we found that the ascidian lectin recognizes only glucose residues, and designated it GBL. Because GBL is associated with ascidian MASPs that activates C3, the sequence data make it seem probable that, despite the lack of a collagenous region, the ascidian lectin is related to mammalian MBL.

An MBL homologue has not been yet identified in invertebrates, although an N-acetyl-galactosamine-binding lectin with a collagen-like domain has been identified as a collectin-like protein in a different species of ascidian, *Stylea plicata* (29). However, its complete structure has not been elucidated, and its binding specificity for carbohydrates is different from that of MBL. Recently, by an affinity chromatography on GlcNac-Sepharose, we isolated ascidian ficolins that have short collagen-like sequences and fibrinogen-like domains (15). As shown in Fig. 1, GBL is a major protein that binds to mannan-Sepharose, and an additional band of 40 kDa was identified as ascidian galactose-specific lectin (Gal-lentin) (30). Because MBL is mainly bound to mannose and GlcNAc, and we failed to isolate an MBL homologue that binds to mannose from ascidian hemolymph, we consider that GBL is the functional counterpart of MBL. Interestingly, GBL has an α-helix structure in its amino-terminal region, which is similar to the configuration of Gly-X-Y repeats. This raises the possibility that GBL has evolved early as a prototype of MBL. During evolution GBL may have acquired the broad binding specificity for carbohydrates and the collagen structure characteristic of MBL. In this regard it will be of interest to determine the structure and function of the lectin associated with MASP in lamprey, one of the most primitive vertebrates, because we have already cloned the cDNA of lamprey MASP (26).

GBL has a unique binding specificity for carbohydrates, because it binds to only glucose residues. Although several lectins that recognize β-1,3-glucan have been reported (24, 25), this is the first report of a lectin that recognizes only the glucose residue. MBL is reported to bind carbohydrates with 3- and 4-hydroxyl groups in the pyranose ring in the presence of Ca$^{2+}$ through the five conserved residues (Glu$^{184}$, Asn$^{186}$, Glu$^{191}$, Asn$^{205}$, and Asp$^{206}$) in the MBL CRD (23). These residues are completely conserved in GBL and mammalian MBLs, supporting the hypothesis that GBL is a prototype of MBL. The structural difference between mannose or GlcNac and glucose is at the site of the 2-hydroxyl group of the pyranose ring. Therefore, it is possible that residues other than the five conserved ones in GBL may be involved in recognizing the 2-hydroxyl group of glucose.

We presented evidence here that AsC3, along with GBL and MASPs, constitute a simple complement system corresponding structurally and functionally to the mammalian lectin pathway. Its activation mechanism, which depends on lectin-based recognition, clearly indicates that this ancestral complement system is part of the innate immune system. Although the origin of the complement system is still not clear, it can be traced back at least to echinoderms, because C3 and factor B have been identified in sea urchin (3). Recently, a C3 receptor on ascidian hemocytes was identified as the homologue of mammalian complement receptor type 3 or 4 (CR3 or CR4) (28). Taken together, our observations strongly suggest that lectin-protease complex, C3, and C3R may have developed as the minimal ancestral components of the primordial complement system in the tunicate lineage.

Another important finding is that an opsonic activity derived from C3 was not observed in GBL-depleted plasma, suggesting that the only activation mechanism of C3 by a lectin-based recognition in the system using yeast involves GBL. Because the alternative pathway was found as the activation mechanism of serum C3 by zymosan without the involvement of any recognition molecules (31), this pathway may not have emerged in the tunicate lineage, although the possibility of a simple role of factor B protein (3) as an amplifier of C3 deposition cannot be excluded completely. The sophisticated recognition mechanism of the alternative pathway to recognize a broad spectrum of pathogens developed more recently. Because the classical activation pathway is part of acquired immunity, the complement lectin pathway seems to have played a central role in host defense against infection for a long time before the evolution of the adaptive immune system in the vertebrate lineage.

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