Activation of the Classical Complement Pathway by Mannose-binding Protein in Association with a Novel Cls-like Serine Protease
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Summary

Serum mannose-binding protein (MBP) is a C-type lectin that binds to terminal mannose and N-acetylglucosamine moieties present on surfaces of certain pathogens and activates the classical complement pathway. In the present study, we describe the mechanism underlying the activation triggered by MBP. The human serum MBP fraction was obtained by sequential affinity chromatography on mannan-Sepharose, anti-IgM-Sepharose and anti-MBP-Sepharose in the presence of calcium ions. This fraction contained a Cls-like serine protease as assessed by C4 consumption. The Cls-like serine protease, designated MBP-associated serine protease (MASP), was separated from MBP by rechromatography on anti-MBP-Sepharose in the presence of ethylenediaminetetra-acetic acid. MASP exhibited both C4- and C2-consuming activities. The molecular mass of MASP was estimated to be 83 kD with two polypeptides of heavy (66 kD) and light (31 kD) chains linked by disulfide bonds. The serine residue responsible for protease activity is located on the L chain. Reconstitution experiments using MASP and MBP revealed that combination of the two components restores C4- and C2-activating capacity on mannan. Based on analyses of molecular size, antigenicity, and 11 NH2-terminal amino acid sequences of the L chain, we conclude that MASP is a novel protein different from Clr or Cls. Our findings are not in accord with a proposed mechanism by which MBP utilizes the Clr2–Cls2 complex to initiate the classical complement pathway.

Serum mannose-binding protein (MBP) is a C-type lectin (1) which binds to terminal mannose and N-acetylglucosamine present on yeast cell walls, gram-negative bacteria, or other cells. MBP has been documented in human (2), rabbit (3), rat (4), and bovine (5) sera, and its apparent molecular mass is about 400–700 kD with a subunit of about 32 kD. Each subunit has a collagenous and a carbohydrate-recognizing region (6, 7). MBP is thought to play a crucial role in host defense against certain pathogens containing mannose or N-acetylglucosamine on their surfaces. Several lines of evidence show that MBP functions as an opsonin. Kuhlman et al. (8) reported that on binding to Salmonella montevideo bearing a mannose-rich LPS, MBP enhances killing by phagocytes. Low levels of MBP in the human serum have been shown to be linked to a defect in opsonization of bakers’ yeast (Saccharomyces cerevisiae) (9). MBP shows structural similarity to Clq, a subcomponent of the first complement component (C1), and can bind to the Clq receptor thereby enhancing phagocytosis (10). MBP also inhibits HIV infection of cells by binding to gp120, which possesses a high mannose oligosaccharide (11, 12). Further, MBP is able to trigger complement activation through both the classical (13, 14) and alternative (15) pathways upon binding to pathogens possessing mannose, leading to direct killing. Complement activation initiated by MBP generates C3b or iC3b, both of which act as opsonins (16).

The mechanism by which MBP activates the classical pathway has been reported by two groups (17, 18) which reached the same conclusion that MBP, like Clq, can associate with the unactivated proenzyme Clr2–Cls2 complex so that the classical pathway proceeds upon binding to mannan. This is based on the finding that isolated MBP and the Clr2–Cls2 proenzyme forms a complex, resulting in activation of C1s. These facts imply that MBP might utilize Clr and C1s for activation in vivo. However, an alternate mechanism is that protease(s) other than Clr and C1s may interact with MBP and initiate the classical pathway. In this study, we obtained human serum MBP in association with a Cls-like serine protease which consumes C4 and C2, resulting in complement
activation. We report here the purification and characterization of the Cls-like serine protease.

Materials and Methods

Reagents. Mannan from S. cerevisiae was purchased from Sigma Chemical Co. (St. Louis, MO). (p-Aminophenyl)methanesulfonylfluoride (p-APMSF), mannose, and tannic acid were from Wako Pure Chemical Industries (Osaka, Japan). CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Polyclonal anti-human Cls serum (rabbit) was from Behringwerke AB (Marburg, Germany). Monoclonal anti-human IgM was prepared in our laboratory. Monoclonal anti-MBP (3E7) was obtained as previously described (19). Coupling of mannan, anti-IgM, or anti-IgG to CNBr-activated Sepharose 4B was performed according to the manufacturer's instructions. Diisopropylfluorophosphate, [1,3-3H] (H-DFP) and [125I]NaI were purchased from New England Nuclear (Boston, MA). C4 was labeled using [125I]NaI and Iodo-Gen from Pierce Chemical Co. (Rockford, IL), according to the manufacturer's instructions. Human Cls (20), C4 (21), C2 (22), and oxidized C2 (CO2) (23) were prepared as described previously. Guinea pig C1 was purchased from Dianmedix Corporation (Miami, FL). Veronal buffered saline (VB) was a standard solution of Veronal buffer containing 0.148 M NaCl (pH 7.4). EDTA-VGB was VB supplemented with 10 mM EDTA and 0.1% gelatin. MGVB was low ionic strength Veronal buffered saline containing 0.1% gelatin, 2.3% mannitol, 2 mM CaCl2 and 0.5 mM MgCl2.

Purification of MBP and MASP. Human serum obtained by recaffecilation of outdated human citrated plasma. Polyethylene glycol 4,000 at a concentration of 7% was added and precipitate dissolved in 50 mM Tris-HCl, 1 M NaCl, 50 mM CaCl2, pH 7.8 (starting buffer), and applied to a yeast mannan-Sepharose column. After washing the column, MBP was eluted with starting buffer containing 300 mM mannose. The eluate was contaminated with IgM and IgG. To remove IgM, the eluted fractions were passed through a monoclonal anti-human IgM-Sepharose column. The pass-through fractions were then applied to an anionic anti-MBP (3E7)-Sepharose column and eluted with 0.1 M glycine-HCl, pH 2.2. After concentration with a YM 10 membrane (Amicon Corp., Danvers, MA), the MBP pool from the 3E7-Sepharose column was dialyzed against 50 mM Tris-HCl, 1 M NaCl, 20 mM EDTA, pH 7.8 and applied to a second 3E7-Sepharose column. The pass-through fractions containing MASP were collected and MBP was eluted with 0.1 M glycine-HCl, pH 2.2.

4C or 2C Consumption. C4 or C2 (2 site-forming units [SFU]) was incubated at 37°C for 30 min with an equal volume of sample or MGVB. For C4, the reaction mixture (100 μl) was further incubated for 60 min with 100 μl of C4-deficient guinea pig serum and 100 μl of sheep erythrocytes sensitized with Ab (EA) (104/ml), followed by addition of 1 ml of EDTA-VGB. The OD of the supernatant was determined at 414 nm. The percentage of C4 consumption was calculated from the hemolytic rate (γ). For C2 consumption, mixtures of C2 and sample were allowed to react with EAC14 cells at 30°C for 5 min, followed by addition of guinea pig serum diluted 1:20 with EDTA-VGB. Incubation was then continued at 37°C for an additional 60 min. Subsequent procedures were the same as in the C4 consumption assay.

C4- and C2-Activating Capacity on E. mannan. Sheep erythrocytes (5 x 104/ml) suspended in PBS were incubated at 37°C for 15 min with an equal volume of tannic acid (0.125 mg/ml). The cells were washed and then incubated with an equal volume of yeast mannan (20 mg/ml) at 37°C for 60 min. After centrifugation, E. mannan were washed with MGVB and suspended at 104/ml. For assay of C4- and C2-activating capacity on E. mannan, 100 μl of sample were incubated with an equal volume of E. mannan (104/ml) at 30°C for 30 min. After washing, the cells were incubated at 30°C for 20 min with 50 μl of human C4 and 4C (20 SFU each). The mixtures were further incubated at 37°C for 60 min with 200 μl of guinea pig serum diluted 1:20 with EDTA-GVB. After adding 1 ml of EDTA-GVB, the hemolytic rate (γ) was calculated spectrophotometrically. The average of hemolytic sites per cell (z) was calculated as z - ln (1-γ).

SDS-PAGE and Western Blotting. SDS-PAGE was performed using the Laemmli system (24). Western blotting was performed as previously described (25) except that an Immobilon-P membrane (Millipore Corp., Bedford, MA) was used.

Cleavage of C4. MASP (100 ng) or Cls (100 ng) in 10 μl of VB was incubated with 10 μl of 125I-C4 (260,000 cpm/50 ng C4) at 37°C for 60 min and subjected to SDS-PAGE followed by autoradiography.

3H-DFP Labeling. 20 μl of 3H-DFP (740 kBq) were incubated with 200 μl of MASP or Cls at 4°C for 17 h. 40 μl of BS (1.6 mg/ml) was added to the reaction mixture as a carrier, and the proteins were then precipitated by the addition of 1 ml of chilled acetone, and kept at -20°C for 2 h. After centrifugation at 15,000 rpm for 10 min, the precipitates were dissolved in SDS-PAGE buffer. After SDS-PAGE, fluorography was carried out at -80°C.

Amino Acid Sequence Analysis. MASP was subjected to SDS-PAGE under reducing conditions and transferred to a PVDF membrane (Bio-Rad Laboratories, Richmond, CA). After staining with Coomassie brilliant blue, the band was excised and subjected to gas-phase protein sequencing (Shimadzu PQS-1; Shimadzu Corporation, Kyoto, Japan).

Results

Cl₁-like Serine Protease Activities in the MBP Fraction. To examine whether MBP contains a protease responsible for activation of the classical complement pathway upon binding to mannan such as Cl₁₂–Cl₁₅, we assumed that an association between MBP and such a protease is facilitated by the presence of calcium ions, as is the case with the C₁ complex. Accordingly, instead of EDTA, which is commonly used, we chose mannose for eluting MBP to keep the complex intact while eluting from mannan-Sepharose column after adsorption. Because the MBP fraction from a mannan-Sepharose contained IgM and IgG as contaminants, it was first passed through a monoclonal anti-IgM-Sepharose column. We then used monoclonal anti-MBP antibody (3E7)-coupled-Sepharose to remove IgG. MBP was eluted with an acidic buffer from a 3E7-Sepharose column. To test the MBP fraction for C4- and C2-activating capacity, E. mannan were sensitized with the MBP fraction from 3E7-Sepharose and incubated with human C4 and C2 followed by guinea pig complement diluted with EDTA-containing buffer as a source of C₃-C₉. As shown in Fig. 1A, the extent of hemolysis of E. mannan depended on the amount of the MBP fraction added, which indicated that the fraction contained a Cl₁-like protease which activates C4 and C2, generating C3 convertase of the classical pathway. Hemolysis was completely impaired when the MBP fraction was preincubated with E. mannan in the presence of p-APMSF (data not shown). These results indicate that the
protease in the MBP fraction responsible for activation of C4 and C2 belongs to the serine protease family. The MBP fraction from 3E7-Sepharose was tested for C4-consuming activity and found to exhibit activity similar to Cls as shown in Fig. 1 B.

Isolation of a Cls-like Serine Protease in the MBP Fraction. To separate the Cls-like serine protease(s) from MBP, the MBP fraction from a 3E7-Sepharose column was dialyzed against buffer containing EDTA and rechromatographed on a 3E7-Sepharose column. C4-consuming activity was recovered in the pass-through, whereas MBP was retained on the column and eluted with an acidic buffer (Fig. 2). C4- and C2-activating capacity, which results in generation of C3 convertase on E.mannan, was not observed either in the pass-through fraction or in the eluate obtained with the acidic buffer.

The SDS-PAGE profile of the Cls-like serine protease in the pass-through which displays C4-consuming activity is shown in Fig. 2. The protease appears under nonreducing conditions as a single band with a molecular mass of 83 kD. We designate this protein MBP-associated serine protease (MASP). Under reducing conditions, MASP shows two bands corresponding to 66 and 31 kD (tentatively termed as H chain for the 66 kD component and L chain for the 31 kD component).

Comparison of MASP with Cls. To examine the possibility that MASP is Cls, we compared their behavior on SDS-PAGE. As shown in Fig. 3 A, although the apparent molecular weight of MASP is slightly larger than that of Cls under nonreducing conditions, both H and L chains of MASP migrated faster than the two corresponding chains of Cls under reducing conditions.

Cl5 is a serine protease whose L chain was labeled with 3H-DFP. 3H-DFP was also found to be incorporated into the L chain of MASP, indicating that its active site is located on the L chain (Fig. 3 B).

To compare antigenicities of MASP and Cls, we performed Western blot analysis using polyclonal anti-Cls serum. MASP at a 100-fold higher concentration than Cls did not react with polyclonal anti-Cls under conditions in which Cls was detected at concentrations as low as 1 ng/ml (data not shown).

Properties of MASP. We then examined whether anti-Cls serum inhibits C4 consumption by MASP. Under conditions in which MASP and Cls exhibit the same C4-consuming activities, MASP or Cls was incubated with C4 in the pres-
ence or absence of anti-C1s serum, and residual C4 activity was assayed. As shown in Fig. 4A, C4 consumption by C1s was inhibited up to 100% by anti-C1s serum. On the other hand, anti-C1s serum did not inhibit C4 consumption by MASP, indicating that C1s is not involved in C4 consumption mediated by MASP.

In experiments shown in Fig. 4B, we examined cleavage of C4 by MASP. Radiolabeled C4 was incubated with MASP and subjected to SDS-PAGE and radioautography. C4 was cleaved by MASP generating a fragment corresponding to C4b with appearance of the α chain.

By analogy to C1s, MASP was expected to have both C2- and C4-consuming activities. C2 was incubated with various amounts of MASP and residual C2 activity was determined. As shown in Fig. 4C, C2 was consumed by MASP in a dose-dependent manner. Furthermore, we compared C4- or C2-consuming activities of MASP and C1s, and found that C1s displays about 150-fold higher C4- or C2-consuming activity than does MASP on a molar basis (data not shown).

Reconstitution of C4- and C2-activating Capacity on E. mannan. As described above, MASP and MBP were separated by an affinity chromatography on a second 3E7-Sepharose column in the presence of EDTA, and C4- and C2-activating capacity was not found in either fraction. Accordingly, we conducted a reconstitution experiment involving a hemolytic assay using isolated MASP and MBP, as shown in Fig. 1, to determine if recombination of MASP and MBP could restore C4- and C2-activating capacity on E. mannan. Preincubation of E. mannan with MBP or MASP alone did not result in lysis. When MBP and MASP were simultaneously preincubated with E. mannan, hemolysis was noted and its rate increased, relative to the amount of MASP used with a fixed concentration of MBP (Fig. 5). These results indicate that MBP and MASP can associate to exhibit C4- and C2-activating capacity on mannan.

**NH2-terminal Amino Acid Sequence of MASP.** The NH2-terminal amino acid sequence of the MASP L chain was determined (Fig. 6). For comparison, the NH2-terminal amino acid sequences (26) of the L chains of human Cls and Clr are aligned. It is clear that MASP is different from Cls or Clr, although it shows some homology with these subunits.

We conducted protein database searches in GenBank (release 72) and found that MASP is a novel protein that has as yet not been reported.

**Discussion**

In reconstitution experiments using MBP and the C1r2-C1s2 complex, Ohta et al. (17) and Lu et al. (18) con-
cluded that MBP can associate in the presence of calcium ions with the unactivated proenzyme complex Clr2–Cls2, resulting in activation of Cls when MBP binds to mannan. If classical complement pathway activation triggered by MBP occurs in serum by this mechanism, one would expect the activated Cls in the MBP fraction obtained from a mannan-Sepharose column after elution with mannoise. Our attempt to isolate the activated Cls from the MBP fraction revealed a Cls-like serine protease with both C4- and C2-consuming activity associated with MBP. We isolated and characterized this Cls-like serine protease termed MASP and found that it consisted of two polypeptides linked by disulfide bond(s), and that the active site for the serine protease is located on the L chain. Structurally and functionally, its properties resemble those of Cls. We concluded, however, that MASP is a novel serine protease, involved in classical complement pathway activation. We based this on the following considerations. First, the molecular size of MASP is different from that of Cls, as judged by results from SDS-PAGE. Under nonreducing conditions, the apparent molecular weight of MASP is slightly larger than that of Cls. Under reducing conditions, the two MASP polypeptides are smaller than the corresponding Cls polypeptides. These discrepancies are probably due to intradisulfide bonds within their molecules. Second, polyclonal antibodies against Cls did not react with MASP as assessed by immunoblotting, indicating that these two proteases are antigenically distinct. Third, providing more direct evidence, the NH2-terminal amino acid sequences of the L chains of MASP and Cls differ. Amino acid analysis also showed that MASP is different from Cls. It is unlikely that both Cls and MASP are involved in classical complement pathway activation initiated by MBP, because anti-Cls serum did not inhibit C4 consumption by the MASP fraction.

Ra-reactive factors (RaRF) are complement-dependent bactericidal substances found in the sera of vertebrates (27), which recognize and bind to mannose and N-acetylglucosamine in the Ra chemotype strain of Salmonella typhimurium in the presence of calcium ions. Mouse RaRF has been most characterized (28-30) and was found to consist of at least two components: a carbohydrate-binding component and a C4- and C2-consuming component. The former component (P28a and P28b) shares structural properties with MBP (31), and the latter is a protease that displays a Cls-like activity in the classical pathway activation by RaRF (32, 33). We recently demonstrated that human MBP is a component of RaRF (19). Taken together, our findings indicate that like mouse RaRF, human RaRF consists of a carbohydrate-binding component (i.e., MBP) and a Cls-like serine protease (i.e., MASP).

Classical complement pathway activation initiated by C1q requires Clr as well as Cls. If a Clr-like serine protease is involved in the activation triggered by MBP, this protease should have coeluted with MASP from the second 3E7-Sepharose column. However, the MASP preparation contained no Clr-like serine protease as determined by SDS-PAGE analysis. In addition, the results of reconstitution experiments using MBP and MASP suggest no requirement for a Clr-like serine protease for classical pathway activation initiated by MBP.

By analogy to Cls (34), it is conceivable that MASP also has an unactivated proenzyme form with a single polypeptide in association with MBP in the presence of calcium ions, and that MASP changes from the unactivated to activated form, accompanied by a cleavage of the single polypeptide into two chains linked by disulfide bond(s) when MBP binds to mannan. We can assume that upon activation, MASP undergoes conformational changes, thereby acquiring autoactivation capacity like that of Cls. If this is the case, MASP may be unique in exhibiting both Clr- and Cls-like activities.

In conclusion, we have isolated and characterized a novel Cls-like serine protease termed MASP which can initiate the classical complement pathway in association with MBP on mannan. Our findings are not in accord with a proposed mechanism by which MBP utilizes the Clr2–Cls2 complex to initiate the activation.

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