Activation of the Classical Complement Pathway by Mannose-binding Protein in Association with a Novel C1s-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 C1s-like serine protease as assessed by C4 consumption. The C1s-like serine protease, designated MBP-associated serine protease (MASP), was separated from MBP by rechromatography on anti–MBP-Sepharose in the presence of ethylenediaminetetraacetic 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 (L) (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 NH_2-terminal amino acid sequences of the L chain, we conclude that MASP is a novel protein different from C1r or C1s. Our findings are not in accord with a proposed mechanism by which MBP utilizes the $C1r_2$–$C1s_2$ complex to initiate the classical complement pathway.

Serum mannose-binding protein (MBP)[1] is a C-type lectin (1) which binds in the presence of calcium ions to 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 opsoniza-

tion of bakers' yeast (*Saccharomyces cerevisiae*) (9). MBP shows structural similarity to C1q, a subcomponent of the first complement component (C1), and can bind to the C1q 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 C1q, can associate with the unactivated proenzyme $C1r_2$–$C1s_2$ complex so that the classical pathway proceeds upon binding to mannan. This is based on the finding that isolated MBP and the $C1r_2$–$C1s_2$ proenzyme forms a complex, resulting in activation of C1s. These facts imply that MBP might utilize C1r and C1s for activation in vivo. However, an alternate mechanism is that protease(s) other than C1r and C1s may interact with MBP and initiate the classical pathway. In this study, we obtained human serum MBP in association with a C1s-like serine protease which consumes C4 and C2, resulting in complement

activation. We report here the purification and characteriza-
tion of the C1s-like serine protease.

Materials and Methods

Reagents. Mannan from *S. cerevisiae* was purchased from Sigma
Chemical Co. (St. Louis, MO). (*p*-amidinophenyl)methanesulfonyl-
fluoride (*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). Poly-
clonal anti–human C1s 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-
MBP to CNBr-activated Sepharose 4B was performed according
to the manufacturer's instructions. Diisopropylfluorophosphate,
[1,3-^{3}H] (^{3}H-DFP) and [^{125}I]NaI were purchased from New En-
gland Nuclear (Boston, MA). C4 was labeled using [^{125}I]NaI and
Iodo-Gen from Pierce Chemical Co. (Rockford, IL), according to
the manufacturer's instructions. Human C1s (20), C4 (21), C2 (22),
and oxidized C2 (^{oxy}C2) (23) were prepared as described previ-
ously. Guinea pig C1 was purchased from Diamedix Corporation
(Miami, FL). Veronal buffered saline (VB) was a standard solution
of Veronal buffer containing 0.148 M NaCl (pH 7.4). EDTA-GVB
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 CaCl$_2$ and 0.5 mM MgCl$_2$.

Purification of MBP and MASP. Human serum obtained by
recalcification 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 CaCl$_2$, 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 a monoclonal 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.

C4 or C2 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 mixtures (100 μl) were further
incubated for 60 min with 100 μl of C4-deficient guinea pig serum
and 100 μl of sheep erythrocytes sensitized with Ab (EA)
(10^8/ml), followed by addition of 1 ml of EDTA-GVB. The OD
of the supernatant was determined at 414 nm. The percentage of
C4 consumption was calculated from the hemolytic rate (*y*). 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-GVB. Incubation was then con-
tinued 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 × 10^8/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 10^8/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
(10^8/ml) at 30°C for 30 min. After washing, the cells were in-
cubated at 30°C for 20 min with 50 μl of human C4 and ^{oxy}C2
(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 (*y*)
was calculated spectrophotometrically. The average number of
hemolytic sites per cell (*z*) was calculated as $z = -\ln(1-y)$.

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 C1s (100 ng) in 10 μl of
VB was incubated with 10 μl of ^{125}I-C4 (260,000 cpm/50 ng C4)
at 37°C for 60 min and subjected to SDS-PAGE followed by auto-
radiography.

^{3}H-DFP Labeling. 20 μl of ^{3}H-DFP (740 kBq) were incubated
with 200 μl of MASP or C1s at 4°C for 17 h. 40 μl of BSA (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 mem-
brane (Bio-Rad Laboratories, Richmond, CA). After staining with
Coomassie brilliant blue, the band was excised and subjected to
gas-phase protein sequencing (Shimadzu PSQ-1; Shimadzu Cor-
poration, Kyoto, Japan).

Results

C1s-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 C1r$_2$–C1s$_2$, we assumed that an associa-
tion 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 adsorp-
tion. 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. 1 *A*, the extent of hemolysis of E.mannan depended
on the amount of the MBP fraction added, which indicated
that the fraction contained a C1s-like protease which acti-
vates 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
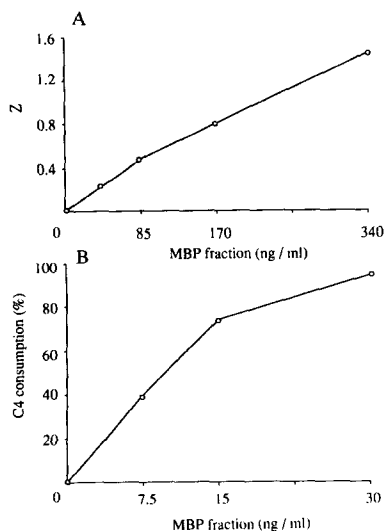*p*-APMSF (data not shown). These results indicate that the

Figure 1. C1s-like protease activities of the MBP fraction from anti-MBP (3E7)-Sepharose. (A) C4- and C2-activation on mannan. E.mannan sensitized with the indicated amounts of the MBP fraction from 3E7-Sepharose was treated with C4 and ^{oxy}C2 and subsequently with C3-C9. The extent of hemolysis is expressed as the z value. (B) C4 consumption. C4 was incubated with the indicated amounts of the MBP fraction from 3E7-Sepharose. Residual C4 activity was determined using C4-deficient guinea pig serum and EA.

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 C1s as shown in Fig. 1 B.

Isolation of a C1s-like Serine Protease in the MBP Fraction. To separate the C1s-like serine protease(s) from MBP, the MBP fraction from a 3E7-Sepharose column was dialyzed against
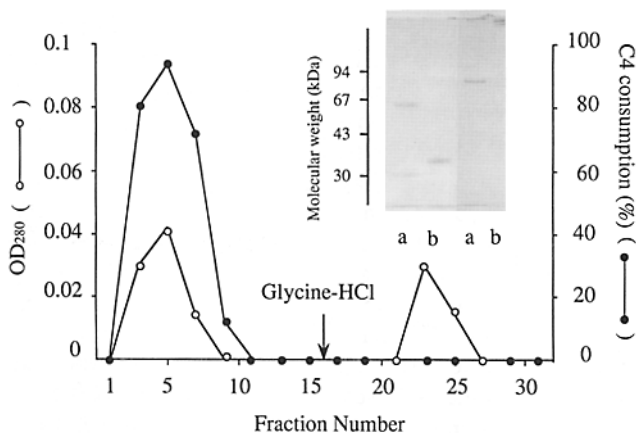
buffer containing EDTA and rechromatographed on a 3E7-Sepharose column. C4-consuming activity was recovered in the pass-through, whereas MBP was retained on the column and eluted with an acidic buffer (Fig. 2). C4- and C2-activating capacity, which results in generation of C3 convertase on E.mannan, was not observed either in the pass-through fraction or in the eluate obtained with the acidic buffer.

The SDS-PAGE profile of the C1s-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 C1s. To examine the possibility that MASP is C1s, 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 C1s under nonreducing conditions, both H and L chains of MASP migrated faster than the two corresponding chains of C1s under reducing conditions.

C1s is a serine protease whose L chain was labeled with ^{3}H-DFP. ^{3}H-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 C1s, we performed Western blot analysis using polyclonal anti-C1s serum. MASP at a 100-fold higher concentration than C1s did not react with polyclonal anti-C1s under conditions in which C1s was detected at concentrations as low as 1 μg/ml (data not shown).

Properties of MASP. We then examined whether anti-C1s serum inhibits C4 consumption by MASP. Under conditions in which MASP and C1s exhibit the same C4-consuming activities, MASP or C1s was incubated with C4 in the pres-
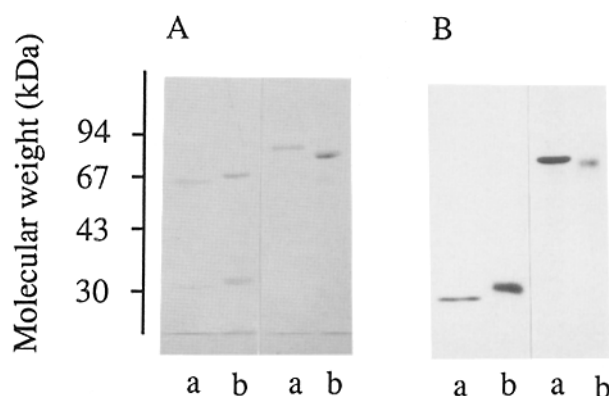

Figure 2. Separation of MASP from MBP by rechromatography on 3E7-Sepharose. Fractions from anti-IgM-Sepharose were dialyzed against EDTA-containing buffer and chromatographed on a second 3E7-Sepharose column. Fractions were diluted 1/500 for the C4 consumption assay. (*Inset*) SDS-PAGE analysis of MASP and MBP. MASP (*fractions 2–9*, lane *a*,) or MBP (*fractions 22–29*, lane *b*) was subjected to SDS-PAGE (8% gel) under reducing (*left*) or nonreducing (*right*) conditions. Proteins were stained with Coomassie brilliant blue R-250.

Figure 3. Comparison between MASP and C1s. (A) SDS-PAGE analysis MASP (lane *a*) or C1s (lane *b*) was subjected to SDS-PAGE (8% gel) under reducing (*left*) or nonreducing (*right*) conditions. Proteins were stained with Coomassie brilliant blue R-250. (B) ^{3}H-DFP labeling. After labeling with ^{3}H-DFP as described in Materials and Methods, MASP (lane *a*) or C1s (lane *b*) was subjected to SDS-PAGE under reducing (*left*) or nonreducing (*right*) conditions, followed by autoradiography.
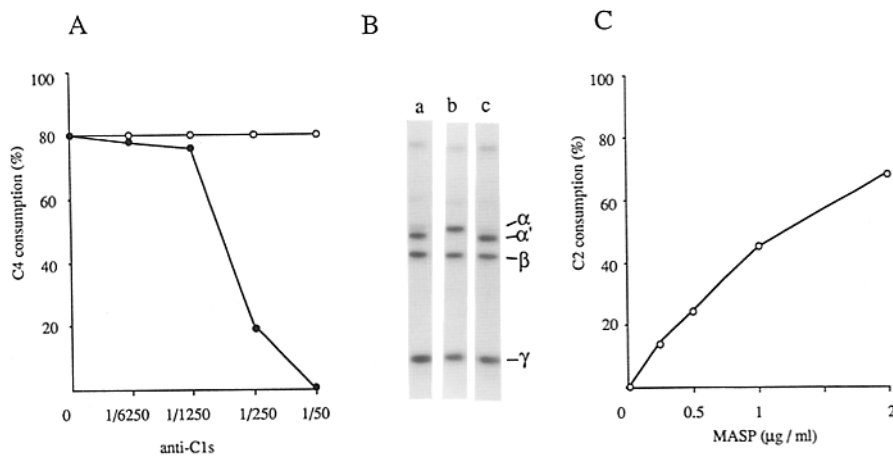
Figure 4. C1s-like activities. (*A*) Effect of anti-C1s on C4 consumption mediated by MASP or C1s. MASP (O) or C1s (●) was incubated with C4 in the presence of the indicated amounts of heat-inactivated anti-C1s serum at 37°C for 30 min and residual C4 activity was determined. (*B*) Cleavage of C4 by MASP. ^{125}I-C4 was incubated with MASP (lane *a*), buffer (lane *b*), or C1s (lane *c*) at 37°C for 60 min and subjected to SDS-PAGE (7.5% gel) followed by autoradiography. (*C*) C2 consumption by MASP. Human C2 was incubated with the indicated amounts of MASP at 37°C for 30 min and residual C2 activity was determined.

ence or absence of anti-C1s serum, and residual C4 activity was assayed. As shown in Fig. 4 *A*, 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. 4 *B*, 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. 4 *C*, 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 11 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 C1s and C1r are aligned. It is clear that MASP is different from C1s or C1r, 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.
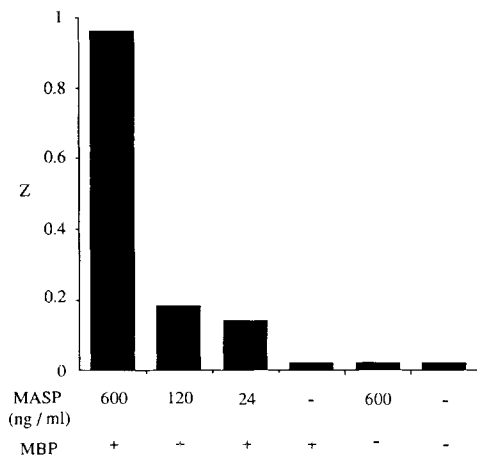
Discussion

In reconstitution experiments using MBP and the C1r₂–C1s₂ complex, Ohta et al. (17) and Lu et al. (18) con-

Figure 5. Reconstitution of MASP and MBP for exhibiting C4 and C2- activating capacity on mannan. 100 μl of E.mannan (10^8/ml), 50 μl of MBP (800 ng/ml), and 50 μl of MASP (600, 120, 24 ng/ml) were incubated at 30°C for 30 min. As a control, MBP alone or MASP alone (600 ng/ml) was incubated with E.mannan. After centrifugation and washing, the cells were suspended in MGVB at 10^8/ml. C4 and C2 activation on E.mannan was assayed as described in Materials and Methods.
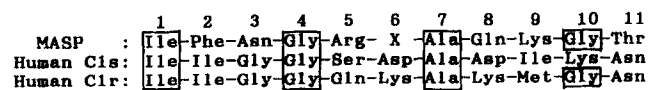

Figure 6. NH₂-terminal amino acid sequences of the L chains of MASP, human C1s, and C1r. Sequences of the L chains of human C1s and C1r have been reported (26). (*Boxed*) Amino acids common to sequences of MASP and the other two proteases.

cluded that MBP can associate in the presence of calcium ions with the unactivated proenzyme complex C1r$_2$-C1s$_2$, resulting in activation of C1s when MBP binds to mannan. If classical complement pathway activation triggered by MBP occurs in serum by this mechanism, one would expect the activated C1s in the MBP fraction obtained from a mannan-Sepharose column after elution with mannose. Our attempt to isolate the activated C1s from the MBP fraction revealed a C1s-like serine protease with both C4- and C2-consuming activity associated with MBP. We isolated and characterized this C1s-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 C1s. 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 C1s, as judged by results from SDS-PAGE. Under nonreducing conditions, the apparent molecular weight of MASP is slightly larger than that of C1s. Under reducing conditions, the two MASP polypeptides are smaller than the corresponding C1s polypeptides. These discrepancies are probably due to intradisulfide bonds within their molecules. Second, polyclonal antibodies against C1s did not react with MASP as assessed by immunoblotting, indicating that these two proteases are antigenically distinct. Third, providing more direct evidence, the NH$_2$-terminal amino acid sequences of the L chains of MASP and C1s differ. Amino acid analysis also showed that MASP is different from C1r. It is unlikely that both C1s and MASP are involved in classical pathway activation initiated by MBP, because anti-C1s 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 pres-ence 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 C1s-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 C1s-like serine protease (i.e., MASP).

Classical complement pathway activation initiated by C1q requires C1r as well as C1s. If a C1r-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 C1r-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 C1r-like serine protease for classical pathway activation initiated by MBP.

By analogy to C1s (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 C1r. If this is the case, MASP may be unique in exhibiting both C1r- and C1s-like activities.

In conclusion, we have isolated and characterized a novel C1s-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 C1r$_2$-C1s$_2$ complex to initiate the activation.

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