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Proteolytic Activities of Two Types of Mannose-Binding Lectin-Associated Serine Protease

Misao Matsushita,1* Steffen Thiel, ‡ Jens C. Jensenius, ‡ Itaru Terai, ‡ and Teizo Fujita*  

Mannose (or mannan)-binding lectin (MBL), a member of the collectins (1), is a Ca2+-dependent serum lectin that recognizes carbohydrates such as mannose and N-acetylglucosamine (GlcNAc) (2) on the surfaces of pathogens and plays a role in innate immunity through activating complement system (3–5). MBL is an oligomer of subunits composed of three identical polypeptide chains comprising a cysteine-rich, a collagen-like, a neck, and a carbohydrate recognition domain. MBL forms a complex with C1r/C1s-like serine proteases termed MASPs (MBL-associated serine proteases), and activates complement via the lectin pathway (6, 7). To date, two types of MASP, MASP-1 (8, 9) and MASP-2 (10, 11), have been identified in human MBL preparations. MASP-1, MASP-2, C1r, and C1s constitute a subfamily of the serine protease family (12). Their structure shares six domains, a first CUB domain, an epidermal growth factor-like domain, a second CUB domain, two complement control protein domains, and a serine protease domain (13, 14). Recently, a protein designated sMAP (small MBL-associated protein; sMAP or MAp19) is complexed with MBL. To clarify the proteolytic activities of MASP-1 and MASP-2 and inhibited their proteolytic activities. The Journal of Immunology, 2000, 165: 2637–2642.

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1 Abbreviations used in this paper: MBL, mannose (or mannan)-binding lectin; GVB, gelatin-containing Veronal-buffered saline; VB, Veronal-buffered saline; EDTA-GVB, gelatin-Veronal buffer containing EDTA; C-EDTA, guinea pig serum diluted with EDTA-GVB; C1 INH, C1 inhibitor; CUB, C1r/C1s/Uegf/bone morphogenetic protein 1; EA, sheep erythrocytes sensitized with Ab; EcAb, EA bearing guinea pig C1 and human C4b; sMAP, small MBL-associated protein; MAP19, MBL-associated plasma protein of 19 kDa; MASP, MBL-associated serine protease; MBL-complex, a complex consisting of MBL, MASP-1, MASP-2, and sMAP; MGVB, gelatin-Veronal buffer containing mannitol, CaCl2, and MgCl2; NPGB, p-nitrophenyl-p-guanidinobenzoate (NPGB) was from Merck (Rahway, NJ); CNBr-activated Sepharose 4B was from Amersham Pharmacia (Uppsala, Sweden); Mouse mAbs against MBL (3E7) (20) and MASP-1 (1E2) (21) and rabbit polyclonal Abs against a synthetic peptide corresponding to the first 20 N-terminal amino acid residues of MASP-2 that was conjugated to a multiple Ag peptide backbone (22). Coupling of mannan, anti-MBL (3E7), or anti-MASP-1 (1E2) to CNBr-activated Sepharose 4B was performed according to the manufacturer’s
Preparation of human MASP-1 and MASP-2

MASP-1 and MASP-2 in proenzyme forms were isolated from human serum as described previously (9, 15, 27). In brief, human serum was passed through a yeast mannan-Sepharose column using a 10 mM imidazole buffer (pH 6.0) containing 0.2 M NaCl, 20 mM CaCl₂, 0.2 mM NPGB, 20 μM p-APMSF, and 2% manniitol. Proenzyme MASP-1 and MASP-2 were separated on mannan-Sepharose and MBL was separated from MASPs and sMAP on a 5-MgCl₂, 1 M NaCl. From the OD determined at 414 nm, the OD of the supernatant was determined at 414 nm. The hemolytic effect of C1 INH on MASP-1 and MASP-2 was expressed as the percentage inhibition by the following formula: % inhibition = (Z₁ - Z₂)/Z₁ × 100, where Z₁ = Z value in the presence of C1 INH and Z₂ = Z value in the presence of MASP-1 or MASP-2.

Separation of MASP-1 and MASP-2

To obtain human MASP-1 and MASP-2 in proenzyme forms, the MBL-complex was first prepared from serum using a mannan column in the presence of serum protease inhibitors. This preparation also contained IgG and IgM. Further purification was achieved using an anti-MBL column. The MBL-complex was bound to the MBL-Sepharose column equilibrated with the same buffer as used for the second Sepharose column. MBL-complexes were eluted from the column with glycine buffer. After dialysis against 50 mM Tris buffer (pH 6.0) containing 0.2 M NaCl, 20 mM CaCl₂, 0.2 mM NPGB, and 20 μM p-APMSF, MBL complexes were eluted with imidazole buffer containing 20 mM EDTA and 1 M NaCl. Finally, proenzymes MASP-1 and MASP-2 were separated by passing through anti-MASP-1-Sepharose in the same buffer as used for the anti-MBL-Sepharose. MASP-2 was recovered in the effluents, whereas MASP-1 was eluted with 0.1 M glycine buffer (pH 2.2).

Human MBL-complexes, in which MASP-1 and MASP-2 were in activated forms, were isolated from serum. For this human serum, was first applied to a mannan-Sepharose column equilibrated with 50 mM Tris buffer (pH 6.0) containing 0.2 M NaCl, 20 mM CaCl₂, 0.2 mM NPGB, and 20 μM p-APMSF. After washing with starting buffer without NPGB and p-APMSF, the incubation was conducted with the same buffer containing 0.3 M manniitol. The MBL-complex eluate was next applied to the anti-MBL-Sepharose column equilibrated with the same buffer. MBL-complexes were eluted from the column with glycine buffer. After dialysis against 50 mM Tris buffer containing 1 M NaCl, 20 mM EDTA, the MBL-complex preparation was applied to an anti-MBL-Sepharose column. The effluent contained a mixture of MASP-1, MASP-2, and sMAP. MASP-1, MASP-2, and sMAP were recovered in the effluents, whereas MBL was retained. After equilibration with glycine buffer, the preparation containing MASP-1, MASP-2, and sMAP was applied to an anti-MASP-1-Sepharose column equilibrated with the same buffer as used for the second anti-MBL-Sepharose. At this step, MASP-2 passed through, whereas MASP-1 was retained on the column and eluted with glycine buffer. sMAP was found in both the MASP-1 and the MASP-2 fractions. The fractions containing MASP-1 or MASP-2 were pooled and used to study the effects of C1 INH on MASP activity.

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to the Laemmli method. After transferring proteins from the gels to polyvinyldene difluoride membranes (Millipore, Bedford, MA), the transferred proteins were probed with anti-MASP-1 peptide or anti-MASP-2 peptide Abs. Peroxidase-conjugated anti-rabbit IgG was used as a second Ab, and the blot was developed with a Konica Immunostain kit (Konica, Tokyo, Japan). Proteolytic activities of MASP-1 and MASP-2

C4 consumption was assayed as described previously (8). In brief, 50 μl of sample containing MASP-1 or MASP-2 diluted in MGVG was incubated with 50 μl of C4 (two site-forming units, SFU) at 37°C for 30 min. The reaction mixtures were further incubated for 60 min with 100 μl of 50-fold-diluted C-deficient guinea pig serum and 100 μl of sheep erythrocytes (10⁹/ml) bearing anti-sheep erythrocytes Abs (EA). The lytic reaction was terminated by the addition of 1 ml of EDTA-GVB. After centrifugation, the OD of the supernatant was determined at 414 nm. The hemolytic rate was calculated as the average number of hemolytic sites per cell (z) defined as: z = ln(1 - y)/y. The experiment was performed under reducing conditions.

C3 activation was assayed as described previously (18). In brief, 10 μl of samples and 10 μl of human C3 (2 μg) in VB were incubated at 37°C for 60 min, and the reaction mixture was subjected to SDS-PAGE (7.5% gel) under reducing conditions.

Complex formation between MASP and C1 INH

MASP-1 or MASP-2 was incubated with C1 INH at 37°C for 30 min. The mixtures were then subjected to SDS-PAGE under nonreducing conditions followed by immunoblotting.

Effect of C1 INH on the proteolytic activities of MASPs

Five microliters of fractions containing MASP-2 in MGVG were incubated with 45 μl of various amounts of C1 INH diluted in MGVG at 37°C for 15 min and then with 50 μl of C4 at 37°C for 30 min. Residual hemolytic activity of C4 was assayed as described above (C4 consumption), and the effect of C1 INH on MASP-1-mediated C4 activation was expressed as the percentage inhibition by the following formula: % inhibition = (Z₁ - Z₂)/Z₁ × 100, where Z₁ = Z value in the absence of MASP-1 or MASP-2 and C1 INH, Z₂ = Z value in the presence of MASP-2, and Z₃ = Z value in the presence of MASP-1 and C1 INH. For inhibition of C2 activation, 25 μl of MASP-1 or MASP-2 were incubated with 25 μl of various amounts of C1 INH, 50 μl of oxidized C2, and 100 μl of EAC4b at 30°C for 10 min and then with C-EDTA at 37°C for 1 h, and the effect of C1 INH on MASP-1 and on MASP-2 was expressed as the percentage inhibition by the following formula: % inhibition = Z₂/Z₁ × 100, where Z₁ = Z value in the absence of MASP-2 and Z₂ = Z value in the presence of MASP-2.

For direct observation of the effect of C1 INH on MASP-1, 10 μl of MASP-1 were incubated with 5 μl of various amounts of C1 INH at 37°C for 30 min and then with 10 μl of C3 (2 μg) for 60 min. The reaction mixtures were analyzed by SDS-PAGE (7.5% gel) under reducing conditions.

Results

Separation of MASP-1 and MASP-2

To obtain human MASP-1 and MASP-2 in proenzyme forms, the MBL-complex was first prepared from serum using a mannan column in the presence of serum protease inhibitors. This preparation also contained IgG and IgM. Further purification was achieved using an anti-MBL column. The MBL-complex was bound to the anti-MBL-Sepharose column equilibrated with the same buffer as used for the second anti-MBL-Sepharose. MASP-1 were incubated with 10 μl of various amounts of C1 INH at 37°C for 30 min and then with 10 μl of C3 (2 μg) for 60 min. The reaction mixtures were analyzed by SDS-PAGE (7.5% gel) under reducing conditions.
anti-MBL column, and MASPs and sMAP were then eluted with EDTA at a high salt concentration, whereas MBL was retained on the column. Finally, the eluate containing MASPs and sMAP was subjected to affinity chromatography on an anti-MASP-1 column. At this step, MASP-2 passed through the column, whereas MASP-1 was retained on the column and could subsequently be eluted with an acidic buffer (Fig. 1). Most of sMAP coeluted with MASP-1. MASP-1 and MASP-2 obtained in this way showed a single band under reducing conditions with molecular size of ~93 kDa and 70 kDa, respectively, indicating that both MASPs were in proenzyme forms.

Based on the results that proenzymes MASP-1 and MASP-2 were separated on the anti-MASP-1 column, we next isolated MASP-1 and MASP-2 in activated forms. The MBL-complex was prepared utilizing mannann and anti-MBL columns. After dialysis against a buffer containing EDTA and 1 M NaCl, the MBL-complex from the anti-MBL column was applied to the anti-MBL column again. In this buffer, MASP-1, MASP-2, and sMAP passed through the column and thus separated from MBL. This preparation was finally fractionated on an anti-MASP-1 column. MASPs were separated on the anti-MASP-1-Sepharose column. The effluent (fractions 1–10) and eluate containing MASPs and sMAP were then eluted with an acidic buffer (Fig. 1). Most of sMAP coeluted with MASP-1 and MASP-2 obtained in this way showed a single band under reducing conditions with molecular size of ~93 kDa and 70 kDa, respectively, indicating that both MASPs were in proenzyme forms.

Proteolytic activities of MASP-1 and MASP-2
Preparations containing either MASP-1 or MASP-2 in activated forms were tested for proteolytic activities against C4, C2, and C3. C4 consumption and C2 activation by MASPs were determined hemolytically as described in Materials and Methods. C3 cleavage by MASP was directly assessed by SDS-PAGE. As shown in Fig. 3, C4 consumption was observed with the fractions containing MASP-2 but not with those containing MASP-1. Both MASP-1 and MASP-2 activated C2. C3 cleavage with an appearance of the α-chain was noted for MASP-1 but not for MASP-2. In contrast with the activated forms of MASPs, proenzymes MASP-1 and MASP-2 showed no proteolytic activities against C4, C2, and C3 (data not shown), indicating that at the conditions of the experiment no activation of the proenzymes occurred.

Complex formation between MASPs and C1 INH
C1 INH forms stable complexes with C1s and C1r in a 1:1 ratio and inhibits their proteolytic activities. To determine the effect of C1 INH on MASP-1 or MASP-2, we first tested for covalent complex formation between C1 INH and MASPs in activated forms. C1 INH was incubated with MASP-1 or MASP-2 in activated forms at 37°C for 1 h and then subjected to SDS-PAGE followed by immunoblotting. As shown in Fig. 4A, a novel band with an apparent m.w. of 196 kDa reacting with anti-MASP-1 Ab appeared after incubation of C1 INH with MASP-1. The molecular size of this band almost matched the sum of MASP-1 (81 kDa) and C1 INH (98 kDa). Similarly, incubation of MASP-2 (63 kDa) with C1 INH resulted in an appearance of a new band (175 kDa) (Fig. 4B). These results indicate that C1 INH formed equimolar complexes with MASP-1 and MASP-2.

Inhibition of MASP function by C1 INH
We next determined whether C1 INH inhibits the proteolytic activities of MASP-1 and MASP-2 in activated forms. The proteolytic activities of MASP-1 against C3 and C2 were examined in the presence of various amounts of C1 INH. As shown in Fig. 5A, C3 cleavage by MASP-1 was inhibited by C1 INH in a dose-dependent manner. Similarly, C1 INH inhibited C2 activation mediated by MASP-1 (Fig. 5B). Fig. 5, C and D, depict the results of the effects of C1 INH on MASP-2 activities against C4 and C2. Both

**FIGURE 2.** Separation of MASP-1 and MASP-2 in activated forms on an anti-MASP-1 column. A preparation of MASPs and sMAP was obtained as the effluent from anti-MBL-Sepharose and applied to an anti-MASP-1 (1E2)-Sepharose column. The effluent (fractions 1–10) and eluate (fractions 12–20) were analyzed for the presence of the components by SDS-PAGE (12% gel) under reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250. (A) After SDS-PAGE, immunoblotting was performed using Abs against MASP-1 peptide (B) or MASP-2 peptide (C). Arrow in B indicates the L chain of MASP-1. Upper and lower arrows in C indicate the H chain of MASP-2 and sMAP, respectively. Right ordinates, Molecular mass markers.

**FIGURE 3.** Proteolytic activities of MASP-1 and MASP-2 against C4, C2, and C3. The effluent fractions from anti-MASP-1-Sepharose containing MASP-2 (fractions 1–4) and the eluate containing MASP-1 (fractions 12–16) from anti-MASP-1-Sepharose were analyzed for the proteolytic activities. Top ( ), C4 consumption. Samples of each fraction were incubated with C4 and residual hemolytic activities of C4 were determined. Middle ( ), C2 activation. Samples of each fraction were incubated with EAC4b and C2 and then with C3 to C9 (guinea pig serum diluted with EDTA-GVB). From hemolytic rates, % consumption was determined as described in Materials and Methods. Bottom (SDS-PAGE), C3 cleavage. Samples of each fraction or buffer (left lane) were incubated with C3 and the reaction mixtures were subjected to SDS-PAGE (7.5% gel) under reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250.
activities of MASP-2 were inhibited by C1 INH in a dose-dependent manner.

Discussion

The binding between C1q, C1r, and C1s in the C1 complex is facilitated by Ca\(^{2+}\), and the complex is formed in a 1:2:2 stoichiometry. On the other hand, the mode of binding and stoichiometry of the complex composed of MBL, MASP-1, MASP-2, and sMAP remains unsolved. In the present report, we showed that MASP-1, MASP-2, and sMAP dissociate from MBL in the presence of EDTA and high concentration of salt (1 M NaCl). Several lines of evidence have revealed that EDTA alone is insufficient, and both EDTA and high concentration of salt are required for the dissociation of MBL from the other components, suggesting that the complex formation is facilitated by a combination of Ca\(^{2+}\) and presumably electrostatic interactions (28, 29). When the mixture of MASP-1, MASP-2, and sMAP from the anti-MBL column was applied to an anti-MASP-1 column with buffer containing EDTA and 1 M NaCl, MASP-2 was recovered in the pass-through fractions, whereas MASP-1 was retained on the column and could be eluted with an acidic buffer. Two explanations can be proposed for the separation of MASP-1 and MASP-2 on this column; MASP-1 and MASP-2 form a complex in a Ca\(^{2+}\)-dependent manner or, alternatively, MASP-1 and MASP-2 are independently complexed with MBL.

As shown in Figs. 1 and 2, sMAP copurified on the anti-MASP-1 column with both MASP-1 and MASP-2 when they were in activated forms, whereas most of the sMAP coeluted with MASP-1 when the MASPs were in proenzyme forms. The reason for this difference in the behavior of sMAP remains unclear. One possibility is that a long exposure of MASP-1-sMAP to EDTA and 1 M NaCl during dialysis in the purification step of activated MASPs allowed some dissociation of MASP-1 and sMAP. Alternatively, it could be suggested that sMAP has a lower affinity for activated MASP-1 than for unactivated MASP-1.

Isolated MASP-1 and MASP-2 in activated forms exhibited proteolytic activities against C3 and C4, respectively. The specificity of MASP-2 for C4 is consistent with a previous report (10). Both
MASPs activated C2. In this respect, the function of MASP-2 resembles C1s in the C1 complex, whereas MASP-1 shows unique proteolytic activities. Analysis of the cDNA of MASP and C1r/C1s serine protease domains from human, mouse, hamster, Xenopus, carp, shark, lamprey, and ascidian revealed that the MASP/C1r/C1s family falls into two groups (30, 31). The first group, termed “TCN type,” where the serine residue in the active center is encoded by TCN, encompasses human C1r, C1s, mouse MASP-2, Xenopus MASP-2, carp MASP, shark MASP, and lamprey MASP. The TCN type possesses a so-called “histidine loop” structure, whereas the AGY type does not. It is speculated that the AGY type diverged from the TCN type in the evolution of the MASP/C1r/C1s family (30, 32).

The ascidians appear to lack the classical pathway C4 and C2, and the function of ascidian MASPs may thus be restricted to cleavage of C3 (33). This type of substrate selectivity has been preserved in human C1-INH, which, like ascidian MASPs, possesses the histidine loop. The C4 cleaving activity of human MASP-2 and C1s could be speculated to be related to the cutout of the histidine loop. The split exon nature of ascidian MASPs and MASP-1 (30), and the TCN codon contrasting to MASP-2 and C1r/C1s are features of no structural consequence to the proteins, but most useful when trying to sort out the phylogeny. Although the stoichiometry of the MBL-complex and activation mechanism remain unsolved, MASP-2 in the complex is likely to possess the same function as C1s, which cleaves C4 and C2 resulting in the formation of C3 convertase, C4b2a. On the other hand, MASP-1 directly cleaves C3 into C3a and C3b, the latter of which initiates the alternative complement pathway (18) and also acts as an opsonin. The physiological significance of the observed C2 activating-capacity of MASP-1 is unclear. It is also unknown which MASP is more active in cleaving C2 on a molar basis, since the presence of sMAP, which has no equivalent in C1, although its role in proteolytic activities. However, several features are different between the MBL-complex and C1. First, the MBL-complex possesses sMAP, which has no equivalent in C1, although its role in the complex remains unsolved. Second, as discussed above, it is possible that MASP-1 and MASP-2 are independently associated with MBL.

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References


