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

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Mannose (or mannan)-binding lectin (MBL), a member of the collectins (1), is a Ca2+-dependent serum lectin that recognizes carbohydrates such as mannos and N-acetylglucosamine (GlcNAc) (2) on the surfaces of pathogens and plays a role in innate immunity by activating the complement system. In human, two types of MBL-associated serine protease (MASP-1 and MASP-2) and a truncated protein of MASP-2 (small MBL-associated protein; sMAP or MAp19) are complexed with MBL. To clarify the proteolytic activities of MASP-1 and MASP-2 against C4, C2, and C3, we isolated these two types of MASP in activated forms from human serum by sequential affinity chromatography. On an anti-MASP-1 column, MASP-2 passed through the column in the presence of EDTA and high salt concentration, whereas MASP-1 was retained. Isolated MASP-1 and MASP-2 exhibited proteolytic activities against C3 and C4, respectively. C2 was activated by both MASPs. C1 inhibitor (C1 INH), an inhibitor for C1r and C1s, formed equimolar complexes with MASP-1 and MASP-2 and inhibited their proteolytic activities. The Journal of Immunology, 2000, 165: 2637–2642.

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3 Abbreviations used in this paper: MBL, mannos (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, C1s/C1r/T1p, bone morpho- genetic protein 1; EA, sheep erythrocytes sensitized with Ab; EAC4b, 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-guanidinobenzoxa; p-APMSF, (aminophenyl)methanesulfonyl fluoride (p-APMSF) and mannos were from Wako Pure Chemical Industries (Osaka, Japan). p-Nitrophenyl-p-guanidinobenzoxa (NPGB) was from Merck (Rahway, NJ). CNBr-activated Sepharose 4B was from Amershams Pharmacal (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 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, C1s/C1r/T1p, bone morpho- genetic protein 1; EA, sheep erythrocytes sensitized with Ab; EAC4b, 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-guanidinobenzoxa; p-APMSF, (aminophenyl)methanesulfonyl fluoride (p-APMSF) and mannos were from Wako Pure Chemical Industries (Osaka, Japan). p-Nitrophenyl-p-guanidinobenzoxa (NPGB) was from Merck (Rahway, NJ). CNBr-activated Sepharose 4B was from Amershams Pharmacal (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).
instructions. Human C3, C4 (23), C2 (24), oxidized C2 (25), and C1 INH (26) were prepared as previously described. Veronal-buffered saline (VB) is a 10-mM solution of Veronal containing 0.148 M NaCl (pH 7.4). EDTA-GVB was supplemented with 10 mM EDTA and 0.1% gelatin. MGVB is a 5-mM solution of Veronal containing 0.074 M NaCl, 0.1% gelatin, 2.3% mannitol, 2 mM CaCl2, and 0.5 mM MgCl2.

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% mannitol. Proenzymes MASP-1 and MASP-2 complexed with MBL were eluted with the above buffer containing 0.3 M mannnose. To separate proenzymes MASP-1 and MASP-2 from MBL, preparations containing the complex were applied to anti-MBL-Sepharose and then MASPs 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 eluents, 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, elution was conducted with the same buffer containing 0.3 M mannnose. 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, whereas MBL was retained and subsequently eluted 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 polyvinylidene difluoride membranes (Millipore, Bedford, MA), blots 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 MGVB 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 C4-deficient guinea pig serum and 100 μl of sheep erythrocytes (106/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, defined as the average number of hemolytic sites per cell (z) defined as

\[ z = -\ln(1 - y) \]

was calculated. The percentage consumption was determined by the following formula: % consumption = (Z1/Z2) × 100, where Z1 = z value in the absence of sample and Z2 = z value in the presence of sample.

EA bearing human C4b (EAC4b) was prepared as described previously (23). Fifty microliters of samples, 50 μl of oxidized human C2 (2 SFU), and 100 μl of EAC4b (107/ml) were incubated in MGVB at 37°C for 10 min, and then 200 μl of 50-fold-diluted guinea pig serum with EDTA-GVB (C-EDTA) was added to the reaction mixture as a source of C3 to C9. After additional incubation at 37°C for 60 min, 1 ml of EDTA-GVB was added to terminate the reaction. From the OD determined at 414 nm, z was calculated as described above.

C3 activation was assayed as described previously (18). In brief, 10 μl of samples and 10 μl of human C3 (2 μg) in VB was 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 MASPs 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 MGVB were incubated with 45 μl of various amounts of C1 INH diluted in MGVB 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 = (Z3 - Z2)/(Z1 - Z2) × 100, where Z1 = z value in the absence of MASP-2 and C1 INH, Z2 = z value in the presence of MASP and C1 INH, and Z3 = z value in the presence of MASP and C1INH. 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 - Z2) × 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 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.

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 serine 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
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 1–4) were analyzed for the proteolytic activities against C4, C2, and C3 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 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.
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

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**FIGURE 4.** Complex formation between MASPs and C1 INH. A, MASP-1 was incubated with buffer (lane a) or with C1 INH (lane b). As a control, C1 INH alone (lane c) was incubated. The reaction mixtures were analyzed by SDS-PAGE (6% gel) under nonreducing conditions, followed by immunoblotting using anti-MASP-1. Upper arrow and lower arrow indicate C1 INH-MASP-1 and MASP-1, respectively. B, MASP-2 was incubated with buffer (lane d) or C1 INH (lane e). Lane f is C1 INH alone, and analyzed by SDS-PAGE (6% gel) followed by immunoblotting using anti-MASP-2. Upper arrow and lower arrow indicate C1 INH-MASP-2 and MASP-2, respectively. Ordinate, Molecular mass markers.

**FIGURE 5.** Inhibition of proteolytic activities of MASPs by C1 INH. A. The effect of C1 INH on the proteolysis of C3 by MASP-1. A fixed mixture of C3 and MASP-1 was incubated with various amounts of C1 INH before SDS-PAGE. The C1 INH concentrations were: lane a, 0 μg/ml; lane b, 20 μg/ml; lane c, 10 μg/ml; lane d, 5 μg/ml; lane e was C3 alone. B and D, The effect of C1 INH on the proteolysis of C2 by MASP-1 (B) or MASP-2 (D). Under the conditions in which both MASP-1 and MASP-2 showed the ε value of 1 in the absence of C1 INH, MASP-1 or MASP-2 was incubated with EAC4b, C2 and various amounts of C1 INH and then with C3 to C9. From hemolytic rates, percent inhibition was determined as described in Materials and Methods. Results are mean ± SD (n = 3). C, The effect of C1 INH on the proteolysis of C4 by MASP-2. Under the conditions in which MASP-2 showed 60% consumption of C4 in the absence of C1 INH, MASP-2 was incubated with various amounts of C1 INH and then with C4. From residual hemolytic activity of C4, percent inhibition was determined as described in Materials and Methods. Results are mean ± SD (n = 3).
MASPs activated C2. In this respect, the function of MASPs resembles C1s in the C1 complex, whereas MASPs-1 shows unique proteolytic activities. Analysis of the cDNA of MASPs and C1r/C1s serine protease domains from human, mouse, hamster, Xenopus, carp, shark, lamprey, and ascidian revealed that the MASPs/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 C1-2M, mouse C1, Xenopus MASPs-1, carp MASPs, and lamprey MASPs. 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 MASPs/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 C1-2M, which, like ascidian MASPs, possesses the histidine loop. The C4 cleaving activity of human MASPs-2 and C1s could be speculated to be related to the cutout of the histidine loop. The split exon nature of ascidian MASPs and AGY-2M (30), and the TCN codon contrasting to MASPs-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, MASPs-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, MASPs-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 MASPs-1 is unclear. It is also unknown which MASPs is more active in cleaving C2 on a molar basis, since the presence of sMAP in both MASPs-1 and MASPs-2 preparations does not allow quantitative analysis of MASPs. However, it should be noted that more C1 INH was required for preventing proteolysis of C2 by MASPs-1 than by MASPs-2, suggesting that the proteolytic activity of MASPs-1 against C2 might be lower than that of MASPs-2.

Unlike C1r or C1s, MASPs-1 forms a complex with α2-macroglobulin (α2M) that is a protease inhibitor in blood (34). The effect of α2M on MASPs-2 is to be elucidated. In this report, we demonstrated that C1 INH inhibited the proteolytic activities of both MASPs-1 and MASPs-2 by forming stable complexes in a 1:1 stoichiometry as do C1r and C1s, indicating a function in regulation of lectin pathway activation. Wong et al. (35) also observed that a mixture of activated MASPs-1 and MASPs-2 interacts with C1 INH, resulting in the formation of complexes between C1 INH and each MASPs. In the C1 complex in blood, C1 INH associates noncovalently with proenzyme C1r to prevent its autoactivation. Upon binding of C1 to immune complexes, C1 INH dissociates from proenzyme C1r, resulting in the autoactivation of C1r and the subsequent activation of C1s. Thus, C1 INH also modulates C1 activation by inhibiting the autoactivation of C1r. If MASPs-1 and/or MASPs-2 autoactivate, it is possible that their autoactivation is also regulated by C1 INH in a manner similar to C1r. The present and previous studies (34, 36) suggest that C1 INH regulates both the classical and lectin pathways and α2M regulates the latter.

The MBL-complex and the C1 complex appear to be similar in that the serine proteases involved in each complex have specific 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 MASPs-1 and MASPs-2 are independently associated with MBL.

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