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Natural Substrates and Inhibitors of Mannan-Binding Lectin-Associated Serine Protease-1 and -2: A Study on Recombinant Catalytic Fragments[1]

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Mannan-binding lectin-associated serine protease (SP) (MASP)-1 and MASP-2 are modular SP and form complexes with mannan-binding lectin, the recognition molecule of the lectin pathway of the complement system. To characterize the enzymatic properties of these proteases we expressed their catalytic region, the C-terminal three domains, in *Escherichia coli*. Both enzymes autoactivated and cleaved synthetic oligopeptide substrates. In a competing oligopeptide substrate library assay, MASP-1 showed extreme Arg selectivity, whereas MASP-2 exhibited a less restricted, trypsin-like specificity. The enzymatic assays with complement components showed that cleavage of intact C3 by MASP-1 and MASP-2 was detectable, but was only $\sim$0.1% of the previously reported efficiency of C3bBb, the alternative pathway C3-convertase. Both enzymes cleaved C3i 10- to 20-fold faster, but still at only $\sim$1% of the efficiency of MASP-2 cleavage of C2. We believe that C3 is not the natural substrate of either enzyme. MASP-2 cleaved C2 and C4 at high rates. To determine the role of the individual domains in the catalytic region of MASP-2, the second complement control protein module together with the SP module and the SP module were also expressed and characterized. We demonstrated that the SP domain alone can autoactivate and cleave C2 as efficiently as the entire catalytic region, while the second complement control protein module is necessary for efficient C4 cleavage. This behavior strongly resembles C1s. Each MASP-1 and MASP-2 fragment reacted with C1-inhibitor, which completely blocked the enzymatic action of the enzymes. Nevertheless, relative rates of reaction with α-2-macroglobulin and C1-inhibitor suggest that α-2-macroglobulin may be a significant physiological inhibitor of MASP-1. *The Journal of Immunology*, 2003, 170: 1374–1382.

The complement system is an important component of the innate immune defense. A prerequisite for the complement system to exert its function is its activation, which can occur through three different routes: the classical, the lectin, and the alternative pathways. The activation of the complement system results in the sequential activation of serine protease (SP)[4] zymogens. The first step in the lectin and the classical pathways is the binding of a specific recognition molecule (mannan-binding lectin (MBL) or C1q) to activator structures, which is followed by the activation of associated SP (1, 2). Although the lectin pathway

was discovered more than a decade ago (3), there are many uncertainties concerning the composition of the activation complex and the substrate specificities of the MBL-associated SP (MASPs). MBL is a member of the collectin family of proteins and binds to specific carbohydrate arrays on the surface of various pathogens through its C type lectin domains (4). To date, three MASPs have been described. First, a single enzyme "MASP" was identified and characterized as the enzyme responsible for the initiation of the complement cascade (i.e., cleaving C2, C4, and possibly C3) (5, 6). Later, it turned out that MASP is in fact a mixture of two proteases: MASP-1 and MASP-2 (7). It was demonstrated that the MBL-MASP-2 complex alone is sufficient for complement activation (8). This is a significant difference from the C1 complex, where the coordinated action of two SP (C1r and C1s, proteases of the classical pathway initiation complex) leads to the activation of the complement system. The role of MASP-1 in the MBL-MASPs complex remains unknown. It was suggested that MASP-1 could directly cleave C3 and thereby activate complement (9, 10), but other laboratories disputed these results (2, 11). Recently, a third novel protease, MASP-3, has been isolated, but its function is yet to be resolved (12). In addition to the SP there is a small nonenzymatic protein (small MBL-associated protein or 19-kDa MBL-associated protein (MAp19)) present in the MBL-MASPs complex (13, 14). The 19-kDa MBL-associated protein is identical with the first two N-terminal domains of MASP-2 except for a unique 4-aa-long sequence at its C-terminal. Several lines of evidence suggest that there are different MBL-MASPs complexes (12, 15) and a

large fraction of the total MASPs in serum is not complexed with MBL (16, 17).

The MASPs, C1r and C1s, form a family of proteases with identical domain organization (18, 19). In these enzymes the first N-terminal C1r/C1s/sea urchin Uegf/bone morphogenic protein (CUB) domain is followed by an epidermal growth factor-like domain and a second CUB domain. A tandem repeat of complement control protein (CCP) modules precedes the C-terminal serine protease domain. Upon activation, an Arg-Ile bond is cleaved in the SP domain of these zymogens.

Although the substrate specificities of MASP-1 and MASP-2 have been studied using natural and recombinant proteins, several important questions remained unanswered. One of the most debated issues is the possible direct C3 cleaving capacity of the MASPs (especially MASP-1). It has been demonstrated that ascidian MASP-1 cleaves ascidian C3 directly (20, 21) for human MASPs; however, various laboratories have reported contradicting data in this field and in none of the cases were kinetic data presented (2, 9–11, 22). Therefore, clarifying the role and significance of MASP-1 and MASP-2 in direct C3 cleavage is of primary importance. If the cleavage of C3 by MASP-1 proves to be insignificant, then the field is still open to assess the biological importance of MASP-1. This could possibly be accomplished by identifying the range of its substrate specificity and the degree of its specific activity. Previous studies showed that MASP-2 cleaved C2 and C4 efficiently, with rates similar to C1s, a classical pathway enzyme (8, 22). However, the contribution of the individual domains to the enzymatic properties of MASP-2 has not yet been determined.

Similar to the substrate specificities of the MASP proteases, their reactivity toward natural inhibitors has already been investigated but not yet in full detail. It is accepted that C1-inhibitor (C1-inh) reacts with both proteases (2, 10), but the rates of the reactions are unknown and the role of another inhibitory protein, α-2-macroglobulin (α_2M), is controversial (2, 22, 23).

We addressed the above questions by defining our objectives as follows: 1) to resolve the controversy about the substrate specificities of MASP-1 and MASP-2 using recombinant MASP-1 and MASP-2 fragments and carefully purified serum complement components; 2) to assess the range of substrate specificity and relative specific activities of MASP-1 and MASP-2 using a competing oligopeptide substrate library; 3) to identify the role of the individual domains of the catalytic region of MASP-2 in C2 and C4 cleavage and compare it with similar studies done on C1r and C1s; 4) to clarify and rank the inhibitory effect of C1-inh and α_2M on MASP-1 and MASP-2.

Materials and Methods

Construction of recombinant plasmids for the expression of the MASP-1 and MASP-2 fragments

For all recombinant constructs the pET-17b expression vector was digested with *Nhe*I and *Eco*RI restriction endonucleases, and PCR products with identical sticky ends were ligated into the plasmid. In the case of the MASP-1 CCP1-CCP2-SP construct, the following forward and reverse primers were used: GCGGCTAGCATGACTGGTAATGAGTGCCCA GAGCTA and GCGGAATTCTCAGTTCCTCACTCCGGTGACCCT (the *Nhe*I and *Eco*RI restriction sites are underlined). Preceding the MASP-1 sequence, the forward primer contained the codon of four amino acids (Ala-Ser-Met-Thr) of the T7-Tag sequence, which increased the efficiency of the recombinant protein expression. As a result of this, the N terminus of the recombinant MASP-1 contained the four extra amino acids and the MASP-1 sequence began with Gly[298] and ended with Asn[699]. The same strategy was followed upon constructing the MASP-2 fragments. The reverse primer for the three MASP-2 constructs was identical: GCGGAAT TCTTAAAAATCACTAATTATGTTCTCG; therefore, all recombinant fragments ended with Phe[686]. The forward primers for MASP-2 CCP1-CCP2-SP, CCP2-SP, and SP were: GCGGCTAGCATGACTGGTTG GAAGATCCACTACACG, GCGGCTAGCATGACTATTGTTGACTGT GGCCCTCCTG, and GCGGCTAGCATGACTCCTGTTTGTGGACTA

TCAGCC, respectively. The recombinant CCP1-CCP2-SP construct contains the Ala-Ser-Met tripeptide before the Thr[287] amino acid of MASP-2 at the N terminus. In the case of the CCP2-SP and SP fragments, the Ala-Ser-Met-Thr extra tetrapeptide is followed by Ile[363] and Pro[432] of MASP-2, respectively.

Expression, renaturation, and purification of the recombinant proteins (patent pending)

The expression plasmids were transformed into BL21(DE3) pLysS host strain, and the transformants were selected on Luria-Bertani medium plates containing ampicillin and chloramphenicol. The expression was conducted according to the manufacturer's instructions (24). After induction with isopropyl-D-thiogalactoside, the cells were collected in a 1/10 volume of Tris-EDTA buffer and frozen at −20°C. The cells were then thawed and the inclusion bodies were collected by centrifugation (12,000 × g, 15 min, 4°C). The supernatant was discarded and the pellet was washed three times with Tris-EDTA buffer (1/10 of the culture volume). The inclusion bodies were solubilized in 6 M GuHCl, 0.1 M Tris-HCl (pH 8.3), 100 mM DTT at room temperature. The solubilized proteins were diluted into the refolding buffers. The refolding buffers contained 50 mM Tris-HCl, 3 mM reduced glutathione (Sigma-Aldrich, St. Louis, MO), 1 mM oxidized glutathione (Sigma-Aldrich), 5 mM EDTA, and 0.5 M arginine. The pH of the solution was adjusted to pH 10.0 in the case of the MASP-2 SP fragment and to pH 9.0 otherwise. The renatured protein solutions were dialyzed against 50 mM Tris-HCl (pH 9.0) and filtered on a 0.45 μm nitrocellulose membrane. The renatured proteins were purified on a Q-Sepharose-Fast Flow column (Amersham Pharmacia Biotech, Piscataway, NJ). The samples were loaded onto the column and the elution was conducted with a linear NaCl gradient from 0–400 mM. Fractions were analyzed by SDS-PAGE. The recombinant proteins were further purified by cation exchange on Mono-S columns (Amersham Pharmacia Biotech). The pH of the fractions containing the MASP-1 CCP1-CCP2-SP fragment and the MASP-2 SP fragment was adjusted to pH 5.0 and dialyzed against 50 mM sodium acetate (pH 5.0). The MASP-2 CCP-SP and MASP-2 CCP1-CCP-2-SP containing fractions were dialyzed against 20 mM Na-phosphate (pH 6.3). In each case, a linear gradient of 0–600 mM NaCl was applied and the fractions containing the protein of interest were pooled. All proteins were estimated to be >90% pure by SDS-PAGE. Individual protein fragments were dialyzed against 20 mM Tris, 140 mM NaCl, pH 7.4, aliquoted, frozen in liquid nitrogen, and kept at −20°C. The concentration of the recombinant proteins was determined by measuring OD$_{280}$ using the calculated absorption coefficients 18.7, 18.5, 19.1, and 14.9 (1%, 1 cm) for the MASP-2 CCP1-CCP2-SP, CCP2-SP, SP, and MASP-1 CCP1-CCP2-SP fragments, respectively. The concentration of the MASP-1 CCP1-CCP2-SP fragment was corrected for its autodegraded, nonfunctional content using relative concentration results obtained by N-terminal sequencing. For calculation of the absorption coefficients we used the method of Gill et al. (25) taking disulfide bridges into account. The molecular masses calculated from the amino acid sequences were 44,017, 35,722, 28,164, and 45,478 Da for the MASP-2 CCP1-CCP2-SP, CCP2-SP, SP, and MASP-1 CCP1-CCP2-SP fragments, respectively.

Purification of C2, C3, C4, factor I, and factor H

Human C2 was prepared by a novel immunoaffinity method (26). It was dialyzed against 20 mM HEPES, 140 mM NaCl, pH 7.4, and frozen in liquid nitrogen. Extinction coefficient (280 nm, 1%, 1 cm) was estimated from its amino acid sequence to be 9.4 for the 102-kDa protein.

Human C3 and C4 were purified according to the method of Dodds (27) with minor modifications. C3 was estimated to be >90% pure by SDS-PAGE and >95% of the C3 preparation was thiolester intact that was shown by the presence of autocatalytic cleavage fragments of C3 α-chain (28) and the lack of cleavage by factor I in the presence of factor H. C4 was determined to be >80% pure by SDS-PAGE, inter-α-trypsin inhibitor is a visible contaminant. C3 and C4 were dialyzed against 20 mM HEPES, 140 mM NaCl, pH 7.4. C3 was used within 3 days; C4 was frozen in liquid nitrogen in 200 μl aliquots and kept at −80°C for storage. Aliquots were thawed only once and then used within 5 days of thawing to ensure the proteins were active. Extinction coefficients, ϵ(280 nm, 1%, 1 cm), of 9.7 and 8.3, and molecular mass of 185 and 205 kDa were used for C3 and C4, respectively (29, 30).

Factors H and I were purified by the method of Sim et al. (31). Extinction coefficients, ϵ(280 nm, 1%, 1 cm), of 12.4 and 14.3, and molecular mass of 150 and 88 kDa were used for Factors H and I, respectively (32).

Purification of C1-inh and $\alpha_2 M$

Cl-inh was purified from human serum using protocols by Sim and Reboul (33) and Pilatte et al. (34). Extinction coefficient, ϵ(280 nm, 1%, 1 cm), of 3.6 and molecular mass of 71.1 kDa were used (35).

Human $\alpha_2 M$ was isolated first as a by-product of the method for C3 and C4 purification (27), then further purified according to Salvesen and Enghild (36). Extinction coefficient, ϵ(280 nm, 1%, 1 cm), of 9.0 and molecular mass of 720 kDa were used (36).

Amidolysis of C3

The intact thiolester bond in C3 was cleaved using ammonium salt as the nucleophile to obtain $C3(NH_3)$, according to Soames and Sim (37) with modifications. C3 was incubated with a final concentration of 0.2 M ammonium hydrogen carbonate for 90 min at 37°C, and assuring that the final pH of the reaction was above 8.0. At the end of the incubation, the material was dialyzed against 20 mM HEPES, 140 mM NaCl, pH 7.4.

N-terminal sequencing

After SDS-PAGE and blotting to polyvinylidene difluoride membrane, the N-terminal amino acid sequences of the recombinant proteins were determined by automated Edman degradation in an Applied Biosystems Procise 494A protein sequencer (PerkinElmer, Beaconsfield, U.K.)

Oligopeptide substrate library

The specificities of the MASP-1 and MASP-2 CCP1-CCP2-SP fragments were tested on a competing oligopeptide library applying the method described by Antal et al. (38). Concentration of individual substrate mixture components was 40 μM, and the enzyme concentrations were 0.3 μM in 550 μl reaction mixture. Fifty microliter aliquots were transferred into the HPLC injection tubes containing 10 μl 5 M acetic acid to stop the reaction at the respective reaction times: 0, 1, 5, 10, 15, and 30 min.

C2, C3, C3(NH$_3$), C4 cleavage

Serial dilutions of MASP-1 and MASP-2 fragments ranging between concentrations of 1 μM and 10 pM were incubated at 37°C in 20 mM HEPES, 140 mM NaCl, pH 7.4 with C2, C3, $C3(NH_3)$, or C4 at typical concentrations of 0.5–1 μM. The cleavage was followed by SDS-PAGE analysis under reducing conditions. Appropriate concentrations of the MASP fragments for further detailed kinetic analysis were chosen to be where half of the protein substrates were hydrolyzed during the first 20 min of incubation. The protein substrates C2, C3, $C3(NH_3)$, or C4 were then incubated with the selected concentrations of the MASP fragments at 37°C in 20 mM HEPES, 140 mM NaCl, pH 7.4. Typically, 11–13 samples were taken at varying time periods but always within 50 min from the beginning of the reaction. Cleavages rates were quantified by measuring the diminution of the cleaved chain visualized by Coomassie-stained SDS-polyacrylamide gels using a GEL DOC 1000 instrument and Molecular Analyst software for denzitometric calculations (Bio-Rad, Hercules, CA). The reactions were assumed to be of the Michaelis-Menten type. In the case of C3 and $C3(NH_3)$ cleavage, a further reasonable assumption of $K_M \gg$[substrate] was made. The constants K_M, k_{cat}, and k_{cat}/K_M were estimated by unbiased, nonlinear regression methods regressing the data on the following equations: $t = ([S_0] - [S] + K_M \times ln([S_0]/[S]))/(k_{cat} \times [E_0])$ or in the case of C3 and $C3(NH_3)$ cleavage, on equation $[S] = [S_0] \times exp(-t \times [E_0] \, k_{cat}/K_M)$ (39).

Radioiodination

The MASP-1 CCP1-CCP2-SP, MASP-2 CCP1-CCP2-SP, CCP2-SP, and SP fragments were labeled with ^{125}I using iodogen (l, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycoluril) (Sigma-Aldrich) as the oxidizing agent (40). Eppendorf tubes (1.5 ml) were coated with 200 μl of iodogen (2 mg/ml in $CHCl_3$) and excess iodogen was washed off with 0.5 ml 20 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4 (HBS). Protein samples were transferred to the iodogen-coated tube and incubated with 10 μl Na^{125}I (1 mCi) (Amersham Pharmacia Biotech) for 10–20 min on ice. Free ^{125}I was removed by desalting on a PD-10 Sephadex G-25 gel filtration column (Amersham Pharmacia Biotech) which was presaturated with 0.1% (v/v) Emulphogene BC720 (Sigma-Aldrich) in HBS. Radiolabeled protein fractions were pooled and stored at 4°C. Specific activity of a protein was determined by measuring on a MiniAssay type 6-20 manual gamma counter (Mini Instruments, Burnham-on-Crouch, Essex, U.K.).

Autoradiography

Dried SDS-PAGE gels of ^{125}I-labeled samples were exposed to x-ray film (Fuji RX; Fuji Photo Film, London, U.K.) at -70°C in autoradiography cassettes with intensifying screens. Films were developed using an X-Ograph imaging system Compact X4 (X-Ograph Imaging System, Malmesbury, U.K.).

Reactivity toward C1-inh and $\alpha_2 M$

Inhibition of the enzymatic activities of the MASP fragments by C1-inh and $\alpha_2 M$ were tested as follows. MASP-1 and MASP-2 fragments at concentrations selected in the C2, C3, $C3(NH_3)$, and C4 cleavage tests were incubated at 37°C with excess molar ratios of either C1-inh, $\alpha_2 M$, or buffer in 20 mM HEPES, 140 mM NaCl, pH 7.4 for 40 min. Complement substrates C2, C3, $C3(NH_3)$, or C4 were added at typical concentrations of 0.5–1 μM and the samples were further incubated for 20 min. The cleaved and uncleaved chains were visualized by reducing SDS-PAGE and the degree of inhibition was compared.

To assess the role of both C1-inh and $\alpha_2 M$ in the regulation of MASP-1 and MASP-2, the following experiment was devised. Each of the ^{125}I-labeled MASP fragments were incubated with either C1-inh or $\alpha_2 M$ or both at physiological relative concentrations ([MASP-1]:[C1-inh]:[$\alpha_2 M$] = 1:30:60; [MASP-2]:[C1-inh]:[$\alpha_2 M$] = 1:90:180) at concentrations [MASP-1 CCP1-CCP2-SP] = 9 nM, [MASP-2 fragments] = 3 nM at 37°C in 20 mM HEPES, 140 mM NaCl, pH 7.4 for 90 min. The samples were further incubated with reducing sample buffer at 37°C for 30 min, run on SDS-PAGE, and visualized by means of autoradiography. The following serum concentrations were used when calculating the relative concentrations: [MASP-1] = 65 nM (16), [MASP-2] = 22 nM (41), [C1-inh] = 2 μM (42), and [$\alpha_2 M$] = 4 μM (43).

To assess the rate of SDS stable complex formation between the MASP-1 and MASP-2 CCP1-CCP2-SP fragments and C1-inh, the ^{125}I-labeled MASP fragments at concentrations ranging between 30 and 100 nM were incubated with 100–350 nM C1-inh at 37°C in 20 mM HEPES, 140 mM NaCl, pH 7.4, for 40 min. Typically, 11–13 samples were taken at varying time periods. The reactions were stopped by reducing sample buffer containing 0.125 M Tris, 4.8% SDS, 8 M urea, 20% glycerol, 720 mM 2-ME, 0.02% bromophenol blue, pH 6.8. The samples were further incubated at 37°C for 30 min and run on reducing SDS-PAGE. Reaction rates were visualized by means of autoradiography and the diminution of the reacted MASP chain was quantified using a GEL DOC 1000 instrument and Molecular Analyst software for denzitometric calculations (Bio-Rad). The kinetic constants were calculated assuming irreversible inhibition preceded by reversible enzyme-inhibitor complex formation. Using steady-state approximation and neglecting the initial enzyme concentration compared with the initial inhibitor concentration ([I_0] $\gg$ [E_0]), the data were regressed on the following equation using nonlinear regression methods: $[E] = [E_0] \times exp(-k_{obs} \times t)$; $k_{obs} = k_2 \times [I_0]/(K_i + [I_0])$, where k_{obs} is the observed pseudo-first order rate of reaction, k_2 is the first order rate constant for inactivation, and K_i is the dissociation constant for the noncovalent E·I complex (44).

Results

Expression, purification, and characterization of the recombinant MASP fragments

The catalytic region of MASP-1 and MASP-2, consisting of the two CCP modules and the SP domain (CCP1-CCP2-SP) (Fig. 1) was expressed in *Escherichia coli* BL-21 cells using the pET-17b expression vector. In the case of MASP-2, truncated fragments of the catalytic region (the SP domain with one CCP module and the SP domain alone) were also expressed in the same expression system (Fig. 1). Because the recombinant proteins accumulated as inclusion bodies inside the bacterial cells, renaturation procedures were needed to restore the native folded structure. The renatured recombinant proteins were purified by ion exchange chromatography on Q-Sepharose Fast Flow and Mono-S columns (Fig. 2). The renaturation and the purification procedures were followed by SDS-PAGE and the purified proteins were subjected to N-terminal sequencing. Before renaturation, the inclusion body proteins yielded single bands on reducing SDS-PAGE corresponding to the zymogen form of the enzymes. After renaturation, the MASP-1 CCP1-CCP2-SP fragment was fully activated and yielded two major bands under reducing conditions, which corresponded to the two-chain structure obtained by the cleavage of the activation site Arg^{448}-Ile^{449} bond, as determined by sequencing. The MASP-1 CCP1-CCP2-SP fragment copurified with a minor contaminant

FIGURE 1. Schematic representation of MASP-1 and MASP-2 and their expressed recombinant fragments. *a*, Schematic representation of the domain structure of MASP-1 and MASP-2. *b*, Fragments of MASP-1 and MASP-2 expressed in an *E. coli* expression system.
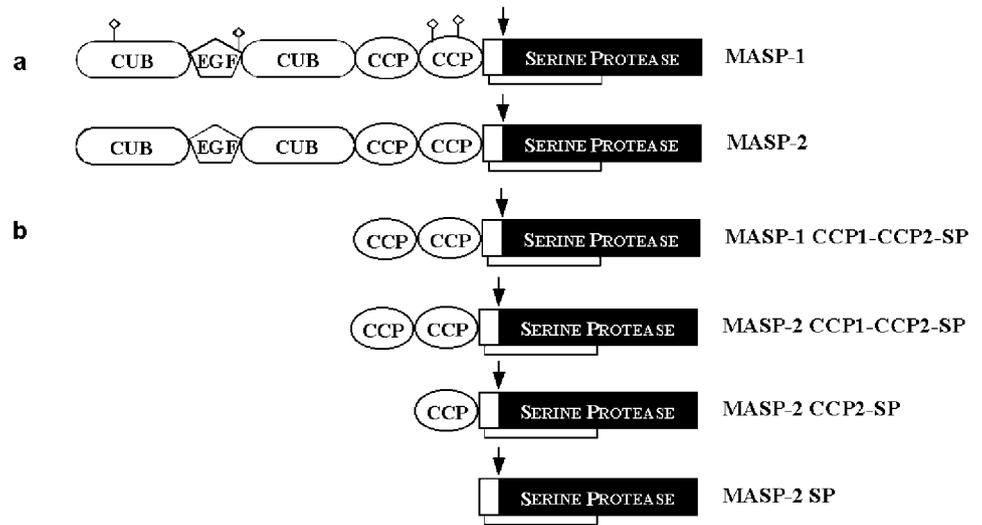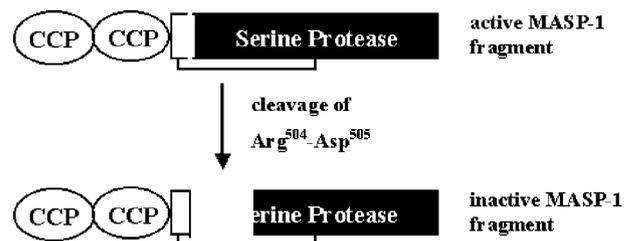
that migrated on SDS-PAGE as a band of 24 kDa under reducing and at 39 kDa under nonreducing conditions. N-terminal sequencing revealed that this fragment is a degraded form of the MASP-1 catalytic region lacking a 6-kDa fragment from its SP domain (Fig. 3). The cleavage occurred at the Arg^{504}-Asp^{505} bond removing the histidine from the catalytic triad and thus causing the loss of enzymatic activity. The MASP-2 fragments migrated as single chain structures under reducing conditions throughout the renaturation procedure; however, they became activated during the purification process. Sequencing analysis confirmed the activation of the MASP-2 fragments to occur through the cleavage of the Arg^{444}-Ile^{445} bond and further modifications were observed in the case of the MASP-2 CCP1-CCP2-SP fragment that displayed Ile^{291} at its N terminus instead of an expected Ala. This indicated that the bond between Lys^{290}-Ile^{291} was cleaved and a heptapeptide (Ala-Ser-Met-Thr-Gly-Trp-Lys) was completely removed. However, this cleavage does not compromise the integrity of the MASP-2 CCP1-CCP2-SP construct, since for technical reasons the original N terminus contained three extra amino acids (Ala-Ser-Met of the T7-Tag) and the CUB2-CCP1 junction is at His^{292}-Tyr^{293}. The yield of proteins produced in the form of inclusion bodies in the cell culture was estimated to be between 10 and 40 mg/l before renaturation. The overall yield of purified proteins recovered from 1 L of cell culture ranged between 0.1 and 0.5 mg.
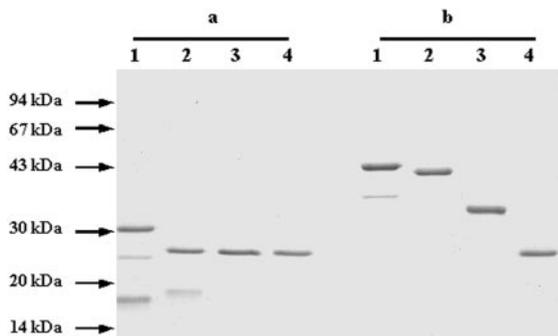
Proteolytic activity of MASP-1 on complement components

The proteolytic activity of the MASP-1 catalytic fragment (CCP1-CCP2-SP) was investigated on protein substrates that are involved in the formation of the C3- and C5-convertase enzyme complexes of the classical and lectin pathways (i.e., on C2, C4, and C3). To resolve the controversy about the C3 cleaving capacity of MASP-1, both C3 with an intact thiolester bond and C3 with a reacted thiolester bond (C3i) were used in the assays. Native C3 with an intact thiolester bond was isolated from fresh human serum and used within 3 days. It was rigorously tested before the proteolytic measurements by treating it with Factor I and Factor H (45) and was found that >95% of the C3 in the preparation had uncleaved thiolester bond. To test the proteolytic activity of MASP-1 on C3i (i.e., on C3 with a cleaved thiolester bond), a batch of the C3 preparation was reacted with ammonia, a small nucleophile, leading to $C3(NH_3)$ formation. The characteristic k_{cat}/K_M values show that the MASP-1 CCP1-CCP2-SP fragment cleaves C3i with a low but significant efficiency, whereas its proteolytic action on C3 is ~20-fold less (Table I and Fig. 4). It is important to note that in control experiments using the C1r CCP1-CCP2-SP fragment expressed in the same expression system as the MASP fragments (46) we observed no cleavage of either C3 or C3i. Our experiments demonstrated that complement component C4, similar to C3, is basically resistant to the proteolytic activity of MASP-1 (Table II). However, C2 was digested by our MASP-1 fragment at a moderate rate (Table II). All cleavages could be inhibited by preincubating MASP-1 CCP1-CCP2-SP with a molar excess (1- to 3-fold) of either C1-inh or $\alpha_2 M$ (see *Materials and Methods*).

FIGURE 2. Coomassie-stained SDS-PAGE of the purified MASP-1 and MASP-2 fragments. *a*, Samples run under reducing conditions. *Lane 1*, MASP-1 CCP1-CCP2-SP; *lane 2*, MASP-2 CCP1-CCP2-SP; *lane 3*, MASP-2 CCP2-SP; and *lane 4*, MASP-2 SP. *b*, Samples run under nonreducing conditions. *Lane 1*, MASP-1 CCP1-CCP2-SP; *lane 2*, MASP-2 CCP1-CCP2-SP; *lane 3*, MASP-2 CCP2-SP; and *lane 4*, MASP-2 SP.

FIGURE 3. An observed autodegradation pattern of the MASP-1 CCP1-CCP2-SP fragment. Cleavage occurs at the Arg^{504}-Asp^{505} bond, which results in the removal of a 6-kDa fragment from the active enzyme. The autolysis of the MASP-1 CCP1-CCP2-SP fragment causes the loss of its enzymatic activity due to the removal of the histidine from the catalytic triad.

Table I. *Cleavage rates of the MASP-1 and MASP-2 CCP1-CCP2-SP fragments on C3 and C3(NH$_3$) (a type of C3i) substrates*[a]

	C3	C3(NH$_3$)
MASP Fragment	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
MASP-1 CCP1-CCP2-SP	300 ± 30	6,100 ± 600
MASP-2 CCP1-CCP2-SP	350 ± 20	3,300 ± 300

[a] Unbiased, nonlinear regression methods were used to determine k_{cat}/K_M values at 37°C from two to four experiments. SEs are indicated after mean values.

Proteolytic activity of MASP-2 on complement components

The proteolytic activity of the MASP-2 CCP1-CCP2-SP fragment on C3 was very similar to that of the MASP-1 fragment. Although MASP-2 CCP1-CCP2-SP had a hardly detectable activity on C3, it exhibited a low but significant enzymatic activity on C3i (Table I). In contrast to the MASP-1 fragment, the CCP1-CCP2-SP, CCP2-SP, and SP fragments of MASP-2 cleaved C2 and C4 very efficiently (Table II) (Figs. 5 and 6). The comparison of the three MASP-2 fragments shows that the SP domain of MASP-2 on its own can cleave C2 with high efficiency. The addition of the CCP modules to the SP domain slightly decreases the proteolytic power in C2 cleavage. When C4 is used as a substrate, the MASP-2 fragments show a different picture. Although the SP domain on its own is capable of cleaving C4, the presence of the CCP2 domain significantly increases the efficiency of the catalysis (a 44-fold increase in the k_{cat}/K_M value). The CCP1-CCP2-SP fragment of MASP-2 is also very efficient in cleaving C4, although its proteolytic power is less than that of the CCP2-SP fragment. All cleavages could be abolished by preincubating the MASP-2 fragments with a molar excess (1- to 3-fold) of C1-inh or with a large molar excess (40-fold) of α_2M.

Proteolytic activities of the catalytic fragments of MASP-1 and MASP-2 on oligopeptide substrates

To further investigate the range of substrate specificity and relative specific activities of MASP-1 and MASP-2 we applied them to a competing oligopeptide substrate library. (38). This library contains a mixture of seven oligopeptides (each 13 aa in length) with different cleavage sites (P1) for both trypsin and chymotrypsin-like enzymes (His-Ala-Ala-Pro-Xxx-Ser-Ala-Asp-Ile-Gln-Ile-Asp-Ile, where Xxx could be Lys, Arg, Tyr, Leu, Phe, Trp, or Pro). The individual peptide substrates compete for the protease under investigation, in our case for either the MASP-1 CCP1-CCP2-SP or the MASP-2 CCP1-CCP2-SP fragment. The results show that both enzymes cleave oligopeptides that contain either Arg or Lys at their P1 positions. The MASP-1 catalytic fragment (CCP1-CCP2-SP) exerted substantially higher activity on the Arg oligopeptide substrate than the MASP-2 fragment (Fig. 7). Comparing the relative specific activities of the two fragments on the P1 Arg oligopeptide under the same conditions, at 15 min MASP-1 showed almost 90% digestion, whereas MASP-2 cleaved <20% of the substrate. This result substantiates that MASP-1 is a potent protease with significant catalytic strength compared with MASP-2. Another essential outcome of the competing oligopeptide substrate library experiments is that MASP-1 possesses extreme Arg selectivity at the P1 site of its substrate. This behavior strongly resembles that of thrombin, which exhibited similar Arg selectivity in the same competing oligopeptide library system (38). MASP-2 also preferred the Arg substrate to the Lys one, but the degree of preference was much less than that of MASP-1. A similar specificity profile was obtained for trypsin in this oligopeptide library system (Fig. 7).
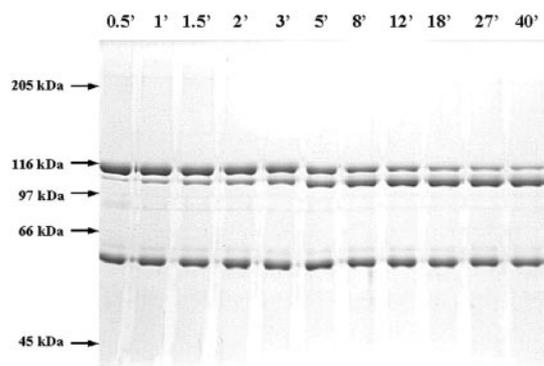

FIGURE 4. Cleavage of C3i by the MASP-1 CCP1-CCP2-SP fragment. Cleavage occurred at 37°C, at an enzyme:substrate molar ratio of 1:5. Samples were taken at various time periods from 0.5–40 min and run under reducing conditions on SDS-PAGE. Incubation times are indicated in minutes. Very similar cleavage patterns were obtained by incubating C3i with MASP-2 CCP1-CCP2-SP, at an enzyme:substrate molar ratio of 2:3, and by incubating C3 with either MASP-1 CCP1-CCP2-SP or MASP-2 CCP1-CCP2-SP, at enzyme:substrate molar ratios of 2:1.

The reaction of the MASP-1 and MASP-2 fragments with C1-inh and α_2M

As mentioned in the previous sections, C1-inh completely abolished the proteolytic activity of both MASP-1 and MASP-2 on all substrates. A marginal molar excess of C1-inh over the enzyme concentration proved to be sufficient for full inhibition. Another inhibitor, α_2M, also inhibited the reactions, but exhibited lower affinity toward MASP-2. A small 1- to 3-fold molar excess of α_2M completely blocked the moderate cleavages of C2 and C3 by the MASP-1 CCP1-CCP2-SP fragment, whereas only a 40-fold molar excess over the MASP-2 fragments was capable of significantly hindering C2 and C4 cleavage. To confirm the inhibition of the MASPs by α_2M and to estimate the relative rates of inhibition by α_2M and C1-inh, the MASP fragments were labeled with [125]I. The radiolabeled MASPs were then mixed with C1-inh, α_2M, or both in serum-like relative concentrations, and the enzyme-inhibitor complexes were analyzed by SDS-PAGE followed by autoradiography (Fig. 8). Both C1-inh and α_2M formed SDS-PAGE stable complexes with all MASP fragments. In the case of the MASP-2 fragments, C1-inh proved to be the primary inhibitor, as it reacted faster with the MASP-2 fragments than α_2M, whereas in the case of MASP-1 α_2M may be a significant physiological inhibitor. The observed pseudo-first order rates of reaction (k_{obs}) with C1-inh were 5-fold less for the MASP-1 CCP1-CCP2-SP than for the MASP-2 CCP1-CCP2-SP fragment. The K_i values for the C1-inh and MASP-2 CCP1-CCP2-SP reaction were in the nanomolar range (Table III).

Discussion

MASP-1 and MASP-2 are the major SP of the lectin pathway. Their functional characterization has been hindered by their low serum concentration ([MASP-1] = 6 μg/ml, [MASP-2] ≈ 2 μg/ml) (16, 41) that rendered their isolation extremely difficult. Most of the work reported on native MASPs (from serum) has involved using mixtures of MASPs, not single, isolated proteins. The recombinant expression of the full-length MASPs has been very difficult. Vorup-Jensen et al. (8) transiently expressed human MASPs in human embryonic kidney 293 cells, but their recombinant MASP-1 had unexpected molecular mass and showed no enzymatic activity. Chen et al. (47) tried to produce rat MASP-1 and MASP-2 in Chinese hamster ovary cells, but the wild-type proteases were cytotoxic to the

Table II. *Cleavage rates of the MASP-1 and MASP-2 fragments on C2 and C4 substrates[a]*

MASP Fragment	C2			C4		
	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
MASP-1 CCP1-CCP2-SP	0.10 ± 0.07	4.8 ± 4.3	30,000 ± 12,000	0.002 ± 0.001	2.7 ± 2.4	690 ± 400
MASP-2 CCP1-CCP2-SP	1.9 ± 0.8	4.0 ± 1.7	500,000 ± 9,000	0.9 ± 0.4	1.6 ± 0.5	550,000 ± 50,000
MASP-2 CCP2-SP	3.0 ± 2.2	2.7 ± 2.0	1,300,000 ± 200,000	2.0 ± 1.0	0.4 ± 0.2	5,700,000 ± 550,000
MASP-2 SP	3.9 ± 3.0	3.2 ± 2.8	2,500,000 ± 1,300,000	0.20 ± 0.06	2.0 ± 1.0	130,000 ± 30,000

[a] Unbiased, nonlinear regression methods were used to determine k_{cat}, K_M, and k_{cat}/K_M values at 37°C from two to four experiments. SEs are indicated after mean values.

cells; therefore, only inactive mutants could be produced. Rossi et al. (22) expressed full-length human MASP-1 and MASP-2 in a baculovirus insect cell system, but due to the very low yield the proteases could not be purified to homogeneity.

To circumvent these problems we decided to express the catalytic fragments of human MASP-1 and MASP-2 in recombinant form. Based on previous experience, we decided to use an *E. coli*-based system, since catalytic fragments of C1r, a homolog of the MASPs, have already been successfully produced in the same system (46). It has long been widely accepted in the literature that the proteolytic γB fragments of C1r and C1s, which consist of three domains, two CCP modules and the SP domain, retain the catalytic activity of the entire molecule both in terms of substrate specificity and catalytic efficiency (48–50). Recent studies with recombinant fragments of C1r and C1s reinforced this view (46, 51, 52), and it is also supported by the recent studies of Rossi and coworkers (22), who expressed CCP1-CCP2-SP fragments of MASP-1 and MASP-2 in the baculovirus insect cell system, which showed comparable enzymatic activities with the full-length molecules toward protein and ester substrates.

Furthermore, to study the role and significance of the CCP modules in the proteolytic function and regulation of MASP-2, the CCP2-SP and SP fragments of MASP-2 were also expressed and characterized (Fig. 1). The inclusion bodies were renatured and the recombinant proteins were purified to homogeneity by ion exchange chromatography (Fig. 2). The enzymatic activity of the purified fragments was tested using various natural and synthetic substrates and inhibitors.

A major, but so far unresolved question is the autoactivation capacity of MASP-1 and MASP-2. The autoactivation process of the MASP protein fragments, which involves the cleavage of an Arg-Ile bond in the SP domain, could be monitored on reducing SDS-PAGE. Upon renaturation the MASP-1 CCP1-CCP2-SP

fragment became fully activated, and gave two bands on PAGE. Also, a less intense band of 24 kDa, most probably an autolytic cleavage product of the SP domain, copurified with the MASP-1 CCP1-CCP2-SP fragment. It seems that MASP-1 has a strong propensity to autoactivate and the activated enzyme is prone to autodegradation upon prolonged incubation. The physiological relevance of this autolysis is yet unknown, but we observed the corresponding autolytic product of the full-length MASP-1 protein in partially purified serum MBL-MASPs complexes (data not shown).

The MASP-2 catalytic fragments revealed somewhat different autoactivation features. The MASP-2 fragments retained their proenzymic form throughout the renaturation procedure. However, post purification they migrated as two separate bands on reducing SDS-PAGE, a characteristic of the activated enzyme. During purification, the protein concentration increases dramatically on the ion exchange columns (up to 1–2 mg/ml), which can facilitate an intermolecular autoactivation process and increase the overall rate of active enzyme formation. Our observations reveal that the smaller MASP-2 fragments (CCP2-SP and SP) can also autoactivate, indicating that the autoactivating ability is an inherent property of the SP domain. In contrast to a previous suggestion (22), but similar to C1r (46), the CCP modules of MASP-2 do not play an essential role in this process. The MASP-2 CCP1-CCP2-SP fragment also showed a sign of autodegradation, because its N-terminal begins with Ile[291] instead of the expected Ala. It looks likely that the short stretch of CUB2 domain fused with the Ala-Ser-Met tripeptide at the N terminus (Ala-Ser-Met-Thr-Gly-Trp-Lys[290]) folded loosely and the Lys[290]-Ile[291] bond in this region was an easy target for a protease with trypsin-like specificity (i.e., MASP-2).

Probably the most controversial issue concerning the substrate specificities of MASP-1 and MASP-2 is their ability to cleave C3.
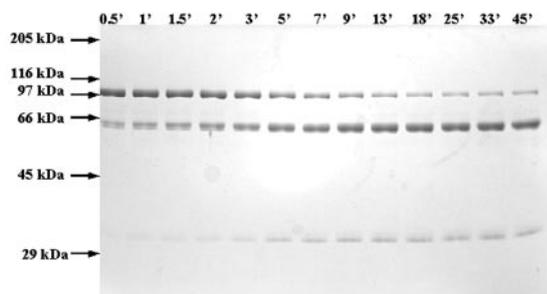
FIGURE 5. Cleavage of C2 by the MASP-2 CCP1-CCP2-SP fragment. Cleavage occurred at 37°C, at an enzyme:substrate molar ratio of 1:500. Samples were taken at various time periods from 0.5–45 min and run under reducing conditions on SDS-PAGE. Incubation times are indicated in minutes. Very similar cleavage patterns were obtained by incubating C2 with either MASP-1 CCP1-CCP2-SP, MASP-2 CCP2-SP, or MASP-2 SP at enzyme:substrate molar ratios of 1:13, 1:550, and 1:500, respectively.
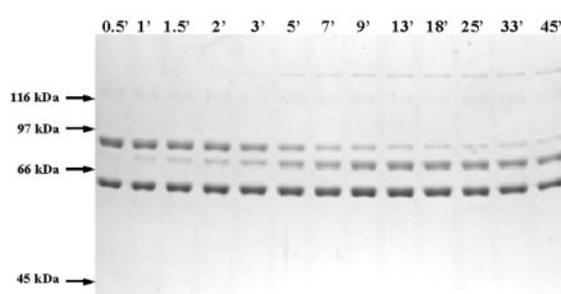
FIGURE 6. Cleavage of C4 by the MASP-2 CCP1-CCP2-SP fragment. Cleavage occurred at 37°C, at an enzyme:substrate molar ratio of 1:320. Samples were taken at various time periods from 0.5–45 min and run under reducing conditions on SDS-PAGE. Incubation times are indicated in minutes. Very similar cleavage patterns were obtained by incubating C4 with either MASP-1 CCP1-CCP2-SP, MASP-2 CCP2-SP, or MASP-2 SP at enzyme:substrate molar ratios of 2:1, 1:1000, and 1:130, respectively.
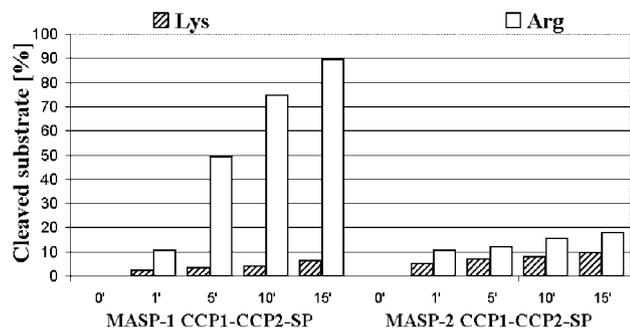
FIGURE 7. Peptidase activities of MASP-1 CCP1-CCP2-SP and MASP-2 CCP1-CCP2-SP. Activities were analyzed on an oligopeptide substrate library containing His-Ala-Ala-Pro-Xxx-Ser-Ala-Asp-Ile-Gln-Ile-Asp-Ile type peptides, where Xxx could be Lys, Arg, Tyr, Leu, Phe, Trp, or Pro. Cleavages were observed only if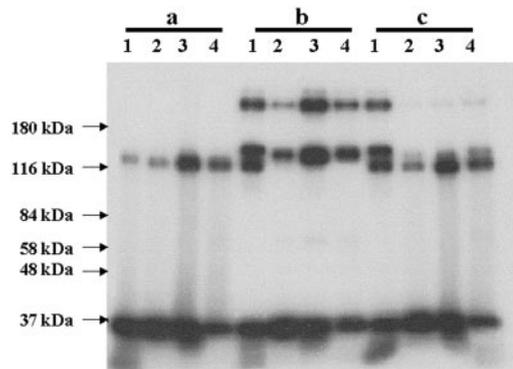 Xxx was either Arg (□) or Lys (▨). Blocks are calculated from the decrease of peak areas after 0, 1, 5, 10, and 15 min reaction times with the respective enzyme.

FIGURE 8. Radiogram of the reaction of the MASP fragments with C1-inh and α_2M. ^{125}I-labeled MASP-1 CCP1-CCP2-SP (*lanes a1, b1,* and *c1*), MASP-2 CCP1-CCP2-SP (*lanes a2, b2,* and *c2*), MASP-2 CCP2-SP (*lanes a3, b3,* and *c3*), and MASP-2 SP (*lanes a4, b4, c4*) fragments were incubated at 37°C with either C1-inh (*lanes a1–a4*), α_2M (*lanes b1–b4*), or both (*lanes c1–c4*) at relative concentrations identical with their relative serum levels. Samples were run under reducing conditions on SDS-PAGE and a radiogram was developed from the gel. Bonds at around 130 kDa correspond to the C1-inh MASP fragment complexes (*lanes a1–a4* and *c1–c4*). Apparent bonds around 320 kDa correspond to one intact subunit of α_2M complexed with a cleaved α_2M subunit plus the MASP fragments (*lanes b1–b4* and *c1–c4*). Bonds observed around 140 kDa correspond to a cleaved α_2M subunit in complex with the MASP fragments (*lanes b1–b4* and *c1–c4*) The bonds around 30 kDa in each lane are unreacted MASP species. Note that when both inhibitors are present (*lanes c1–c4*) the MASP-1 fragment (*lane c1*) displays the pattern observable with α_2M (*lane b1*), whereas the MASP-2 fragments (*lanes c2–c4*) show patterns similar to their reaction with C1-inh (*lanes a2–a4*).

C3 contains a thiolester group inside the molecule that becomes exposed after the cleavage of C3 by the C3 convertase enzymes. The exposed thiolester group is then rapidly hydrolyzed or reacts with a nucleophile on the cell surface. Nevertheless, uncleaved C3 is also prone to spontaneous hydrolysis or attack by low molecular mass nucleophiles yielding nonfunctional C3i, which may also occur during the purification process and upon prolonged storage. It is important to differentiate between C3 (i.e., C3 with an intact thiolester bond) and C3i (C3 with a cleaved thiolester bond) as they respond differently to proteolysis, although they migrate identically on SDS-PAGE. We measured the kinetic parameters of C3 cleavage by using both freshly prepared C3 and C3i. Our results demonstrate that the k_{cat}/K_M values of both MASP-1 and MASP-2 on the C3 substrate were very low both in absolute terms (~300 $M^{-1}s^{-1}$), and relative to the alternative and classical pathway C3-convertases. The alternative pathway C3-convertase, C3bBb, was measured earlier to cleave C3 with a k_{cat}/K_M value of 3.1×10^5 $M^{-1}s^{-1}$ (53), which is three orders of magnitude higher than the corresponding rates of C3 cleavage by MASP-1 and MASP-2, reported herein. Previous reports showed that the classical pathway C3-convertase, C4b2a, also exhibited high affinity toward C3 and displayed a K_M value of 1.8×10^{-6} M^{-1} for C3 cleavage, which is in the same range as measured for C3bBb (5.6×10^{-6} M^{-1}) (53, 54). Therefore, we strongly believe that C3 is not the natural substrate of either MASP-1 or MASP-2. Nevertheless, this marginal C3 cleaving activity might be sufficient for complement cascade activation. It can be hypothesized that despite their low catalytic efficiency, MASP-1 or MASP-2 could initiate the alternative pathway of complement activation at a single site of a target cell, and in accordance with the one-hit theory of immune hemolysis (55), it could result in the destruction of this cell through alternative pathway amplification.

C3i was cleaved with a low but significant efficiency: the k_{cat}/K_M values were ~10- to 20-fold higher than in the case of C3 cleavage. Therefore, it is possible that the C3 cleaving activities of the MASPs, reported earlier in the literature, were mostly due to the presence of C3i in C3 preparations. Also, since MASP-1 and MASP-2 cleave C3 at a similar rate, this might correspond to the rate at which C3 converts to C3i, which would mean that C3 is not cleaved at all in our assays. It is unlikely that the C3i cleaving ability of the MASPs plays a significant physiological role, as C3i is degraded by Factor I in the presence of Factor H at a much higher rate.

C4, similar to C3, was basically resistant against the proteolytic activity of MASP-1. However, C2 was digested by the recombinant MASP-1 fragment at a moderate rate. The k_{cat}/K_M value for the C2 cleavage was two orders of magnitude higher than that of the C4 cleavage. However, the C2 cleavage alone is not sufficient to initiate the complement cascade, since physiologically relevant C2 cleavage occurs on the C2C4b complex only. It should also be stressed that this moderate k_{cat}/K_M value is smaller by an order of magnitude than the corresponding values for MASP-2 or C1s.

The recombinant MASP-2 fragments cleaved C2 and C4 efficiently. This and the autoactivating capacity of MASP-2 is in accordance with the observation that MBL-MASP-2 complex can activate the complement cascade (8). Experiments with the three different functionally active truncated catalytic fragments of MASP-2 (i.e., CCP1-CCP2-SP, CCP2-SP, and SP) showed that C2 cleavage is mediated entirely by the SP domain. Therefore, the SP domain probably contains all necessary contact sites for efficient C2 binding and cleavage, and the CCP domains do not contribute to this reaction. This phenomenon closely resembles C1s, where the SP domain alone is enough to mediate C2 cleavage (51). On the contrary, C4 digestion of the MASP-2 fragments is influenced by the presence of the CCP domains. Although the single SP domain can cleave C4 at a moderate efficiency, the addition of the

Table III. *The rates of reaction of radioiodinated MASP-1 and MASP-2 CCP1-CCP2-SP fragments with C1-inh*[a]

MASP Fragment	k_{obs} (s^{-1})[a]	K_i (nM)	k_2 (s^{-1})
MASP-1 CCP1-CCP2-SP	1.4×10^{-3}	NA	NA
MASP-2 CCP1-CCP2-SP	7.8×10^{-3}	8.8	8×10^{-3}

[a] Observed pseudo first order rates of reaction (k_{obs}) of ^{125}I-labeled MASP-1 and MASP-2 CCP1-CCP2-SP fragments reacting with C1-inh ([C1-inh] = 350 nM). K_i and k_2 values are not available (NA) for MASP-1 CCP1-CCP2-SP.

Table IV. *The role of the domains in the C-terminal catalytic region of C1r, C1s, and MASP-2*[a]

Domain	C1r	C1s	MASP-2
SP	Autoactivation	C2 cleavage	Autoactivation C2 cleavage
CCP2-SP	C1s cleavage	C4 cleavage	C4 cleavage
CCP1	Dimerization	C4 cleavage	ND

[a] The role of the domains were studied using recombinantly expressed deletion mutants of C1r (46), C1s (51), and MASP-2 (this study).

Table V. *Comparative characterization of the serine proteases of the lectin and classical pathway initiation complexes*[a]

Feature	Enzymes
Autoactivation capacity	C1r $\sim$ MASP-1 > MASP-2
C2 cleaving capacity	C1s $\sim$ MASP-2 $\gg$ MASP-1
C4 cleaving capacity	C1s $\sim$ MASP-2
C3 and C3i cleaving capacity	MASP-1 $\sim$ MASP-2
Reactivity with C1-inhibitor	MASP-2 > C1s > C1r $\sim$ MASP-1
Reactivity with α_2M	MASP-1 $\gg$ MASP-2

[a] Data shown based on present and previous studies (46, 51, 56).

CCP2 module to the SP domain increases the k_{cat}/K_M value dramatically ($\sim$44-fold increase). It seems very likely that the CCP2 domain contains additional binding sites for the protein substrate C4. This is reflected in the decrease of the K_M value (from 2.0–0.4 μM), which indicates a stronger binding of the substrate. Our results are similar to previous results of Rossi et al. (51), who demonstrated the essential role of the CCP2 module in C4 cleavage by C1s. Previously, we have revealed a similar phenomenon in the case of C1r, where the CCP2 domain significantly increased the proteolytic power of the SP against the C1s substrate (46). Apparently, the CCP2 domains play an essential role in determining the enzymatic properties of the C1r, C1s, and MASP-2 proteases (Table IV).

This paper provides the first comprehensive analysis of the substrate specificities and relative activities of MASP-1 and MASP-2 using a competing oligopeptide substrate library. In this assay MASP-1 showed an extreme Arg selectivity at the P1 site of the substrates, acting like thrombin or mouse endoproteinase Arg-C (38), whereas the substrate specificity of MASP-2 resembles trypsin. Thrombin cleaves the P1 Arg peptide $\sim$20-fold more efficiently than the MASP-1 fragment (38). Nevertheless, the 4- to 5-fold higher catalytic potential of MASP-1 on the Arg oligopeptide compared with MASP-2 suggests that a natural substrate for MASP-1 may exist that is cleaved more efficiently than C3. Indeed, in recent studies Hajela et al.[5] showed that MASP-1 cleaves fibrinogen and Factor XIII at much faster rates than C3 and is thus, similar to thrombin, able to catalyze the formation of cross-linked fibrin.

C1-inh was able to block the proteolytic activities of both MASP-1 and MASP-2. The observed pseudo-first order rates of reaction (k_{obs}) with C1-inh were 5-fold less for the MASP-1 than for the MASP-2 fragment. The K_i values for the C1-inh and MASP-2 CCP1-CCP2-SP reaction were in the nanomolar range, which is an order of magnitude less than those of the C1r and C1s interactions with C1-inh and is an indicator of strong binding (56). The pseudo-first order rates of inhibition of C1r, C1s, MASP-1, and MASP-2 by C1-inh can be ranked using previously published results for C1r and C1s (56) and results presented in this study for MASP-1 and MASP-2 (Table V).

A marginal molar excess of α_2M was enough to completely block the proteolytic activity of MASP-1, whereas only a 40-fold molar excess over the MASP-2 fragments was capable of significantly hindering C2 and C4 cleavage. When both α_2M and C1-inh competed for either the MASP-1 or the MASP-2 catalytic fragment, C1-inh proved to be a better inhibitor of MASP-2, while MASP-1 reacted readily with both C1-inh and α_2M. There is an evolutionary aspect of these observations: MASP-1 represents an ancient type of SP, since the active site Ser[646] is encoded by a TCN type codon, where T stands for thymidine, C stands for cytidine,

and N stands for any of the four nucleotides (57). Other indicators of this are a highly conserved Ser residue downstream the active site (Ser[667]), also encoded by TCN (58), a histidine loop around the active site, and the six-exon-encoded SP domain (59). It is presumed that an α_2M-like molecule could be the precursor of C3 during the evolution of the complement cascade (60). It is interesting to note that the ancient type protease (MASP-1) reacts more efficiently with the ancient type substrate (α_2M) than the more modern protease (MASP-2).

Several lines of evidence indicate that the complement and the blood clotting cascades evolved from a common ancestral protease cascade (60). It has also been reported that sequence homologs of fibrinogen served immunologic roles. MASP-1 shows characteristics of both cascades (complement-like features: interaction with MBL, inhibition by C1-inh; clotting-like feature: arginine selectivity at P1) and it can be speculated that as an ancient protease it participates in both cascades.

Using the results of the present study and data obtained by other laboratories, we can compare some basic characteristics of the C1r, C1s, MASP-1, and MASP-2 proteases (Table V). All of these proteases, except C1s, are capable of autoactivating (46). The capacity to autoactivate enables C1r through cleaving C1s and MASP-2 through cleaving C2 and C4 to initiate the complement cascade after having received an activation signal from a recognition molecule (e.g., C1q, MBL, or ficolin). As in vivo natural substrates of MASP-1 are not yet well characterized, the physiological consequences of its autoactivating capacity is hard to predict. In the case of C1s and MASP-2, the SP domains alone cleave C2 as efficiently as the whole molecules. Nevertheless, for efficient C1s cleavage by C1r and efficient C4 cleavage by C1s and MASP-2, the CCP2 module is essential. This could be partly attributed to the feature conserved during evolution that the CCP2 module forms a compact structural and functional unit with the SP domain (61–63). All four proteases can be inhibited by the serpin C1-inh, but MASP-1 is much more sensitive to α_2M.

In our view, the most intriguing problem that remains to be solved is the identification of the natural substrate of MASP-1. Our work provided an important detail toward answering this question. We showed that MASP-1 is a relatively potent peptidase and similar to thrombin, has an extreme Arg specificity at the P1 site.

Acknowledgments

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