Two Mechanisms for Mannose-binding Protein Modulation of the Activity of Its Associated Serine Proteases*

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Serum mannose-binding protein (MBP) neutralizes invading microorganisms by binding to cell surface carbohydrates and activating MBP-associated serine proteases-1, -2, and -3 (MASPs). MASP-2 subsequently cleaves complement components C2 and C4 to activate the complement cascade. To analyze the mechanisms of activation and substrate recognition by MASP-2, zymogen and activated forms have been produced, and MBP-MASP-2 complexes have been created. These preparations have been used to show that MBP modulates MASP-2 activity in two ways. First, MBP stimulates MASP-2 autoactivation by increasing the rate of autocatalysis when MBP-MASP-2 complexes bind to a glycan-coated surface. Second, MBP occludes accessory C4-binding sites on MASP-2 until activation occurs. Once these sites become exposed, MASP-2 binds to C4 while separate structural changes create a functional catalytic site able to cleave C4. Only activated MASP-2 binds to C2, suggesting that this substrate interacts only near the catalytic site and not at accessory sites. MASP-1 cleaves C2 almost as efficiently as MASP-2 does, but it does not cleave C4. Thus MASP-1 probably enhances complement activation triggered by MBP-MASP-2 complexes, but it cannot initiate activation itself.

Serum mannose-binding protein (MBP) or mann-binding lectin is the recognition component of the lectin branch of the complement cascade (1–3). It selectively targets invading microorganisms for neutralization by binding to cell surface mannos and N-acetylglucosamine residues and activating MBP-associated serine proteases (MASPs).

The different MASPs, designated MASP-1, -2, and -3, are homologs of C1r and C1s of the classical pathway of complement activation. In each MASP, an N-terminal segment, consisting of two CUB domains separated by an EGF-like domain, mediates both dimerization and MBP binding (7). The protease domain is linked to the N-terminal segment by two CCP modules (see Fig. 1A). MASPs are synthesized as zymogens and autoactivate through cleavage of the polypeptide at the N-terminal end of the serine protease domain. Only MASP-2 has a clearly defined role in complement fixation (8, 9). It cleaves C3, releasing the peptide anaphylatoxin C3a and exposing a highly labile thioester bond in the C4b fragment, which forms a covalent attachment to the target surface. Complement component C2 binds to C4b and is also cleaved by MASP-2. The C-terminal portion, designated C2a, remains attached to the C4b fragment and forms the catalytic subunit of the C3 convertase (C4bC2a). Cleavage of C3 releases the anaphylatoxin C3a and deposits C3b on the cell surface, which in turn targets the cell for phagocytosis or lysis. Human MASP-2 cleaves C4 more efficiently than C2 (8). Complement activation must be tightly regulated to prevent damage to host tissues.

Relatively little is known about the mechanisms that control activation in the lectin pathway, although C1 inhibitor inhibits the catalytic activity by binding to MASP-2. MASP-1 and MASP-3 are alternatively spliced products from the same gene (4). They share the same N-terminal domains but have different protease domains. The role of MASP-1 is unknown. It has been reported to cleave complement components C2 and C3 (10). However, only low activities were detected in separate studies (8), suggesting that these proteins might not be physiological ligands. The role of MASP-3 is also not known.

MBP is a mixture of oligomers assembled from subunits that are formed from three identical polypeptide chains (11). Surface recognition is mediated through C-terminal carbohydrate recognition domains, which are linked to collagenous stems by a short coiled-coil of α-helices. MASPs bind at the C-terminal side of an interruption in the collagenous domain which causes the collagenous stems to splay apart (12). Each MASP dimer bridges two MBP subunits so that MBP dimers bind single MASP dimers, whereas MBP trimers and tetramers can bind up to two MASP’s (13, 14).

In the present study, MASP-2 activation has been analyzed by comparing the activities of zymogen and activated forms of MASP-2 alone and in complex with MBP. The data show that MBP modulates the activity of MASP-2 through multiple mechanisms.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and peptide N-glycosidase F were purchased from New England Biolabs. The rat liver cDNA library and cDNA Marathon kit were from Clontech. N-α-Carbobenzoxy-L-lysine p-nitrophenyl ester (ZLNE), trichloroacetic acid, benzoyl-L-arginine p-nitroanilide (BAPNA), dimethyl sulfoxide, and protein molecular mass markers were from Sigma Chemical Co. Polyvinylidene difluoride membranes were from Applied Biosystems. Tissue culture media were from...
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Invitrogen. Affi-Gel 10 matrix was from Bio-Rad Laboratories. Iminodiacetic acid-Sepharose resin, Mono Q, and Mono S HR5/5 columns were obtained from Amersham Biosciences.

Analytical Methods—SDS-PAGE was performed as described by Laimlin (15). Amino acid sequencing was carried out in a Beckman LP3000 protein sequencer. Polypeptide samples for sequence analysis were separated by SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) filters followed by silver staining.

Production of Modified Forms of MASP-2 and MASP-1—Full-size catalytically inactive MASP-2, called MASP-2A, in which the active site serine residue at position 613 is changed to an alanine residue, was produced by expression in Chinese hamster ovary cells and purified on MBP-agarose affinity columns as described previously (14). A catalytically active form of MASP-2, MASP-2K, was created by replacing the arginine residue, the cleavage site for zymogen activation, with a lysine residue. Protein was expressed and purified as described for the alanine mutant. It was activated by incubating protein in 50 mM Tris-Cl, pH 7.5, containing 150 mM NaCl and 25 mM CaCl₂ at 37 °C for up to 16 h. The extent of cleavage of the MASP-2 polypeptide was monitored by SDS-PAGE.

A catalytically active form of MASP-1, designated MASP-1ent, was produced by substituting residues 315-Lys-His-Ile-Ser-Arg, immediately preceding the cleavage site forzymogen activation, with the recognition sequence of the serine protease enterokinase, Asp-Asp-Asp-Lys. Protein was purified and characterized as described for modified forms of MASP-2. It was activated by incubation with enterokinase (0.01% w/w) in 20 mM Tris-Cl, pH 7.4, containing 50 mM NaCl and 2 mM CaCl₂ at 37 °C. Enterokinase was removed by passing the reaction mixture over an MBP-agarose affinity column.

Cloning of Rat C2 and C4 cDNAs—The cDNA encoding rat complement component C2 was amplified from a liver cDNA library using the PCR. The 5'-oligonucleotide ATGGGCCCCATCAGATGTTTTCATGCC-CTGC contains the start codon (underlined) and encodes the first 9 residues of the signal sequence of mouse C2. The 3'-oligonucleotide binds to the untranslated region of the cDNA, 240 nucleotides downstream of the stop codon. Fragments of the rat C4 cDNA were amplified by rapid amplification of cDNA ends using a rat liver cDNA library. In each case, three different cDNA clones were produced and sequenced, using each strand as a template. Single nucleotide mutations introduced by the reverse transcription or amplification steps were corrected by assembling restriction fragments. Standard molecular biological techniques were performed as described (17).

Production and Purification of Recombinant C2 and C4—The cDNAs encoding the mature C2 and C4 polypeptides were ligated to the cDNA of the dog preproinsulin signal sequence followed by six histidine residues of the signal sequence of mouse C2. The 3'-oligonucleotide binds to the untranslated region of the cDNA, 240 nucleotides downstream of the stop codon. Fragments of the rat C4 cDNA were amplified by rapid amplification of cDNA ends using a rat liver cDNA library. In each case, three different cDNA clones were produced and sequenced, using each strand as a template. Single nucleotide mutations introduced by the reverse transcription or amplification steps were corrected by assembling restriction fragments. Standard molecular biological techniques were performed as described (17).

Analytical Ultracentrifugation—Equilibrium experiments were carried out as described previously in a Beckman XLA-70 centrifigure using Epon charcoal-filled six-hole centerpieces (19, 20). For analysis of the interaction between zymogen MASP-2A and C4, proteins were mixed at a 1:1, 1:2, and 2:1 molar ratios to give initial absorbances of 0.2 at 280 nm in 50 mM Tris-Cl, pH 7.5, containing 150 mM NaCl, 5 mM CaCl₂, and 5 mM MgCl₂, at 37 °C. At various times, aliquots were removed and the reaction was stopped by boiling in gel-loading buffer. The extent of C2 cleavage was determined by scanning SDS-polyacrylamide gels, using a Densitometer SI from Molecular Dynamics. A similar assay was used to measure cleavage of C4, except that the concentration of activated MASP-2 was 0.25 mM, and the concentration of C4 was between 0.05 and 1 mM. Data were fitted to the Michaelis-Menten equation using nonlinear regression. Kinetic parameters represent average values from three separate experiments. Similar assays were used to measure the catalytic activities of MASP-1ent. The concentration of C2 was 0.3–15 μM.

RESULTS

Mechanism of MBP Stimulation of MASP-2 Autocatalysis—To investigate the mechanism of MASP-2 zymogen activation, it was necessary to start with a homogeneous preparation of zymogen. In previous studies, a zymogen preparation was obtained by substituting alanine for the active site serine residue, thus creating a catalytically inactive protein (MASP-2A) that cannot autoactivate. In a modification of this strategy, an alternative mutant form of MASP-2 was created by replacing the arginine residue at the autocleavage site with lysine (MASP-2K). This change reduces the rate of proteolytic cleavage and prevents activation of the zymogen during synthesis, secretion, and purification on MBP-agarose, allowing prepara-
glycosidase F is indicated by an arrow with peptide bands with apparent molecular masses of 67, 30, and 24 kDa. Digestion under reducing conditions, activated MASP-2K migrated as three 15% polyacrylamide gels and detected by staining with Coomassie Blue. Under nonreducing conditions, activated MASP-2K migrates as a single band, indicating that a disulfide bond links the two fragments of activated MASP-2K. No cleavage of MASP-2A was detected after incubation at 37 °C. The migration position of peptide:N-glycosidase F (PNGaseF) to remove N-linked oligosaccharides showed that the 30-kDa fragment was a glycosylated form of the smaller fragment. Edman degradation of the smaller C-terminal fragment confirmed that cleavage occurs at the expected position for MASP activation. Under nonreducing conditions, activated MASP-2K migrates as a single band, indicating that a disulfide bond links the two fragments of activated MASP-2K. No cleavage of MASP-2A was detected after incubation at 37 °C. The migration position of peptide:N-glycosidase F is indicated by an arrow.

Fig. 1. SDS-PAGE of activated and zymogen MASP-2. A, domain organization of MASP-2. B, analysis of expressed proteins separated on 15% polyacrylamide gels and detected by staining with Coomassie Blue. Under reducing conditions, activated MASP-2K migrates as three bands with apparent molecular masses of 67, 30, and 24 kDa. Digestion with peptide:N-glycosidase F (PNGaseF) to remove N-linked oligosaccharides showed that the 30-kDa fragment was a glycosylated form of the smaller fragment. Edman degradation of the smaller C-terminal fragment confirmed that cleavage occurs at the expected position for MASP activation. Under nonreducing conditions, activated MASP-2K migrates as a single band, indicating that a disulfide bond links the two fragments of activated MASP-2K. No cleavage of MASP-2A was detected after incubation at 37 °C. The migration position of peptide:N-glycosidase F is indicated by an arrow.

Fig. 2. Kinetics of MASP-2 catalysis were analyzed alone and in complex with MBP (Fig. 2). If MBP inhibited zymogen activation in the MASP-2K–MBP complex, the lower activation rate would be expected in this complex relative to zymogen MASP-2K alone. However, the rate of activation is higher in the complex, so MBP binding apparently induces a conformational change in the MASP which readies it for activation. An even higher rate of activation was measured when complexes were incubated with mannan-Sepharose, so ligand binding by MBP must promote additional conformational changes that trigger activation. Thus, MBP controls MASP-2 activation by increasing the rate of autocatalysis on binding to a target cell. Although autocatalysis of wild-type MASP-2 is likely to be faster than activation of the MASP-2K mutant, MBP stimulation probably follows the same pathway, reflecting the underlying role of MBP in the activation process.

Disrupted Catalytic Site in Zymogen MASP-2—The lower catalytic activities of various serine protease zymogens relative to the activated forms can be explained by two mechanisms (25). In one mechanism, all or part of the active site is disrupted, preventing substrate binding or one of the early steps of catalysis. In the other mechanism, the catalytic site is preformed, but access to macromolecular substrates is blocked by part of the polypeptide chain. Small synthetic substrates can often discriminate between these two mechanisms because they can gain access to a preformed active site in a zymogen.
and serve as substrates even when the larger natural substrates are excluded. However, they will not be cleaved efficiently if the active site is disrupted in the zymogen.

To probe the accessibility of the catalytic site, the catalytic activities of the zymogen and activated forms of MASP-2K were compared using a small ester substrate, ZLNE. Hydrolysis of ZLNE catalyzed by activated MASP-2K displays Michaelis-Menten kinetics, with a $K_m$ of $0.31 \pm 0.04$ mM and a $k_{cat}$ of $131 \pm 3$ s$^{-1}$. In contrast, <1% activity is detected using zymogen MASP-2K, under all conditions tested (Fig. 3). The relative inactivity of the zymogen for even a small substrate indicates that the active site must be disrupted, thereby preventing catalysis. Indeed, the $K_m$ determined using a 100-fold higher concentration of the zymogen preparation was broadly comparable with the $K_m$ of the activated enzyme, suggesting that the activity observed in the preparation of zymogen was caused by trace amounts of activated MASP-2K, resulting from autocatalysis during the assay.

**Differences in Substrate Specificities between MASP-1 and MASP-2**—The catalytic activities of MASPs for physiological substrates have been tested previously using material purified from mammalian sera (8, 10). However, conflicting activities have been reported for MASP-1, possibly as a result of contamination by other serine proteases or by protease inhibitors that are normally present in serum. To avoid the chance of such contamination in these studies, assays were undertaken using recombinant substrates.

The cDNAs for rat C2 and C4 were amplified by the PCR, using oligonucleotide primers derived from the sequence of the mouse cDNAs. Recombinant proteins were produced with N-terminal histidine tags and were purified by affinity chromatography on immobilized nickel columns. Purified rat C2 migrates as a single broad band on SDS-polyacrylamide gels (Fig. 4B) (26, 27). As expected, rat C4 consists of three polypeptide chains linked by disulfide bonds (28) (Fig. 5B). Autolytic cleavage under denaturing conditions (29) confirmed that the thioester bond is formed in C4 (Fig 5B). Activated MASP-2K cleaves C2 into its characteristic C2a and C2b fragments (Fig. 4B). Separate experiments showed that it also cleaves C4, releasing the N-terminal portion of the $\alpha$-chain (C4a) to form the activated form, C4b (Fig 4B). Cleavage of both substrates is relatively efficient and follows Michaelis-Menten kinetics. The $k_{cat}$ values are comparable, consistent with a common catalytic mechanism. However, the $K_m$ for C4 is >20-fold lower than the $K_m$ for C2 (Table 1). Similar kinetic properties have been reported previously for human MASP-2 (8). The simplest explanation to account for these differences is that activated MASP-2 has a higher affinity for C4 than for C2.

**Fig. 3. Hydrolysis of ZLNE by activated and zymogen MASP-2K.** Activated and zymogen MASP-2K (3.2 nm) were incubated with 0.8 mM ZLNE. The increase in absorbance is caused by the release of the product p-nitrophenol.

**Fig. 4. Cleavage of recombinant C2 by activated MASP-2.** A, domain organization of C2. The N-terminal sequence of the C2a fragment, determined by Edman degradation, is shaded. Sites of N-linked glycosylation are shown by balls and sticks. Based on the sequence of the cDNA, rat C2 shares 76 and 86% sequence identity with the human and mouse proteins (26, 27). Amino acid residues corresponding to the cleavage site for activation, the consensus motif for Mg$^{2+}$ binding in the von Willebrand factor type A (vWFA) domain, and the catalytic triad in the serine protease domain are conserved. B, detection of C2 cleavage by SDS-PAGE. 2.5 mM recombinant rat C2 was incubated with 1 nM activated MASP-2K at 37 °C. A 15% gel was used under reducing conditions and stained with Coomassie Blue. The apparent molecular mass of 92 kDa for C2 is greater than the value calculated from the amino acid sequence (82 kDa) probably because of the presence of glycans. Ovalbumin was included in the reaction buffer to prevent nonspecific interactions.

Initial attempts to produce MASP-1 using the same strategy as described for MASP-2 were unsuccessful. Instead, recombinant protein was produced by completely replacing the sequence immediately prior to the cleavage site with the recognition sequence for the serine protease enterokinase. As expected, the resulting protein, designated MASP-1ent, is secreted as zymogen (Fig. 6). Upon treatment with limiting amounts of enterokinase, it is cleaved at the expected position to generate the activated protease. Trace amounts of enterokinase were removed from the MASP-1ent preparation by reapplying the activated MASP to an MBP-agarose column and washing the column extensively before elution.

Small synthetic substrates were used to probe the specificity of activated MASP-1ent. The activated protein hydrolyzes the p-nitroaniline derivative, BAPNA, following Michaelis-Menten kinetics, with a $K_m$ of $8.9 \pm 1.5$ mM and a $k_{cat}$ of $4.3 \pm 0.4$ s$^{-1}$. Interestingly, MASP-1ent does not hydrolyze ZLNE, even at high enzyme concentrations, demonstrating that MASP-1 has different substrate specificity from MASP-2. Typically, ZLNE is cleaved most efficiently by proteases with a preference for a lysine residue at the P1 position, whereas BAPNA is cleaved by arginine-specific proteases. Only 3–7% activity for BAPNA was detected using zymogen MASP-1ent, under all conditions tested, indicating that all or part of the active site is disrupted in the zymogen of MASP-1 as it is in MASP-2.

Activated MASP-1ent also cleaves recombinant rat C2 into its characteristic fragments following Michaelis-Menten kinetics (Fig. 7). The kinetic parameters are broadly comparable with C2 cleavage by MASP-2K (Table 1), indicating that C2 is likely to be a natural substrate of MASP-1. MASP-1 does not cleave C4, even after prolonged incubation with high concentrations of enzyme. Thus, both MASP-1 and MASP-2 cleave C2,
but only MASP-2 cleaves C4 to initiate complement activation. MASP-1 probably augments the activities of MASP-2 by promoting complement fixation through C2 cleavage when MBP-MASP complexes bind to target cells.

Accessory C4 Binding Sites on MASP-2: Modulation of Interactions by MBP—Efficient cleavage of C4 by MASP-2 could result from accessory binding sites on MASP-2 which increase the affinity for C4, thereby lowering the $K_m$ for the reaction. To

**Fig. 5. Cleavage of recombinant C4 by activated MASP-2.** A, domain organization of rat C4. N-terminal sequences of the $\alpha$, $\beta$, and $\gamma$ chains were determined by Edman degradation. The pattern of disulfide bonds is based on the arrangement of bonds in human C4 [28]. Residues that form the thioester linkage are boxed. The peptide bond that is cleaved by MASP-2 to activate C4 is indicated by an arrow. Rat C4 shares 77 and 85% sequence identity with human C4B and mouse C4. Key amino acid residues are conserved, including residues at the cleavage site for activation, residues that form the thioester bond, and two clusters of acidic residues that are implicated in forming the binding site for C2. Of the two isotypes in humans, rat C4 is more similar to the human C4B isotype. B, analysis of C4 cleavage by SDS-PAGE. Proteins were separated on 10% gels and detected by staining with Coomassie Blue. Sample marked with an asterisk was heated at 100 °C for 10 min prior to loading on the gel to promote autolysis of the $\alpha$ chain. N- and C-terminal products are indicated by $\alpha N$ and $\alpha C$, respectively. C, cleavage of 500 nM C4 by 0.5 nM activated MASP-2K. Proteins were separated on a 10% gel and visualized by staining with Coomassie Blue. Edman degradation of the cleaved $\alpha$ chain ($\alpha'$) confirmed that MASP-2 cleaved C4 at the expected site for activation.

**Table I**

| Kinetic properties of activated MASP-2K and MASP-1ent |
|------------------------------------------------------|
| MASP-2K                                               | MASP-1ent                                      |
| $K_m$ [M]                                            | $k_{cat}$ [s$^{-1}$]                           | $k_{cat}/K_m$ [s$^{-1}$] | $K_m$ [M] | $k_{cat}$ [s$^{-1}$] | $k_{cat}/K_m$ [s$^{-1}$] |
| C4                                                    | 0.23 ± 0.04                                    | 6.2 ± 1.1                     | 28 ± 9   | —                      | —                      |
| C2                                                    | 4.5 ± 0.1                                      | 5.6 ± 1.8                     | 1.3 ± 0.4| 13 ± 3                 | 3.9 ± 0.5              | 0.34 ± 0.12            |

*MASP-1ent did not cleave C4 under any of the conditions tested.*
The interaction of zymogen MASP-2A and C4 is rapidly exchanging between bound and unbound species during the course of these experiments. In a mixture of zymogen MASP-2A and C4, the amount of unassociated MASP-2 decreased, and the average $s^*$ increased relative to the individual components analyzed separately, indicating that a complex is formed between the two proteins (Fig. 8). However, most of the MASP-2A is still unassociated, so the $K_D$ for the interaction must be higher than the loading concentration of the components (0.7 μM). Also, the complex does not sediment as a distinct peak with a higher $s^*$ than each component, indicating that zymogen MASP-2A and C4 are rapidly exchanging between bound and unbound species during the course of the experiment.

To measure the affinity of the interaction between MASP-2A and C4, equilibrium distributions were measured at three different loading concentrations using different ratios of C4 and MASP-2A. Data fitted well to a model assuming formation of a 1:1 complex, in which the $K_D$ is 6.8 ± 2.0 μM (Fig. 9). Because the catalytic site is disrupted in the zymogen, binding to C4 must occur through accessory sites on MASP-2 which are preserved in the zymogen. These interactions would facilitate recognition of C4, accounting for the low $K_m$ value of activated enzyme.

Interactions between zymogen MASP-2 and C4 in serum would be expected to increase the likelihood of spontaneous complement activation, which would be detrimental to the host. MASP-2 circulates as complexes with MBP, so it was of interest to determine whether such complexes still bind to C4. MBP-MASP-2A complexes were formed by mixing purified MBP trimers with zymogen MASP-2A and were analyzed using sedimentation velocity. Most of the protein appears as a single stable species that sediments faster than either component (0.7 μM). The $s^*$ values for zymogen MASP-2A and C4 are 5.6 S and 7.9 S, corrected for the effects of temperature on buffer density and viscosity.
that C4 and MASP-2 fail to interact when MASP-2 is bound to MBP. Thus, MBP blocks the interaction of MASP-2 with C4 until binding to a suitable carbohydrate ligand activates the MBP. Thus, MBP blocks the interaction of MASP-2 with C4 that C4 and MASP-2 fail to interact when MASP-2 is bound to MBP and carbohydrate moieties on the C4 polypeptide.

**DISCUSSION**

The data presented here reveal two mechanisms by which MBP regulates MASP activity and substrate recognition in the lectin pathway of complement activation (Fig. 12). In circulating complexes, MBP averts spontaneous complement activation by preventing zymogen MASP-2 binding to C4. When complexes bind to the surface of a microorganism, MBP triggers activation by increasing the rate of MASP-2 autocatalysis. Conformational changes in the immobilized MBP-MASP-2 complexes expose accessory binding sites on the MASP which would enable transient interactions with its primary substrate, C4. Separate changes form the catalytic site and allow access to C4. The relatively high affinity of MASP-2 for C4 probably ensures that covalent attachment of C4 to the cell surface occurs near the MBP-MASP-2 complex. Consequently, once C2 associates with C4b, it is cleaved efficiently by nearby MASP-2 molecules to form C4bC2a, which is the C3 convertase. Cleavage of C4 by MASP-2 represents a potential amplification step because each MASP can cleave and activate multiple C4 molecules. Indeed, C4 and C2 are present at much higher concentrations in serum than is MASP-2, so it is likely that each MBP-MASP-2 complex becomes surrounded by multiple activated C4b molecules. In addition to MASP-2, activated MBP-MASP-1 complexes can cleave these C2 molecules to enhance the efficiency of complement activation.

Kinetic and thermodynamic data show that MASP-2 binds to C4 with considerably higher affinity than it binds to C2, indicating that there is an accessory C4 binding site on MASP-2. This extra binding site may be located within the serine protease domain itself, as occurs in the serine protease domains of certain clotting factors as well as in other complement components (25). The additional interactions often involve extended loops that ensure specific interactions with physiological substrates. Kinetic data using truncated forms of MASP-2 have shown that a fragment of human MASP-2 containing the CCP-2 domain as well as the serine protease domain cleaves C4 more efficiently than the serine protease domain alone (30). Thus, the CCP-2 domain might bind directly to C4, or it could indirectly affect catalysis by stabilizing the serine protease domain.

The accessory binding site for C4 is largely preformed in zymogen MASP-2 but only becomes exposed once MBP-MASP complexes have been activated. MBP might disrupt the interaction between MASP-2 and C4 by changing the conformation of the MASP. Alternatively, it could simply block the interaction by preventing access to C4. C2 does not bind to zymogen...
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MASP-2, implying that it binds only near the catalytic site, and there is no assembly binding site for C2. Differences in the interactions of MASP-2 with its natural substrates probably reflect the order of events that lead to complement activation (31). Cleavage of C4 by activated MASP-2 results in attachment of C4b to the cell surface. Subsequent cleavage of C2 occurs only after it has bound to the C4b fragment. Thus, cleavage of C2 occurs on the cell surface where the close proximity of the MBP-MASP-2 complex would allow cleavage of C2 by the same or nearby MASP molecules.

Typical serum concentrations of C4 in healthy individuals (~3 μM) are considerably higher than the $K_m$ for binding to MASP-2, so cleavage of C4 proceeds at near maximum velocity for the catalyzed reaction. The concentration of circulating C4 is subject to wide variation in certain disease conditions (32). Expression is up-regulated during acute infection as part of the acute phase response, whereas C4 concentrations are depleted in hereditary conditions and can become reduced in chronic inflammatory disorders such as rheumatoid arthritis or systemic lupus erythematosus. The low $K_m$ would ensure that complement activation can still occur over the wide range of serum concentrations that might be encountered.

MASP-1 cleaves C2 with a catalytic efficiency that is only ~3-fold lower than MASP-2, suggesting that C2 is likely to be a physiological substrate for MASP-1. The lower catalytic efficiency is mainly the result of a higher $K_m$ for C2 as substrate for MASP-1 compared with MASP-2. Previous studies have shown that the activity of a fragment of human MASP-1, consisting of the CCP modules and the serine protease domain, was <10% of the activity of the corresponding fragment of MASP-2, mainly caused by a lower $k_{cat}$ (30). Although these differences could be caused by differences in the catalytic activities of human and rat proteins, they might also reflect differences in the catalytic properties of full size and truncated proteins if the N-terminal CUB-1 and EGF-like domains modulate the activity of the protease domain in MASP-1.

MASP-1 and MASP-2 compete for binding sites on MBP molecules. MBP dimers bind single MASP dimers, whereas larger MBP oligomers bind up to two MASP dimers, so they could form complexes containing both MASP-1 and MASP-2 (14). MASP-1 probably competes in complement activation by cleaving C2 after it has bound to the C4b fragment. Because MASP-1 does not cleave C4 itself, however, this process could only occur in MBP-MASP complexes that contain MASP-1 and MASP-2 or where MBP-MASP-1 complexes bind to the cell surface near enough to a C4bC2 complex. Thus, MASP-1 probably augments complement activation but cannot initiate complement fixation in the absence of MASP-2.

The relative inactivity of zymogen MASP-2 toward the small substrate ZLNE shows that the catalytic site is disrupted. The molecular changes that prevent enzyme activity are likely to be relatively subtle and are probably similar to those that prevent catalysis in chymotrypsinogen and other related serine proteases (25). The disordered catalytic site of zymogen MASP-2 provides another inherent control mechanism that safeguards against nonspecific proteolysis during biosynthesis and secretion as well as preventing spontaneous complement activation in serum. Thus, multiple mechanisms modulate MASP activity in the lectin pathway of complement activation.

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