Mannose-binding lectin (MBL) Mutants Are Susceptible to Matrix Metalloproteinase Proteolysis

POTENTIAL ROLE IN HUMAN MBL DEFICIENCY*

Mannose-binding lectin (MBL) plays a critical role in innate immunity. Point mutations in the collag en-like domain (R32C, G34D, or G37E) of MBL cause a serum deficiency, predisposing patients to infections and diseases such as rheumatoid arthritis. We examined whether MBL mutants show enhanced susceptibility to proteolysis by matrix metalloproteinases (MMPs), which are important mediators in inflammatory tissue destruction. Human and rat MBL were resistant to proteolysis in the native state but were cleaved selectively within the collagen-like domain by multiple MMPs after heat denaturation. In contrast, rat MBL with mutations homologous to those of the human variants (R23C, G25D, or G28E) was cleaved efficiently without denaturation in the collagen-like domain by MMP-2 and MMP-9 (gelatinases A and B) and MMP-14 (membrane type-I MMP), as well as by MMP-1 (collagenase-1), MMP-8 (neutrophil collagenase), MMP-3 (stromelysin-1), neutrophil elastase, and bacterial collagenase. Sites and order of cleavage of the rat MBL mutants for MMP-2 and MMP-9 were: Gly46-Lys47 → Gly33-Ser34 → Gly63-Gln64 → Asn86-Asp87, Met81 which differed from that of MMP-14, Gly36-Leu37 → Asn80-Met81, revealing that the MMPs were not functionally interchangeable. These sites were homologous to those cleaved in denatured human MBL. Hence, perturbation of the collagen-like structure of MBL by natural mutations or by denaturation renders MBL susceptible to MMP cleavage. MMPs are likely to contribute to MBL deficiency in individuals with variant alleles and may also be involved in clearance of MBL and modulation of the host response in normal individuals.

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The abbreviations used are: MBL, mannose-binding lectin; CBD, collagen binding domain; C domain, carbohydrate domain; MASP, MBL-associated serine protease; MMP, matrix metalloproteinase; MT, membrane-type; TIMP, tissue inhibitor of metalloproteinases; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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Mannose-binding lectin (MBL) in the pathogenesis of these diseases. Variant human MBL and recombinant rat MBL with homologous mutations show decreased formation of the higher order oligomers (15, 24, 25). Replacing glycine with relatively bulky acidic side chains or introducing a cysteine reduces hydroxylation and glycosylation of proximal proline and lysine residues. This disrupts the disulfide bond arrangement of the NH2-terminal cysteine-rich domain, which perturbs the local structure of the collagen triple helix (24–26). These structural defects prevent MASPs from binding to the collagen-like domain (27, 28), and together with defective oligomerization, result in impaired complement activation (24, 25). It is unclear whether MBL serum deficiency is the result of decreased MBL secretion (24–26), enhanced turnover, or a combination of these. We speculated that variant MBL with its perturbed collagen triple helix may be more susceptible to proteolysis than wild-type protein, but this has yet to be addressed.

Collagens are substrates for a family of zinc-dependent endoproteinases, the matrix metalloproteinases (MMPs) (29, 30). MBL binds mannose and N-acetylgalactosamine residues on microbial surfaces, whereupon MBL-associated serine proteinases (MASPs) initiate the complement cascade by the lectin pathway resulting in cell lysis (2). Opsonization of microorganisms by MBL can also result in phagocytosis mediated by cell surface receptors on polymorphonuclear cells or macrophages (3).
These enzymes exhibit broad substrate specificity and in addition to extracellular matrix components, process a wide range of bioactive molecules including cell surface receptors, growth factors, and chemokines (31). Because MBL has a collagen-like domain, we evaluated the susceptibility of human serum MBL to MMP collagenolytic activity and compared this with recombinant rat MBL with mutations homologous to those found in human MBL (25). Our studies revealed that denaturation or mutations in MBL that perturb the collagen-like structure render MBL susceptible to cleavage by multiple MMPs. These findings support the hypothesis that MMPs could contribute to low serum levels of MBL in diseases marked by MBL deficiency.

EXPERIMENTAL PROCEDURES

Purification of Human MBL—Human MBL was purified as described with modification (32). Human serum was obtained from volunteer donors who had provided written informed consent. The study was performed under an approval for human subjects research obtained from the University of British Columbia. A crude preparation of MBL was obtained by passage of human serum through manrose-Sepharose and maltose-Sepharose columns. MBL was purified further using Sephacryl S-300, equilibrated in acetate buffer (100 mM sodium acetate, 100 mM NaCl, 5 mM EDTA, pH 5.0), to remove MASPs. α2-Macroglobulin (which can inhibit MMPs) was removed using an anti-α2-macroglobulin IgG (Enzyme Research, South Bend, IN) coupled to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences), and unreacted sites were blocked with glycine. The resin was equilibrated in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). MBL was recovered in the column flow-through, and purity was assessed by SDS-PAGE and silver staining.

Production of Rat MBL—Wild-type rat MBL (serum form, MBP-A) and MBL containing mutations homologous to those occurring in human MBL deficiency, R23C (R23C-MBL), G25D (G25D-MBL), and G28E (G28E-MBL), were generated and expressed in Chinese hamster ovary cells and purified as described (8, 25). CaCl2 was added to a final concentration of 5 mM before use.

Source of Enzymes and Domains—Human MMP-9 and human MMP-3 were gifts from Dr. R. Fridman (Wayne State University, Detroit, MI) and Dr. H. Nagase (Kennedy Institute, Hammersmith Hospital, London, UK), respectively. Human MMP-1, MMP-7, MMP-8, MMP-13, and MMP-14 were expressed and purified (33) or generously supplied by British Biotech Pharmaceuticals, Oxford, UK. Human MMP-2 was expressed in Timp2−/− myc/ras-transformed fibroblasts (34) and purified using gelatin-Sepharose (Amersham Biosciences) for lower molecular weight-Sepharose (Sigma) to remove human MBL. Human MMP-9 (35) were activated where necessary with 2 mM 4-aminophenylmercuric chloride, followed by lentil lectin-Sepharose (Sigma) to remove MMP-9 (35). MMPs and purified using gelatin-Sepharose (Amersham Biosciences) followed by native carboxypeptidase Y (Sigma) to remove TIMPs. TIMPs were expressed in Chinese hamster ovary cells and purified as described previously (35).

Production of Fab fragments was carried out with human MBL (25). CaCl2 was added to the incubation to a final concentration of 5 mM before use.

NH2-terminal Sequencing—Protease-digested MBL samples (nonreduced) were electrophoresed on 12.5% Tris-Tricine gels and silver stained. Monomers, 31-kDa polypeptides released by reduction of the oligomeric forms of MBL, are indicated by arrows.

RESULTS

Human MBL Is Cleaved Differentially by MMPs—Native and denatured collagen (gelatin) are cleaved differentially by members of the MMP family. To determine whether collagenolytic MMPs have the potential to regulate MBL levels in vivo by cleavage in the collagen-like domain, we tested the susceptibility of native or denatured human serum MBL to proteolysis. Upon reduction, oligomers of native and denatured MBL dissociate and electrophorese as ~31-kDa monomers (Fig. 1). None of the MMPs tested cleaved native human MBL when incubated at 28 °C (to ensure triple helicity of the collagen-like domain) or 37 °C (data not shown), including the collagenases MMP-1 and MMP-13 (Fig. 1A). However, denatured MBL was

**Fig. 1.** Heat-denatured human MBL is cleaved differentially by MMPs. 18 pmol of human serum MBL with (D) or without (N) heat denaturation (60 °C, 20 min) was incubated overnight at 28 °C in the absence or presence of 1.4 pmol of MMP, or enzyme was incubated alone (−). Samples were reduced and electrophoresed on 12.5% Tris-Tricine gels and silver stained. Monomers, 31-kDa polypeptides released by reduction of the oligomeric forms of MBL, are indicated by arrows.

body (Bio-Rad). Immunoreactive protein was detected by enhanced chemiluminescence (Amersham Biosciences).

NH2-terminal Sequencing—Protease-digested MBL samples (nonreduced) were electrophoresed on 12.5% Tris-Tricine gels and blotted to polyvinylidene fluoride membrane. Membranes were stained with Coomassie Brilliant Blue R-250, destained with 50% methanol, and bands of interest were excised. NH2-terminal sequences were determined by Edman Chemistry using an Applied Biosystems Procise Sequencer. Standard derivatives were not detected for proline and lysine residues, which have been shown to be modified to hydroxyproline or glucosylgalactosylhydroxlysine in rat MBL (8).

2 E. Tam and C. M. Overall, manuscript in preparation.
Fig. 2. MMPs release fragments from disulfide cross-linked oligomers of human MBL. A and B, 250 pmol of heat-denatured human MBL was incubated at 37°C overnight alone or with 16 pmol of MMP-2, MMP-9, MMP-14, or bacterial collagenase (BC) (+), or enzymes were incubated alone (−). C, 83.2 pmol of heat-denatured MBL was incubated alone or with 5.3 pmol of MMP-2 overnight at 37°C in the absence or presence of 250 nM BB94, 482 nM TIMP-2, or 2.5 mM phenylmethylsulfonyl fluoride (PMSF). Nonreduced samples were electrophoresed on 12.5% Tris-Tricine gels and silver stained (A) or Western blotted with monoclonal antibody raised against the human MBL sequence. Cleavage sites were designated hP1-P1′, which form a proteolytic unit at the cell surface (35). Both MMP-2 and MMP-14 reduced the intensity of the 31-kDa denatured MBL band and produced new fragments of 25.3 and 21.1 kDa, respectively. Where native MBL was incubated with MMP-2, a band below the 31-kDa MBL monomer represents a MMP-2 breakdown product, which is faintly visible in the MMP-2 controls. The leukocytic MMPs, MMP-8 (neutrophil collagenase) and MMP-9, MMP-14 (gelatinase B), processed denatured MBL to fragments of 23.9 and 21 kDa (MMP-8) and 23.6 and 20 kDa (MMP-9) (Fig. 1C). MMP-7 (matrilysin), a protease that cleaves gelatin, only weakly cleaved denatured MBL to a 23.9-kDa fragment (Fig. 1D).

Because MMP-2, MMP-9, and MMP-14 were the most efficient at cleaving denatured human MBL, these enzymes were subjected to NH2-terminal sequencing (Table I). Cleavage sites are designated hP1-P1′, which indicates a cleavage site at the NH2-terminal. Cleavage sites determined by NH2-terminal sequencing are indicated.

cleaved weakly by MMP-1 (decreased 31-kDa band, Fig. 1A) and more completely by MMP-2 (gelatinase A) and MMP-14 (MT1-MMP) (Fig. 1B), which form a proteolytic unit at the cell surface (35). Both MMP-2 and MMP-14 reduced the intensity of the 31-kDa denatured MBL band and produced new fragments of 25.3 and 21.1 kDa, respectively. Where native MBL was incubated with MMP-2, a band below the 31-kDa MBL monomer represents a MMP-2 breakdown product, which is faintly visible in the MMP-2 controls. The leukocytic MMPs, MMP-8 (neutrophil collagenase) and MMP-9, MMP-14 (gelatinase B), processed denatured MBL to fragments of 23.9 and 21 kDa (MMP-8) and 23.6 and 20 kDa (MMP-9) (Fig. 1C). MMP-7 (matrilysin), a protease that cleaves gelatin, only weakly cleaved denatured MBL to a 23.9-kDa fragment (Fig. 1D).

Because MMP-2, MMP-9, and MMP-14 were the most efficient at cleaving denatured human MBL, these enzymes were analyzed further. On nonreducing SDS-PAGE, MBL oligomers migrate near the top of the gel, whereas cleavage fragments, no longer bound to the oligomer, separate in the gel and are distinguished readily. After overnight incubation, MMP-2 generated two fragments from denatured human MBL of 24.8 and 22.2 kDa (Fig. 2A). The 22.2-kDa fragment was also produced by MMP-9, whereas a fragment of 26.3 kDa was released by MMP-14. To determine the sites of MMP cleavage, fragments were subjected to NH2-terminal sequencing (Table I). Cleavage sites are designated hP1-P1′, which indicates a cleavage site at the NH2-terminal. Cleavage sites determined by NH2-terminal sequencing are indicated.

Because neutrophils are present at sites of infection and inflammation where MBL is localized, we examined the ability of neutrophil elastase to cleave human MBL. Unlike neutrophil-specific MMP-8 (Fig. 1C), neutrophil elastase partially cleaved native MBL to fragments of 26.2 and 22.4 kDa (Fig. 4A, −DTT), visible as a band of 27.7 kDa under reducing conditions (Fig. 4B, +DTT). The intensity of these bands was enhanced after denaturation of MBL, indicating that although elastase could cleave native MBL, proteolysis was limited by the triple helical fold of the collagen-like domain. No synergistic effect on MBL proteolysis was detected by the addition of MMPs and not by any MASPs remaining in the preparation.

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To determine the efficiency of MMP activity, increasing amounts of MMP-2, MMP-9, or MMP-14 were added to denatured human MBL (42 pmol). Cleavage fragments were generated in a concentration-dependent manner (Fig. 5). The cleavage at hGly54-Lys55 occurred first with 1.5 pmol of MMP-2, whereas significant quantities of the lower molecular mass fragment, hGly72-Gln73, were produced at higher enzyme concentrations (Fig. 5A). Both MMP-9 and MMP-14 produced single fragments, hGly72-Gln73 and hGly48-Leu49 respectively, even at the lowest enzyme concentration used (0.4 pmol) (Fig. 5, B and C). Cleavage of denatured human MBL by MMP-2, MMP-9, and MMP-14 was rapid, occurring within 1 h (data not shown). Proteases often localize to substrates via specific binding
sites, termed exosites, which also increase catalytic activity (40). Gelatinases bind to collagen via a domain inserted into the MMP catalytic domain, the CBD (37), comprised of three fibronectin type II modules, and to the chemokines monocyte chemotactrant protein-3 (41) and stromal cell-derived factor-1 (33) via the hemopexin C domain. To determine the role of the CBD and hemopexin C domain in the cleavage of MBL, recombinant domains were used in competition assays. The addition of 500:1 molar ratio of CBD:MMP-2 resulted in an increase in the 24.8 and 22.2 kDa bands (Fig. 6A, lower panel). This appeared to be the result of stimulation of cleavage at hGly54-Lys55 and hGly72-Gln73 because the oligomeric forms of MBL decreased with increasing CBD (Fig. 6A, upper panel).

This appeared to be the result of stimulation of cleavage at hGly54-Lys55 and hGly72-Gln73 because the oligomeric forms of MBL decreased with increasing CBD (Fig. 6A, upper panel). Neither CBD nor MMP-2 was detected by the -MBL monoclonal antibody (data not shown). The addition of exogenous MMP-2 hemopexin C domain also resulted in an increase of the 24.8-kDa product at 100:1 C domain:MMP-2 (Fig. 6B). However, in this case there was a concomitant decrease in the...
The hemopexin C domain of MT1-MMP with (Fig. 6) the “linker” that connects this domain to the catalytic domain had the same effect on cleavage of MBL as the MMP-2 hemopexin C domain at a similar concentration. However, cleavage of MBL by MT1-MMP was not affected significantly by the addition of the MMP-2 or MT1-MMP hemopexin C domain (data not shown). Hence, native human MBL was resistant to MMP activity, but upon denaturation it was cleaved efficiently in the collagen-like domain by MMPs. In the case of MMP-2, the mechanism involves both the CBD and hemopexin C domains, whereas MT1-MMP appears less dependent on the hemopexin C domain for MBL cleavage.

**Fig. 6. The influence of MMP exosite domains on MBL cleavage.** Increasing amounts of (A) recombinant MMP-2 CBD, (B) MMP-2 hemopexin C domain (CD), (C) MMP-14 hemopexin C domain (CD), (D) MMP-14 hemopexin C domain minus linker (hinge region that connects to the catalytic domain) (CDL) were added to overnight incubations of heat-denatured human MBL and MMP-2 (MBL:2 molar ratio 15:1) at the exosite domain:MBP-2 molar ratios shown above each lane. Incubations of MMP-2 and exosite domain without MBL were included (S). Nonreduced samples were electrophoresed on 12.5% Tris-Tricine gels and Western blotted with α-MBL monoclonal antibody 131-1. The upper and lower panels of A are from two different exposures of the same Western blot. The upper panel shows the MBL oligomers remaining after the incubation, and the lower panel shows the generation of lower molecular mass fragments.

**Fig. 7. Engineered MBL containing naturally occurring human mutations is cleaved readily by MMPs.** 64 pmol of recombinant rat MBL (wt) or mutant G28E-MBL, G25D-MBL, or R23C-MBL was incubated overnight at 37 °C with 5.3 pmol of MMP-2 (A), MMP-9 (B), or MMP-14 (C). Nonreduced samples were electrophoresed on 12.5% Tris-Tricine SDS-PAGE and silver stained. Fragments are indicated by arrows with molecular mass (× 10−3 Da), and cleavage sites were determined by NH2-terminal sequencing.

22.2-kDa product and no detectable effect on the remaining oligomers, suggesting inhibition of cleavage at hGly72-Gln73. The hemopexin C domain of MT1-MMP with (Fig. 6D) or without (Fig. 6D) the “linker” that connects this domain to the catalytic domain had the same effect on cleavage of MBL as the MMP-2 hemopexin C domain at a similar concentration. However, cleavage of MBL by MT1-MMP was not affected significantly by the addition of the MMP-2 or MT1-MMP hemopexin C domain (data not shown). Hence, native human MBL was resistant to MMP activity, but upon denaturation it was cleaved efficiently in the collagen-like domain by MMPs. In the case of MMP-2, the mechanism involves both the CBD and hemopexin C domains, whereas MT1-MMP appears less dependent on the hemopexin C domain for MBL cleavage.

Rat MBL Containing Naturally Occurring Human Mutations Is Cleaved Readily by MMPs—Naturally occurring mutations in MBL are known to perturb the helical structure of the collagen-like domain. Because denatured human MBL was cleaved efficiently by MMPs, we hypothesized that proteolysis of variant MBL proteins might contribute to low serum levels in these patients. To address this, we examined the MMP susceptibility of recombinant rat MBL containing point mutations corresponding to natural human mutations, R23C-MBL, G25D-MBL, and G28E-MBL (Fig. 3). Nonreduced recombinant wild-type rat MBL electrophoresed at the top of gels consistent with higher order oligomers (Fig. 7, first lane from left). In contrast, because of defective oligomerization (25), R23C-MBL, G25D-MBL, and G28E-MBL electrophoresed as multiple bands (Fig. 7A–C, first two lanes) unless heat-denatured (data not shown), whereas the nonadenatured mutant MBL proteins were processed efficiently by MMP-2, MMP-9, and MMP-14 (Fig. 7, A–C). Virtually all polypeptide forms of R23C-MBL, G25D-MBL, and G28E-MBL were degraded, although the 100–200-kDa complexes of R23C-MBL were more resistant than those of the other two mutants. NH2-terminal sequencing of major fragments was performed to determine the sites of cleavage, designated rP1-P1′, where r signifies rat MBL (Table II). After incubation, MMP-2 degraded the three mutants to a doublet with a major component of 19.5 kDa and a second product of 19 kDa (Fig. 7A). Degradation of R23C-MBL by MMP-2 resulted in an additional minor product of 17.2 kDa. The 19.5-kDa product for all three mutants resulted from cleavage at rAsn80-Met81 (Fig. 7C). Cleavage by MMP-2 was inhibited by the MMP inhibitors BB94 and TIMP-2, but not by phenylmethylsulfonyl fluoride, ruling out the involvement of MASPs (Table II and Fig. 3). Cleavage of MBL by MMP-2 was inhibited by the MMP inhibitors BB94 and TIMP-2, but not by phenylmethylsulfonyl fluoride, ruling out the involvement of MASPs (data not shown). Incubation with MMP-9 resulted in major products of 23 and 19.5 kDa with minor products of 24.4 and 17.8 kDa for all three mutants (Fig. 7B). The 19.5-kDa product was identical to that generated by MMP-2, rAsn80-Met81, and the 23-kDa fragment resulted from cleavage at rGly63-Gln64 (Table II and Fig. 3). MMP-14 cleaved the three mutants to a unique 27.5-kDa fragment by cleavage at rGly39-Leu40 and the 19.5-kDa product by cleavage at rAsn60-Met81 (Fig. 7C and Table II).

Other MMPs were less efficient at cleavage. MMP-3 cleaved the monomers of R23C-MBL, G25D-MBL, and G28E-MBL to products of 19.5 and 17.8 kDa, but it was selective in its cleavage of the 100–200 kDa bands (data not shown), suggesting that oligomerization inhibited cleavage by this enzyme. There was a small amount of cleavage of the mutants to lower molecular mass forms by MMP-1 (18.9 and 16.7 kDa) and an enhancement of the 27.5 kDa band by MMP-8, but no cleavage by MMP-7 or MMP-13 (data not shown).

Although the human collagenases (MMP-1, MMP-8, and MMP-13) were ineffective against wild-type rat MBL and only weakly active against the MBL mutants, native wild-type rat
MBL was partially degraded by bacterial collagenase to fragments of 19.4, 18.2, and 17.3 kDa (Fig. 8A). The three MBL mutants were less resistant to cleavage, with bacterial collagenase generating high levels of these fragments together with an additional product of 16.7 kDa. The latter was most likely generated from the 17.3-kDa fragment, which was depleted. Neutrophil elastase also partially cleaved native wild-type rat MBL to a prominent product at 16.9 kDa and a faint band at 29.3 kDa (Fig. 8B). The mutants were cleaved by neutrophil elastase to a doublet of 17.7 and 16.9 kDa, and R23C-MBL was processed to an additional product of 29.3 kDa.

### Efficacy of MBL Proteolysis

To determine the MMP potency and cleavage pattern of MBL, MBL mutants were incubated with MMP-2, MMP-9, or MMP-14 for various periods of time and at different enzyme concentrations (Fig. 9). A series of intermediates was observed over time when G25D-MBL was incubated with MMP-2, MMP-9, or MMP-14 at a constant enzyme:substrate ratio of 1:12 (Fig. 9A). Cleavage by MMP-2 was highly efficient (Fig. 9A). A 23.7-kDa fragment appeared rapidly after a 1-min incubation at 37 °C. This initial fragment resulted from cleavage at rGly51-Ser52 (Table II and Fig. 3). After 10 min of incubation, an intermediate band of 23 kDa was produced by cleavage at rGly63-Gln64. The rAsn80-Met81 fragment was produced after 10 min and was the major stable product that accumulated after overnight incubation together with a minor product at 19 kDa as found previously (Fig. 7A).

**TABLE II**

| MMP  | Fragment size | NH₂-terminal sequence | Deduced sequence | Cleavage site | Homologous to |
|------|---------------|-----------------------|------------------|--------------|---------------|
| 14   | 58.0, 39.6, 30| SGSQTXᵃ | SGSQTC | NH₂-terminus of MBL | hG⁴⁶-L⁴⁹ |
| 14   | 27.5          | LQGPXG             | LQGPPOG         | rG⁴⁶-L⁴⁹    | hG⁴⁶-K⁵⁵ |
| 9(2) | 24.4          | KLGPGX             | KLGPGPO         | rG⁴⁶-K⁵⁵    | hG⁴⁶-K⁵⁵ |
| 2(9) | 23.7          | SVGAXG             | SVGAXPOG        | rG⁴⁶-K⁵⁵    | hG⁴⁶-K⁵⁵ |
| 2, 9 | 23.0          | QXGDRG             | QXGDRGPO        | rG⁴⁶-Q⁵⁴    | hG⁴⁶-Q⁵⁴ |
| 2, 9 | 19.5          | MEARIN             | MEARINPO        | rG⁴⁶-M⁵¹     | hG⁴⁶-M⁵¹ |

ᵃ X, O, and K are the same as in Table I. Parentheses indicate minor cleavage products for the MMP indicated.

**Fig. 8.** MBL mutant proteins are cleaved by bacterial collagenase and neutrophil elastase. 64 pmol of recombinant rat MBL (wt) or mutant G28E-MBL, G25D-MBL, or R23C-MBL was incubated overnight at 37 °C with 5.3 pmol of (A) bacterial collagenase (BC) or (B) neutrophil elastase (NE). Nonreduced samples were electrophoresed on 12.5% Tris-Tricine SDS-PAGE and silver stained. Fragments are indicated by arrows and molecular masses (> 10⁻³ Da).

**Fig. 9.** Determination of the sequence of cleavage of MBL mutant proteins. A, 42 pmol of active MMP was added to 512 pmol of G25D-MBL and incubated at 37 °C. Samples were removed at the times indicated. Unincubated G25D-MBL (S) and MMP were run as controls. B, 64 pmol of G28E-MBL was incubated at 37 °C overnight alone or with various concentrations (indicated in pmol) of MMP, or 5.3 pmol of MMPs was incubated alone. Nonreduced samples were electrophoresed on 12.5% Tris-Tricine SDS-PAGE and silver stained: MMP-2 (i), MMP-9 (ii), or MMP-14 (iii). Fragments are indicated by arrows with molecular mass (> 10⁻³ Da) or NH₂-terminal sequence, where determined.
stable product (Fig. 9Aii). The rAsn\textsuperscript{80}-Met\textsuperscript{81} fragment began to appear after 1 h but was a minor product compared with the MMP-14-specific rGly\textsuperscript{39}-Leu\textsuperscript{40} fragment. Transient bands at 58, 39.6, and 30 kDa (indicated by arrows in Fig. 9Aii) began with the sequence SGSQTX, which corresponds to the NH\textsubscript{2} terminus of full-length rat MBL. These likely consist of disulfide cross-linked fragments that are separated by cleavage at rAsn\textsuperscript{80}-Met\textsuperscript{81}. The polypeptide complexes persisted up to 4 h of incubation with this enzyme.

With all three MMPs, cleavage was concentration-dependent and proceeded through a series of intermediates that corresponded to those generated in the time course experiments (Fig. 9B). After overnight incubation with 0.2 pmol of MMP-2, all of the forms of G28E-MBL were degraded except for the 24.9 kDa band, which remained along with a new band of 23.7 kDa (Fig. 9Bi). This initial cleavage product likely corresponds to the rGly\textsuperscript{51}-Ser\textsuperscript{52} fragment of Fig. 9Ai. At higher concentrations of MMP-2, an intermediate band at 23 kDa corresponding to the rGly\textsuperscript{63}-Gln\textsuperscript{64} fragment was replaced by the prominent rAsn\textsuperscript{80}-Met\textsuperscript{81} fragment. At the highest concentrations of MMP-2, faint bands at 19 and 16.9 kDa were apparent. After overnight incubation with 0.2 pmol of MMP-9, all of the polypeptide complexes in the original preparation were converted to a doublet of 24.4 and 23 kDa, probably by cleavage at rGly\textsuperscript{45}-Lys\textsuperscript{46} and rGly\textsuperscript{63}-Gln\textsuperscript{64}, respectively (Fig. 9Bi). Disappearance of the 24.4-kDa product coincided with generation of the rAsn\textsuperscript{80}-Met\textsuperscript{81} fragment (19.5 kDa) and a band of 16.2 kDa at higher concentrations of MMP-9. The rGly\textsuperscript{39}-Leu\textsuperscript{40} fragment was a major product of MMP-14 activity (Fig. 9Bi). Higher molecular mass bands were degraded progressively with increasing enzyme concentration, and transient bands appeared similar to those in Fig. 9Aiii, likely representing the NH\textsubscript{2} terminus of the protein. As well as the rAsn\textsuperscript{80}-Met\textsuperscript{81} fragment, minor products of 18.4 and 17.2 kDa were produced at the highest concentrations of MMP-14. Thus, the order of cleavage of the MBL mutants G25D-MBL and G28E-MBL appears to be: MMP-2 (rGly\textsuperscript{45}-Lys\textsuperscript{46} → rGly\textsuperscript{51}-Ser\textsuperscript{52} → rGly\textsuperscript{63}-Gln\textsuperscript{64} → rAsn\textsuperscript{80}-Met\textsuperscript{81}; MMP-9, rGly\textsuperscript{45}-Lys\textsuperscript{46} → (rGly\textsuperscript{51}-Ser\textsuperscript{52}) → rGly\textsuperscript{63}. Glu\textsuperscript{64} → rAsn\textsuperscript{80}-Met\textsuperscript{81}; MMP-14, rGly\textsuperscript{39}-Leu\textsuperscript{40} → rAsn\textsuperscript{80}-Met\textsuperscript{81}). Cleavage by MMP-2 at rGly\textsuperscript{45}-Lys\textsuperscript{46} and by MMP-9 at rGly\textsuperscript{51}-Ser\textsuperscript{52} are either infrequent occurrences, or these products are rapidly processed further. Each cleavage likely destabilizes the triple helix, allowing progressive cleavage of the variant MBL.

DISCUSSION

Humans with MBL mutations have reduced levels of serum MBL, a condition predisposing patients to severe microbial infections and diseases such as rheumatoid arthritis, systemic lupus erythematosus, and cystic fibrosis. Here we have demonstrated the markedly increased susceptibility of MBL mutants to proteolytic cleavage by MMPs, a family of proteinases with important roles in inflammatory and immune responses.

Both human serum MBL and recombinant rat MBL were resistant to MMP activity in vitro unless denatured, indicating that the triple helical collagen-like domain masks protease-sensitive sites on the individual polypeptide chains. However, recombinant rat MBL proteins with mutations homologous to those found in human variant MBL were rapidly and concentration-dependently cleaved at specific sites by the gelatinolytic MMPs, MMP-2, MMP-9, and the MMP-2 activator MMP-14 and less efficiently by MMP-3, MMP-1, and MMP-8. Neutrophil elastase and bacterial collagenase also cleaved at specific sites within the collagen-like domain. Cleavage of variant rat MBL occurred at sites homologous to those cleaved in human MBL (Table 1), except for rAsn\textsuperscript{80}-Met\textsuperscript{81} and rGly\textsuperscript{51}-Ser\textsuperscript{52} which are sites unique to rat MBL. Digestion of denatured wild-type rat MBL by MMP-2, MMP-9, and MMP-14 produced the same fragments as digestion of G28E-MBL (data not shown). Although it is possible that the mutations have generated a sequence that is now recognized in the native state by the hemopexin C domain exosites, this is unlikely because the location of the Gly residues in the core of the triple helix renders them relatively inaccessible to solvent, and the three mutations are different and occur independently. Hence, we suggest that MBL mutations structurally destabilize the collagen-like domain of MBL, permitting cleavage by MMPs through the exposure of cleavage sites and exosite binding regions on individual strands within the perturbed triple helix. The processing of MBL mutants at sites identical to those cleaved in heat-denatured human or wild-type rat MBL supports previous studies reporting that the structure of the collagen-like domain of rat MBL mutants R23C-MBL, G25D-MBL, and G28E-MBL is perturbed (25, 26). Cleavage of variant MBL and denatured human MBL was rapid and efficient, occurring within minutes of exposure to MMP-2, MMP-9, and MMP-14 at low enzyme:substrate ratios, suggesting that MMPs are likely to cleave the mutants in vivo. Effective MMP cleavage of mutant MBL but resistance of wild-type MBL provides an explanation for the absence of MBL in homozygotes but persistence of low levels of MBL in heterozygotes (12, 13, 15).

The MBL binding site for the receptor C1qRp, which is proposed to mediate phagocytosis (3), lies in the collagen-like domain (42) (see Fig. 3) encompassing the sequence G\textsuperscript{52}EKGEP which includes Gly\textsuperscript{52} and is near the other MBL mutations. Hence, receptor binding may already be disrupted in variant MBL, but cleavage of MBL by MMPs between the receptor binding site and carbohydrate recognition domain (Fig. 3) would separate the recognition and effector functions of the molecule. However, the biological effects of MMP cleavage on MBL function in vitro or in vivo cannot be assessed because both mutant MBL and denatured MBL have a perturbed collagen domain structure that alone disrupts MASP binding and oligomerization, which are requirements for complement activation (25, 26, 28). This also suggests that MMP inhibitors would be of little therapeutic benefit for patients with variant forms of MBL. Hence it is likely that in addition to any effects of the mutations in the collagen-like domain on secretion, increased turnover of variant MBL by members of the MMP family contributes to the low serum levels of variant MBL in vivo.

With regard to wild-type human MBL, there was no evidence for cleavage of the purified protein in vitro by MMPs without heat denaturation. A role for MMPs in the clearance of MBL cannot be ruled out however, because additional factors may be involved in vivo. MBL is part of a complex with MASPs which, like ligand binding (2), may alter the conformation of MBL rendering it susceptible to cleavage. Oxidation by free radicals derived from activated neutrophils and macrophages, which impairs the function of the structurally homologous molecule C1q (43), may affect the structure of MBL. Finally, other proteolytic events may occur, initiating a cascade resulting in degradation of MBL by MMPs. However, using purified MASPs free MBL in vitro, neither binding to mannose-Sepharose or bacterial cell fragments nor oxidation by hydrogen peroxide increased the susceptibility of native MBL to MMP cleavage.3 Further studies are required to elucidate the clearance pathway for MBL in vivo and to assess the involvement of MMPs.

Degradation of MBL by MMPs might be a mechanism to evade MBL-mediated host detection and destruction. Both mi-

3 G. S. Butler and C. M. Overall, unpublished observations.
Croorganisms and tumor cells, which express mannose-containing proteins on their surface and are killed or growth-inhibited by MBL (44, 45), express collagenases (46–48) and can induce host cells to secrete MMPs (49–51). Cleavage of native MBL by bacterial collagenases, some of which are reported to activate MMPs (52), supports the idea that these collagenases could act as virulence factors in this regard. Tumor cells sequester MMPs at the cell surface (for review, see Ref. 53), where they may be poised to degrade MBL bound to mannose-containing cell surface proteins, potentially enabling the tumor to escape host detection.

The mechanism of collagen cleavage by MMPs has yet to be elucidated fully, although a role for exosites on the hemopexin C domain, acting in concert with specific residues in the catalytic domain in the case of MMP-1, is established (40, 54). Competition experiments confirmed that exosite domains are important for cleavage of MBL. The apparent stimulation of cleavage of denatured human MBL at hGly54–Lys55 by the CBD of MMP-2 (for review, see Ref. 53) may be a common target for cleavage of these proteins by MMPs. Indeed pepsin-treated C1q is cleaved in the collagen-like domain by several MMPs (62). New members and functions of this modular protein family are being discovered continually, and it will be interesting to determine whether MMP processing plays a role in their regulation.

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Cleavage of Mannose-binding Lectin by MMPs and phagocytosis via C1qRp (60), suggesting involvement in termination of the inflammatory response. A newly discovered group of homologous proteins, the EMILINs, have C1q and collagen-like domains and are found in extracellular matrix where they may mediate elastogenesis and cell adhesion (59). The presence of homologous collagen-like regions suggests that this may be a common target for cleavage of these proteins by MMPs. Indeed pepsin-treated C1q is cleaved in the collagen-like domain by several MMPs (62). New members and functions of this modular protein family are being discovered continually, and it will be interesting to determine whether MMP processing plays a role in their regulation.

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Mannose-binding Lectin (MBL) Mutants Are Susceptible to Matrix Metalloproteinase Proteolysis: POTENTIAL ROLE IN HUMAN MBL DEFICIENCY

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