The Gly-54 → Asp allelic form of human mannose-binding protein (MBP) fails to bind MBP-associated serine protease

Misao MATSUSHITA*, R. Alan B. EZEKOWITZ† and Teizo FUJITA*‡

*Department of Biochemistry, Fukushima Medical College, Fukushima 960-12, Japan, and †Division of Hematology/Oncology, Harvard Department of Pediatrics, Children's Hospital and Dana Faber Cancer Institute, Boston, MA 02115, U.S.A.

The human mannose-binding protein (MBP) is a pattern recognition molecule that appears to play a role in initial host defence. MBP activates the complement cascade and it may act as an opsonin both in the absence and in the presence of complement. A number of distinct MBP allelic forms exist in different population groups. An allele that occurs in 5–7% of Caucasians was identified by an inability to activate the complement system. A homozygous mutation at base pair 230 of the MBP gene results in a Gly-to-Asp substitution at the fifth collagen repeat. It appears that the resultant protein, MBP_D, is able to form high-order multimers that bind bacteria but do not support complement activation. Recently a novel serine protease,

the MBP-associated serine protease (MASP), has been described. MBP–MASP complexes circulate in serum and result in the direct activation of a novel complement pathway (lectin pathway) in the absence of the first complement components. In this study we demonstrate that MASP and its proenzyme proMASP are unable to bind to recombinant (r)MBP_D. This lack of a MASP–rMBP_D association corresponds to a failure of the Gly-54 → Asp form of MBP to activate complement. Our results provide a biochemical basis for the functional deficit in the Gly-54 → Asp allelic form of MBP and suggest that the proMASP/MASP binding site maps to the fifth collagen repeat of MBP.

INTRODUCTION

Serum mannose-binding protein (MBP) is a C-type lectin [1] which recognizes the patterns of oligosaccharides that decorate yeast, certain Gram-negative and -positive bacteria, the envelope glycoprotein of the human immunodeficiency virus, and the haemagglutinin of some strains of influenza virus. MBP appears to play an important role in the first line of host defence against a broad range of pathogens. MBP is considered to be a member of the collectins, a group of proteins that contain a collagen region as well as globular, lectin-like heads [2,3]. The MBP may act as an opsonin [4] through binding to the collectin receptor (C1q receptor) on phagocytes. In addition, when bound to its ligand *in vitro*, MBP can destroy bacteria directly by activating the complement cascade [5–7]. Previous reports suggested that MBP initiates complement activation by binding the C1r₂C1s₂ complex and generating active C1s [8,9]. However, we have demonstrated that human serum MBP can associate with a C1s-like serine protease, designated MASP (MBP-associated serine protease), that activates the complement cascade by consuming C4 and C2 [10]. Thus complement activation via the formation of an MBP–MASP complex (termed the lectin pathway) represents a new mechanism for regulating this cascade [11].

It has been reported that low to absent baseline levels of serum MBP may be associated with a complement-dependent opsonic defect that results in recurrent infections in infants [12]. These patients have an MBP allele in which guanine is replaced by adenine at base 230. This base pair substitution results in an aspartic acid residue instead of a glycine at the fifth collagen repeat [13]. Although this substitution was predicted to disrupt the collagen helix leading to dysfunctional MBP, studies with recombinant MBPs have shown that both recombinant (r)MBP_G

(wild-type) and rMBP_D (Gly-54 → Asp form) assemble into multimers and exhibit opsonic activities [14]. Unlike recombinant MBP_G, however, recombinant MBP_D is unable to initiate complement activation as determined by C4 activation in human serum. In this paper we report the molecular basis of the failure of complement activation by recombinant MBP_D.

MATERIALS AND METHODS

Reagents

Mannan from *Saccharomyces cerevisiae* was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human serum MBP, MASP [10], recombinant MBPs (rMBP_G and rMBP_D) [14], and human C4 [15], C2 [16] and oxidized C2 [17] were prepared as described previously. Human MASP proenzyme (proMASP) was purified using the purification method for MASP [10], with modifications. Briefly, human serum was chromatographed on mannan–Sepharose followed by anti-MBP–Sepharose in the presence of *p*-nitrophenyl *p*-guanidinobenzoate to prevent auto-activation of proMASP. Veronal buffered saline (VB) was a standard solution of Veronal buffer containing 0.148 M NaCl (pH 7.4). EDTA–GVB was VB supplemented with 10 mM EDTA and 0.1% gelatin. MGVB was low-ionic-strength VB containing 0.1% gelatin, 2.3% mannitol, 2 mM CaCl₂ and 0.5 mM MgCl₂. Na¹²⁵I was purchased from Amersham International.

Haemolytic assay

The C4- and C2-activating capacities of MASP and MBP on mannan were assayed as described previously [10]. Briefly, 100 μl of mannan-coated sheep erythrocytes (E_{mannan}; 10⁸/ml of MGVB)

Abbreviations used: E_{mannan}, sheep erythrocytes coated with yeast mannan; (r)MBP, (recombinant) mannose-binding protein; MBP_G, wild-type MBP; MBP_D, Gly-54 → Asp MBP form; MASP, MBP-associated serine protease; proMASP, proenzyme of MASP; VB, Veronal buffered saline; EDTA–GVB, VB supplemented with 10 mM EDTA and 0.1% gelatin; MGVB, low-ionic-strength VB containing 0.1% gelatin, 2.3% mannitol, 2 mM CaCl₂ and 0.5 mM MgCl₂.

‡To whom correspondence should be addressed.

was incubated with 50 μ l of MASP or proMASP and 50 μ l of MBP at 30 °C for 30 min. After washing, the cells were suspended in MGVB at 10^8 /ml and incubated with 50 μ l of C4 and 50 μ l of oxidized C2 at 30 °C for 20 min, and then with 200 μ l of guinea pig serum diluted with EDTA-GVB at 37 °C for 1 h. After incubation, 1 ml of EDTA-GVB was added to the reaction mixtures and they were centrifuged. The haemolytic rate (y) was determined spectrophotometrically. The average number of haemolytic sites per cell (z) was calculated as $z = -\ln(1 - y)$.

Binding of 125 I-MASP to MBP on mannan

MASP and MBPs were labelled using Na 125 I and Iodo-Gen from Pierce Chemical Co., Rockford, IL, U.S.A. The specific radioactivities of MASP, serum MBP, rMBP_G and rMBP_D were 0.38, 0.33, 0.30 and 0.25 MBq/ μ g respectively. Samples of 400 μ l of E_{mannan} (5×10^8 /ml of MGVB) were incubated with 100 μ l of MBP (80 μ g/ml) at 30 °C for 1 h. As a control, E_{mannan} were also incubated with MGVB alone. After incubation, the cells were washed with MGVB and suspended in 400 μ l of MGVB. A 100 μ l aliquot of the cell suspension was then incubated with 100 μ l of various concentrations of 125 I-MASP at 30 °C for 1 h. After washing, the radioactivity of the pellets was determined. Specific binding of 125 I-MASP was calculated from the binding to the E_{mannan} -MBP complex by subtracting the binding to E_{mannan} . In the above experiments, the amounts of MBP bound to E_{mannan} were determined using 125 I-MBP as a tracer.

RESULTS AND DISCUSSION

In an earlier study we had described an MBP genotype in which glycine is substituted by aspartic acid at codon 54; this encoded a functional recombinant protein, rMBP_D [14]. Like rMBP_G, rMBP_D is able to bind micro-organisms with similar avidity and act as an opsonin, but unlike rMBP_G, rMBP_D lacks the ability to activate complement. Thus we set out to determine whether the failure of rMBP_D to activate C4 and C2 could be related to a defect in its ability to associate with MASP. First, we wished to establish that rMBP_G, like serum MBP, can bind to E_{mannan} , associate with MASP and initiate C4 and C2 activation (monitored as complement-dependent haemolysis). As shown in Figure 1, in the presence of MASP, C4 and C2 as well as C3 and the terminal complement components, rMBP_G induced the dose-dependent lysis of E_{mannan} . The results obtained with rMBP_G were similar to those obtained with MBP purified from serum. We next set out to determine whether rMBP_D was able to mediate MASP-dependent complement lysis of E_{mannan} . Under identical conditions, however, rMBP_D failed to mediate E_{mannan} lysis at all concentrations tested (Figure 1a).

In the experiments shown in Figure 1(a), a proteolytically active form of MASP was used. We have observed a pro-form of MASP (proMASP) that circulates in the serum (M. Matsushita and T. Fujita, unpublished work). ProMASP has no proteolytic activity until it associates with an MBP-ligand complex; it then acquires the ability to activate C4 and C2. Nonetheless, proMASP was also able to initiate E_{mannan} lysis in the presence of either serum-derived MBP or rMBP_G, but not in the presence of rMBP_D (Figure 1b). Together, these results indicate that serum-derived MBP-ligand complexes as well as rMBP_G-ligand complexes (but not rMBP_D-ligand complexes) can convert proMASP to MASP.

We favoured the idea that the failure of rMBP_D- E_{mannan} complexes to activate complement was the result of a lack of MASP binding to rMBP_D. Thus the relative binding of rMBP_D to E_{mannan} was determined. Following the addition of 8 μ g of

Figure 1 Reconstitution of MBP and MASP or proMASP showing C4- and C2-activating capacity on mannan

A 100 μ l sample of E_{mannan} (1×10^8 /ml of MGVB) was simultaneously reacted with various amounts of serum MBP, rMBP_G or rMBP_D and 50 μ l of MASP (a) or proMASP (b). After incubation at 30 °C for 30 min, the cells were washed with MGVB, suspended at the same concentration and further incubated with 50 μ l each of C4 and oxidized C2 at 30 °C for 20 min, followed by incubation with 200 μ l of guinea pig serum diluted with EDTA-GVB at 37 °C for 60 min. After addition of 1 ml of EDTA-GVB, the haemolytic rate (y) was determined spectrophotometrically. The average number of haemolytic sites per cell (z) was calculated as $z = -\ln(1 - y)$. All assays were performed in triplicate; results are means $\pm$ S.E.M.

Figure 2 Binding of MASP to MBP on mannan

Following incubation of 100 μ l of E_{mannan} (5×10^8 /ml of MGVB) with 2 μ g of serum MBP (○), rMBP_G (□), rMBP_D (△) or buffer at 30 °C for 30 min, the cells were further incubated at 30 °C for 30 min with 100 μ l of various concentrations of 125 I-labelled MASP. After washing, the specific binding of 125 I-MASP was determined. All assays were performed in triplicate; results are means $\pm$ S.E.M.

serum-derived MBP, rMBP_G or rMBP_D to 2×10^8 E_{mannan} , 1.6 ± 0.3 , 2.1 ± 0.2 and 1.9 ± 0.3 μ g of the respective ligands were detected bound to the erythrocyte targets. These results are consistent with previous work showing equivalent binding of the two forms of rMBP to micro-organisms [14]. We next incubated the MBP- E_{mannan} complexes with various amounts of radio-labelled MASP. Figure 2 shows a dose-dependent increase in the

amount of MASP bound to serum MBP and rMBP_G, whereas MASP did not bind to rMBP_D. To exclude the possibility that MASP or proMASP may cleave rMBP_D and attenuate binding, we performed the above experiments in the presence of 1 mM (*p*-amidophenyl)methanesulphonyl fluoride, an inhibitor of serine proteases. We found similar results in the presence or absence of this inhibitor. In addition, we attempted to prepare labelled proMASP but found that, during preparation, proMASP was partly converted to MASP. Thus 100 ng of radiolabelled proMASP added to serum MBP-, rMBP_G- and rMBP_D-complexed E_{mannan} was in fact a mixture of proMASP and MASP. With this caveat, we found that 20 ± 3 ng and 29 ± 4 ng bound to serum MBP-E_{mannan} and rMBP_G-E_{mannan} respectively. By contrast, no binding to rMBP_D was noted.

Our results suggest that the failure of rMBP_D to activate complement is the result of an inability to bind proMASP/MASP. Serum MBP and rMBP_G are both active in haemolytic assays, as shown in Figure 1. However, the serum MBP activity reaches a plateau at 1 µg/ml, whereas rMBP_G activity increases in a dose-dependent manner. The reason for this difference is not clear. One possible explanation is that the relative concentrations of oligomers may differ in the two preparations. Complement activation appears to be dependent on the available hexamers of trimers that form the 'bunch of tulips' configuration [9]. Serum MBP is known to consist of a mixture of oligomeric forms of hexamers of trimers, pentamers of trimers and trimers of trimers, whereas rMBP_G forms only hexamers of trimers and pentamers of trimers [14]. Perhaps at higher concentrations of rMBP_G, hexamer of trimer formation is favoured and the enrichment of this form of the protein accounts for the enhanced activity.

Our findings bring into focus the role of the collagen domain of MBP. Until recently it was thought that the collagen domain was instrumental in trimer formation of MBP subunits. However, recent studies showed that a 148-amino-acid peptide that consists of only the 'neck' and carbohydrate recognition regions of human MBP forms trimers in solution and in crystal. A second crystal form revealed how the trimers might associate. The 'neck' region forms a helical coiled-coil whereby each α helix interacts with a neighbouring carbohydrate recognition domain [18]. Although the collagen domain is likely to influence the stability of high-order multimers of MBP, our results suggest other roles for the collagen domain. The implication of our findings in the present study is that the MASP binding site maps at or close to the fifth collagen repeat. This concept is supported by results showing that truncated forms of MBP that lack the collagen domain do not activate complement [19]. In addition, C1q, the first complement component, has, like MBP collagen, tails that interact with C1r₂C1s₂, the functional equivalents of MASP [20]. Sellar et al. [21] have speculated that the aberration of a 'bend' in the regular collagen repeat that occurs in wild-type MBP and C1q is important for the binding of C1r₂C1s₂. Our results support the idea that MASP binds to MBP at the same site as these homologous serine proteases. We predict that the Gly-to-Asp substitution is likely to alter the phasing of the collagen helix, and this would allow for the disruption of the MASP binding site.

It is of interest that rMBP_D retains its ability to act as an opsonin [14]. This indicates that the Gly-to-Asp change at the fifth collagen repeat does not disturb the interaction with the

collectin receptor. In fact, according to a scheme proposed by Sim and colleagues, the addition of a positively charged amino acid at this position may be expected to enhance binding to the collectin receptor [22]. Their proposal is that the positively charged patch of amino acids that precedes the 'bend' in the collagen region that occurs in C1q, MBP, the surfactant apoprotein A and conglutinin marks the cell binding domain of the collectins. In human MBP the 'bend' occurs at the eight collagen repeat. Another interesting question is whether MASP binding to the predominant MBP allelic form masks the cell binding site, thereby favouring assembly of the terminal complement components of the membrane attack complex rather than clearance of complexes by cells. For MBP_D, the failure to bind proMASP/MASP and the resultant inability to activate complement may allow enhanced uptake of MBP_D-ligand complexes by cells that express collectin receptors.

We thank Ms. Mihoko Mogi for technical assistance. This work was supported by Grants-in-Aid for Scientific Research (B) (06454223), for Scientific Research (C) (05670307), and for the International Scientific Research Program from the Ministry of Education, Science and Culture, the government of Japan and the Ryoichi Naito Foundation for Medical Research (to M.M. and T.F.). R.A.B.E. is an Established Investigator of the American Association and is supported by a Grant-in-Aid from the Bristol Myers Squibb Medical Foundation.

REFERENCES

- Drickamer, K. (1988) *J. Biol. Chem.* **263**, 9557–9560
- Sastry, K. and Ezekowitz, R. A. (1993) *Curr. Opin. Immunol.* **5**, 59–66
- Weis, W. I., Drickamer, K. and Hendrickson, W. A. (1992) *Nature (London)* **360**, 127–134
- Kuhlman, M., Joiner, K. and Ezekowitz, R. A. B. (1990) *J. Exp. Med.* **169**, 1733–1745
- Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T. and Yamashina, I. (1989) *J. Biol. Chem.* **262**, 7451–7454
- Kawasaki, N., Kawasaki, T. and Yamashina, I. (1989) *J. Biochem. (Tokyo)* **106**, 483–489
- Schweinle, J. E., Ezekowitz, R. A. B., Tenner, A. J., Kuhlman, M. and Joiner, K. A. (1989) *J. Clin. Invest.* **84**, 1821–1829
- Ohta, M., Okada, M., Yamashina, I. and Kawasaki, T. (1990) *J. Biol. Chem.* **265**, 1980–1984
- Lu, J., Thiel, S., Wiedemann, H., Timpl, R. and Reid, K. B. M. (1990) *J. Immunol.* **144**, 2287–2294
- Matsushita, M. and Fujita, T. (1992) *J. Exp. Med.* **176**, 1497–1502
- Sato, T., Endo, Y., Matsushita, M. and Fujita, T. (1994) *Int. Immunol.* **6**, 665–669
- Super, M., Thiel, S., Lu, J., Levinsky, R. J. and Turner, M. W. (1989) *Lancet*, **ii**, 1236–1239
- Sumiya, M., Super, M., Tabona, P., Levinsky, R. J., Arai, T., Turner, M. W. M. and Summerfield, J. A. (1991) *Lancet* **337**, 1569–1570
- Super, M., Gillies, S. D., Foley, S., Sastry, K., Schweinle, J. E., Silverman, V. J. and Ezekowitz, R. A. B. (1992) *Nature Genet.* **2**, 50–55
- Matsushita, M. and Okada, H. (1986) *J. Immunol.* **136**, 2994–2998
- Nagasawa, S. and Stroud, R. M. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2998–3001
- Polly, M. J. and Müller-Eberhard, H. J. (1967) *J. Exp. Med.* **126**, 1013–1025
- Sherrif, S., Chang, C.-Y. and Ezekowitz, R. A. B. (1994) *Nature Struct. Biol.* **1**, 789–793
- Schweinle, J. E., Nishiyasu, M., Ding, T. Q., Sastry, K., Gillies, S. D. and Ezekowitz, R. A. B. (1993) *J. Biol. Chem.* **268**, 364–370
- Knobel, H. R., Heysser, C., Rodrick, C. and Isliker, H. (1974) *J. Immunol.* **112**, 2094–2101
- Sellar, G. C., Blake, D. J. and Reid, K. B. M. (1991) *Biochem. J.* **274**, 481–490
- Malhotra, R., Laursen, S. B., Willis, A. C. and Sim, R. B. (1993) *Biochem. J.* **293**, 15–19