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Identification of the Site of Human Mannan-Binding Lectin Involved in the Interaction with Its Partner Serine Proteases: The Essential Role of Lys55

Florence Teillet,*†‡§ Monique Lacroix,*†‡§ Steffen Thiel,¶ Dietmar Weilguny,|| Teit Agger,|| Gérard J. Arlaud,*†‡§ and Nicole M. Thielens‡*†‡§

Mannan-binding lectin (MBL), a member of the collectin family, is an oligomeric C-type lectin that recognizes and binds patterns of neutral carbohydrates such as mannose and N-acetylgalactosamine present on the surface of pathogens (1, 2). It is a major actor of innate immunity, due to its ability to opsonize pathogens and thereby enhance their phagocytosis, and to activate the complement cascade via the lectin pathway (3). The latter activity results from the ability of MBL to associate with MBL-associated serine proteases MASP-2, a protease that shares with complement protease C1s the specific property of cleaving complement proteins C4 and C2 (4, 5). In addition to MASP-2, MBL binds to its homologs MASP-1 and MASP-3, as well as to MBL-associated protein 19 (MAp19) (6–9).

MASP-1 and -3, whereas mutations at residues Leu49 and Leu56 were ineffective. In conclusion, the MASP binding site of MBL is not identical. The Journal of Immunology, 2007, 178: 5710–5716.

Human MBL is assembled from a single polypeptide chain, consisting of a 21-residue cysteine-containing N-terminal stretch, a collagen-like region comprising 19 repeating Gly-X-Y triplets with one interruption causing a bend in the structure, a 34-residue hydrophobic stretch, and a 112-residue C-terminal C-type lectin carbohydrate recognition domain. The chains associate to form a homotrimeric structural unit comprising a collagen-like triple helix, an α helical coiled-coil or “neck” region, and three carbohydrate recognition domains (10). The structure is stabilized by interchain disulfide bonds that allow formation of bouquet-like oligomers comprising two to six or more structural units (11). The triple helices associate at their N-terminal end to form a stalk and then diverge at the level of the interruption in the Gly-X-Y sequence, defining a hinge in the molecule. The two major oligomeric forms of MBL isolated from human plasma have been identified as trimers and tetramers of the structural unit (12). A recombinant form of human MBL has been produced in human cells and shown to exhibit an oligomerization pattern similar to that of plasma-derived MBL (13, 14).

MASP-1, MASP-2, and MASP-3 each exhibit homologous modular structures, with an N-terminal CUB module (15), an epidermal growth factor (EGF)-like module (16), a second CUB module, two complement control protein (CCP) modules (17), and a serine protease domain. MASP-1 and MASP-3, which are alternative splicing products of the MASP1/3 gene, comprise identical CUB₁-EGF-CUB₂-CCP₁-CCP₂ segments but different serine protease domains (7). MAp19 results from alternative splicing of the MASP-2 gene and comprises the same N-terminal CUB₁-EGF segment as MASP-2, followed by four C-terminal residues unique to MAp19 (8, 9). Studies using recombinant human (18–20) and rat (21) proteins have shown that the MASPs and MAp19 each form
homodimers through their N-terminal CUB2 domain. In turn, each homodimer forms Ca\(^{2+}\)-dependent complexes with MBL and with ficolins L and H through interactions involving primarily the CUB1-EGF moiety of each protein, but strengthened by the following CUB2 module (19, 20, 22).

Studies mainly based on the use of synthetic peptides have led to the conclusion that the MASPs bind on the C-terminal side of the hinge region of rat MBL (23). In line with these experiments, a three-dimensional model has been proposed for the interaction between human MAp19 and MBL, featuring major interactions between acidic residues of MAp19 and basic residues of MBL, including Lys\(^{55}\) (24). The purpose of this work was to test the validity of this model by generating a series of recombinant MBL point mutants to analyze their ability to interact with the MASPs and MAp19.

Materials and Methods

**Proteins**

Human MASP-1, the MASP-1/3 CUB\(_2\)-EGF-CUB\(_2\) segment, and MAp19 were expressed using a baculovirus/insect cells system and purified as described previously (18, 19). Recombinant MASP-3 was expressed using the same system and purified as described by Zundel et al. (20), with some modifications as follows. After the first anion-exchange chromatography step, fractions containing MASP-3 were concentrated by ultrafiltration and final purification was achieved by high-pressure gel permeation on a TSK G-3000 SWG column (7.5 × 600 mm; Tosohaas) equilibrated in 145 mM NaCl, 1 mM CaCl\(_2\), and 50 mM triethanolamine-HCl, and run at 1 ml/min. Recombinant MASP-2 used for surface plasmon resonance analyses was also produced using a baculovirus/insect cell system (18). Other functional assays were performed using recombinant MASP-2 expressed in mammalian cells (4).

The concentrations of purified recombinant proteins were determined using the following absorption coefficients \((A_{190}, 1\text{ cm at } 280\text{ nm and molecular weights: MASP-3, 12.9 and 87.600 (20); MAp19, 11.6 and 19.086 (18); and MASP-1 CUB\(_2\)-EGF-CUB\(_2\), segment, 10.0 and 34,300 (19). Due to the low amount of material recovered, estimation of the concentration of recombinant MASP-1 and MASP-2 was based on Coomassie blue staining after SDS-PAGE analysis using appropriate internal standards and molecular masses of 82,000 and 75,100, respectively (18).**

**Site-directed mutagenesis**

The expression plasmids coding for all MBL mutants were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. A pC1-MBL expression plasmid coding for wild-type (WT) MBL (25) was used as a template.

Mutagenic oligonucleotides were purchased from MWG-BIOTECH. The sequences of all mutants were confirmed by dsDNA sequencing (Genome Express).

**Production and purification of recombinant MBL variants**

Two different methods were used for production of recombinant MBL. In most cases, plasmids containing the mutated MBL cDNA inserts were used for transfection of Freestyle 293-F cells (Invitrogen Life Technologies). Briefly, plasmids (1 μg/ml) were mixed with Lipofectamine 2000 (Invitrogen Life Technologies) and OptiMEM (Invitrogen Life Technologies) according to the manufacturer’s instructions and used for transfection of 293F cells (10\(^6\) cells/ml). Cells were cultured for 4 days in Freestyle expression medium (Invitrogen Life Technologies) and supernatants were collected by centrifugation and stored in the presence of 0.01% sodium merthiolate. Supernatants were diluted 1:2 with 10 mM Tris-HCl, 145 mM NaCl, 5 mM CaCl\(_2\), 0.01% Tween 20 (pH 7.4), and passed through a column containing 0.5 ml of glucose-Sepharose beads (glucosamine-Sepharose 4FF; GE Healthcare). The beads were washed with the column buffer and bound MBL was eluted with 10 mM Tris, 145 mM NaCl, 5 mM EDTA, and 0.01% Tween 20 (pH 7.4). Fractions were collected and tested for MBL content.

Some of the MBL variants, as listed in Table I, were produced using CHO-K1 cells (American Type Culture Collection). Cells were cultured in D-MEM/F12 (1/1) with GlutaMAX I medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS. Cells were transfected with the pC1-MBL constructs using Lipofectamine 2000 (Invitrogen Life Technologies) as described by the manufacturer. Selection of gene-resistant cell lines was carried out after transfection, in the presence of 600 μg/ml geneticin sulfate (G418; Invitrogen Life Technologies) and 50 μg/ml ascorbic acid (Sigma-Aldrich). After 3–4 wk, selected colonies were subcloned in tissue culture plates and supernatants were tested for MBL production. The highest producer clone was selected and expanded in 175-cm\(^2\) flasks. Once confluence was reached, the medium was replaced with CD-CHO-A serum-free medium (Invitrogen Life Technologies) and supernatants were tested for MBL content.

**Table I. Kinetic and dissociation constants for binding of MASP-1, MASP-3, MASP-1/3 CUB\(_1\)-EGF-CUB\(_2\), and MAp19 to immobilized MBL variants**

<table>
<thead>
<tr>
<th>MBL Variant</th>
<th>(k_m) (M(^{-1}) s(^{-1}))</th>
<th>(k_{d}) (s(^{-1}))</th>
<th>(K_D) (nM)</th>
<th>(k_m) (M(^{-1}) s(^{-1}))</th>
<th>(k_d) (s(^{-1}))</th>
<th>(K_D) (nM)</th>
<th>(k_m) (M(^{-1}) s(^{-1}))</th>
<th>(k_d) (s(^{-1}))</th>
<th>(K_D) (nM)</th>
<th>(K_D) (nM)</th>
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</thead>
<tbody>
<tr>
<td>WT(^{a})</td>
<td>1.8 × 10(^5)</td>
<td>1.7 × 10(^{-3})</td>
<td>9.4</td>
<td>2.1 × 10(^5)</td>
<td>1.6 × 10(^{-3})</td>
<td>4.3</td>
<td>2.0 × 10(^5)</td>
<td>1.4 × 10(^{-3})</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>WT(^{b})</td>
<td>1.7 × 10(^5)</td>
<td>1.8 × 10(^{-3})</td>
<td>10.6</td>
<td>2.5 × 10(^5)</td>
<td>1.9 × 10(^{-3})</td>
<td>7.6</td>
<td>2.5 × 10(^5)</td>
<td>2.0 × 10(^{-3})</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>K55A(^{a})</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K55E(^{a})</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
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<tr>
<td>K55Q(^{a})</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
<td>___ ___ ___ ___</td>
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<tr>
<td>R47A(^{b})</td>
<td>1.4 × 10(^5)</td>
<td>2.0 × 10(^{-3})</td>
<td>14.3</td>
<td>1.3 × 10(^5)</td>
<td>1.2 × 10(^{-3})</td>
<td>9.2</td>
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<td>2.4 × 10(^{-3})</td>
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<td>1.9 × 10(^{-3})</td>
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<td>L49A(^{a})</td>
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<td>1.1 × 10(^{-3})</td>
<td>7.3</td>
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<td>3.7</td>
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<td>6.5</td>
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<tr>
<td>L49G(^{b})</td>
<td>1.4 × 10(^5)</td>
<td>1.9 × 10(^{-3})</td>
<td>13.6</td>
<td>1.7 × 10(^5)</td>
<td>1.4 × 10(^{-3})</td>
<td>8.2</td>
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<td>2.5 × 10(^{-3})</td>
<td>11.9</td>
<td></td>
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<tr>
<td>L56A(^{a})</td>
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<td>1.9 × 10(^{-3})</td>
<td>11.2</td>
<td>2.1 × 10(^5)</td>
<td>1.4 × 10(^{-3})</td>
<td>6.7</td>
<td>2.5 × 10(^5)</td>
<td>2.2 × 10(^{-3})</td>
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<tr>
<td>L56G(^{a})</td>
<td>1.7 × 10(^5)</td>
<td>1.9 × 10(^{-3})</td>
<td>11.2</td>
<td>2.1 × 10(^5)</td>
<td>1.4 × 10(^{-3})</td>
<td>6.7</td>
<td>2.5 × 10(^5)</td>
<td>2.2 × 10(^{-3})</td>
<td>8.8</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Material expressed in 293-F cells.

\(^{b}\) Material expressed in CHO cells.

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SDS-PAGE analysis of the MBL oligomerization state

MBL samples were analyzed by SDS-PAGE under nonreducing conditions using Tris-acetate gels containing a 3–8% polyacrylamide gradient. Proteins were transferred to a polyvinylidene difluoride membrane and detected by Western blotting using the mouse monoclonal anti-human MBL Ab Hyb131-01 (AntibodyShop) and aHRP-conjugated polyclonal rabbit anti-mouse Ab (DakoCytomation) as primary and secondary Abs, respectively.

Surface plasmon resonance spectroscopy and data evaluation

Analyses were performed using a Biacore 3000 instrument (Biacore). The MBL variants were diluted to 20 μg/ml in 10 mM sodium acetate (pH 4.0) and immobilized at 4000–7000 resonance units on the surface of CM5 microtiter wells and incubated overnight at 4°C. Wells were washed with 10 mM Tris-HCl, 145 mM NaCl, 5 mM CaCl2, and 0.05% Tween 20 (pH 7.4) and then incubated for 90 min at 37°C with 2 μg/ml complement component C4 purified according to Dodds (28). The wells were washed and a mixture of two biotin-labeled anti-human C4 Abs (162.2 and 162.1 from AntibodyShop) was added. After incubation for 2 h, wells were washed and europium-labeled streptavidin, diluted in 10 mM Tris-HCl, 145 mM NaCl, and 25 μM EDTA (pH 7.4), was added. After incubation for 1 h, wells were washed and the amount of europium in the wells was measured by time-resolved fluorometry. Results are expressed relative to a standard curve obtained by dilution of a standard serum as described by Petersen et al. (29).

Assay of the ability of MBL variants to trigger the lectin pathway of complement

Recombinant MBL variants in 4 mM sodium barbital, 145 mM NaCl, 2 mM CaCl2, and 1 mM MgCl2 (pH 7.4) were mixed with one volume of MBL-deficient serum diluted 1/25 in the same buffer. Samples were added to mannan-coated microtiter wells and incubated overnight at 4°C. Wells were washed with 10 mM Tris-HCI, 145 mM NaCl, 5 mM CaCl2, and 0.05% Tween 20 (pH 7.4) and samples were tested for their ability to deposit C4 fragments onto the mannan-coated surface as described above.

Results

To locate the site(s) of MBL involved in the interaction with the MASPs, a series of recombinant point mutants were produced to analyze their binding properties. Several residues from the collagen-like region of MBL, located on the C-terminal side of the hinge region, were targeted for this purpose. Lys55 appeared as a major candidate because 1) it belongs to the sequence stretch proposed to be involved in MASP binding in rat MBL (23); 2) it does not undergo posttranslational hydroxylation in human MBL (30); 3) it does not undergo posttranslational hydroxylation in human MBL (30; 24), was also selected as a target. Leu50, Pro53, and Leu56 were also subjected to site-directed mutagenesis, mainly to test their implication in a possible hydrophobic component of the interaction, as suggested in the model proposed by Gregory et al. (24). All target residues were mutated to Ala and/or to other amino acids as listed in Table I. The resulting MBL variants were produced by means of two different mammalian expression systems, each known to achieve proper posttranslational modifications of the collagen-like sequence of MBL (4, 13), and purified to homogeneity by affinity chromatography on either glucosamine-Sepharose or N-acetyl-glucosamine-agarose beads as specified in Materials and Methods. All MBL variants were produced at yields comparable to that of the WT.

**FIGURE 1.** Sequence alignment of the collagen-like regions of MBL and the ficolins. Hydroxyproline residues and lysine residues known or predicted to be hydroxylated and glycosylated (23, 30) are shown in red. Residues submitted to point mutation are highlighted in yellow. The residue numbering shown is that of mature human MBL.
protein, except mutant Lys$^{55}$Arg, which was consistently obtained at lower yields.

Analysis of the interaction properties of the MBL variants by surface plasmon resonance spectroscopy

The ability of each MBL variant to associate with the MASPs was analyzed by surface plasmon resonance spectroscopy using immobilized MBL and either MASP-1, MASP-2, MASP-3, or MAp19 as soluble ligands. In contrast to MASP-2 and MAp19, MASP-1 and MASP-3 each contain several N-linked oligosaccharides. Due to the use of a baculovirus/insect cells expression system, these will belong to the high-mannose type and are therefore possible targets for the C-type lectin carbohydrate recognition domain of MBL (31). To prevent this unwanted interaction, binding of MASP-1 and MASP-3 to immobilized MBL was systematically conducted in the presence of excess free mannose as described in Materials and Methods. Comparative binding experiments at varying mannose concentrations indicated that recognition of MASP-1 and MASP-3 through the lectin domain of MBL was prevented in the presence of 10 mM mannose. In contrast, similar kinetic constants for binding of MAp19 to MBL were determined in the presence or absence of mannose (data not shown).

The MASPs and MAp19 each readily associated with immobilized WT MBL in the presence of Ca$^{2+}$ ions, as illustrated by the representative binding curves shown in Fig. 2 (MASP-1, B (MASP-2), and C (MAp19). The kinetic parameters for the interaction with MASP-1, MASP-3, and MAp19 were determined, yielding comparable $K_D$ values for both MBL preparations, ranging from 4.3 to 14.6 nM (Table I), in keeping with previous measurements (18–20). Unfortunately, due to the low amounts of recombiant material available, the binding parameters could not be determined in the case of MASP-2.

Replacement of Lys$^{55}$ by Ala, Gin, or Glu either abolished or strongly inhibited interaction of MBL with the MASPs and MAp19. In the case of mutants Lys$^{55}$Gin and Lys$^{55}$Glu, a faint residual binding to MAp19 could still be measured, but the $K_D$ values increased dramatically, due to decreased $k_{on}$ values (Table I). Conversely, whereas mutation of Lys$^{55}$ to Arg abolished interaction with MASP-2 and MAp19 (Fig. 2, B and C), it did not prevent binding to MASP-1 and MASP-3, with only 1.5- and 2.1-fold increases in $K_D$, resulting from both a decrease in $k_{on}$ and an increase in $k_{off}$ (Fig. 2A and Table I). The Lys$^{55}$Ala mutant showed no detectable interaction with either of the MASPs or MAp19.

Arg$^{47}$ was modified to Ala, Lys, or Glu. The Arg$^{47}$Glu mutant consistently showed a decreased affinity for MASP-1, MASP-3, and MAp19. However, the inhibitory effect was much more pronounced for MASP-1, with a 13-fold increase in $K_D$, resulting from both decreased $k_{on}$ and increased $k_{off}$ values (Table I). In the case of MASP-1 and MASP-3, the $K_D$ values increased 3.0- and 4.0-fold, respectively, and a comparable 2.2-fold increase was observed for the MASP-1/3 CUB$_1$-EGF-CUB$_2$ interaction domain (Table I). Surprisingly, the Arg$^{47}$Lys mutant showed no detectable interaction with either of the MASPs or MAp19.

The Arg$^{47}$Ala mutation had a slight impact on the interaction with MASP-1, as judged from the 2.3-fold increase in the $K_D$ value, but no significant effect on the interactions with MASP-3, the MASP-1/3 CUB$_1$-EGF-CUB$_2$ segment, and MAp19 (Table I).

Leu$^{49}$ and Leu$^{56}$ were mutated to Ala and Gly and to Ala, Gly, and Ser, respectively. As listed in Table I, none of these mutations had a significant effect on the interaction between MBL and either MASP-1, MASP-3, the MASP-1/3 CUB$_1$-EGF-CUB$_2$ segment, or MAp19. Replacement of Pro$^{57}$ by Ala slightly increased the $K_D$ of the interaction with MASP-1 and MASP-3 due to increased $k_{off}$ values, but had no impact on the interaction with MAp19 (Table I).

Effect of MBL mutations on interaction with MASP-2

To further analyze their ability to associate with MASP-2, selected MBL mutants were mixed with recombinant MASP-2, incubated in mannan-coated wells, and bound MBL-MASP-2 complexes were revealed by reaction with an anti-MASP-2 Ab (see Materials and Methods). The amount of MBL bound to the mannan-coated wells was similar for all variants as detected by an anti-MBL Ab. As illustrated in Fig. 3, increasing the concentration of WT MBL resulted in increased MASP-2 binding, and similar binding curves were obtained using point mutants at Leu$^{49}$ and Leu$^{56}$. In contrast, no detectable binding was observed for mutants Lys$^{55}$Ala and Lys$^{55}$Glu, providing additional evidence that both mutations abolished MBL interaction with MASP-2, as also observed by surface plasmon resonance spectroscopy.

Effect of MBL mutations on its ability to trigger activation of the lectin pathway

To further assess the ability of the MBL mutants to form a functional MBL-MASP-2 complex, selected mutants were mixed with a MBL-deficient serum, incubated in mannan-coated wells, and tested for their ability to induce deposition of C4 fragments on the...
mannan-coated surface. Again, in keeping with the above binding experiments, no C4 deposition was detected in the case of mutants Lys55Ala and Lys55Glu (Fig. 4). Mutation Arg47Glu also resulted in a marked decrease in C4-cleaving activity, in agreement with the pronounced inhibition of the MBL-MAp19 interaction observed by surface plasmon resonance spectroscopy (Table I and Fig. 2C). The mutants at positions Leu49 and Leu56, as well as mutants Arg47Ala and Pro53Ala had C4-cleaving activities similar to that of WT MBL.

The functionality of certain MBL variants was further assessed by testing their ability to activate purified C4 upon incubation with MASP-2 using the assay described in Materials and Methods. Again, mutants at positions Leu49 and Leu56 retained a C4-cleaving activity similar to that of WT MBL. In contrast, mutants Lys55Ala, Lys55Glu, and Lys55Arg failed to cleave C4 (data not shown).

**Effect of point mutations on the oligomerization state of MBL**

To check the effect of point mutations on the oligomeric state of the protein, the MBL mutants were submitted to SDS-PAGE analysis. As illustrated in Fig. 5, analysis under nonreducing conditions of mutants Lys55Ala and Lys55Arg yielded ladder-like patterns similar to those characteristic of WT MBL. In each instance, bands with apparent molecular masses of 26, 52, 78, and 156 kDa were observed, with major species at ~235 and 310 kDa corresponding to disulfide-linked trimers and tetramers of the structural unit, respectively, as described previously for serum-derived and recombinant WT MBL (12, 14). Among the different MBL variants produced in the course of this study, only the Arg47Lys mutant yielded a different electrophoretic pattern, with a significant decrease in the proportion of higher oligomers (data not shown). All other mutants yielded patterns similar to the WT protein, indicating that the corresponding mutations had no significant impact on the folding and oligomerization of MBL. Under reducing conditions, all variants, including the Arg47Lys mutant, yielded a single band of apparent molecular mass ~32 kDa, characteristic of the single polypeptide chain of MBL (12, 14).

**Discussion**

The objective of this study was to gain precise information about the amino acid residues of human MBL involved in the interaction with the MASPs. For this purpose, a series of MBL variants targeting residues located on the C-terminal side of the hinge region of the protein were produced using two alternative mammalian expression systems and tested for their ability to associate with the MASPs and MAp19 using surface plasmon resonance spectroscopy and functional assays. Using either expression system, all variants except Lys55Arg were produced at yields comparable to that of WT MBL. In the same way, all mutants could be purified by affinity chromatography on N-acetylglucosamine- or glucosamine-derivatized beads, implying that they essentially retained the carbohydrate-binding properties characteristic of WT MBL. Further analyses by surface plasmon resonance spectroscopy were performed, indicating that all variants tested bound to immobilized mannos-BSA in similar ways (data not shown). This was confirmed by analysis of the binding of the MBL variants to mannan-coated microtiter wells. However, mutant Arg47Lys showed a significantly decreased binding efficiency, consistent with a decreased content in higher oligomers as seen by SDS-PAGE analysis.

The major conclusion from this study is that Lys55 plays an essential role in the association of MBL with its partner serine proteases. As illustrated in Fig. 4, analysis under nonreducing conditions of mutants Lys55Ala and Lys55Arg yielded ladder-like patterns similar to those characteristic of WT MBL. In each instance, bands with apparent molecular masses of 26, 52, 78, and 156 kDa were observed, with major species at ~235 and 310 kDa corresponding to disulfide-linked trimers and tetramers of the structural unit, respectively, as described previously for serum-derived and recombinant WT MBL (12, 14). Among the different MBL variants produced in the course of this study, only the Arg47Lys mutant yielded a different electrophoretic pattern, with a significant decrease in the proportion of higher oligomers (data not shown). All other mutants yielded patterns similar to the WT protein, indicating that the corresponding mutations had no significant impact on the folding and oligomerization of MBL. Under reducing conditions, all variants, including the Arg47Lys mutant, yielded a single band of apparent molecular mass ~32 kDa, characteristic of the single polypeptide chain of MBL (12, 14).
proteases. This is clearly illustrated by the fact that mutation of Lys55 to Ala abolishes interaction with all three MASPs and MAp19 as measured by surface plasmon resonance spectroscopy and prevents formation of enzymatically active MBL/MASP-2 complexes as demonstrated by different functional assays. Nevertheless, the other mutations at Lys55 have differential effects on the interaction properties of MBL. Thus, mutations Lys55Gln and Lys55Glu abolish binding to MASP-1 and MASP-3, but only slightly inhibit interaction with MASP-19. Conversely, replacement of Lys55 by Arg abolishes interaction with MASP-2 and MASP19, but only slightly weakens interaction with MASP-1 and MASP-3, suggesting that, whereas Arg is tolerated at position 55 for binding to MASP-1 and MASP-3, there is a strict requirement for Lys for interaction with MASP-2 and MASP19. In line with the observations by Wallis et al. (23), the above findings provide a strong indication that the MBL binding sites for MASP-2/MASP19 and for MASP-1/3 are structurally similar but not strictly identical.

Our identification of Lys55 as a key player in the interaction of human MBL with its partner proteases is in keeping with the report by Wallis et al. (23) indicating that the MASP binding site of rat MBL involves a sequence stretch surrounding this residue. It should also be emphasized that Lys55 is conserved in all MBL species of known sequence except porcine MBL (Fig. 1). In addition, along with Lys46, Lys55 is one of the two conserved Lys residues of the human MBL collagen-like region that do not undergo posttranslational hydroxylation (23, 30). Thus, contrary to other Lys residues that carry disaccharide moieties, Lys55 is fully accessible and available for an ionic interaction with an acidic residue contributed by the MASPs. Indeed, in support of this hypothesis, preliminary chemical cross-linking experiments based on the use of 1-ethyl-3-(3-(dimethylamino-propyl)carbodiimide (a reagent able to convert salt bridges between carboxyl and amino groups into covalent bonds) provide support for the involvement of a salt bridge at the interface between MBL and MASP-3 (data not shown).

Analysis of the interaction properties of the MBL variants with point mutations at residue Arg47 yields more complex results. Thus, substitution of Glu for Arg has a significant inhibitory effect on the interaction with MASP19, and to a lesser extent, with MASP-1 and MASP-3. These observations, and the fact that the Arg47Glu mutation markedly decreases the ability of MBL to trigger the lectin pathway, suggest at first sight a direct involvement of Arg47 in the interaction with MASP-2. However, the Arg47Ala mutation has very little effect on the interaction with MASP-1, no significant impact on the interaction with MASP-3 and MASP19, and no effect on the ability of MBL to trigger the lectin pathway. Based on these considerations, we propose that Arg47 is likely not directly involved in the MBL-MASP interface, but rather plays an indirect role in the long-range charge attraction between these proteins. Thus, replacement of Arg by Glu would have a repulsive effect, whereas Ala would have little or no impact. Finally, our finding that the Arg47Lys mutant shows no detectable interaction with the MASPs or MASP19 should be interpreted with great caution, considering that this mutant is the only one produced in this study showing an abnormal oligomerization pattern, characterized by a greatly reduced proportion of higher oligomers. Why this particular mutation results in defects in the oligomerization process remains to be elucidated. Nevertheless, because substitution of Lys for Arg at position 47 creates a Xaa-Lys-Gly consensus sequence for hydroxylation (32), it is tempting to hypothesize that subsequent glycosylation of Lys47 generates steric hindrance, thereby impairing oligomerization. At any rate, this side effect, per se, provides a sound explanation for the observed lack of interaction with the MASPs, since lower MBL oligomers are known to be less efficient in this respect than higher oligomers (22, 23, 33). Thus, from our point of view, the lack of interaction of the Arg47Lys mutant as observed in this study has no bearing on the implication of Arg47 at the MBL-MASP interface.

Although mutation of Pro53 to Ala has a slight effect on the interaction of MBL with MASP-1 and MASP-3, it has no detectable impact on the interaction with MAp19 and no effect on MBL ability to trigger activation of the lectin pathway. It appears unlikely, therefore, that Pro53 (which in human MBL is modified to hydroxyproline (23, 30)) contributes directly to the MBL-MASP interaction. Considering that this residue is located in close vicinity of Lys55, the slight effects resulting from the Pro53Ala mutation may arise from local structural modifications within the sequence stretch surrounding Lys55. Finally, none of the mutations performed at Leu49 and Leu56 has a detectable effect on the interaction of MBL with the MASPs and MAp19, nor on its ability to form a functional complex with MASP-2. This possibly reflects the fact that these residues do not contribute to a great extent to the interaction with the MASPs. However, it cannot be excluded that they participate in hydrophobic contacts that are not totally destabilized by the mutations (to Ala, Gly, Ser) performed in this study. Whatever is the role of Leu56 in the MBL-MASP interaction, it is clearly not critical, and therefore it is likely that the lack of binding observed by others (23) for a rat MBL-A mutant, where Lys46-Leu47, the counterpart in rat MBL of the human Lys55-Leu56 stretch, was replaced by Pro-Hyp, was due for the most part to the mutation of Lys46.

In summary, it may be concluded from these experiments that the MASP binding site of MBL involves a sequence stretch centered on Lys55, which lies on the C-terminal side of the hinge region of MBL, about half-way along the collagen-like region of this protein. This particular residue is likely to be directly involved in an ionic bond with an acidic residue contributed by the MASPs, the resulting interaction representing a major component of the MBL/MASP assembly. These findings provide experimental support to the three-dimensional model proposed for the interaction between MBL and MAp19 (24). Finally, it should be emphasized that Lys55 is also conserved in all ficolins of known sequence except pig α-ficolin (Fig. 1). In keeping with our previous finding that mutations on MAp19 have comparable effects on the interaction with MBL and L-ficolin (24), it appears likely therefore that MBL and the ficolins share homologous MASP binding sites. Conversely, it is interesting to note that lung surfactant protein A, a member of the collectin family that has an overall structure similar to that of MBL, but does not interact with the MASPs (34), features an Asn residue at the position corresponding to Lys55 of MBL (Fig. 1).

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Disclosures

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References


