Localization and Characterization of the Mannose-Binding Lectin (MBL)-Associated-Serine Protease-2 Binding Site in Rat Ficolin-A: Equivalent Binding Sites within the Collagenous Domains of MBLs and Ficolins

Umakanth Venkatraman Girija, Alister W. Dodds, Silke Roscher, Kenneth B. M. Reid and Russell Wallis

*J Immunol* 2007; 179:455-462; doi: 10.4049/jimmunol.179.1.455

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Localization and Characterization of the Mannose-Binding Lectin (MBL)-Associated-Serine Protease-2 Binding Site in Rat Ficolin-A: Equivalent Binding Sites within the Collagenous Domains of MBLs and Ficolins

Umakhant Girija, Alister W. Dodds, Silke Roscher, Kenneth B. M. Reid, and Russell Wallis

Ficolins and mannose-binding lectins (MBLs) are the first components of the lectin branch of the complement system. They comprise N-terminal collagen-like domains and C-terminal pathogen-recognition domains (fibrinogen-like domains in ficolins and C-type carbohydrate-recognition domains in MBLs), which target surface-exposed N-acetyl groups or mannose-like sugars on microbial cell walls. Binding leads to activation of MBL-associated serine protease-2 (MASP-2) to initiate complement activation and pathogen neutralization. Recent studies have shown that MASP-2 binds to a short segment of the collagen-like domain of MBL. However, the interaction between ficolins and MASP-2 is relatively poorly understood. In this study, we show that the MASP-2 binding site on rat ficolin-A is also located within the collagen-like domain and encompasses a conserved motif that is present in both MBLs and ficolins. Characterization of this motif using site-directed mutagenesis reveals that a lysine residue in the X position of the Gly-X-Y collagen repeat, Lys56 in ficolin-A, which is present in all ficolins and MBLs known to activate complement, is essential for MASP-2 binding. Adjacent residues also make important contributions to binding as well as to MASP activation probably by stabilizing the local collagen helix. Equivalent binding sites and comparable activation kinetics of MASP-2 suggest that complement activation by ficolins and MBLs proceeds by analogous mechanisms. The Journal of Immunology, 2007, 179: 455-462.

The lectin pathway of complement provides a front-line defense within the immune system by neutralizing pathogens via an Ab-independent mechanism (1). The recognition components of this pathway, serum mannose-binding lectins (MBLs) and ficolins bind directly to surface-exposed carbohydrates and N-acetyl groups on pathogens and activate the complement cascade via MBL-associated serine protease-2 (MASP-2). Complement activation leads to host-mediated lysis and phagocytosis of pathogens. It also stimulates inflammatory and adaptive immune responses via arrays of complement receptors on host cells (2).

The lectin pathway is associated with health and disease on a number of levels. Point mutations in the human MBL gene lead to a common immunodeficiency, which is especially important when the adaptive immune system is immature or compromised, for example, during HIV infection or following chemotherapy (3, 4). The lectin pathway is also important in the pathogenesis of inflammatory disorders, such as cystic fibrosis and rheumatoid arthritis, where variant MBLs are associated with more severe disease (5). Although normally protective, inappropriate lectin pathway activation is sometimes associated with host damage. For example, activation upon reperfusion of tissues following ischemia causes considerable tissue damage (6–8), thereby, exacerbating conditions where blood supply is suboptimal or temporarily blocked.

MBLs and ficolins both consist of oligomers of trimeric subunits, each composed of a collagen-like domain linked to a cluster of three C-terminal pathogen-recognition domains (9, 10): C-type carbohydrate-recognition domains in MBLs and fibrinogen-like domains in ficolins. The carbohydrate-recognition domains of MBLs are joined to the collagen-like domain via an α-helical coiled coil neck which is absent in ficolins. Individual subunits in both families are linked together at the N-terminal ends of polypeptides via disulfide bonds and splay apart either at a break in the Gly-X-Y consensus repeat (called the kink), present in all mammalian MBLs and some ficolins, or at the junction between the short N-terminal domain and the collagen-like domain. Humans produce two serum ficolins designated L- and H-ficolins, together with a leukocyte-associated protein called M-ficolin, whereas rats, mice, and some other mammals produce only two ficolins, ficolins-A and -B, orthologs of human L- and M-ficolin, respectively.

Three MASP (-1, -2, and -3) circulate with MBL and ficolins (11–13). However, MASP-2 alone is sufficient to activate the lectin...
Specific inhibitors of lectin pathway activation have important therapeutic potential for treatment of disorders involving transient ischemia, such as heart and kidney disease. Understanding the interactions of components of this pathway at the molecular level is of great interest not only from a biochemical perspective but also as a basis for rational drug design. Our previous studies therefore indicate that the proline residues in the Y positions are represented as hydroxyproline (O) based on the sequences of rat MBL-A and MBL-C, in which all such residues are at least partially derivatized, except for the proline residue preceding the kink, which is unmodified in each case.

Compared with MBLs, relatively little is known about the interactions between ficolins and MASPs. Alignment of the collagen-like domains reveals that sequences akin to the MASP-binding motif of MBLs are also present in the collagenous domains of most ficolins (Fig. 1). In this study, we demonstrate that a lysine residue (Lys66) within the conserved motif is essential for MASP-2 binding and probably mediates the specificity of the ficolin-MASP interaction. Mutations to adjacent residues within the motif also disrupt binding to different extents, indicating that these residues also play important roles in complement activation.

Materials and Methods

DNA restriction and modifying enzymes were obtained from New England Biolabs or Roche. Tissue culture medium and protein molecular mass markers for SDS-PAGE were purchased from Invitrogen Life Technologies. Sepharose-6B, methotrexate, and N-acetyl glucosamine were obtained from Sigma-Aldrich. Oligonucleotide primers were purchased from Sigma-Aldrich and Eurogentech. Nickel-Sepharose 6 fast flow was purchased from GE Healthcare.

Cloning of rat ficolin-A

The cDNA of rat ficolin-A was amplified from a rat cDNA library using PCR (20). Forward and reverse oligonucleotide primers were: GAAGGCAGGCAATGTTAGTTGCTAGTGTAG and AGAGACTCTTTCCTTCAGCCAGAC. The PCR product was cloned into the polylinker of mammalian expression plasmid pED-4 (21), which contains the dihydrofolate reductase gene as a selectable marker. The resulting plasmid was used to transfect the dihydrofolate reductase deficient, Chinese hamster ovary cell line DXB11, using the calcium phosphate precipitation method (22). We have shown previously that this expression system facilitates expression of plasmid plasmids during biosynthesis, including dihydrofolate reductase formation, hydroxylation of proline, and hydroxylation and glycosylation of lysine residues within the collagen-like domain (22, 23). Protein expression was amplified using increasing concentrations of methotrexate.
(up to 0.5 μM), an inhibitor of dihydrofolate reductase, as described previously (22). Mutant forms of ficolin-A were created by introducing changes to the cDNA using a PCR-based site-directed mutagenesis strategy (20). Mutant cDNAs were cloned into pED-4 in the same way as for the wild-type cDNA.

Protein production and purification

Recombinant proteins were produced from Chinese hamster ovary cells in serum-free medium as described previously (23), and were purified by affinity chromatography on GlcNAc-Sepharose columns. In brief, culture medium (300 ml) containing ficolin-A was diluted with an equal volume of high-salt loading buffer (50 mM Tris-HCl (pH 7.5) containing 25 mM CaCl₂ and 1.25 M NaCl) and loaded onto a GlcNAc-Sepharose column (1 ml) pre-equilibrated with high-salt loading buffer. After washes with high- and low-salt loading buffer (10 ml each; 50 mM Tris-HCl (pH 7.5) containing 25 mM CaCl₂ and 150 mM NaCl), protein was eluted with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 300 mM GlcNAc. Before subsequent characterization, GlcNAc was removed by dialysis.

Catalytically active and inactive forms of rat MASP-2, called MASP-2K and MASP-2A, respectively, were produced by expression in Chinese hamster ovary cells and purified from serum-free medium as described (17, 24). MASP-2A is full-length MASP-2, in which the active site serine residue at position 613 is changed to an alanine. MASP-2A cannot autoactivate, so it is secreted in the zymogen form. MASP-2K is full-length MASP-2 in which the active site residue (Arg424) is replaced by a lysine residue (17). This change reduces the rate of autocatalysis and thereby prevents activation of the zymogen during biosynthesis, secretion, and purification, allowing preparation of pure zymogen. Lectin-MASP-2K complexes autoactivate more slowly than wild-type complexes when bound to a carbohydrate-coated surface to generate catalytically active MASP.

MASP-2 activation kinetics

MASP-2K was incubated alone, with wild-type or mutant ficolin-A in the presence and absence of GlcNAc-Sepharose (5 μl of a 1:1 v/v suspension in a total volume of 30 μl), in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 5 mM CaCl₂, at 37°C with mixing. A 1:2-fold molar excess of ficolin was used to ensure that MASP-2K was fully bound. At various times, aliquots of the suspension were removed from the reaction mix, and proteins were separated by SDS-PAGE under reducing conditions. The amount of MASP activation was quantified from the amount of MASP-2 cleavage by scanning gels using a ChemiGenius (Syngene).

Gel filtration chromatography

Gel filtration chromatography was conducted on a Superdex 200 column (10 mm × 30 cm; GE Healthcare) equilibrated in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl at a flow rate of 0.5 ml/min, at room temperature. Bovine thyroglobulin (8 nm), horse spleen apoferritin (5.9 nm), sweet potato β-amylase (4.15 nm), yeast alcohol dehydrogenase (3.72 nm), BSA (3.52 nm), and bovine carbonic anhydrase (2.39 nm) were used as standards. Values in brackets are the Stoke’s radii of protein standards. The partial specific volume of ficolin-A (0.721 ml/g) was calculated from the ratio of the dissociation and association rate constants

\[ K_{\text{on}} = \frac{(V_e - V_0)}{(V - V_0)} \]

Analytical ultracentrifugation

All experiments were conducted in a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics using an An60Ti rotor. Before setting up the cells, ficolin-A was dialyzed overnight against 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. Experiments were conducted at 40,000 rpm and at 20°C using aluminum centerpieces. Scans were collected at 2-min intervals at 230 nm. Data were analyzed by calculating the sedimentation coefficient distribution c(\(s\)) using the software package SEDFIT (25). Sedimentation coefficients are expressed as \(s_{20,w}\) by correcting for the effects of buffers (26).

Determination of molecular mass of ficolin-A

The molecular masses (M) of ficolin-A oligomers were calculated from their sedimentation coefficients and Stoke’s radii (RS), where N is Avo-
**Results**

*Production and characterization of rat serum ficolin*

To produce pure ficolin-A that was not contaminated by ficolin-B or other serum lectins, protein was produced using a well-characterized mammalian expression system and was purified by affinity chromatography on immobilized GlcNAc columns. Rat ficolin-A has not been characterized before, so to begin with we examined its structure and biochemical properties. Four separate species were detected using gel filtration chromatography (Fig. 2), which eluted from the column as one distinct peak and three partially overlapping peaks, implying that ficolin-A exists as multiple oligomeric forms. Four separate species were also detected by velocity sedimentation ultracentrifugation, enabling measurement of their sedimentation coefficients (Fig. 3). By comparing the shape-independent molecular mass of each oligomer, calculated from its Stokes radius (from gel filtration) and sedimentation coefficient (see equation in Materials and Methods), with the predicted masses of oligomers based on the ficolin-A amino acid sequence (calculated mass ~34 kDa), we concluded that ficolin-A consists of monomers, dimers, trimers, and tetramers of subunits (with three, six, nine, and twelve polypeptide chains, respectively). The proportion of oligomers in fresh protein preparations is shown in Table I. When re-applied to the gel filtration column, individual gel-filtration fractions eluted at their previous positions, revealing that oligomers are stable and do not dissociate into smaller forms upon dilution (data not shown). Further analysis of gel filtration fractions showed that most polypeptides in oligomers are linked together by disulfide bonds, but that the bonding pattern is heterogeneous (Fig. 2B). For example, fraction 1 (Fig. 2A) comprises mainly tetramers of subunits together with smaller amounts of trimers of subunits. However, a ladder of covalently linked polypeptides is observed on SDS-polyacrylamide gels under nonreducing conditions (Fig. 2B), ranging from 1 to 12 polypeptides, implying that although some tetramers are assembled from 12 disulfide-linked polypeptides, others must be formed from combinations of smaller covalently linked structures, associated through non-covalent interactions. Similar heterogeneity has previously been described in both native and recombinant ficolin preparations from a variety of species (27, 28), as well as in some MBLs, including rat MBL-A and -C (22, 23).

To measure the interaction between ficolin-A and MASP-2, ficolin was immobilized on a sensor chip and binding by soluble MASP was monitored, using surface plasmon resonance (Fig. 4). The binding kinetics were complex, probably reflecting the heterogeneous nature of ficolin-A. Nevertheless, reasonable fits were achieved using a two-complex, parallel reaction binding model in which apparent dissociation constants $K_D$ were 5.0 ± 0.5 and 110 ± 10 nM, with association rate constants of 0.6 ± 0.2 and 0.35 ± 0.02 × 10^5 M⁻¹ s⁻¹ and dissociation rate constants of 3.0 ± 0.2 and 3.8 ± 0.1 × 10⁻³ s⁻¹. As expected, binding between ficolin-A and MASP-2 was Ca²⁺-dependent with no binding detected in the presence of EDTA.

Although ficolins are known to activate MASPs when they bind to a suitable substrate, such as a bacterial cell wall or on immobilized GlcNAc, the mechanism of activation is poorly understood. To characterize this process in more detail, the kinetics of ficolin-MASP activation was measured by following autolysis of the MASP-2 polypeptide. As shown in Fig. 5, in the absence of an activating substrate, ficolin-MASP-2K complexes activate no faster thanzymogen MASP-2K alone. However, upon addition of GlcNAc-Sepharose, the rate of MASP-2 cleavage increases markedly. Thus, ficolin-A must trigger complement activation by increasing the rate of MASP autocatalysis, but only when complexes bind to a substrate.

**Characterization of the MASP-binding site in ficolin-A**

Although serum MBLs and ficolins are functionally analogous in several respects, the domains are different, with the exception of the collagen-like domain (Fig. 1). Because the C-terminal portion of the collagen-like domain of MBL mediates binding to MASP-2, we therefore focused on the equivalent portion of ficolin-A, which contains the motif: OGKXGP that is present in all MBLs and ficolins known to activate complement (Fig. 1). To test whether this sequence encompasses the MASP-binding site in ficolin-A, we produced a recombinant protein containing the double substitution

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**Table I. Biophysical properties of ficolin-A oligomers measured by gel filtration chromatography and velocity analytical ultracentrifugation**

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Stoke’s Radius (R$_S$) (Å)</th>
<th>Sedimentation Coefficient (v$_{20,w}$) (S)</th>
<th>Calculated Molecular Mass (kDa)</th>
<th>Total Ficolin-A (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>49 ± 2</td>
<td>4.25 ± 0.12</td>
<td>96 ± 6</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>Dimer</td>
<td>70 ± 3</td>
<td>5.93 ± 0.08</td>
<td>194 ± 10</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>Trimer</td>
<td>82 ± 4</td>
<td>7.85 ± 0.10</td>
<td>299 ± 16</td>
<td>47 ± 6</td>
</tr>
<tr>
<td>Tetramer</td>
<td>94 ± 5</td>
<td>9.46 ± 0.06</td>
<td>412 ± 22</td>
<td>20 ± 1</td>
</tr>
</tbody>
</table>

*From gel filtration chromatography.*
K56P and M57O and compared its properties with those of the wild-type protein. The double mutant failed to bind to MASP-2A under any of the conditions examined (Fig. 4B). As expected from the binding data, it also failed to activate MASP-2 in the presence of GlcNAc-Sepharose (Fig. 5B). Loss of activity was not due to disruption of the collagen-like domain during biosynthesis, because the mutant resembled wild-type ficolin-A, both in terms of its oligomeric and its covalent structure (Fig. 6). Nor could it be explained by loss of carbohydrate binding, because the mutant still bound to GlcNAc-Sepharose, enabling purification by affinity chromatography using the same protocol as for the wild-type protein. Rather, the data suggest that the observed interactions probably reflect MASP-2 binding to immobilized ficolin dimers, trimers, and tetramers.

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aliphatic side chain or by a methionine residue. To examine the effects of replacing the hydrophobic side chain, typically found in this position with a polar or an acidic side chain, we changed Met58 to a serine residue or a glutamic acid residue. Interestingly, recombinant mouse ficolin-B, which naturally has a glutamic acid residue at the position equivalent to Met57 in rat ficolin-A, neither binds to MASP-2 nor activates complement (29).

The mutant ficolins were initially tested for MASP-2-binding (Table II). The K56A mutant failed to bind to the MASP when

FIGURE 4. Binding of MASP-2 to immobilized ficolin-A analyzed by surface plasmon resonance. Comparable amounts of wild-type (A) and K56P, M57O ficolin-A (B) (4489 and 4331 response units, respectively) were immobilized on separate channels of the same sensor chip, and MASP-2A was injected at 0.04, 0.08, 0.16, and 0.33 μM. In A, dotted lines show the fit to a two-complex, parallel reaction-binding model and dashed lines show the global fit to a single component binding model. Residuals to the fits are shown in the panels below. The symbol to the left of the residual plots corresponds to 20 response units. The χ² values were 2.54 and 22.1, respectively. Because ficolin-A consists of four different oligomers, more than two different ficolin-MASP complexes are probably formed on the sensor chip. Nevertheless, reasonable fits were achieved using the relatively simple parallel reaction-binding model, implying either that certain ficolin oligomers bind to MASP-2 only weakly or that different oligomeric forms bind to the MASP with comparable kinetics. It has been shown previously that trimers and tetramers of MBL subunits bind to MASP-2 with similar affinities, dimers bind ~2-fold more weakly whereas single subunits have very low affinities (24). By analogy, we suggest that the observed interactions probably reflect MASP-2 binding to immobilized ficolin dimers, trimers, and tetramers.

FIGURE 5. Kinetics of ficolin-MASP-2 activation analyzed by SDS-PAGE. A, SDS-polyacrylamide gel (4–12% linear gradient gel) of ficolin-MASP-2K complexes, incubated with GlcNAc-Sepharose. Proteins were separated under reducing conditions and were stained with Coomassie blue. The N-terminal fragment of MASP-2K runs as a double band due to differential glycosylation. Activation was measured by quantifying cleavage of the MASP polypeptide. B, Comparison of MASP-2K activation in wild-type and ficolin-A K56P, M57O complexes. There was no detectable difference in activation of zymogen MASP-2K in the presence or absence of GlcNAc-Sepharose.

Detailed characterization of the putative MASP-binding motif has not been conducted previously in either MBLs or ficolins. To determine which residues contribute to MASP-2 binding, an additional set of site-directed mutants was created, in which individual residues were replaced throughout the conserved motif. Sequence alignment shows that Hyp54, Lys56, and Pro59 are strictly conserved in MBLs and in ficolins, therefore, each residue was changed to an alanine residue. With the exception of mouse ficolin-B and porcine ficolin-B, position 58 is occupied either by an

either the MASP or the ficolin was immobilized onto the sensor chip (Fig. 7 and Table II), despite having comparable oligomeric and covalent structures as wild-type ficolin-A (Fig. 6). As expected, it also failed to activate MASP-2 on the GlcNAc-Sepharose substrate (Fig. 8). Thus, Lys<sup>56</sup> must be essential for efficient MASP-2 binding and complement activation, and the basic side chain of the lysine residue probably forms direct contacts with the MASP-2 polypeptide. All of the other ficolin mutants bound to MASP-2 to some extent (Fig. 7 and Table II) and the kinetic data were analyzed using the two complex, parallel reaction model that was used for analysis of wild-type complexes. MASP-2 binding was most impaired in the M57E mutant, in which the $K_d$ values were reduced by ~10- and ~20-fold. Interestingly, much of the differences in binding were due to slower association rates (5- and 8-fold slower compared with the wild-type interactions), implying that electrostatic interactions are important for MASP-2 binding by ficolin-A and that introduction of an acidic side chain near the binding site impairs binding significantly. In contrast, the M57S mutant bound to MASP-2 with similar affinity as wild-type ficolin.

Although ficolins and MASP complexes normally circulate as complexes, the key step in the activation process is when complexes bind to an activating surface. To reproduce this process, we examined ficolin-MASP complex formation on GlcNAc-Sepharose. As seen in Fig. 7B, MASP-2A only bound to GlcNAc-Sepharose through its interactions with ficolin-A. Moreover, the amount of MASP associated with each mutant ficolin correlated well with the affinity of the interactions measured by surface plasmon resonance (Table II), confirming that the kinetic measurements are a reliable measure of the strength of the interactions.

MASP-2 activation by the ficolin-A mutants was generally in line with their MASP-2 binding properties, suggesting that defective activation was a result of reduced binding. Thus, activation by the M57E mutant was reduced by ~8-fold, whereas P59A and O54A mutants were 3- and 2-fold lower than wild-type protein, respectively. These effects cannot be attributed to large conformational changes, because the gel filtration profiles and covalent structures of the proteins were normal (Fig. 6). Nevertheless, a Pro → Ala substitution at the X position, or a Hyp → Ala

![FIGURE 7](image)

**FIGURE 7.** Binding of wild-type and mutant ficolins to MASP-2. A. Surface plasmon resonance of wild-type and mutant ficolins binding to immobilized MASP-2A. Ficolins (at 0.05 mg/ml) were injected sequentially onto the same sensor chip. B. Ficolin-MASP-2A complexes were pelleted on GlcNAc-Sepharose and separated by SDS-PAGE (4–12% linear gradient gel), under reducing conditions. Relative amounts of MASP-2 bound to the ficolin (compared with wild-type complexes) were: K56P, 0.00; O54A—0.69; K56A—0.00; M57E—0.11; M57S—1.09; P59A—0.38, from two separate experiments. MASP marker is the starting amount of MASP-2A used in each experiment. MASP-no ficolin shows the amount of MASP-2A associated with GlcNAc-Sepharose in the absence of ficolin, demonstrating that MASP-2 only binds to GlcNAc-Sepharose through its interaction with ficolin-A.

![FIGURE 8](image)

**FIGURE 8.** MASP-2 activation by wild-type and mutant ficolins. Proteins were incubated with GlcNAc-Sepharose and were separated by gel electrophoresis.

<p>| Table II. Kinetic properties of MASP-2 interactions and MASP-2 activation by wild-type and mutant ficolins |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Ficolin</th>
<th>$k_{on}$&lt;sup&gt;a&lt;/sup&gt; (×10&lt;sup&gt;3&lt;/sup&gt;) M&lt;sup&gt;—1&lt;/sup&gt;s&lt;sup&gt;—1&lt;/sup&gt;</th>
<th>$k_{off}$&lt;sup&gt;a&lt;/sup&gt; (×10&lt;sup&gt;3&lt;/sup&gt;) s&lt;sup&gt;—1&lt;/sup&gt;</th>
<th>$K_d$&lt;sup&gt;a&lt;/sup&gt; (nM)</th>
<th>Relative $K_d$ Values&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Relative MASP-2 Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6.0 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>5.0 ± 0.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>K56P, M57O</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>O54A</td>
<td>5.0 ± 0.6</td>
<td>2.2 ± 0.8</td>
<td>4.7 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.04 ± 0.01</td>
<td>5.2 ± 0.7</td>
<td>153 ± 43</td>
<td>0.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>K56A</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.B.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>M57E</td>
<td>1.0 ± 0.1</td>
<td>4.8 ± 0.5</td>
<td>49.0 ± 10</td>
<td>0.11 ± 0.03</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.03 ± 0.003</td>
<td>9.4 ± 1.9</td>
<td>3230 ± 960</td>
<td>0.039 ± 0.014</td>
<td></td>
</tr>
<tr>
<td>M57S</td>
<td>4.1 ± 1.3</td>
<td>4.0 ± 0.9</td>
<td>116 ± 6</td>
<td>0.62 ± 0.37</td>
<td>0.96 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.37 ± 0.03</td>
<td>4.8 ± 1.2</td>
<td>133 ± 43</td>
<td>0.95 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>P59A</td>
<td>11.3 ± 3.0</td>
<td>6.5 ± 0.1</td>
<td>6.8 ± 2.4</td>
<td>0.87 ± 0.39</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.42 ± 0.2</td>
<td>7.5 ± 1.0</td>
<td>253 ± 41</td>
<td>0.45 ± 0.11</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on fits to a two complex, parallel-binding reaction of MASP-2A binding to immobilized ficolin-A. For each ficolin mutant, most of the binding signal (70–80%) was due to interactions described by the parameter in the lower part of the panel.

<sup>b</sup> N.B., No binding was detected due to weakness of the interaction.
change at the Y position, typically lowers the stability of collagen peptides (30), so the phenotypes of the P59A and O54A mutants might be caused by local destabilization of the collagen helix.

Discussion
Structures of ficolins
The biophysical data show that rat ficolin-A consists of mixtures of oligomers ranging from monomers to tetramers of subunits, each assembled from three identical polypeptide chains. The oligomeric structure is very similar to rat MBL-A, in which dimers, trimers, and tetramers are all capable of activating complement, but monomers have little or no activity (23). In MBL, the relationship between structure and activity can be explained because there are binding sites for two MBL subunits on each MASP-2 dimer (31), so monomers bind to MASPs only weakly and consequently are unlikely to undergo the conformational changes that lead to activation. Given the similarities between the structures of ficolins and MBLs and their MASP-binding sites, described here, it is likely that ficolin oligomers have similar properties to those of MBL oligomers.

Although human L-ficolin is usually described as a tetramer of subunits, smaller oligomers are also detected in serum preparations (32). It is worth noting that dimers, trimers, and tetramers of ficolin subunits have similar Stoke’s radii, due to their asymmetry (Table I), so are likely to coelute on gel filtration columns of low resolution. Furthermore, their sedimentation coefficients are also similar and are unlikely to be resolved using techniques such as gradient ultracentrifugation. Indeed, in this study, the different ficolin oligomers could only be identified from calculations of the Lamell equation solutions $c(s)$, and not by other approaches for determining distributions, including $g(s^*)$ or $I_g(s^*)$. For these reasons, it is likely that ficolin preparations previously described as pure tetramers might also contain some other oligomeric forms.

Equivalent binding sites within the collagenous domains of MBLs and ficolins
The data show that MASPs bind to equivalent sites within the central portions of the collagenous domains of ficolins and MBLs. In particular, a conserved lysine residue (Lys$^{56}$ in ficolin-A) in the X position of the Gly-X-Y collagen repeat is critical for binding and probably forms key contacts with the MASP. Although other adjacent residues might also bind to MASP-2 through main chain interactions, few additional interactions are likely to mediate binding specificity, because elsewhere in the collagen-like domains the sequences are either different in MBLs and ficolins or have been shown to be unimportant for MASP binding. For example, residues N-terminal to the kink or in triplets 6 and 7 of rat MBL-A (Fig. 1), play only minor roles in MASP-2 binding (19). In addition, both the N- and C-terminal portions of the collagenous domains of human MBL and rat MBL-A and -C contain glycosylated-hydroxylsine residues that are likely to block protein-collagen interactions (19). Based on their sequences, derivatized lysine residues are likely to play a similar role in other MBLs and ficolins (Fig. 1).

In theory, Lys$^{56}$ residues in more than one chain of the collagen triple helix could interact with the MASP. Such a binding mechanism is seen in the structure of a triple helical integrin-binding collagen peptide in complex with the integrin $\alpha_2$ domain, in which phenylalanine and arginine residues in two separate chains of the helix within the sequence GFOGER, bind to the integrin (33). The three GFOGER strands adopt a unique orientation in the crystal structure of the complex, probably because of the one-residue stagger of polypeptides in the collagen helix. Interactions with residues in more than one chain, as seen in the integrin-peptide complex, would increase the binding affinity of the interaction, which involves a relatively short segment of collagen helix.

It is of interest to note that C1q also contains sequences similar to the MASP-binding motif, at equivalent positions of all three polypeptide chains (19). For example, the A, B, and C chains of human C1q contain the sequences: OGKVGY, OGKVGP, and OGKNGP, respectively. These sites probably form part of the binding sites for the MASP homologs, C1r and C1s. In vivo, there is no apparent cross-reactivity between C1q and MASPs or between MBLs/ficolins and C1r/C1s, although interactions between MBL and C1r/C1s have been described in vitro (34). Differences in sequences and in protein architectures might both contribute to binding specificities of the recognition and activation components of lectin and classical pathways.

Mouse ficolin-B naturally contains a glutamic acid residue in the position equivalent to Met$^{57}$ in rat ficolin-A (Fig. 1), and the recombinant mouse protein does not bind to MASP-2 or activate complement, despite otherwise having the required sequences for binding (29). Ficolin-B is relatively poorly characterized compared with its serum homolog, so absence of complement activity might be caused by structural differences other than those at the MASP-binding site. However, the data presented here suggest that the glutamate side chain in mouse ficolin-B, is likely to disrupt MASP binding and activation. From this perspective, it will be interesting to examine the activity of native rat ficolin-B, which has a methionine in the equivalent position, so it is predicted to bind to MASP-2 based on our proposed model. Most other MBLs and ficolins contain the full MASP-binding motif. Exceptions are porcine MBL-C and ficolin-A, in which the lysine residue is absent in each case. By contrast, porcine MBL-A contains the standard binding motif, whereas ficolin-B contains the similar sequence: VGGKAGP. As far as we are aware, complement activation by porcine MBLs and ficolins has not been analyzed. However, based on their sequences, we would predict that these proteins either bind to MASP-2 differently from other members of the MBL or ficolin families or that they have lower complement fixing activities than their other mammalian homologs.

Acknowledgment
We thank Wilhelm Schwaeble for providing the rat liver cDNA library.

Disclosures
The authors have no financial conflict of interest.

References


