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Localization and Characterisation of the MASP-2 binding site in rat ficolin-A: Equivalent binding sites within the collagenous domains of Mannose-binding Lectins and Ficolins

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Abstract

Ficolins and mannan-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 neutralisation. 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. Characterisation of this motif using site-directed mutagenesis reveals that a lysine residue in the X position of the Gly-X-Y collagen repeat, (Lys^{56} 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.

INTRODUCTION

The lectin pathway of complement provides a frontline defence within the immune system by neutralizing pathogens via an antibody independent mechanism (1). The recognition components of this pathway, serum mannose-binding lectins (MBLs[1]) 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

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[1]The abbreviations used are:

MBL mannose-binding lectin

MASPMBL-associated serine protease

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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 MASPs (-1, -2 and -3) circulate with MBL and ficolins (11-13). However, MASP-2 alone is sufficient to activate the lectin pathway of complement. Once activated it cleaves C4 and then C4b2 to generate the C3 convertase (C4b2a), the enzyme responsible for activating C3, which is the next component of the pathway (14). MASPs are homologues of C1r and C1s of the classical pathway of complement and comprise two CUB domains (domain found in complement component Clr/Cls, Uegf, and bone morphogenic protein 1) separated by a Ca^{2+}-binding epidermal growth factor-like domain, followed by two complement-control protein modules and a serine protease domain. MASPs are homodimers that circulate as zymogens and only become activated once the lectin component binds to a target surface, such as a bacterial cell (15). Activation leads to cleavage of the MASP polypeptide chain, within a linker, at the N-terminal end of the serine protease domain. MASPs-1 and -2 autoactivate, whereas MASP-3 is activated by an unknown serum protease (16). The roles of MASP-1 and MASP-3 are not known. MASP-1 cleaves C2 but not C4, so might enhance complement activation triggered by lectin·MASP-2 complexes, but cannot initiate activation itself (17). It also possesses thrombin-like activity, so its major role might be within the clotting cascade (18).

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 therefore of great interest not only from a biochemical perspective but also as a basis for rational drug design. Our previous studies have shown that the MASP-binding site in rat MBL-A is located entirely within a portion of the collagenous domain, C-terminal to the hinge (19) (Fig. 1). Specifically, one or both of Lys^{46} and Val^{47} are essential for MASP-binding and complement activation. These residues form part of a short sequence: OGKXGP (where O is hydroxyproline, and X an aliphatic residue other than glycine, or a methionine

residue) that is present in almost all MBLs, and that we have termed the putative MASP-binding motif.

Compared to 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 (Lys^{56}) 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.

MATERIAL AND METHODS

Materials

DNA restriction and modifying enzymes were obtained from New England Biolabs or Roche. Tissue culture medium and protein molecular weight markers for SDS-polyacrylamide gel electrophoresis were purchased from Life Technologies. Sepharose-6B, methotrexate and N-acetyl glucosamine were from Sigma. Oligonucleotide primers were from Sigma and Eurogentech. Ni-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 the polymerase chain reaction (20). Forward and reverse oligonucleotide primers were: GAACTCGAGGCCACCATGTGGTGGCCTATGCTG and AGAGAATTCTTATGATGCCCGGATTTTCAT. Start and stop codons are underlined. The cDNA 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 faithfully carries out essential post-translational modifications of polypeptides during biosynthesis, including disulfide-bond formation, hydroxylation of prolines and hydroxylation and glycosylation of lysines 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 polymerase chain reaction-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. Briefly, 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 $CaCl_2$ and 1.25 M NaCl) and loaded onto a GlcNAc-Sepharose column (1 ml) pre-equilibrated with high-salt loading buffer. After washes with of high- and low-salt loading buffer (10 ml each; 50 mM Tris-HCl, pH 7.5, containing 25 mM $CaCl_2$ and 150 mM NaCl), protein was eluted with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 300 mM GlcNAc. Prior to 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 is secreted in the zymogen form. MASP-2K is full-length MASP-2 in which the arginine residue at the cleavage site for zymogen activation (Arg^{424}) 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 $CaCl_2$, 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-polyacrylamide gel electrophoresis, under reducing conditions. The amount of MASP activation was quantified from the amount of MASP-2 cleavage by scanning gels, using a ChemiGenius, from Syngene.

Gel-filtration chromatography

Gel filtration chromatography was carried out 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), bovine serum albumin (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 Stoke's radii of ficolin-A oligomers were determined from a graph of $[-\log(K_{av})]^{\frac{1}{2}}$ against the Stokes radius of protein standards, where K_{av} is the partition coefficient, V_e is the elution volume of the protein and V_0 and V_t are the void and total volumes of the gel filtration column, respectively.

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)} \quad \text{eqn. 1}$$

Analytical ultracentrifugation

All experiments were carried out in a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics using an An60Ti rotor. Before setting up the cells, ficolin-A was dialysed overnight against 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl. Experiments were carried out at 40,000 rpm and at 20 °C, using aluminium centrepieces. 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 Avogadro's number, η and ρ are the solvent viscosity and density and $\bar{\nu}$ is the partial specific volume of the protein.

$$M = \frac{s_{20,w}.N6\pi.\eta.RS}{\left(1-\bar{\nu}\rho\right)} \quad \text{eqn. 2}$$

The partial specific volume of ficolin-A (0.721 ml/g) was calculated from its amino acid composition as described (26).

Surface plasmon resonance

Measurements were performed using a BIAcore 2000 instrument (BIAcore). Protein ligands were diluted to 25 μg/ml in 10 mM sodium acetate, pH 4.0 and immobilized (4000 - 5000 response units) onto the carboxymethylated dextran surface of a CM5 sensor chip (BIAcore) using amine coupling chemistry (BIAcore amine coupling kit). Binding was measured in 10 mM HEPES pH 7.4, containing 150 mM NaCl and 5 mM $CaCl_2$ at a flow rate of 5 μl/min and at 25 °C. After injection of 20 μl of ligand, the protein surface was regenerated by injection of 10 μl of 10 mM HEPES buffer, pH 7.4, containing 1M NaCl and 5 mM EDTA. Data were analyzed by fitting association and dissociation curves to Langmuir binding models for several protein concentrations simultaneously, using BIAevaluation 4.1 software (BIAcore). Increasingly complex models were tested until a satisfactory fit to the data was achieved. Apparent equilibrium dissociation constants (K_D) were calculated from the ratio of the dissociation and association rate constants (k_{off}/k_{on}).

Ficolin·MASP complex formation on GlcNAc-Sepharose

MASP-2A was incubated alone, with wild-type or mutant ficolin-A in the presence of GlcNAc-Sepharose (5 μl of a 1:1 *v/v* suspension in a total volume of 30 μl) in 10 mM HEPES pH 7.4 containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.005% (v/v) Surfactant P20. After incubation at 25 °C for 15 minutes, the GlcNAc-Sepharose was pelleted and was washed 5 times using 300 μl of buffer, before resuspension in gel loading buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis. The amount of MASP and ficolin retained with the GlcNAc-Sepharose pellet were quantified by scanning their band intensities using a ChemiGenius, from Syngene.

RESULTS

Production and characterization of rat serum ficolin

In order to produce pure ficolin-A that was not contaminated by ficolin-B or other serum lectins, protein was produced using a well-characterised mammalian expression system and was purified by affinity chromatography on immobilized GlcNAc columns. Rat ficolin-A has not been characterised 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 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 (Eqn. 2. in Experimental Procedures), with the predicted masses of oligomers based on the ficolin-A amino acid sequence (calculated mass ~ 34 kDa), we conclude 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

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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 2.B). 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 non-reducing conditions (Fig. 2B), ranging from 1 to twelve polypeptides, implying that while 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).

In order 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_{D1} and K_{D2} were 5.0 ± 0.5 and 110 ± 10 nM, with association rate constants of 6.0 ± 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 Ca^{2+}-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. In order 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 than zymogen 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 if this sequence encompasses the MASP-binding site in ficolin-A, we produced a recombinant protein containing the double substitution 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 Lys^{56} and/or Met^{57} bind to MASP-2, and that these interactions are essential for efficient MASP activation. In rat MBL, equivalent mutations (K46P and V47O) also abolished MASP-2 binding and complement activation (19), so the MASP recognizes comparable sequences within the collagen-like domains of rat ficolin-A and MBL-A.

Detailed characterisation of the putative MASP-binding motif has not been carried out previously in either MBLs or ficolins, so in order 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 Hyp^{54}, Lys^{56} and Pro^{59} are strictly conserved in MBLs and in ficolins, so 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 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 Met^{58} 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 Met^{57} 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 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^{56} 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 analysed 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_Ds were reduced by ~10- and ~20-fold. Interestingly, much of the differences in binding were due to slower associations rates (5- and 8-fold slower compared to 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. By contrast, the M57S mutant bound to MASP-2 with similar affinity as wild-type ficolin.

Whilst ficolins and MASP complexes normally circulate as complexes, the key step in the activation process is when complexes bind to an activating surface. In order to reproduce this process, we examined ficolin·MASP complex formation on GlcNAc-Sepharose. As seen in Figure 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 can not 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 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 destabilisation 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 co-elute 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 Lamm equation solutions *c(s)*, and not by other approaches for determining distributions, including *g(s*)* or *ls-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 mannose-binding lectins 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. Whilst other adjacent residues might also bind to MASP-2 through main chain contacts, 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 hinge 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 MBLs-A and -C contain glycosylated-hydroxylysine 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 α2-I 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 C1qs also contain 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 homologues, 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 M^{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 characterised compared to its serum homologue, 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 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, while ficolin-B contains the similar sequence: VGKAGP. As far as we are aware, complement activation by porcine MBLs and ficolins has not been analysed. 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 homologues.

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REFERENCES

1. Turner MW. Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunology Today. 1996; 17:532–540. [PubMed: 8961631]
2. Carroll MC. The complement system in regulation of adaptive immunity. Nat. Immunol. 2004; 5:981–986. [PubMed: 15454921]
3. Garred P, Madsen HO, Balslev U, Hofmann B, Pedersen C, Gerstoft J, Svejgaard A. Susceptibility to HIV infection and progression of AIDS in relation to varient alleles of mannose-binding lectin. Lancet. 1997; 349:236–240. [PubMed: 9014910]
4. Neth O, Hann I, Turner MW, Klein NJ. Deficiency of mannose-binding lectin and burden of infection in children with malignancy: a prospective study. The Lancet. 2001; 358:614–618. [PubMed: 11530147]
5. Kilpatrick DC. Mannan-binding lectin: clinical significance and applications. Biochim. Biophys. Acta. 2002; 1572:401–413. [PubMed: 12223282]
6. Hart ML, Ceonzo KA, Shaffer LA, Takahashi K, Rother RP, Reenstra WR, Buras JA, Stahl GL. Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q. J. Immunol. 2005; 174:6373–6380. [PubMed: 15879138]
7. Moller-Kristensen M, Wang W, Ruseva M, Thiel S, Nielsen S, Takahashi K, Shi L, Ezekowitz A, Jensenius JC, Gadjeva M. Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. Scand. J. Immunol. 2005; 61:426–434. [PubMed: 15882434]

8. Walsh MC, Bourcier T, Takahashi K, Shi L, Busche MN, Rother RP, Solomon SD, Ezekowitz RA, Stahl GL. Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. J. Immunol. 2005; 175:541–546. [PubMed: 15972690]
9. Fujita T, Matsushita M, Endo Y. The lectin-complement pathway - its role in innate immunity and evolution. Immunol. Rev. 2004; 198:185–202. [PubMed: 15199963]
10. Garlatti V, Belloy N, Martin L, Lacroix M, Matsushita M, Endo Y, Fujita T, Fontecilla-Camps JC, Arlaud GJ, Thielens NM, Gaboriaud C. Structural insights into the innate immune recognition specificities of L- and H-ficolins. Embo J. 2007; 26:623–633. [PubMed: 17215869]
11. Dahl MD, Thiel S, Matsushita M, Fujita T, Willis AC, Christensen T, Vorup-Jensen T, Jensenius JC. MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. Immunity. 2001; 15:127–135. [PubMed: 11485744]
12. Sato T, Endo Y, Matsushita M, Fujita T. Molecular characterization of a novel serine protease involved in activation of the complement system by mannose-binding protein. International Immunology. 1994; 6:665–669. [PubMed: 8018603]
13. Thiel S, Vorup-Jensen T, Stover CM, Schwaeble W, Laursen SB, Poulsen K, Willis AC, Eggleton P, Hansen S, Holmskov U, Reid KBM, Jensenius JC. A second serine protease associated with mannan-binding lectin that activates complement. Nature. 1997; 386:506–510. [PubMed: 9087411]
14. Wallis R, Dodds AW, Mitchell DA, Sim RB, Reid KB, Schwaeble WJ. Molecular Interactions between MASP-2, C4, and C2 and Their Activation Fragments Leading to Complement Activation via the Lectin Pathway. J. Biol. Chem. 2007; 282:7844–7851. [PubMed: 17204478]
15. Wallis R, Dodd RB. Interaction of mannose-binding protein with associated serine proteases: effects of naturally occurring mutations. J. Biol. Chem. 2000; 275:30962–30969. [PubMed: 10913141]
16. Zundel S, Cseh S, Lacroix M, Dahl MR, Matsushita M, Andrieu JP, Schwaeble WJ, Jensenius JC, Fujita T, Arlaud GJ, Thielens NM. Characterization of recombinant mannan-binding lectin-associated serine protease (MASP)-3 suggests an activation mechanism different from that of MASP-1 and MASP-2. J. Immunol. 2004; 172:4342–4350. [PubMed: 15034049]
17. Chen CB, Wallis R. Two mechanisms for mannose-binding protein modulation of the activity of its associated serine proteases. J. Biol. Chem. 2004; 279:26058–26065. [PubMed: 15060079]
18. Presanis JS, Hajela K, Ambrus G, Gal P, Sim RB. Differential substrate and inhibitor profiles for human MASP-1 and MASP-2. Mol. Immunol. 2004; 40:921–929. [PubMed: 14725788]
19. Wallis R, Shaw JM, Uitdehaag J, Chen CB, Torgersen D, Drickamer K. Localization of the serine protease-binding sites in the collagen-like domain of mannose-binding protein: indirect effects of naturally occurring mutations on protease binding and activation. J. Biol. Chem. 2004; 279:14065–14073. [PubMed: 14724269]
20. Sambrook, J.; Fritsch, EF.; Manatis, T. Book Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 1989. Cold Spring Harbor Laboratory Press, Vol. Editor, ed^edsPages
21. Kaufman RJ, Davies MV, Wasley LC, Michnick D. Improved vectors for stable expression of foriegn genes in mammalian cells by use of the untranslated leader sequence from EMC virus. Nucleic Acids Research. 1991; 19:4485–4490. [PubMed: 1653417]
22. Wallis R, Drickamer K. Asymmetry adjacent to the collagen-like domain in rat liver mannose-binding protein. Biochem. J. 1997; 325(Pt 2):391–400. [PubMed: 9230118]
23. Wallis R, Drickamer K. Molecular determinants of oligomer formation and complement fixation in mannose-binding proteins. J. Biol. Chem. 1999; 274:3580–3589. [PubMed: 9920905]
24. Chen CB, Wallis R. Stoichiometry of complexes between mannose-binding protein and its associated serine proteases. Defining functional units for complement activation. J. Biol. Chem. 2001; 276:25894–25902. [PubMed: 11337510]
25. Brown PH, Schuck P. Macromolecular size-and-shape distributions by sedimentation velocity analytical ultracentrifugation. Biophys. J. 2006; 90:4651–4661. [PubMed: 16565040]
26. Laue, TM.; Bhairavi, DS.; Ridgeway, TM.; Pelletier, SL.; Harding, SE.; Rowe, AJ. Analytical Ultracentrifugation in Biochemistry and Polymers Science. Horton, JC., editor. Royal Society of Chemistry; 1992. p. 90-125.

27. Hummelshoj T, Thielens NM, Madsen HO, Arlaud GJ, Sim RB, Garred P. Molecular organization of human Ficolin-2. Mol. Immunol. 2007; 44:401–411. [PubMed: 16595153]

28. Ohashi T, Erickson HP. Oligomeric structure and tissue distribution of ficolins from mouse, pig and human. Arch. Biochem. Biophys. 1998; 360:223–232. [PubMed: 9851834]

29. Endo Y, Nakazawa N, Liu Y, Iwaki D, Takahashi M, Fujita T, Nakata M, Matsushita M. Carbohydrate-binding specificities of mouse ficolin A, a splicing variant of ficolin A and ficolin B and their complex formation with MASP-2 and sMAP. Immunogenetics. 2005; 57:837–844. [PubMed: 16328467]

30. Persikov AV, Ramshaw JAM, Kirkpatrick A, Brodsky B. Amino acid propensities for the collagen triple helix. Biochemistry. 2000; 39:14960–14967. [PubMed: 11101312]

31. Feinberg H, Uitdehaag JC, Davies JM, Wallis R, Drickamer K, Weis WI. Crystal structure of the CUB1-EGF-CUB2 region of mannose-binding protein associated serine protease-2. Embo J. 2003; 22:2348–2359. [PubMed: 12743029]

32. Krarup A, Thiel S, Hansen A, Fujita T, Jensenius JC. L-ficolin is a pattern recognition molecule specific for acetyl groups. J. Biol. Chem. 2004; 279:47513–47519. [PubMed: 15331601]

33. Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by integrin alpha2beta1. Cell. 2000; 101:47–56. [PubMed: 10778855]

34. Lu J, Thiel S, Wiedemann H, Timpl R, Reid KBM. Binding of the pentamer/hexamer forms of a mannan-binding protein to zymosan activates the proenzyme $C1r_2C1s_2$ complex of the classical pathway of complement, without involvement of C1q. J. Immunol. 1990; 144:2287–2294. [PubMed: 2313094]

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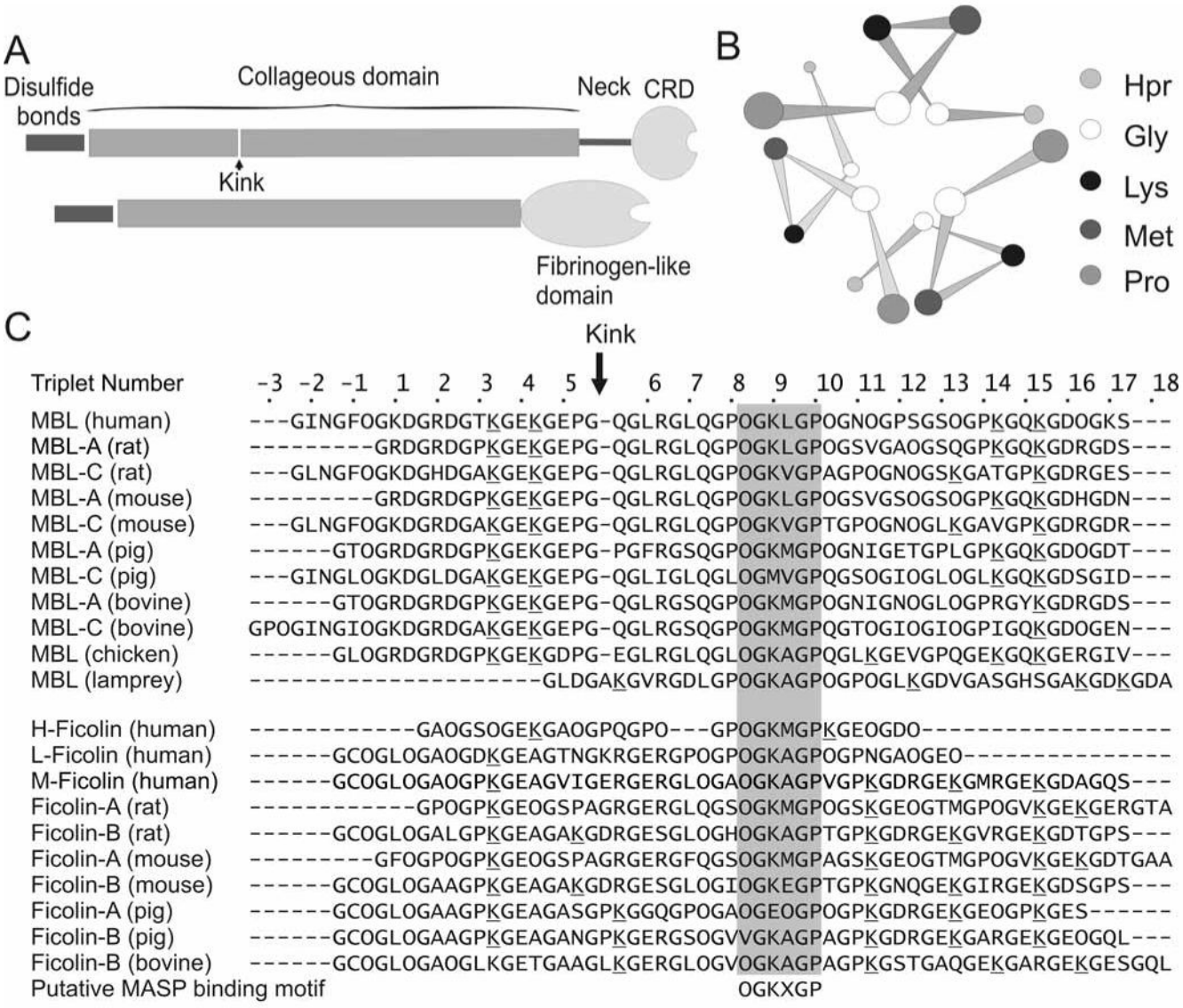

Figure 1. Aligned sequences of the collagenous domains of MBLs and ficolins

A, Domain organization of MBLs (top) and ficolins (bottom). The carbohydrate-recognition domain of MBL is labeled as *CRD*. All mammalian MBLs, but only certain ficolins have an interruption or kink within the Gly-X-Y repeat of the collagen-like domain. B, Collagen helical wheel of the putative MASP-binding motif. C, Aligned sequences of the collagen-like domains of MBLs and ficolins. Numbering of triplets is based on the sequence of rat MBL-A. The putative MASP-binding motif is shaded. Lysine residues within the Y position of the Gly-X-Y repeat are underlined. All such residues are at least partially hydroxylated and glycosylated in rat MBL-A and MBL-C (22, 23). Most of 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 immediately preceding the kink, which is unmodified in each case.

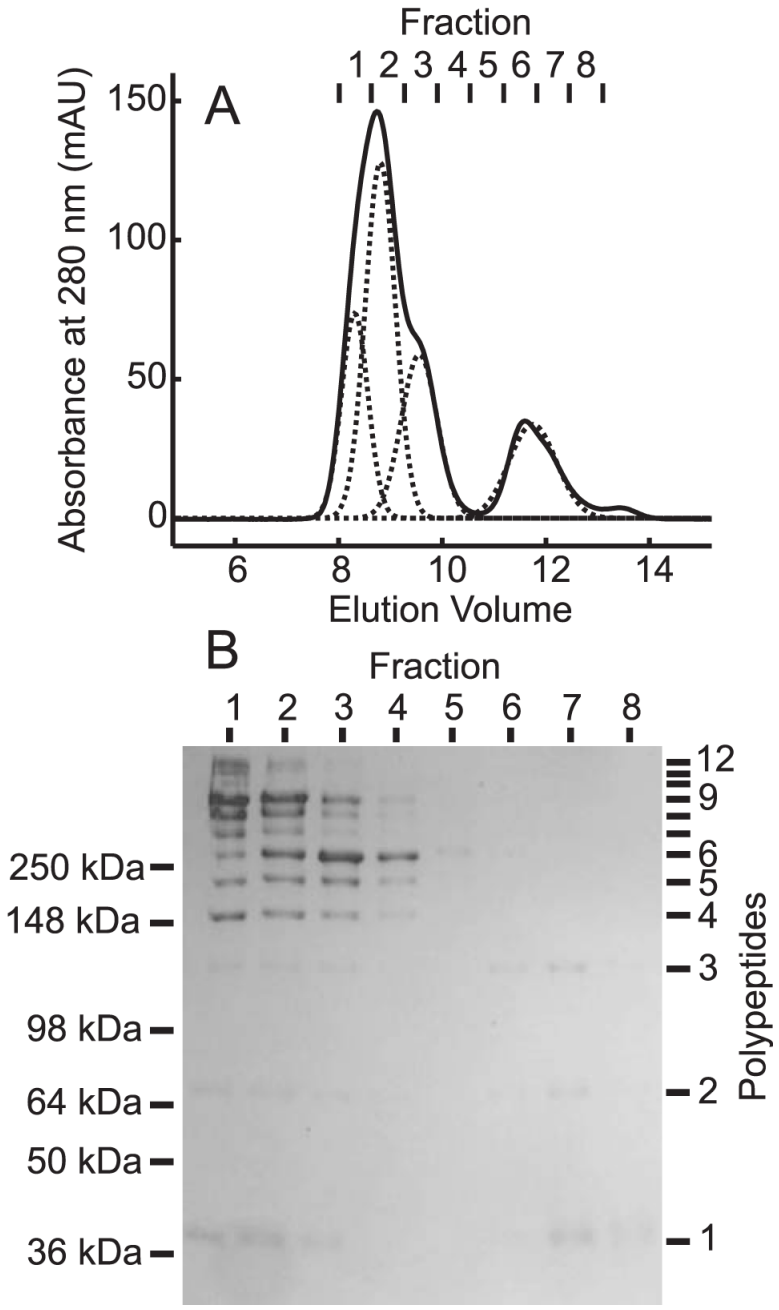

Figure 2. Gel filtration and SDS-polyacrylamide gel electrophoresis of recombinant ficolin-A

A, Gel filtration elution profile of purified rat ficolin-A. Data were fitted to Gaussian curves, using the software Origin (Microcal). The best fit was achieved with four curves, which are shown by *dotted lines*. Addition of extra parameters did not improve the fits significantly. *B*, SDS-polyacrylamide gel (4-12% linear gradient gel) under non-reducing conditions. Proteins were stained with Coomassie blue. Numbers above the gel correspond to elution fractions from the gel filtration column (in *A* above).

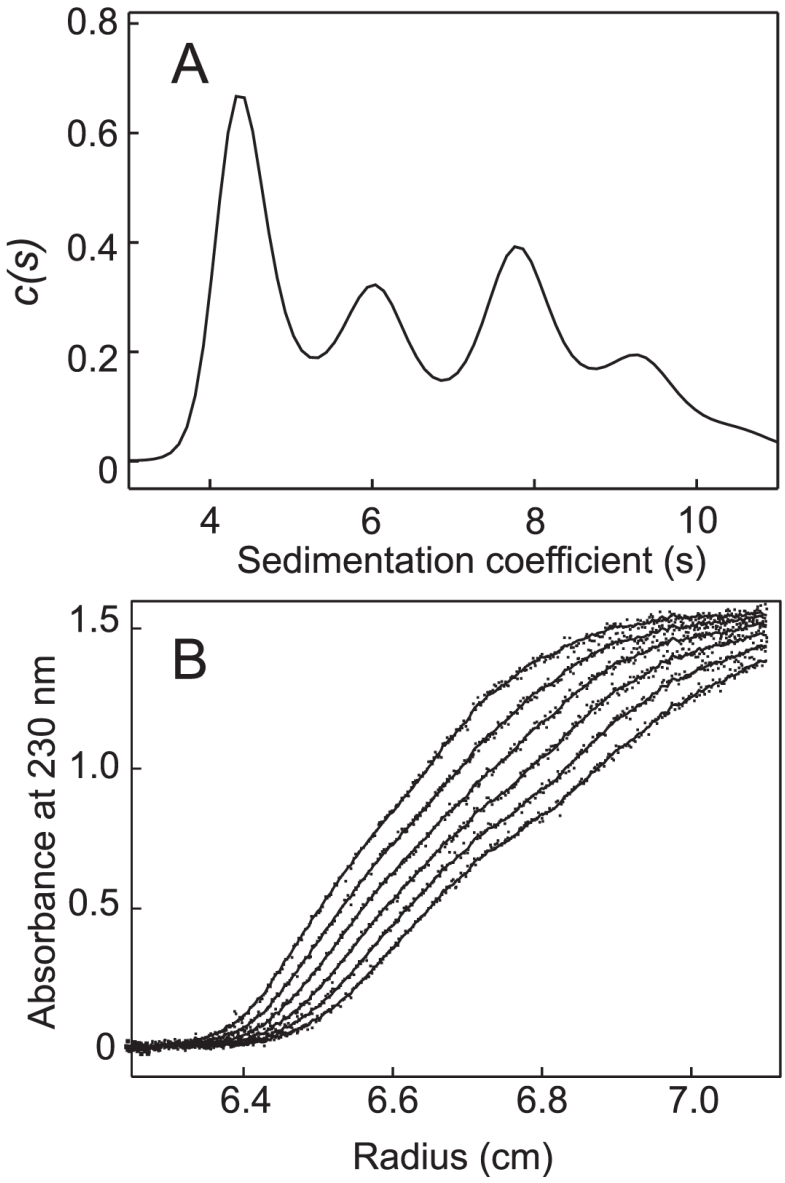

Figure 3. Velocity sedimentation ultracentrifugation of ficolin-A

A, Distribution of sedimenting species *c(s)* analysed using the software package SEDFIT. Minor differences in the proportions of ficolin oligomers from those detected by gel filtration (in Fig. 2) are probably due to differences in the extinction coefficients of oligomers at 280 nm (gel filtration) and 230 nm (sedimentation). *B*, Example of data from five absorbance scans. Solid lines show the global fit to the data.

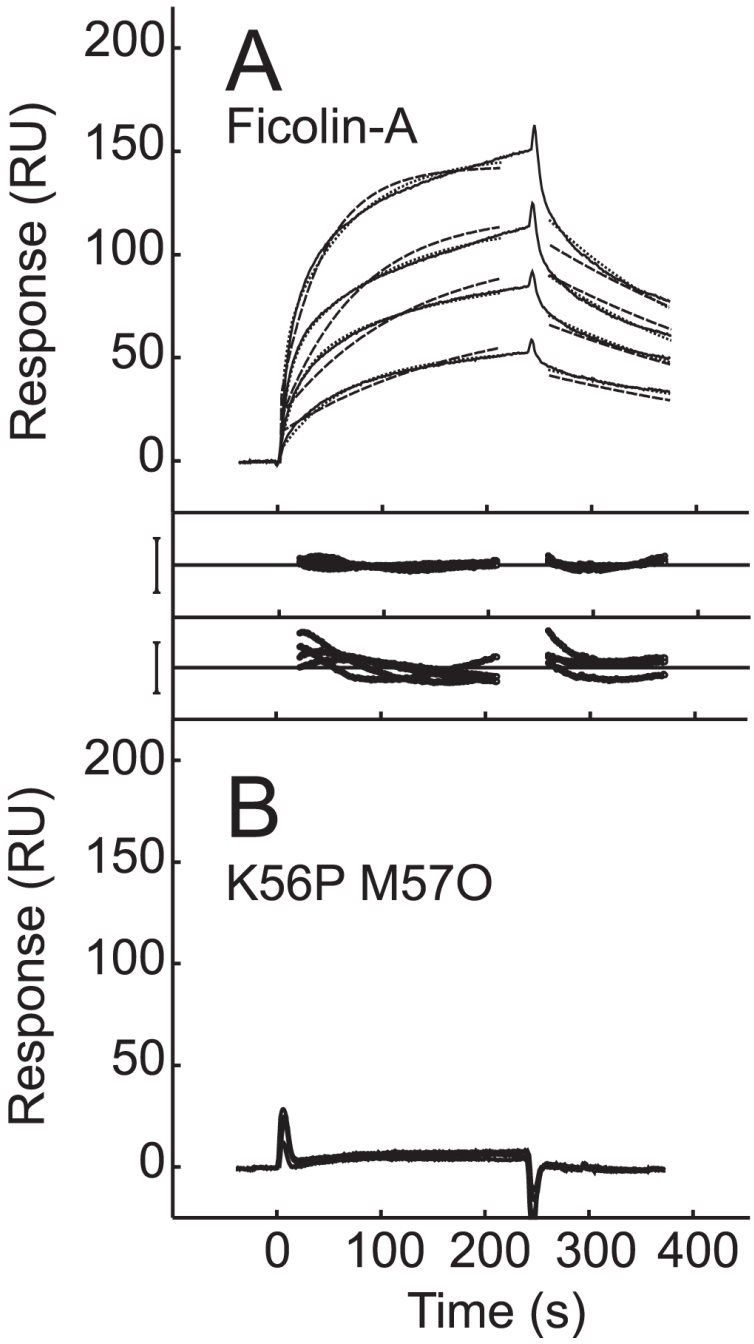

Figure 4. Binding of MASP-2 to immobilised ficolin-A analysed by surface plasmon resonance

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 Chi-squared 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.

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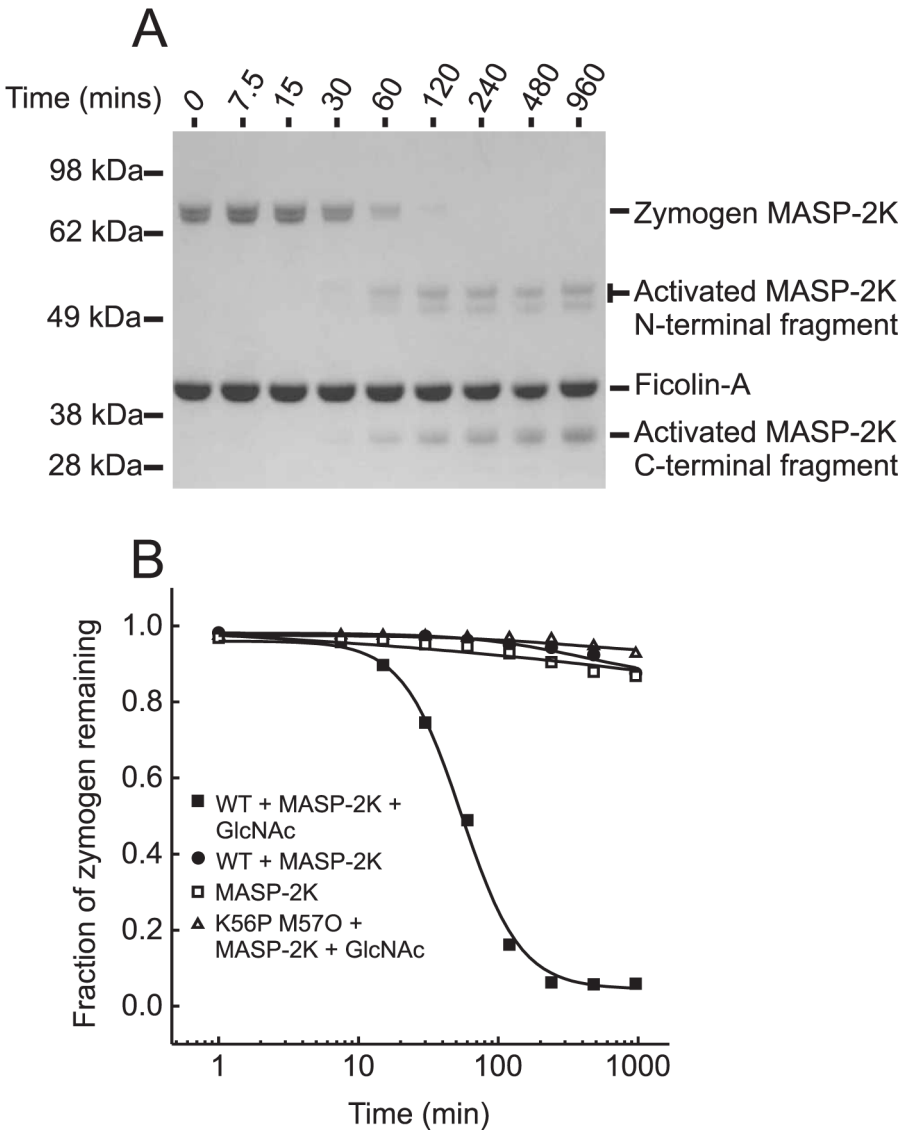

Figure 5. Kinetics of ficolin·MASP-2 activation analysed by SDS-polyacrylamide gel electrophoresis

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.

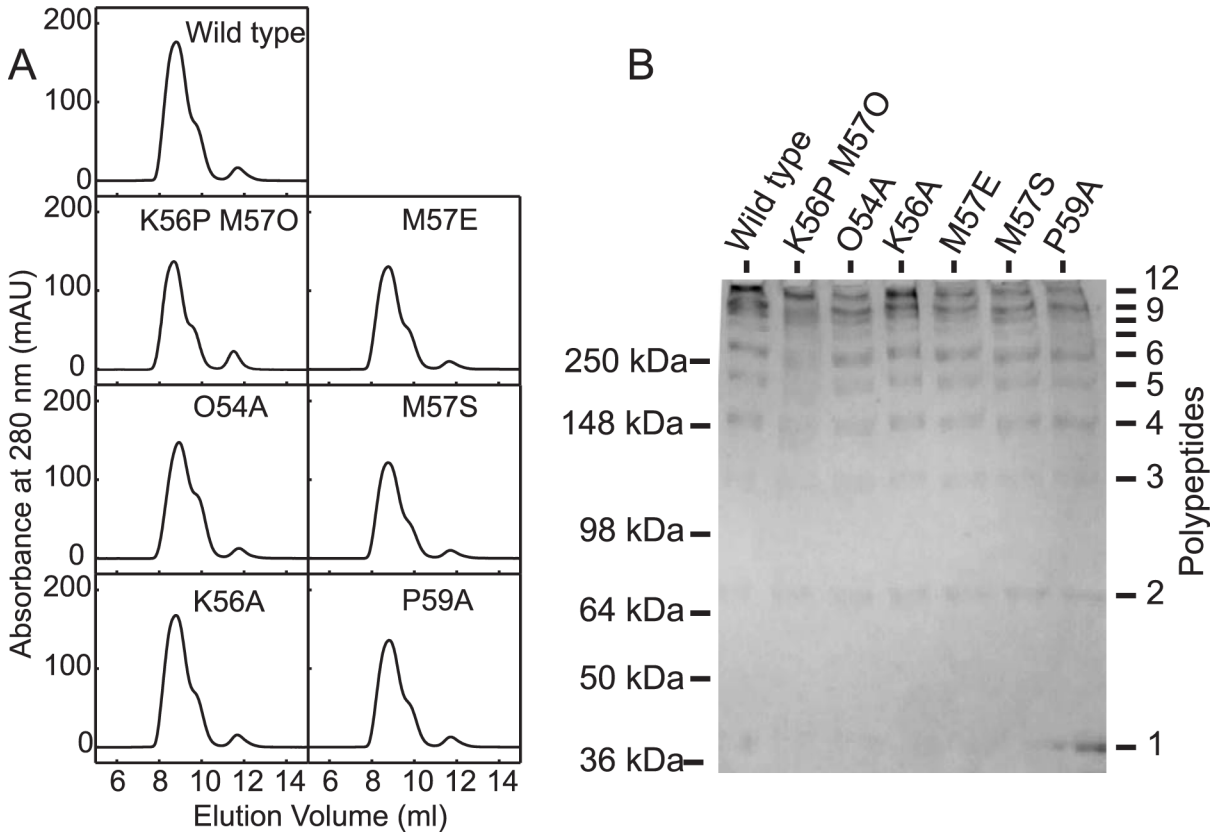

Figure 6. Biophysical properties of ficolin-A mutants

A, Gel filtration of wild type and mutant ficolins. *B*, Covalent structure of ficolin-A mutants by SDS-polyacrylamide gel electrophoresis, under non-reducing conditions.

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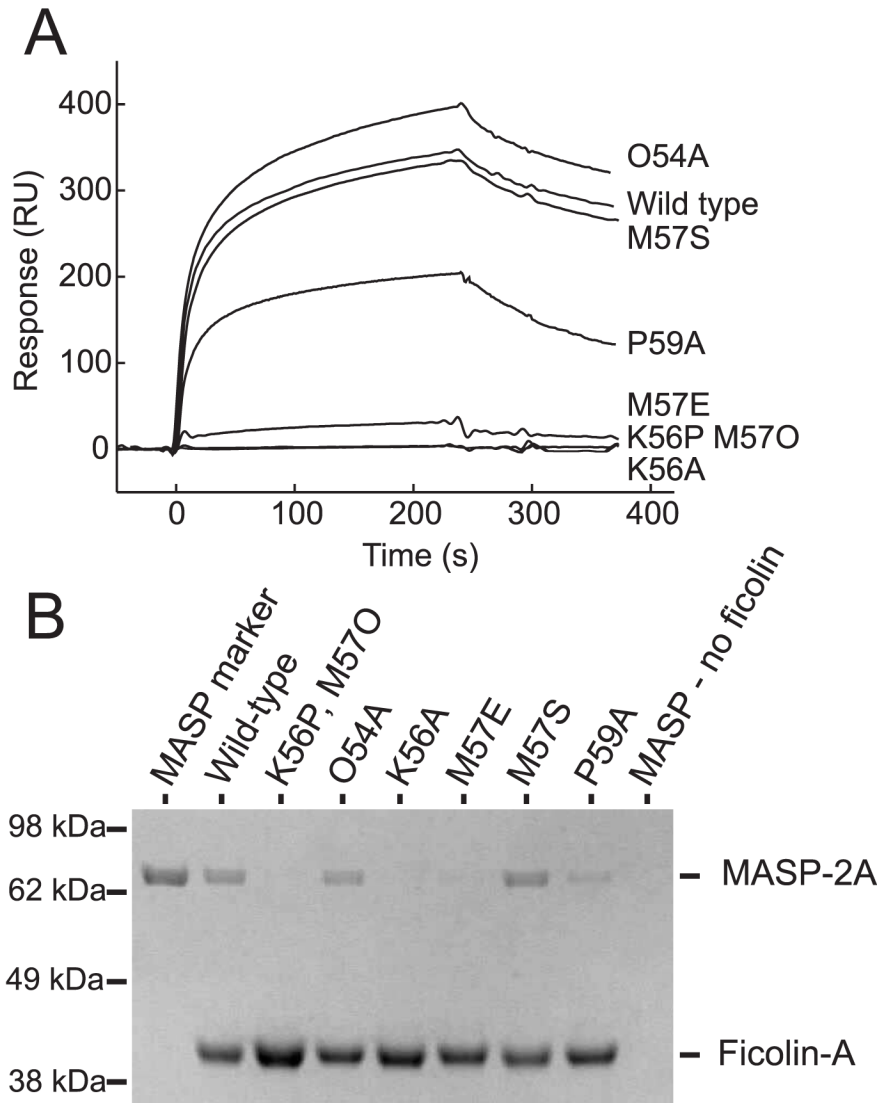

Figure 7. Binding of wild-type and mutant ficolins to MASP-2

A, Surface plasmon resonance of wild-type and mutant ficolins binding to immobilized MASP-2A. Ficolins (at 0.05 mg/ml) were injected sequentially on to the same sensor chip. *B*, Ficolin·MASP-2A complexes were pelleted on GlcNAc-Sepharose and separated by SDS-polyacrylamide gel electrophoresis (4-12% linear gradient gel), under reducing conditions. Relative amounts of MASP-2 bound to the ficolin (compared to wild-type complexes) were: K56P, M57O - 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.

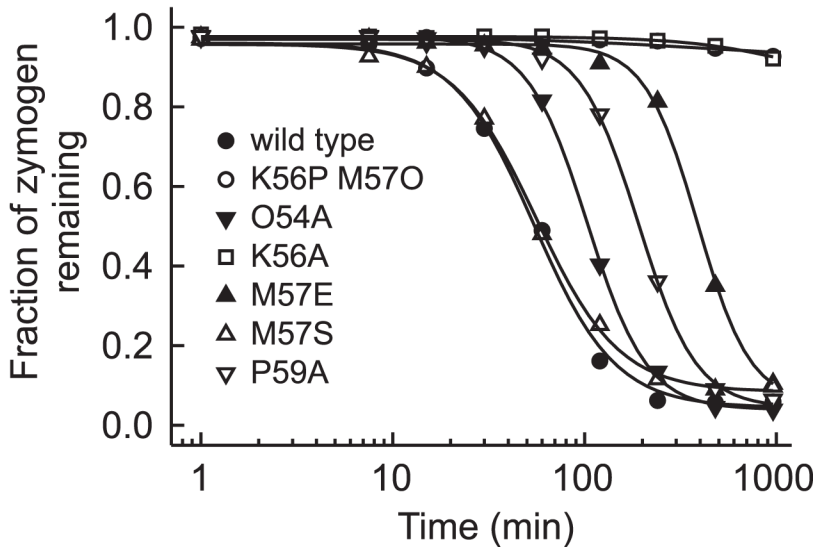

Figure 8. MASP-2 activation by wild-type and mutant ficolins

Proteins were incubated with GlcNAc-Sepharose and were separated by gel electrophoresis.

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Table I

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

Subunits	Stoke's Radius (*RS*)	Sedimentation coefficient ($s_{20,w}$)	Calculated molecular mass	% of total ficolin-A[a]
	(Å)	S	k Da	%
Monomer	49 ± 2	4.25 ± 0.12	96 ± 6	10 ± 6
Dimer	70 ± 3	5.93 ± 0.08	194 ± 10	24 ± 1
Trimer	82 ± 4	7.85 ± 0.10	299 ± 16	47 ± 6
Tetramer	94 ± 5	9.46 ± 0.06	412 ± 22	20 ± 1

[a]From gel filtration chromatography

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Table II

Kinetic properties of MASP-2 interactions and MASP-2 activation by wild type and mutant ficolins

Ficolin	k_{on} [a]	k_{off} [a]	K_D [a]	Relative K_{DS} [a]	Relative MASP-2 activation
	(× 10^{-5}) $M^{-1}s^{-1}$	**(× 10^{3}) s^{-1}**	**nM**		
Wild type	6.0 ± 0.2 0.35 ± 0.02	3.0 ± 0.2 3.8 ± 0.1	5.0 ± 0.5 110 ± 10	1.0 1.0	1.0
K56P, M57O	N.B.[b]	N.B.[b]	N.B.[b]	N.B.[b]	≪0.05
O54A	5.0 ± 0.6 0.40 ± 0.01	2.2 ± 0.8 5.2 ± 0.7	4.7 ± 0.5 153 ± 43	1.1 ± 0.2 0.8 ± 0.3	0.53 ± 0.03
K56A	N.B.[b]	N.B.[b]	N.B.[b]	N.B.[b]	≪0.05
M57E	1.0 ± 0.1 0.03 ± 0.003	4.8 ± 0.5 9.4 ± 1.9	49.0 ± 10 3230 ± 960	0.11 ± 0.03 0.039 ± 0.014	0.14 ± 0.01
M57S	4.1 ± 1.3 0.37 ± 0.03	4.0 ± 0.9 4.8 ± 1.2	11.6 ± 6 133 ± 43	0.62 ± 0.37 0.95 ± 0.39	0.96 ± 0.03
P59A	11.3 ± 3.0 0.42 ± 0.2	6.5 ± 0.1 7.5 ± 1.0	6.8 ± 2.4 253 ± 41	0.87 ± 0.39 0.45 ± 0.11	0.29 ± 0.02

[a] 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.

[b] No binding was detected due to weakness of the interaction.