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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 14, pp. 10546–10552, April 2, 2010

Engineering Novel Complement Activity into a Pulmonary Surfactant Protein*

Received for publication, December 21, 2009, and in revised form, January 21, 2010 Published, JBC Papers in Press, January 29, 2010, DOI 10.1074/jbc.M109.097493

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Complement neutralizes invading pathogens, stimulates inflammatory and adaptive immune responses, and targets non- or altered-self structures for clearance. In the classical and lectin activation pathways, it is initiated when complexes composed of separate recognition and activation subcomponents bind to a pathogen surface. Despite its apparent complexity, recognition-mediated activation has evolved independently in three separate protein families, C1q, mannose-binding lectins (MBLs), and serum ficolins. Although unrelated, all have bouquet-like architectures and associate with complement-specific serine proteases: MBLs and ficolins with MBL-associated serine protease-2 (MASP-2) and C1q with C1r and C1s. To examine the structural requirements for complement activation, we have created a number of novel recombinant rat MBLs in which the position and orientation of the MASP-binding sites have been changed. We have also engineered MASP binding into a pulmonary surfactant protein (SP-A), which has the same domain structure and architecture as MBL but lacks any intrinsic complement activity. The data reveal that complement activity is remarkably tolerant to changes in the size and orientation of the collagenous stalks of MBL, implying considerable rotational and conformational flexibility in unbound MBL. Furthermore, novel complement activity is introduced concurrently with MASP binding in SP-A but is uncontrolled and occurs even in the absence of a carbohydrate target. Thus, the active rather than the zymogen state is default in lectin·MASP complexes and must be inhibited through additional regions in circulating MBLs until triggered by pathogen recognition.

Complement is a central part of the immune system that neutralizes pathogens via antibody-dependent and -independent mechanisms and stimulates a variety of protective responses including phagocytosis, inflammation, and adaptive immunity (1). It is triggered via three routes called the classical, lectin, and alternative pathways. The classical and lectin pathways both selectively target pathogen-associated molecular patterns via circulating complexes composed of recognition and zymogen protease subcomponents (2). Upon binding to a target, conformational changes in the recognition subcomponent trigger activation of the protease subcomponent, which in turn activates the downstream complement cascade. In the classical pathway, C1q binds to microorganisms, immune complexes, apoptotic and necrotic cells as well as amyloids to initiate the stepwise activation of C1r and C1s. In the lectin pathway, mannose-binding lectin (MBL)[5] and serum ficolins bind to terminal mannose-like epitopes or *N*-acetyl groups on pathogens to activate MBL-associated serine protease-2 (MASP-2).

Initiating complexes of the classical and lectin pathways have a number of properties in common (2). Although unrelated, recognition subcomponents have similar domain organizations consisting of a short cysteine-containing domain followed by a collagen-like domain linked to a C-terminal recognition domain (Fig. 1) (3). During biosynthesis, polypeptides associate into trimeric subunits (4), which in turn assemble to form larger oligomers resembling bouquets (5, 6). In C1q, the collagenous stalks associate with each other through their N-terminal portions and splay apart at a short interruption within the repeating Gly-Xaa-Yaa sequence, called the kink. Although MBL and some ficolins also possess a kink-like region, recent measurements have revealed that the stalks probably diverge nearer the N terminus, at the junction with the cysteine-containing domain, and thus form spider-like structures rather than the classical bouquets of C1q (7). Recognition subcomponents possess different numbers of subunits. For example, human C1q is a hexamer assembled from three different polypeptide chains (8), and rat MBLs are homo-oligomers comprising dimers, trimers, and tetramers of subunits (9).

The protease subcomponents, C1r and C1s of the classical pathway and MASP-2 of the lectin pathway, are homologous and bind to the collagenous domains of their respective recognition subcomponent through Ca^{2+}-dependent interactions (10–13). They each comprise two Ca^{2+}-binding CUB domains (domain found in complement component C1r/C1s, Uegf, and bone morphogenic protein 1), separated by a Ca^{2+}-binding epidermal growth factor-like domain (EGF), followed by two complement-control protein modules and a serine protease domain

* This work was supported by Grant G0501425 from the Medical Research Council and Grant 077400 from The Wellcome Trust.

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[5] The abbreviations used are: MBL, mannose- or mannan-binding lectin; MASP, MBL-associated serine protease; CUB, domain found in complement component C1r/C1s, Uegf, and bone morphogenic protein 1; EGF, epidermal growth factor; SP-A, pulmonary surfactant protein-A; Hyp, hydroxyproline.

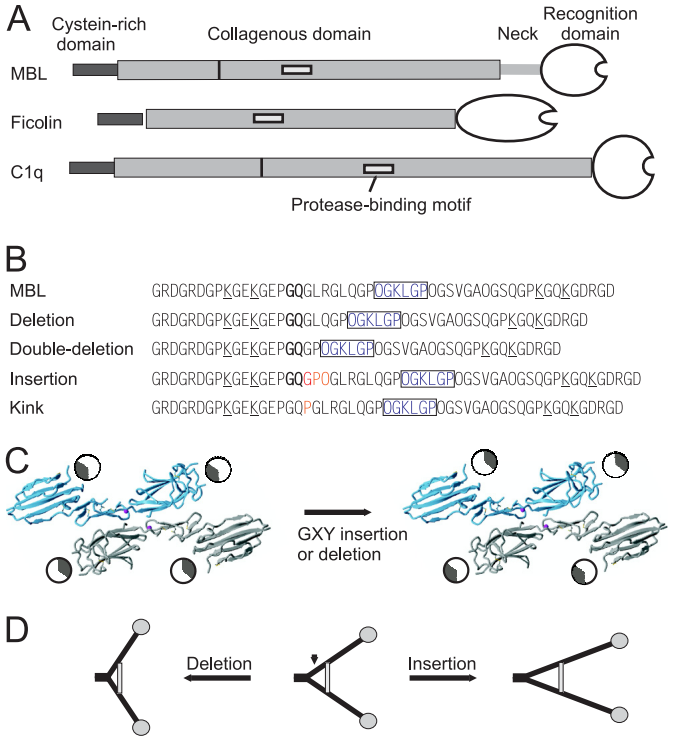

FIGURE 1. **Changes to the collagenous domains of MBLs.** *A,* domain organizations of MBL, ficolins, and C1q. Interruptions within the collagenous domains of C1q and MBL are marked by a *bold line*. The binding sites for MASPs and C1r/C1s within the collagenous domains of MBL, ficolins, and C1q are indicated by *light gray boxes*. The recognition domains are: carbohydrate-recognition domain in MBL, fibrinogen-like domain in ficolins, and C1q-globular domain in C1q. *B*, aligned sequences of the collagenous domains of wild-type and modified MBLs. Residues forming the protease-binding motif are in shown in *blue* and are *boxed*. The GQ residues that form the kink-like region are in *bold*. Residues inserted into the collagenous domain are shown in *red*. Glycosylated hydroxylysine residues are *underlined*. *C*, predicted changes to the alignment of the MASP-binding sites on MBL subunits as a result of a single insertion or deletion of a Gly-Xaa-Yaa into the collagenous domain based upon a pitch of 3.5 for a collagen helix. The figure shows a cross-section through the four collagenous stalks, which are represented by *circles*. Each binding site is shaded in *gray*. The structure of the CUB1-EGF-CUB2 domains of MASP-2 is from Ref. 14. *D*, schematic representation of the effects of changes to the position of the MASP-binding sites on MBL. Only two MBL stalks are shown for clarity. The MASP is shown as a *box* and is *shaded* in *light gray*. Carbohydrate recognition domains are represented as *gray* circles. The *arrow* shows the position of the insertion/deletion. To accommodate the MASP, the collagenous stalks would have to move closer together for an insertion or splay further apart for a deletion.

(14, 15). C1r and C1s assemble to form heterotetramers (C1s-C1r-C1r-C1s), and MASPs are homodimers. Although the stoichiometries of the resulting C1 and MBL·MASP complexes differ, interactions between subcomponents are analogous, with equivalent contacts between the CUB domains of the proteases and the collagenous domains of the recognition subcomponents (2). Overall, each C1rs tetramer presents a total of six binding sites, one for each of the collagenous domains of C1q (one site on each CUB1 of C1r and C1s and each CUB2 of C1r). Each MASP-2 dimer presents four binding sites to MBL and ficolins (one site on each CUB domain).

A number of other proteins have bouquet-like architectures, such as adiponectin (16), emilins (17), and some collectins (18), including pulmonary surfactant protein-A (SP-A). Nevertheless, only MBLs, ficolins, and C1q molecules are able to activate complement. As yet, the only established difference between these proteins and their structural analogues is their ability to bind to MASPs or C1rs tetramers. The MASP-binding site in MBLs and ficolins is characterized by a distinct motif within the collagenous domain: Hyp-Gly-Lys-Xaa-Gly-Pro, where Hyp is hydroxyproline and Xaa is generally an aliphatic residue. Point mutations in this sequence disrupt MASP binding (10, 13). C1q also possesses a similar motif, and we have proposed that this region forms the binding sites for C1r and C1s (2).

In this paper, we have further probed the functions of the recognition subcomponents by examining the structural requirements for complement activation. Initially, we modified the collagenous domain of MBL to change the position and relative orientations of the MASP-binding site on the collagenous stalks. The data reveal that MASP binding and activation are surprisingly tolerant to such changes, implying considerable rotational and conformational flexibility. To take these studies further, we have engineered the MASP-binding motif into SP-A, which also has a C-type lectin domain and an architecture similar to that of MBL but cannot bind MASPs or activate complement. We find that MASP binding, comparable with that by MBL, can be engineered through just three amino acid substitutions to the collagenous domain of SP-A. The resulting proteins also activate the MASP but lack the control mechanisms necessary to target carbohydrate surfaces selectively.

EXPERIMENTAL PROCEDURES

Protein Components—Recombinant rat MBL and modified forms of rat MASP-2 were produced in a Chinese hamster ovary cell expression system and purified as described previously by affinity chromatography on mannose-Sepharose and nickel-Sepharose columns, respectively (9, 19). Recombinant wild-type rat MASP-2 is toxic to producing Chinese hamster ovary cells and autoactivates during biosynthesis. We therefore used two modified forms of MASP-2, both of which are secreted as zymogens and have been characterized extensively with regard to their structures, activation, and catalytic properties (19, 20). MASP-2A is a catalytically inactive form in which the active site serine at position 613 has been replaced by an alanine. MASP-2K is a catalytically active form, in which the arginine residue at the cleavage site for zymogen activation (Arg^{424}) has been changed to a lysine residue to slow down the rate of spontaneous autoactivation during biosynthesis. Binding and activation assays were carried out using MASP-2A and MASP-2K, respectively. Mutant forms of MBL were created by PCR and expressed in the same way as the wild-type protein. All mutations were verified by DNA sequencing of the entire cDNA within the expression vector, prior to transfection of the Chinese hamster ovary cells.

Cloning and Production of Recombinant Wild-type and Mutant SP-As—The cDNA of SP-A isoform A1 was cloned into the polylinker of the eukaryotic expression vector pED-4 (21), which contains the dihydrofolate reductase cDNA as a selectable marker. Proteins were produced by expression in the Chinese hamster ovary cell line DXB11 following amplification using the dihydrofolate reductase inhibitor, methotrexate, to a final concentration of 25 μM. Culture medium was harvested using a protocol that has been described previously for produc-

tion of recombinant MBL (9, 22). Protein was purified by affinity chromatography on mannose-Sepharose columns (1-ml column for 250 ml of medium). Following washes with high salt (10 mM Tris-HCl (pH 7.4), containing 500 mM NaCl and 5 mM $CaCl_2$), Triton (10 mM Tris-HCl (pH 7.5), containing 0.1% Triton X-100 and 5 mM $CaCl_2$), and low salt buffers (10 mM Tris-HCl (pH 7.4), containing 5 mM $CaCl_2$), protein was eluted in 0.5-ml fractions of 10 mM Tris-HCl (pH 7.4), containing 5 mM EDTA. Wild-type SP-A and all mutants bound to the affinity matrix, confirming that they were folded correctly. Fractions containing recombinant protein were identified by SDS-PAGE. Mutations were introduced into the cDNA of SP-A by PCR, and the encoded proteins were produced in the same way as the wild-type protein. Wild-type and mutant SP-As were stored in low ionic strength buffer (10 mM Tris (pH 7.4), containing 2 mM $CaCl_2$) because they tended to aggregate at high protein concentrations (>0.1 mg/ml) in salt concentrations >10 mM.

Surface Plasmon Resonance—Measurements were performed using a BIAcore 2000 instrument (GE Healthcare) or a Bio-Rad ProteOn XPR36 biosensor. Protein ligands were diluted into 10 mM sodium acetate (pH 4.5 for MBL or pH 5.0 for SP-A) and immobilized onto the carboxymethylated dextran surface of a CM5 sensor chip (GE Healthcare) or a GLM chip (Bio-Rad), using amine coupling chemistry. Binding was measured in 10 mM Tris-HCl (pH 7.4), containing 140 mM NaCl, 2 mM $CaCl_2$, and 0.005% surfactant SP40, at a flow rate of 25 μl/min and at 25 °C. After injection of ligand, the protein surface was regenerated by injection of 10 μl of 10 mM Tris-HCl buffer (pH 7.4), containing 1 M 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 (GE Healthcare). 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}). MBL was immobilized on the chip surface rather than used as a soluble ligand because it bound to the chip, thereby masking analysis of the protein-protein interactions. SP-A was also immobilized because it tended to precipitate on the chip surface.

MASP-2 Activation Assays—Activation was measured by following MASP autolysis using a modified version of the protocol described previously (20). Briefly, MASP-2K was mixed with wild-type or mutant MBL or SP-A and added to a suspension of mannose- or fucose-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 MBL or SP-A was used to ensure that all of the MASP-2K was bound to the recognition molecule. The mixture was incubated at 37 °C with shaking, and aliquots of the suspension were removed from the reaction mix at various times and immediately frozen on dry ice to quench the reaction. Proteins were separated by SDS-PAGE under reducing conditions, and the amount of MASP cleaved was quantified by densitometry. Data are the mean ± S.E. from at least two separate experiments using different protein preparations, unless otherwise stated.

RESULTS

MASP Binding and Activation Are Tolerant of Changes in the Position and Orientation of Binding Sites on MBL—MASPs bind inside the cone created by the collagenous stalks of MBL and bridge up to four stalks simultaneously (Fig. 1) (3). Consequently, during complex formation, the binding sites on the MBL stalks must either face inward or reorient so that the correct collagen-CUB domain interactions are formed. If the stalks are relatively rigid or are unable to reorient independently, any change in their position or alignment would lead to loss of function. To examine the tolerance of MBL to such changes, we made a series of modified MBLs in which the collagenous domain was modified. Because the pitch of collagen is typically ~3.5 residues (24), deletion or insertion of a Gly-Xaa-Yaa triplet would change both the position of each binding site and its relative orientation. For example, deletion of a triplet would effectively rotate the orientation of the binding site on the collagen by ~180° as well as shift the position of further toward the N terminus (Fig. 1). Deletion of a second triplet would restore the original alignment but move the binding sites even further toward the N terminus. To retain the ability to bind MASP, the collagenous stalks would not only have to splay further apart (for a deletion) or move closer together (for an insertion), but also rotate independently of each other. Thus, analysis of MASP binding and activation by the modified MBLs provides a useful probe of the conformational and rotational flexibility of MBL.

Two deletion mutants were created in which either one or two Gly-Xaa-Yaa triplets were removed from the collagenous domain, thus moving the MASP-binding motif closer to the N terminus of MBL (Fig. 1). In addition, a single Gly-Pro-Hyp was inserted near the kink-like region to move the MASP-binding motif further toward the C terminus. We also made an MBL in which the kink-like region itself was removed by insertion of a proline residue to restore the Gly-Xaa-Yaa tandem repeats. A previous study has demonstrated that the kink-like region is not essential for complement activation by MBL (25). However, the kinetics of activation were not investigated in this work, so more subtle changes might have been missed. Analysis of the purified, recombinant MBLs by SDS-PAGE under nonreducing conditions, shown previously to be a sensitive indicator of MBL assembly (26), demonstrated that all assembled correctly during biosynthesis (Fig. 2).

Initially, MBLs were tested for their abilities to bind to MASP-2A, using surface plasmon resonance. As expected, wild-type bound with complex kinetics compatible with the formation of 1:1 complexes, together with binding of a second MASP at relatively high protein concentrations (19). The K_D values were 3.7 and 164.5 nM, consistent with previous measurements (2). Surprisingly, the entire set of mutant MBLs bound to MASP-2A with similar affinities and kinetics (Table 1), indicating that none of the changes perturbed MASP binding (Fig. 2). We next examined their complement activities by monitoring MBL-dependent MASP-2K autoactivation with mannose-Sepharose as an activating target (Fig. 3). In the presence of the target, all mutant MBLs activated the MASP with rates comparable to that of wild-type ($t_{½}$ ~20 min). The greatest difference observed was for the double-deletion mutant, which activated

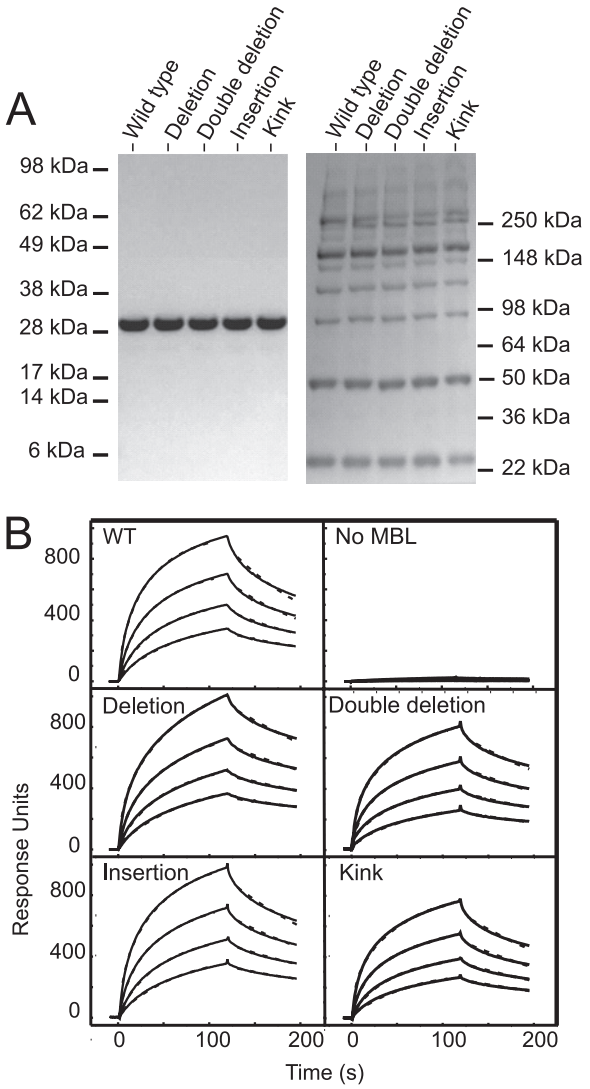

FIGURE 2. **MASP-binding by modified MBLs.** *A*, SDS-PAGE of wild-type and mutant MBLs under reducing (*left*) and nonreducing (*right*) conditions. Mutant MBLs are assembled from single polypeptides chains, which migrate as a ladder of covalently linked polypeptides under nonreducing conditions, characteristic of the heterogeneous nature of oligomers of wild-type MBL (9). *B*, binding of MASP-2A to immobilized MBLs by surface plasmon resonance. MASP-2A was injected at 333, 167, 83, and 42 nM over immobilized MBL (~7000 response units). A sensor chip blocked by treatment with ethanolamine was used as a negative control (*No MBL*). All data were fitted to a two-complex parallel binding model, and the fits are shown by the *dotted lines*. *WT*, wild-type.

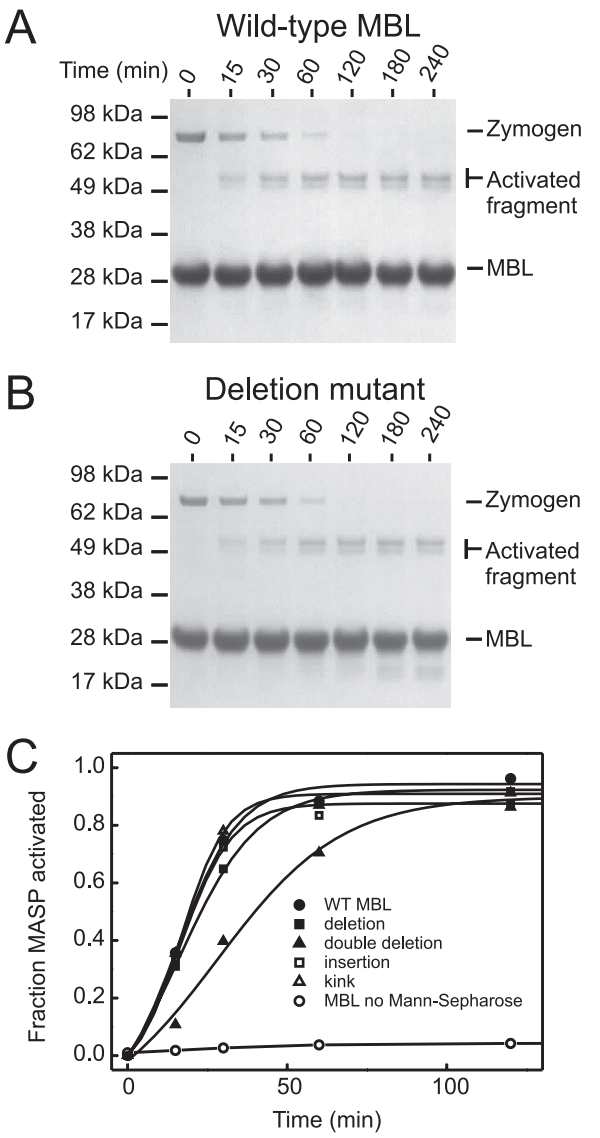

FIGURE 3. **MASP activation by modified MBLs.** Kinetics of MBL·MASP-2 activation analyzed by SDS-PAGE. *A* and *B*, wild-type MBL and the deletion mutant were incubated with MASP-2K together with mannose-Sepharose as an activating target. Proteins were separated on a 4–12% linear gradient gel under reducing conditions and were stained with Coomassie Blue. The N-terminal fragment of MASP-2K runs as a double band due to differential glycosylation. MASP activation was measured by quantifying cleavage of the MASP polypeptide. The other mutants were tested in the same way (data not shown). *C*, comparison of MASP-2K activation by wild-type (*WT*) and mutant MBLs. Data for all mutants are shown.

TABLE 1
Kinetic properties of modified MBLs

MBL modification	k_{on} ($\times 10^{-4}$)	k_{off} ($\times 10^{3}$)	K_D[a]	Half-time for MASP-2K activation
	$M^{-1}\,s^{-1}$	s^{-1}	nM	Min
Wild-type	40 ± 1	1.5 ± 0.1	3.7 ± 0.1	20 ± 1
	2.9 ± 0.1	4.75 ± 0.1	165 ± 2	
Deletion	38 ± 4	3.8 ± 1.7	11 ± 6	22 ± 1
	3.1 ± 0.7	5.4 ± 1.3	175 ± 2	
Double deletion	39 ± 1	3.1 ± 0.1	8.2 ± 0.3	39 ± 3
	3.3 ± 0	6.4 ± 0.1	195 ± 3	
Insertion	44 ± 3	2.2 ± 0.6	4.9 ± 1.2	21 ± 1
	4.6 ± 0	9.1 ± 0.2	199 ± 5	
Kink	57 ± 1	6.2 ± 2.7	10 ± 2	21 ± 1
	5.2 ± 0.2	6.3 ± 0.2	136 ± 9	

[a] K_D values were calculated from k_{off}/k_{on} for each experiment and were averaged from two separate experiments.

the MASP just 2-fold more slowly ($t_{1/2}$ ~40 min), despite removal of six amino acid residues from the collagenous domain. In the absence of the target, activation was relatively slow and similar to autoactivation by MASP-2 alone (>1000 min), indicating that all MBLs activate only on a carbohydrate target. Thus, neither insertions, deletions, nor removal of the kink-like region disrupts MASP-2 binding or activation, despite large changes to the position and relative orientations of the MASP-binding sites. The tolerance of MBL to these changes must reflect significant adjustment and realignment of the collagenous stalks. Given that collagenous domains themselves are relatively rigid and inflexible (27), changes must occur at the N terminus, where the stalks converge, probably at

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MBL                                  GRDGRDGPKGEKGEPGQG     LRGLQGPOGKLGPOGSVGAOGSQGPKGQKGDRGD
SP-A      GSOGIOGTOGSHGLOGRDGRDGLKGDOGPOGPMGPOGEMPCPPGNDGLOGAOGIOGECGEKGEOGERGPOGLO
SPA-KL                                                                  KL
SPA-KLP                                                                 KL P
SPA-KLPO                                                                KL P0
SPA-KLPOdel                           ----                              KL P0
SPA-KLPOS                             S                                 KL P0
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FIGURE 4. **Design of modified SP-As.** Sequences of the collagenous domains of MBL (*top*) and SP-A (*bottom*) Sequences were crudely aligned based on the positions of the kink of SP-A and the kink-like region of MBL (*boxed*). The MASP-binding motif is shaded *gray*. Changes to the sequence of SP-A are indicated.

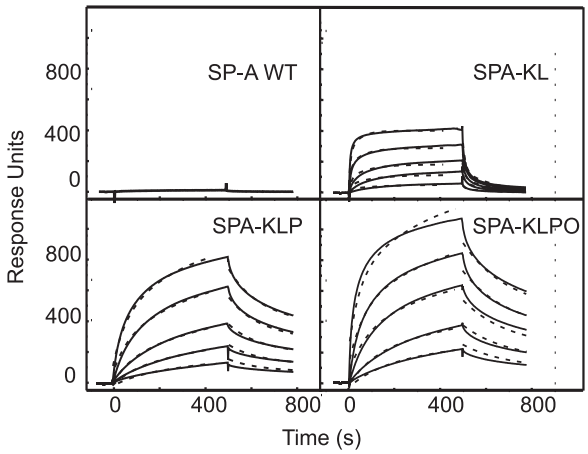

FIGURE 5. **MASP-binding by modified SP-As.** Binding of MASP-2A to immobilized SP-As by surface plasmon resonance. MASP-2A was injected at 764, 447, 261, 152, and 89 nM over each of the immobilized SP-As (~6000 response units). All data were fitted to a two-complex parallel binding model, and the fits are shown by the *dotted lines*.

TABLE 2
Kinetic properties of engineered SP-As

Engineered SP-A	k_{on} ($\times 10^{-4}$)	k_{off} ($\times 10^{3}$)	K_D[a]	Half-time for MASP-2K activation[b]
	$M^{-1}\,s^{-1}$	s^{-1}	nM	Min
Wild-type	N.B.[c]	N.B.[c]	N.B.[c]	>1000[d]
SPA-KL	1.2 ± 0.2	7.0 ± 0.1	600 ± 110	>1000[d]
	4.3 ± 0.1	91 ± 2	2120 ± 100	
SPA-KLP	5.4 ± 2.9	1.4 ± 0.1	36 ± 20	270 ± 56
	36 ± 24	17.6 ± 1.0	104 ± 77	
SPA-KLPO	2.6 ± 0.8	2.5 ± 0.6	97 ± 7	256 ± 96
	9.6 ± 1.0	22 ± 3.1	232 ± 9	
SPA-KLPOdel	5.3 ± 2.3	1.7 ± 0.1	29 ± 17	316 ± 171
	18 ± 0.0	21 ± 1	114 ± 2	
SPA-KLPOS	4.5 ± 2.6	4.6 ± 1.2	99 ± 18	166 ± 43
	21 ± 2	37 ± 15	203 ± 31	

[a] K_D values were calculated from k_{off}/k_{on} for each experiment and were averaged from two separate experiments.
[b] Averaged data from three separate experiments.
[c] No binding detected.
[d] Activation occurred a rate similar to autoactivation of MASP-2 alone.

the junction between the cysteine-rich domain and the collagenous domain.

Engineering Complement Activity into a Pulmonary Surfactant Protein—Given its surprising tolerance to even large changes in MBL structure, we sought to characterize MASP activation further by engineering novel activity into a bouquet-like template. We chose SP-A for these studies because of its similar structure but lack of innate complement activity. SP-A, like MBL, is a member of the collectin family of animal lectins with an N-terminal collagen-like domain and a C-terminal carbohydrate-binding domain (28). It has sugar specificity similar to that of MBL (but with a preference for L-fucose over mannose) (29), allowing purification by affinity chromatography and assessment of complement activity using similar activation assays. Although structurally analogous to MBL, the collagenous domains of SP-A and MBL are of different lengths and share no apparent sequence identity (other than the repeating Gly-Xaa-Yaa motif characteristic of all collagens) (Fig. 4). SP-A possesses a natural kink near the middle of the collagenous domain where the collagenous stalks splay apart, similar to C1q (30). Previous studies have shown that upon removal of the kink, the stalks diverge nearer the N terminus of polypeptides, at the junction between the N-terminal domain and the collagenous domain, thus becoming more like MBL and ficolins (30).

Three new recombinant constructs were created in which all or part of the MASP-binding motif was introduced into the collagenous domain of SP-A (Fig. 4). To allow enough space for the MASP to bind within the SP-A bouquet, changes were made five Gly-Xaa-Yaa triplets toward the C-terminal side of the kink. In SPA-KL, Glu^{63} and Cys^{64} were replaced by lysine and leucine residues, respectively. Lysine in the Xaa position is known to be essential for MASP binding and complement activation in both MBLs and ficolins. The adjacent cysteine residue was also replaced to avoid any potential disulfide bond formation that might interfere with MASP binding. In SPA-KLP, a proline residue was introduced in place of the glutamate at position 66 to optimize the MASP-binding motif further: Hyp-Gly-Lys-Leu-Gly-Pro. One further change was made in SPA-KLPO, replacing Lys^{67} by a hydroxyproline residue. Lysine residues in the Yaa position of collagen are often hydroxylated and glycosylated during biosynthesis, so this change was designed to prevent any potential steric inhibition of MASP binding by the sugar residues (24). We created two further variants of SPA-KLPO, in which the kink was removed or modified to change the point at which the stalks splay apart (30) and thus more resemble MBL and ficolins. In one mutant, SPA-KLPOdel the kink sequence (PCPP) was removed completely, and in the other, called SPA-KLPOS, the cysteine residue was replaced by a serine residue to remove the potential for disulfide bond formation, which is believed to tether the separate stalks together in native SP-A (30).

Following production in Chinese hamster ovary cells, all proteins bound to mannose- or fucose-Sepharose columns, demonstrating that they were folded correctly. As expected, wild-type SP-A did not bind to MASP-2A at any of the concentrations tested (up to 1 μM). Surprisingly, however, SPA-KL bound with appreciable affinity (Fig. 5). The data best fitted a two-complex, parallel-reaction model, with apparent dissociation constants K_{D1} and K_{D2} of 600 and 2120 nM, respectively, compared with 3.8 and 166 nM for MBL·MASP (Table 2). SPA-KLP bound with even higher affinity, with K_D values of 36 and 104 nM, only slightly weaker than MBL. Additional changes had little effect on MASP binding, so the naturally occurring lysine residue in SP-A (or its potential glycosylated derivative) does not prevent MASP access. Removal of the kink region also had little effect, indicating that the point at which the stalks splay

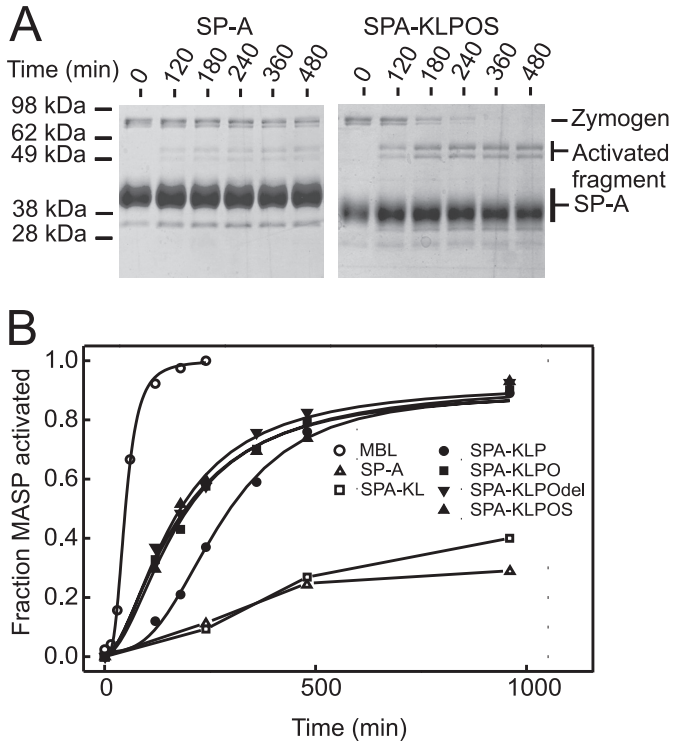

FIGURE 6. **MASP-2 activation by modified SP-As.** Fucose-Sepharose was used as the activating ligand. Proteins were separated on a 15% SDS-polyacrylamide gel and were stained with Coomassie Blue. *B*, MASP-2K activation by wild-type and mutant SP-As. Averaged data from three separate experiments are shown.

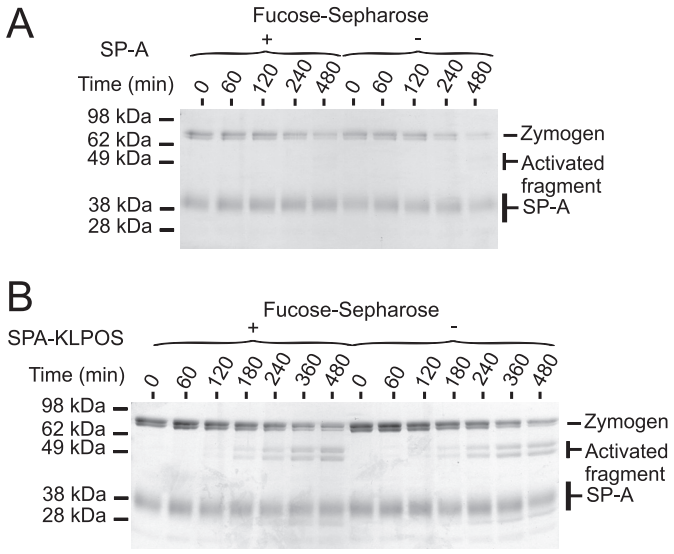

FIGURE 7. **MASP-2 activation in the presence and absence of a carbohydrate target.** Proteins were separated on a 15% SDS-polyacrylamide gel and were stained with Coomassie Blue. MASP-2K activation by wild-type SP-A (*A*) and SPA-KLPOS (*B*) in the presence and absence of a carbohydrate target.

apart and thus the angle between adjacent stalks are not limiting for MASP binding. Thus, just three amino acid changes to the collagenous domain are sufficient to introduce MASP binding to SP-A almost comparable with MBL itself.

We next examined whether the modified SP-As could activate MASP-2K. The half-time for activation by MBL was ~50 min, using fucose-Sepharose as a target. As expected, wild-type SP-A, which does not bind to the MASP at all, and SPA-KL, which binds only weakly, did not activate the MASP (Fig. 6). In each case, the measured rate was similar to the intrinsic autoactivation rate of MASP-2K alone ($t_{½}$ >1000 min). Surprisingly, however, SPA-KLP and SPA-KLPO activated the MASP significantly faster than the basal rate ($t_{½}$ ~260 min). Similar rates were also measured for SPA-KLPOdel and SPA-KLPOS, demonstrating that the kink is neither necessary for MASP activation nor modulates the rate of activation significantly. To determine whether activation was target-dependent, assays were repeated in the absence of fucose-Sepharose. Surprisingly, comparable rates were observed (Fig. 7). Activation was still much faster than the basal rate, so it must be driven by SP-A binding, but was constitutive, occurring even in the absence of a carbohydrate target. Thus, modified SP-As lack the control mechanisms required to activate MASP selectively.

DISCUSSION

The data presented here reveal that novel MASP binding and complement activation can be introduced into a bouquet-like template through relatively minor changes to the chemical makeup of the collagenous stalks. Just three substitutions: Glu[63] to Lys, Cys[64] to Leu, and Glu[66] to Pro, establish MASP binding in SP-A, which is almost equivalent to that of MBL. Of these residues, only the lysine is essential for binding (10, 13). Generally, the adjacent residue in the Yaa position (leucine in rat MBL and the SP-A mutants) has an aliphatic side chain, although a small polar residue such as serine can also be accommodated, but glutamate cannot (10). The proline residue in the Xaa position of the next Gly-Xaa-Yaa triplet is also not essential for MASP binding and can be replaced by an alanine with only a small decrease in affinity. The main function of the proline is probably to help stabilize the binding region (10). It is notable that a significant component of the difference in MASP binding by SPA-KLP relative to SPA-KL is due to faster association, so it probably reflects removal of the unfavorable electrostatic interactions mediated by the glutamate, rather than the introduction of the proline residue itself. The presence of any additional binding sites for MASP on MBL can be completely ruled out from the data presented here. Overall, therefore, specificity of the MBL-MASP interaction appears to be driven mainly by a single lysine residue in the collagen-like domain.

Several other binding motifs have been identified in collagens and typically extend across two or more Gly-Xaa-Yaa triplets. For example, the I domain of α2 integrin recognizes the sequence GFOGER (31), and the extracellular matrix protein SPARC binds to the sequence GVMGFO (32). Thus, the less stringent binding requirements of MASP-2 are relatively unusual. Nevertheless, it is notable that almost all of the other lysine residues in the collagenous domains of MBL and ficolins are in the Yaa position of the Gly-Xaa-Yaa repeat, so they are likely to be post-translationally modified by hydroxylation and glycosylation, which probably blocks any potentially incorrect MASP interactions. Perhaps even more importantly, MASP binds to MBL and ficolins through multiple relatively weak interactions involving up to four separate CUB-collagen contacts. Thus, much of the binding specificity is probably medi-

ated through the architectures of the subcomponents and the multivalent nature of the interactions.

Complement activation is thought to be triggered by changes in the angle between collagenous stalks when complexes bind to an activating target (3, 23). The data presented here indicate that significant conformational and rotational flexibility of the stalks must be permitted, implying that unbound MBL is probably quite flexible. Both relaxed-to-strained and strained-to-relaxed mechanisms could explain the activation process. The traditional (relaxed-to-strained) view is that activation is transmitted to the MASP through a change in the structure of MBL that induces a high energy strained state. However, in an alternative but equally valid possibility, binding of the zymogen MASP to MBL (or ficolin) induces strain into circulating complexes, which releases upon pathogen binding to trigger activation. A major difference between these mechanisms is that the relaxed or default states of the complexes would differ: inactive in a relaxed-to-strained mechanism as activation is reliant on surface binding, but active in a strained-to-relaxed mechanism. In the work described here, MASP binding leads to constitutive activation by SP-A, even in the absence of a target. Thus, activation is the default state, as would be expected in a strained-to-relaxed mechanism. The possibility that modified SP-As are locked into a high energy conformation that activates MASP constitutively cannot be completely excluded. However, SP-A must also be flexible to bind to the MASP multivalently, which would seem to be at odds with this possibility. Furthermore, large structural changes (through removal of the kink) do not affect MASP activation by SP-A, which also seems incompatible with such a mechanism.

Based on these data, we propose that MBL·MASPs also activate via a strained-to-relaxed mechanism. We have recently suggested that activation of the C1 complex also operates in this way (2) and have proposed that strain is induced into the zymogen complex through the interactions between the protease domains of C1r. In this respect, the mechanism must differ in MBL·MASP complexes because the SP domains of a MASP dimer do not interact significantly until the moment of autocatalysis. Interestingly, however, recent analysis suggests that the MBL stalks are not evenly distributed, but rather are highly asymmetrical and separated by ~35–40° between nearest neighbors (7). In this case, significant strain would be induced when MBL binds to a MASP. Release of this strain upon target recognition might drive the changes that initiate complement activation.

From an evolutionary perspective, these data suggest that recognition-mediated complement activation could have arisen relatively rapidly in a collectin-like molecule such as SP-A as result of a small number of changes to the collagen-like domain. It would also explain how similar activation mechanisms could have arisen multiple times in structurally analogous but unrelated recognition components. Clearly however, regulation of activation necessitates additional sequences, and we are currently undertaking studies to identify these regions in MBL and ficolins.

Acknowledgments—We thank Robert Freedman and Katrine Wallis, of the Department of Biological Sciences, University of Warwick, for use of the departmental BIAcore facility.

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