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*J Immunol* 2009; 182:1518-1525; doi: 10.4049/jimmunol.182.3.1518
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Complement Inhibitor C4b-Binding Protein Interacts Directly with Small Glycoproteins of the Extracellular Matrix

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Components derived from cartilage have been suggested to maintain the inflammation in joints in arthritis. Small leucine-rich repeat proteins (SLRPs) are structural components of cartilage important in organizing the meshwork of extracellular matrix components. It has recently been shown that the SLRP fibromodulin interacts with complement initiator C1q, leading to complement activation. The complement response is limited since fibromodulin also interacts with the complement inhibitor factor H. We have now found that osteoadherin, chondroadherin, fibromodulin, and proline arginine-rich end leucine-rich repeat protein bind to the complement inhibitor C4b-binding protein (C4BP). Using direct binding assays with C4BP fragments and C4BP mutants lacking individual domains in combination with electron microscopy, we have demonstrated that mainly the central core of C4BP mediated binding to SLRPs. Binding of SLRPs to C4BP did not affect its ability to inhibit complement. Osteoadherin, fibromodulin, and chondroadherin, which bind C1q and activate complement, were found to cause significantly higher C9 deposition in C4BP-depleted serum compared with Igs, indicating that the level of complement activation initiated by SLRPs is regulated by simultaneous binding to C4BP. A similar dual binding of C1q and complement inhibitors was observed previously for other endogenous ligands (amyloid, prions, C-reactive protein, and apoptotic cells) but not for exogenous activators (bacteria-bound Igs). These interactions can be significant during inflammatory joint diseases, such as rheumatoid arthritis, where cartilage is degraded, and cartilage components are released into synovial fluid, where they can interact with factors of the complement system. The Journal of Immunology, 2009, 182: 1518–1525.

The complement system carries out important roles in immunity and consists of ~35 proteins, which become activated in a cascade-like manner through proteolytic cleavages. Complement has an important role in defense against pathogens, removal of apoptotic and necrotic cells, and regulation of Ab responses of the adaptive immune system. Complement can be activated through three different pathways. In the classical pathway, the triggering factor is immune complexes, whereas the lectin pathway is initiated by specific carbohydrates on bacterial surfaces. The alternative pathway is initiated by autoactivation of the complement component C3 or directly by properdin and serves as an amplification loop to the other two pathways. Since excessive or misguided activation of complement may lead to chronic or acute inflammation, complement is tightly regulated by several soluble and membrane-bound inhibitors.

C4b-binding protein (C4BP) is a large glycoprotein (570 kDa), and one of the main soluble inhibitors of complement. Due to binding of C4b, C4BP inhibits the formation of the classical pathway C3 convertase, C4b2a, and increases its decay (1). Furthermore, C4BP serves as a cofactor to the serine protease factor I (FI) in the proteolytic degradation of C4b (2) and C3b (3). The main isoform of C4BP contains seven identical α-chains and one β-chain, forming a spider-like polymer. C4BP is almost entirely composed of 60-aa long complement control protein (CCP) domains. Each α-chain contains 8 and the β-chain 3 CCP domains (4, 5). In addition, both chains have ~60 aa at the C terminus that are involved in chain polymerization.

The three outermost CCP domains of the α-chain of C4BP are required for complement inhibition and bind both heparin and C4b (6). An especially important site for these interactions is a positively charged cluster of amino acids at the interface of CCP 1–2 (7). Due to steric hindrance, only four C4b molecules can bind simultaneously to one C4BP molecule (8).

All β-chain-containing C4BP in the circulation is bound to vitamin K-dependent anticoagulant protein S (PS), forming a C4BP:PS complex (9) but in acute-phase response, the level of C4BP lacking a β-chain rises in the plasma (10). Other known ligands for C4BP are C3b (3), DNA (11), C-reactive protein (CRP) (12), and several proteins on bacterial surfaces (for review, see Ref. 13).

Abbreviations used in this paper: C4BP, C4b-binding protein; CCP, complement control protein (domain); CHAD, chondroadherin; CRP, C-reactive protein; FH, factor H; FI, factor I; FM, fibromodulin; NHS, normal human serum; OPD, o-phenylendiamine; OSAD, osteoadherin; PRELP, proline arginine-rich end leucine-rich repeat protein; PS, protein S; RT, room temperature; SLRP, small leucine-rich repeat protein.
Because inflammation in joint diseases can be resolved by joint replacement, it would seem that components that can propagate inflammation are derived from cartilage. Cartilage consists of a small number of cells surrounded by an extensive extracellular matrix. Molecules, which regulate the assembly and function of this matrix, include members of the small leucine-rich repeat protein (SLRP) family such as proline arginine-rich end leucine-rich repeat protein (PRELP) (14), chondroadherin (CHAD) (15), osteoadherin (OSAD) (16) and fibromodulin (FM) (17). As a general characteristic, these proteins contain 10–11 leucine-rich repeat motifs of 25-aa residues, arranged in a tandem array flanked by cysteine loops (18). The N terminus of PRELP differs from the other SLRPs in that it is basic and rich in arginine and proline (19). FM and OSAD have nine and six tyrosine sulfates in the N-terminal region, respectively, which contribute to the acidic properties of these proteins (20). OSAD has, in addition, a large and acidic C terminus that distinguishes it from other members of the family (21).

Some members of the SLRP family have been shown to interact with complement components and regulate complement activity. FM (22), OSAD and CHAD (23) are all able to bind to the globular heads of C1q and trigger complement activation and, at the same time, decrease the release of C5a and deposition of C9 through binding to the complement inhibitor factor H (FH). Laminin (24) and fibromectin (25) bind to C1q as well, although neither of these interactions lead to complement activation. The SLRP proteins decorin and biglycan also bind to C1q but inhibit complement interactions leading to complement activation. The SLRP proteins decorin and biglycan also bind to C1q but inhibit complement activation via the classical pathway (26). In this study, we investigate the binding of the complement inhibitor C4BP to four SLRP proteins: PRELP, OSAD, CHAD, and FM.

### Materials and Methods

#### Proteins

c4BP (27), mutants of C4BP lacking single CCP domains (6), and single, nonpolymerized C4BP α-chains (6) were expressed and purified as described previously. The central core of C4BP was prepared by limited digestion with chymotrypsin (27) and consists of C-terminal extensions of α-chains together with CCP8 and a small fragment of CCP7 (see Fig. 1). C4BP-PS complex was purified from plasma as described previously (28). C4met, which corresponds functionally to C4b, was prepared by treatment with m-Chymotrypsin as described in Ref. 7. C4b (Complement Technology) was labeled with 125I using the chloramine T method. PRELP, FM (14), decorin, and biglycan were purified from bovine cartilage according to previously published methods (29). Recombinant human OSAD and CHAD with N-terminal His tags were expressed from the pQE8 vector in M15(pREP4) and purified on a Ni2+–NTA agarose column (Qiagen). Alternatively, recombinant human OSAD and CHAD were expressed in EBNA 293 cells as described previously (20, 30).

Normal human serum (NHS) was prepared from freshly drawn blood of six healthy volunteers with a permit of a local ethical committee. The pooled blood was allowed to clot for 30 min at room temperature (RT) and then 1 h on ice. After two centrifugations, the serum fraction was frozen in aliquots and stored at −80°C or used for preparing C4BP-depleted serum. For C4BP depletion, fresh serum was passed through a HiTrap column (GE Healthcare) coupled with mAb 104 (31). The flow through was analyzed by ELISA, and the fractions lacking C4BP were pooled and frozen at −80°C. C1q purified from plasma was added to the depleted serum to compensate for C1q that bound to the mAb-coupled column. The concentration of C1q in NHS and C4BP-depleted NHS was assayed by ELISA.

#### Direct binding assay

Microtiter plates (Maxisorp; Nunc) were coated with 50 μl of SLRPs at a concentration of 5.0 μg/ml (PRELP, OSAD), 10 μg/ml (FM), or 2.5 μg/ml (CHAD) in 75 mM sodium carbonate buffer (pH 9.6) overnight at +4°C. Wells coated with 1% BSA (Sigma-Aldrich), 2 μg/ml C4b (Complement Technology), or 7 μg/ml CRP (Sigma-Aldrich) were used as controls. Blocking was performed with 200 μl of 1% BSA in PBS (blocking buffer) for 2 h at RT. C4BP was diluted in binding buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl2, and 50 μg/ml BSA) and incubated with the immobilized SLRPs at concentrations indicated in figures. For investigations of the effect of ionic strength on C4BP-SLRP interactions, the binding buffer was supplemented with NaCl to concentrations ranging from 150 to 300 mM. To assess the binding of various forms and fragments of C4BP (rC4BP wild-type and mutants lacking individual domains, plasma-purified C4BP-PS, and C4BP core) to the SLRPs, 12 nM (corresponding to 5 μg/ml recC4BPwt) of each C4BP variant was incubated with SLRPs immobilized at 5 μg/ml. The plates were washed with 50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl2, and 0.1% Tween 20 between each step. Bound C4BP was detected with a rabbit anti-C4BP 9008 polyclonal Ab, followed by a goat anti-rabbit HRP conjugate (DakoCytomation). The plates were developed with o-phenylenediamine (OPD) substrate and H2O2 and the absorbance at 490 nm was measured (ELX808IU; Bio-Tek Instruments). Kd values (dissociation equilibrium constants) for the binding of rC4BP to the SLRPs were calculated from the binding curves using non-linear regression analysis, applying the equation for one-site binding (GraphPad Prism).

#### Surface plasmon resonance (Biacore)

The interaction between C4BP and SLRPs was analyzed using surface plasmon resonance (Biacore 2000; Biacore). The individual flow cells of a CMS sensor chip were activated, each with 20 μl of a mixture of 0.2 M L-ethyl-3-(3 dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysulfosuccinimide at a flow rate of 5 μl/min, after which FM, PRELP, OSAD, and CHAD diluted to 30 μg/ml in 10 mM sodium acetate buffer (pH 4.5) were injected over individual flow cells to reach 8000 resonance units. Remaining active groups were blocked with 20 μl of 1 M ethanolamine (pH 8.5). A negative control was prepared for each chip by activating and subsequently blocking the surface of flow cell 1. The association kinetics was studied for various concentrations of purified C4BP (3–750 nM) in 10 mM HEPES (pH 7.5) supplemented with 100 mM NaCl, 2.5 mM CaCl2, and 0.005% Tween 20. Protein solutions were injected for 100 s during the association phase at a constant flow rate of 30 μl/min. The dissociation kinetics was studied for various concentrations of purified C4BP (3–750 nM) in 10 mM HEPES (pH 7.5) supplemented with 100 mM NaCl, 2.5 mM CaCl2, and 0.005% Tween 20. Protein solutions were injected for 100 s during the association phase at a constant flow rate of 30 μl/min. The sample was injected first over the negative control surface and then over immobilized SLRPs. Signal from the control surface was subtracted. The dissociation after omitting the C4BP from the injected solution was followed for 200 s at the same flow rate. In all experiments, two 15-μl injections of 2 M NaCl were used to remove bound ligands during a regeneration step. The BiaEvaluation 3.0 software (Biacore) was used to analyze sensorgrams obtained. For FM and OSAD, response units obtained at plateau sensorgrams were plotted against concentrations of injected protein and used for calculations of equilibrium affinity constants. For PRELP and CHAD, we used the 1:1 Langmuir binding model with drifting baseline (BiaEvaluation).

#### Deposition of complement factors from serum

Microtiter plates were coated with OSAD or FM at 2.5 μg/ml or CHAD at 5 μg/ml in 75 mM sodium carbonate buffer (pH 9.6), O/N at +4°C.
Aggregated human IgG (2.5 µg/ml) and BSA (1%) were used as positive and negative controls, respectively. After incubation with blocking buffer for 2 h at RT, NHS, NHS depleted of C4BP, or depleted serum reconstituted with 200 µg/ml C4BP-PS, all at dilutions of 1/200 (IgG), 1/75 (OSAD, FM), or 1/25 (CHAD) were added to the wells. All serum samples were diluted in GVB buffer (5 mM veronal buffer (pH 7.35), 144 mM NaCl, 1 mM MgCl2, 0.15 mM CaCl2, and 1% gelatin). After incubation for 40 min at 37°C, deposited C9 was detected using a goat anti-C9 Ab (Complement Technology), followed by a HRP-conjugated rabbit anti-goat Ab (DakoCytomation). The plates were developed with OPD substrate and H2O2, and the absorbance at 490 nm was measured.

C4b degradation assay

Microtiter plates were coated with OSAD, CHAD, PRELP, and FM at 10 µg/ml in 75 mM sodium carbonate buffer (pH 9.6), O/N at +4°C. The wells were blocked for 2 h at RT with 1% BSA in PBS, C4BP-PS at several dilutions in binding buffer (50 mM HEPES (pH 7.4), 75 mM NaCl, 2 mM CaCl2, and 50 µg/ml BSA) was added to the wells and allowed to bind overnight at RT. After three washes with 50 mM Tris, 75 mM NaCl, 2 mM CaCl2, 0.1% Tween 20, 60 mM FI, and trace amounts of 125I-C4b diluted in TBS (50 mM Tris-HCl (pH 7.5) and 75 mM NaCl) were added to the plates and incubated for 10 h at 37°C. Reducing SDS-PAGE sample buffer was added directly to the wells for 10 min, and the samples were boiled at 95°C for 5 min, after which, they were analyzed by 10–15% gradient SDS-PAGE. The protein bands were visualized using a PhosphorImager (Molecular Dynamics), and their intensities quantified with ImageGauge 4.1 (Fuji Photo Film).

In solution, C4BP-PS (100 nM) was incubated with 250 nM C4met, 60 nM FI, and trace amounts of 125I-labeled C4b in TBS together with OSAD, CHAD, PRELP, or FM at 1 µM. As a control, mAb 104 against C4BP was used in place of the SLRPs. As positive and negative controls, reactions were prepared without added SLRPs or FI. After 1.5 h of incubation at +37°C, the samples were analyzed as described above.

Electron microscopy

Complexes between plasma purified C4BP-PS and OSAD, CHAD, PRELP, and FM were analyzed by negative staining and electron microscopy as described previously (32). Protein concentrations were adjusted to 10 nM in 50 mM Tris-HCl, 150 mM NaCl (pH 7.4), and 5-µl aliquots were adsorbed onto carbon-coated grids for 1 min, washed with two drops of water, and stained with two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. The SLRPs were identified by labeling with colloidal thiocyanate gold (33). Specimens were observed in a Jeol JEM 1230 electron microscope operated at a 60 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 charge-coupled device camera. For quantitative analysis, 100 different fields were evaluated, and ~1500 gold-labeled conjugates were counted for each SLRP.

Statistics

All assays were conducted at least on three separate occasions. An unpaired Student’s t test was used to compare the means between the groups, and values of p < 0.05 were considered significant.

Results

C4BP binds to PRELP, OSAD, CHAD, and FM

In our initial screening, we found that both rC4BP consisting of only α-chains and the C4BP-PS complex purified from plasma
bound to some of the SLRPs. Because the $\beta$-chain did not appear to be necessary for these interactions, we continued to characterize the binding using rC4BP. The different forms of C4BP used in this study are represented schematically in Fig. 1. To investigate the interactions between C4BP and the SLRPs we used a microtiter plate solid-phase assay in which rC4BP at various concentrations was allowed to bind to immobilized SLRPs, after which, bound C4BP was detected with a polyclonal Ab. We found that rC4BP bound to PRELP, OSAD, CHAD, and FM in a concentration-dependent manner (Fig. 2) and that binding was even more pronounced than to the positive control, CRP (12). Since the OSAD and CHAD used in this study were of bacterial origin, binding of rC4BP to eukaryotic OSAD and CHAD was verified as well (Fig. 2 C).

To assess the affinity of the interactions between the SLRPs and C4BP-PS, we used surface plasmon resonance (Biacore). Increasing concentrations of fluid-phase C4BP-PS were injected over a CM5 sensor chip with covalently coupled OSAD, CHAD, PRELP, or FM. For OSAD and FM, $K_D$ values were calculated based on the steady-state affinity model and yielded values of 18.9 nM and 15 nM, respectively. The steady-state binding curve and the sensorgrams for the C4BP-OSAD interaction is illustrated in Fig. 3A. The sensorgrams for the interactions between C4BP and PRELP and CHAD were evaluated by using the 1:1 Langmuir model of binding, and the calculated $K_D$ values were 63.2 nM for PRELP and 80 nM for CHAD. As an example, the obtained sensorgrams (black) and fitted curves (red) for the C4BP-PRELP interaction are plotted in Fig. 3B. The $K_D$ values as well as the association and dissociation rate constants are summarized in Table I.

Table I. Affinity constants for interactions between C4BP and SLRPs

<table>
<thead>
<tr>
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<th>Solid-Phase Binding</th>
<th>Surface Plasmon Resonance</th>
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<tr>
<td></td>
<td>Assay with Immobilized</td>
<td>SLRPs, $K_D$</td>
</tr>
<tr>
<td>OSAD</td>
<td>$K_D$: 8.43 e-8</td>
<td>$K_D$: 1.27 e-9</td>
</tr>
<tr>
<td>CHAD</td>
<td>$K_D$: 1.27 e-9</td>
<td>$K_D$: 0.72 e-9</td>
</tr>
<tr>
<td>PRELP</td>
<td>$K_D$: 1.62 e-9</td>
<td>$K_D$: 0.72 e-9</td>
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$*$ The binding constants were determined from the solid-phase assays presented in Fig. 2 by nonlinear regression analysis as well as by using surface plasmon resonance (Biacore 2000). For FM and OSAD, only $K_D$ values could be calculated as described in Fig 3. For CHAD and PRELP, the binding curves were evaluated using 1:1 Langmuir binding model and both $K_D$ as well as association ($k_a$) and dissociation ($k_d$) rate constants. $K_D$ values for the binding of rC4BP to the SLRPs immobilized on plates were calculated from the binding curves presented in Fig. 2 using nonlinear regression analysis, applying the equation for one-site binding.

To determine whether C4BP and the SLRPs bind via hydrophobic or ionic interactions, the binding assay was conducted in the presence of increasing NaCl concentrations. The interaction between rC4BP and PRELP was completely abolished already at 25 mM NaCl (Fig. 4).

FIGURE 4. The SLRP-C4BP interaction is mainly ionic in character. rC4BP (12 nM) was allowed to bind to immobilized OSAD, CHAD (A), PRELP, and FM (B) in binding buffer supplemented with NaCl to final ionic strengths ranging from 150 to 300 mM. C4met and BSA were coated as positive and negative controls, respectively. The amount of bound C4BP was detected with a polyclonal Ab. The data are presented as the mean and SD of three separate experiments.

FIGURE 5. Binding of C4BP deletion mutants to OSAD, CHAD, PRELP, and FM. Fluid-phase rC4BP (control) or C4BP deletion mutants lacking single CCP domains of the $\alpha$-chain (all used at 12 nM) were allowed to bind to immobilized OSAD (A), CHAD (B), PRELP (C), and FM (D) and the amount of bound protein was detected using a polyclonal Ab. The data are given as the mean and SD from three separate experiments. The statistical significance of differences was calculated compared with recC4BPwt; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The binding of C4BP to the SLRPs is ionic in character
To determine whether C4BP and the SLRPs bind via hydrophobic or ionic interactions, the binding assay was conducted in the presence of increasing NaCl concentrations. The interaction between rC4BP and PRELP was completely abolished already at 25 mM NaCl.
NaCl above the normal physiological concentration, indicating a prominent charge dependence (Fig. 4A). The binding of rC4BP to FM decreased with increasing NaCl concentration (Fig. 4A), but the ionic strength of 600 mM was required to completely inhibit the interaction (data not shown). The interaction between C4BP and OSAD also decreased with increasing ionic strength and was abolished at 300 mM NaCl (Fig. 4B). The binding of C4BP to CHAD could only be reduced by 50% by augmenting the ionic strength, which indicated that the interaction is dependent on both ionic and hydrophobic forces (Fig. 4B).

OSAD, CHAD, PRELP, and FM bind to the core of C4BP

To determine which parts of C4BP are involved in SLRP binding, we first evaluated binding of the deletion mutants of C4BP lacking single CCP domains to immobilized OSAD, CHAD, PRELP, and FM. Deletion of CCP8, close to the core of C4BP, decreased the binding to all of the SLRPs most prominently (Fig. 5). Deletion of CCP1, 2, 4, 5, 7, or 8 resulted in a significant decrease in binding of C4BP to OSAD, indicating several possible binding sites along the α-chain (Fig. 5A). Binding to CHAD was affected to some extent by the removal of CCP2, CCP4, and CCP7, although the effect was not as marked as when CCP8 was deleted (Fig. 5B). The binding of C4BP to FM was most strongly inhibited by the removal of CCP8, as for the other SLRPs, while deletion of CCP2 yielded only a slight reduction in binding (Fig. 5D). Interestingly, removal of the central CCP domains (CCP3, 4, 5, or 6) increased FM binding affinity. A similar effect was seen in the binding of C4BP mutants lacking any of the central CCP domains to PRELP (Fig. 5C). Taken together, the main binding site for SLRPs on C4BP appears to reside in CCP8.

To verify that the major binding site on C4BP was located in the center of the protein, including CCP8, we tested the ability of the isolated core of C4BP to bind to immobilized SLRPs. The core fragment containing CCP8 and the 60 C-terminal residues of seven polymeric α-chains was able to bind to all of the SLRPs (Fig. 6). Furthermore, the C4BP-PS complex did not bind the SLRPs as well as rC4BP (Fig. 6B), which might be due to steric hindrance at the core where the β-chain and the associated PS are attached.

To further verify the core of C4BP as the SLRP binding site, we studied the interaction of gold-labeled SLRPs with C4BP-PS using electron microscopy. Under the conditions used, the C4BP-PS molecule resembles a spider with the α-chains pointing out from the center of the molecule (indicated by arrows), but some images showed a more condensed form. Selected particles of C4BP-PS at a higher magnification. C, Single molecules of C4BP-PS with gold labeled OSAD. D, C4BP-PS and gold-labeled CHAD. E, C4BP-PS and gold-labeled PRELP. F, C4BP-PS and gold-labeled FM. Scale bars are 100 nm (A) and 50 nm (B–F).

The SLRPs do not inhibit the cofactor activity of C4BP

One of the main functions of C4BP is to act as a cofactor for FI in the proteolytic degradation of C4b (1). To assess the functionality of C4BP bound to the SLRPs, we measured the C4BP cofactor...
Complement activation in C4BP-depleted serum

OSAD, CHAD, and FM have previously been shown to bind C1q and activate the classical pathway of complement. We hypothesized that the binding of C4BP to the same ligands would limit this activation. To evaluate this hypothesis, we measured the deposition of activated complement components from NHS and serum depleted of C4BP on plates coated with OSAD, CHAD, FM, or aggregated human IgGs as the positive control. There was significantly higher C9 deposition seen for the C4BP-depleted serum compared with NHS for all three of the coated SLRPs (Fig. 9). Some effect of C4BP depletion from serum could be seen for the IgG control also, but it was not as dramatic as that seen for the SLRPs.

Discussion

Rheumatoid arthritis is an autoimmune disease characterized by joint inflammation leading to cartilage breakdown. The complement system has been shown to play a role in both initiating the inflammatory state as well as in maintaining the inflammation during chronic disease. Complement activation products, such as C3d (34), C1q-C4 complexes (35), sC5b-9, and the fragment Bb (36), can be found in the synovial fluid of patients with active arthritis, indicating ongoing activation of complement. Mice deficient in C3, factor B, and C5 are resistant to collagen-induced arthritis, an experimental model of rheumatoid arthritis, showing the importance of complement in the effector phase of chronic arthritis (37–39).

As both rheumatoid arthritis and osteoarthritis lead to cartilage damage and breakdown, proteins, including members of the SLRP family, normally retained in the cartilage are released into the synovial fluid. Several SLRPs have been shown to interact with complement and either activate or inhibit the cascade (22, 26). Much work still remains to elucidate these processes and their net outcome in various tissues and pathological situations. In this study, we investigated the binding of several SLRPs to the complement inhibitor C4BP. We found that OSAD, CHAD, PRELP, and FM bound to C4BP in a concentration-dependent manner. Furthermore, we showed that the binding of PRELP, FM, and OSAD to C4BP was ionic in nature, whereas the binding of C4BP to CHAD and PRELP and OSAD have overall basic and acidic properties, respectively, which are likely to contribute to their binding properties. A cluster of tyrosine sulfate residues in the N terminus of FM contributes the activity in a FI-mediated C4b degradation assay using $^{125}$I-labeled C4b. C4BP-PS bound to immobilized OSAD, CHAD, PRELP, or FM was able to induce cleavage of the C4b α-chain yielding a C4d cleavage product in the presence of FI (Fig. 8A). The amount of generated product was dependent on the concentration of C4BP-PS bound to SLRPs. Furthermore, C4BP-PS bound to immobilized SLRPs was able to interact with $^{125}$I-labeled C4b (data not shown).

The addition of SLRPs, at up to a 10-fold molar excess compared with C4BP, to the mixture of C4BP-PS, C4b, and FI in a solution did not have any effect on degradation of the C4b α-chain. At these conditions, >90% of the C4BP-PS in the solution should be bound to the SLRPs as calculated from the $K_d$ values obtained in the Biacore experiments. Addition of mAb 104, which binds to CCP1 of C4BP and thus inhibits the interaction of C4BP with C4b, caused a complete inhibition of C4BP cofactor activity (Fig. 8B).

These results indicate that binding of OSAD, CHAD, PRELP, or FM to C4BP does not affect its cofactor activity.
anionic character of this SLRP, which may be important for the interactions studied. Being basic, CHAD in contrast may use hydrophobic patches to bind C4BP as well as clusters of charged residues.

In an attempt to determine binding sites, we found that the isolated core of C4BP binds the four investigated SLRPs. The importance of this region for SLRP binding was further supported by the fact that removal of CCP8 from C4BP resulted in the most significant decrease in binding. Interestingly, deletion of central α-chain CCP domains increased the binding of C4BP to PRELP and FM. A similar phenomenon was previously observed for the interaction between C4BP and Haemophilus influenzae (40). Deletion of these central domains may therefore result in discreet conformational changes or alterations in flexibility that affects only some ligand interactions. A confirmatory result supporting the involvement of CCP8 in SLRP binding was obtained using electron microscopy, where gold-labeled SLRPs were shown to interact specifically with the central core region of C4BP-PS. Even though deletion of other CCP domains in the α-chain of C4BP showed some ability to decrease interactions, little or no binding of OSAD, CHAD, PRELP, or FM to the α-chains of C4BP could be observed using electron microscopy, indicating that any additional binding site along the α-chains must have weak affinity. In accordance with the hypothesis that CCP8 harbors the main binding site for SLRPs, we found that SLRP binding does not affect the ability of C4BP to act as a cofactor in the degradation of C4b, which we have previously shown to require CCPI–3 (6).

Previously, it has been suggested that FM regulates local complement activation at sites of joint inflammation, due to both its complement activating and inhibiting properties (22). Similar complement activating ability has been shown for OSAD (23), suggesting a wider role for these proteins in the disease process. Therefore, it is tempting to suggest that the interactions of PRELP, OSAD, FM, and CHAD with C4BP might play a physiological role in the local regulation of complement activity. In this study, we show that C4BP maintains its regulatory properties as a cofactor for FI when bound to these four SLRPs and that removal of C4BP from serum significantly increases the degree of complement activation initiated by OSAD, CHAD, and FM. This means that the SLRPs exposed on the degrading cartilage or SLRP fragments released into the synovial fluid of patients with active disease might locally inhibit complement by binding to C4BP. An interesting observation is that those SLRPs that are able to both bind and activate C1q are also capable of binding to FH and C4BP, whereas those SLRPs that are able to only bind but not activate C1q do to bind complement inhibitors (22, 26). C1q and FH do not compete with C4BP for binding to OSAD, CHAD, PRELP, or FM (data not shown), indicating that different complement proteins bind to the SLRPs at different sites. This implies that these interactions are not coincidental but serve some functional purpose. Furthermore, CRP that has been shown to activate complement through binding to the heads of C1q (41) also binds to both FH and C4BP (12, 42). This most likely serves to regulate the level of complement response, preventing excessive complement activation that would be deleterious for the joints. In general, an entirely novel concept emerges regarding interactions of complement with self/alterned self. It appears that many of the endogenous ligands that are able to bind the complement activator C1q also interact with complement inhibitors such as C4BP and FH (in some cases both). This phenomenon allows phagocytosis of the material labeled with C1q, which functions as an opsonin. The low level of complement activation, which occurs as a consequence of these interactions, can also give rise to phagocytosis via receptors for fragments of C3. Importantly, the whole process is kept under tight control by inhibitors of the later stages of the complement cascade (C4BP and FH), which prevent release of C5a and formation of the membrane attack complex and therefore down-regulate inflammation. This is the case for several extracellular matrix proteins as shown in this and other reports (22, 23) but also for apoptotic (43) and necrotic cells, CRP, amyloid deposits in Alzheimer’s disease (44), and human prion proteins (45). This is in contrast with the situation when C1q is recognizing pathogens either via direct binding or, most often, via specific IgG and IgM Abs. These molecules do not capture complement inhibitors and allow full activation.

Taken together, the complement system and the SLRPs appear to engage in many intricate and interdependent interactions that need to be studied in detail to develop therapeutics that would inhibit the proinflammatory potential of the SLRPs.

Acknowledgments

We thank Maria Baumgartner for skilful technical assistance and Viveka Tilgreen for advice.

Disclosures

The authors have no financial conflict of interest.

References
