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Regulation of Complement Activation by C-Reactive Protein: Targeting of the Inhibitory Activity of C4b-Binding Protein

Andreas P. Sjöberg,* Leendert A. Trouw,* Fabian D. G. McGrath,† C. Erik Hack,‡ and Anna M. Blom2*†

C-reactive protein (CRP) is the major acute phase protein in humans. It has been shown that CRP interacts with factor H, an inhibitor of the alternative pathway of complement, and now we demonstrate binding of CRP to the fluid-phase inhibitor of the classical pathway, C4b-binding protein (C4BP). C4BP bound to directly immobilized recombinant CRP as well as CRP attached to phosphocholine. The binding was sensitive to ionic strength and was enhanced in the presence of calcium. C4BP lacking β-chain and protein S, which is a form of C4BP increasing upon inflammation, bound CRP with higher affinity than the C4BP-protein S complex. The binding could not be blocked with mAbs directed against peripheral parts of the α-chains of C4BP while the isolated central core of C4BP obtained by partial proteolytic digestion bound CRP, indicating that the binding site for CRP is localized in the central core of the C4BP molecule. Furthermore, we found complexes in serum from a patient with an elevated CRP level and trace amounts of CRP were also identified in a plasma-derived C4BP preparation. We were also able to detect C4BP-CRP complexes in solution and established that C4BP retains full complement regulatory activity in the presence of CRP. In addition, we found that C4BP can compete with C1q for binding to immobilized CRP and that it inhibits complement activation locally. We hypothesize that CRP limits excessive complement activation on targets via its interactions with both factor H and C4BP. The Journal of Immunology, 2006, 176: 7612–7620.

The human complement system is an important branch of innate immunity and comprises ~35 known plasma and membrane-bound proteins involved in efficient activation and tight regulation of the system. Proper activation of the complement system is crucial for defense against pathogens, removal of apoptotic and necrotic cells, and development of correct Ab responses, whereas excessive or misguided activation contributes to the pathogenesis of most chronic and acute inflammatory diseases. Complement proteins form multimolecular complexes and limited proteolysis is central for both its activation and regulation. The potentially harmful complement system must be carefully regulated and therefore several soluble and membrane-bound proteins are dedicated to this function.

C4b-binding protein (C4BP) is a high molecular mass (570-kDa) plasma glycoprotein, which efficiently inhibits the activation of both the classical and lectin pathways of complement. Apart from preventing the assembly of the C3 convertase (C4b2a complex), it also accelerates the natural decay of the complex (1). In addition, C4BP binds C4b and serves as a cofactor to the plasma serine protease factor I (FI) in the cleavage of C4b both in fluid phase and when C4b is deposited on cell surfaces (2). C4BP is also able to present soluble C3b for cleavage by FI (3). C4BP belongs to a gene family of related proteins named the regulators of complement activation (RCA), which also includes soluble inhibitor factor H (FH) and the membrane-attached proteins complement receptor 1, membrane cofactor protein, and decay accelerating factor (4). Each of the proteins of the RCA family binds C4b and/or C3b and is important for the inhibition of the classical and/or alternative pathways of complement activation. All RCA proteins contain variable numbers of tandemly arranged domains, which are denoted complement control protein (CCP) repeats. The major form of C4BP in plasma consists of seven identical α-chains, each composed of eight CCPs, and one β-chain (three CCPs), all chains being linked together by disulfide bridges in the central core of the molecule formed by C-terminal extensions following the CCPs (2, 5). All β-chain-containing C4BP molecules in the circulation are complexed with protein S (PS), a vitamin K-dependent protein involved in the coagulation system. C4BP is classified as an acute phase reactant, because its plasma level increases during inflammation and after trauma (6–8). It is mainly produced by hepatocytes and the expression of α- and β-chains is differentially regulated by cytokines (9). Furthermore, the plasma level of the isoform of C4BP lacking β-chain, and hence PS, increases to a higher extent in acute phase response as compared with the levels of β-chain-containing C4BP (10).

C-reactive protein (CRP) is a 120-kDa major acute phase protein, which belongs to the family of pentraxins. It consists of five identical subunits and binds a broad variety of ligands including phosphocholine, chromatin, and bacterial Ags (11, 12). Upon inflammation the plasma concentration of CRP may rise >550-fold to reach 0.5 mg/ml. CRP is known to bind bacterial surfaces and to bind the globular heads of C1q and activates the classical

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pathway of complement as a result of this interaction. Furthermore, it has been observed that C3b and C4b are fixed to CRP during complement activation induced by CRP (13). CRP-complement complexes were formed only during CRP-dependent activation and not during activation by other factors, even in the presence of high CRP levels. In the same study, it was observed that most of the C4b deposited on CRP was in a cleaved form, which requires cleavage by FI in the presence of cofactor C4BP.

This observation, along with the reported interaction between CRP and another complement inhibitor FH (14, 15), formed the basis for the present study. In the current article, we show that also C4BP, particularly the form, which is increased in acute phase inflammation, binds CRP, preferably in immobilized form but also in solution. This ionic interaction is enhanced by calcium and mediated by the central core region of C4BP. In addition, we show that CRP can be copurified with C4BP and that C4BP-CRP complexes are present in patient serum known to contain high levels of CRP. Importantly, we found that C4BP decreased complement activation initiated by surface-bound CRP.

Materials and Methods

Proteins and sera

C4BP (16), C1q (17), C4 (18), FH (3), and FI (19) were purified from human plasma as described. Recombinant C4BP (20), monomeric C4BP α-chains (21), and the C4BP central core region (22) were expressed in HEK 293 cells and purified by affinity chromatography using mAb 104 directed against CCP1 of the C4BP α-chain (20). C3b and C4b were purchased from Advanced Research Technologies and recombinant CRP was obtained from Calbiochem. All of the proteins were at least 95% pure, as judged by Coomassie brilliant blue (Serva) staining of proteins separated by SDS-PAGE. All proteins were stored at −80°C. PC-BSA was prepared by coupling p-aminophenylphosphoryl-choline to BSA according to the procedure described by Padilla et al. (23). CRP was labeled with 125I using the chloramine-T method reaching a specific activity of 0.4–0.5 MBq/μg of protein. Normal human serum (NHS) was obtained from freshly drawn blood from healthy volunteers and was allowed to clot for 30 min at room temperature (RT) with all further steps on ice; individual sera were pooled and stored in aliquots at −70°C or used to generate depleted serum. Human serum deficient in C4BP-PS was prepared by passing fresh serum through a HiTrap column (GE Healthcare) coupled with mAb 104 (24). The flow-through was collected and the depleted serum was stored in aliquots at −70°C.

Direct ligand-binding assay

To test the binding of several ligands to immobilized CRP, microtiter plates (Maxisorb; Nunc) were incubated overnight at 4°C with 50 μl of solution containing 7 μg/ml CRP or 2.5 μg/ml phosphorylcholine conjugated with BSA (PC-BSA) in 75 mM sodium carbonate (pH 9.6). Coating buffer only was used as blank. The wells were washed three times with 150 mM veronal buffer (VB)-BSA (150 mM VB, 2 mM CaCl2, 0.05% BSA (Sigma-Aldrich) (w/v), 0.1% Tween 20 (w/v), pH 7.5) or 50 mM VB-BSA and then blocked for 2 h at RT with 200 μl of VB-BSA. CRP (7 μg/ml) in VB-BSA was used to coat the PC-BSA-coated wells for 4 h at RT. After another three washes with VB-BSA, increasing concentrations of C4BPrec, C4BP-PS, FH, or C1q were added in VB-BSA and the plates were incubated for 3 h at RT. The wells were then washed three times with VB-BSA and incubated with Abs against C4BP (mAb 104), FH (goat; Quidel), or C1q (rabbit; DakoCytomation) diluted in VB-BSA, followed by species-matched, HRP-conjugated secondary Abs (DakoCytomation). The plates were then washed and developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (DakoCytomation) and H2O2 and absorbance at 490 nm was determined. For assessment of ionic strength dependence, the same basic assay was used. However, 30 μg/ml of each binding protein was incubated in VB-BSA of varying NaCl concentrations, ranging from 25 to 350 mM, and all steps after blocking were conducted in buffers of corresponding ionic strength. The binding assay assessing calcium dependence was performed following the same protocol but the VB-BSA was either devoid of CaCl2 or supplemented with 2 mM CaCl2 or 5 mM EDTA. For this assay, 10 μg/ml CRP was immobilized and 20 μg/ml C4BP was incubated in the wells.

To test the binding of fluid-phase CRP to immobilized C4BP, the mAb 104 directed against CCP1 of C4BP was coated onto microtiter plates at 5 μg/ml in coating buffer. After blocking, 10 μg/ml C4BP-PS or C4BP-prec was incubated in 50 mM VB-BSA. Buffer only was used as negative control. The wells were incubated with increasing concentrations of CRP, after which detection followed using polyclonal Abs against CRP (DakoCytomation), HRP-conjugated secondary rabbit Abs, and the OPD enzyme system. Washing and incubation times were the same as described for the binding assay above. With 10 μg/ml C4BP-prec, 10 μg/ml of the central core portion or monovalent α-chain was immobilized directly on the plate or via anti-C4BP mAb 104, respectively.

Surface plasmon resonance (Biacore)

The interaction between CRP and C4BP was analyzed using surface plasmon resonance (Biacore 2000; Biacore). Two flow cells of a CM5 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-hydroxy-succinimide at a flow rate of 5 μl/min, after which recombinant CRP (10 μg/ml in 10 mM sodium acetate buffer, pH 4.5) was injected over flow cell 2 to reach 2000 resonance units (RU). Unreacted groups were blocked with 20 μl of 1 M ethanolamine (pH 8.5). A negative control was prepared by activating and subsequently blocking the surface of flow cell 1. The association kinetics were studied for various concentrations of the C4BP variants (C4BP-rec, C4BP-prec) and its truncated substrate (DakoCytomation) and H2O2 and absorbance at 490 nm was determined. For assessment of ionic strength dependence, the same basic assay was used for the following steps. After blocking, the binding of the proteins was performed in two steps. The wells were either incubated separately with 16 μg/ml C4BP-prec followed by 10 μg/ml CRP or first incubated with buffer only (50 mM VB-BSA) and then with C4BPrec, which had been coincubated with CRP at 16 and 10 μg/ml respectively. Both steps were conducted for 1.5 h at 37°C. A biotinylated mAb against CRP (13) in combination with HRP-conjugated StreptABC complex (DakoCytomation) and the OPD substrate was used for detection and development.

Competition assay

The ability for C4BP and C1q to compete for the binding to immobilized CRP was assessed by using a modified binding assay protocol as described above. The changes were as follows: in the binding step, 5 μg/ml C1q or 10 μg/ml C4BP-prec was incubated in the CRP-coated wells along with increasing concentrations of C4BP or C1q, respectively. Monoclonal anti-C4BP Abs (mAb104) or polyclonal anti-C1q Abs (DakoCytomation), in combination with species-specific HRP-conjugated secondary Abs were used to detect bound protein.

Complement deposition on immobilized CRP

CRP and aggregated hlgG were coated onto Maxisorb microtiter plates in 75 mM sodium carbonate buffer (pH 9.6) overnight at 4°C. Wells incubated with buffer only were used as negative controls. The concentrations used for CRP and hlgG were 7 and 5 μg/ml, respectively. Between each step, the plates were washed four times with 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5). The wells were blocked with 1% BSA (Sigma-Aldrich) in PBS for 2 h at RT. Normal human serum or C4BP-depleted serum was diluted in GVB + buffer (2.5 mM VB (pH 7.3), 150 mM NaCl, 0.1% gelatin, 1 mM MgCl2, and 0.15 mM CaCl2) and incubated in the wells for 30 min at 37°C with shaking. Complement activation was assessed by detecting deposited C3b with polyclonal anti-C3d Abs (DakoCytomation) diluted in block buffer. Bound Ab was revealed with HRP-labeled anti-rabbit Abs and the OPD substrate.

C4b degradation assay

C4BPrec (100 μM) was mixed with 250 mM C4b, 60 mM FL and trace amounts of 125I-labeled C4b in 30 mM Tris-HCl (pH 7.4). CRP was added to final concentrations ranging from 0 to 100 μg/ml. A negative control
lacking FI was also included. The samples were incubated for 1.5 h at 37°C and the reaction was terminated by the addition of SDS-PAGE sample buffer with reducing agent (dithiothreitol). The samples were then incubated at 95°C for 3 min and applied to a 10–15% gradient SDS-PAGE. The separated proteins were visualized and quantified using a PhosphoImager (Molecular Dynamics).

Detection of CRP-C4BP complexes in serum

To test whether elevated C4BP level would correlate with circulating CRP-C4BP complexes, we used serum from a sarcoma patient receiving TNF-α limb perfusion (23). The serum had an elevated C4BP level (53 μg/ml) and was subjected to gel filtration (Superose 6, Akta Explorer; GE Health Care) in 50 mM Tris-HCl and 150 mM NaCl (pH 8.5) supplemented with 2 mM CaCl₂ to assay for CRP-C4BP complexes. Fractions of 200 μl were collected and CRP as well as C4BP levels were quantified and compared with the levels of CRP-C4BP complexes. Size markers were applied to the column in separate runs: blue dextran (void volume), albumin (67 kDa), catalase (232 kDa), and thyroglobulin (669 kDa). C4BP concentration in the fractions was determined by ELISA performed as follows: a polyclonal rabbit anti-C4BP Ab (in-house) was coated onto microtiter plates at 2.5 μg/ml in 75 mM sodium carbonate (pH 9.6) at 4°C overnight. Between each step, the plates were washed four times with 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5). The wells were blocked for 1 h at 37°C with blocking buffer (3% fish gelatin in wash buffer) followed by incubation with the gel filtration fractions at 1/1000 in blocking buffer. Bound C4BP was detected with anti-C4BP mAb in 104 blocking buffer, 1 h at 37°C, and HRP-conjugated anti-rabbit Abs in blocking buffer, 1 h at 37°C. Bound secondary Abs were visualized using the OPD substrate.

CRP was detected using an identical protocol except for the catching and detection Abs used. The plates were coated with polyclonal rabbit Abs against CRP (DakoCytomation) and bound CRP was detected with the monoclonal CRP Ab 5G4 (13).

CRP-C4BP complexes were detected with a similar ELISA protocol as for C4BP, except for the following changes. Rabbit CRP Abs were used for coating, the fractions were diluted 1/20, and biotinylated mAb 104 against C4BP in combination with HRP-conjugated StreptABC complex were used to detect bound C4BP.

Furthermore, we performed a Western blot detecting C4b in gel filtration fractions containing C4BP. Proteins were separated on 12% SDS-PAGE under reducing conditions. The gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Pall), which was blocked and stained for C4 using an anti-C4c Ab (DakoCytomation) followed by HRP-conjugated anti-rabbit Ab.

Detection of CRP in a C4BP preparation purified from human plasma

Affinity purified plasma-derived C4BP-PS (25 μg), C4BPrec (25 μg), and CRP (0.1 μg) were separated on a 12% SDS-PAGE under reducing conditions. The gel was blotted onto a PVDF membrane (Pall) and the membrane was incubated with blocking buffer for 1 h at RT. Goat anti-CRP Abs (DakoCytomation) diluted in blocking buffer and HRP-conjugated goat anti-rabbit Abs in blocking buffer were used to detect CRP. Each incubation was performed for 1 h at RT and between each step the membrane was washed four times, 5 min per wash in 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5). The membrane was developed with 0.1% (w/v) diaminobenzidine (Sigma-Aldrich), 0.03% (w/v) H₂O₂, and 0.06% (w/v) NiCl₂ in TBS and the reaction was stopped with repeated washes in H₂O. In parallel, an identical gel run with less protein (C4BP-PS and C4BPrec: 4 μg, CRP: 1 μg) was stained with Coomasie brilliant blue (Serva).

Statistics

Differences between conditions were analyzed for significance by using Student’s t test. A value of p < 0.05 was considered significant, and p < 0.01 is labeled with ** in the figures.

Results

C4BP binds CRP

To study direct binding between CRP and C4BP, we have used a microtiter plate-based assay where the plates were coated with CRP directly or with CRP bound to its natural ligand PC. Throughout the study, we have used four different forms of C4BP varying in subunit composition as presented in Fig. 1. First, we detected binding between CRP and both C4BP-PS isolated from human plasma (seven α-chains, one β-chain, bound PS) and recombinant C4BPrec (α6β0) at physiological ionic strength. CRP was directly immobilized and increasing concentrations of C4BPrec, C4BP-PS as well as two established ligands of CRP, C1q and FH, were then added (Fig. 2, upper panel). Of the four tested proteins, C1q bound CRP strongest followed by the two forms of C4BP and the weak binding of FH. This indicates that the binding of C4BP to CRP is not mediated by the β-chain. To study even the relatively weak interactions such as the one between CRP and FH, we performed additional experiments in a buffer of 50 mM ionic strength. We found that both C4BP forms bound strongly to CRP immobilized directly on the plates (Fig. 2, middle panels) whereas the binding to CRP bound to PC-BSA was less pronounced although significant. C1q bound equally well as C4BP to CRP-PC-BSA and directly immobilized CRP and it also showed significant binding to PC-BSA alone (Fig. 2, lower left panel). FH bound well to immobilized CRP but its binding to the CRP-PC-BSA complex was identical as to PC-BSA alone (Fig. 2, middle lower right panel).

Since there was a significant difference in binding of several ligands to CRP vs CRP-PC-BSA, we also performed experiments with the purpose of establishing how much CRP is bound to microtiter wells directly as compared with wells precoated with PC-BSA. We found that CRP appears to bind only slightly better to empty wells than to PC-BSA (36% vs 19% of 7 μg/ml applied CRP becomes immobilized, respectively; data not shown). We therefore conclude that the differences we noted in the assays determining binding between C4BP and CRP provide an accurate account of the differences in capacity for C4BP to bind CRP in the presence or absence of PC-BSA.

Next, we studied whether the interaction also could occur between immobilized C4BP and CRP in the fluid phase. Microtiter plates were coated with mAb directed against CCP1 (mAb 104), the most peripheral of the tandemly arranged CCP domains making up the C4BP α-chain. Both plasma-derived C4BP-PS and C4BPrec were allowed to bind to the Abs and their respective interaction to CRP was evaluated. The C4BP-PS complex showed clear binding at higher CRP concentrations, and we could detect an even stronger interaction with C4BPrec (Fig. 3A).

C4BP binds CRP via the C-terminal region of the α-chain (central core)

To further elucidate the characteristics of the C4BP-CRP interaction, we tested the ability of fragments of the C4BP α-chain to bind

![FIGURE 1. The various forms of C4BP used in this study. The majority of C4BP molecules in circulation consist of seven α-chains and one β-chain in complex with PS (α7β1 + PS). C4BPrec is made up of six α-chains but contains no β-chain (α6β0). A variant that does not exist naturally in the circulation is plasma-derived C4BP with artificially removed PS (α7β1). A minor fraction of C4BP in plasma, which becomes up-regulated upon inflammation, lacks the β-chain and hence also PS (α7β0).](http://www.jimmunol.org/)

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to CRP. Recombinant polymeric C4BP and a shortened, monomeric variant of C4BP lacking the H9251 C-terminus were bound to mAb 104 immobilized on microtiter plates. CRP interacted only with the polymeric and not the monomeric form of C4BP. Furthermore, we have generated the central core region of C4BP/H9251 chains by partial proteolytic cleavage and purification of obtained fragments. Fig. 3 shows that CRP binds the central core of C4BP directly immobilized on microtiter plates. These results indicate that CRP interacts with the central core of C4BP formed by the C-terminal extensions of the H9251 chains rather than with its extended arms.

Surface plasmon resonance studies of the C4BP-CRP interaction

CRP was immobilized covalently on a CM5 chip and the various forms of C4BP (outlined in Fig. 1) were injected over the surface in a buffer containing 50 mM NaCl. The association and dissociation phases of binding were recorded and used for calculation of equilibrium affinity constants ($K_D$). Representative sensograms used for construction of a binding curve used for calculations with Biacore software are shown in Fig. 4. The affinity was highest for both recombinant C4BP composed of six $\alpha$-chains and plasma C4BP without $\beta$-chain and PS (see table in Fig. 4). C4BP containing the $\beta$-chain but no PS bound weaker and finally the major form of C4BP in plasma, with $\beta$-chain and in complex with PS, bound weakest. This provided further evidence that the binding site for CRP is localized near the central core of the molecule where all subunits are held together. Accordingly we could not block the binding with mAbs directed against extended parts of $\alpha$-chains of C4BP and deletion mutants lacking individual domains (CCP1 to CCP8) bound well (data not shown).

The C4BP-CRP interaction is electrostatic in nature and is enhanced by calcium

Many interactions in which CRP is involved are based on ionic contacts between charged amino acids. To evaluate the nature of the C4BP-CRP binding in this sense C4BP-PS, C4Bprec, C1q, and

![Figure 2. Binding of C4BP, C1q, and FH to immobilized CRP. Upper panel, CRP was coated directly in microtiter plates and C4Bprec, plasma-derived C4BP-PS, C1q, and FH were incubated in a buffer with physiological ionic strength (150 mM). Bound proteins were detected with monoclonal (C4BP) or polyvalent Abs (FH, C1q). Middle and lower panels, CRP was either directly immobilized in microtiter plates or bound to PC-BSA covalently attached to the plates. C4Bprec, C4BP-PS, C1q, and FH were then incubated at various concentrations in a buffer with low ionic strength (50 mM), and the amounts of bound proteins were detected with Abs. Data were standardized, except in the upper panel and are given as means and SD of triplicates.](http://www.jimmunol.org/)

![Figure 3. Binding of CRP in the fluid phase to immobilized intact and truncated C4BP. A, C4Bprec and plasma-derived C4BP-PS were bound to microtiter plates coated with mAb 104 directed against CCP1 of the C4BP $\alpha$-chain. CRP was then introduced and the amount of CRP bound to C4BP was detected with a polyclonal Ab. B, Monomeric C4BP $\alpha$-chains comprising all parts of the chain (CCP1–8) except for the C-terminal extension were immobilized on the plates via mAb 104. The center core portion of C4BP composed of CCP8 and C-terminal extensions was coated directly onto the plate. CRP was incubated at various concentrations and bound protein was detected using a polyclonal Ab. Data are standardized and are given as means and SD of triplicates.](http://www.jimmunol.org/)

![Figure 4. Biacore analysis of CRP-C4BP interaction. A typical sensogram showing the result of the injection of increasing concentrations (in milligrams per milliliter) of C4Bprec over a Biacore CM5 chip with covalently coupled CRP. The detected binding is expressed in arbitrary resonance units. Presented in the table are the equilibrium affinity constants for the binding between CRP and the various C4BP variants determined using Biacore.](http://www.jimmunol.org/)
FH were all incubated in buffers of varying ionic strength on CRP immobilized directly on plates. Bound proteins were detected with specific Abs and the results clearly show that all four interactions are of ionic nature (Fig. 5A). Interestingly, the FH-CRP interaction was the one most sensitive to an increase in ionic strength. We next performed the assay with CRP bound to PC-BSA with similar results (Fig. 5B). The binding between C1q or CRP and PC-BSA, however, proved to be much more resistant to increased NaCl concentration than the interactions with C4BP variants and FH.

We have also tested whether the interaction between C4BP and CRP is calcium dependent and we found that the interaction is strongest in buffer containing calcium and that there is a significant although not dramatic decrease using a buffer without calcium or a buffer with EDTA (Fig. 5C).

CRP forms complexes with C4BP in solution

The ability of C4BP to bind to immobilized CRP may already be a valuable tool for modulation of complement activation during the acute phase of inflammation, but we also wanted to examine whether complexes can be formed in a solution. To achieve this, we coincubated CRP and C4BPprec in solution, added this solution to microtiter plates coated with Abs specific for C4BP, and then detected the CRP attached to C4BP using Abs against CRP. In parallel, using the same coating and detection conditions, we incubated the plates with only C4BP, washed them, and next incubated them with only CRP followed by detection of bound CRP. When comparing the data from separate incubations with coincubated proteins, no difference could be detected in binding efficiency (Fig. 6). This indicates that CRP and C4BP can form complexes in solution.

C4BP competes with C1q for binding to CRP

To determine whether C1q and C4BP can bind CRP simultaneously or whether they share binding sites, we used a competition assay in which CRP was immobilized in microtiter plates and C1q and C4BP were added together. One of the proteins was added at a constant concentration while titration of the other protein was performed. The experiments showed that even though we added significant amounts of C1q to a solution with fixed C4BP concentration, no decrease in C4BP-binding to CRP could be detected. However, under reversed conditions we were able to detect a significant decrease in C1q binding to CRP in the presence of C4BP (Fig. 7A).

C4BP decreases complement activation triggered by CRP

To assess functional consequences of the interaction between CRP and C4BP, we devised a complement activation assay where NHS or the same serum depleted of C4BP was allowed to react in the presence of CRP. We measured the amount of C3b deposited in CRP-coated microtiter plates and compared the values determined...
CRP does not inhibit the ability of C4BP to serve as cofactor for FI

The ability of C4BP to act as a cofactor for FI mediated degradation of C4b in the presence of CRP was tested using I125-labeled C4b. C4b is degraded to C4d and C4c fragments in the presence of FI and a cofactor such as C4BP. Detection of radioactively labeled C4b. C4b is degraded to C4d and C4c fragments in the presence of CRP does not inhibit the cofactor activity of C4BP (Fig. 8). This indicates that the binding of CRP to C4BP does not influence the capacity of C4BP to function as a cofactor for FI-mediated degradation of C4b in the fluid phase. The experiment was repeated with the same outcome using the highest CRP concentration and various incubation buffers such as Biacore buffer or 150 mM VB-BSA (data not shown).

CRP-C4BP complexes detected in serum with elevated CRP level

We wanted to establish whether complexes between CRP and C4BP are detectable in the circulation. To this end, we collected serum from a patient with a CRP level of ~50 μg/ml. The serum was subjected to gel filtration in a buffer with physiological ionic strength, and the collected fractions were analyzed for total protein content as well as presence of C4BP, CRP, and CRP-C4BP complexes. As expected, C4BP, which is a large protein of 570 kDa, migrated quickly through the column as a rather narrow peak in the initial section of the A280 nm plot (Fig. 9A). When comparing these data with the results from the CRP-C4BP complex ELISA (Abs against C4BP serving as capture and Abs against CRP used for detection), a partially overlapping pattern was observed (Fig. 9A).

**FIGURE 8.** CRP does not inhibit FI cofactor activity of C4BP. C4BP-PS (200 nM) was incubated with 250 nM C4b, 60 nM FI, trace amounts of I125-labeled C4b, and increasing concentrations of CRP. Immediately after the reaction, sample buffer with reducing agent was added, samples were heated at 95°C, and the proteins were separated by SDS-PAGE (10–15%). The gel was dried and subjected to autoradiography. As a negative control, FI was omitted in the incubation mixture (denoted –FI). The lane denoted 0 included FI but no CRP. Arrows indicate bands corresponding to the three C4b subunits (α, β, and γ) and the degradation product C4d.

Free CRP eluted from the column as a broad peak with the major part eluting later than the major C4BP peak, but partially overlapping the C4b-containing fractions. Most of the C4BP-CRP complexes eluted earlier than free C4BP, indicating that it was of higher molecular mass. We also performed a Western blot for C4b content in the gel filtration fractions containing C4BP (data not shown). This was to assess whether a part of the CRP-C4BP complexes might be mediated by C4BP bound to C4b deposited on CRP. We were able to detect minute amounts of C4b in most of the complex containing fractions but these traces cannot account for the bulk of the C4BP-CRP complexes. Taken together, these results imply that a fraction of CRP in serum is indeed bound to C4BP and elutes as a high molecular complex. We have been able to detect similar complexes in several sera with increased CRP levels but not in NHS (data not shown).

CRP can be copurified with C4BP from human plasma

Further evidence for the presence of CRP-C4BP complexes in vivo comes from the detection of small quantities of CRP in a preparation of C4BP from pooled human plasma purchased from a local blood bank. C4BP was purified using affinity chromatography with mAb 104 against C4BP-α-chains coupled to a Bio-Rad affigel column. The column was washed with 1 M NaCl before elution and bound protein was eluted with guanidinium chloride (16). Using Western blot detecting CRP, we were able to detect a positive signal for CRP in plasma-derived C4BP-PS but not in recombinantly expressed C4BP (Fig. 9B). A Coomassie-stained gel ran in parallel showed that the preparations were 95% pure and the band corresponding to CRP could not be detected with this staining (data not shown). We have assayed several preparations of C4BP purified in our laboratory and they contained varying small amounts of CRP, most probably dependent on CRP level in plasma used for purification.

**Discussion**

During the first stages of the acute phase response, a multitude of systemic events are triggered, most of which promote a proinflammatory state. The increased expression and release of acute phase reactants such as CRP, serum amyloid A component, and various complement components mediate many of these proinflammatory effects. To prevent adverse consequences for host tissues, counteracting mechanisms function to balance the inflammatory actions. As a result of its complexity, the regulation of the acute phase response is only partially understood. The serum levels of several complement components of importance to both the classical and alternative pathways are increased during the acute phase of inflammation, although to varying degrees. C3, for instance, is up-regulated by 50–100%, while C4BP levels have been reported to increase by almost 300% (8) during the initiation of an acute phase response.

The alternative pathway complement regulator FH has previously been shown to bind to CRP (15, 25), leading to the hypothesis that CRP could help to control the disproportionate complement attack during the early stages of inflammation. This could be achieved by attenuation of the alternative pathway amplification loop and inhibition of the C5 convertase. However, since CRP also binds and activates C1, triggering the classical pathway, it would be reasonable to assume that CRP could need a helping hand to regulate also this route of complement activation. Because of the general nature of the enhancement of immune efficiency during acute phase response, not only invading microorganisms run a higher risk of being attacked. Already compromised areas of host
well as CRP-C4BP complexes using ELISA. The maximum value in each assay was set to 1 and the data are standardized against this. The CRP-C4BP complexes obtained during gel filtration of serum. Therefore, it

FIGURE 9. Complexes between CRP and C4BP detected in serum and CRP detected in purified plasma-derived C4BP-PS. A. Serum with ~50 μg/ml CRP was subjected to gel filtration on a Superose 6 column, and the collected fractions were analyzed for total protein content (A280) and CRP, C4BP, as well as CRP-C4BP complexes using ELISA. The maximum value in each assay was set to 1 and the data are standardized against this. The CRP-C4BP complex graph represents the mean of triplicate readings. Elution volumes of molecular mass standards run separately are indicated with arrows. B. CRP, C4BP, C4BP-prec, and C4BP-PS were separated on 12% SDS-PAGE under reducing conditions, blotted onto a PVDF membrane, and CRP was detected with specific polyclonal Abs. Sizes of molecular mass standards are indicated on the left.

tissue can turn apoptotic or even necrotic, and unchecked complement attack may exacerbate the damage further. CRP binds apoptotic cell membranes via exposed PC moieties in a calcium-dependent manner (26). A number of pathogenic microorganisms express PC and the interactions with CRP have been implicated in opsonization for phagocytosis as well as in the initiation of complement-mediated lysis (27).

In the present study, we propose a novel regulating mechanism where C4BP, a major inhibitor of the classical and lectin pathways of complement, via direct binding to CRP may inhibit excessive complement activation during the acute phase response.

C4BP has previously been reported to bind to serum amyloid P component (SAP) (22), which is a member of the pentraxin family and has an overall structure strikingly similar to that of CRP. SAP is one of the major acute phase reactants in mice. Interestingly, the plasma levels of mouse CRP rise only slightly during early inflammation while SAP only plays a minor role in the human acute phase response. SAP is a universal constituent of amyloid deposits and is hypothesized to take part in clearance of cellular debris and innate immunity. It has been reported to bind a wide variety of ligands including various bacteria, influenza virus, LPS, and a number of matrix components (28). Similar to our findings for CRP, the C4BP binding site for SAP is located in the central core region of the protein, suggesting an analogous interaction between the two pentraxins and C4BP. Contrary to what we have found in the present study, previously published data indicated that SAP and CRP from rat interact with C4BP while human CRP is unable to bind human C4BP (29). The initial study did not investigate complexes between C4BP and CRP in a situation when one of the ligands is immobilized. In our hands, such complexes were easier to detect than C4BP-CRP complexes in a solution, which was the focus of the previous study. Apparently the analytical centrifugation used in the initial study was not a suitably sensitive method for detection of complexes in a solution.

We could easily detect binding of C4BP to immobilized CRP in physiological ionic strength (Fig. 2). We have also performed several experiments at low ionic strength (50 mM) to be able to pick up even interactions of low affinity such as the FH-CRP interaction. C4BP-CRP interaction occurs readily when CRP is immobilized but we have not been successful in repeating the results shown in Fig. 6 (complex formation in a solution) in a buffer of physiological ionic strength. Potentially, in the pure in vitro setting in this binding assay, some of the requirements for an efficient interaction between CRP and C4BP are lost. However, we believe that the interaction between CRP and C4BP will be most important when CRP is immobilized on a solid surface such as the cell membrane of an apoptotic cell. In this context, complexes formed in solution are of secondary importance. Moreover, as presented in Fig. 9, we have been able to detect complexes in serum with elevated CRP levels, thus providing proof that the interaction does in fact occur in the fluid phase or could be secondary to an event where C4BP and CRP interacted while in the solid phase.

Interestingly, we observed that C4BP could compete with the binding of C1q to immobilized CRP. The reverse effect was not seen. These results may be explained by differences in binding properties between the two proteins. Possibly C1q needs areas surrounding its binding site for a stable interaction while C4BP is bound tightly even in the nearby presence of C1q. CRP may also be able to bind more than one C4BP molecule and since only one C1q molecule can bind each CRP molecule this property may be influential in a competing situation. It remains uncertain where the binding site for C4BP is located on the CRP molecule. On one side, C4BP interacts better with CRP directly immobilized on plates than when it is bound to its ligand PC-BSA, implying that perhaps the binding site for C4BP is overlapping with that for PC-BSA. In contrast, C4BP is able to compete with C1q and C1q is known to interact with CRP’s opposite side. Perhaps C4BP binds to the rim of CRP partially interacting with both sides of CRP.

Most importantly, the inhibitory effect of C4BP on C1q binding to CRP may be an additional factor decreasing complement activation on CRP-coated surfaces. When assessing the ability of CRP to trigger complement activation, we were able to detect a significant increase in C3b deposition in the absence of C4BP as compared with deposition in NHS. This supports our hypothesis that C4BP may indeed serve to decrease complement attack on surfaces covered with CRP. We were also able to detect complexes between CRP and C4BP in human serum with increased CRP levels. In addition to this, we identified CRP in an affinity-purified sample of plasma-derived C4BP-PS. The existence of C4BP-CRP complexes in the circulation further supports an in vivo function for the binding during acute phase inflammation. We have detected small amounts of C4b in the fractions containing C4BP-CRP complexes obtained during gel filtration of serum. Therefore, it
is possible that in vivo the C4BP-CRP binding is further strengthened by interaction between CRP-bound C4b and C4BP. However, one has to keep in mind that we can clearly detect CRP-C4BP in the absence of C4b or serum.

Previously, we have shown the binding of the C4BP-PS complex to apoptotic (30) as well as necrotic (31) cells. The binding of this complement inhibitor was shown to have several biologically important functions such as limiting the overall proinflammatory potential of apoptotic and especially necrotic cells. More specifically, the interaction between the C4BP-PS complex and apoptotic cells was shown to be dependent on PS. During an acute phase response especially the C4BP form without β-chain and PS is produced to ensure stable-free PS levels. This particular form of C4BP does not have the capacity to directly bind to apoptotic cells. Our results lead us to hypothesize that CRP can act as a bridge between apoptotic cells and the inflammatory form of C4BP, providing a safety mechanism against even further deleterious complement attack on injured host cells. However, we were unable to show increased binding of purified C4BP of any molecular form to apoptotic cells by coinubcation in the presence of CRP (data not shown).

CRP has in the recent years gained importance as a key player in the formation and maintenance of atherosclerotic plaques and as a major risk factor for cardiovascular disease (reviewed in Refs. 32 and 33). Via interaction with the FcγII receptor, CRP induces expression of metalloproteinases, which may render atherosclerotic plaques unstable. Increased leukocyte chemotaxis has also been associated with CRP in atherosclerotic arterial intima. Although the origin of CRP deposited in atherosclerotic lesions is disputed at the moment (34, 35), it is clear that the elevated CRP concentration constitutes a detrimental factor in cardiovascular disease. The complement-activating properties of CRP are in this context highly interesting. A number of recent studies show connections among complement activity, atherosclerosis, and cardiovascular disease (36). Complement activation into the terminal pathway with membrane attack complex formation has been indicated to be required for full maturation of the atherosclerotic plaques. Serum C3 and increased C5a levels serve as inflammatory predictors of cardiovascular disease (37, 38). Furthermore, sublytic assembly of membrane attack complex induces activation and proliferation of endothelial cells, which can lead to thickening of the vascular wall. CRP activates the classical pathway of complement via its interaction with C1q while the binding to FH has been postulated to reduce terminal pathway activation (39). Moreover, CRP has been reported to increase the endothelial expression of membrane-bound complement regulators (40). Additional regulation of complement activation on endothelial cells may be mediated by the interaction between CRP and C4BP reported in this study. We speculate that the interaction between CRP and C4BP may be of importance in the very acute phase of inflammation and in foci of tissue degeneration.

In conclusion, we have found a strong interaction between CRP and the complement regulator C4BP. The interaction is localized to the central core of C4BP and the C4BP variant predominantly expressed during inflammation binds to CRP with the highest affinity. Moreover, we show that C4BP limits complement activation on CRP. We propose that while CRP binding to targets will enhance C1q binding and opsonization, binding of fluid-phase complement regulators FH and C4BP will limit the complement-mediated damage to host tissue.

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Disclosures
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References