A Cluster of Positively Charged Amino Acids in the C4BP α-Chain Is Crucial for C4b Binding and Factor I Cofactor Function*°

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C4b-binding protein (C4BP) is a regulator of the classical complement pathway, acting as a cofactor to factor I in the degradation of C4b. Computer modeling and structural analysis predicted a cluster of positively charged amino acids at the interface between complement control protein modules 1 and 2 of the C4BP α-chain, which is linked in C4b binding. Three C4BP mutants, R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q, were expressed and assayed for their ability to bind C4b and to function as factor I cofactors. The apparent affinities of R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q for immobilized C4b were 15-, 50-, and 140-fold lower, respectively, than that of recombinant wild type C4BP. The C4b binding site demonstrated herein was also found to be a specific heparin binding site. In C4b degradation, the mutants demonstrated decreased ability to serve as factor I cofactors. In particular, the R39Q/R64Q/R66Q mutant was inefficient as cofactor for cleavage of the Arg1317–Thr1318 peptide bond in C4b. In contrast, the factor I mediated cleavage of Arg1317–Asn1318 bond was less affected by the C4BP mutations. In conclusion, we identify a cluster of amino acids that is part of a C4b binding site involved in the regulation of the complement system.

The human complement system comprises about 35 known plasma- and membrane-bound proteins involved in efficient activation and tight regulation of the system. Complement proteins form multi-molecular complexes, and limited proteolysis is central for both activation and regulation. Factor I is a serine protease responsible for down-regulation of both classical and alternative pathways of complement. Factor I degrades C1r, C1s, and C4b-binding protein (C4b),1 respectively (1, 2). The human complement system comprises about 35 known plasma- and membrane-bound proteins involved in efficient activation and tight regulation of the system. Complement proteins form multi-molecular complexes, and limited proteolysis is central for both activation and regulation. Factor I is a serine protease responsible for down-regulation of both classical and alternative pathways of complement. Factor I degrades C1r, C1s, and C4b-binding protein (C4b),1 respectively (1, 2). C4BP is a large plasma protein (molecular mass, ∼570 kDa) consisting of seven identical α-chains and a unique β-chain linked together by disulfide bridges (3, 4). The α- and β-chains contain eight and three complement control protein (CCP) modules, respectively (5). CCP modules consist of approximately 60 amino acids forming a compact hydrophobic core surrounded by five or more β-strands organized into β-sheets (6). Electron microscopy of C4BP demonstrated a spider-like conformation, with the seven α-chains forming extended tentacles (7, 8). S. pyogenes bacterial surface proteins (12, 26), and recent work has suggested that Arg 66 and/or His67 is involved in the C4bC2a complex, which is the classical pathway C3 convertase (14). C4, which is the precursor of C4b, is composed of a 93-kDa α-chain, a β-chain (75 kDa), and a 32-kDa γ-chain, which are linked by disulfide bridges (15–17). During complement activation, C1s cleaves a 9-kDa fragment (C4a) from the N terminus of the α-chain (18, 19). The remaining part of the molecule (C4b) acquires transient capability to bind amino and hydroxyl groups via a reactive glutamyl residue that is exposed upon cleavage of an internal thiolester bond (20). C4b-like molecules can also be generated from C4 by treatment with amines, chaotropes, or repeated freezing and thawing, which result in the cleavage of the internal thiolester bond without liberation of C4a (21).

Each α-chain of C4BP contains a C4b binding site, but, most likely due to steric hindrance, only up to four C4b molecules can bind to one C4BP molecule (22). Several different regions of the α-chains have been suggested to be involved in C4b binding. Initially, an N-terminal 48-kDa α-chain fragment, formed by chymotrypsin digestion, was found to bind C4b and to express factor I cofactor activity (23, 24). This agreed well with electron microscopy results, which demonstrated C4b binding to the peripheral end of each C4BP tentacle (7). Several reports agree with the concept that the three most N-terminal CCPs are necessary and sufficient for C4b binding (25–27). The C4b binding site in human C4BP partially overlaps with the binding site for the S. pyogenes surface proteins (12, 26), and recently, it was suggested that Arg66 and/or His67 is involved in these interactions (26).

The elucidation of the regulatory mechanisms of complement is hindered by the lack of structural information. To overcome these limitations, we have used a combination of molecular modeling and site-directed mutagenesis to study the interaction between C4b and C4BP. In a recent study, we identified a cluster of positively charged amino acids present at the surface of the CCP1–2 modules of the C4BP α-chain as a potential binding site for C4b and heparin (28). We now report results...
obtained with a series of C4BP mutants (R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q), which show this cluster to be crucial for binding of both C4b and heparin.

EXPERIMENTAL PROCEDURES

Materials—The sensor chip CM5 and amine coupling kit were from Biacore AB. Molecular weight markers for electrophoresis were from Amersham Pharmacia Biotech. Lipofectin, Opti-MEM, and Dulbecco’s modified Eagle’s medium were from Life Technologies, Inc. Chondroitin sulfate and streptavidin were from Sigma, fractionated heparin was from Innovagel, and low molecular weight heparin (Pragrum) and hyaluronan (Healon) were from Amersham Pharmacia Biotech.

Protein Modeling and Geometry Optimization—The reported C4BP model (28) was based on the structure of a CCP pair from factor H. Since then, NMR coordinates of another CCP pair (CCP3-4 of the vaccinia virus complement protein VCP) (29) have become available. However, based on additional modeling work and analysis of our experimental data, we conclude that CCP15 and CCP16 of factor H (30) were the best starting templates to build CCP1 and CCP2 of C4BP, respectively. Our initial model was refined via molecular dynamics simulation using five different simulation protocols (e.g. different set of partial charges, different dielectric or tethering constants). The same procedure was also applied to the VCP and factor H NMR pairs. We performed calculations in order to select the best set of parameters. The structural refinement of the initial model included energy minimization in vacuo using Discover (Biosym-MSI). All calculations were carried out using the CVFF force field parameters and a 20-Å cut-off distance for nonbonded interactions. Hydrogen atoms were added to the model, and partial charges were assigned to all atoms. In an effort to account for the lack of solvent and ions, potentially charged residues were given appropriate parameters to obtain electrostatic neutrality (31). A total of 10 consensus residues belonging to the central core regions of CCP1 and CCP2 were initially tethered during the minimization and molecular dynamics simulation procedures (the force constant, K, added on all heavy atoms was 5 kcal-Å²). This constant was subsequently relaxed (K = 0) in the final energy minimization. A 100-ns dynamics simulation at 300 K for each model was performed these calculations in order to select the best set of parameters.

Proteins—C4BP (8), C4 (32), and factor I (33) were purified from human plasma as described previously. The concentrations were determined by measurement of absorbance at 280 nm, and extinction coefficients (1%, 1 cm) were used. Aliquots of C4BP stock solutions (0.6–2 μg/ml) were diluted in flow buffer (0.33 ml) was estimated with an enzyme-linked immunosorbent assay. The amount of C4BP in collected fractions (0.33 ml) was estimated with an enzyme-linked immunosorbent assay. NaCl concentration in the samples was calculated from the measurement of conductivity. The interaction between heparin and C4BP was determined by the delfluoride membrane (Millipore). Membranes were washed with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 3% fish gelatin, and 0.1% Tween 20. They were then incubated with various antibodies diluted in above buffer. Membranes were washed with the same buffer without fish gelatin and incubated with anti-immunoglobulins conjugated with alkaline phosphatase, washed again, and developed.

C4b Ligation Binding Assay—Microtiters plates (Maxisorp, Nunc) were incubated overnight at 4 °C with 50 μl of solution containing 10 μg/ml C4b for reasons of clarity. Proteins were added in TBS supplemented with 10% fetal calf serum, 3.4 mM glutamine, 100 μg/ml G418. Medium was injected during the association phase and the amount of C4BP in collected fractions was measured in a γ-counter.

Heparin Affinity—About 200 μg of C4BP or one of the three mutant proteins were added at a flow of 0.5 ml/min on 2 ml heparin-Sepharose (Amersham Pharmacia Biotech) equilibrated in 20 mM Tris-HCl, pH 7.4. The column was washed with 5 volumes of the starting buffer, and the proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M NaCl at a flow of 1 ml/min. The amount of C4BP in collected fractions (0.33 ml) was estimated with an enzyme-linked immunosorbent assay. NaCl concentration in the samples was calculated from the measurement of conductivity. The interaction between heparin and C4BP was also studied using a BIACore biosensor system (Biacore AB). Two flow cells of CM5 sensor chip were activated with 20 μl of a mixture of 0.2 μl 1-ethyl-3(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxy-sulfosuccinimide at a flow rate of 5 μl/min, after which 45 μl of streptavidin (0.1 mg/ml in 10 mM acetate buffer, pH 4.5) was injected. Unreacted groups were blocked with 20 μl of 1 M ethanolamine, pH 8.5. Approximately 4000 resonance units of streptavidin were fixed on the surface of each chip. Biotinylated heparin (kind gift of Dr. Markku Salminen; Department of Medical Biochemistry and Microbiology, Uppsala University) was injected over one of the two streptavidin surfaces, the other being a negative control. About 600 resonance units of heparin was immobilized. The association kinetics were studied for recombinant C4BP and the three mutants at various concentrations (0–500 mM). The flow buffer was 10 mM Hepes-KOH, pH 7.4, supplemented with 75 mM NaCl, 0.005% Tween 20, and 3.4 mM EDTA. Aliquots of C4BP stock solutions (0.6–2 μM in flow buffer) were diluted in the flow buffer, and 45 μl was injected during the association phase.
at constant flow rate of 20 μl/min. The dissociation was followed for 6 min at the same flow rate. In all experiments, 10 μl of 2 M NaCl was used to remove bound ligands.

**Synthetic Peptide**—An 18-mer peptide, EILQEEDLIDEDDIPVRS, corresponding to residues 740–757 of human C4, was purified by high pressure liquid chromatography after synthesis and lyophilized. It was dissolved in phosphate-buffered saline at a 100 mM concentration. To test the effect of the peptide on the C4b-C4BP interaction, 20 nM C4BP in 20 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 0.1% bovine serum albumin was mixed with increasing amounts of the peptide (0.1 nM to 50 mM). The samples were then added to microtiter plates with immobilized C4b and the binding of C4BP estimated as described for the C4b ligand binding assay. Alternatively, the microtiter plates were incubated overnight at 4 °C with 50 μl of solution containing 20 μg/ml of the peptide in 75 mM sodium carbonate, pH 9.6, and the binding of 125I-C4BP was assayed as described for the competition assay.

**RESULTS**

**Refined Molecular Model of CCP1–2 and Strategy for Site-directed Mutagenesis**—The three-dimensional structures of two pairs of CCP modules have been determined with NMR so far: CCP15–16 of factor H and CCP3–4 of VCP. The CCP pairs of these two structures present different intermodular angles (29, 30). Our initial model for the C4BP α-chain (28) was based on the structure of the factor H pair. The NMR structure of factor H CCP15 was found to be the best starting conformation to build C4BP CCP1 because the number of insertions and deletions between the sequence of the model to build and the CCP15 template was small as compared with other CCPs of known structure. Also, when using CCP15 of factor H as a template, the amino acid side chains in the resulting C4BP CCP1 model run according to rules deduced from analysis of experimentally determined structures. For instance, the positively charged side chain of Arg39 is oriented toward the solvent, mimicking Arg41 of factor H (numbering according to the Protein Data Bank file, entry 1hfh). In contrast, when VCP CCP2 was used as a template, Arg39 of C4BP pointed directly into the hydrophobic core of the module. Even though some other rotamers for Arg39 could be used to avoid this problem (37), the guanidinium group of Arg39 would be 15 Å away from R64 and on a different face of the molecule. However, Arg39 and Arg64 are most likely topological neighbors (see below) because heparin binds to this region of C4BP. Similar observations were made for the second CCP of the C4BP α-chain, suggesting that factor H CCP16 is an appropriate template to build this C4BP module. Moreover, when the VCP intermodular angle was used to build the C4BP CCP1–2 model, Lys63 of C4BP was “locked” into an aromatic/hydrophobic pocket formed at the interface between the modules. Such problems did not occur when the factor H pair was used as the initial template. The angle between C4BP CCP1 and CCP2 based on the one present in factor H was consistent with appropriate amino acid distribution of C4BP (e.g., charged residues were exposed) and with the presence of a positively charged cluster on one face of the molecule. However, because the α-chain of C4BP has an extended shape as shown by electron microscopy and x-ray scattering data, we suggest that the intermodule angle of factor H has to be stretched to represent more closely a native C4BP tentacle in a solution.

The geometry of the initial CCP1–2 model was refined via molecular dynamics simulation and the resulting model is shown in a simplified representation in Fig. 1A. The stereo-
chemistry of the final model was analyzed using ProStat ( Biosym-MSI). We investigated bond lengths, backbone $\phi$-angles, side chain $\chi_1$ and $\chi_2$ angles, and chirality. All were found to be in agreement with values obtained for experimentally determined structures. A Ramachandran plot is presented in Fig. 1B and shows that most $\phi$-$\psi$ angles are within the energetically favored regions supporting the quality of the model. The few peptide bonds of the model that are found in unfavored regions were found to involve mostly glycines (indicated by arrows in Fig. 1B), which is a well known feature of this amino acid. Moreover, Ramachandran plots using NMR structures of other CCP modules yielded a similar percentage of peptide bonds outside the allowed regions, again mainly due to Gly residues (not shown).

The three-dimensional model of CCP1–2 was used as basis for selection of mutations. In order to probe the functional importance of interface between CCP1 and CCP2, three mutants were generated, R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q. The C4BP mutants were expressed in eucaryotic cells and purified from cell culture media using mAb 104 affinity chromatography (Fig. 2). The epitope for mAb 104 is located in CCP1 or at the interface between CCP1 and CCP2 (27). The expression levels of wild type and mutant C4BP were similar and approximately 2 mg of pure protein was obtained from 1 liter of culture medium. Without reduction (Fig. 2, B and D), recombinant wild type and mutant C4BP demonstrated molecular weights slightly lower than that of plasma derived C4BP, which was due to the lack of the $\beta$-chain in recombinant C4BP (35). On immunoblotting, the reduced proteins were visualized with a rabbit antibody against human C4BP (Fig. 2C), whereas unreduced mutants were recognized by mAb 67 (Fig. 2D), the epitope of which is located in the middle part of the $\alpha$-chain (27). The surface exposed mutations that were introduced in C4BP did not cause folding problems affecting expression levels, reactivities with various antibodies, and electrophoretic mobilities. The three arginine residues that were the targets for the mutagenesis were solvent exposed and not involved in any clear stabilizing ionic interactions (i.e. salt bridges) and the amino acid substitutions were therefore expected to be well tolerated. The replacement of positively charged Arg by polar Gln is conservative in term of overall size and ability to form hydrogen bonds with the solvent. Collectively, these data strongly suggest that the proteins were well folded.

**C4BP Mutagenesis Resulting in Decreased Binding of C4b to C4BP**—The interaction between C4b and recombinant C4BP mutants was analyzed with a direct binding assay as well as in a competition assay. Each C4BP molecule contains up to seven C4b binding sites, each of which binds C4b with relatively low affinity (22). The fast dissociation of C4b from C4BP prevented the use of microtiter plate based assays with immobilized C4BP. As an alternative approach to investigate the effects of the mutations, binding of C4BP mutants to immobilized C4b was tested. Due to the multimeric nature of C4BP and the multiple C4b binding sites on each C4BP, this approach only allowed a qualitative analysis of the effect of the mutations on the C4b-C4BP interaction but did not permit calculation of affinity constants. We chose to estimate the apparent affinity from the midpoint of the binding curve in the direct binding assay using purified C4BP and immobilized C4b. The binding was estimated both at physiological NaCl concentration (150 mM) and at 50 mM NaCl in order to elucidate the influence of ionic strength on the interaction. Plasma purified and recombinant wild type C4BP bound to immobilized C4b with similar apparent affinities, as shown in Fig. 3. At physiological NaCl concentrations (Fig. 3B), R39Q and recombinant wild type C4BP reached 50% of maximal binding at 37 and 3 nM (12.3-fold difference), respectively. R64Q/R66Q and R39Q/R64Q/R66Q reached 50% binding at 168 and 400 nM, which is 56 and 133 times higher, respectively, than the 3 nM found for recombinant wild type C4BP (Fig. 3D). At the lower ionic strength, 0.5 mM of recombinant wild type C4BP was required to give 50% compared with physiological conditions (data not shown). At low ionic strength, R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q reached 50% binding at concentrations that were 19 (9.5 nM), 46 (23 nM), and 200 (100 nM) times higher, respectively, than that of recombinant wild type C4BP (see Fig. 3A).

In the competition assay, the recombinant proteins were allowed to compete with $^{125}$I-labeled C4BP tracer for binding to immobilized C4b (Fig. 3C). In the absence of competitor, 18–24% of the added $^{125}$I-labeled C4BP tracer bound to the immobilized C4b, and the binding of radiolabeled tracer could be competed out by unlabeled C4BP. Recombinant wild type and plasma C4BP were equally efficient in displacing the $^{125}$I-C4BP tracer from the immobilized C4b, the half maximal competition being reached at 1 nM concentration of C4BP (experiments done at 50 mM NaCl). The concentrations of R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q required to obtain 50% inhibition were 15, 67, and 94-fold higher, respectively, than that of recombinant wild type C4BP.

A sequence in C4 rich in negatively charged amino acids residues, $^{74}$EILQEEIFIEDDDIPVRS$^{757}$, has been proposed...
to be a binding site for C4BP (38). As the binding site for C4b in C4BP is rich in positively charged amino acid residues, we decided to test whether a synthetic peptide corresponding to this C4b sequence was able to inhibit the C4b-C4BP interaction. However, no inhibition of the binding between C4b and C4BP was observed, even in the presence of 50 μM peptide (data not shown). Furthermore, no binding of 125I-labeled C4BP to immobilized peptide (in microtiter plates) was observed (data not shown). Even though the results obtained with the peptide were negative, they do not exclude the possibility that the proposed region in C4b is part of the C4BP binding site because the peptide in solution may not have adopted the proper conformation.

**Mutagenesis of C4BP and Loss of Factor I Cofactor Function**—To elucidate whether the impaired C4b binding was matched by a decrease in factor I cofactor activity, the C4BP variants were incubated with C4b, factor I and trace amounts of 125I-labeled C4b. Proteins were then separated by SDS-PAGE and C4b visualized by autoradiography (Fig. 4A). In the presence of both C4BP and factor I, C4b was degraded, and C4d and α4 molecules appeared as described (21). The α3 + α4 fragment had the same mobility as the β-chain, which is why the two polypeptides cannot be distinguished. Recombinant wild type C4BP was equally efficient as factor I cofactor as plasma purified C4BP (Fig. 4, lanes 3, 4, 9, and 10). The three C4BP mutants as compared with recombinant wild type C4BP demonstrated decreased cofactor activity. To quantify the loss in cofactor activity, the intensities of bands corresponding to C4d and α4 were estimated by densitometry (Molecular Dynamis). As judged from the amount of C4d released, the R39Q/R64Q/R66Q mutant functioned poorly as factor I cofactor (Table I). In contrast, generation of α4 was less affected by the introduced mutations, and the level of α4 generated in the presence of the R39Q/R64Q/R66Q mutant was only 30% lower than that formed in presence of recombinant wild type C4BP. The time course of the two cleavages in the presence of recombinant wild type C4BP or the R39Q/R64Q/R66Q mutant is shown in Fig. 5. During its inactivation, C4b is cleaved at two positions by factor I, at Arg937-Thr938 and Arg1317-Asn1318 (Fig. 4A). In the presence of recombinant wild type C4BP (Fig. 5, open circles), the Arg1317-Asn1318 cleavage resulting in the ap-
The intensities of bands corresponding to the C4d and the α4 fragment as shown in Fig. 4B were determined by densitometry and are represented as mean values of 3–6 determinations ± S.D.

|        | C4d | α4 |
|--------|-----|----|
| Factor I | 7 ± 8 | 13 ± 7 |
| Plasma C4BP | 100 | 100 |
| Recombinant wild type | 103 ± 7 | 98 ± 14 |
| R39Q | 63 ± 11 | 97 ± 9 |
| R64Q/R66Q | 59 ± 11 | 88 ± 6 |
| R39Q/R64Q/R66Q | 17 ± 9 | 69 ± 7 |

Fig. 5. Time course of C4b degradation. Samples containing 250 nm C4b, trace amounts of 125I-labeled C4b, 60 nm factor I, and 200 nm of wild type or R39Q/R64Q/R66Q C4BP were incubated at 37 °C for the times indicated. They were then mixed with a sample buffer and heated, and the proteins were separated by 7.5–15% SDS-PAGE. The gels were then subjected to autoradiography, and intensities of bands corresponding to the C4d and the α4 fragment were estimated by densitometry. Shown is the time course for generation of the α4 fragment (A) and the C4d (B); open circles, recombinant wild type; closed circles, R39Q/R64Q/R66Q mutant.

Mutagenesis of C4BP and the Loss of Heparin Binding Site—C4BP is known to bind to heparin (13, 39), but the binding site on C4BP has not been localized. Inhibition of the interaction between C4BP and C4b by heparin (40) suggests that the C4b and heparin binding sites overlap. Unfractionated heparin, which contains molecules varying in size from 4 to 30 kDa (mostly 13 kDa), and low molecular weight heparin, prepared by deaminative cleavage, consisting of molecules in a range of 2 to 6 kDa (mostly 4 kDa), were found to be equally efficient in inhibiting the C4b-C4BP interaction (Fig. 6). Under the same conditions, chondroitin sulfate or hyaluronan had no effect.

Affinity chromatography was used to assess the capacity of the mutant C4BP molecules to bind to heparin. Recombinant wild type C4BP eluted as a broad peak with maximum at 273 ± 7 mrt NaCl (Fig. 7). Similar results were obtained with plasma purified C4BP eluting at 266 ± 9 mrt NaCl (not shown). The heparin binding abilities of C4BP were compromised by the mutations R39Q and R64Q/R66Q, eluting at 216 ± 5 and 181 ± 4 mrt, respectively. The R39Q/R64Q/R66Q mutant eluted from the heparin-Sepharose already at 129 ± 3 mrt NaCl, indicating that it would not bind to heparin under physiological ionic strength.

To further demonstrate the decrease in heparin affinity as a result of the introduced mutations, surface plasmon resonance technique (BIAcore) was used to monitor the formation and dissociation of surface-bound complexes between the recombinant C4BP variants and immobilized heparin (Fig. 8). Each C4BP protein sample was injected over the surface carrying immobilized streptavidin-biotinylated heparin complexes and also over a chip containing streptavidin alone as control. The sensograms presented were obtained after subtraction of the unspecific binding of C4BP to the streptavidin containing chip. Wild type recombinant C4BP demonstrated specific, dose-dependent binding to the immobilized heparin. The rate of association was high and the dissociation rate was low, suggesting high affinity interaction. However, as the interaction between the fluid phase multimeric C4BP and the immobilized heterogeneous heparin is very complex, it was not possible to calculate meaningful affinity constants for the interaction. The three mutant C4BP molecules yielded weak binding signals, suggesting that most of the specific heparin binding site was lost as a result of the mutagenesis (Fig. 8B).
A, C4BP (0–500 nM) were injected over chips containing biotinylated heparin-Sepharose and eluted with a gradient of NaCl from 0.0 to 0.5 M; 0.33-ml fractions were collected. Concentration of C4BP in each fraction was measured by an enzyme-linked immunosorbent assay, and salt concentration was estimated by measurement of conductivity. Recombinant wild type C4BP is represented by closed circles; R39Q mutant, open squares; R64Q/R66Q, closed squares; R39Q/R64Q/R66Q, open diamonds.

Fig. 7. Affinity chromatography on heparin-Sepharose. Recombinant wild type C4BP or its mutants were applied on 2 ml of heparin-Sepharose and eluted with a gradient of NaCl from 0.0 to 0.5 M; 0.33-ml fractions were collected. Concentration of C4BP in each fraction was measured by an enzyme-linked immunosorbent assay, and salt concentration was estimated by measurement of conductivity. Recombinant wild type C4BP is represented by closed circles; R39Q mutant, open squares; R64Q/R66Q, closed squares; R39Q/R64Q/R66Q, open diamonds.

Discussion

The factor I cofactor function of C4BP resulting in the degradation of C4b is a physiologically important regulatory mechanism of the complement system. Due to the complexity and heterogeneity of the interacting proteins, C4BP, C4b, and factor I, three-dimensional structures of the individual proteins and of protein complexes have not been determined experimentally and are unlikely to become available in the near future. However, molecular modeling and computer-guided site-directed mutagenesis are efficient tools for the identification of binding sites and elucidation of structure-function relationships. These techniques have proven successful in other studies, e.g. in the investigation of two CCP modules from CD46 (41).

Based on the sequence similarities between CCP modules in C4BP and various CCPs with known three-dimensional structures, we constructed a three-dimensional model for the α-chain of human C4BP (28). Theoretical and interactive analysis of potential binding sites suggested the interface between C4BP CCP1 and CCP2, displaying a cluster of positively charged amino acids, to be of putative functional importance. Sequences of five cDNAs coding for C4BP α-chains have been reported: human (42), bovine (43), rat (44), mouse (45), and rabbit (46). Analysis of interspecies similarities of amino acid sequences showed the N-terminal region of the α-chain containing the positively charged cluster to be highly conserved, suggesting physiological importance (46). Taken together, these observations prompted us to construct several α-chain mutants in which arginines in CCP1 and CCP2 were replaced by glutamine residues. Three mutants, R39Q, R64Q/R66Q, and R39Q/R64Q/R66Q, were expressed in the eucaryotic system and purified. From the results of direct binding studies and a competition assay, it is clear that the suggested electropositive cluster indeed represents a key interaction site for C4b. Our data agree with a previous report focusing on the S. pyogenes C4BP interaction by Accardo et al. (26), which suggested the binding site for C4b to span CCP1–3 and pointed out the importance of the Arg66-His67 pair. A mutant carrying the combined change of Arg66 to Gla and His67 to Thr demonstrated decreased binding to C4b-Sepharose at physiological ionic strength. However, at 40 mM NaCl, no difference in binding (estimated as retention on a C4b-Sepharose) between mutant and recombinant wild type C4BP could be observed (26).

The C4b-C4BP interaction is highly sensitive to salt concentration, and already at 200 mM NaCl, the binding decreased by about 50%. This stands in sharp contrast to the C4BP-protein S interaction, which is essentially unaffected by high salt concentrations (47). Protein S binds to the most N-terminal CCP of the β-chain (48). Structural analysis of a β-chain model revealed a cluster of solvent exposed hydrophobic residues, which are potentially involved in the binding of protein S (47). Such clusters of residues with hydrophobic characteristics favor tight and stable interactions and may therefore have been maintained during evolution.

Even though most reports suggest the C4b binding site to be located within CCPs 1–3, other regions of the α-chain have been proposed to be involved as well. Thus, proteolytic fragments of C4BP were used to map the C4b binding and factor I cofactor activities to distinct regions spanning CCP6–7 and CCP3–6, respectively (49). In addition, a monoclonal antibody directed to CCP6 was found to block C4b binding (50), which is difficult to reconcile with the observation that mouse C4BP lacking CCP5–6 binds human C4b (45). Recently, it was shown that a cryptic binding site for C3b is located near the C terminus of the α-chain (51). This site was only exposed in recombinant, monomeric cell-bound C4BP, which makes its physiological significance unclear. However, such a cryptic C3b binding site may also have the ability to interact with C4b, which could explain some of the disagreement in published reports. When all available data are taken into consideration, it appears clear that the interface between CCP1 and CCP2 forms a crucial binding site for C4b. However, other regions within CCP1–3 are presumably required for expression of the full binding
capacity between C4BP and C4b and for expression of factor I cofactor activity.

The C4b-C4BP complex is not the first example of an interaction in which the interface area between two CCP modules plays a pivotal role in forming a binding site. Thus, the binding sites for measles virus on CD46 involves the interface between CCP1 and CCP2 together with parts of CCP2 (41). Moreover, insertion of two and four amino acids between CCP1 and CCP2 and between CCP3 and CCP4 of factor H, respectively, entirely abolished its factor I cofactor function (52). Recently, it has been shown that an antibody blocking the interaction between CR2 and C3dg is directed against an epitope on a recess formed between CCP1 and CCP2 (53).

The decreased binding of mutated C4BP to C4b was matched by loss of its factor I cofactor activity. However, the mutations introduced in C4BP had differential effects on the two cleavage sites in C4b, such as the cleavage of the Arg397, Thr398 peptide bond was more severely affected as compared with the Arg1317, Asn1318 bond. The mechanism by which C4BP and factor H function as factor I cofactors in the degradation of C3b and C4b is unknown. Recently, it was suggested that factor H causes conformational changes in C3b, facilitating its interaction with factor I (54). Thus, it was proposed that factor I binds both factor H and C3b and that the binding sites do not overlap. Possibly, a similar mechanism is involved in the C4BP/factor I mediated C4b degradation. This would be consistent with the presence of a secondary C4b binding site located outside the electropositive cluster in CCP1–2, which possibly is more involved in the cleavage of the Arg1317, Asn1318 peptide bond than the Arg397–Thr398 bond. This could explain the selective impairment of one of the cleavage reactions.

Heparin interacts with several complement proteins, but the affinity of the binding of heparin to C4BP is the highest among the complement proteins, most likely due to the multimeric nature of C4BP (39). We now show that the C4b binding site also interacts with the negatively charged heparin but not with chondroitin sulfate or hyaluronan that is negatively charged due to a presence of carboxyl groups. The pattern, composition, and spacing of basic amino acids in heparin binding peptides and proteins have been investigated in detail (55, 56). From its location, it is known that heparin (characterized by a high charge density) interacts tightly with peptides displaying a series of at least three to five positively charged residues and that the binding is stronger when the cluster contains arginine residues rather than lysines. Our results are fully consistent with these observations as we show that Arg39, Arg64, and Arg66 play a key role in heparin binding. In addition, because of its location, we suggest that Lys63 is also involved in the heparin binding site. Indeed, after numerous molecular modeling trials (i.e., using different intermodule angles), the most rational location for the Lys63 side chain is in direct vicinity to the three guanidinium groups of Arg39, Arg64, and Arg66. The mutations were found to abrogate the binding of C4BP to heparin almost entirely, which suggested that the electropositive cluster in CCP1–2 constitutes the major heparin binding site in C4BP. Analysis of the three-dimensional model of the whole α-chain of C4BP showed that the electropositive cluster between CCP1 and CCP2 was the only one presenting the key characteristics of a heparin binding site. It is noteworthy that heparin binding sites have also been found at the surface of factor H CCP7, CCP13 and CCP20 (57–59). However, the mechanism of action of heparin in the case of factor H seems to be different than the one for C4BP, because heparin enhances binding of factor H to surface-bound C3b, thereby helping in the inactivation of C3b by factor I (60). Furthermore, low molecular weight heparin (5 kDa) was ineffective in the factor H-C3b system, whereas it still inhibits C4b-C4BP interaction.

In a C4b degradation assay, similar concentrations of heparin were required to inhibit factor I cofactor activity as the ones needed for blocking C4BP binding to immobilized C4b (not shown). The multimeric nature of C4BP makes the heparin interaction potentially interesting from a physiological perspective. Binding of C4BP to the host cells via for example three heparin binding sites present on three different α-chains would favor degradation of C4b close to the cell surface as the other four α-chains would be free to interact with C4b and factor I. Thus, interaction of C4BP with heparin-like molecules, present at the surface of some cells, could protect them from the destructive and inflammatory consequences of complement activation. The localization of the heparin binding site at the tip of the C4BP tentacles is consistent with this hypothesis because if such a site was located closer to the central core region of C4BP, it would not be as easily accessible for binding.

In recent years, several complement components have gained recognition as potential therapeutics, e.g. complement components may have cardioprotective roles, have anti-inflammatory actions, or help to overcome hyper acute rejection during xenotransplantation (reviewed in Ref. 61). For instance, a surface-bound form of human C4BP consisting of only one α-chain (CCP1 to CCP8) has been constructed (51). This engineered C4BP molecule efficiently blocked complement-mediated endothelial cell lysis, suggesting that C4BP could be useful for clinical xenotransplantation. However, although complement regulators have therapeutic potential, our knowledge of the intermolecular interactions involving complement regulatory proteins is still rudimentary. The now identified binding site for C4b on the C4BP molecule is part of an effort not only to describe sites for protein-protein interactions but also to enhance our understanding of molecular mechanisms involved in regulation of the complement system by factor I and its cofactor proteins.

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