Topology and Structure of the C1q-Binding Site on C-Reactive Protein

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Topology and Structure of the C1q-Binding Site on C-Reactive Protein

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The host defense functions of human C-reactive protein (CRP) depend to a great extent on its ability to activate the classical complement pathway. The aim of this study was to define the topology and structure of the CRP site that binds C1q, the recognition protein of the classical pathway. We have previously reported that residue Asp112 of CRP plays a major role in the formation of the C1q-binding site, while the neighboring Lys114 hinders C1q binding. The three-dimensional structure of CRP shows the presence of a deep, extended cleft in each protomer on the face of the pentamer opposite that containing the phosphocholine-binding sites. Asp112 is part of this marked cleft that is deep at its origin but becomes wider and shallower close to the inner edge of the protomer and the central pore of the pentamer. The shallow end of the pocket is bounded by the 112–114 loop, residues 86–92 (the inner loop), the C terminus of the protomer, and the C terminus of the pentraxin α-helix 169–176, particularly Tyr175. Mutational analysis of residues participating in the formation of this pocket demonstrates that Asp112 and Tyr175 are important contact residues for C1q binding, that Gln88 influences the conformational change in C1q necessary for complement activation, and that Asn158 and His38 probably contribute to the correct geometry of the binding site. Thus, it appears that the pocket at the open end of the cleft is the C1q-binding site of CRP. The Journal of Immunology, 2001, 166: 3998–4004.

-reactive protein (CRP), a member of the phylogenetically ancient pentraxin family, is a major acute phase plasma protein in man (1). CRP is composed of five identical noncovalently bound subunits (2, 3) and has Ca2+-dependent binding specificity for phosphocholine (PCh) and the PCh residues of bacterial polysaccharides (4), exemplified by pneumococcal C-polysaccharide (5). CRP also binds PCh polar head groups of membrane phospholipids (6) as well as certain nuclear constituents that do not contain PCh, such as small ribonucleoprotein particles (7, 8). Ligation-complexed CRP is recognized by C1q and efficiently activates the classical pathway of human complement (9). Complexed CRP can also elicit responses from phagocytic cells, probably through binding to the FcγRI and FcγRIIa receptors (10, 11). Thus, CRP represents an important first line of innate host defense.

Our previous mutational studies have shown that residue Asp112 of CRP plays a critical role in the formation of the C1q-binding site, while Lys114 appears to hinder binding of C1q to CRP (12). Subsequent determination of the crystal structure of human CRP (2, 3) revealed a cyclic 5-fold symmetric aggregation of protomers, each having a flattened jellyroll appearance, consisting of anti-parallel β-strands arranged into two β-sheets. Two calcium ions are ligated in close proximity to each other to side chains and main chain carbonyls of each protomer. They are integral structural elements of the PCh binding site of the protomer (13) and all are located on the same recognition face of the CRP pentamer. Because complement activation and opsonization by CRP require its binding to an appropriate multivalent ligand through the PCh binding site, it seems that the structural elements involved in CRP-C1q and CRP-FcγR interactions must be located on the face of the pentamer opposite the ligand-binding site. This effector face is characterized by an unusual extended deep cleft (2), which starts at about the center of each protomer and extends to the central pore of the pentamer. Residue Asp112 is located within the relatively open end of the cleft toward the center of the pentamer, suggesting that this region of the cleft may be involved in C1q binding.

In the present study we have used site-directed mutagenesis to define the topology and structure of the C1q binding site of CRP. The results indicate that the site is located at the shallow end of the cleft toward the pentamer’s central pore and that in addition to Asp112, Tyr175 is critical for C1q binding and complement activation.

Materials and Methods

Construction and expression of mutant CRP

The CRP cDNA clone HLCRP-23 in the expression vector p91023 (14) was used as template for construction of mutant CRP cDNA. Mutagenesis was conducted using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Mutagenic oligonucleotides were synthesized on a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). All mutations were verified by nucleotide sequencing using the chain termination method (15). Two independent clones for each mutant were purified by two successive CsCl gradient ultracentrifugations, sequenced, and used for protein expression.

COS cells used for transfection were maintained in culture as reported previously (14). Forty micrometers of each recombinant plasmid containing wild-type (wt) or mutant CRP cDNA was transfected into 4 × 106 COS cells by electroporation at 350 V/500 μF using a Gene Pulser apparatus (Bio-Rad, Richmond, CA). Transfected cells were replated in tissue culture dishes containing 10 ml of medium. The culture media were changed after 16 h and harvested 96 h after electroporation.
Purification of CRP and C1q

Native human CRP was purified from ascitic fluid by affinity chromatography on a P-Ch-conjugated agarose column (Pierce, Rockford, IL) as described previously (16). After affinity chromatography, CRP was further purified by fast protein liquid chromatography anion exchange chromatography on a MonoQ column (Pharmacia, Piscataway, NJ) eluted with a 0.15- to 1.0-M NaCl gradient in 20 mM Tris buffer, pH 7.8. Recombinant wt and mutant CRP were purified from COS cell culture supernatants using a single P-Ch affinity chromatography step as reported previously (13). C1q was purified from human plasma according to the method of Tenner et al. (17). The purity of all protein preparations was assessed by 5–20% polyacrylamide gradient SDS-gel electrophoresis.

CRP ELISA and ligand binding assays

The concentration of CRP was measured by a solid phase sandwich-type ELISA, using a sheep anti-human CRP serum (Cappel, Durham, NC) as capture Ab and the anti-CRP mAb HD2.4 (18) as reporter. Standard curves were constructed using purified native CRP (6.25–200 ng/ml). Wells were developed with affinity-purified alkaline phosphate-conjugated goat anti-mouse IgG (Pierce) followed by Sigma 104 phosphate substrate. Color development was measured at 405 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA).

The binding avidity of CRP for P-Ch ligands was measured by two assays: a P-Ch-BSA or PnC attached to the solid phase in P-Ch-BSA (9 mol of P-Ch/mol BSA) was synthesized according to the method of Chesebro and Metzger (19). PnC was isolated from S. pneumoniae, strain R36A, as described by Liu and Gotschlich (20), except that the final enzymatic step was omitted. Both assays used protein A-purified HD2.4 mAb as reporter. Tris-buffered saline, pH 7.2, containing 0.1% BSA, 0.01% Nonidet P-40, 0.5 mM CaCl2, and 0.02% NaN3 (P-Ch binding buffer) was used throughout the assays. Standard curves were constructed by using purified native CRP (6.25–200 ng/ml). Purified wt and mutant CRP were diluted to a concentration of 50 ng/ml and tested in duplicate. Wells were developed as in the ELISA, using affinity-purified alkaline phosphate-conjugated goat anti-mouse IgG and Sigma 104 phosphate substrate.

C1q binding and C3 deposition assays

C1q binding assays used either P-Ch-BSA or PnC in the solid phase. Microtiter wells were coated with 100 μl of either P-Ch-BSA or PnC at a concentration of 10 μg/ml in Tris-buffered saline, pH 7.2. Standard curves were constructed using serial dilutions of purified native CRP, covering the concentration range from 50–1600 ng/ml in P-Ch binding buffer. Purified wt and mutant CRP were diluted to appropriate concentrations in the P-Ch binding buffer and tested in duplicate. Wells accounting for nonspecific binding received buffer B without CRP. After overnight incubation at 4°C, the wells were washed, washed twice with P-Ch binding buffer, and rinsed once with C1q binding buffer (10 mM Tris-HCl (pH 7.2), 100 mM NaCl, 5 mM CaCl2, 0.1% BSA, 0.01% Nonidet P-40, and 0.02% NaN3). Then 100 μl of 0.5 or 50 μg/ml of purified C1q in C1q binding buffer was added to wells coated with P-Ch-BSA or PnC, respectively. After 3-h incubation at room temperature, the wells were washed once with C1q binding buffer and twice with P-Ch binding buffer. Then, 100 μl of 5 μg/ml casein acid-purified rabbit anti-C1q IgG in P-Ch binding buffer were added to each well and incubated for 1 h at room temperature. Rabbit anti-human C1q sera were raised in this laboratory. After washing, the plates were developed using alkaline phosphate-conjugated goat anti-rabbit IgG (Pierce) and Sigma 104 phosphate substrate.

For C3 deposition assays, microtiter wells were coated with 100 μl of either P-Ch-BSA or PnC at a concentration of 10 μg/ml in Tris-buffered saline, pH 7.2. Standard curves were constructed using serial dilutions of purified native CRP, covering the concentration range from 50–1600 ng/ml in P-Ch binding buffer. Purified wt and mutant CRP were diluted to appropriate concentrations in the P-Ch binding buffer and tested in duplicate. Wells accounting for nonspecific binding received buffer B without CRP. After overnight incubation at 4°C, the wells were washed, washed twice with P-Ch binding buffer, and rinsed once with C1q binding buffer (10 mM Tris-HCl (pH 7.2), 100 mM NaCl, 5 mM CaCl2, 0.1% BSA, 0.01% Nonidet P-40, and 0.02% NaN3). Then 100 μl of 0.5 or 50 μg/ml of purified C1q in C1q binding buffer was added to wells coated with P-Ch-BSA or PnC, respectively. After 3-h incubation at room temperature, the wells were washed once with C1q binding buffer and twice with P-Ch binding buffer. Then, 100 μl of 5 μg/ml casein acid-purified rabbit anti-C1q IgG in P-Ch binding buffer were added to each well and incubated for 1 h at room temperature. Rabbit anti-human C1q sera were raised in this laboratory. After washing, the plates were developed using alkaline phosphate-conjugated goat anti-rabbit IgG (Pierce) and Sigma 104 phosphate substrate.

Results

To investigate the topology of the C1q-binding site, we constructed mutants of residues Tyr175, His38, Asn158, Thr90, and Glu88, which are located in the vicinity of Asp112 and the cleft on the effector face of each CRP protomer (Fig. 1). Asp112, Lys114, Tyr175, Thr90, and Glu88 describe the shallow end of the cleft, while His38 and Asn158 are located deeper within the cleft. The previously described mutants D112N, D112A, and K114A (12) were included in the present study for completeness. All mutant CRP cDNAs were expressed successfully and purified by affinity chromatography on P-Ch-conjugated agarose. SDS-PAGE demonstrated that the apparent m.w. of the subunits of recombinant wt and mutant CRP was identical with that of native CRP. Similarly, the elution volume of all CRP species from gel filtration columns was identical with that of native CRP, indicating a pentameric structure. The functional integrity of the mutants was evaluated by ligand binding assays, using P-Ch-BSA or PnC in the solid phase. Known differences between the two ligands suggested the use of both. Compared with BSA-P-Ch, PnC has a higher density of P-Ch residues arranged in a symmetrical array, thus allowing multipoint high affinity attachment of CRP. However, PnC also could bind CRP through sugar residues and, therefore, BSA-P-Ch provides a better model for ligands binding CRP only through P-Ch residues. The results are summarized in Fig. 2. Values shown are binding activities relative to native CRP and reflect specific avidity for P-Ch-BSA or PnC. In this and all subsequent assays two independent clones for each mutant were tested. The data shown are for one clone; the second clone gave similar results. Each mutant CRP was purified from two different transfections and was assayed three to eight times. As shown, the P-Ch and PnC binding activities of wt and all mutant CRPs, except E88R, did not differ from each other, and all were close to unity. The small observed differences among these CRP species were not significant. E88R bound to both ligands poorly, which we interpret to indicate a gross conformational change apparently affecting the P-Ch-binding site. For this reason E88R was not tested further.

The C1q-binding activities of the various CRP species were also evaluated using P-Ch-BSA and PnC in the solid phase. As shown in Fig. 3, mutants D112N, D112A, and K114A gave results similar to those published previously (12), i.e., mutation of Asp112 or Lys114 resulted in a significant decrease or increase, respectively, in C1q binding. Substitution of Ala for Tyr175 resulted in a decrease in C1q binding similar to that observed for the Asp112 mutants. Substituting Ala for His38 did not affect the C1q-binding activity of either P-Ch-BSA- or PnC-complexed CRP. However, substituting Glu for His38 resulted in a significant, although <50%, decrease in C1q-binding activity. The most dramatic change in C1q-binding activity occurred when His38 was replaced by Arg. H38R CRP bound 5- to 10-fold less C1q than wt CRP. Mutations of residues Glu88, Thr90, and Asn158 did not cause significant changes in C1q binding. To further evaluate the C1q-binding activity of selected mutants, CRP dose-response experiments were performed. Fig. 4 shows the results of a representative experiment. As shown, no significant C1q binding to D112N,
have less than half the complement activating ability of wt CRP. Similarly, E88Q, E88A, N158A, and C1q-binding activity than wt, are at least 100-fold less efficient at Y175A, and H38R mutants, which express pronounced in the C3 deposition assay. Thus, the D112N, D112A, assay (Fig. 3), although the effects of mutations were more pronounced in Fig. 5 are consistent with those of the C1q-binding result of complement activation by CRP-bound C1. The results summarize the number of C3 convertase complexes (C4b2a) formed as a reposition of C3 fragments to the solid phase provides a measure of in the solid phase. No substantial differences in C1q binding activity were observed among native, recombinant wt, and E88A CRP bound to either PCh-BSA or PnC.

The complement-activating efficiency of ligand-bound CRP mutants was assessed by the C3 deposition assay. The assay uses PnC as a complement source. Deposition of C3 fragments to the solid phase provides a measure of the number of C3 convertase complexes (C4b2a) formed as a result of complement activation by CRP-bound C1. The results summarized in Fig. 5 are consistent with those of the C1q-binding assay (Fig. 3), although the effects of mutations were more pronounced in the C3 deposition assay. Thus, the D112N, D112A, Y175A, and H38R mutants, which express ∼5- to 10-fold lower C1q-binding activity than wt, are at least 100-fold less efficient at complement activation. Similarly, E88Q, E88A, N158A, and H38Q, which display only 20–40% reduction in C1q binding, have less than half the complement activating ability of wt CRP.

Conversely, K114A, which binds ∼4.5 times more C1q than wt, has >15-fold increased complement-activating efficiency. To ensure that the results of the C3 deposition assay truly reflected the complement-activating potential of the CRP mutants, dose-response experiments were performed. As shown in Fig. 6, Y175A CRP did not activate complement at any of the concentrations used, while D112N and H38R activated complement negligibly and only at concentrations of >2 μg/ml. The dose-response curves of H38Q, E88A, E88Q, and N158A are parallel to each other and to native and wt CRP, indicating that differences among them were maintained throughout the dose-response range.

Discussion
The goal of the present study was to elucidate the topology and structure of the C1q-binding site of CRP. Two sets of data from our previous work guided the investigation: mutational data on the location of the site and the crystal structure of the protein. Construction and testing of a series of CRP mutants indicated that Asp112 is a major determinant of C1q binding (12). It was also shown that the positively charged side chain of Lys114 hinders
binding of C1q, because substitution of negatively charged, neutral, or nonpolar amino acids for this residue resulted in greatly enhanced C1q binding and complement activation. Determination of the three-dimensional structure of CRP at 3.0 Å resolution demonstrated that Asp$^{112}$ is part of an unusual cleft that extends from about the center of each protomer to its edge at the central pore of the CRP pentamer (2). The cleft is deep and narrow at its origin, but it opens up, becoming wider and shallow toward the pentamer’s center. It is situated on the effector face of each protomer, opposite the PCh-binding site, and its conformation is not affected by the presence or the absence of calcium at the PCh-binding site (21). Asp$^{112}$ is at the open end of the cleft, where a well-defined shallow pocket is formed, bounded by the 112–114 loop, residues 86–92 (the inner loop), the C terminus of the protomer and the C terminus of the pentraxin α-helix 169–176, particularly Tyr$^{175}$ (Fig. 1). Residues His$^{38}$ and His$^{95}$ line the floor of the pocket, while Asp$^{158}$ is located upstream toward the center of the cleft. The topology and structure of the shallow end of the cleft led to the suggestion that it may be the C1q-binding site of the protomer (2). The fact that Asp$^{112}$ is both an integral part of this structural feature and is critical for C1q binding is certainly consistent with this view. The present data further support this proposal.

Our mutational analysis demonstrates that in addition to Asp$^{112}$, Tyr$^{175}$ is a major determinant for C1q binding to complexed CRP. Substitution of Ala for this residue led to almost complete loss of C1q-binding activity when PCh-BSA was the CRP ligand and greatly reduced activity when PnC was the ligand (Figs. 3 and 4). A similar difference between the two ligands was observed for the D112N mutant and can probably be attributed to the regularly

![FIGURE 4. Dose-response curves of the binding of C1q to ligand-complexed CRP. Experimental details are given in Fig. 3. Values on the y-axis represent binding of anti-C1q to CRP-complexed C1q.](image)

![FIGURE 5. Complement activation by PnC-complexed wt and mutant CRP. PnC-absorbed NHS (1/10 dilution) was added to microtiter wells coated with PnC and reacted with CRP. Deposited C3 fragments were detected using anti-C3d mAb. Values on the horizontal axis represent the ratio of the concentration of each CRP species measured by C3 deposition assay to that measured by ELISA. Results are the average of at least three different experiments. All values are normalized for native CRP. Asterisks mark significant differences from wt (*, p < 0.005).](image)

![FIGURE 6. Dose-response of complement activation by PnC-complexed CRP. Binding of C3 fragments to complexes of PnC with native (n), wt (r), or mutant CRP. Experimental details are given in Fig. 5. Values on the y-axis represent binding of anti-C3d to C3 deposited on PnC-CRP complexes.](image)
arrayed and more closely spaced PCh groups of PnC compared with PCh-BSA, a feature that allows a higher number of the CRP pentamer’s PCh-binders to engage. The implication, then, is that multipoint attachment leads to a conformational change or a tilting of the protomers, exposing the C1q-binding site to the surface. His38 presents a special case. The H38R mutant expresses greatly reduced C1q binding, while the H38Q mutant only moderately reduces binding, and H38A has essentially wt binding activity. A likely explanation of these results is that the Arg substitution at this position places the long, positively charged, side chain in a position that favors the formation of a 3-Å salt bridge with the negatively charged side chain of Asp112. Neutralization of the negative charge of Asp112 obviously has the same effect on C1q binding as the D112N mutation. Alternatively, the Arg side chain of the H38R mutant could adopt the same configuration as the corresponding residue of serum amyloid P (22), forming a hydrogen bond to the main chain of Tyr175, again interfering with C1q binding. Because residue H38 contributes to the topology of the floor of the putative C1q-binding site, the reduction of binding observed for the Gln substitution can perhaps be attributed to a less favorable stereochemistry than that offered by the side chain of His, while the small side chain of Ala apparently does not interfere with the geometry of the C1q-binding site. Finally, as noted before, substitution of Ala for Lys at position 114, results in a 4-fold increase in C1q binding. We interpret this result to indicate that the positive charge of the Lys side chain interferes with C1q binding. Observation of the structure of the proposed binding site (Fig. 1) leads us to propose further that the effect is exercised by the side chain of Lys114 from the neighboring protomer.

Investigation of the ability of the PnC-complexed CRP mutants to form an effective C3 convertase gave an interesting picture in which all the effects observed for C1q binding were greatly amplified (Figs. 5 and 6). Thus, D112N, D112A, Y175A, and H38R essentially failed to form a C3 convertase even when very large amounts of CRP were used, while cleavage of C3 by the convertase formed by K114A was increased by 15-fold over that effected by the wt control. More instructive were the results for mutants that did not significantly affect C1q binding. E88Q, E88A, and N185A had significantly reduced ability to form the C3 convertase, while their C1q-binding ability was only modestly decreased (compare Figs. 3 and 5). In contrast, T90A, N158S, and H38A, all of which bound C1q with an affinity similar to that of wt CRP, also expressed wt C3 cleaving activity. Two nonmutually exclusive explanations can be offered for the observed amplification effect of C3 cleavage compared with the C1q binding. Firstly, several enzymatic steps separate the results of the two assays, accounting at least in part for the observed amplification. Secondly, formation of a C3 convertase requires binding of C1q in a way that induces a conformational change that, in turn, causes the reorientation and mutual activation of the two C1r zymogens, which then activate the two C1s zymogens to active serine proteases. It seems possible that certain mutations inhibit C1q binding to complexed CRP only minimally, but prevent or attenuate the induction of the fully active conformation of the bound C1q, affecting the kinetics of C1r and C1s activation and resulting in the formation of smaller amounts of C3 convertase.

Taken together, the results of C1q binding and C3 activation assays strongly indicate that the pocket at the cleft upstream of the proposed C1q-binding site, results in significantly reduced binding of CRP to FcyRII (10). Thus, it appears that the C1q- and FcyRII-binding sites partially overlap each other and that they are both located within the vicinity of the CRP cleft.

C1q is a large (460-kDa) molecule composed of six identical subunits, each consisting of three structurally similar, but distinct, polypeptide chains, A, B, and C (reviewed in Ref. 23). Each subunit has a triple-helical collagen-like region and a globular head region. The C1r2C1s2 proenzyme complex is associated with the collagenous part of the molecule, while the heterotrimeric globular heads have been shown to contain binding sites for IgG and IgM (24). The exact topology and structure of the Ig-binding site(s) is not known, but ionic bonds are probably involved, and Arg residues of C1q have been implicated (25). For evolutionary and structural reasons we propose that the CRP-binding site is also located on the globular heads of C1q, although evidence for its location on the collagen part of the molecule has been presented (26, 27). C1q is a member of a large family of proteins, termed collectins (28), which share a similar structure consisting of globular heads and collagen stems. In all collectins the globular heads mediate recognition functions through ligand-binding sites, while the collagen parts carry out effector functions. Evidence for the presence of the CRP-binding site on the neck or tail collagen region of C1q (26, 27) was presented before determination of the structure of CRP (2, 3) and did not benefit from the knowledge that the PCh-binding sites are on one face of the pentamer rather than on the perimeter of the ring. The only physically reasonable access to a CRP pentamer positioned with its recognition face against a cell wall is then provided by the opposite, effector face, and this would be effectively inaccessible to the neck or tail region of C1q.

Assuming there is a CRP-binding site on each C1q globular head, the number of functional C1q-binding sites per CRP pentamer is determined by the relative size of the reacting partners. The diameter of the C1q globular head has been estimated from electron micrographs to be about 50 Å (29), while the overall dimensions of the CRP pentamer are ~102 Å outside diameter with a 30 Å diameter central pore and a protomer diameter of 36 Å. Therefore, it would appear that only a single globular C1q head from a C1q molecule can bind to a CRP pentamer bound flat on the surface of a ligand. Consequently, more than one CRP molecule in close proximity to each other would be necessary for complement activation, which is also a condition for complement activation by complexed IgG. The question then arises as to whether there is one or more C1q-binding site per CRP pentamer. The Co of the five Tyr175 of the pentamer lie on a circle of 38 Å diameter, and the Co of the five Asp112 are at a similar distance. Although it seems possible that a single binding site is formed by these residues, which are perhaps brought closer together or exposed more fully to the solvent following binding of CRP to an effector ligand, such a site would have to be pentameric and symmetrical, probably requiring a similarly symmetrical array of contact residues on the C1q globular head. This seems unlikely. It seems more likely that there are five possible C1q binding sites per pentamer, but that only one of these sites, possibly formed by two adjacent protomers, is able to engage. This alternative possibility would offer much more flexibility and would match size requirements more closely. Thus, an array of CRP molecules bound flat on the surface of a pathogen or damaged cell would present a wide array of possible C1q-binding site locations and orientations. This would allow binding of multiple C1q heads to multiple pentamers on a one-to-one basis, without the need for significant structural rearrangement in either molecule. The pentameric symmetry of CRP enhances...
this flexibility, but may also be required to provide the correct interprotomer geometry for C1q binding at the protomer interface.

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