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A C-Reactive Protein Mutant That Does Not Bind to Phosphocholine and Pneumococcal C-Polysaccharide

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C-reactive protein (CRP), the major human acute-phase plasma protein, binds to phosphocholine (PCh) residues present in pneumococcal C-polysaccharide (PnC) of Streptococcus pneumoniae and to PCh exposed on damaged and apoptotic cells. CRP also binds, in a PCh-inhibitable manner, to ligands that do not contain PCh, such as fibronectin (Fn). Crystallographic data on CRP-PCh complexes indicate that Phe 66 and Glu 81 contribute to the formation of the PCh binding site of CRP. We used site-directed mutagenesis to analyze the contribution of Phe 66 and Glu 81 to the binding of CRP to PCh, and to generate a CRP mutant that does not bind to PCh-containing ligands. Five CRP mutants, F66A, F66Y, E81A, E81K, and F66A/E81A, were constructed, expressed in COS cells, purified, and characterized for their binding to PnC, PCh-BSA, and Fn. Wild-type and F66Y CRP bound to PnC with similar avidities, while binding of E81A and E81K mutants to PnC was substantially reduced. The F66A and F66A/E81A mutants did not bind to PnC. Identical results were obtained with PCh-BSA. In contrast, all five CRP mutants bound to Fn as did wild-type CRP. We conclude that Phe 66 is the major determinant of CRP-PCh interaction and is critical for binding of CRP to PnC. The data also suggest that the binding sites for PCh and Fn on CRP are distinct. A CRP mutant incapable of binding to PCh provides a tool to assess PCh-inhibitable interactions of CRP with its other biologically significant ligands, and to further investigate the functions of CRP in host defense and inflammation. The Journal of Immunology, 2002, 169: 3217–3222.

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2 Address correspondence and reprint requests to Dr. Alok Agrawal, Department of Biochemistry, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106; E-mail address: axa144@pc.case.edu

3 Abbreviations used in this paper: CRP, C-reactive protein; SAP, serum amyloid P; PnC, pneumococcal C-polysaccharide; PCh, phosphocholine; PEt, phosphoethanolamine; Fn, fibronectin; wt, wild type; CHO, Chinese hamster ovary.

Thus, an investigation of the PCh binding site and the generation of a mutant incapable of binding to PCh are crucial to understand the structure-function relationships of CRP.

CRP is composed of five identical noncovalently bound subunits of 206 aa with a molecular mass of ~23 kDa (1, 7). All five subunits have the same orientation in the pentamer, with a PCh binding site located on one face of each subunit (9). The PCh binding site consists of a hydrophobic pocket formed by residues Leu 64 , Phe 66 , and Thr 76 , and two calcium ions which are bound to CRP by interactions with the side chains and main chain carbonyls of amino acids from different parts of the primary structure (9). Crystallographic analysis of CRP-PCh complexes (Fig. 1) has demonstrated that the phosphate group of PCh directly coordinates with the two calcium ions (21). The choline moiety of PCh lies within the hydrophobic pocket. The exposed face of Phe 66 provides hydrophobic interactions with the methyl groups of choline, while the side chain of Glu 81 , which is located on the other side of the pocket, interacts with the positively charged quaternary nitrogen of choline (21). Previous mutational analyses of Thr 76 in CRP have confirmed the significance of the hydrophobic pocket for PCh binding (22).

In the present study, we have used site-directed mutagenesis to produce CRP with mutations of Phe 66 and Glu 81 . The goal was to determine the contribution of these two amino acids to PCh binding and to generate a CRP mutant incapable of binding to PCh-containing ligands. The results indicate that Phe 66 is the major determinant of PCh binding, although both Phe 66 and Glu 81 participate. The F66A mutant CRP was found incapable of binding to PCh or PnC.

Materials and Methods

Construction of mutant CRP cDNA

We constructed five CRP cDNA encoding F66A, F66Y, E81A, E81K, and F66A/E81A mutants. The substitutions of Phe 66 to Tyr and Glu 81 to Lys were based on the corresponding amino acids in SAP (7). The wild-type (wt) CRP cDNA clone HLCRP-23 in the eukaryotic expression vector p91023 (23–25) was used as template for construction of mutant cDNA for

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Native human CRP was purified from ascitic fluid by calcium-dependent affinity chromatography on a Pch-conjugated agarose column (Pierce, Rockford, IL) followed by HPLC anion-exchange chromatography on a MonoQ column (Amersham Pharmacia Biotech, Piscataway, NJ) as described previously (20, 26). The recombinant wt and all five mutant CRP were purified from culture media by a single affinity chromatography step using a PCh-conjugated agarose column (22). Briefly, 2 ml of culture media containing CRP was diluted to 8 ml in 0.1 M borate buffer saline (pH 8.3) containing 5 mM CaCl2 and passed twice through a 1.0 ml column. After collecting the flow-through fractions and washing with the same buffer (20 ml), bound CRP was eluted with EDTA (10 mM)-containing borate buffer (8 ml). The wt, F66A, and F66A/E81A CRP were also purified by immunoaffinity chromatography using polyclonal anti-CRP Ab-conjugated agarose column for the purposes of recovering fractions containing concentrated CRP. The culture media (2 ml) containing CRP was diluted to 4 ml in TBS and passed twice through the immunoaffinity column (1.0 ml). After collecting the flow-through fractions and washing with the same buffer (8 ml), bound CRP was eluted with 50 mM glycine buffer (pH 3.0) and neutralized immediately with 1 M Tris (pH 9.0). All purified CRP preparations were immediately dialyzed against TBS.

The polyclonal anti-CRP Ab was purified from rabbit anti-human CRP antiserum (Sigma-Aldrich, St. Louis, MO) by affinity chromatography on a CRP-conjugated agarose column. The conjugation of CRP or anti-CRP Ab to agarose was performed using the AminoLink Immobilization kit (Pierce).

**CRP ELISA**

The concentration of CRP was estimated using ELISA as described previously (20). Affinity-purified polyclonal anti-CRP was used as the capture Ab (2 μg/ml), and the monoclonal anti-CRP Ab HD2.4 (5 μg/ml) as reporter. The HD2.4 mAb (27) was affinity-purified from ascitic fluid generated from a hybridoma cell line obtained from American Type Culture Collection (Manassas, VA). Standard curves were constructed with purified native CRP (6.25–200 ng/ml in TBS containing 0.1% BSA and 0.01% Nonidet P-40). HRP-conjugated goat-anti-mouse IgG (Pierce) was used as the secondary Ab, and the color was developed using the HRP substrate kit (Bio-Rad, Hercules, CA). Color was measured at 405 nm in an ELISA plate reader ( Molecular Devices, Menlo Park, CA).

**Pch binding assay**

Binding activity of CRP for Pch-containing ligands was evaluated by two assays using Pch-BSA or PnC in the solid phase as described previously (20). Pch-BSA (9 mol Pch/mol BSA) was synthesized according to a published method (28). PnC was purchased from Statens Seruminstitut (Copenhagen, Denmark). Microtiter wells were coated with either PCh-BSA or PnC at 10 μg/ml in TBS. Because CRP-Pch interaction requires calcium, the culture media or the purified wt and mutant CRP were diluted to appropriate concentrations in calcium-containing buffer (TBS containing 0.1% BSA, 0.01% Nonidet P-40, and 5 mM CaCl2; TBS-Ca), and the same buffer was used throughout the assay. Purified native CRP (0.25–200 μg/ml) was used to construct standard curves. The assays used HD2.4 mAb as a reporter and the wells were developed as in the ELISA. In some experiments, binding curves were constructed by using serial dilutions of purified wt and mutant CRP, covering the concentration range from 1–1000 ng/ml.

**E4A.1 binding assay**

Microtiter wells were coated with caprylic acid-purified anti-CRP mAb E4A.1 at 10 μg/ml in TBS. Because E4A.1 mAb detects a calcium-dependent epitope on CRP (27, 29), the TBS-Ca buffer was used throughout the assay. The binding curves were constructed by using serial dilutions of purified wt and mutant CRP, covering the concentration range from 10–1000 ng/ml. Bound CRP was detected by using affinity-purified polyclonal anti-CRP Ab as reporter. Wells were developed with HRP-conjugated goat anti-rabbit IgG (Pierce) followed by the steps as in the ELISA.

**Fn binding assay**

The binding of CRP to Fn was assessed as described previously (14, 17) with some modifications reported earlier (23). In brief, microtiter wells were coated with 2 μg/ml Fn (Roche) in PBS. The binding curves were constructed by using serial dilutions of purified wt, and mutant CRP, covering the concentration range from 10–5000 ng/ml in PBS (pH 5.0) containing 0.01% Nonidet P-40 and 2% polyethylene glycol-6000. Affinity-purified rabbit anti-CRP was used as reporter to detect bound CRP. The wells were developed as in the E4A.1 binding assay.

**Gel filtration and SDS-PAGE**

Gel filtration of wt and mutant CRP was conducted by HPLC on a Superose 12 PC 3.2/30 column (Amersham Pharmacia Biotech) as described.
previously (23). The affinity-purified CRP preparations (50 µl, 0.4–1.2 µg depending upon the concentrations of the stocks) were injected into the column and eluted with TBS at a flow rate of 40 µl/min. Twenty fractions (3 min each) were collected and CRP-containing fractions were located by ELISA. Purified native CRP was used as molecular size standard. SDS-PAGE of purified CRP (10 µl, 80–240 ng depending upon the concentrations of the stocks) was performed under reducing conditions.

Results

Construction, expression, and PCh-binding activity of CRP

All mutant CRP cDNA were expressed successfully following transient transfections in COS cells, as determined by ELISA. The PCh binding assay, using PCh-BSA as a ligand, was performed on culture media containing wt and mutant CRP (Fig. 2). The wt and F66Y CRP bound to PCh-BSA with similar avidities, as indicated by the ratio of CRP concentrations measured by the PCh binding assay to that measured by ELISA. This ratio reflects specific apparent avidity of individual CRP species for PCh-BSA. The binding of E81A CRP to PCh-BSA was reduced by more than half compared with wt CRP. The three other mutants, F66A, E81K, and F66A/E81A failed to bind PCh-BSA. Two independent clones for each mutant gave identical results.

Purification of CRP by PEt-affinity chromatography

To exclude the possibility that the culture media may interfere with the binding of CRP to PCh-BSA, we purified wt and mutant CRP from the culture media of transiently transfected COS cells. The purification profiles from affinity chromatography on a PEt-conjugated agarose column are shown in Fig. 3. The wt, F66Y, E81A, and E81K CRP bound to PEt-agarose in the presence of calcium and could be eluted by EDTA, producing similar elution profiles. Interestingly, F66A and F66A/E81A mutants bound to PEt poorly, as indicated by the shallow peaks in the unbound fractions, but enough could be purified for use in the solid-phase PCh binding assays. Variation in the peaks for unbound CRP and eluted CRP was due to variations in fraction size and flow rate. Thus, the substitution of Phe66 with Ala abolished the binding of CRP to PCh, but did not abolish the binding of CRP to PEt. Because CRP also binds to other phosphate monoesters such as dAMP (30), we performed affinity chromatography of various CRP species on a dAMP-conjugated agarose column (Sigma-Aldrich). The results (data not shown) were similar to those obtained from chromatography on PEt-agarose column. Assuming that the PEt- and the PCh binding sites of CRP are same, we conclude that the presence of culture media in the binding assays does not interfere with CRP-PCh interactions because all the mutant CRP bound to PEt-agarose.

Dose-response curves of binding of purified CRP to PCh-BSA and PnC

PCh binding assays using purified CRP were performed using two different PCh-containing ligands: PCh-BSA (Fig. 4A) and PnC (Fig. 4B). The wt (native or recombinant) and F66Y CRP bound to PCh-BSA and PnC in a dose-dependent manner and produced essentially overlapping curves, indicating that their PCh-binding activities did not differ from each other. Substitution of Glu81 with Ala reduced binding to both the ligands and the reduction was more pronounced toward PnC. The substitution of Glu81 with Lys substantially reduced binding to both the ligands. F66A and the F66A/E81A CRP mutants did not bind to either PCh-BSA or PnC throughout the dose-response range. Each mutant CRP was purified from two independent transfection experiments and was assayed three times. The data shown in this study represent CRP purified by PEt-affinity chromatography and that obtained for one
Characterization of PCh binding site of CRP using an mAb

We also investigated the relative avidities of various CRP for the mAb EA4.1 (Fig. 5). The binding of this mAb to CRP is calcium-dependent, and can be inhibited by PCh, indicating that EA4.1 binds at or near the PCh binding site (27). As shown, the wt, F66Y, and E81A CRP bound to EA4.1 in a dose-dependent manner and produced overlapping curves, indicating that their EA4.1-binding epitopes, and hence the PCh binding sites, are almost identical. Substitution of Phe66 with Ala or the substitution of Glu81 with Lys drastically decreased binding to EA4.1. The double mutant F66A/E81A was similar to the single mutant F66A in binding to EA4.1 mAb. Therefore, both Phe66 and Glu81 are parts of the EA4.1-binding epitopes on CRP. Because the binding of F66A and E81K CRP mutant to EA4.1 reflected their PCh-binding activity, we interpret the data to indicate and confirm the presence of a structurally altered PCh binding site in these CRP mutants.

Effect of mutated PCh binding sites on CRP-Fn interaction

Because it was previously shown that PCh inhibits CRP-Fn interaction, and it was proposed that CRP binds to Fn via the PCh binding site (17), we tested the CRP mutants for binding to Fn (Fig. 6). All of the mutants bound to Fn as well as did the native and recombinant wt CRP, suggesting that Phe66 and Glu81 do not participate, directly or indirectly, in the formation of the Fn binding site. The observed differences between the binding curves for each CRP were within experimental error. The experiment shown in this study used F66A and F66A/E81A CRP mutants which were purified by immunoaffinity chromatography. A second experiment using PEt-affinity purified F66A and F66A/E81A mutant CRP in the range of 10–200 ng/ml gave similar results (data not shown).

Assessment of the overall structure of CRP

We performed gel filtration chromatography to assess the pentameric nature of the affinity-purified mutant CRP (Fig. 7A), although the results of the Fn binding assay, combined with the finding that all CRP mutants bound PEt in a calcium-dependent manner, indicated that the structure of the CRP mutants was likely to be similar to native CRP. The elution profile of all CRP species from the gel filtration column was similar to that of native CRP. The data are shown only for wt, F66A, and F66A/E81A CRP. The latter two are the ones that did not bind PCh. These results demonstrated that mutant CRP had the same size as native CRP, indicating that they all had a pentameric structure. SDS-PAGE analysis (Fig. 7B) of purified CRP showed a single band and demonstrated that the apparent molecular mass of the subunits of wt and mutant CRP was also identical with that of native CRP. Pentameric CRP-containing fractions from the gel filtration chromatography were again tested for binding to PnC and the results (data not shown) were comparable to the results obtained from affinity-purified CRP and from CRP in the media. Thus, F66A CRP mutant is pentameric and does not bind PCh or PnC.

Discussion

The results indicate that both Phe66 and Glu81 participate in the formation of the PCh binding site, although Phe66 is the major determinant of CRP-PCh and CRP-PnC interactions. The PCh binding site of CRP contains a hydrophobic pocket, two calcium ions, and Glu81, which is located on the other side of the hydrophobic pocket. The hydrophobic pocket consists of Leu64, Phe66, and Thr76 (9). The exposed face of Phe66 provides hydrophobic interactions with the methyl groups of choline, while the side chain of Glu81 interacts with the positively charged quaternary nitrogen of choline (21). The contrasting PCh-binding activities of F66A and F66Y CRP mutants, combined with previously reported findings on the mutational analysis of Thr76 (22), indicate that the topology of the hydrophobic pocket and the interaction between Phe66 and the methyl groups of choline are more important than the interaction between Glu81 and choline nitrogen for binding of CRP to PCh-containing ligands. The importance of the methyl groups of choline in CRP-PCh interaction has been indicated previously by hapten inhibition experiments (30, 31). It has been shown that PCh, which contains three methyl groups, is ~50-fold more powerful an inhibitor of CRP-PnC interaction than PEt, which contains no methyl groups (30, 31), although PCh and PEt are structural analogs (13), and presumably both bind to the same site on CRP.

Our findings on the mutational analysis of the PCh binding site are also informative about the carbohydrate binding site of CRP. CRP binds to deoxyribulose pneumococcal type IV capsular polysaccharide that does not contain PCh (31). Because galactose is the
Only common group between PnC and depruvylated pneumococcal type IV capsular polysaccharide, it has been proposed that CRP interacts with PnC via galactose residues as well (31). However, it was shown that the binding of CRP to carbohydrates occurs with much lower avidity than binding to PCh (30–33). Because the F66A and F66A/E81A mutant CRP did not differentiate between PCh-BSA and PnC for binding, we propose that the PCh binding site also participates in binding of CRP to carbohydrate moieties. This conclusion is supported by the fact that CRP-carbohydrate interactions are also calcium-dependent and PCh inhibitable (31–33). Indeed in SAP, it has been shown that both PEt and the carbohydrates bind to a common site (8).

The finding that the CRP mutants F66A and F66A/E81A, incapable of binding to PCh, bound to Fn was unexpected because PCh is known to inhibit CRP-Fn interaction (17). Our results indicate that the PCh binding and Fn binding sites on CRP are distinct. Because the binding of CRP to Fn is inhibited by high concentrations of calcium (14, 17) that would probably result in occupancy of both the calcium binding sites, we hypothesize that the binding of Fn requires amino acids in CRP that bind calcium ions. Therefore, the inhibition of CRP-Fn interaction by PCh (17) in the presence of calcium could be due to occupancy of the calcium binding sites by calcium. The binding of CRP to Fn is similar to the binding of CRP to polycations; both being inhibited by calcium (30, 34). Our data are consistent with the proposal that the Fn and polycation binding sites on CRP are overlapping (14, 23) and that the PCh binding and polycation binding sites are distinct (30, 34). We favor the statement (30) that the inhibition of binding of CRP to a ligand by PCh, which reflects the combination of PCh and calcium, does not necessarily mean that the PCh binding site is involved. It could be the involvement of one or both of the calcium binding sites. Our data are also compatible with the finding that SAP, that contains a Tyr at position 66 and Lys at position 81, also binds to Fn (7, 35).

Besides binding to bacterial cell wall polysaccharides including PnC, CRP is known to bind to a variety of other biologically significant PCh-containing molecules including enzymatically degraded low density lipoprotein (36), membrane phospholipids like phosphatidylcholine and sphingomyelin (18), and pulmonary surfactant lipids (37). In addition, CRP has been shown to bind to damaged and necrotic cells (38–41) and to apoptotic cells (42), probably as a result of binding to exposed PCh moieties. CRP has the capacity to bind to a variety of non-PCh ligands also, including chromatin, histones and small nuclear ribonucleoproteins (15, 16, 43, 44), polycations (45), and Fn (14). Binding of CRP to most of these ligands is PCh inhibitable. This suggested an important functional role of the PCh binding site in binding to these ligands. However, our finding that Fn does not bind CRP via the PCh binding site raises the need to reevaluate the precise role of the PCh binding site in the interactions of CRP to its known ligands including the non-PCh ligands.

CRP binds to several bacterial species (46) including S. pneumoniae (47), Hemophilus influenzae (48), and Neisseriae spp. (49) most likely through PCh groups. The binding of CRP to S. pneumoniae has been shown to be PCh inhibitable (47). CRP also binds, in a calcium-dependent manner, to fungi (50), yeast (51), and certain parasites such as Plasmodium falciparum (52) and Leishmania donovani (53) through either PCh or non-PCh ligands on their surfaces. In mouse models of bacterial infections, human CRP has been shown to be protective against infection with S. pneumoniae and Salmonella typhimurium, perhaps due to binding of CRP to bacteria through PCh groups present on their surface (54–57). In animal models of myocardial infarction, CRP is found deposited on myocardial cells within the infarcted area (58). Administration of human CRP into a rat model of myocardial infarction resulted in deposition of CRP on the surfaces of damaged myocardial cells in and around the infarct (59) and enhanced the extent of damage by C activation. Based on these findings, it has been proposed that the PCh binding site could be used as a therapeutic target in coronary heart disease. We propose that experiments involving F66A CRP mutant in vitro binding studies with bacteria and the experiments involving administration of F66A CRP mutant in the animal models of human diseases would shed light on the role of PCh binding site of CRP. The mutants reported in this paper, available for use in in vivo experiments, will help prove if the binding of CRP to bacteria via PCh groups is required for protection, and if deposition of CRP on damaged cells and subsequent C activation depend on an intact PCh binding site.

Taken together, our results demonstrate that PhE66F is the major determinant of CRP-PCh interaction and is critical for binding of CRP to PnC. The data also suggest that PCh binding and Fn binding sites on CRP are distinct. A CRP mutant, F66A, incapable of binding to PCh provides a tool to assess PCh-inhibitable interactions of CRP with its other biologically significant ligands in vitro, and to investigate the functions of CRP in host defense and inflammation in vivo.

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