ANALYSIS OF BINDING SITES IN HUMAN C-REACTIVE PROTEIN FOR FcγRI, FcγRIIa AND C1q BY SITE-DIRECTED MUTAGENESIS1

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Running title: Mapping of CRP binding sites to FcγR and C1q

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Human C-reactive protein (CRP) is a classical, acute phase serum protein synthesized by the liver in response to infection, inflammation, or trauma. CRP binds to microbial antigens and damaged cells, opsonizes particles for phagocytosis and regulates the inflammatory response by the induction of cytokine synthesis. These activities of CRP depend on its ability to activate complement and to bind to Fcγ receptors (FcγR). The goal of this study was to elucidate amino acid residues important for the interaction of CRP with human FcγRI (CD64) and FcγRIIa (CD32). Several mutations of the CRP structure were studied based on the published crystal structure of CRP. Mutant and wild-type recombinant CRP molecules were expressed in the baculovirus system and their interactions with FcγR and C1q were determined. A previous study by our laboratory identified an amino acid position, Leu176, critical for CRP binding to FcγRI and work by others determined several residues important for C1q binding. The amino acid residues important to CRP binding to FcγRIIa were previously unknown. This study newly identifies residues Thr173 and Asn186 as important for the binding of CRP to FcγRIIa and work by others determined several residues important for C1q binding. The amino acid residues important to CRP binding to FcγRIIa were previously unknown. This study newly identifies residues Thr173 and Asn186 as important for the binding of CRP to FcγRIIa and FcγRI. Lys114, like Leu176, was implicated in binding to FcγRI, but not FcγRIIa. Single mutations at amino acid positions Lys114, Asp169, Thr173, Tyr175 and Leu176 affected C1q binding to CRP. These results further identify amino acids involved in the binding sites on CRP for FcγRI, FcγRIIa, and C1q and indicate that these sites are overlapping.

C-reactive protein (CRP)1 is a member of the ancient pentraxin protein family (1). Classical pentraxins share several features including a cyclic arrangement of five (or in a few cases six) identical non-covalently bound subunits, calcium-dependent ligand binding sites in each subunit, and high sequence similarity (2). Although there is little overall structural resemblance between CRP and immunoglobulin, CRP exhibits multiple functional similarities to antibodies, reviewed in (3). These include recognition of ligands, activation of complement via the classical pathway, binding to receptors on phagocytic cells, induction of cytokine synthesis and enhancement of phagocytosis. These functional similarities are explained by the shared ability of CRP and IgG to interact with complement component C1q and with Fcγ receptors (FcγR) I and II. Conservation of its cyclic pentameric structure, calcium-dependent binding, and phosphocholine (PC) binding capability throughout evolution (4) suggest an important biological role for CRP. In vivo experiments have implicated CRP in protection against infection, regulation of systemic and local inflammatory reactions, clearance of autoantigens, and prevention of systemic autoimmune disease, reviewed in (3,5).

FcγR are well characterized as important to antibody-mediated immunity and when cross-linked by bound IgG antibodies, trigger cellular responses. It has been previously demonstrated that CRP binds to human FcγRI on U-937 cells and transfected COS-7 cells (6,7). This finding has
recently been confirmed by surface plasmon resonance studies, which established a high affinity interaction between FcγRI and CRP \((K_D=0.81 \times 10^{-9} \text{M})\) \((8)\). Our laboratory has recently shown that FcγRII is the second receptor for CRP on human leukocytes and myeloid cells \((9,10)\). The binding of CRP to FcγRIIa was recently confirmed by confocal fluorescence microscopy \((11)\) and the rosetting of CRP opsonized erythrocytes by FcγRIIa-transfected COS-7 cells \((12)\).

Interactions between IgG of different subclasses and FcγR have been extensively characterized by using chimeric antibodies, site-directed mutagenesis, and most recently co-crystallization of the human IgG1 Fc fragment with soluble FcγRIII \((13-16)\). The selection of the sites to be mutated on CRP was based on the current knowledge regarding the sites in IgG that interact with FcγR, the assumption of functional homology of the similar amino acid motifs in IgG and CRP and the published crystal structure of CRP \((17)\). Because aggregated IgG completely inhibits CRP binding to FcγR on cells, and human IgG1 completely inhibits CRP binding to FcγRI in surface plasmon resonance \((8)\), it is likely that the sites on FcγR that bind IgG and CRP are similar. Based on sequence homology with IgG \((18)\), we previously identified a specific amino acid position, Leu176 that is important for CRP binding to FcγRI. Mutation of Leu176 to Glu eliminates CRP binding to FcγRI while preserving binding to FcγRIIa. Another group has identified several amino acid residues in CRP important in binding to C1q \((7,19,20)\).

CRP is an important molecule involved in multiple aspects of the response to infection and other inflammatory stimuli. Increasing our understanding of the basic biology of the interaction of CRP with receptors on inflammatory cells will increase our understanding of the inflammatory process and may lead to the development of new anti-inflammatory molecules, warranting investigation of CRP’s interactions at the molecular level. The homogeneity of human CRP in the human population dictates that any investigation into the structure-function relationships of CRP requires alternate methods than studying naturally occurring polymorphisms. This study uses oligonucleotide-directed mutagenesis to characterize the interaction of CRP with FcγRI and FcγRIIa, and with C1q. The mutant and wild-type (wt) recombinant proteins were expressed in the baculovirus system \((21)\) and were purified and tested for binding to FcγR and C1q. This strategy enabled us to identify important residues in FcγRI and FcγRIIa binding. The results indicate that the residues, Lys114, Thr173, Leu176 and Asn186 in CRP are important for binding to FcγRI. Thr173 and Asn186 are important for binding to FcγRIIa and mutation of Lys114, Leu176 or Thr173 affected C1q binding as well. Additional amino acid positions Asp169 and Tyr175 were also demonstrated to be important to CRP binding of C1q. Thus, it is evident that the binding sites on CRP for FcγRI, FcγRIIa, and C1q are discrete but must overlap.

Materials and Methods

**Mutagenesis**—Mutant CRP DNAs were generated with the PCR-mediated QuikChange mutagenesis kit (Stratagene, La Jolla, CA). A 1.01 kb BamHI-BglIII fragment was subcloned from \(\lambda\)HLCRP23 (a generous gift from J.E. Volanakis, University of Alabama at Birmingham), into the baculovirus transfer vector pBlueBac4 \((pBB4, \text{Invitrogen, Carlsbad, CA})\) and maintained in DH5-\(\alpha\) E. coli \((\text{Promega, Madison, WI})\). This construct was used as the CRP template for all PCR reactions, except L176A which was subcloned into pFastBac1 \((\text{Invitrogen, Carlsbad, CA})\).

Primer sequences were designed based on the published genomic DNA sequence for CRP \((22)\). Primer pairs were designed with single, double, or triple base changes to produce single amino acid changes upon translation. The primers were synthesized either on an Oligo 1000 DNA Synthesizer \((\text{Beckman Instruments, Inc., Fullerton, CA})\) or by the Center for Genetics in Medicine at the University of New Mexico \((\text{Albuquerque, NM})\). Mutant CRP plasmid DNA was produced by PCR with the CRP containing plasmid as template. In order to remove the template DNA (which consists of wild-type sequence), PCR products were then subjected to \(Dpn\ I\) digestion at 37°C for 3 h to preferentially eliminate bacterial DNA, i.e., methylated and hemimethylated DNA. Mutant CRP DNAs were used to transform DH5-\(\alpha\) E. coli \((\text{Promega, Madison, WI})\) for clonal expansion according to manufacturer’s directions.
The identities of mutant DNAs were confirmed via direct sequencing (Sequenase V. 2.0, Amersham Biosciences, Piscataway, NJ) or by cycle sequencing at the Center for Genetics in Medicine at the University of New Mexico (Albuquerque, NM). Large-scale DNA plasmid preparations were then performed by cesium chloride gradient centrifugation followed by phenol-chloroform extraction and ethanol precipitation.

Recombinant protein expression: Mutant CRP DNAs in the baculovirus transfer vector were co-transfected with baculovirus DNA (Bac-N-Blue, Invitrogen) into SF-9 insect cells (Invitrogen, Carlsbad, CA) using the MaxBac Transfection Kit (Invitrogen), except for L176A which was constructed with the BactoBac Expression System (Invitrogen). SF-9 insect cells were maintained in supplemented Grace’s Media (Invitrogen), according to manufacturer’s instructions, and additionally supplemented with heat-inactivated 10% fetal calf serum (Hyclone Labs, Ogden, UT). Transfection stocks were plaque purified according to the Transfection Kit manufacturer’s directions as described (23). Recombinant plaques were selected based on phenotype and β-galactosidase reporter gene expression. Individual recombinant plaques were picked and used to infect additional SF-9 cells plated at a density of 5 x 10^5 cells/well in 12-well tissue culture plates. Viral DNA was isolated from the culture supernatants according to manufacturer’s directions and verified by PCR. Viral stocks were re-purified by plaque assay if they contained non-recombinant as well as recombinant virus. Viral stocks comprised of pure, recombinant virus were used to inoculate successively larger cultures of SF-9 cells to create high-titer stocks of recombinant virus. The titers of the viral stocks were determined by plaque assay on SF-9 cells. The high-titer viral stocks were then used to inject *Trichoplusia ni* (Lepidoptera) larvae (Entopath, Easton, PA) for large scale mutant protein expression as previously described (7,21), except that the proteins were not metabolically labeled. Larvae were processed for CRP recovery as previously described (21). Briefly, a crude larval lysate containing protease inhibitors was applied to a PC-Sepharose affinity chromatography column in the presence of 2 mM calcium. The bound CRP was eluted from the column with a pH 7.4, 10 mM Tris, 75 mM citrate buffer and dialyzed into pH 7.4, 20 mM Tris, 0.15 M NaCl, 10 mM CaCl_2 (TNC). The purity of the CRP was evaluated with 5-20% polyacrylamide gradient SDS-gel electrophoresis.

Reagents and Antibodies: Human CRP was prepared from human pleural fluids as previously described (24). PC-conjugated BSA (15:1 molar ratio) (PC-BSA) and C-polysaccharide (PnC) were prepared in our laboratory as described (25,26). The anti-CRP monoclonal antibody (mAb) 2C10 was graciously provided by Dr. Larry Potempa (Immtech, Evanston, IL) and used as a hybridoma tissue culture supernatant. 8C10 and 9H8 are anti-CRP mAb that recognize denatured or “neo” determinants on CRP (27) and were kindly provided in purified form by Dr. Henry Gewurz (Rush Medical College, Chicago, IL). C1q was purchased from Sigma (St. Louis, MO). The following antibodies were purchased: HRP-sheep anti-human CRP from The Binding Site (San Diego, CA); sheep anti-human CRP, HRP-goat anti-human C3 and HRP-goat anti-mouse IgG from ICN (Irvine, CA); FITC-goat anti-mouse (GAM) IgG F(ab’)_2 affinity purified PE-GAM IgG F(ab’)_2, PE-C1KM5 (anti-CD32 mAb) and PE-10.1 (anti-CD64 mAb) from Caltag (Burlingame, CA); mouse mAb to human C1q from Quidel (San Diego, CA); AT10 (an anti-CD32 mAb) and FITC-AT10 from Serotec (Raleigh, NC); FITC-FL18.26 (an anti-CD32 mAb) from BD-Pharmingen (San Diego); anti-CD64 mAb 197 and anti-CD32 mAbs IV.3 and 32.2 from Medarex (Annandale, NJ). FITC-FL18.26 was pre-absorbed against mock-transfected COS-7 cells for 30 minutes on ice prior to use, in order to reduce non-specific binding. Enzyme-linked immunosorbent assays (ELISA): Immulon 2 microtiter plates (Dynatech Laboratories, Alexandria, VA) were used throughout. The blocking buffer was 0.5% BSA in TNC, the dilution buffer was 0.1% BSA in TNC, and the washing buffer was 0.05% Tween 20 in TNC. The plates were developed with ABTS substrate (1 mg/ml 2,2’-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) and 0.005% H_2O_2 in McIlvaine’s citric acid-phosphate buffer, pH 4.6) and absorbance measured at 405 nm using an ELISA plate reader.

Isolated CRP was quantitated by sandwich
ELISA using a human CRP standard curve as described (21) as well as by BCA assay (Pierce Chemical Co., Rockford, IL). Wt and mutant CRP were tested for native structure by ELISA as previously described (21). Briefly, ELISA plates were coated with 5 µg/ml PC-BSA, blocked and incubated with 5 µg/ml CRP. The mAb 2C10, which recognizes native determinants, was used to determine PC-BSA binding ability. The mAb 8C10 and 9H8 were used to determine the exposure of denatured determinants, which are not detectable on ligand-bound human CRP (27). All mAb were detected using HRP-goat-anti-mouse IgG and ABTS substrate.

Human, wt and mutant CRP were compared for ligand binding using an inhibition assay. ELISA plates were coated with 10 µg/ml PC-BSA or PnC and blocked. CRP (20 ng/ml) was mixed with free PC (10 to 100 µM) and applied to the wells. CRP binding was detected using HRP-anti-CRP and ABTS substrate.

Wt and mutant CRP were compared for C1q binding and complement activation on PC-BSA-coated ELISA wells. Plates were coated with PC-BSA at 5 µg/ml in TNC. The plates were blocked and incubated with CRP samples, diluted from 0.10 to 3.0 µg/ml. The plates were washed and incubated with C1q at 5 µg/ml for 1 h at room temperature. Binding was detected using anti-C1q mAb at 5 µg/ml, HRP-conjugated anti-mouse IgG and ABTS substrate. For the complement activation assay normal human serum diluted 1/20 was added in place of C1q, and plates were incubated for 15 min at 37°C. Plates were developed with HRP-rabbit-anti-human C3 and substrate. Heat-inactivated (56°C, 30 min) normal human serum was used as a control.

Cell Culture---The COS-7 cell line (American Type Culture Collection, Bethesda, MD) was used to express either FcγRI or FcγRIIa. The cells were grown in a humidified incubator at 37°C in 5% CO₂ in DMEM (Sigma, St. Louis, MO) supplemented with HEPES and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). K-562 cells were also obtained from the American Type Culture Collection and grown in RPMI-1640 media (Sigma) supplemented with 10% calf serum (HyClone, Logan, UT), 2mM L-glutamine and 50 µg/ml gentamycin.

Receptor Expression---For the cell transfections, the cDNA clone for the R131 allele of human FcγRIIa in the pCDSRα296 transient transfection vector (28) was obtained from Dr. Kevin Moore (DNAx Research Institute, Palo Alto, CA). The plasmid DNA pRC/CMV-FcγRIaI (the IgG high-affinity receptor) was a gift from Dr. Robert P. Kimberly (University of Alabama at Birmingham, Birmingham, AL). Cells were transfected with the GenePORTER transfection reagent from Gene Therapy Systems, Inc. (San Diego, CA) as described (29) or with the Gene Juice transfection reagent from Novagen (Madison, WI) according to the manufacturer’s instruction. Control cells received transfection reagent only. Flow cytometric assays were performed 66-78 h after transfection. The percentage of transfected cells was determined using either FITC-FLI8.26 or PE-C1KM5 for FcγRII and PE-10.1 or mAb32.2 and PE-GAM IgG F(ab')2 for FcγRI.

CRP receptor binding assay---The CRP receptor binding assay has been described previously (29). Cells in PBS containing 0.05% azide and 0.1% globulin-free BSA (PAB) were incubated with CRP diluted in PAB for 1 h on ice, then washed twice and incubated with mAb2C10 culture supernatant for 30 min on ice. Cells were washed twice with PAB, and incubated with PE-GAM IgG F(ab')2 (1 µg/10⁶ cells) or FITC-GAM IgG F(ab')2 for 30 min on ice. The cells were then washed and resuspended in PAB for analysis by flow cytometry on a Becton-Dickinson FACSCalibur flow cytometer equipped with Cell Quest software (Becton-Dickinson, Mountain View, CA). The samples were analyzed by forward and side scatter to exclude dead cells, and a minimum of 20,000 cells was collected.

The mutants were tested for binding to both FcγRI and FcγRII with transfected COS-7 cells expressing FcγRI or FcγRIIa and K562 cells, a human erythroleukemia cell line that expresses FcγRIIa as its only FcγR. K562 cells were used for initial screening of mutants for FcγRIIa binding. Those mutant proteins showing a change from wild type for receptor binding were verified by binding assay to transfected COS-7 cells. Several recombinants showing no change from the wild type were, however, initially tested in both the cell culture and transfection systems, in order to ensure predictive value of the cell culture system before transfections with FcγRIIa were performed.
Data for receptor binding were collected on a Becton-Dickinson FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson, Mountain View, CA). Analysis was performed by nonlinear regression analysis using GraphPad PRISM (GraphPad Software, Inc., San Diego, CA). For all measurements of CRP binding, the binding of 2C10 and the anti-mouse secondary antibody were subtracted. Results are presented as the change in geometric mean channel fluorescence (ΔGMCF) relative to secondary antibody controls.

**Molecular models**- Models shown in Figures 1 and 7 were produced with the program WebLab ViewerLite (Molecular Simulations, Inc.) using the crystal structure of CRP complexed with PC (1B09)(15) and human IgG1 Fc region obtained from co-crystallization with FcγRIII (1E4K)(15).

**RESULTS**

**Design of mutants.** We have established that CRP binds to FcγRI and FcγRIIa on human leukocytes, myeloid cells and transfected COS cells (7,10,29). The particular sites to be mutated were chosen on the basis of sequence similarity between CRP and the regions of IgG shown to be important for FcγR binding (30-32). In IgG two regions were identified as important for IgG binding to FcγRI and FcγRII by comparison between IgG from different species and subclasses (31). The first region defined was 234LLGGPS239 for human IgG1 and FLGGPS for IgG4. The importance of this region in binding to FcγRI and FcγRII was confirmed by mutagenesis experiments (13,18,30). A second region in IgG 327ALPAPI333 was initially identified by sequence homology (18) and more recently confirmed by site-directed mutagenesis (14) and co-crystallization (15,16). This region also contains residues (Pro 329 and Pro 331), important for human IgG1 binding to C1q (33). Sequence homologies between CRP and serum amyloid P component (SAP) and both of these regions of IgG have been identified (Table 1). For the human pentraxins, there is a greater resemblance between CRP and the 234-237 region of IgG, whereas SAP shows greater sequence homology with the 328-333 region of IgG. Interestingly, these regions that are separated by a large stretch of amino acids in IgG are contiguous in CRP and SAP. The location of this proposed binding region on the A face of the CRP pentamer is shown on the bottom of Fig. 1 and a comparison of this region with the homologous regions on human IgG1 is shown on the top of Fig. 1. Both regions show an exposed proline (colored brown). In IgG this proline is proposed to interact with FcγRIII in a proline sandwich with tryptophans 87 and 110 (15).

Additional positions were chosen based on sequence differences between CRP and SAP, since SAP was expected to demonstrate a different binding activity with respect to CRP at the outset of this study. However, since the inception of the study, we have determined that SAP also binds to human FcγRI, FcγRIIa, and FcγRIIb (34). CRP is postulated to possess a binding groove, bordered on one side by an α-helix comprised of the residues Glu169 to Leu176 and a loop comprised of the residues Gly177 to Asn183 and toward the center of the pentamer by another loop comprised of the residues Glu112 to Lys114. This cleft is not present in SAP due to differences in amino acid identities in the homologous positions in the sequences of the two proteins (35). Therefore, the mutagenesis encompassed some SAP-like mutants, N158D, L176Q, D169E, E170N and N186D at individual residues within the putative binding cleft.

**Verification of mutant molecule integrity.** Mutants were tested for preservation of conformational integrity by ELISA with mAb specific for native and denatured determinants. Mutants were further tested for ligand binding by measuring PC inhibition of binding to wells coated with PC-BSA and PnC. All of the mutants, except E170N, bound to PC-BSA and reacted with mAb 2C10, which recognizes native and denatured determinants (27) and those tested all showed nearly identical PC inhibition of binding to PC-BSA (not shown) and PnC (Fig. 2B).

**Cell binding.** The mutant CRPs were tested for binding to FcγRI and FcγRIIa-transfected COS-7 cells. Representative binding assays are shown in Fig. 3-5. The most dramatic change in binding was shown by T173A, which
mutation eliminated binding to FcγRI (Fig. 3) and drastically reduced binding to FcγRIIa (Fig. 4A), when compared to the binding of wt recombinant CRP. Mutant T173A represents the first identification of a residue involved in binding of CRP to FcγRIIa. It also identifies another residue involved in CRP binding to FcγRI to add to the previous demonstration of the contribution of residue 176 in CRP binding to FcγRI (7). The loss of binding of T173A to FcγRIIa was confirmed using K562 cells, which express FcγRIIa (Fig. 5).

Mutant L176Q (Fig. 3), and mutants D169E, D169A, Y175L and N158D (Fig. 4) effected little discernible change in binding to either FcγRI or FcγRIIa. E170N was shown to have reduced binding to FcγRIIa, but the structural integrity of the protein was questionable based on low reactivity in the PC-BSA binding assay (Fig. 2A). L176Q, unlike the mutant L176E (7) and L176A (Table 2) showed no appreciable change in binding to FcγRI or FcγRIIa (Fig. 4B), indicating a requirement for both a neutral charge and a large side chain for the interaction with the corresponding residue on FcγRI. The results of binding assays with other CRP mutants are summarized in Table 2. Notably, K114A showed reduced binding to FcγRI, but normal binding to K562 cells, and mutant N186D showed reduced binding to both FcγRI and K562 cells. The Y175L mutation was predicted to increase binding to FcγR based on sequence homology. This mutant did show increased binding to mouse FcγR, based on binding studies with peritoneal macrophages from FcγRIIb-deficient mice (not shown). Binding to human and mouse FcγRII was unchanged.

C1q binding. Mutants K114A, D112A (7,19), and E88A (20), have previously been identified as corresponding amino acid residues important in C1q binding. Because the C1q and FcγR binding sites of CRP are postulated to be on the same face of the molecule (20), it is reasonable that certain important residues for binding may be shared not only by the FcγR, but among FcγRI and FcγRIIa and C1q. Our group also examined mutant K114 and found C1q binding to be high versus wt CRP (Fig. 6), congruent with the previous findings of Agrawal et al. (19). Mutants N158D and E88A exhibited normal binding to C1q, in comparison to the previous demonstration of no change in C1q binding by mutant N158S and a slight decrease for N158A and E88A and E88Q (19). Mutant T173A (Fig. 6) and mutants D169A, Y175L, L176Q, L176A, L176E, and N186D (Table 2), however, no longer bound C1q. The results of the complement activation assay measuring C3 deposition after incubation in normal human serum agreed with the C1q binding assays (not shown).

E170N appeared to be a possible expression-defective variant, based on dramatically reduced binding of PC-BSA. Although binding curves were initially generated for E170N and both FcγR in this study, the protein was unable to bind PC-BSA soon after the flow cytometry experiments, thus calling into question the stability of the mutant. The change of this amino acid may have induced a large conformational change to disrupt the tertiary structure of the expressed protein. This mutant was, therefore, not pursued further.

The amino acid positions at which mutations were made are depicted in red on one subunit in Fig. 7. The residues implicated in FcγR binding site are depicted in cyan on a second subunit, and the proposed C1q binding site is depicted in green on a third subunit, showing considerable overlap. The homology region residues 175-185 between CRP and IgG is shown in yellow.

DISCUSSION

The goals of the present study were to examine the binding sites on CRP for FcγRI and FcγRIIa and to develop mutant CRPs lacking individual binding activities. Modification of Leu176 to Glu in a prior study by our laboratory completely abrogated CRP binding to FcγRI without affecting its binding to FcγRIIa (7). We examined this particular amino acid position again and changed Leu176 to either Gln or Ala. CRP with Leu176 changed to Ala had binding properties that were similar to the change to glutamic acid, while the change to glutamine resulted in no discernible change of binding interaction with FcγRI or FcγRIIa. Because the glutamine mutation changes the amino acid to the same identity as that of SAP, the observation is consistent with the finding that SAP also binds murine and human FcγR (7,34,36).
However, binding to C1q was absent in the Gln variant, as in the Glu and Ala variants of position 176. These observations support the hypothesis of Agrawal et al. (20) that an α-helix at amino acids 169-176 at the C-terminus of the protein—and part of a large cleft on CRP (opposite its PC-binding face) contributes a part of the C1q binding site.

The amino acid residues involved in CRP binding to FcγRIIa were completely unknown prior to this study. In this study, we have been able to identify Thr173 and Asn186 as residues affecting CRP binding to both FcγRI and FcγRIIa. The fact that residue 176 should exhibit different binding capability dependent upon the type of amino acid change indicates that at this time we cannot rule out a large degree of overlap in the FcγRI and FcγRIIa binding sites on CRP that would include Leu176 in the FcγRIIa binding site. It has been demonstrated that, in IgG, the LLGG motif (234-237 EU numbering, see Kabat et al. (37)) is part of the active site for both FcγRIIa binding and FcγRI binding (13,14,18,30). Yet, the interactions for each residue are not the same for IgG and each receptor (14,30), as in our observation of Leu176 in CRP. The fact that the type of amino acid change made alters the binding capability to FcγR indicates that our mutagenesis screen in all likelihood provides only a limited, first estimation of the identification of residues involved in CRP binding. We can, however, state that Thr173 and Leu176 must be important to CRP binding to FcγRI. Both of these residues are within the C-terminus of the pentraxin α-helix (aa169-aa176) that borders a shallow pocket on the A face of CRP (35). It was recently demonstrated that the residues involved in binding of IgG1 to FcγRI are within a subset of a group that are involved in binding to FcγRII (and FcγRIII)(14). At the current time, with only a small number of CRP residues investigated, we can say that the same is not true for CRP binding to the different FcγR, because although mutation of Thr173 and Asn186 affected binding to both FcγRI and FcγRII, while mutation of Lys114 and Leu176 only affected FcγRI binding.

In this study we produced mutants D169A, T173A, Y175L, and L176Q, which all eliminated C1q binding (Fig. 6). These are within the α-helix at amino acid positions 169-176 at the C-terminus of the protein that is proposed to be a major component of a C1q binding pocket (20). N158D, D169E, and E88A did not show any difference in C1q or FcγR binding. It was previously reported that N158S bound normally to C1q and that N158A, E88A and E88Q showed slightly decreased binding (20). While D169E had normal binding, mutation of D169 to alanine, eliminated binding, providing support for the hypothesis that charge is important for CRP binding to C1q (18). We further confirmed increased C1q binding and complement activation for the K114A mutant located in the Asp-Lys loop toward the center of the CRP pentamer. These observations help to further refine the identification of the C1q binding site by Agrawal et al. (19,20), which was supported by modeling (38).

Interestingly, the K114A mutant lacked FcγRI binding, but retained FcγRIIa binding. To the degree that there appear to be multiple important residues in common among the FcγRI and FcγRIIa and C1q binding sites, one may postulate that a portion of the C1q binding site has been co-opted for binding to FcγR some time during the course of the evolution of the vertebrate lineage or perhaps of the mammalian lineage. The complement system existed before the emergence of adaptive immunity and has recently been suggested as being present in the dipteran insect Anopheles gambiae, based on the identification of a novel protein that is both structurally and functionally similar to complement factor C3 (39), the central component of the complement activation pathways. Members of the family of pentraxin molecules had been identified in another arthropod, horseshoe crabs (40,41), and recently a putative and novel Drosophila pentraxin homologue, b6, has been characterized (42). CRP is viewed as a prototypical acute phase protein and is part of a nonspecific, initial response to tissue insult or invasion by pathogens, which would likely represent an evolutionary precursor to the innovation of a clonal host response seen in most vertebrates. CRP’s ability to activate complement would surely have conferred sufficient selective advantage to maintain its expression since the protostome-deuterostome divergence to persist through the emergence of the vertebrate lineage, in order to be available for co-option for use in the FcγR-mediated adaptive immunity response.

The modulation of binding activity
through site-directed mutagenesis of CRP may be re-examined in light of recent claims that CRP does not bind to FcγRIIa (45) and not to FcγRI nor FcγRIIa (43). These findings have been addressed elsewhere (8,34,44), but the observations in this study provide additional evidence for FcγR binding by CRP. These claims argue that CRP binding to these receptors previously described by our laboratory and others was likely due to binding of the Fc portion of the antibodies used for detection (45) or contaminating IgG in CRP preparations used in binding studies (43). If the prior claim were correct, then we should have been able to expect that mutagenesis of CRP would effect no change in demonstrable binding avidity in conjunction with an antibody-based binding detection method. The ability to reduce, enhance, and/or eliminate binding ability by mutating recombinant CRP in this study indicates that the CRP must be able to bind FcγR, since the lack of binding was detected with an antibody-based detection system (2C10 mAb). Additionally, to address the latter claim, we demonstrate in this study several recombinant mutant CRP’s that show minimal to no change in FcγR specificity. This is germane because insects do not have the capacity to express IgG that might otherwise be present in the protein preparation and detected as binding to FcγR.

The co-crystallization of the human IgG1 Fc fragment with FcγRIII (15,16) and studies of the mouse IgG2b Fc fragment and mouse FcγRII interactions in solution using NMR (46) have led to a model of asymmetric binding of one FcγR to two CH2 domains. Several other FcR have been crystallized (47,48) and these structures were used to form a general model for Fc-FcγR interactions (49). In this model, 234Leu-Ser239 residues in both hinge regions of the CH2 domains provide important contacts with hydrophobic regions of FcγR. A second major contact is a “proline sandwich” formed between Pro239 of one CH2 domain and two Trp residues (87 and 110) on FcγR. Pro329 is part of the second homology region identified in Fig. 1. Based on these models and the data obtained from the CRP crystal structure and mutagenesis experiments, we propose that the hydrophobic region formed by the α-helix (Glu169 to Leu176) and adjacent loop (177Gly-Asn186) on the A face of CRP provide the major area of contact between CRP and FcγR (see Fig. 1). The location of a “proline sandwich” motif to CRP binding is speculative, but Pro182 is exposed on the binding face in a homologous position to Pro329 in the Leu-Glu333 region of IgG. Mutation of residues Thr175, Leu176 and Asn186 within the homology region all decreased binding of CRP to one or both FcγR. A peptide derived from this region of CRP (174Ile-Leu185) has been reported to activate human and mouse macrophages for tumor cell killing and cytokine production (50-52). Based on spatial considerations, we propose that a single CRP subunit contacts the D2 domain of FcγR. Although co-crystallization of Fc-FcγRIII shows contact by both IgG CH2 domains, experiments using hybrid antibodies demonstrated that a single IgG1 CH2 domain containing the LLGGP motif is sufficient for high affinity binding of IgG to FcγRI (53). The C1q binding site is proposed to include charged residues (Glu88 and Asp112) toward the central pore of the CRP pentamer. This explains the increased C1q binding ability of the K114A mutant. The role of this region in FcγR binding is less clear. The E88A mutant bound to both FcγR normally, and the K114A mutant eliminated FcγRI binding without altering FcγRIIa binding. There is no obvious corresponding residue in the IgG1 Fc-FcγRIII structure (15). However, FcγRII has an additional domain compared to FcγRII and FcγRIII, and the interactions between this domain and IgG have not been fully characterized (14,15). The carbohydrate residue NAG1 at Asn297 of IgG1 is proposed to contact FcγRIII (15) or to stabilize the hinge region of IgG1 (16). CRP is not glycosylated and therefore a directly homologous interaction cannot be present.

Identification of important residues responsible for the binding interaction can guide design of CRP for modulating immune recruitment through FcγR, as well as by complement activation. The FcγR binding-mutants presented here can provide additional molecular tools for future functional studies, in order to better understand CRP’s role in immune function through FcγR-mediated signaling. The mutants will allow for the study of interactions of CRP with individual FcγR on leukocytes. CRP is an important molecule that is involved in many aspects of the inflammatory response to infection and inflammatory stimuli. Increasing our
understanding of the basic biology of the interaction of CRP with receptors on inflammatory cells will increase our understanding of the inflammatory process and may facilitate the generation of new therapeutic molecules.

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FOOTNOTES

1 The abbreviations used are: ABTS, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); CRP, C-reactive protein; ΔGMCF, change in geometric mean channel fluorescence; ELISA, enzyme-linked immunosorbent assays; FcγR, Fcγ receptors; mAb, monoclonal antibody; GAM, goat anti-mouse IgG, PAB, PBS containing 0.05% azide and 0.1% globulin-free BSA; PC, phosphocholine; wt, wild type; PnC, Streptococcus pneumoniae C-polysaccharide; TNC, pH 7.4, 20 mM Tris, 0.15 M NaCl, 10 mM CaCl2; SAP, serum amyloid P component.

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FIGURE LEGENDS

Fig. 1. Location of the homology regions on the A face of the human CRP pentamer. A model of the crystal structure of the human CRP pentamer A face is shown at the bottom with residues 175-185 shown as space-filling with colors as defined in the table within the figure. The top of the figure shows residues 175-185 of CRP overlaid onto a model of human IgG1 (showing residues 229–341 of both Cγ2 domains of human FcγRI) with the corresponding region (residues 234-238,328-333) shown as space-filling.

Fig. 2. A. Binding of mutant CRP to PC-BSA and PnC. CRP mutants were bound to ELISA wells coated with PC-BSA and detected with mAb 2C10 as a measure of ligand-binding activity. B. CRP mutants were incubated with increasing amounts of PC and binding to ELISA wells coated with PnC was determined using HRP-sheep anti-CRP. Additional mutants that were tested (Y175L, L176E, L176A) also showed identical PC inhibition curves compared to human CRP.

Fig. 3. Decreased binding of CRP mutant T173A to FcγRIIa-transfected COS-7 cells. COS-7 cells were transfected with a plasmid encoding human FcγRIIa and binding of human and recombinant CRP was determined by flow cytometry. The ΔGMCF is the geometric mean channel fluorescence after subtracting the second antibody control. Data were fitted to a single binding site model for receptor binding. Binding of mutant T173A is greatly reduced compared to human CRP, wt recombinant CRP or L176Q. Mock indicates binding of human CRP to mock-transfected cells.

Fig. 4. Decreased binding of CRP mutant T173A to FcγRIIa-transfected COS-7 cells. COS-7 cells were transfected with a plasmid encoding human FcγRIIa (R131 allele) and binding of human and recombinant CRP was determined by flow cytometry. The ΔGMCF is the geometric mean channel fluorescence after subtracting the second antibody control. Data were fitted to a single binding site model for receptor binding. Results from two representative experiments are shown in panels A and B. Binding of mutant T173A is greatly reduced compared to human CRP, wt recombinant CRP or the mutants D169A, D169E, N158D or Y175L. Mock indicates binding of human CRP to mock-transfected cells.

Fig. 5. Decreased binding of CRP mutant T173A to K562 cells expressing FcγRIIa. Recombinant CRP were tested for K562 binding in order to corroborate the results from binding assays for FcγRIIa in the transfection system. Binding assays were carried out as described for the transfected-COS-7 cells, except using the K562 cell line which expresses FcγRIIa as its only FcγR. Data were fitted.
to a single binding site model for receptor binding. Results for other mutant CRP are summarized in Table 2.

**Fig. 6.** Decreased binding of several CRP mutants to C1q. Recombinant CRP were bound to PC-BSA coated wells and tested for C1q binding using purified human C1q and a mAb to C1q for detection. The mean absorbance of duplicate wells from a representative experiment is shown. Results indicate that mutant K114A has increased C1q binding. Mutants N158D and D169E are within the range of human and recombinant wt CRP. Mutants T173A, Y175L, L176Q and D169A did not bind C1q. Results for other mutant CRP are summarized in Table 2.

**Fig. 7.** Structure of CRP with proposed FcγR and C1q binding sites. The CRP pentamer is shown with residues 175-185 in yellow on one subunit. The residues investigated are indicated in red on a second subunit. On a third subunit the residues directly implicated by mutagenesis experiments to be involved in binding to FcγR are shown in cyan. The proposed C1q binding site is shown on a fourth subunit in green, based on data presented by Agrawal *et al.* (19,20) and the current findings. The structure was rendered in Weblab ViewerLite.
Table I. *Sequence homology between CRP and IgG binding region*<sup>a</sup>

|                | 175 | 176 | 177 | 178 | 179 | 180 | 181 | 182 | 183 | 184 | 185 |
|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Human SAP      | Y   | Q   | G   | T   | P   | L   | P   | A   | N   | I   | L   |
| Human CRP      | Y   | G   | G   | G   | P   | F   | S   | P   | N   | V   | L   |
| Human IgG1     | L   | L   | G   | G   | P   | L   | P   | A   | P   | I   | E   |
| Human IgG3     | L   | L   | G   | G   | P   | L   | P   | A   | P   | I   | E   |
| Human IgG4     | F   | L   | G   | G   | P   | L   | P   | S   | S   | I   | E   |
| Human IgG2     | V   | A   | -   | G   | P   | L   | P   | P   | I   | E   |     |
| IgG sequence (EU system) | 234 | 235 | 236 | 237 | 238 | 239 | 330 | 331 | 332 | 333 |     |

<sup>a</sup>Amino acid identities are represented by bold letters and shading.
Table 2. Binding properties of mutant CRP molecules

| Mutant  | FcγRI binding | FcγRII binding | K562 binding | C1q binding |
|---------|---------------|----------------|--------------|-------------|
| E88A    | Normal        | ND\textsuperscript{a} | Normal       | Normal      |
| K114A   | Low           | ND             | Normal       | Increased   |
| N158D   | Normal        | Normal         | Normal       | Normal      |
| D169A   | Normal        | Normal         | Normal       | No binding  |
| D169E   | ND            | Normal         | ND           | Normal      |
| N172L   | ND            | Normal         | Normal       | ND          |
| T173A   | No binding    | Low            | Low          | No binding  |
| Y175L   | ND            | Normal         | Normal       | No binding  |
| L176A   | No binding    | ND             | Normal       | No binding  |
| L176Q   | Normal        | ND             | Normal       | No binding  |
| L176E   | No binding    | ND             | Normal       | No binding  |
| F180A   | Normal        | ND             | Normal       | ND          |
| N186D   | No binding    | ND             | Low          | Normal      |
| R188A   | ND            | ND             | Normal       | ND          |

ND\textsuperscript{a} not done
Figure 2

A

Binding of mAb 2C10

wt CRP
E88A
K114A
N158D
D169A
E170N
T173A
Y175L
L176E
N186D

B

Binding to PnC (% of Control)

human CRP
K114A
N158D
D169E
T173A
L176Q

PC (µM)
Figure 4

A

- human CRP
- D169A
- D169E
- wt CRP
- T173A
- Mock

ΔGMCF

CRP (µg/ml)

B

- Human CRP
- N158D
- Y175L
- wt CRP
- Mock

ΔGMCF

CRP (µg/ml)
Figure 5

- human CRP
- T173A
Figure 6

- K114A
- human CRP
- wt CRP
- N158D
- D169E
- T173A
- Y175L
- L176Q
- D169A
Analysis of binding sites in human C-reactive protein for FcγRI, FcγRIIa and C1q by site-directed mutagenesis
Ranhy Bang, Lorraine Marnell, Carolyn Mold, Mary-Pat Stein, Kevin T. Du Clos, Corinn Chivington-Buck and Terry W. Du Clos

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