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Structure and function studies of functional domains in human C-reactive protein

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The Ohio State University, 1993
STRUCTURE AND FUNCTION STUDIES OF FUNCTIONAL DOMAINS IN HUMAN C-REACTIVE PROTEIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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* * * * *

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Department of Microbiology
To Carolyn.

For five years of complete support and for our future.
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LIST OF ABBREVIATIONS

APR    Acute phase reactant
BSA    Bovine serum albumin
B-CRP  Biotinylated C-reactive protein
CB-Pep Cell binding peptide
CD     Circular dichroism spectrophotometry
CPS    C-polysaccharide
CRP    C-reactive protein
CRP-R  CRP-receptor
DTT    Dithiothreitol
ER     Endoplasmic reticulum
FP     Hamster female protein
FcR    IgG Fc receptor
H-APF  Hepatocyte-specific nuclear protein
IL     Interleukin
KLH    Keyhole limpet hemocyanin
LGL    Large granular lymphocyte
LPS    Lipopolysaccharide
mAb    Monoclonal antibody
MTT    3-(4,5-dimethylthiazol-2-yl)-2,5-
       diphenyltetrazolium bromide
NF     Nuclear factor
NK     Natural killer
PAF    Platelet activating factor
PBL    Peripheral blood lymphocyte
PC     Phosphorylcholine
PMA    Phorbol 12-myristate 13-acetate
PMN    Polymorphonuclear neutrophils
rhIL-6 Recombinant human interleukin-6
SAA    Serum amyloid A
SAP    Serum amyloid-P component
snRNP  Small nuclear ribonucleoprotein
TGF-β  Transforming growth factor beta
TMB    TMB, 3,3’, 5,5’-tetramethylbenzidine
TNF-α  Tumor necrosis factor alpha
[θ]    Molar elipticity
INTRODUCTION

The acute systemic inflammatory response to tissue damage or bacterial infection is accompanied by increased hepatocyte production of a large and functionally diverse group of serum glycoproteins termed acute phase reactants (APR). The acute phase response collectively refers to the production of APRs and the associated increase in leukocyte specific activities that begins almost immediately and continues two days thereafter. In most mammals, the prototypical APR is C-reactive protein (CRP). CRP concentrations in the blood often increase several thousand fold above endogenous levels during inflammatory responses reaching nearly mg/ml concentrations in the serum. CRP is composed of five identical, noncovalently linked subunits arranged as a cyclic pentamer and is therefore classified as a Pentraxin.

CRP possesses many biological activities that include complement activation, platelet activation, opsonization, and modulation of various leukocyte responses. Although a role for CRP in the specific immune responses is poorly defined, the biological activities ascribed to CRP are primarily associated with non-specific host resistance. Most of the biological activities of CRP are initiated by ligand binding
through the single phosphorylcholine (PC)-binding site on each subunit. PC-binding is in turn dependent upon the Ca\textsuperscript{2+}-induced conformational change that occurs in the molecule allowing access to the PC-binding sites. Therefore, in the interest of understanding Ca\textsuperscript{2+}'s influence on CRP's biological activities one purpose of this investigation was to locate the Ca\textsuperscript{2+}-binding sites within the CRP amino acid sequence and to determine whether the regions are involved in the Ca\textsuperscript{2+}-induced conformational change.

The increase in blood CRP concentrations during the acute phase is followed by the deposition of CRP at sites of tissue damage. CRP binding through its PC-binding site to fibronectin, laminin and exposed phospholipids in damaged cell membranes probably accounts for its deposition at sites of tissue damage. Sequence comparison studies between the homologous pentraxin family member serum amyloid P (SAP) and the extracellular matrix proteins fibronectin and laminin lead to experiments that identified a region in the SAP sequence that was similar to a sequence in laminin that supports cell attachment. Although a synthetic peptide containing the laminin-like sequence was shown to be inactive, a second, novel synthetic peptide from SAP was discovered that was capable of supporting the attachment of all adherent cell types. The corresponding peptide in CRP and intact CRP itself were also shown to support cell attachment in later experiments. These observations suggest a potential role for
CRP in inflammatory responses and eventual wound healing. The second purpose of this investigation was to determine the minimal sequence and critical residues in the cell attachment peptide from CRP.
CHAPTER I.
LITERATURE REVIEW

A. C-reactive Protein and the Acute Phase Response. Tissue damage and acute bacterial infections initiate a systemic and rapid (within 24hrs) increase in the synthesis of a large, diverse group of blood proteins termed acute phase reactants (APR). Protease inhibitors, transport proteins, complement factors, and coagulation proteins are examples of acute phase reactants. The major acute phase proteins in most mammals are C-reactive protein (CRP) and serum amyloid A (SAA). CRP is the prototypical acute phase protein in humans and often increases by more than 1000-fold in concentration within the first 24 hrs of an acute inflammatory response (1,2,3). CRP and several of the other APR are associated with inducible, heightened nonspecific host defenses. CRP is the focus of the studies described within and therefore a description of its properties follows.

B. Early History of CRP. CRP was first defined in 1930 by Tillet and Francis by its ability to precipitate the C-fraction of polysaccharides of cell walls and the capsule of Streptococcus pneumoniae (4). CRP production during various acute microbial infections involving gram negative as well as
gram positive bacteria was also examined by Ash et. al. (5) who found increased levels of CRP in both typhoid fever and E. coli pyelitis patients. CRP was first classified as a protein by Abernathy and Avery (6) on the basis of heat inactivation at 65°C, a procedure that resulted in its association with serum albumin. Oxalated serum of patients was shown not to react with C-polysaccharide and was the very first indication of the involvement of calcium in CRP binding activities (6). McCarty et al. (7) used crystallized CRP to immunize rabbits and found that the antisera reacted only with an antigen in acute phase sera. The development of rabbit models by Lofstrom et al. for the study of CRP in the acute phase response was a major advance leading to experiments identifying the liver as the site of CRP synthesis (8). Rabbits injected with turpentine, endotoxin, or paratyphoid incorporated 14C-labeled amino acids into CRP in the liver, but not other tissues or organs (9).

C. Molecular Properties. CRP consists of an aggregate of identical subunits, each with a m.w. of 23,000 Da each (10). Amino acid analysis suggested that each subunit contains a single intrachain disulfide bond (10). Electron microscopy revealed that CRP consists of 5 subunits arranged cyclicly classifying it as a Pentraxin protein (11). Amino acid sequencing of purified protein suggested that CRP consisted of a single polypeptide of 187 amino acids (12). Cell free translation of liver mRNA demonstrated that the primary
translation product was larger than native CRP by 18 amino acids, suggesting the presence of a leader sequence (13). DNA sequencing later revealed that CRP actually consists of 206 amino acids (14,15). The discrepancy between protein and cDNA sequencing occurred because of a 19 amino acid repeat not discerned by Edman degradation (14,15). The disulfide bond was shown to occur between residues 36 and 97 (14,15).

D. Biosynthesis. Immunostaining techniques showed that CRP synthesis in the liver occurs only in hepatocytes (16). Some evidence was also presented for extrahepatic CRP synthesis by peripheral blood lymphocytes which express the CRP on their cell surface (17,18). NK cells also appear to express CRP on their cell surface, where it has been implicated in the lytic function (19,20,21).

Studies of inflammatory responses in rabbits revealed that CRP synthesis in the liver reaches its peak 38 hrs after the onset of inflammation with the periportal production detectable at 8 hrs reaching the peripheral hepatic lobules by 16 hours (16). By 38 hours, CRP producing cells are found in clumps around central, mid-lobular, and periportal veins (16). Electron microscopy showed that CRP accumulates on both rough and smooth endoplasmic reticulum, and in cytoplasmic vacuoles filled with secretory vesicles, the latter rapidly merge with the membrane for secretion of serum proteins (22). An early theory suggested de novo synthesis of CRP does not occur during inflammation, but that CRP may exist as a precursor in
the serum which is cleaved during inflammatory responses to provide the biologically active native molecule (23). Gel filtration experiments and density gradient centrifugation techniques revealed this was not the case and showed that CRP newly synthesized by the liver is the same as native biologically active CRP (24). Kushner (16) suggested that CRP secretion is faster during inflammation in experiments revealing that the increase in intracellular CRP is less than the increase in CRP in serum during inflammation. CRP has a serum half life of 5.8 to 6 hours in rabbits (25) and 4 hours in mice (26). Pulse labeling techniques revealed that CRP secretion rates in primary hepatocytes from rabbits undergoing acute inflammatory responses are increased and that CRP is preferentially retained in the ER of hepatocytes from non-inflamed animals (27). Experiments with hepatoma cell lines transfected with a fusion gene containing the metallothionein I promoter linked to the CRP protein coding sequence showed that increasing the rate of CRP synthesis does not increase the rate of secretion (28). The results suggest normal rabbit hepatocytes retard the secretion of CRP and that the inhibition lessens during the acute phase response (28). Recently, CRP was shown to bind specifically to the lumenal surface of permeabilized rough microsomes and not to Golgi fractions (29). The binding was shown to occur through a 60 kDa protein from extracts of rough microsomes isolated from normal rabbit hepatocytes (29). The 60 kDa high affinity form
of the binding protein was not present in the rough microsomes from rabbits undergoing inflammatory responses suggesting a novel way of obtaining more rapid production during the acute phase (29).

E. Cytokine Regulation of CRP Synthesis. The role of specific mediators for induction of CRP synthesis by various hepatic cell types was extensively examined. The very first observation for cytokine regulation of CRP synthesis was a macrophage product, specifically leukocyte endogenous mediator (30). Goldman et. al. (31) reported induction of CRP synthesis in hepatocyte cell lines with the addition of supernatants from LPS stimulated macrophages. In similar experiments, Hep3B2 cells stimulated with conditioned medium from normal peripheral blood monocytes also increased synthesis of CRP as measured by Northern analysis and radioimmunoassay (32). Prior to secretion, CRP was processed to its final size as shown in previous experiments with primary hepatocytes (24,31).

Difficulties were at first encountered in identifying the specific inflammatory cytokine(s) required for CRP induction. Goldman (31) first reported that IL-1 alpha and beta, interferon gamma, IL-2, and TNF alpha did not increase CRP synthesis. However, Goldman reported that a 30,000 mw protein in monocyte conditioned medium could induce CRP synthesis (31). In addition, Ganapathi et al. (33) was unable to demonstrate CRP induction by IL-1 or TNF, but did report that
an 18,000 mw protein from monocyte conditioned media could induce CRP production. Later, the same group demonstrated that the activity in the conditioned medium could be inhibited by the addition of anti-IL-6 antibodies (34). It was subsequently shown that IL-6 alone could induce CRP production in NPLC/PRF/5 cells while both IL-6 and either IL-1 alpha or beta were required for CRP synthesis in Hep 3B cells (34). Later, Mosage et al. (35) demonstrated that rhIL-1 and rhIL-6 could both induce CRP synthesis by hepatocytes. It was also shown that anti-IL-6 neutralizing antibody could decrease IL-1 stimulation of CRP synthesis indicating that IL-1 induction of CRP may in part be dependent upon IL-1 induction of IL-6 (35). Although, other studies clearly show that hepatocytes only respond to IL-6 but do not produce it (36). In experiments with primary hepatocytes, CRP could be induced by rhIL-6 resulting in a 20-fold increase in CRP synthesis (37). Finally, Taylor et al. (38) reported that either IL-1 beta or IL-6 alone could induce CRP synthesis by PLC/PRF/5 cells and that the combinations were additive.

Transforming growth factor-beta (TGF-beta) was reported to modify IL-1-beta and IL-6 induced CRP synthesis in the hepatoma line PLC/PRF/5 (38). TGF-beta at concentrations of above 0.1 pg/ml inhibited IL-1 or IL-6 induction of CRP; whereas concentrations below 0.1 pg/ml slightly enhanced CRP synthesis (38). Tumor necrosis factor-α (TNF-α), at 30 U/ml, also inhibits IL-1 and IL-6 stimulated synthesis of CRP by
hepatocytes (39).

The role of corticosteroids in the form of dexamethasone and prednisolone in the hepatic production of CRP has been examined in vitro (40,41). Incubation of HepG2 cells simultaneously with IL-6 and dexamethasone increases the magnitude of CRP release significantly above that of IL-6 induction alone (40). Dexamethasone alone had no effect on CRP synthesis (40). Conditioned medium from prednisolone treated monocytes stimulated with LPS produced less CRP synthesis than conditioned medium from monocytes stimulated with LPS alone (41). Prednisolone inhibited monocyte production of IL-1 alpha and beta and prednisolone alone did not induce the synthesis of CRP suggesting it prevents IL-1 alpha and beta induction of CRP synthesis (41).

F. Molecular Genetics and Regulation of CRP Expression.

1. Gene Structure of CRP. The human CRP gene exists as a single copy on chromosome 1 (42). The gene was cloned by two labs simultaneously and was found to contain a 278 nucleotide (nt) intron within the codon for the third residue of mature CRP (14,15). The intron contains a poly A sequence and a repetitive sequence (GT)_{15} G(GT), potentially capable of forming Z-DNA (14). The 5' flanking region of the gene has 3 regions containing the Drosophila heat shock consensus sequences (14). The promoter region of the CRP gene contains a CAAT box at -81 and TATA box at -29 from the mature mRNA cap site (14,15). A single pseudogene within 16 Kb from the CRP
The pseudogene demonstrated 50-80% region specific homology with the expressed gene (31).

Samols et al. (43) first reported an increase in hepatic CRP mRNA after an inflammatory signal and went on to show that the mRNA could form a stable secondary structure that either prevented or decreased its translation in a cell free system, however, there is no evidence that the formation of the secondary structure occurs in cell mediated translation events (43). Time course studies later revealed that intramuscular injection of sterile turpentine in rabbits results in an increase in CRP mRNA which peaks at 33 hrs and remain elevated until 72 hrs (44). Nuclear run on experiments showed that CRP regulation does occur at the level of transcription, but these studies did not rule out post-transcriptional control mechanisms (45).

2. Cytokine Regulation of the CRP Promoter. The role of cytokines in regulation of the CRP promoter was demonstrated in experiments using cells transfected with reporter gene constructs containing the 5′-flanking region of the human CRP gene. The transfected cells increased CRP production when stimulated with conditioned medium from monocytes treated with LPS (45). The addition of IL-6 to monocyte conditioned medium further increased the level of CRP synthesis above that of IL-6 alone, indicating the involvement of other cytokines in addition to IL-6 (46). CRP gene constructs used in expression
experiments clearly demonstrated that IL-6 was the major or most potent inducer of CRP and that IL-1 beta and IL-6 together induced the best expression (47). Deletions from the 5' -end and 3' -end of the cis-acting promoter region of the CRP gene located regions responsive to IL-6 upstream from the TATA box (47). The IL-1 effect was shown to occur at sequences most likely important for optimal mRNA translatability or nuclear cytoplasmic transport (47). The 5' flanking region contains two acute phase response elements responsive to IL-6 at positions -94 to -50 and -137 to -106 (45, 46).

Gantner proposed that transcription factors present in hepatocytes are activated by IL-6 (47). Experiments using reporter gene constructs driven by various regions of the CRP promoter lead to the identification of promoter elements responsive to IL-6 and to hepatocyte specific nuclear proteins that bind to the promoter region (48). IL-6 was shown to induce two factors, H-APF-1 and H-APF-2, that bind to subregions (alpha and beta) in the CRP promoter which are absent in other non-hepatic cell types (49). The hepatocyte specific nuclear protein, H-APF-2, that binds at -90 to -46 is both quantitatively and qualitatively changed by IL-6 and appears to work cooperatively with H-APF-1 to promote CRP expression (49). Point mutations in the promoter that reduce binding of either nuclear protein drastically decrease CRP gene expression (49). Nuclear protein binding studies revealed that binding to the beta region is identical in cell
extracts from uninduced or IL-6 induced Hep3B cells (49). By contrast, nuclear protein binding to the alpha region of the promoter is increased in the cells stimulated with IL-6 (49). More recent work has established that the two IL-6 responsive elements in the CRP promoter are identical to the IL-6 responsive elements recognized by the transcription factor, NF-IL-6, in a number of genes (50).

Two elements capable of binding a liver specific transcription factor HNF-1 are also located in the CRP promoter and are separated by approximately 80 bp; mutations at either site abolish HNF-1 recognition (51). Other nuclear factors may very well be involved in CRP expression as demonstrated by experiments in which the region from -120 to -90 that does not bind H-APF-1 or 2, allowed inducibility of the SV40 promoter (49).

G. Binding Specificities of CRP.

1. Phosphorylcholine binding. CRP was discovered on the basis of its ability to precipitate the C-fraction of acidic extracts of the pneumococcal capsule and cell wall preparations (4). The C-fraction is the ribitol teichoic acid fraction of the capsular material common to all of pneumococci and is now referred to as the C-polysaccharide (CPS). Gotsclich et al. (52) demonstrated that the CRP-CPS precipitation reaction could be inhibited by various low m.w. phosphate monoesters. The studies showed that CRP bound 1 molecule of phosphate monoester per subunit and that the
interaction required the presence of calcium as Ca$^{2+}$ ions (52). Phosphorylcholine (PC) inhibited the precipitation reaction considerably better on a molar basis than CMP or AMP and thus was considered to be the major reactive moiety in the CPS (53). Since PC exists as a zwitterion at pH 7, the suggestion was made that CRP binding to PC occurs between both the negatively charged phosphate and the positively charged choline groups in PC (54). This conclusion is supported by Young and Williams who reported that replacement of the phosphate with a carboxylate group in analogues of PC resulted in decreased inhibition of the CRP-CPS precipitation (55).

The PC binding region is located within the first of two highly conserved regions in CRP and corresponds to residues 51-66 (14,15,55). The Lys-57 and Arg-58 were proposed to bind to the negatively charged phosphate group and Asp-60 and Glu-62 were proposed to bind to the positively charged choline moiety (56). A peptide corresponding to residues 47-63 of CRP was synthesized and shown to bind directly to PC and to compete with CRP for binding to PC (57). Peptide 47-63 was recognized by monoclonal antibodies (mAb) that bind the T-15 idiotope present on a mouse myeloma protein (TEPC-15) specific for PC (57). In addition, one mAb generated against peptide 47-63 was capable of binding to the TEPC (T-15) myeloma protein (58). Peptides with neutral amino acid substitutions at the charged residues showed decreased PC-binding activity and reduced recognition by anti-peptide 47-63 mAb (58). Site
directed mutagenesis of CRP which substituted amino acids at residues within the PC-binding region revealed the location of several critical residues by measuring the binding avidity of mutant CRPs to PC-substituted bovine serum albumin (PC-BSA)(59). Mutant CRPs with single amino acid substitutions bound PC with avidities similar to wild type, however, one mutant showed decreased avidity with a Lys substitution for Trp at residue 67 (59). A triple substituted mutant with Gln substituted for Lys at residue 57, Gly substituted for Arg at residue 58, and Lys substituted for Trp at residue 67 failed to bind PC-BSA (59). Inhibition experiments using PC and dAMP to inhibit peptide binding to PC-BSA indicated that residues Lys-57, Arg-58 and Trp-67 contribute to PC-binding avidity (59).

The interaction between phospholipids and CRP was studied in a variety of lipid membrane systems (60). CRP was shown to bind best to positively charged liposomes (61,62). $^{125}$I-CRP binding studies using positively charged liposomes showed that the interaction is calcium independent and not inhibited by PC (63). In experiments with phosphatidylcholine containing liposomes CRP was shown to bind specifically to the PC moiety and to activate complement as determined by the release of liposomally trapped glucose (60,61,62). The binding to PC polar head groups does not occur without relaxation of the lipid bilayer, suggesting that CRP does not bind to the lipid bilayer of intact cells, but requires disruption of the lipid
bilayer (64,65). Rabbit experiments showed that CRP is selectively deposited at sites of tissue damage (66) where it presumably binds via the PC binding region to a variety of physiological substrates that include fibronectin (67,68), laminin (69), phospholipids on damaged membranes (65), chromatin (70,71,72) and histones (72). The interactions suggest a mechanism by which the biological activities of CRP can be focused at sites of tissue damage.

CRP was shown to bind to platelet activating factor (PAF) through its PC-binding region and inhibit PAF induced aggregation of human platelets in a time and dose dependent manner (74). These results suggest that by depressing the bioavailability of the PAF, CRP may serve as an important modulator of platelet activation during acute inflammatory reactions.

2. Binding to Polycations. When CRP binding to polycations was examined by DiCamelli et al., the findings suggested separate binding sites for PC and polycations (73). In addition, CRP precipitation of poly-L-lysine occurred in the presence and absence of Ca\(^{2+}\) and is inhibited by PC only in the absence of Ca\(^{2+}\) (75). The results suggest a separate polycation binding site that either resides partially within the PC binding site of CRP or is influenced by the PC-binding site (63,75). Rabbit CRP binding to lysine- and arginine-rich proteins using binding and precipitation reactions was shown to occur at pH 6.0, in the presence of calcium, and was
inhibited by PC (76). The CRP specificity for the Arg-rich cationic molecules was proposed to be linked to CRP's chromatin scavenging biological activity (76).

3. Chromatin Binding. CRP association with the nucleus was first observed using immunofluorescence staining of synovial histiocytes from patients with rheumatoid arthritis (77). The association was specific for synovial histiocytes and occurred only with damaged cells (77). Robey et al. (70) first characterized CRP binding to chromatin and demonstrated that the binding was saturable, Ca$^{2+}$ dependent, and appeared to involve both the PC and polycation binding site(s). Interestingly, the interaction occurred only once in every 160 base pairs of DNA indicating that the interaction did not involve the DNA itself, but the histones (70). CRP-chromatin complexes activate the classical complement pathway (79), which is then is amplified by the alternative pathway resulting in the solubilization of the CRP-chromatin complex (79).

Concurrently, both Pepys et al. and DuClos et al. showed CRP did not bind directly to DNA and the binding to chromatin decreased or did not occur at all after depletion of histone H1 (80,81). DuClos went on to show that CRP binds directly to purified histone H1, H2A and to a lesser extent histone H2B, but did not bind specifically to histone H3 or H4 (80). The CRP-histone binding interaction was calcium dependent and occurred via the PC binding region of CRP (80). CRP binding
to proteolytic cleavage fragments and synthetic peptides from histones revealed that CRP binds to the C-terminal region of histone H1 and to a 15 amino acid N-terminal sequence in histone H2A (71). Reconstitution of individual histones with DNA inhibited CRP binding and indicated that CRP binding to native chromatin is not mediated by binding to the individual core histones (71). However, these experiments did not rule out the possibility that CRP could bind to differentially exposed determinants on histones H2A and H2B. DuClos et al. (82) demonstrated that CRP is capable of binding and precipitating the U1 snRNP complex. The specific interaction of CRP and chromatin suggests that CRP functions as a scavenger for chromatin fragments released from damaged cells (78). The hypothesis generated by these observations is that CRP and complement mediated scavenging and solubilization of chromatin prevent autoimmunity by inhibiting the formation of antibodies to chromatin.

4. Calcium Binding. CRP has two Ca$^{2+}$-binding sites of equal affinity ($K_d = 6 \times 10^{-5}$ M) per subunit (10,83). Circular dichroism and monoclonal antibody binding experiments revealed that calcium induces a conformational change in CRP that is required for PC binding (55,84). The precise location of both Ca$^{2+}$ binding sites within the CRP subunit has not yet been determined and is the subject of chapter II. However, the highly conserved acidic stretch of amino acids between residues 134-148 present in all Pentraxins was proposed to be
a Ca\textsuperscript{2+}-binding region (56). Residues 134-148 were also shown to have sequence similarity to Ca\textsuperscript{2+} binding regions in other proteins and proteolytic cleavage at two sites within this sequence results in decreased Ca\textsuperscript{2+} binding (83). Anti-peptide 134-148 mAb were shown to bind to CRP and allosterically alter both the PC-binding activity of CRP and the expression of epitopes within the PC-binding region of CRP (85).

Recently the location of the second calcium binding site was suggested to occur between residues 157-171 in CRP on the basis of secondary structure predictions provided by infrared spectroscopy studies of the hamster female protein (FP), another member of the Pentraxin family (86). Residues 157-171 show limited sequence similarity with calcium binding sequences from other proteins (86). Direct calcium binding within this region in CRP is examined in chapter II.

H. Biological Activities of CRP. Most of the reported biological activities for CRP are associated with nonspecific host resistance linking CRP to various roles in acute inflammatory responses. However, CRP is also highly conserved throughout evolution, with CRP or CRP-like molecules being found in species as primitive as the horseshoe crab, \textit{Limulus polyphemus} (87). The biological activities for CRP were most extensively studied in higher mammals where the acute phase response is more pronounced and far more complex. Although the underlying function(s) for CRP remains to be determined, numerous biological activities were documented for CRP and can
be placed into two simple categories:

(1) antibody-like activities of CRP;

(2) Leukocyte specific effects of CRP;

1. Antibody-like Activities of CRP

   a. Complement Activation. Complement (C) activation by CRP was first observed with the addition of pneumococcal CPS to human acute phase sera containing CRP (88). Complement components C1-C9 were shown to be depleted indicating activation of the classical C pathway (88,89,90). Complement activation by CRP-polycation complexes was observed by Siegel et al. (90,91). Protamine addition to CRP resulted in the consumption of C1, C4, and C2 while C3-9 were only minimally depleted (90). Additional synthetic polycations and the naturally occurring leukocyte granule basic proteins were shown to initiate complement activation through the classical pathway with the depletion of C1-6 (91). CRP bound to liposomes containing cholesterol and phosphotidylcholine or sphingomyelin were also able to activate complement (88). More recently, CRP by itself was shown to activate complement under mildly acidic conditions (pH 6.3) even in the absence of its ligands (92).

The analogy between human CRP and antigen-antibody complexes was demonstrated by experiments showing that human CRP failed to activate guinea pig (GP) complement due to an
incompatibility with GP Clq (93). Therefore, CRP activation of C is initiated by an interaction with Clq in a manner similar to Ag-Ab complex mediated C activation (93). More recently, Western blot and ELISA experiments showed that CRP binding to Clq is not inhibited by aggregated IgG and therefore binds to a separate site on Clq (94). CRP was shown to bind to the collagen like region (CLR) or the "stalks" of Clq while aggregated IgG bound to the globular head region of Clq (94). Similar experiment using peptides from potential binding sites in the CLR of Clq revealed that CRP binds to the regions in the Clq A chain between residues 14-26 and 76-92 (95).

CRP was shown under specific conditions to inhibit the alternative C pathway while activating the classical C pathway (63,96). This inhibition occurred with CRP bound to either S. pneumoniae or to liposomes containing lecithin or sphingomyelin (63). Inhibition occurred at the level of C3b and involved direct binding of factor H to C3b (96). Liposomes themselves have been shown to induce the alternative C pathway and the addition of CRP to liposomes results in a shift to the classical C pathway (97,98).

b. Opsonic Properties. The addition of CRP containing sera to bacterial pathogens was shown to enhance nonspecific phagocytosis by leukocytes and was the first evidence of a role for CRP in modulating leukocyte functions (99,100). Since CRP and Igs appear to share an ability to
utilize complement proteins, CRP was tested for opsonization activity. Mortensen et al. (101) demonstrated that in the presence of complement, CRP coated CPS-erythrocytes were readily ingested. The studies demonstrated that CRP initiates the deposition of opsonic C cleavage products (C3b, C4b) and that both C and CRP were required for phagocytosis (101). The possibility of FcR involvement in CRP-mediated phagocytosis was proposed because aggregated IgG could inhibit ingestion of CRP-CPS coated erythrocytes (102). Edwards et al. (103) showed the phagocytosis of S. pneumonieae coated with CRP increased after the activation of the complement cascade.

An additional heat stable mediator with a molecular weight <10,000 Da released by monocytes appears to play a role in CRP initiated phagocytosis (104). In the presence of the mediator, stimulated PMNs phagocytized CRP coated erythrocytes (104). These observations all support the proposal that both CRP and C are required for CRP specific opsonization.

CRP was also shown to serve as an opsonin for neutrophil phagocytosis of two species of fungi (105,106). Addition of CRP increased neutrophil phagocytosis of metabolically active conidia and to a lesser extent dormant spores from Aspergillus fumigatus (106). In a separate study, a virulent strain of Candida albicans resisted CRP mediated opsonization better than an attenuated strain of the same fungi (105). These experiments suggest that CRP may play a protective role in preventing fungal infections.
2. CRP Modulation of Leukocyte Responses. Most of the effects of CRP on leukocytes have been shown to increase nonspecific general functions such as cytokine production, bacteriocidal and tumoricidal activities, and chemotaxis. IL-1 production by monocytes was shown to increase with the addition of CRP or CRP-platelet-activating factor (PAF) complexes (107,108). The results suggest that CRP and CRP-PAF complexes interact with specific receptors that signal the production of immunostimulatory cytokines (107,108).

CRP induced tumoricidal activity in both human monocytes and mouse macrophages (109,110,111). CRP liposomes stimulated tumoricidal activity by mouse inflammatory macrophages using in vivo assays with a syngeneic fibrosarcoma target (109). The same group later demonstrated that CRP mediated monocyte/macrophage cytotoxicity in a dose-dependent manner against various human tumor cell lines (111). CRP incorporated into liposomes was able to inhibit metastasis of the fibrosarcoma in mice (111). Zahedi et al. showed that CRP could induce antitumor activity in some macrophage cell lines as well as in both elicited and resident mouse macrophages independent of T-cell cytokines or LPS (110). CRP delivered in liposomes was shown to induce tumoricidal activity in peritoneal exudate cells against the T241 fibrosarcoma and MCA-38 colon carcinoma (111). A synthetic peptide from CRP (RS-83277) was shown to induce anti-tumor activity and secretion of IL-1 and IL-6 by macrophages (112).
The use of anti-neo-CRP antibodies demonstrated cell surface staining of both NK cells and B cells (113,114). Neo-CRP is a form of CRP that results from the unfolding of CRP in the presence of heat, urea, or weak acid to expose neo-antigenic epitopes not available on native CRP (113,115). Cell surface CRP was shown to be required for NK cell-mediated cytotoxicity without the addition of fluid phase CRP (19). Anti-CRP antibodies can inhibit NK cell activity and were shown to block effector-target cell interaction or facilitate capping of an important cell surface effector molecule (20). The addition of soluble CRP or CRP ligands did not inhibit NK cell mediated lysis indicating that CRP is not involved in binding to target cells, but rather a subsequent lytic event (20). In addition, complement mediated removal of surface CRP positive cells from peripheral blood removes NK cell mediated cytotoxicity (116). More recent findings document the presence of very small amounts of CRP mRNA in NK-cells (117).

In general, the oxidative metabolism of macrophages is increased in response to CRP while the same response in PMNs is decreased (115, 118-123). Experiments by Barna et al. demonstrated that the delivery of CRP in liposomes was 10 to 100x better than CRP alone at inducing superoxide production by macrophages (109). The addition of CRP alone could not induce the oxidative burst in monocytes and PMNs as detected by luminol chemiluminescence (CL) (118,119). Aggregated CRP (Agg-CRP) however, could increase Fc receptor mediated
stimulation of oxidative metabolism by aggregated IgG (Agg-IgG)(119). The Agg-CRP increase of Agg-IgG stimulation of the oxidative burst in monocytes and neutrophils was recently shown to be limited to intracellular responses (124). These experiments used phenol red oxidation to measure extracellular release of hydrogen peroxide and flow cytometric measurement of dichlorofluorescin oxidation to measure intracellular peroxide production revealing that the Agg-CRP enhancement of Agg-IgG induced oxidative burst occurs intracellularly without extracellular release of oxidative products (124). PMA stimulation of the oxidative response in PMNs increased with simultaneous CRP addition although preincubation of PMNs with CRP inhibits the response to PMA (123). Miyagawa (121) reported that guinea pig macrophages increase superoxide metabolism with low doses of CRP while high doses of CRP inhibited the response. Finally, Tebo et al. (125) reported that CRP induced weak superoxide production by the human monocytic line, U937.

The potential role for the release of biologically active peptides from proteolytic digests of CRP in superoxide production was examined (115,122,123,126). Synthetic peptides and neutrophil mediated digests of CRP were shown to induce IL-1 production by monocytes suggesting that cells can degrade CRP into biologically active peptides (122). Synthetic peptides from CRP containing tuftsin tetrapeptide (Thr-Lys-Pro-Arg)-like sequences were shown to increase $O_2^\cdot$ production
in phagocytic leukocytes (122). Conversely, peptides consisting of residues 51-58 and 181-187 of CRP partially inhibited $O_2^-$ production in macrophages (123). PMA stimulated neutrophils were shown to degrade CRP into biologically active peptides capable of inhibiting $O_2^-$ production in zymosan-activated neutrophils (126). Ying et al. (115) demonstrated that soluble peptides with a mw <14,000 generated by PMN lysosomal enzyme degradation of CRP could inhibit neutrophil $O_2^-$ production. In addition, sequencing of superoxide inhibiting peptides from neutrophil mediated proteolytic digests of CRP revealed that the inhibitory peptides corresponded to residues 201-206, 83-90 and 77-82 of the CRP sequence (127). Recently, a 600 kDa CRP-degrading protease from PMA stimulated neutrophils was purified using size exclusion chromatography and shown to migrate as four separate bands on SDS-PAGE with m.w. of 209, 316, 398, and 501 kDa (128).

Various other effects of CRP on leukocyte activities have been reported. CRP and CRP complexes have been shown to partially increase the response in mixed lymphocyte experiments and more specifically increased cell mediated cytotoxicity (129). CRP is also shown to induce a limited blastogenic response in resting lymphocytes (129). CRP is shown to effect the chemotactic response of both monocytes and neutrophils. In monocytes, purified CRP increased both chemotaxis and procoagulant activity (130). CRP at 1 ug/ml
produced the maximum chemotactic response in neutrophils while concentrations above 1μg/ml resulted in decreased chemotaxis (131).

CRP binding by leukocytes was first examined in experiments that showed peripheral blood lymphocytes (PBLs) could bind to CRP-CPS complexes, but not the CRP alone (132). The same laboratory went on to show that heat aggregated CRP and CRP coated CPS-erythrocytes also bound to PBLs (133). In addition, FcR positive cells accounted for 70% of the PBLs that bound to CRP (133). Examination of FcR+ cells demonstrated that only 12 percent were capable of binding CRP and the CRP binding cells demonstrated the characteristics of large granular lymphocytes (LGL) (133).

Fluorescein labeled heat aggregated CRP was used to demonstrate CRP binding to both neutrophils and monocytes in flow cytometry experiments (118,134). Flow cytometry demonstrated that 36% of the PMNs, 70% of the monocytes and 8% of the lymphocytes bound aggregated CRP (118,119).

Highly purified 125I-labeled CRP allowed precise examination of CRP binding to human leukocytes as well to mouse macrophages and led to the discovery that CRP binding was a receptor mediated event (131,135-138). Specific and saturable receptor binding by human neutrophils was shown to occur with an affinity of $K_d = 1 \times 10^8$ M (135,131). More recent experiments show that CRP binding to neutrophils is most consistent with a two site receptor model, i.e.
demonstrating both a high-affinity receptor population ($K_d = 3.7 \times 10^{-10}$ M) and a low-affinity population ($K_d = 2.5 \times 10^{-8}$ M) (139).

The role of Fc receptors in CRP binding was examined after aggregated IgG was shown to displace labeled CRP bound to neutrophils (131,135). The use of IgG immobilized monocytes demonstrated that 20% of the IgG binding cells were capable of binding CRP coated erythrocytes (140). The cells that bound CRP bound the same amount of IgG as the non-CRP binding cells (140). Experiments by Ballou et al. (136) and Tebo et al. (137) demonstrated specific binding of CRP to human monocytes and the human monocyte cell line U-937 with similar affinities ($K_d = 10^{-7}$ M). Ballou et al. (136) reported that 100 fold excess of human IgG failed to inhibit $^{125}$I-CRP binding to U-937 cells whereas Tebo et al. (137) demonstrated that human polyclonal IgG and a human IgG, myeloma protein blocked CRP binding to U-937 cells (137). In the reverse experiment CRP could not block IgG binding to the same cells (137). The CRP receptor from the mouse macrophage-like cell line PU51.8 was shown to be a 57-60 kDa protein in experiment that used CRP affinity chromatography for purification of solubilized cell membranes (138). The same approach was used with U-937 cells which expressed a receptor that involves two proteins with m.w. of 38-40 and 58-60 kDa (137). Both receptor proteins were shown to be distinct from FcRI and FcRII by isolating CRP binding proteins and FcR(s) from the
same $^{125}$I labeled cell extract (137,138). Cross-linking and precipitation experiments using lysates from surface-labeled U-937 revealed a 43-45 kDa protein associated with CRP (141), similar to the previously described CRP receptor (137). In the same experiments a second CRP associated membrane protein was identified and shown to be the Fc-RI (141). The results indicate that the receptors for CRP are distinct membrane proteins closely associated with IgG FcR(s).

Once bound to receptors on U-937 cells, $^{125}$I-labeled CRP was shown to be internalized and degraded into TCA soluble radiolabeled products that accumulate extracellularly (125). The addition of monensin, a carboxylic ionophore which prevents receptor recycling, resulted in the accumulation of internalized CRP indicating that the CRP-R is recycled (125). CRP also initiated the differentiation of U-937 cells so that they acquired the ability to produce $\text{H}_2\text{O}_2$, further supporting the hypothesis that CRP internalization and degradation leads to the activation of monocytes during inflammation (125).

I. Calcium (Ca$^{2+}$)-Binding Proteins and CRP. Ca$^{2+}$ binding regulates many essential biological functions of extracellular and intracellular proteins. Ca$^{2+}$ induced conformational changes in proteins allow Ca$^{2+}$ levels to control many cellular processes. A few examples of the range of intracellular processes controlled by Ca$^{2+}$-binding proteins are protein phosphorylation by protein kinase C (142), regulation of muscle contraction by troponin-C (143), and polymerization of
Several general properties of Ca\(^{2+}\)-binding proteins are relevant to the design of experiments described in the following report. The first property of Ca\(^{2+}\)-binding proteins is the presence of polar donor groups brought together within grooves, cavities or on the surface of the protein itself. The major donor groups are the oxygen bearing carbonyl, carboxylate, and hydroxyl groups (145). The second property of Ca\(^{2+}\)-binding proteins is that fold energy is required to bring together the polar donor groups at the binding site to overcome electrostatic repulsion (145). Fold energy is also important for providing the proper conformation within the polar donor groups for coordination of the calcium ion (145).

Ca\(^{2+}\)-binding sites in proteins can be separated into three classes on the basis of structural and functional differences. The first class of Ca\(^{2+}\)-binding sites bind Ca\(^{2+}\) via sequential amino acids with the appropriate donor groups on the side chains. Ca\(^{2+}\)-binding in sequential sites dissipates the electrostatic repulsion of negative charges in adjacent residues and is often accompanied by an overall conformational change in the intact protein. The second class consists of nonsequential Ca\(^{2+}\)-binding sites usually within a preformed groove in the protein that brings the donor groups to the site during protein folding. Little or no conformational change takes place in this second class of Ca\(^{2+}\)-binding proteins. The third class of Ca\(^{2+}\)-binding sites
consists of a very long series of anionic residues brought together both sequentially and through folding. In this class, Ca\textsuperscript{2+} is proposed to travel rapidly along the series of anionic residues. This is the proposed model for the mode of action found in the ATPase calcium ion pump found in the inner membrane of eukaryotic mitochondria and chloroplasts (146).

CRP appears to belong to the class of sequential Ca\textsuperscript{2+}-binding proteins for two reasons; first, Ca\textsuperscript{2+}-binding induces a conformational change in the protein and second, CRP is not known to be involved in Ca\textsuperscript{2+} transport. The majority of Ca\textsuperscript{2+}-binding proteins belong to this category. Calcium triggering of the conformation change in these proteins allows several of them to serve as sensitive mediators of Ca\textsuperscript{2+}-dependent signal transduction pathways. The first X-ray crystallography studies of a protein from this group were carried out using the carp muscle Ca\textsuperscript{2+}-binding protein parvalbumin (147,148). Parvalbumin consists of six helical regions (A-F) with two Ca\textsuperscript{2+}-binding sites occurring in short 12 residue loops between helical sections C and D and helical sections E and F. The Ca\textsuperscript{2+}-binding sequence between perpendicular helical regions E and F coordinate Ca\textsuperscript{2+} with 8 of the 12 residues in the loop sequence and is referred to as the "EF-hand" (148). The EF-hand designation refers to the similarity of the perpendicular E and F helical regions to a right hand with only the thumb and index finger extended. In this model, the E helix is represented by the index finger, the F helix is represented by
the thumb and Ca$^{2+}$-binding loop is represented by the unextended second finger. The EF-hand model is also known as the helix-loop-helix motif and has been used to classify over 100 Ca$^{2+}$-binding proteins containing over 276 Ca$^{2+}$-binding sites (149). Of these 276 Ca$^{2+}$-binding sites, 165 have unique sequences within the 12 amino acid Ca$^{2+}$-binding loop region and all 20 amino acids are represented (149). The most highly conserved residues are found at the ligand positions at 1, 3, 5 and 12, and at the nonligand positions 6 and 8 (149). The similarities between helix-loop-helix calcium binding sequences and Ca$^{2+}$-binding sequences in CRP are addressed in chapter II.

J. Cell Binding Activity of CRP.

1. Cell Binding Peptide of SAP and CRP. Studies comparing the sequence of SAP to the extracellular matrix proteins fibronectin and laminin detected a tetrapeptide, YIGR that closely resembled the YIGSR cell attachment peptide from laminin (150). Contrary to the expected results, the YIGR containing SAP peptides did not support cell attachment however, a control peptide consisting of residues 27-38 EKPLQNFTLCFR, was shown to support cell attachment of various adhesive cell types including osteoblasts, fibroblasts, and various tumor cell lines (150). The cell attachment activity was localized to a six residue peptide consisting of residues 33-38 which supported 83% of the cell attachment to the original peptide (150). Reversing the sequence of the peptide
resulted in a complete loss of activity (150). The results suggest that a specific receptor for the peptide exists on adherent cell types although intact SAP did not support cell adhesion (150).

Proteolysis of CRP by neutrophils releases biologically active peptides capable of inducing activation responses such as chemotaxis and superoxide production in monocytes and neutrophils (122,127). Three biologically active peptides from CRP contain sequences similar to the Tuftsin tetrapeptide (Thr-Lys-Pro-Arg) found in IgG (122). While characterizing the receptor for CRP on macrophages (137), a peptide (r27-38 of CRP) containing one of the tuftsin-like sequences was synthesized and found to be capable of supporting cell attachment (151). This peptide is now referred to as the cell binding (CB-Pep) (151).

2. Characterization of the CB-peptide of CRP. Fernandez et al characterized the cell attachment to CB-Pep comparing its cell binding activity to that of the active peptides from fibronectin (Fn), laminin, and the CB-Peptide from SAP (151). The pentraxin peptides supported similar levels of cell attachment and were not inhibited by soluble cell attachment peptides from Fn, laminin, or Tuftsin (151). Intact CRP, but not intact SAP, was also capable of supporting cell attachment (151). The recently described cell attachment activity of CRP suggests a new role for CRP that might involve the early stages of wound healing during the inflammation. The
determination of the minimal sequence and critical residues within the CB-peptide is the topic of chapter III.
CHAPTER II.

CALCIUM ION BINDING REGIONS IN C-REACTIVE PROTEIN: LOCATION, CONFORMATIONAL CHANGES, AND ALLOSTERIC REGULATION

A. Introduction

During a systemic acute inflammatory response the pattern of synthesis by hepatocytes of several blood proteins is reorchestrated in response to cytokines released as a result of tissue damage or infection (152). These blood proteins are termed acute phase reactants and their increased synthesis is controlled at the transcriptional level primarily by interleukin (IL)-1 and IL-6 (2,153), and also by cytokines acting via the IL-6 receptor transducing apparatus such as IL-11 (154), leukocyte inhibitory factor (155), and oncostatin M (156). C-reactive protein (CRP) is the prototype acute phase reactant in humans because its concentration increases several thousand-fold (1,2,157). CRP is classified as a Pentraxin based on its composition of five identical, noncovalently linked subunits arranged in a flat pentameric disc (1,11). Based on the cDNA, each CRP subunit has 206 residues in a single polypeptide chain that is non-glycosylated with one internal disulfide bond (14,15). The predicted secondary
structure for CRP is 34% α-helix and 45% β-sheet based on circular dichroism (CD) spectroscopy (55). The tertiary structure of CRP is not yet known; however, low resolution x-ray diffraction analysis of CRP crystals is consistent with a planar pentagonal arrangement (158,159). Although a precise physiological role for CRP remains to be defined, many of its \textit{in vitro} biological activities are associated with nonspecific host defense events and include complement activation, opsonization, and leukocyte activation (reviewed in 1,3,157,160). The definitive characteristic for CRPs from all species is its lectin-like Ca$^{2+}$-dependent binding to phosphorylcholine (PC), as well as PC-bearing substrates (1,52,53).

Most of the biological activities of CRP are triggered upon binding to PC-bearing substrates which requires a Ca$^{2+}$-induced conformational change in CRP that is initiated at a separate site (84,161). Therefore, it was of interest to localize the Ca$^{2+}$-binding regions within each CRP subunit and determine their influence on the single PC-binding site per subunit (53,161). CRP has two Ca$^{2+}$-binding sites of equal affinity per subunit ($K_d = 6 \times 10^{-5}$ M) (52,83). Although the precise location of both Ca$^{2+}$-binding sites has not yet been determined, the highly conserved amino acids between 134-148 in all pentraxins was proposed as one of the Ca$^{2+}$-binding regions on the basis of sequence similarity to Ca$^{2+}$-binding regions in other proteins (56), as well as the demonstration
of a decrease in Ca\textsuperscript{2+} binding after proteolytic cleavage of intact CRP at two sites within this region (83). In the experiments reported herein the location of both Ca\textsuperscript{2+}-binding regions in CRP were determined using synthetic peptides. Conformational changes in both the peptides, and in CRP itself, mediated by these regions were demonstrated. The results suggest a mechanism by which these two Ca\textsuperscript{2+} binding sites regulate the PC-binding activity of CRP and in turn influence its biological activities.
B. Materials and methods

1. Purification of CRP. CRP was purified from human ascites fluids by Ca$^{2+}$-dependent affinity chromatography on PC-phenyl sepharose (Pierce Chemical Co., Rockford, IL), after removal of serum amyloid-P component (SAP) as described elsewhere (85,58).

2. CRP Synthetic Peptides. The following peptides numbered according to the derived sequence of CRP (10,11) were used:

peptide 134-148 (r134)I-L-G-Q-E-Q-D-S-F-G-G-N-F-E-G;

peptide 152-176 (r152)L-V-G-D-I-G-N-V-N-M-W-D-F-V-L-S-P-D-E-I-N-T-I-Y-L. Both peptides were synthesized by The Ohio State University Biochemical Instrument Center using t-boc synthesis on a Model 9500 Peptide Synthesizer (Milligen/Biosearch, Millipore, Burlington MA).

3. Generation of mAbs to Peptide 134-148. BALB/c mice were immunized with 100ug of peptide 134-148-KLH dissolved 1:1 in CFA by injecting 0.2ml i.p. Mice were rechallenged with 50 ug peptide 134-148-KLH in IFA every 4 wk thereafter for a total of 4 injections. A final injection of 100 ug peptide 134-148-KLH was given i.v. and 3 days later the spleen cells from mice displaying an ELISA titer against peptide 134-148-BSA >1:2000 were fused with P3X63.AG853 myeloma cells. The initial screening for antibody-producing clones was by a direct ELISA against peptide 134-148-BSA immobilized onto immulon II plates (Dynatech, Chantilly, VA) with 20 mM
carbonate/bicarbonate buffer pH 9.6. Clones producing antibody against the peptide were subcloned twice by limiting dilution. The isotype of each clone was determined with a Mono AB ID EIA kit from Zymed Laboratories (San Francisco, CA). The mAb-secreting cells were adapted to grow in DMEM containing 5% horse serum. Approximately 5 x 10^6 cells were inoculated i.p. into BALB/c mice treated with three weekly doses of pristane. The IgM in the ascites was partially purified using an AVID-AL column (Bioprobe, Tustin, CA).

4. Generation of Polyclonal Ab to Peptide 152-176. One NZW rabbit was injected subcutaneously at 8 sites with a total of 500 ug of the peptide emulsified in Freund’s complete adjuvant. After two weeks, the rabbit was rechallenged intramuscularly with an additional 200 ug of peptide in saline. After 4 weeks, the serum was collected and the IgG was purified using protein A affinity chromatography (Pierce). Specific binding of the Ab to both the peptide and intact purified CRP was determined by an indirect ELISA.

5. MAb to Human CRP. The mAb EA4-1 that recognizes an epitope within the PC-binding region of CRP was a generous gift from Drs. Michael Kilpatrick and John Volanakis (University of Alabama Medical Center, Birmingham AL) (84,161). The mAb HD2-4 was obtained from the American Type Culture Collection (Rockville, MD) and recognizes an epitope on CRP on the plane opposite the PC-binding region (161). Additional mAb to both intact CRP and synthetic peptides of CRP were
generated previously by us and described elsewhere (58,85).

6. ELISA. The specificity of the anti-peptide 152-176 polyclonal Ab was determined by an indirect ELISA. Briefly, peptide 152-176 or intact purified CRP were coated onto Immulon II plates in carbonate buffer at pH 9.6 overnight at 4°C. The plates were washed and blocked with 1% BSA in 20 mM TBS (pH 7.4) containing 2 mM Ca\(^{2+}\). The amount of bound Ab was detected with HRP-labeled goat anti-rabbit IgG followed by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Labs. Inc., Gaithersburg MD). The absorbance was read at 450 nm after the addition of 1M HCl.

The alteration in the availability of epitopes in the PC binding region of CRP was determined by first coating an Immulon II plate with mAb EA4-1 at 100 ng/well. Biotinylated CRP (B-CRP), at 250 ng/ml was incubated for 30 min with serial two fold dilutions of the anti-peptide Ab (100 to 0.5 ug/ml) before adding the mixture to the EA4-1 mAb coated wells. Bound B-CRP was detected using streptavidin-HRP and TMB substrate.

7. Equilibrium Dialysis Measurement of Calcium Binding. Direct Ca\(^{2+}\) binding to peptide 134-148 and peptide 152-176 was measured using equilibrium dialysis with \(^{45}\)Ca\(^{2+}\). Equilibrium dialysis was performed using a 10 well microdialysis cell (PGC Scientific) equipped with 100 ul chambers separated by a spectropore 7 dialysis membrane with a M.W. cutoff of 1000 Da (Spectrum Medical Industries, Houston TX). One half of the
dialysis cell was loaded with 100 ul of a 1 mg/ml solution of peptide in tris buffered saline (TBS) passed through a chelex column (Biorad) to remove trace Ca\(^{2+}\). The second half of the cell was loaded with various concentrations of \(^{45}\)Ca\(^{2+}\) plus carrier CaCl\(_2\). The concentration of CaCl\(_2\) in the carrier solution was determined by atomic absorption spectrophotometry. Equilibrium was reached over a 48 h interval by rotating the cell to assure mixing. Samples of 50 ul from each well were added to 2 ml of scintillation fluid, and counted using a \(^{32}\)P window on Beta-scintillation counter (BS-3000, Beckman Instruments). The data was plotted on a binding curve and subjected to scatchard analysis to calculate the dissociation constant (\(K_d\)).

8. Detection of \(^{45}\)Ca\(^{2+}\)-binding to CRP Fragments. Pronase cleavage of CRP was carried out as previously described (83,85). After proteolytic cleavage, CRP fragments were separated by SDS-PAGE on a 15% gel and transferred to nitrocellulose membranes. The membranes were exposed to \(^{45}\)Ca\(^{2+}\) and autoradiographed to detect Ca\(^{2+}\) binding by the cleavage fragments by a method described elsewhere (162).

9. Circular Dichroism. CD spectra of peptide 134-148 and peptide 152-176 were measured on a Jobin Yvon dichrograph model CD6 (Jobin Yvon Longjumeau, France) in the presence and absence of Ca\(^{2+}\). Ca\(^{2+}\) free peptide samples were prepared at 0.1 mg/ml in PBS containing 0.1 mM EDTA. The results are presented as the molar ellipticity [\(\theta\)] in degree cm\(^2\) dmole\(^{-1}\).
The Ca$^{2+}$ concentration was gradually brought up to 2 mM with the addition of a small volume from a 1M CaCl$_2$ stock solution (total volume change of <1%).
C. Results

1. Calcium Binding by Peptide 134-148 of CRP. Indirect evidence suggested that residues 134-148 of CRP are involved in Ca\(^{2+}\) binding (58,83,85). Equilibrium dialysis experiments were done to directly test whether peptide 134-148 binds Ca\(^{2+}\). \(^{45}\text{Ca}^{2+}\) binding by peptide 134-148 approached saturation at approximately 0.6 mM Ca\(^{2+}\) (Fig. 1). Scatchard analysis of the binding data revealed that the peptide binds 1 Ca\(^{2+}\) ion per peptide molecule with a \(K_d = 5.2 \times 10^{-4}\) M (Fig. 1, inset). \(^{45}\text{Ca}^{2+}\) binding to synthetic peptides corresponding to residues 1-15 and 47-63 of CRP was not detected under identical conditions. The results indicate that residues 134-148 probably represent one of the two Ca\(^{2+}\) binding regions within each CRP subunit.

2. Effect of Anti-Peptide 134-148 mAb on PC-binding Epitope. The mAb EA4-1 was generated against intact CRP and characterized as a PC-binding site specific mAb by Kilpatrick et al. (84). This mAb was used to detect changes in the availability of a PC-binding region epitope after treatment of the native intact pentraxin with each of the various mAb to peptide 134-148. The EA4-1 epitope is available only in the presence of Ca\(^{2+}\) ions (84). Each mAb was allowed to react with B-CRP (25ng) before adding the B-CRP to ELISA plate wells containing surface bound EA4-1 mAb. Four of the five mAb to peptide 134-148 inhibited the expression or availability of the EA4-1 epitope within the PC-binding site (Fig. 2). Only
Figure 1. Ca\(^{2+}\)-binding to the CRP synthetic peptide 134-148 using equilibrium dialysis. Peptide 134-148 at 100 μg/well (1.0 mg/ml) was dialyzed against various concentrations of Ca\(^{2+}\) containing a trace of \(^{45}\)Ca\(^{2+}\) in chambers separated by 1000 m.w. cutoff membranes that retain the peptide. The chambers were rotated until equilibrium was reached (48 h). Inset. Scatchard analysis of the Ca\(^{2+}\)-binding data for peptide 134-148. V represents the molar ratio of Ca\(^{2+}\) bound/peptide. The peptide binds 1 Ca\(^{2+}\) ion per molecule with a \(K_d = 5.2 \times 10^{-4}\) M.
Figure 1

Molar Ratio of Bound Ca$^{++}$ to Peptide

[Graph depicting the molar ratio of bound Ca$^{++}$ to peptide against [Ca$^{++}$] mM.]
Figure 2. Effect of mAb specific for peptide 134-148 on the binding of biotinylated-CRP to the PC-binding site specific mAb EA4-1. Various concentrations of each of the mAb was allowed to react with 100 ng of biotinylated-CRP before adding it to EA4-1 mAb coated wells (50 ng) well. The HD2-4 and 4B10 mAb were included as controls. Mean values from four experiments are shown for each mAb.
Inhibition of Binding of the EA 4-1 mAb to CRP

Figure 2
one of the mAb to peptide 134-148 (CaF8) failed to induce an alteration in the availability of the EA4-1 epitope on CRP (Fig. 2). The mAb 4B10, which reacts with the N-terminal region of CRP also failed to influence recognition of CRP by EA4-1. Figure 2 clearly reveals a difference in the inhibition induced by the anti-peptide 134-148 mAb vs the inhibition by the PC-site specific mAb EA4-1. The results are consistent with the interpretation that the epitope(s) recognized in the Ca\textsuperscript{2+}-binding region of CRP influence the conformation of the protein.

3. Binding of \textsuperscript{45}Ca\textsuperscript{2+} to Protease Fragments of CRP. Previous studies failed to reveal the location of the second Ca\textsuperscript{2+} binding region within CRP. To localize the second Ca\textsuperscript{2+} binding site, CRP was subjected to limited proteolysis with pronase which selectively cleaves each polypeptide subunit of CRP between residues 146 and 147 in the absence of Ca\textsuperscript{2+} (83), and then the intact CRP subunit and pronase cleavage fragments were tested for Ca\textsuperscript{2+} binding. Following separation by SDS-PAGE under reducing conditions and transfer to nitrocellulose membranes, the CRP fragments were exposed to \textsuperscript{45}Ca\textsuperscript{2+} (Fig. 3). The resulting bands on the autoradiograph of the transferred proteins in figure 3B indicate the presence of Ca\textsuperscript{2+}-binding sites in the intact CRP subunit, as well as the 16 and 6.5 kDa fragments from the pronase digestion. The 16 kDa band may represent the Ca\textsuperscript{2+}-binding activity of residues 134-148 (56,83). The 6.5 kDa band indicates the presence of a second
Figure 3. Ca\textsuperscript{2+} binding to fragments of CRP generated by protease cleavage. Limited proteolysis by pronase cleaves CRP between residues 146 and 147. Intact CRP (lane 2A) and fragments generated by pronase cleavage (lane 1A) were separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane and stained with amido black. An identical, unstained membrane was then exposed to a solution of \textsuperscript{45}Ca\textsuperscript{2+} (1 mCi/ml), rinsed in 50% ethanol, and autoradiographed (B). The bands in lane 1B correspond to the 16 and 6.5 kDa fragments and reveal the presence of Ca\textsuperscript{2+} binding sites. Lane 2B shows the binding of \textsuperscript{45}Ca\textsuperscript{2+} by an equivalent amount of the intact subunit of CRP.
Figure 3
Ca\textsuperscript{2+}-binding region between residues 147 and 206 of CRP.

4. Calcium Binding by Peptide 152-176. Since the \textsuperscript{45}Ca\textsuperscript{2+}
binding to the C-terminal fragment of CRP indicated that the second Ca\textsuperscript{2+}-binding region of CRP lies within the C-terminal 60 residues, a 25 amino acid peptide consisting of residues 152-176 from within this region was synthesized on the basis of the presence of acidic residues and sequence similarity to other Ca\textsuperscript{2+}-binding regions (149). Equilibrium dialysis experiments were done in the same manner as with peptide 134-148 to test whether Ca\textsuperscript{2+} binding occurred with this peptide. The peptide bound Ca\textsuperscript{2+} in solution and saturation was approached at approximately 0.4 mM (Fig. 4). Scatchard analysis of the binding data revealed that peptide 152-176 binds 1 Ca\textsuperscript{2+} ion per molecule with a \( K_d = 1.7 \times 10^{-4} \) (Fig. 4, inset). Therefore, the affinity of peptide 152-176 for Ca\textsuperscript{2+} is approximately three-fold greater than peptide 134-148 under identical conditions.

5. Circular Dichroism (CD). Ca\textsuperscript{2+} induced conformational changes in well-characterized Ca\textsuperscript{2+}-binding proteins such as calmodulin are driven by conformational changes that occur at the calcium binding site itself (163,164). CD spectroscopy has been used to detect Ca\textsuperscript{2+}-induced conformational changes in peptides corresponding to Ca\textsuperscript{2+}-binding sequences of other proteins (165). To determine if a conformational change occurs in the CRP peptides the CD spectra of peptides 134-148 and 152-176 were measured in the presence and absence of Ca\textsuperscript{2+}.
Figure 4. Binding of Ca\(^{2+}\) to synthetic peptide 152-176 of CRP using equilibrium dialysis. Peptide 152-176 at 100 ug/well (1 mg/ml) was dialyzed against various concentrations of Ca\(^{2+}\) containing trace amounts \(^{45}\)Ca\(^{2+}\) in chambers separated by a 1000 m.w. cutoff membrane that retains the peptide. The chamber was rotated until equilibrium was reached (48 h). **Inset.** Scatchard analysis of the Ca\(^{2+}\)-binding data for peptide 152-176. \(V\) represents the molar ratio of Ca\(^{2+}\)/peptide. The peptide binds 1 Ca\(^{2+}\) per molecule with a \(K_d = 1.7 \times 10^{-4}\) M.
With the addition of 2 mM Ca\(^{2+}\), peptide 152-176 showed a positive shift in molar ellipticity \([\theta]\) between 230 and 210 nm indicating an overall change in the conformation of the peptide backbone. Over the same wavelengths, the shorter peptide 134-148 showed very little change in its CD spectrum in the presence and absence of physiological concentrations of Ca\(^{2+}\). Although the region in CRP defined by peptide 134-148 was previously shown to influence epitope expression in the PC binding region (85), CD and spectroscopic methods in general may not be sensitive enough to detect a conformational change in a 15 residue peptide.

6. Effect of Antibodies to Ca\(^{2+}\)-Binding Peptides on the PC-Binding Site. In a previous experiment, a mouse mAb (EA4-1) which is specific for a Ca\(^{2+}\)-dependent epitope in the PC-binding sites within CRP, was used as a sensitive indicator of an allosteric change in the intact pentraxin (84). Several of the mAb generated against peptide 134-148 bind to intact CRP and induce an allosteric change that inhibits the expression of the EA4-1 epitope in the PC binding region (85). A similar assay was used to determine whether the peptide 152-176 is also involved in the allosteric control of epitope expression by the PC-binding region. Incubation of soluble CRP with a polyclonal Ab to peptide 152-176 inhibited the binding of CRP to the EA4-1 mAb in a "capture" ELISA (Fig. 6). Binding of another mAb, HD2-4, that recognizes an epitope on the opposite plane of the CRP pentamer (161), was not affected by the
Figure 5. The effects of Ca$^{2+}$ on the far UV region of the CD spectrum of peptides 134-148 and 152-176. Peptides (0.1mg/ml) were dissolved in 10 mM phosphate buffer (pH 7.2) with 0.15 M NaCl and 0.1 mM EDTA. CD measurements were made using a 1 cm cell and the results are presented as molar ellipticity ([θ]) in deg x cm$^2$ x dmole$^{-1}$. Ca$^{2+}$ concentrations were increased to 2 mM by adding 10 ul of a 200 mM stock solution of CaCl$_2$ to 1 ml of peptide 134-148 (A) or peptide 152-176 (B). Note the difference between the molar ellipticity scales in (A) and (B).
Figure 5
Figure 6. Effect of an Ab to peptide 152-176 and peptide 134-148 on the recognition of CRP by the anti-PC binding region mAb EA4-1. B-CRP (250 ng/ml) was incubated with serial dilutions of the anti-peptide 152-176 Ab and then added to plates coated with EA4-1 (50 ng/well). The effect of adding antibodies to both Ca\(^{2+}\)-binding peptides (134-148 and 152-176) is also shown. The percentage of EA4-1 captured CRP was calculated on the basis of the amount of B-CRP bound in the absence of anti-peptide Ab.
Figure 6

% Inhibition of CRP Capture by EA4-1

\[ \mu g/ml, \text{Anti-152-176} \]

- ▲ — Anti-152-176
- □ — Anti-152-176
- ■ — Anti-134-148
- ○ — HD2-4
addition of Ab to peptide 152-176. Incubation of CRP with a constant amount of mAb to peptide 134-148 and various amounts of Ab to peptide 152-176 restored most of the recognition of CRP by the PC-binding site specific EA4-1 mAb (Fig. 6). The mAb to peptide 134-148 and the polyclonal Ab to peptide 152-176 bind to CRP equally well in the presence and absence of Ca^{2+} (data not shown), indicating that the coordination of Ca^{2+} by side chain interactions does not affect Ab binding. The results suggest that the Ca^{2+}-binding region in peptide 152-176 is directly involved in the allosteric regulation of epitope expression in the PC-binding region. The finding that the simultaneous binding of Ab to both Ca^{2+}-binding sites results in a conformation within the PC-binding site that is different from that induced after binding of either Ab alone, suggests that calcium binding at both sites in the subunit is probably required to maintain the conformation of CRP needed for biological activity.
D. Discussion

The $\text{Ca}^{2+}$-dependent binding properties of all the pentraxins suggest that $\text{Ca}^{2+}$ is likely to play a significant role in maintaining a conformation suitable for biological reactions. The structural basis for the $\text{Ca}^{2+}$-induced conformational change within the CRP pentraxin required for subsequent binding to PC is still poorly understood. Since most of the pro-inflammatory activities ascribed to CRP occur after engagement of its PC-binding site that is initiated only in the presence of $\text{Ca}^{2+}$, studies to locate the two $\text{Ca}^{2+}$-binding sites within each CRP subunit were undertaken using synthetic peptides spanning each potential region and examining whether those sequences are involved in both $\text{Ca}^{2+}$-binding and conformational changes. Such studies should lead to an understanding of the mechanism whereby CRP functions in a variety of biological reactions.

The precise location of the two $\text{Ca}^{2+}$-binding sites of CRP has not been determined. However, the highly conserved region in all pentraxins consisting of residues 134-148 was suggested as a $\text{Ca}^{2+}$-binding site by Kinoshita et. al. (83) on the basis of sequence similarity to $\text{Ca}^{2+}$-binding regions in other proteins and the demonstration of a substantial decrease in $\text{Ca}^{2+}$ binding after proteolytic cleavage of intact CRP within this region. To directly demonstrate $\text{Ca}^{2+}$-binding by this region we tested the synthetic peptide consisting of residues 134-148 in equilibrium dialysis experiments and found that it
was capable of binding 1 Ca\(^{2+}\) ion per molecule. The decreased affinity for Ca\(^{2+}\) by this peptide \((K_d = 5.2 \times 10^{-4}\) M\) when compared to the intact CRP molecule \((K_d = 6 \times 10^{-5}\) M\) is likely a limitation the short peptide has in maintaining the conformation needed for high affinity Ca\(^{2+}\) binding \((165)\). Nonetheless, the measured affinity of this peptide falls within the range of affinities of other Ca\(^{2+}\)-binding peptides \((165)\). To localize the second Ca\(^{2+}\)-binding site, CRP was cleaved between residues 146 and 147 by limited proteolysis, the fragments of 16 and 6.5 kDa separated, and exposed to \(^{45}\)Ca\(^{2+}\) which clearly demonstrated the presence of Ca\(^{2+}\)-binding sites in both fragments. Ca\(^{2+}\) binding by the 16 kDa fragment most likely represents the Ca\(^{2+}\)-binding site composed of residues 134-148 \((83)\). The Ca\(^{2+}\)-binding activity of the 6.5 kDa fragment corresponding to the C-terminal 60 amino acids of the 206 residue CRP subunit indicates the presence of a second, distinct Ca\(^{2+}\)-binding region. Therefore, a site for Ca\(^{2+}\)-binding within the C-terminal 60 residues was sought.

The frequencies of the 20 amino acids at each position within Ca\(^{2+}\)-binding sequences in 165 Ca\(^{2+}\)-binding loop sequences from EF-hand proteins were provided in a review by Marsden et al. \((149)\) and used to align the Ca\(^{2+}\)-binding sequence within the last 60 amino acids in the CRP sequence. The best alignment was achieved by beginning the Ca\(^{2+}\)-binding sequence with residue 158 in CRP and considering the Trp at position 162 as an insert \((Table 1)\). Using this alignment,
Table 1

Frequency of Each Amino Acid at Sequential Positions Within 165 Different Ca\(^{2+}\)-Binding Sites\(^a\) and the Alignment of Residues 158 to 170 of CRP\(^b\)

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Consensus Sequence

Asp --- Asp Gly Asp Gly --- Ile --- --- Asp Glu

CRP Sequence\(^c\)

Asn Val Asn Met Asp Phe Val Leu Ser Pro Asp Glu

158 159 160 161 163 164 165 166 167 168 169 170

\(^a\)The frequencies at the positions within the 165 different Ca\(^{2+}\)-binding sites were provided in a review by Marsden et al. 1989 ( ).

\(^b\)The figures represent the number of times out of 165 different Ca\(^{2+}\)-binding sequences that each amino acid occurs at the indicated position.

\(^c\)In order to provide the best fitting sequence comparison, the Trp at residue 162 in the CRP sequence was removed.
the Phe at position 6 is the only amino acid in a position not found in EF-hand Ca$^{2+}$-binding sequences. However, it should be pointed out that Phe is the most commonly found amino acid at position 7 of the EF-hand Ca$^{2+}$-binding sequences (Table 1). A comparison of the consensus sequence at positions 1-12 of the 165 EF-hand Ca$^{2+}$-binding sequences vs. the CRP sequence between 158 and 170 reveals some similarity especially at positions 5, 11, and 12 with conservative substitutions at positions 3 and 8 (Table 1). Therefore, to directly test for Ca$^{2+}$-binding in this region, we synthesized a peptide consisting of residues 152-176 and used equilibrium dialysis to measure Ca$^{2+}$-binding. Peptide 152-176 bound 1 Ca$^{2+}$ ion/molecule and reached saturation around 0.4 mM Ca$^{2+}$ with a $K_d = 1.4 \times 10^{-4}$. Since CRP is a blood protein, the affinity of the two sites for Ca$^{2+}$ should be sufficient at serum levels of 1-2 mM Ca$^{2+}$.

During the course of this work, experiments published by Dong, et al. (86) using infrared spectrophotometry derived a predicted secondary structure for the hamster female protein (FP), a member of the pentraxin protein family. Sequence similarity to EF-hand type Ca$^{2+}$-binding sequences and the estimated secondary structure were used to predict the location of Ca$^{2+}$-binding sites in all the pentraxins (86). One of the predicted Ca$^{2+}$-binding sites consisted of residues 154-168 in FP and corresponds to the residues 157-171 of the CRP, essentially the same sequence that binds Ca$^{2+}$ in this
study (86). Dong, et al. (86) also predicted that the second 
Ca$^{2+}$-binding site was located between residues 98-113 in CRP. 
This prediction contrasts with the results shown here that 
peptide 134-148 binds Ca$^{2+}$. There are two additional reasons 
for thinking that residues 98-113 do not compose the second 
site. The sequence between residues 98-113 contains two Trp 
at residues 100 and 110; however, Trp was shown to occur only 
twice in 165 different calcium binding sequences (Table I) and 
is therefore not likely to be found in Ca$^{2+}$-binding sites 
(149). Dong, et al. (86) state that the presence of Phe at 
the potential ligand binding positions between residues 134- 
148 could interfere with Ca$^{2+}$-binding; however, Phe is the 
most frequently occurring amino acid at positions 7 and 10 
within the 165 calcium binding sequences (Table I).

After locating the two Ca$^{2+}$-binding sites in CRP, it was 
important to determine whether both regions contribute to the 
Ca$^{2+}$-induced conformational change that occurs in the intact 
pentraxin. CD measurements with the 25 amino acid peptide 
152-176 indicated that a conformational change occurs upon 
Ca$^{2+}$ binding and suggested that the same region within the 
intact CRP subunit may undergo a similar conformational 
change. By contrast, CD measurements detected only a very 
small Ca$^{2+}$-induced conformational change in the 15 residue 
peptide 134-148. The negative finding may reflect the 
limitations of CD for measuring conformational changes in 
short peptides or indicate that no conformational change
occurs at this site. Both a polyclonal anti-peptide 152-176 Ab and several anti-peptide 134-148 mAb were shown to bind to intact CRP and decreased the availability of an epitope in the PC-binding region recognized by mAb EA4-1, a mAb selected for its ability to inhibit the PC-binding of intact CRP (84). The PC-binding region is known to occur within only one of the two planes of the pentraxin (161). Simultaneous binding of the polyclonal anti-peptide 152-176 Ab and an anti-peptide 134-148 mAb to intact CRP restored the availability of EA4-1 epitope suggesting that when both Ca\(^{2+}\)-binding sites are engaged a conformational change occurs that permits PC binding.

The best characterized proteins that undergo Ca\(^{2+}\)-induced conformational changes belong to the EF-hand family (166). The Ca\(^{2+}\)-binding sites in this group of proteins consist of 12 amino acid Ca\(^{2+}\)-binding loops that are joined at both ends to \(\alpha\)-helices (167). For example, in calmodulin, the Ca\(^{2+}\) binding event induces the loop region to twist moving the adjoining helical regions and altering the tertiary structure of the molecule (163,164). We propose a similar model for the Ca\(^{2+}\)-induced conformation change in CRP with one important difference, i.e. Ca\(^{2+}\)-binding occurs at two proximal, nearly adjacent sites rather than to sequentially separated Ca\(^{2+}\)-binding sites as in typical EF-hand proteins. The two Ca\(^{2+}\)-binding regions in CRP occur nearly sequentially along the peptide (residues 134-148 and 152-176) and the Garnier and Robsen (168) secondary structure prediction indicates that
these two sequential Ca\textsuperscript{2+}-binding regions are preceded and followed by regions of predicted $\alpha$-helicity. Like calmodulin, the conformational change initiated at these two Ca\textsuperscript{2+}-binding sites may be propagated to the adjoining regions of predicted $\alpha$-helicity, with an overall result of allosterically changing CRP to accommodate PC-bearing substrates. The potential importance of these results is that it may provide an explanation of the change in CRP's binding activities and structural stability during inflammation.

ACKNOWLEDGEMENT. The authors thank Dr. Wayne Bechtel, Dept. of Biochemistry, for his advise on CD spectroscopy.
CHAPTER III.

THE CELL ATTACHMENT PEPTIDE OF C-REACTIVE PROTEIN:
CRITICAL AMINO ACIDS AND MINIMUM LENGTH

A. Introduction

The acute phase of the systemic inflammatory response to tissue injury or infection is characterized by a rapid reorchestration in the pattern of blood proteins synthesized by liver hepatocytes in response to several cytokines (152). C-reactive protein (CRP) is the prototype acute phase reactant since its concentration can increase several thousand-fold in humans and most vertebrates (1,2,3). CRP is composed of five identical noncovalently associated subunits of 206 amino acids each (11,15) and displays Ca²⁺-dependent binding to monophosphate esters, especially phosphorylcholine (PC) (52,157). The homologous protein, serum amyloid P-component (SAP), and CRP are classified as Pentraxins, proteins with a highly conserved secondary structure and gene organization (2,15,56). Human CRP mediates several inflammatory biological activities including leukocyte activation (124,131,137) and initiation of the complement cascade (65,94). One of CRP's unique properties is that it is selectively deposited at sites of
tissue damage suggesting a mechanism for focusing its biological activities (22,66,77,169). CRP also interacts via its PC-binding region with the extracellular matrix proteins fibronectin (Fn) (67,68), and laminin (69), and to phospholipids on damaged membranes (65), substances that are likely to be more accessible in inflamed and damaged tissues. Neutrophil proteolysis of CRP generates peptides that also activate monocytes and neutrophils (122,126,127,170). The active peptides contain tuftsin tetrapeptide (Thr-Lys-Pro-Arg)-like sequences (122). The analysis of one of the tuftsin-bearing peptides composed of residues 27-38 for its interaction with the monocyte CRP-receptor (CRP-R) (137) resulted in the finding that the peptide supports cell attachment in vitro and therefore the peptide is referred to as the cell-binding peptide or CB-Pep (151). Characterization of the interaction between cells and the CB-Pep revealed that attachment occurs at physiological concentrations, is distinct from the attachment to the extracellular matrix proteins, Fn and laminin, and is not inhibited by the tuftsin tetrapeptide (151). The interaction with the CB-Pep indicated that cell attachment was very different from that supported by RGDS-bearing peptides from Fn (151).

To determine the relationship between the receptor for CB-Pep to other known cell attachment receptors it became important to determine both the critical residues involved
and the minimal length of the peptide that supports cell attachment. Our approach was to synthesize substituted peptides for determining the critical residues involved and truncated peptides to determine the minimal length required. The experiments described herein using both direct cell attachment and inhibition of cell attachment to the CB-Pep by the various synthetic peptides show that the critical residues are present within a five residue minimal sequence in the C-terminal half of the dodecapeptide. The findings suggest that CRP, and especially a peptide derived from CRP, may contribute to the wound repair process by supporting cell attachment via receptors distinct from the integrin receptors for extracellular matrix proteins.
B. Materials and Methods

1. Peptide Synthesis. The list below shows the sequence of the CB-Pep of CRP, as well as the substituted and truncated peptides that were synthesized. The numbering of the residues is based on the published sequence by Woo, et al. (15). The substituted amino acid is shown in bold and its position is part of the designation for each of their peptides. The truncated peptides with amino acids deleted from the C- and N-terminus are designated by the remaining residues.

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<tr>
<td>CB29-38</td>
<td>P-L-K-A-F-T-V-C-L-H</td>
</tr>
<tr>
<td>CB30-38</td>
<td>L-K-A-F-T-V-C-L-H</td>
</tr>
<tr>
<td>CB31-38</td>
<td>K-A-F-T-V-C-L-H</td>
</tr>
<tr>
<td>CB32-38</td>
<td>A-F-T-V-C-L-H</td>
</tr>
<tr>
<td>CB33-38</td>
<td>F-T-V-C-L-H</td>
</tr>
<tr>
<td>CB33-37</td>
<td>F-T-V-C-L</td>
</tr>
<tr>
<td>CB27-37</td>
<td>T-K-P-L-K-A-F-T-V-C-L</td>
</tr>
<tr>
<td>CB27-36</td>
<td>T-K-P-L-K-A-F-T-V-C</td>
</tr>
<tr>
<td>CB27-35</td>
<td>T-K-P-L-K-A-F-T-V</td>
</tr>
<tr>
<td>CB27-34</td>
<td>T-K-P-L-K-A-F-T</td>
</tr>
</tbody>
</table>

The substituted and truncated peptides were synthesized by FMoc-tert-butyl solid phase synthesis strategy on an advanced chemtech model 350 multiple peptide synthesizer. The solid support consisted of 4-methylbenzhydrylamine resin.
(0.54 mmol of Cl/g) using an acid labile linkage agent 4-(hydroxymethyl)phenoxyacetic acid (171,172). Side chain protection was as follows: S-trityl for cysteine, tert-butyl ethers for serine, threonine and tyrosine, N-im-trityl for histidine, tert-butyloxycarbonyl (Boc) for lysine, and N°-PMC for arginine. The resin support was neutralized with 10% diisopropylethylamine (DIEA) in dichloromethane (DCM). The linkage agent was activated as its pentafluorophenyl (pfp) ester (5 fold) and attached to the resin by double coupling (30 min each). During the last 15 min, 3 equivalents of catalyst [1-hydroxybenzotriazole (HOBt)] were added. Ninhydrin Kaiser test indicated that no amino groups were left unreacted. The C-terminal amino acids were esterified to the linker-activated support by double coupling of the preformed pfp ester (6 fold excess) for 1 hour in the presence of dimethylaminopyridine (0.1 equiv.) as the catalyst. A typical coupling cycle was as follows: the N-protected peptide resin was washed once and treated for 12 min with 50% piperidine in DMF followed by 10 DMF washes. Duplicate 30 min couplings for each amino acid were preformed using a 6 fold excess of FMoc amino acid, HOBt and 1,3-diisopropyl-carbodiimide (DIC). After the chain was elongated the N-terminal FMoc group was removed and the resin washed transferred, and dried in a desiccator. The side chain protecting groups and the peptide resin anchoring bond were cleaved by treatment with trifluoroacetic acid
(TFA) containing 5\% anisole, 3\% thioanisole and 2\% ethanedithiol. The resins were washed several times with neat TFA and the combined washings were rotary-evaporated to an oily residue. The crude peptides were precipitated with diethyl ether and taken up in 0.1 M acetic acid. Several ether extractions were carried out to remove scavengers and the aqueous acidic phase was lyophilized to a dry powder. One of the full-length substituted peptides was N-terminally sequenced on a milligen prosequencer model 6600 and the analysis confirmed the sequence accuracy. The substituted and truncated peptides were >70\% pure on the basis of reverse phase HPLC profiles.

The first CB-Pep was synthesized by the Ohio State University Biochemical Instrument Center using t-boc synthesis on a model 9500 peptide synthesizer (Millegen/Millipore, Burlinton, MA) and was estimated to be ~90\% pure on the basis of amino acid composition.

2. Cell Attachment Assays. Polystyrene microplates (Immuron-2, Dynatech) were coated with 10 nmoles of CB-Pep, 2 nmoles of intact CRP or 0.5 pmoles of fibronectin (Fn) in carbonate-bicarbonate buffer (Ph 9.6) as described previously (69,151). The wells were washed 3x in phosphate-buffered saline (PBS) and blocked for 1 h with 1\% BSA in PBS. Normal rat kidney fibroblasts (NRK-49F) from American Type Culture Collection) between passages 2 and 10 were added at 10^5/well and allowed to adhere for 30 min in RPMI
medium containing 5% calf serum. For inhibition of cell attachment soluble CB-Pep at 100 nmoles/well was added at the same time as the cells. The plates were washed 3x with PBS to remove unattached cells and the percentage of attached cells determined by the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay (173). Briefly, 100 μl of medium and 20 μl of a 5 mg/ml MTT in PBS was added to the attached cells in each well and incubated at 37°C in 5% CO₂ for two hours. The reduced MTT crystals formed by viable cells were dissolved in 0.01 M HCl in isopropanol and the amount of MTT dye incorporated was determined by reading its absorbance at 550 nm. The absorbance of the converted dye was proportional to the number of viable cells remaining in the wells. The percentage of cell attachment was calculated by dividing the average absorbance of three wells by the average absorbance of three wells coated with Fn.

3. Reduction of Cys in CB-Pep. Dithiothreitol (DTT) reduction of the CB-Pep was done by dissolving 5 mg of the peptide in 0.1 M acetic acid and 0.05 M DTT. DTT was removed from the peptide solution by gel filtration through a sephadex G15 column by collecting fractions at the void volume. DTT removal was assayed by the addition of Ellman's reagent. The peptide was then dialyzed against PBS (pH 7.4) to remove the acetic acid. All buffers were purged with nitrogen gas prior to use in any steps involving the reduced
peptide.

4. Direct Cell Attachment to Substituted and Truncated Peptides. Serial two-fold dilutions of substituted peptides and peptides with deleted residues were diluted in carbonate buffer (pH 9.6) and coated onto Immulon-II microplate wells. The dilution range of the coated peptides was from 20 to 0.16 nmoles/well. Cell attachment was determined as described above.

5. Inhibition of Cell Attachment to CB Peptide. Microplates were coated with 10 nmoles/well of CB-Pep washed and blocked as described above. Serial 2-fold dilutions of either substituted or deleted peptides at 100 to 12.5 nmoles/well were added with $10^5$ NRK-49F cells and allowed adhere for 30 min in RPMI medium plus 5% calf serum. The plates were washed 3x with PBS to remove the unattached cells and the percentage of attached cells was determined by the MTT viability assay as described above.
C. Results

1. Cell Attachment to Intact CRP, Fn and the CB-Pep.

To compare the relative efficiency of cell attachment to both the CB-Pep of CRP and the intact protein we compared cell attachment to equal molar amounts of CRP and CB-Pep vs. plasma Fn. Similar levels of cell attachment were observed when comparing CB-Pep or intact CRP at 10 nmoles/well (Fig. 7). The molar concentration for the intact CRP was calculated using a m.w. of 23 kDa/subunit and therefore 10 nmoles of CRP subunits is equivalent to 2 nmoles of the intact pentraxin. The 2 nmoles of CRP are equal to 230 ug. A variety of moderate systemic inflammatory responses are associated with CRP concentrations >230 ug/ml (rev. in 3). CRP would be further concentrated as it deposits at sites of tissue damage. A dose-response relationship was observed between cell attachment activity and the amount of intact, immobilized CRP expressed in terms of nmoles of CRP subunits (Fig. 8). The results suggest that a threshold concentration of CRP is required for efficient cell attachment.

Fn at only 0.5 pmoles/well supported a similar level of cell attachment as that of 10 nmoles of CB-Pep indicating that attachment to Fn is much more efficient. The addition of soluble CB-Pep at 100 nmoles/ml as a competing peptide to the fibroblasts significantly inhibited cell attachment to both the CB-Pep and intact CRP suggesting that the cell
FIGURE 7. Efficiency of cell attachment to Fn, CRP and the CB-Pep of CRP. Soluble CB-Pep at 100 nmoles/0.1 ml was added to the cells before allowing them to attach to either CB-Pep or CRP coated directly onto the plate. The amount of CRP is expressed as nmoles of CRP subunits. Data are the mean (+/- SD) values from a representative experiment in which cell attachment was measured in triplicate.
Figure 7

![Graph showing % of Cell Attachment to Fn with different conditions: 0.5 pmoles ofFn, 10 nmoles CB-Pep, 10 nmoles CB-Pep and 100 nmoles Free CB-Pep, 10 nmoles CRP, and 10 nmoles CRP and 100 nmoles Free CB-Pep. The graph includes error bars for each condition.](image)
FIGURE 8. Cell attachment to different amounts of immobilized CRP. The amount of CRP is expressed in terms of nmoles of CRP subunits. The data are the mean values (+/- SD) from three experiments.
Figure 8

% Cell Attachment to Fn

nmoles of CRP Subunits

0 20 40 60 80 100

.5 1 2 5 10
interaction with CRP occurs at this region. The CB-Pep was shown previously by us not to inhibit cell attachment to Fn (151). The specificity of the cell-binding interaction with the CB-Pep was explored further.

2. Cell Attachment to Reduced CB-Pep. Since dimer formation through the Cys-SH at position 36 in the CB-Pep readily occurs in solution, the possibility that only dimers support cell attachment was considered. A comparison of reduced vs. non-reduced CB-Pep failed to detect any difference over a range of concentrations (1-20 nmoles/well) in the level of cell attachment (data not shown). These results clearly indicated that dimer formation does not affect the cell attachment activity of the peptide.

3. Cell Attachment to Truncated Peptides. To determine the minimal amino acid sequence required for cell attachment to the CB-Pep, a series of synthetic peptides with sequential N-terminal and C-terminal deletions were used in direct cell attachment assays. Two of the ten truncated peptides, CB27-34 and CB27-35, failed to support cell attachment, indicating that residues 33-36 are required for cell attachment (Table 2 and Fig. 9). A comparison of the relative cell attachment activity of different amounts of four truncated peptides shown in figure 9 suggests that the critical residues for activity are: 33 through 36.

4. Inhibition of Cell Attachment to CB-Peptide by Truncated Peptides. Since attaching the truncated peptides
### Table 2

Cell Attachment to Truncated Synthetic Peptides vs. the CB-Pep of CRP

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>% of Attachment to Fibronectin&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB-Pep</td>
<td>T-K-P-L-K-A-F-T-V-C-L-H</td>
<td>100</td>
</tr>
<tr>
<td>CB27-37</td>
<td>T-K-P-L-K-A-F-T-V-C-L</td>
<td>95</td>
</tr>
<tr>
<td>CB27-35</td>
<td>T-K-P-L-K-A-F-T-V</td>
<td>42</td>
</tr>
<tr>
<td>CB27-34</td>
<td>T-K-P-L-K-A-F-T</td>
<td>38</td>
</tr>
<tr>
<td>CB29-38</td>
<td>P-L-K-A-F-T-V-C-L-H</td>
<td>NS&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CB30-38</td>
<td>L-K-A-F-T-V-C-L-H</td>
<td>90</td>
</tr>
<tr>
<td>CB31-38</td>
<td>K-A-F-T-V-C-L-H</td>
<td>96</td>
</tr>
<tr>
<td>CB32-38</td>
<td>A-F-T-V-C-L-H</td>
<td>82</td>
</tr>
<tr>
<td>CB33-38</td>
<td>F-T-V-C-L-H</td>
<td>85</td>
</tr>
</tbody>
</table>

*The peptides were coated at 10 nmoles/well. Normal rat kidney fibroblasts were added to the wells at 10<sup>3</sup> cells/well and the percentage of cell attachment calculated vs. the attachment to 0.5 pmoles Fn.*

*Results are mean values of three experiments.*

*Peptides that were not soluble (NS) under the conditions of the experiment.*
FIGURE 9. Direct cell attachment to modified peptides based on the CB-Pep with N-terminal and C-terminal amino acid deletions. Serial two fold dilutions of the peptides were coated onto plates and fibroblasts were added at $10^5$ cells/well. The percentage of cell attachment vs. the attachment to 0.5 pmoles of Fn was measured. Results are mean values of 3 experiments.
Figure 9

- △ - CB27-36
- ○ - CB27-35
- ◊ - CB27-34
- □ - CB33-38

% of Attachment to Fn

nmoles/Well of Peptide

20  40  60  80  100

0.62  1.25  2.5  5  10  20
to the plate may force a non-native conformation and may not result in a quantitative attachment of the peptide to the well, an inhibition assay of cell attachment was performed to test each peptide in solution. The data in Figure 10 demonstrate that peptides containing residues 33-37 inhibited cell attachment to the immobilized CB-Pep, whereas any peptide truncated to within residues 33-37 failed to inhibit attachment. Peptide 33-38 partially inhibited cell attachment, whereas peptide 32-38 completely inhibited attachment, indicating that residue 32 may also be involved in cell attachment (Fig. 10). As expected, the longer N-terminally truncated peptides CB28-38, CB29-38, CB30-38, and CB31-38 also inhibited cell attachment. The shorter N-terminally truncated peptides CB32-38 and CB33-38 still significantly inhibited attachment. The two shorter C-terminally truncated peptides CB27-34 and CB27-35 did not inhibit attachment to CB-Pep (Table 3). The findings from both the direct attachment and attachment inhibition assays suggests that the minimal sequence required for cell attachment consist of residues 33-37: Phe-Thr-Val-Cys-Leu.

5. Cell Attachment to Substituted Peptides. To identify the critical residues within the CB-Pep responsible for direct cell attachment activity, experiments were done using synthetic peptides containing a single substitution at each residue. Only the CB33Y and CB34S peptides of the eight substituted peptides failed to support cell attachment
Table 3

Inhibition of Cell Attachment to CB-Peptide by Peptides
Truncated From the N-Terminus and C-Terminus

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>% of Attachment to Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB29-38</td>
<td>P-L-K-A-F-T-V-C-L-H</td>
<td>NSc</td>
</tr>
<tr>
<td>CB30-38</td>
<td>L-K-A-F-T-V-C-L-H</td>
<td>0</td>
</tr>
<tr>
<td>CB31-38</td>
<td>K-A-F-T-V-C-L-H</td>
<td>14</td>
</tr>
<tr>
<td>CB32-38</td>
<td>A-F-T-V-C-L-H</td>
<td>23</td>
</tr>
<tr>
<td>CB33-38</td>
<td>F-T-V-C-L-H</td>
<td>55</td>
</tr>
<tr>
<td>CB27-36</td>
<td>T-K-P-L-K-A-F-T-V-C</td>
<td>89</td>
</tr>
<tr>
<td>CB27-34</td>
<td>T-K-P-L-K-A-F-T</td>
<td>91</td>
</tr>
</tbody>
</table>

The synthetic peptides were tested for inhibition of cell attachment at 125-1000 nmoles/ml against CB-Pep coated at 10 nmoles/well. The data shown is for 1000 nmoles/ml.

Results are mean values of three experiments.

Peptides that were not soluble under the conditions of the experiment.
FIGURE 10. Inhibition of cell attachment to the CB-Pep by modified peptides with residues deleted from the N- or C-terminus. Serial two-fold dilutions of the soluble peptides were added to $10^5$ fibroblasts before addition to wells coated with 10 nmoles/well of the CB-Pep. The percentage of the cell attachment to 10 nmoles of CB-Pep is indicated. The ability of a peptide to inhibit cell attachment by >50% was considered significant. Data are mean values of three experiments.
Figure 10

% Attachment

nmoles/ml of free Peptide

- CB-Pep
- CB27-36
- CB32-38
- CB33-38
- CB27-37

Figure 10
(Table 4 and Fig. 11). Titration of the substituted peptides over a range of relevant concentrations revealed that the loss of activity did not reflect a significant change in the dose-response curve (Fig. 11). The results clearly indicate a requirement for both Phe and Thr at positions 33 and 34 of the CB-Pep.

Substitutions for each of the residues between 33 and 37 of the CB-Pep were chosen to maintain charge, hydrophobicity, and size. The substitutions were: Phe to Tyr at residue 33, Thr to Ser at residue 34, Val to Leu at residue 35, and Leu to Ile at residue 37. The Cys at residue 36 was not substituted since it is part of the intrachain disulfide bond with the Cys-97. The tuftsin tetrapeptide (TKPR) which is identical to the first three residues of CB-Pep was previously shown by us not to affect cell attachment to CB-Pep (151), and therefore residues 27 through 29 were not substituted. The conservative nature of these substitutions may have prevented identification of additional critical residues.

6. Inhibition of Cell Attachment to CB-Pep by Substituted Peptides. Synthetic peptides with single conservative substitutions at each of the residues beginning with amino acid 30 of the CB-Pep were tested for their ability to inhibit fibroblast attachment to the CB-Pep. All the substituted peptides inhibited cell attachment to the CB-Pep except peptide CB33Y which has a Tyr replacing the
Table 4

Cell Attachment to CB-Pep with Single Amino Acid Substitutions

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>% of Attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB31R</td>
<td>T-K-P-L-R-A-F-T-V-C-L-H</td>
<td>96</td>
</tr>
<tr>
<td>CB32L</td>
<td>T-K-P-L-K-L-F-T-V-C-L-H</td>
<td>82</td>
</tr>
<tr>
<td>CB34S</td>
<td>T-K-P-L-K-A-F-R-V-C-L-H</td>
<td>16</td>
</tr>
<tr>
<td>CB35L</td>
<td>T-K-P-L-K-A-F-T-L-C-L-H</td>
<td>81</td>
</tr>
<tr>
<td>CB37I</td>
<td>T-K-P-L-K-A-F-T-L-C-I-H</td>
<td>100</td>
</tr>
<tr>
<td>CB38K</td>
<td>T-K-P-L-K-A-F-T-L-C-L-K</td>
<td>83</td>
</tr>
</tbody>
</table>

*The peptides were coated at 10 nmoles/well. Fibroblasts were added at $10^5$ cells/well and allowed to adhere for 30 min. The percentage of the cell attachment vs. attachment to 10 nmoles/well of CB-Pep was measured for each peptide. Results are mean values of three experiments.

*Peptides are designated by the substituted residue which is underlined and in bold.
FIGURE 11. Direct cell attachment to peptides containing a single conservative substitution at each of several different residues within the CB-Pep. The substituted amino acid is indicated by the number of the residue. Fibroblasts (10^5/well) were added to wells coated with peptide. The percentage of the cell attachment for each peptide vs. attachment to 0.5 pmole of Fn is indicated. Results are mean values of three experiments.
Figure 11

- △ - CB30I
- ○ - CB32L
- □ - CB33Y
- ◇ - CB34S
- ▲ - CB35L

% of Attachment to Fn

nmoles/well of Peptide

0.08 0.16 0.31 0.625 1.25 2.5 5 10 20
Phe at position 33 (Table 5 and Fig. 12). The inability of peptide CB33Y to inhibit cell attachment confirms that the Phe at 33 is critical for cell attachment. Titration of the substituted peptides in the attachment inhibition assay showed a dose-response effect (Fig. 12). The peptides with conservative substitutions for residues 34 through 37 inhibited cell attachment as well as the CB-Pep itself (Table 5). The results from both the direct attachment and attachment inhibition experiments with substituted peptides reveal that Phe and Thr at residues 33 and 34 are critical residues within the CB-Pep.

7. Cell Attachment to CB33-37 and CB-Pep. Cell Attachment to the truncated peptides indicated that the minimal sequence required for the cell attachment activity of the CB-Pep is F-T-V-C-L (residues 33-37). To confirm this prediction, the peptide itself was synthesized and compared to the CB-Pep in direct cell attachment assays. The pentapeptide (CB33-37) supported cell attachment over a range of concentrations similar to that of the CB-Pep (Fig. 13) although cell attachment to the CB33-37 was considerably less efficient than the cell attachment to CB-Pep. The limited solubility of peptide CB33-37 precluded its use in attachment inhibition experiments.
Table 5

Inhibition of Cell Attachment to CB-Pep by Synthetic Peptides With Single Amino Acid Substitutions* 

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>% of Attachment to CB-Pep</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB31R</td>
<td>T-K-P-L-R-A-F-T-V-C-L-H</td>
<td>5</td>
</tr>
<tr>
<td>CB32L</td>
<td>T-K-P-L-L-F-T-V-C-L-H</td>
<td>23</td>
</tr>
<tr>
<td>CB34S</td>
<td>T-K-P-L-K-A-F-R-V-C-L-H</td>
<td>8</td>
</tr>
<tr>
<td>CB35L</td>
<td>T-K-P-L-K-A-F-T-L-C-L-H</td>
<td>15</td>
</tr>
<tr>
<td>CB37I</td>
<td>T-K-P-L-K-A-F-T-L-C-I-H</td>
<td>0</td>
</tr>
<tr>
<td>CB38K</td>
<td>T-K-P-L-K-A-F-T-L-C-L-K</td>
<td>45</td>
</tr>
</tbody>
</table>

*Soluble peptides with single residue substitutions at 1000 nmoles/ml were added along with 10⁵ fibroblasts to wells coated with 10 nmoles/well of the CB-Pep. Peptides are designated by the substituted residue which is underlined and in bold.

b The percentage of the cell attachment to 10 nmoles of CB-Pep is indicated for each of the competing peptides. Results are mean values from three experiments.
FIGURE 12. Inhibition of cell attachment to CB-Pep by peptides with substituted residues. Serial two-fold dilutions of soluble peptides were added along with $10^5$ fibroblasts to wells coated with 10 nmoles of CB-Pep. The percentage of cell attachment to 10 nmoles of CB-Pep is shown.
Figure 12

% Attachment

nmoles/ml of Free Peptide

- ▲ - CB-Pep
- ● - CB32L
- ● - CB33Y
- ■ - CB38K
FIGURE 13. Direct cell attachment to peptide CB33-37 and the CB-Pep. Serial two fold dilutions of the peptides were coated onto plates and fibroblasts were added at $10^5$ cells/well. The percentage of cell attachment vs. cell attachment to 0.5 pmoles of Fn was measured. Results are mean values of 3 experiments.
Figure 13

% Cell Attachment to Fn

nmoles/well

CB-Pep

CB33-37
D. Discussion

Blood levels of the acute phase reactant CRP become greatly elevated during the first 24-48 h as part of the systemic inflammatory response to acute microbial infections, major trauma, vasculitis, and connective tissue diseases, reaching levels up to 1.0 mg/ml (2). Thereafter, CRP levels rapidly decline and the protein is deposited at inflamed sites (22,66,77,169) where the CRP is presumably degraded by neutrophils based on observations of the in vitro degradation of CRP by PMNs (122,126,127,170). The CRP-derived peptides generated by PMNs elicit a monocyte chemotactic response, as well as cytokine and superoxide production (22,30,31). Membrane receptors from human monocytic cell lines for both monomers of CRP and CRP-complexes that mediate binding, phagocytosis, and activation have been partially characterized (137,141); however, the site(s) on CRP recognized by monocytes or neutrophils have not been defined. During the course of studies of the ligand specificity of the CRP-R we found that one of several synthetic peptides tested supported cell attachment in vitro (151). The active peptide is composed of residues 27-38 and is therefore found in each of the 5 identical subunits of the intact pentameric protein. A peptide composed of residues 27-38 from the homologous human pentraxin SAP was reported by Dhawan et al. (150) to mediate cell attachment.
The cell binding activity of the CB-Pep of CRP was not inhibited by the cell adhesive peptides of the extracellular matrix proteins of Fn and laminin, i.e. the RGDS-bearing peptides of Fn and the YIGSR-bearing peptides of laminin (151). Attachment to the CRP-peptide does not occur via integrin receptors since the post-attachment cell "spreading" is not observed indicating that cell attachment to the CB-Pep does not involve the cytoskeleton (174). In addition, the cell binding to the CRP-derived peptide appears to involve recognition of a unique sequence motif since the 12 residue sequence of the CB-Pep was not found in a search of proteins in the sequence data banks at the level of >55% homology. The CRP cell attachment sequence is not similar to any of the known adhesive recognition sequences (175). Therefore, identification of the critical residues and the minimal recognition sequence within the 12 residue CB-Pep of CRP may allow us to eventually define a potentially novel receptor for selective cell adhesion at inflamed sites.

Since CRP is in general analogous to Fn in that both blood proteins are acute phase reactants and found at sites of tissue damage, we compared the efficiency of cell attachment of both the CB-Pep and intact CRP vs Fn. Although Fn mediates cell attachment at least $10^4$ times more efficiently than CRP on a molar basis, both the CB-Pep and intact CRP supported similar levels of cell attachment.
Since the peptide blocked the activity of the intact CRP, the peptide residues are probably accessible on the intact, immobilized form of CRP. However, CRP in solution failed to block cell attachment to the CB-Pep, clearly suggesting distinct conformations for CRP in the fluid phase vs. the immobilized form. Furthermore, anti-CB-Pep antibodies bind to the intact CRP when it is bound to a substrate on a surface, but not when it is in solution (Mullenix & Mortensen, unpublished). These observations are consistent with those of others documenting the appearance of new epitopes on CRP upon binding to a solid phase (176).

Substrate adsorption artifacts with the CB-Pep were not a major consideration since soluble peptide also actively inhibited attachment and because similar levels of cell adhesion to a carrier (BSA)-CB-Pep conjugate were observed previously (151,174). Cell attachment to CRP deposited at sites of tissue damage might be substantial even if the process is much less efficient than with extracellular matrix proteins like Fn since CRP often attains uM levels in the blood (122,151) and therefore nmole amounts of CRP could easily accumulate at damaged sites (2,66,126). A recent study of the fate of radiolabeled CRP in patients with mild inflammatory conditions failed to detect CRP deposition at inflamed sites, but did not exclude CRP localization to sites of tissue necrosis (177). Nonetheless, if CRP deposits are degraded in vivo in a manner consistent with
the neutrophil-mediated proteolysis documented in vitro (122, 126, 127), then the active peptides generated may mediate cell adhesion at or near the site. Despite the in vitro studies of CRP degradation by neutrophils into biologically active peptides, recovery of a cell-attachment peptide from such a digestion has not been achieved (126, 127). However, the CRP cell attachment peptide could be generated since neutrophil elastase and tryptic cleavage sites exist at residues 25 and 31, respectively. Such cleavage would result in peptides of sufficient length to contain the critical residues for cell attachment.

Our approach of using modified synthetic peptides to identify both the critical residues and minimal length of the CRP cell-attachment peptide was the same as that originally used to characterize the major cell recognition sequence of Fn (178). Experiments using both direct cell attachment to immobilized peptides and inhibition of cell attachment by soluble peptides were conducted to minimize the structural constraints imposed by a "solid-phase" presentation of the peptide. The CRP CB-Pep in solution lacks definitive secondary structure measurable by circular dichroism analysis (Mullenix, et al., unpublished); however, there is no evidence that any specific conformation is required for cell attachment activity. Definitive three-dimensional structural information on CRP is not yet available (158), and therefore it is not possible to
determine whether the active sequence is on the surface of CRP or becomes available with a conformational change induced by either binding to a matrix (68,69,176) or physiological levels of Ca$^{2+}$ (84,85). Results from both experimental approaches indicated that the minimal sequence required for cell adhesion is: (r33) **Phe-Thr-Val-Cys-Leu** (r37). The experiments with substituted peptides clearly indicated that the hydrophobic, nonpolar Phe at position 33 and the adjacent polar Thr residue at 34 are the critical residues within the peptide. The conservative substitutions for the small hydrophobic residues at 35 (Ile for Leu) and at 37 (Ile for Val) did not influence activity. Although the Cys at residue 36 of the CB-Pep allows homodimer formation, the reduced peptide supported the same level of cell attachment indicating that cleavage within the CRP subunit is not necessary for activity and is consistent with the cell binding activity observed with the intact pentraxin.

Since the homologous SAP peptide composed of residues 27 through 38 possesses similar cell-binding activity, the critical residues within the minimal sequence would be expected to be conserved. A comparison of the relevant sequence below shows that the only significant difference is the presence of the large, hydrophobic Phe at position 37 of
SAP vs. the smaller, hydrophobic Leu in the CRP sequence:

SAP (33-37): F-T-L-C-F

CRP (33-37): F-T-V-C-L

It is noteworthy that the C-terminal half of the SAP dodecapeptide was found to be the active portion of the peptide in cell attachment assays, suggesting that this function may be conserved among the pentraxin family members (56,150). The SAP peptide has recently been shown to bind heparin (179); however, the CRP CB-Pep failed to bind heparin in preliminary studies, although the peptide may interact with other proteoglycans that are widely distributed on cell surfaces (180). The receptor interacting with the CB-Pep may be similar or identical to the CRP-R on both monocytes and neutrophils already characterized by several investigators (137,141); however, the only basis for proposing that the CRP-R and the cell adhesion receptor for CB-Pep are similar is that CB-Pep itself partially inhibits specific ligand (CRP) binding to human monocytic and granulocytic cell lines (181). The potential for cellular interactions with sites containing CRP and SAP deposits are much greater than heretofore appreciated and suggest a potential role for CRP in inflammatory responses and eventual wound healing.
SUMMARY

Despite the numerous biological activities ascribed to CRP, its exact physiological role in the response to tissue injury and as a part of the host's inflammatory response is not known. The magnitude of the increase in serum levels of CRP during inflammatory responses in most mammals and CRP's conservation throughout vertebrate evolution suggest an important physiological role. One approach to this difficult problem is to define and identify regions in the CRP sequence that are responsible for the various biological activities of CRP. Once identified, these sites can be targeted in experiments designed to identify the loss of specific biological activities of CRP. These experiments would allow investigators to approach the goal of identifying CRP's specific role in the inflammatory response.

A few of the biological activities associated with CRP include complement activation, opsonization, platelet activation, and modulation of leukocyte responses. All of these activities are dependent upon CRP's ability to bind specific ligands through its PC-binding region. Because CRP must undergo a Ca\(^{2+}\)-induced conformational change in order to bind to PC, all of its biological activities are also
dependent upon the molecule's ability to bind Ca\textsuperscript{2+}. CRP was previously shown to contain two equal affinity (K\textsubscript{d} = 6 \times 10^{-5} M) Ca\textsuperscript{2+}-binding sites per subunit. Residues 134-148 of CRP were proposed to contain one of the two Ca\textsuperscript{2+}-binding regions on the basis of its conserved sequence among all the pentraxins and proteolytic digest experiments that show that cleavage at a residue in this sequence in intact CRP result in markedly decreased Ca\textsuperscript{2+}-binding.

Autoradiography with \textsuperscript{45}Ca\textsuperscript{2+} detected the presence of a second Ca\textsuperscript{2+}-binding site in a proteolytic cleavage fragment that corresponds to the C-terminal 60 amino acids of the CRP sequence. The most likely calcium binding sequence in the C-terminal 60 amino acids of CRP occurs between residues 152-176 since it possesses a high concentration of acidic residues and sequence similarities to Ca\textsuperscript{2+}-binding sites in other proteins.

Direct evidence for the location of calcium binding sites in CRP was provided by \textsuperscript{43}Ca\textsuperscript{2+}-binding studies using equilibrium dialysis. The experiments demonstrated saturable Ca\textsuperscript{2+} binding by synthetic peptides corresponding to residues 134-148 and 152-176. Both peptides bound a single Ca\textsuperscript{2+} ion with dissociation constants (K\textsubscript{d}) on the order of 10^{-4} M. In addition, mAb and polyclonal Ab binding to both calcium binding regions was shown to alter epitope expression within the PC binding region of intact CRP. Circular dichroism spectrophotometry also detected a
conformational change in peptide 152-176 induced by Ca$^{2+}$. The results indicate that both Ca$^{2+}$-binding regions in CRP are involved in the Ca$^{2+}$-induced conformational change in CRP that allows access to the PC-binding site. We suggest that a mechanism similar to that of EF-hand Ca$^{2+}$-binding proteins exists in CRP in which localized Ca$^{2+}$-induced conformational changes within the calcium binding sites themselves are propagated to adjacent helical regions resulting in an overall change in the conformation of the intact molecule.

The identification of a region in the CRP sequence capable of supporting attachment of various adhesive cell types suggested a unique role in inflammation for CRP that could eventually contribute to wound healing. The cell adhesion sequence occurs between residues 27-38 of the CRP sequence and is distinctly different from the cell adhesion sequences in the extracellular matrix proteins fibronectin and laminin. To determine the minimal sequence and critical residues within the cell adhesion sequence in CRP, a similar approach to that taken to determine the cell attachment sequences of the extracellular matrix proteins fibronectin and laminin was used. This approach involved synthesizing N-terminally and C-terminally truncated peptides and peptides containing single conservative amino acid substitutions for use in direct cell attachment experiments. The truncated peptides identified the minimal sequence for
cell attachment to be: \( (r33) \text{Phe-Thr-Val-Cys-Leu-37} (r37) \). The substituted peptides identified the Phe at residue 33 and Thr at residue 34 to be critical residues for cell attachment activity. The conservative nature of our amino acid substitutions may have prevented the identification of additional critical residues. Non-conservative substitutions involving changes in charge, hydrophobicity and size were not made because in our judgement, any change in the peptide's activity after a non-conservative substitution would be less significant than a loss in the peptide's activity after a conservative substitution.

Both of the investigations involved the identification of sequences within CRP that mediate specific biological activities. The studies identify regions within CRP to be targeted in future experiments designed to identify an underlying role for CRP in inflammatory responses.
REFERENCES


140. Zeller, J., B. Kubak, and H. Gewurz. 1989. binding sites for C-reactive protein on human monocytes are distinct from IgG Fc receptors. Immunology 67:51.


Human C-Reactive Protein

PCA-Thr-Asp-Met-Ser-Arg-Lys-Ala-Phe-Val-Phe-Pro-Lys-Glu-Ser
1 5 10 15
Asp-Thr-Ser-Tyr-Val-Ser-Leu-Lys-Ala-Pro-Leu-Thr-Lys-Pro-Leu
16 20 25 30
Lys-Ala-Phe-Thr-Val-Cys-Leu-His-Phe-Tyr-Thr-Glu-Leu-Ser-Ser
31 35 40 45
Thr-Arg-Gly-Tyr-Ser-Ile-Phe-Ser-Tyr-Ala-Thr-Lys-Arg-Gln-Asp
46 50 55 60
Asn-Glu-Ile-Leu-Ile-Phe-Trp-Ser-Lys-Asp-Ile-Gly-Tyr-Ser-Phe
61 65 70 75
Thr-Val-Gly-Gly-Ser-Ile-Leu-Phe-Glu-Val-Pro-Glu-Val-Thr
76 80 85 90
Val-Ala-Pro-Val-His-Ile-Cys-Thr-Ser-Trp-Glu-Ser-Ala-Ser-Gly
91 95 100 105
Ile-Val-Glu-Phe-Trp-Val-Asp-Gly-Lys-Pro-Arg-Val-Arg-Lys-Ser
106 110 115 120
Leu-Lys-Gly-Tyr-Thr-Val-Gly-Ala-Glu-Ala-Ser-Ile-Ile-Leu
121 125 130 135
Gly-Gln-Glu-Gln-Asp-Ser-Phe-Gly-Gly-Asn-Phe-Glu-Gly-Ser-Gln
136 140 145 150
151 155 160 165
Leu-Ser-Pro-Asp-Glu-Ile-Asn-Thr-Ile-Tyr-Leu-Gly-Gly-Pro-Phe
166 170 175 180
Ser-Pro-Asn-Leu-Asn-Trp-Arg-Ala-Leu-Lys-Tyr-Glu-Val-Gln
181 185 190 195
Gly-Glu-Val-Phe-Thr-Lys-Pro-Gln-Leu-Trp-Pro-OH
196 200 205

Figure 14. Human CRP Amino Acid Sequence