Definition of a C-reactive Protein Binding Determinant on Histones*

(Received for publication, September 14, 1990)

Terry W. Du Clos†, Lorna T. Zlock, and Lorraine Marnell‡

From the Veterans Administration Medical Center and the University of New Mexico, Albuquerque, New Mexico 87108

C-reactive protein (CRP) is an acute phase inflammatory protein in man which binds to phosphocholine, chromatin, histones, and the 70-kDa protein of the U1 small nuclear ribonucleoprotein particle in a calcium-dependent, phosphocholine-inhibitable manner. CRP also binds to other proteins including fibronectin. The determinants involved in CRP binding to these diverse proteins have not been identified. The binding of CRP to histones was examined as these proteins are available in large quantity at high purity and subject to protease digestion with well characterized products. Histone H1 was digested with thrombin and trypsin to produce three distinct fragments, N-terminal, central globular, and C-terminal. CRP was shown only to bind to the C-terminal fragment. Binding to histone H2A was also examined. CRP binding was not diminished by cleavage of the C-terminal fragment but was greatly decreased when the central globular region of H2A was tested. Peptides were prepared to be identical to the N- and C-terminal fragments of H2A. The N-terminal (15 amino acid) fragment of H2A blocked CRP-induced precipitation of phosphocholine-coupled bovine serum albumin and histone H2A, whereas the C-terminal fragment showed no inhibition. Thus we have defined the first reported CRP binding determinant on a protein.

C-reactive protein (CRP)† is an acute phase serum protein in man which has been shown to possess many properties in common with immunoglobulin including complement activation (Kaplan and Volanakis, 1974; Siegel et al., 1974), enhancement of phagocytosis (Mortensen et al., 1976; Nakayama et al., 1982), and protection from infection (Mold et al., 1981a). It has been proposed that CRP may play an important role in the recognition and clearance of nuclear material from damaged cells. CRP was originally identified by its ability to bind to pneumococcal C-polysaccharide in a calcium-dependent manner (Tillett and Francis, 1930). This binding was determined to occur through the phosphocholine (PC) moiety of pneumococcal polysaccharide (Volanakis and Kaplan, 1971). CRP exhibits binding to polycationic molecules as well (DiCamelli et al., 1980). Unlike the binding to the C-polysaccharide this reaction is not inhibited by PC and does not require calcium (Potempa et al., 1981). More recently, CRP has been demonstrated to bind to other substrates including chromatin (Du Clos et al., 1988; Robey et al., 1984), histones (Du Clos et al., 1988), and small nuclear ribonucleoproteins (Du Clos, 1989). This binding is specific for the protein moiety and not the nucleic acid. CRP binding to fibronectin (Salonen et al., 1984) and laminin (Swanson et al., 1989) has also been reported, although the determinants involved in the binding to these proteins have not been determined. It is unknown whether a conserved sequence is recognized in all these proteins or whether a specific charge density is involved. In each case, the binding of CRP to these proteins is inhibitable by free PC and is calcium-dependent, indicating binding to the PC-binding region on CRP rather than the polycation-binding region.

In a previous publication, we examined the interactions of CRP with histones and chromatin and determined that CRP binding to chromatin is dependent on the presence of histone H1 (Du Clos et al., 1988). We also found that CRP bound to purified histones H2A and H2B. We now report that binding to H1 occurs through the C-terminal region of H1, the site which is responsible for condensation of chromatin (Allan et al., 1966; Moran et al., 1985). Furthermore, we localize the binding determinant on histone H2A to the N-terminal 15 amino acids. This represents the first definition of a CRP binding determinant on a protein. The significance of this finding in relation to the postulated ability of CRP to mediate chromatin unfolding is discussed.

EXPERIMENTAL PROCEDURES

Purification of CRP—CRP was isolated from human pleural fluid and plasmapheresis samples. Plasma was first processed for isolation of Factor XII (Miletich et al., 1981) and then by affinity chromatography and gel filtration exactly as described previously (Mold et al., 1981b). Purity of CRP preparations was determined both by silver staining of 15% SDS-PAGE gels and by fast protein liquid chromatography/ion exchange chromatography on a Mono Q column (Pharmacia LKB Biotechnology Inc.) with a 0.15-0.5 M NaCl gradient in 20 mM Tris, pH 7.8. CRP elutes as a single distinct peak off the Mono Q column and migrates as a single band on SDS-PAGE, distinct from serum amyloid P component and IgG light chain controls. All preparations were negative when tested for serum amyloid P component by Ouchterlony.

Radiolabeling—CRP was radiolabeled with Na⁹⁹ᵐT by the solid state lactoperoxidase method as previously described using lactoperoxidase-Sepharose (Mold et al., 1981b) (Worthington). Radiolabeled CRP was re-isolated by affinity chromatography from PC-Sepharose.

* This work was supported by the Veterans Administration and in part by Grant BRSG S07 RR-05583-24 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence and reprint requests should be addressed: VA Medical Ctr., 2100 Ridgecrest Dr. S.E., Albuquerque, NM 87108. Tel.: 505-265-1711 (Ext. 4411).

‡ Supported by NIH Training Grant 5T32AR07-173.

1 The abbreviations used are: CRP, C-reactive protein; CR2A, the digestion product of H2A produced by the endogenous nuclease of chromatin; PC, phosphocholine; G-H1, the globular region of H1; NG-H1, the N-terminal thrombin digestion fragment of H1; C-H1, the C-terminal thrombin digestion fragment of H1; PC-BSA, phosphocholine-coupled bovine serum albumin; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

3 T. W. Du Clos, L. T. Zlock, and L. Marnell, unpublished data.
to ensure high retention of PC binding activity following radiolabeling.

**Preparation of Chromatin and cH2A**—Chromatin was prepared from calf thymus (Pel-Freeze) according to the method of Hoffman and Chalkley (1978). To prepare cH2A, chromatin was incubated according to the general method of Eckbush et al. (1978). Specifically, chromatin (0.5 M NaCl, 0.02 M Tris, pH 8.0, 1 mm EDTA, for 30 min at 37° C. Histones were then extracted with 0.4 N H4SO4 and the DNA removed by centrifugation at 10,000 × g for 15 min. Histones in the supernatant were precipitated with 20% trichloroacetic acid and collected by centrifugation at 3,000 × g. The pellets were washed once with acetic acid (200 ml of acetate, 0.1 ml of concentrated HCl). 3 times in acetone and then lyophilized.

**Preparation of H2A—**H2A was a generous gift of Dr. Joe Gatewood, Division of Life Sciences, Los Alamos National Laboratory Los Alamos, NM. It was purified from HeLa cells by chromatography on a C18 reverse phase column (Waters, Milford, MA) with a gradient of 25-65% acetone in H2O.

**Isolation of Histone H1—**H1 was isolated from calf thymus (Pel-Freeze) according to the method of Johns (1964). Minced calf thymus was homogenized in 0.9% NaCl, 0.05 M NaHSO4, pH 6.0, in a Waring blender at top speed for 2 min. The homogenate was centrifuged at 110,000 × g for 45 min and the pellet was resuspended in 0.05 M NaPO4, pH 6.8. They were washed with PBS-Tween and then blocked with 1 mg/ml of BSA for 1 h at 4° C. The blots were incubated with peroxidase-conjugated sheep anti-human CRP (Organon Teknika-Cappel, West Chester, PA). Plates were washed again and then were developed with 0.005% H2O2 and 1 mg/ml 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) in Methylvaine's citrate phosphate buffer, pH 4.6. The plates were read on a Bio-Rad model 3550 microplate reader at 415 nm.

**Dimethylenethylenediamine for Coating Efficiency of ELISA Plates—**Protein binding to microtiter plates coated with H1 or H1 digestion fragments as above was measured using an assay developed for this purpose. Coated plates were washed 3 times with 0.05% Tween 20 in PBS, pH 7.4, and then incubated 10 min with 0.00125% trinitrobenzenesulfonyl acid (Sigma) in 0.05 M borate buffer, pH 9.0, 0.1 M NaCl. They were washed again and then were incubated with 0.005% H2O2 and 1 mg/ml 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) in Methylvaine's citrate phosphate buffer, pH 4.6. The plates were then incubated with rabbit anti-dinitrophenyl-BSA (ICN Biomedicals, Lisle, IL) for 1.5 h. After washing, the plates were incubated with peroxidase-conjugated goat anti-rabbit IgG (Tago, Burlingame, CA), developed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and H2O2, and read on the microplate reader at 415 nm.

**RESULTS**

CRP binds to histone H1 in a calcium-dependent, PC-inhibitable manner (Du Clos et al., 1988). To localize the binding site for CRP on H1, digestion fragments of H1 were prepared. These fragments have been well characterized with respect to sequence and electrophoretic mobility on SDS-PAGE (Chapman et al., 1976; Hartman et al., 1977) (Fig. 1). H1 is composed of three domains: a small, unstructured, already well characterized (Fig. 1) designated NG-H1 and C-H1 (Hardin and Thomas, 1983). Microtiter wells were coated with C-H1, NG-H1, or C-H1, and CRP binding was measured by an ELISA.

The efficiency of protein binding to the microtiter wells was assessed as described under "Experimental Procedures." In all cases the efficiency of coating with the different fragments was similar. As shown in Fig. 2, CRP binding to G-H1 and NG-H1 was far less than the binding of CRP to C-H1. CRP
C-reactive Protein Binding Determinant

C-reactive Protein Binding Determinant

binding to C-H1 was similar to binding to H1 (data not shown). Binding to C-H1, like binding to the intact H1, was eliminated in the presence of 10^{-4} M PC (data not shown). Therefore the determinants recognized by CRP appeared to reside in the C-terminal region of H1.

CRP binding to C-H1 was also tested in a blotting assay. The digestion products obtained by hydrolysis of H1 with thrombin were separated by 15% SDS-PAGE and transferred to PVDF membranes. As shown in Fig. 3, CRP bound to undigested H1 and to C-H1. H1 normally runs as a doublet as does C-H1. The C-H1 band consistently appears as a diffuse doublet, possibly due to the high charge of the C terminus reflecting its high lysine content. No binding of NG-H1 to PVDF was detected by Amido Black staining so CRP binding could not be adequately measured by blotting. However we showed above (Fig. 2) in the ELISA format that CRP did not bind NG-H1.

C-H1 is composed of a repetitive sequence composed primarily of lysine, proline, and alanine. A peptide (KA-PK) was prepared from this region which was identical to amino acids 197–206 of bovine H1.1 and which has a similar sequence to much of the C terminus of H1 (Wu et al., 1984). This peptide was then tested for its ability to inhibit the precipitation of PC-BSA by CRP. KA-PK failed to produce inhibition at concentrations as high as 10^{-4} M (Table I) which suggested that binding to C-H1 was probably not simply due to high lysine content and that this sequence of C-H1 is not the binding site.

CRP binding to H2A by blotting and by ELISA was equivalent to or greater than its binding to H1 (Du Clos et al., 1988). If, as in the case of H1, CRP were to bind to the charged tail region of histone H2A, it would be easier to determine the sequence involved, as H2A has much shorter nonglobular regions than H1. When chromatin is incubated in the presence of high salt, the C-terminal region of H2A is cleaved off by an endogenous H2A-specific protease between amino acids 114 and 115 (Eickbush et al., 1976). CRP binding to the major 114-amino acid product, known as cH2A, was tested in a blotting format. As seen in Fig. 4, CRP binding to cH2A was approximately equivalent to the intact H2A indicating that the C-terminal region is not required for binding. We had previously determined that CRP binding to the

**Table I**

Inhibition of CRP-induced precipitation of PC-BSA and H2A by synthetic histone peptides

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ligand precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC-BSA</td>
</tr>
<tr>
<td>PC</td>
<td>2.0 × 10^{-5} M</td>
</tr>
<tr>
<td>SG-AR</td>
<td>3.3 × 10^{-4} M</td>
</tr>
<tr>
<td>SG-AK</td>
<td>4.5 × 10^{-4} M</td>
</tr>
<tr>
<td>GQ-AK</td>
<td>5.0 × 10^{-4} M</td>
</tr>
<tr>
<td>KT-GK</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

*Concentration required for 50% inhibition of precipitation.

<ref>Fig. 3. CRP binding to NG-H1 versus C-H1 by blotting. A, 15% SDS-PAGE analysis of samples used in the blotting experiment, stained with Coomassie Blue. Lane 1, C-H1-containing fraction of CM-cellulose of thrombin-digested H1. Lane 2, purified H1. B, ^125I-CRP binding to: lane 1, purified H1; lane 2, C-H1 fraction of thrombin-digested H1, separated by 15% SDS-PAGE, and transferred to PVDF.

<ref>Fig. 4. CRP binding to cH2A by blotting. A, Amido Black stain of histones. Lane 1, histones from untreated chromatin. Lane 2, chromatin treated to produce cH2A. B, ^125I-CRP binding in the presence of 2 mM CaCl2 to: lane 1, untreated chromatin; lane 2, cH2A-containing chromatin. ^125I-CRP binding in the presence of 10^{-4} M PC to: lane 3, untreated chromatin; lane 4, cH2A-containing chromatin.

<ref>Fig. 2. CRP binding to G-H1, C-H1, and NG-H1 by ELISA. Microtiter wells were coated with either G-H1, C-H1, or NG-H1 at 1 μg/ml and tested for CRP binding in the presence of 2 mM CaCl2 as described under “Experimental Procedures.” □, CRP binding to G-H1; ○, CRP binding to C-H1; △, CRP binding to NG-H1.

<ref>Fig. 1. H1 digestion fragments.
To compare the relative avidities of CRP for H2A and PC-BSA a quantitative precipitin curve was established. Increasing amounts of PC-BSA and H2A were added to a fixed amount of CRP and 125I-CRP. The amount of CRP precipitated was measured as the number of counts/min precipitated. As shown in Fig. 5, nearly equivalent precipitin curves were obtained for PC-BSA and H2A. Values are expressed in terms of nanomoles of PC and of H2A.

Peptides were then synthesized corresponding to the N- and C-terminal regions of H2A which are removed from H2A by trypsin digestion of chromatin (Bohm and Crane-Robinson, 1984). These peptides were tested for their ability to inhibit CRP-induced precipitation of PC-BSA. As shown in Table I, a 50% inhibition of CRP-induced precipitation of PC-BSA was produced by about 2 \times 10^{-6} \text{ M} PC. This agrees well with the figure obtained by others (Oliviera et al., 1980). The C-terminal H2A peptide KT-GK produced no inhibition at concentrations as high as 10^{-3} \text{ M}. On the other hand, the N-terminal peptide SG-AR produced 50% inhibition of CRP binding at about 3.3 \times 10^{-3} \text{ M} and over 90% inhibition at 10^{-2} \text{ M}. The longer N-terminal peptide, SG-AK, produced 50% inhibition of CRP binding at about 4.5 \times 10^{-4} \text{ M}. The peptide QG-AK, identical to SG-AK but lacking the N-terminal five amino acids, produced inhibition similar to SG-AR (5 \times 10^{-3} \text{ M} for 50% inhibition). Thus these peptides appear to be effective inhibitors of CRP binding to PC. This experiment was repeated with nearly identical results. In these experiments the peptides were 25-200-fold less potent inhibitors than PC, with the longer peptide about 10 times more effective than either of the shorter peptides.

Similiar results were obtained for the inhibition of CRP-induced precipitation of H2A. SA-AK produced approximately 25-fold less inhibition as compared to PC in both cases. However, the two shorter N-terminal peptides SG-AR and QG-AK were more effective inhibitors of CRP binding to H2A than to PC-BSA. Equal concentrations of the N-terminal peptides were required for inhibition of CRP-induced precipitation, about 5 \times 10^{-4} \text{ M} (Table I). Inhibition by PC was similar for PC-BSA (2 \times 10^{-6} \text{ M}) and H2A (1.5 \times 10^{-5} \text{ M}). Therefore, CRP binds to peptides of 11–15 amino acids derived from the N terminus of H2A thus defining a peptide binding site on histones.

Discussion
In a previous publication we demonstrated the specific binding of CRP to histones H2A, H2B, and H1 (Du Clos et al., 1988). Binding was inhibited by PC and by EDTA, indicating binding through the site on CRP which interacts with PC. The PC-binding site on CRP has been determined to require the presence of an amino group separated from a phosphate group by a specific distance (Oliviera et al., 1980). Because several proteins have been identified which bind CRP through this site it was of interest to determine how a protein or peptide sequence could fulfill the binding requirements.

Phosphorylation could be considered a strong possibility for CRP recognition. However, we have demonstrated a high affinity of the CRP-PC-binding site for a synthetic peptide which is unmodified so that phosphorylation is not necessary for binding. Furthermore, we found no significant influence of alkaline phosphatase treatment of histones and purified H1 on CRP binding as measured by blotting and ELISA.

We have shown in this paper that CRP binds to H1 within C-H1, the C-terminal thrombin digestion product. No significant binding was detected to either the globular region of H1 or the N terminus. Thus, the binding of CRP to this lysine-rich histone is not due to the fact that this is a lysine-rich protein which had been suggested by earlier studies. Rather, CRP binds to a specific domain of this protein. We attempted to determine the binding site within these 90 amino acids by using a synthesized peptide identical to the repetitive region of C-H1 composed primarily of lysine, proline, and alanine (KA-PK). However, CRP failed to bind to this peptide, indicating a lack of interaction with a short, lysine-rich peptide. Thus, CRP does not simply recognize a lysine-rich sequence but requires a relatively unique sequence for binding.

The interaction between CRP and the C-terminal region of H1 could be important in vivo. It has previously been demonstrated that CRP bound to chromatin (Robey et al., 1984), and we demonstrated that this binding requires the presence of H1 (Du Clos et al., 1988). It has been determined that the C-terminal domain of H1 is responsible for maintaining the compact structure of condensed chromatin perhaps by neutralizing the charge on the internucleosomal DNA or by binding to core histones on adjacent nucleosomes (Allan et al., 1986; Moran et al., 1985). CRP binding to H1 could potentially unfold the chromatin, allowing endogenous nucleases to degrade the DNA. In fact, CRP has been shown to increase the sensitivity of chromatin to nuclease digestion *in vitro* (Shephard et al., 1986).

An important question is how CRP gains access to nuclear material *in vivo*. CRP binding to damaged tissue at sites of inflammation or injury has been shown by immunofluorescence (Du Clos et al., 1981; Kushner and Kaplan, 1961). In addition, deposition in the nuclei of cells from synovial biopsies of patients with rheumatoid arthritis has been demonstrated (Gitlin et al., 1977). We have shown that CRP contains a nuclear localization sequence and when microinjected into cells localizes in the nucleus (Du Clos et al., 1990). It has been demonstrated that treatment of cells with membrane perturbing agents such as bradykinin, phospholipase A2, and lysocitrien leads to CRP binding in the nucleus (Robey et al., 1984). The conditions at sites of inflammation may cause similar cell membrane damage and should then allow CRP to enter the damaged cells and localize in the nucleus where it could bind to chromatin.

Due to the repetitive nature of C-H1 and the lack of smaller C-terminal cleavage fragments we were unable to further localize the binding site on C-H1 for CRP. However, because CRP binds to H2A, another relatively lysine-rich histone, we were able to study another potential peptide-binding site. The sequence of H2A has been determined and the enzymatic digestion patterns have been defined. When chromatin is treated with trypsin, five histone fragments, P1–P5, are pro-

**Fig. 5.** Quantitative precipitation of CRP by H2A and PC-BSA. Precipitation of 125I-CRP by H2A (■) and PC-BSA (○).
duced which lack 11–26 N-terminal amino acids (Bohm and Crane-Robinson, 1984). We previously tested CRP binding to P1–P6 by blotting (Du Clos et al., 1988). CRP binding to P2, the central globular region of H2A lacking both N- and C-terminal amino acids, was much lower than intact H2A. Therefore, CRP binding to H2A was significantly decreased when the charged N- and C-terminal regions were removed by trypsin treatment of chromatin.

We next examined the binding of CRP to ch2A, a cleavage product which lacks the C-terminal 11 amino acids of H2A. This modification did not appear to significantly decrease CRP binding as measured by blotting. Thus we reasoned that the N-terminal region was very likely to contain a binding epitope. This hypothesis was supported by the findings we obtained with synthetic peptides identical to the N- and C-terminal regions of H2A, SG-AK, and KT-GK, respectively. The C-terminal peptide, which had proved unnecessary for CRP binding to H2A, also failed to inhibit CRP binding in a precipitation assay. In contrast, the N-terminal peptide was an effective inhibitor of CRP binding to PC-BSA and H2A.

The degree of inhibition produced by the H2A N-terminal peptides SG-AR, SG-AK, and QG-AK was compared with that of PC. Nearly equal concentrations of PC were required to inhibit CRP binding to PC-BSA or H2A by 50%. However, only the longest peptide from the N-terminus of H2A, SG-AK, was as effective an inhibitor for PC-BSA as it was for H2A. The two shorter peptides, SG-AR and QG-AK, were less effective inhibitors for CRP-induced precipitation of PC-BSA as compared to H2A. Thus the peptide which most effectively mimics PC is SG-AK. Inhibition of CRP binding to H2A by the peptides as compared to PC required very similar concentrations. Thus the peptides appear to bind with similar affinity as does H2A to the CRP binding site. The affinity constant for CRP as measured by equilibrium dialysis is \( K = 0.19 \times 10^6 \text{M}^{-1} \) at 5°C. Using the present technique we are unable to determine a true association constant. However, we are able to determine a relative association constant for the peptides if we assume that the affinity constant for CRP for PC is 0.19 \( \times 10^6 \text{M}^{-1} \). The association constant for the peptides is 25–100-fold less or 0.76 \( \times 10^4 \) to 0.19 \( \times 10^4 \). These peptides are therefore likely to contain the same binding determinant as the native H2A molecule and the determinant is likely to be continuous in nature. This is the first description of a CRP binding determinant on a protein.

It is of interest to note that CRP fails to bind to H4 by blotting or ELISA (Du Clos et al., 1988). Since H4 and H2A share an identical N-terminal five amino acids, SGRGK, this determinant seems not to be sufficient for binding. The peptide QG-AK was significantly less effective than the complete 15-amino acid peptide SG-AK. It is therefore more likely that CRP binding occurs within the region of residues 6–15 of H2A but requires the complete sequence for effective binding. There are several similar but no identical sequences in the C terminus of H1.

The definition of a short peptide sequence to which CRP is capable of binding may be valuable in identifying additional epitopes on other proteins to which CRP binds, including fibronectin (Salonen et al., 1984) and the 70-kDa protein of the U1 small nuclear ribonucleoprotein (Du Clos, 1989). Comparison with other known CRP binding proteins and by alterations of this peptide should provide information concerning the essential features of CRP-binding proteins. Since many of the proteins to which CRP binds are targets of autoantibodies it may provide information concerning the nature of proteins which stimulate autoantibody formation.

**REFERENCES**


Tillett, W. S., and Francis, T., Jr. (1930) J. Exp. Med. 52, 561–571
