Specificity of the Binding Interaction between Human Serum Amyloid P-component and Immobilized Human C-reactive Protein*

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C-reactive protein (CRP) and serum amyloid P-component (SAP) are two members of a small group of conserved blood proteins termed pentraxins, proteins that possess a common structural feature of five identical non-covalently linked subunits arranged in a flat planar disc (1–3). CRP is the archetypical acute-phase reactant in most vertebrates, and its increased synthesis by hepatocytes in response to inflammatory cytokines may result in a several thousandfold increase in blood levels within 24 h after tissue damage or infection, whereas SAP levels increase by approximately 50% in humans and most other mammals (4). CRP and SAP share extensive amino acid sequence similarity (~70%) and possess the same gene organization, suggesting a common ancestral gene (5–7). CRP has 206 residues/subunit and is not glycosylated (5, 6), whereas SAP has 294 amino acids/subunit with 11% N-linked carbohydrate (7, 8). CRP circulates as a single pentraxin, while human SAP is composed of two pentraxins bound face-to-face (9).

The two pentraxins share several molecular and biochemical properties. They both bind free Ca2+ ions which induce a conformational change that allows binding to a variety of substrates (10–12). CRP and SAP bind to chromatin (13, 14), histones H1 and H2A (15), and fibronectin (16–18). Both CRP and SAP activate the classical complement cascade by interacting with the collagen-like stem region of C1q (19–21). The two proteins also have unique binding reactivities. SAP appears to bind to DNA itself (22), whereas CRP binds uniquely to the U1 small nuclear RNP (23). The defining characteristic for CRP from all species is its Ca2+-dependent binding to the phosphorylcholine (PC) moiety (1) on certain polysaccharides and phospholipids (24, 25). By contrast, SAP does not bind to PC, but does bind to agarose (26), zymosan (11), the glycosaminoglycans heparin and dermatan sulfate (27), a variety of mannosyl-rich glycoproteins (28), and to C4b-binding protein (16). SAP was originally named on the basis of its identity to the P-component that is present in all amyloid deposits (29) regardless of the composition of the amyloid fibrils (1). SAP binds to both isolated amyloid fibrils (30), as well as to amyloid in vivo (31). Despite investigations of the molecular and binding properties of these two proteins, a unique biological role for either pentraxin has yet to be established.

While investigating functional regions within CRP we observed that SAP binds selectively to immobilized, but not soluble CRP, in a Ca2+-dependent manner in buffers of physiological ionic strength (32). The purpose of the experiments described here was to characterize this binding interaction for its relative affinity and its specificity in terms of the recognition site on the immobilized CRP and the binding site on SAP for CRP. The interaction between these pentraxins may underlie events in inflammation leading to localization of these pentraxins to sites of tissue damage and subsequent repair.

EXPERIMENTAL PROCEDURES

Purification and Labeling of CRP and SAP—CRP was purified from human ascites fluids by Ca2+-dependent affinity chromatography on PC-phenyl-Sepharose (Pierce Chemical Co.) exactly as described elsewhere (32, 33). Human SAP was purified by affinity chromatography on agarose beads as described elsewhere (11, 32). Protein concentration was estimated using the reported extinction coefficients at 280 nm of 1.82 for SAP and 1.95 for CRP (11, 32). The concentration of SAP was also determined by rocket immunoelectrophoresis using a nonspecific rabbit anti-human SAP antibody (Ab) and a single sample of normal human (male) serum at 40 µg/ml of SAP. Biotin succinimidyl ester (N-hydroxy succinimidyl-Biotin, Pierce Chemical Co.) was added at 250 µg of biotin/mg of purified pentraxin protein in 0.1 M sodium borate buffer, pH 8.8, containing 5 mM EDTA and mixed gently for 4 h. The biotinylated (B) proteins were diazylated against Tris-buffered saline (TBS),
pH 7.6, containing 10 mM EDTA to remove excess biotin. The biotinylated SAP (B-SAP) migrated in SDS-PAGE as a single band of 31 kDa, corresponding to the 204-amino-acid subunit with 11% (w/w) carbohydrate (8). B-CRP migrated as a 25-kDa protein by SDS-PAGE and retained its PC binding activity.

B-SAP was also labeled externally with 125I by mixing 0.25 mCi of Na125I (Amersham Corp.) with 0.5 mg of protein in 0.5 ml of TBS plus 10 mM EDTA in a glass tube coated with Iodogen (Pierce Chemical Co.). The specific activity of the labeled SAP after resolation by agarse affinity chromatography was 0.3–0.4 μCi/μg protein. 125I-Labeled SAP was prepared as described previously and was reisolated by PC affinity chromatography (33).

CRP Synthetic Peptides—The following peptides were synthesized and correspond to the designated residues within the 204-amino-acid subunit of mature human CRP: Pep 134–148 (r134) ILQEQDFSGGN-PEG (r148); Pep 191–206 (r191) KYVEQVGGYKGLW (r206); Pep 24–39 (r25) RYGRGSFYYTKQKO (r39); Pep 13–15 (r15) QTDMSRRKAPVPP (r15); Pep 27–38 (r27) TPK1KAPTCC (r38); Pep 152–178 (r152) LYGIVGNWMDVFLSPDEINTIRL (r178).

The Pep 47–63 corresponds to the highly conserved PC-binding region of CRP (34) that by itself binds PC (35). The Pep 134–148 corresponds to a highly conserved region of both SAP and CRP (36) that contained specific PC-binding sites. Crude enzyme digestion of the peptide fractions corresponded to residues 1–146 and 147–206, respectively (36). The separated proteins were blotted onto a polyvinylidene difluoride membrane. The membranes were resolved by SDS-PAGE (15% gel) under reducing conditions to reveal 16- and 7-kDa fragments corresponding to residues 1–146 and 147–206, respectively (36). The separated proteins were blotted onto a polyvinylidene difluoride membrane for analysis of binding of B-SAP to the CRP fragments.

SAP Deglycosylation—B-SAP was deglycosylated with neuraminidase as described elsewhere (40) or recombinant peptide-N-glycosidase F (PNGaseF) (New England BioLabs) according to the supplier's specifications with some modifications. Briefly, 0.5 units of neuraminidase were added to 400 μg of SAP in 0.5 μl sodium acetate with 10 mM EDTA, pH 5.0, and incubated overnight at 37 °C (40). Prior to PNGaseF treatment, 20 μg of SAP was denatured by boiling for 10 min in the presence of 0.5% SDS and 1% 2-mercaptoethanol. SAP was deglycosylated with 15,000 units of PNGaseF in 1% Nonidet P-40 overnight at 37 °C. Deglycosylation was confirmed by a mobility shift of the SAP subunit on SDS-PAGE (15% gel) (8).

ELISA Measure of SAP Binding—CRP, SAP, and their ligands were coated overnight at 4 °C onto Immulon I1 plates (Dynatech, Inc.) using a carbonate-bicarbonate buffer, pH 9.6: CRP, 200 ng/well; SAP, 100 ng/well; PC-KLH, 50 ng/well; and agaran or zymosan at 1 μg/well. The plates were washed and blocked with 10 μg/ml bovine serum albumin in TBS containing 2 mM Ca2+ and 0.05% Tween-20. Human CRP (2 μg/ml) and equivalent amounts of other proteins were added to bind to their ligands for 1 h at 37 °C. B-SAP in TBS plus 1 μg Ca2+ at 2-fold dilutions over the range of 5 to 1000 ng/well (200 μl/well) was allowed to bind to immobilized CRP and other ligands for 1 h at 37 °C. The bound B-SAP was detected with horseradish peroxidase-streptavidin (1:2000 dilution) followed by the 3,3',5,5'-tetramethylbenzidine substrate for horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and reading the absorbance at 450 nm on an ELISA plate reader (Dynatech). Inhibition of SAP binding to CRP was performed by allowing 200 ng of B-SAP to bind to the immobilized CRP in the presence of CRP peptides, monosaccharides, disaccharides, or after reacting the CRP with region-specific Ab.

Western Blotting—Purified SAP, CRP, and Pronase-digested CRP were resolved by SDS-PAGE (15% gel) under reducing conditions and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 2% nonfat dry milk in TBS with 2 μg Ca2+ for 1 h followed by washing in TBS/Ca2+. The transferred proteins were incubated with B-CRP or B-SAP at 5 μg/ml in a TBS/Ca2+ buffer with 1% nonfat dry milk overnight. Binding of the biotinylated proteins was detected with streptavidin-horseradish peroxidase and the substrate 3,3',5,5'-tetramethylbenzidine.

Radioimmunoassay of Specific Binding—Human CRP, at 200 ng/well, was coated at 4 °C overnight in carbonate-bicarbonate buffer onto Immulon I1 Removewells. The amount of the immobilized CRP was determined using trace amounts of 125I-labeled CRP; approximately 40% of the 125I directly immobilized CRP was bound/well (0.64 pmol) and 35% of the 200 ng of the CRP (0.48 pmol) was bound to the PC-KLH. Wells were blocked and radiolabeled SAP was allowed to bind to 200 μl at 1 h at 37 °C. After washing, the bound SAP was measured by counting the entire well in a Beckman-3005 gamma counter. Specific binding of SAP was >90% of the total binding at 0.5 saturation based on the amount of bound 125I-SAP inhabitable by a 100-fold excess of unlabeled SAP.

Trypsin Digestion of B-SAP—Purified B-SAP was dialyzed into water, lyophilized, and denatured by incubation for 2 h in 8 μl urea, 100 mM EDTA, 100 mM NH,HCO,; 100 μM 2-phenylethyl chloromethyl ketone treated-trypsin-Sepharose beads were washed in 100 mM NH,HCO, and added at 100 μl of beads/200 μg of SAP in 0.4 ml and incubated for 18 h at 37 °C. The SAP was recovered by centrifugation, frozen at -20 °C for sequencing, or used immediately for binding. The digested SAP was analyzed by SDS-PAGE (12.5% gel) run under reducing conditions.

HPLC Analysis of Protease-generated Peptides from SAP—Analysis of the peptides generated from SAP was done by reverse-phase HPLC on a Waters model 600E chromatograph with a model 486 detector. Initial characterization of the peptides was done on a Regis Val-U Pak HP analytical C18 column using a 40-min gradient of 1% Buffer A (0.1% trifluoroacetic acid in water) to 100% Buffer B (100% acetonitrile with 0.1% trifluoroacetic acid). After purification, the peptide fractions were analyzed on a Waters Pico-Tag amino acid analyzer. The peptide fractions were sequenced by automated Edman degradation using an Applied Biosystems model 470A sequencer, a model 120A phenylthiohydantoin analyzer, and a model 900A data module.

RESULTS

Binding of Labeled SAP to Immobilized CRP—To obtain quantitative data on the specific binding interaction between SAP and immobilized CRP, purified human SAP was externally labeled with 125I and its binding to directly coated CRP or to CRP captured on microplate wells via binding to PC as PC-KLH was measured in buffers at physiological ionic strength. The use of an approach to reach Imnuched saturation when 1000 ng (4.3 pmol) of 125I-SAP was added to 80 ng (0.6 ml) of directly immobilized CRP, or a molar ratio of soluble SAP/immobilized CRP of approximately 7, based on a molecular mass of 230,000 for SAP and 125,000 for CRP (Fig. 1A). Scatchard analysis of the binding data yielded a Kd = 5 × 10^-9 M (5 nM) and a molar ratio of specifically bound SAP/CRP = 0.28 at saturation.
Fig. 1. Binding of $^{125}$I-labeled SAP to immobilized CRP. CRP was immobilized by either direct coating (A) or by capture on PC-KLH (B). Labeled SAP was allowed to bind for 60 min at 37 °C, and the amount of specifically bound SAP was measured. The binding curve data represent the average of four experiments with a S.E. of <20% for each value. Inset, Scatchard analysis of the binding data resulted in a calculated $K_D = 5 \times 10^{-8}$ M for CRP directly coated and a molar ratio of bound SAP/CRP of 0.28 (A). Scatchard plot of the data for SAP binding to CRP already bound to its major ligand PC (PC-KLH), resulted in a $K_D = 10^{-9}$ M and a molar ratio of SAP/CRP = 0.25 (B).

The binding of SAP to CRP captured via PC as PC-KLH was also assessed. The binding interaction of SAP to the bound CRP-PC-KLH also approached saturation (Fig. 1B) and had a $K_D = 10^{-8}$ M (10 nM), with a molar ratio of specifically bound SAP/immobilized CRP = 0.25 (Fig. 1B, inset). The binding curves, as well as the derived measures of affinity and the molar ratios of the bound reactants were not significantly different for the two forms of immobilized CRP. The binding interaction with the labeled SAP reached equilibrium by 20 min. The results demonstrate that the binding of SAP to immobilized CRP is saturable, rapid, and of relatively high affinity.

Since most of the known specific binding reactions of both SAP and CRP are dependent on the presence of physiological levels of Ca$^{2+}$ ions (1–2 mM), the Ca$^{2+}$ dependence of the binding of SAP to directly immobilized CRP was examined. SAP was not capable of binding to CRP in the presence of 10 mM EDTA and the addition of >20 mM CaCl$_2$ was required to completely restore the binding to CRP. Since SAP requires Ca$^{2+}$ ions for binding to ligands (11, 26, 41) and CRP undergoes a Ca$^{2+}$-mediated conformational change (10, 12, 37), it is not apparent whether one, or both, of the reactants requires Ca$^{2+}$ for the binding interaction.

SAP Binding to CRP in Solution—Although CRP does not bind to directly immobilized SAP (32), whether SAP binds to CRP when both reactants are in solution remained to be tested. Preliminary experiments showed that neither agarose nor zymosan in suspension at 1 µg/ml inhibited the binding of SAP to immobilized CRP. Therefore, any complexes formed between SAP and B-CRP in solution would be easily detected by using bound forms of the two SAP substrates: agarose (26) and zymosan (11), respectively. Various amounts of B-CRP were allowed to interact with a single concentration of SAP that displayed substantial binding to the two substrates. SAP did not bind to CRP (B-CRP) in solution since labeled complexes could not be detected with either of the SAP substrates (Fig. 2). CRP at even a 5-fold molar excess did not inhibit B-SAP binding to agarose or zymosan, nor did B-CRP bind to the SAP substrates (Fig. 2).

Effect of CRP Peptides on SAP Binding to Immobilized CRP—Direct binding of B-SAP to CRP synthetic peptides coated onto microplates indicated that binding was specific for the peptide corresponding to residues 134–148 of CRP (32), a region that participates in Ca$^{2+}$ binding (36). A more sensitive
A. Various amounts of B-CRP were tested for binding to SAP in solution (2.5 μg/ml) and then allowed to bind to agarose. B-SAP and B-CRP binding to the agarose substrate were included as controls.

B. Various amounts of B-CRP were allowed to react with SAP in solution (2.5 μg/ml) and then allowed to bind to the SAP substrate zymosan. A, B-CRP; •, B-SAP.

Identification of the SAP-binding Site on CRP—The region on CRP that serves as the recognition site for SAP was also explored using Ab specific for different surface epitopes of CRP. Purified mAb or polyclonal Ab to CRP were allowed to react with the immobilized CRP and the extent of inhibition of binding of B-SAP measured. Of the Ab surveyed, only mAb 3H12, which is specific for the COOH-terminal region of CRP (39), was capable of significant blocking (Fig. 4). At a 10-fold molar excess of added mAb 3H12 (3 μg/ml) to CRP, 80% of the SAP binding was inhibited (Fig. 4). Inhibition was not detected using Ab directed toward residues 134-148 of CRP (Ca2-binding region (EA4-1) (12), the cell-attachment peptide (CB02) (38), or to the NH₂ terminus (4B10) (39). Additional evidence showing the binding of SAP to the COOH-terminal region of CRP was obtained by probing a blot of two fragments of the CRP subunit generated by digestion with Pronase to yield 16- and 6.5-kDa fragments by cleavage between residues 146 and 147 (36). SAP bound to both the intact 25-kDa CRP subunit and the 6.5-kDa fragment corresponding to residues 147-206, but not to the 16-kDa fragment (Fig. 5). This result supports the hypothesis that the SAP-binding site is located within the COOH-terminal 60 residues of CRP.

Effect of Deglycosylation of SAP on Added Sugars on Binding to CRP—CRP displays weak lectin-like binding reactivity with either phosphate monoesters (42) or the sugars present on some glycoproteins (43). To test the possibility that CRP binds to the carbohydrate on SAP, sugar moieties that were identified as ligands for either SAP or CRP (43, 44) were tested as potential inhibitors of SAP binding to immobilized CRP. The disaccharides β-D-Gal-(1-3)-α-D-GalNAc and β-D-Gal-(1-4)-D-GlcNAc (N-acetyl-lactosamine) that inhibit the binding activity of immobilized CRP for IgG (43) failed to alter SAP binding to the CRP when added up to 10 mM. Man-1-PO₂ and Man-6-PO₄ were also tested because they inhibit SAP binding reactivity to zymosan and a variety of polysaccharides (44); however, neither of these monosaccharides mediated significant inhibition of
Ab were removed and 100 ng of B-SAP was allowed to bind. Bound SAP was detected by ELISA, and the percentage inhibition of binding was calculated on the basis of SAP bound in the absence of antibody. Data were from experiments. A, 3H12; O, EA4–1; V, CaC2; C, 4B10; □, CB02.

FIG. 4. Blocking of SAP binding to immobilized CRP by anti-CRP Ab. Purified mAb and polyclonal Ab to different surface regions of CRP were allowed to bind to 200 ng of immobilized CRP. The unbound Ab were removed and 100 ng of B-SAP was allowed to bind. Bound SAP was detected by ELISA, and the percentage inhibition of binding was calculated on the basis of SAP bound in the absence of antibody. Data are from experiments. A, 3H12; O, EA4–1; V, CaC2; C, 4B10; □, CB02.

FIG. 5. Binding of SAP to fragments of CRP. Purified CRP was partially digested with Pronase-Sepharose beads to yield 16- and 6.5-kDa fragments by selective cleavage between residues 146 and 147. Undigested CRP (lanes 1 and 2) and cleaved CRP (lanes 2 and 4) were separated by SDS-PAGE on a 15% gel run under reducing conditions and transferred to polyvinylidene difluoride membranes. The transferred protein was stained with Amido Black (lanes 1 and 2) or probed with 5 μg/ml of B-SAP (lanes 3 and 4).

SAP binding to CRP. The results suggest that the sugar moieties on SAP and the lectin-binding site of CRP are not involved in the SAP to CRP interaction.

CRP binding to serum glycoproteins was shown to be increased by removal of terminal sialic acids and abrogated by deglycosylation of the glycoproteins with PNGaseF (43). The role of the N-linked carbohydrate of SAP in the binding of SAP to CRP was tested by deglycosylating SAP with either neuraminidase or PNGaseF. The two forms of deglycosylated SAP, which displayed the predicted increases in mobility on SDS-PAGE (8), bound as efficiently to the immobilized CRP as did the unmodified SAP (Fig. 6). The two deglycosylated preparations of SAP had equivalent amounts of biotin when compared with unmodified SAP as determined by a direct ELISA. Therefore, the carbohydrate moieties present on SAP are not likely to participate in the binding interaction with CRP.

Identification of the CRP-binding Sequence on SAP—To localize the CRP-binding site within SAP, polypeptide fragments from SAP digested with trypsin were evaluated for their binding activity toward immobilized CRP. Trypsin digestion produced four large fragments of 16, 20, 29, and 4.5 kDa that were resolved by reversed-phase HPLC and recovered as B-polypeptides. One fragment with a relative molecular mass of 4.5 kDa displayed substantially more binding reactivity for immobilized CRP than the 16- and 20-kDa fragments when assayed at 2 μg/ml. This CRP-binding fragment was analyzed by SDS-PAGE and isoelectric focusing and found to be sufficiently homogeneous for sequencing. Amino acid analysis was performed on the isolated 4.5-kDa fragment and then it was sequenced. The NH2-terminal 11 residues were an exact match for residues 144–154 of the derived sequence for SAP (7) (Table 1). The entire fragment most likely spans residues 144–199 since Lys-199 is the next trypsin cleavage and biontylation site.

DISCUSSION

The experiments described here were done to quantitate and to characterize the specificity of the unidirectional binding interaction between the two human pentraxins SAP and CRP. Our initial report of this interaction showed that the CRP must be immobilized and occurred in a Ca2+-dependent manner even in the presence of serum (32). The new findings described herein show that binding of soluble SAP to two distinct forms of immobilized CRP is saturable, of relative high affinity with a Kd in the 5–10 nM range of SAP, and specific for residues within the COOH-terminal third of the 206-amino-acid CRP subunit. These properties taken together clearly distinguish the SAP to CRP interaction from the autoaggregation of SAP which is not saturated and readily prevented in the presence of carrier proteins, such as the 10 mg/ml of bovine serum albumin present in the binding studies described herein (41). Whether the directly immobilized CRP recognized by SAP is the equivalent of the...
conformation represented by neo-CRP is uncertain (45); nonetheless, the native CRP conformation “captured” by immobilized PC-KLH was still recognized by SAP suggesting that binding is not dependent on any single conformation of CRP. The calculated valence of 0.25–0.3 SAP molecules bound/CRP molecule suggests that SAP may bridge or cross-link several CRP molecules to form a matrix. Although the biological significance of this matrix formation remains to be elucidated, it is possible that the binding event itself may lead to the development of a nucleating site for the localization of both pentraxins to a single focus, especially during tissue repair (46–48).

The results clearly show that SAP recognizes a site within the COOH-terminal region of each of the CRP subunits on the basis of three different observations. The first is that SAP binding to CRP was selectively inhibited by a mAb (3H12) specific to an intact CRP molecule and there is considerable evidence that SAP can bind certain CRP peptides generated at inflamed sites with specificity for residues 199–206 in this region of CRP (49). The second is that binding event itself may lead to the development of a matrix. Although this notion is very speculative, the overall structure of SAP of two flat pentameric discs with 10 identical glycosylated subunits is compatible with a process requiring cross-linking of protein ligands.

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**REFERENCES**


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Binding of SAP to CRP

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