Protein A – a new ligand for human C-reactive protein

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Abstract Phosphorylcholine (PC) is a classical ligand of C-reactive protein (CRP), a clinically important acute phase protein. In search of new ligands, CRPs were affinity-purified from several pathological samples, which exhibited distinct molecular variants induced in different diseases. Both glycosylated and non-glycosylated CRPs showed calcium-independent differential-binding to *Staphylococcus aureus* cell-surface Protein A. CRP possesses separate binding sites for Protein A and PC with different binding constants. We have demonstrated that Protein A is another ligand in addition to PC establishing an extended definition of CRP. Protein A binding may impart immunomodulatory roles of CRP in combating microorganisms or other foreign materials.

Keywords: C-reactive protein; Glycosylated human C-reactive protein; Molecular variant; Phosphorylcholine; Protein A binding; *Staphylococcus aureus* cell-surface Protein A

1. Introduction

C-reactive protein (CRP) is a phylogenetically ancient and highly conserved major acute-phase homopentameric plasma protein. According to the classic definition of CRP, proclaimed in 1930, it binds specifically to phosphorylcholine (PC) moiety of pneumococcal C-polysaccharide in a characteristic calcium dependent manner [1]. Most of the reactivity of CRP mediates through binding of surface structure of a number of different microorganisms by complement activation. Although, the gram-positive bacteria are generally resistant to complement-mediated lysis, CRP, after association with its ligands, is able to opsonize these cells and thus facilitates the clearance of encapsulated bacteria from the circulation [2,3]. Therefore, it is worthwhile to search for new physiological ligands, which may provide information on the specific biological roles of CRP.

CRP and immunoglobulin G (IgG) share many functions like complement activation, opsonization and phagocytosis [4] and also have similar type of amino acid composition, suggesting a common evolutionary origin [5]. The binding specificity of Fc region of IgG with *Staphylococcus aureus* Protein A (SpA), a 42 kDa well-characterized cell wall protein, has been exploited in numerous immunochemical studies. Considering the similarity between CRP and IgG, it is interesting to explore whether Protein A behaves as a ligand also for CRP.

Different molecular variants of CRP are reported to be induced under different toxic aquatic conditions in fresh water fishes [6–10]. Recently, we have convincingly demonstrated that human CRP is glycosylated and distinct molecular variants are induced in several pathological conditions [11].

The main aim of the current investigation is to explore the ligand binding property of CRP for better assessment of the structural aspects of this group of proteins. Therefore, CRPs are purified in different pathological conditions (Table 1). Subsequently, existence of distinct molecular variants have been demonstrated with differential binding to Protein A, which has been established as a new ligand for CRP. Existence of separate binding sites and differential affinity of CRP for Protein A and PC allowed us to propose an extended definition of CRP.

2. Materials and methods

Sera from patients (Table 1) showing detectable levels of CRP by agglutination were used for experiments with the approval of Institutional human ethical clearance committee. Informed consent was taken from all individuals.

2.1. Purification and characterization of human CRP

CRPs were purified following a standard protocol as described by Volanakis et al. [12,13]. Typically, human serum (approx. 4 ml) was passed through a column of agarose beads (1 cm × 5 cm) in the presence of Tris-buffered saline (TBS; 50 mM Tris/HCl and 150 mM NaCl), pH 7.5, with $CaCl_2$ (5 mM) to remove the serum amyloid-P component. It was immediately applied to Sepharose–PC (1 cm × 10 cm) affinity matrix, washed with TBS (20 mM Tris/HCl) and $CaCl_2$ (10 mM), pH 7.5 and bound protein was eluted with EDTA (10 mM) containing $CaCl_2$ (1 mM). Protein fractions were pooled, dialyzed against TBS (20 mM Tris/HCl), before adjusting the calcium concentration to 10 mM, and were allowed again to bind to another Sepharose–PC affinity matrix. The column was washed with TBS containing $CaCl_2$ (10 mM) and pure CRP was eluted with PC (2 mM) in TBS (20 mM Tris/HCl), pH 7.5, containing $CaCl_2$ (0.5 mM). The eluted protein was extensively dialyzed against HEPES (20 mM) with saline containing EDTA (2 mM), followed by same buffer without EDTA and stored at −20 °C. Concentration of CRP was measured by a competitive-ELISA using polyclonal anti-human CRP antibody with reference to a standard curve generated using known concentrations of CRP [14].

Purified CRPs were analyzed by native- (5%) [15], and SDS–PAGE (7.5–10%) [16] under reducing and non-reducing conditions. Western

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Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ammonium salt; CRP, C-reactive protein; IgG, Immunoglobulin G; PC, Phosphorylcholine; *S. aureus*, *Staphylococcus aureus*; SpA, *Staphylococcus aureus* cell-surface Protein A

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Table 1
Concentration and molecular profile of CRPs

No.	Clinical samples	CRP in crude serum		Fold increase[b]	Sugar[@]	Sialic acid[$]	Linkage specific sialic acid[#]	
		Agglutination[a]	Competitive ELISA (μg/ml)				SNA	MAA
1	Rheumatic fever	+++	17.0–42.4	34–84	1+	–	ND	ND
2	Alcoholic hepatitis	+	35.2–58.3	70–116	3+	1+	1+	–
3	Acute appendicitis (post surgery)	++	55.0–62.4	110–124	1+	2+	–	1+
4	Liver malignancy	+	22.5–44.0	45–88	2+	4+	–	4+
5	Snake bite	+++	39.3–47.2	79–94	3+	2+	3+	–
6	Rheumatoid arthritis (RA)	+++	53.3–67.0	106–134	2+	1+	2+	–
7	Brain abscess	++	8.1–28.0	16–56	–	–	ND	ND
8	Meningitis	+++	18.3–41.4	37–83	2+	2+	–	2+

[@, $, #] The extent of glycosylation, sialylation and linkage specific sialic acid was determined (Section 2.1) and graded by '+' based on densitometric scanning.
'–', Absence of total sugars and sialic acid; ND, not determined.
[a] Polystyrene latex particles coated with monoclonal anti-CRPantibodies were incubated with crude serum and graded as weak (+), medium (++) and strong (+++), depending on degree of visible agglutination.
[b] Fold increase was calculated considering 0.5 μg/ml as normal level.

blot [17] analysis was done separately by using anti-human CRP and anti-human IgG antibodies.

In addition, two ELISA were used for further detection of any possible contamination of human IgG in the CRP sample. Human IgG (0–500 ng/100 μl/well) were separately coated on the same 96-well ELISA plate. After non-specific blocking, HRP-anti-human IgG was added, washed extensively and bound complex was detected by 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ammonium salt (ABTS) and read at $OD_{405\,nm}$ by ELISA reader (Win Read V.2.1, Anthos Labtec, UK). A standard graph was generated by plotting different concentrations of human IgG vs $OD_{405\,nm}$; linearity was observed between 0 and 100 ng concentrations of IgG. In parallel, under identical conditions, CRP (250 ng/100 μl/well) was coated separately in the same plate and processed similarly.

Additionally, a known amount 0, 5, 10, 25, 50 and 100 ng of human IgG was mixed with 250, 245, 240, 225, 200 and 150 ng of CRP, respectively, maintaining final protein concentration at 250 ng and coated on wells (250 ng/100 μl/well), blocked and HRP-anti-human IgG was added. The wells were processed as described above.

The degree of glycosylation, sialylation and linkage specific sialic acid was detected using equal amounts of CRP by DIG-glycan detection and differentiation kits according to manufacturer's instructions (Roche Molecular Biochemicals, Cat Nos. 1142372 and 1210238, respectively). To reconfirm the presence of sugars in the induced CRPs, they were deglycosylated using deglycosylation kit (Roche Molecular Biochemicals, Cat Nos. 1836552 and 1347101) and analyzed by SDS–PAGE.

2.2. ELISA for CRP binding to Protein A

The binding of both glycosylated and non-glycosylated CRPs with Protein A was analyzed by a two-step ELISA. The microtitre plate was coated with fixed or increasing concentration of CRPs by keeping overnight, washed, blocked with BSA (2%, TBS–BSA) at 25 °C for 2 h and incubated with HRP–Protein A (dilution 1:10,000) for 30 min at 37 °C. After extensive washing the bound complex was detected by ABTS and read at $OD_{405\,nm}$ by ELISA reader.

In order to quantitate the contribution of IgG contamination (if any) in CRP, in its Protein A binding, another ELISA was designed, in which human IgG (0–500 ng/100 μl/well) were coated on a 96-well ELISA plate and HRP–Protein A was added. In parallel, under identical condition, CRP (250 ng/100 μl/well) was coated separately in the same plate and processed similarly.

2.3. ELISA for CRP binding to Protein A in presence of PC and vice versa

In order to distinguish the binding sites of CRP for both PC and Protein A, an inhibition study was performed. Either fixed amount of CRP pre-incubated with PC (50–150 mM in 0.5 mM $CaCl_2$) were immobilized onto the microtitre wells or PC was allowed to bind with CRP-coated wells at 4 °C overnight, washed and incubated with HRP–Protein A. In parallel, PC and HRP–Protein A were added together to the CRP-coated wells and processed as above.

2.4. Binding of CRPs to Protein A–sepharose 4B

CRPs were iodinated [18] and fixed amounts of ^{125}I-CRPs were separately incubated with Sepharose–Protein A (20 μl) in presence of $CaCl_2$ (0–50 mM) overnight at 4 °C. Equal volume of buffer lacking $CaCl_2$ was used as control.

To assess the nature of CRP–Protein A binding, a fixed amount of Sepharose–Protein A matrix (20 μl) was incubated with increasing amounts of ^{125}I-CRPs in the presence or absence of $CaCl_2$ (0.5 mM) overnight at 4 °C. Un-conjugated Sepharose-4B along with CRP pre-incubated with galactose (50 mM) was used as control to exclude the non-specific binding. Measurements were done in triplicates. The binding constants were calculated from the Scatchard plot [19]. For comparison of CRP–Protein A binding with CRP–PC, Sepharose–Protein A was replaced with Sepharose–PC and processed similarly.

2.5. Binding of CRP to S. aureus bacterial cells

To demonstrate the binding of CRP to Protein A, *S. aureus*, rich source of cell surface protein A (SpA) was used. ^{125}I-CRPs, with or without pre-incubation with PC (150 mM in TBS–$CaCl_2$) to block the PC binding sites at 4 °C overnight, were allowed to react with different amounts of bacterial cells ($OD_{600\,nm}$ = 0.2–0.7) at 37 °C for 30 min, washed with TBS–BSA and bound radioactivity was measured.

Similarly, ^{125}I-CRPs were pre-incubated with Protein A (molar ratio of CRP/Protein A = 1:5) to block all the Protein A binding sites on CRPs and incubated with the bacteria ($OD_{600\,nm}$ = 0.5), washed and the bound counts for Protein A-incubated-CRP were compared with the counts exhibited by free CRP.

2.6. Flow cytometric analysis of binding of FITC-CRP to S. aureus

Cells ($OD_{600\,nm}$ = 0.5 in TBS–BSA) were incubated with FITC-CRPs (0.1–50 μg/ml) or FITC-CRPs pre-incubated with Protein A at 37 °C for 30 min, washed twice, fixed with 4% paraformaldehyde and analyzed on a Becton Dickinson FACScan using CELL QUEST software. Only Cells, FITC-BSA or cells incubated with unconjugated CRP served as controls.

3. Results

3.1. Purification and molecular characterization of CRPs

CRPs were purified separately from individual patients having several pathological conditions (Table 1). Crude sera (lane 1) and serially purified fractions were electrophoretically analyzed (Fig. 1A). Amongst the major proteins found in the eluate from aga-

rose column (lane 2), three were eluted with EDTA from 1st PC (lane 3) and, therefore, considered as calcium binding proteins. Single band in PC elution from 2nd PC column was taken as pure CRP (lane 4). Single band in Western blot with anti-human CRP further confirmed its homogeneity (lane 7). As assayed by immunoblotting of the gel using polyclonal anti-human IgG antiserum, the preparation of purified CRP showed no detectable amounts of IgG confirming its absence as demonstrated by the lack of binding with anti-human IgG (lane 5) as compared to lane 6 in which standard human IgG showed strong bands.

No IgG (lane 4) was detected even with higher amount of CRP, when pure human IgG (lane 1) along with three different concentrations of CRP_{VL} (lanes 2–4) was analyzed on SDS–PAGE (Fig. 1B). In order to examine any contamination of human IgG in the CRP sample, an ELISA was performed in which CRP (250 ng) was allowed to bind with anti-human IgG. Under identical condition, negligible absorbance ($OD_{405\,nm} = 0.013 \pm 0.002$) was observed in CRP-coated wells in comparison with equal amount of pure IgG was coated on the same plate (Fig. 2A). Considering the limit of sensitivity of this ELISA, the OD in CRP coated wells may be considered as the basal level and IgG contamination would be less than 1% (Fig. 2A). In parallel, when a known amount 0, 5, 10, 25, 50 and 100 ng of human IgG was mixed with 250, 245, 240, 225, 200 and 150 ng of CRP, respectively, maintaining the final protein concentration at 250 ng, IgG alone or IgG-CRP mixture showed almost same $OD_{405\,nm}$ (Fig. 2A).

The induced CRP levels, as determined by ELISA from crude serum, varied significantly from 8–67 μg/ml of serum (Table 1), corresponded to 16–134-fold increase above the normal level (0.5 μg/ml) confirming that CRP was an acute phase reactant in these pathological conditions.

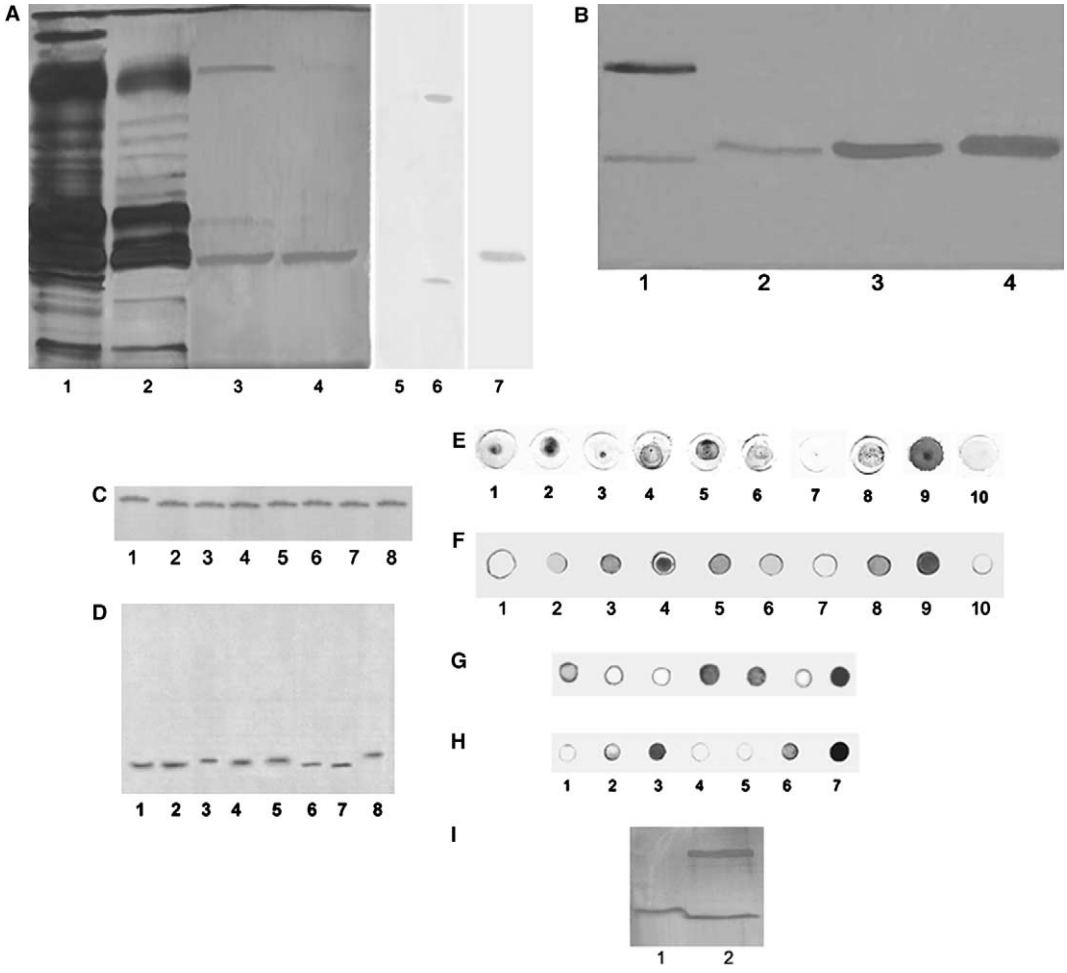

Fig. 1. Purification and characterization of CRPs. (A) *Electrophoretic analysis*. Equal amounts of serially purified fractions from serum of a patient with rheumatoid arthritis were electrophoretically analyzed on a SDS–PAGE (10%). Lane 1, crude serum; lane 2, unbound fraction from agarose column; lane 3, EDTA eluates from 1st PC column and lane 4, PC eluates from 2nd PC column; Western blotting of equal amount (14 μg) of PC eluted fraction from a patient (lane 5) and standard human IgG (lane 6) probed with HRP-anti-human IgG; lane 7, PC eluted fraction probed with murine anti-human CRP and detected by HRP-anti-murine IgG. (B) An increasing amount of purified human CRP_{VL} (1, 2.5, 5 μg/well in lanes 2, 3, 4, respectively), along with human IgG (1 μg/well, lane 1) were analyzed by SDS/PAGE. Gel was fixed and stained for protein with silver nitrate. Following analysis were carried out using purified CRPs (1 μg/lane or spot). Bands/spots 1–8 correspond to the CRPs in the same order as in Table 1. (C)–(D) *Native and SDS–PAGE*. CRPs were electrophoretically analyzed. (E)–(F) *Detection of neutral sugar and sialic acid*. CRPs were blotted on nitrocellulose strips and processed as described in Section 2.1. Columns 9 and 10 represent positive and negative controls, respectively. (G)–(H) *Detection of α2-6 and α2-3 linked sialic acid*. $CRP_{alcoholic\ hepatitis}$, $CRP_{acute\ appendicitis}$, $CRP_{liver\ malignancy}$, $CRP_{snakebite}$, CRP_{RA}, $CRP_{meningitis}$ (columns 1–6) were analyzed by DIG glycan differentiation kit using α2-6 (SNA) and α2-3 (MAA) linked sialic acid binding lectins. (I) *SDS–PAGE before and after treatment with N-glycosidase F*. A representative profile of a glycosylated $CRP_{acute\ appendicitis}$ before (lane 1) and after *N*-glycosidase F digestion (lane 2) at 37 °C overnight were electrophoresed (Section 2.1). The upper band in lane 2 corresponds to *N*-glycosidase F (34.6 kDa).

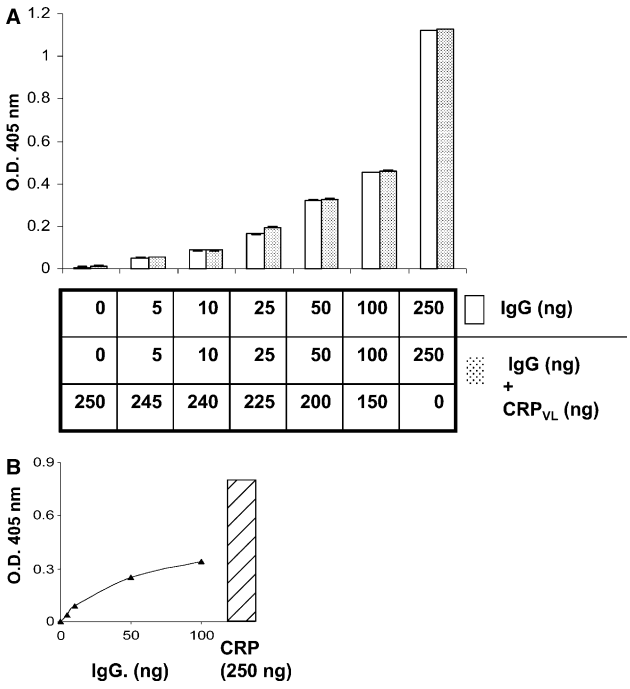

Fig. 2. Evaluation of contaminating IgG (if any) in purified CRP by ELISA. (A) A known amount 0, 5, 10, 25, 50 and 100 ng of human IgG was mixed with 250, 245, 240, 225, 200 and 150 ng of CRP, respectively, maintaining final protein concentration at 250 ng. The IgG alone (□) or IgG-CRP mixture (▨) was coated on wells (250 ng/100 μl/well) separately, blocked. HRP-anti-human IgG was added and bound complex was detected by ABTS. (B) Purified human IgG (0–100 ng/100 μl/well) and a fixed concentration of CRP (250 ng/100 μl/well) was coated on a 96-well ELISA plate. HRP–Protein A was added separately and processed as described in Section 2.

Purified CRPs showed single bands in native gel (Fig. 1C) confirming their electrophoretic homogeneity with small but distinct differences in mobility. Single subunit in SDS–PAGE (Fig. 1D) supported again their purity with variations in their mol. wt (27–31 kDa) indicating the presence of molecular variations in different clinical samples.

DIG enzyme-immunoassay clearly demonstrated that out of eight CRPs (Table 1), seven are glycosylated (Fig. 1E). $CRP_{brain\ abscess}$ (column 7) showed no reactivity indicating the absence of carbohydrates. Among seven glycosylated CRPs, six of them are differentially sialylated (Fig. 1F, columns 2–6 and 8). $CRP_{rheumatic\ fever}$ (column 1) showed no color indicating absence of sialylation. Notably, the extents of glycosylation and sialylation prominently differed from each other, as evident from the intensities of the spots by densitometric scanning.

The presence of linkage specific sialic acid in these six sialylated CRPs was determined with the help of *Sambucus nigra* agglutinin (SNA) (Fig. 1G) and *Maackia amurensis* agglutinin (MAA) (Fig. 1H). $CRP_{alcoholic\ hepatitis}$, $CRP_{snakebite}$ and CRP_{RA} showed positive reactivity with SNA (Fig. 1G, columns 1, 4, and 5) indicating existence of α2-6 linked sialic acids. In contrast, $CRP_{acute\ appendicitis}$, $CRP_{liver\ malignancy}$ and $CRP_{meningitis}$ were reactive to MAA (Fig. 1H, columns 2, 3 and 6) suggesting the presence of α2-3 linked sialic acids.

A representative gel picture shows a shift in the mobility of $CRP_{acute\ appendicitis}$, a glycosylated CRP, indicating decrease in the molecular mass of three kDa after *N*-glycosidase F-digestion (Fig. 1I). No change in the electrophoretic mobility was observed following *O*–glycosidase digestion indicating that CRP is glycosylated possibly through *N*- and not *O*-linkage.

3.2. CRP binds to Protein A

The exponential titration curve of the binding of Protein A with increasing concentration (0–4 μg/ml) of CRP is shown in Fig. 3, inset. The curve reached a plateau at about 2.5 μg/ml. This is the first demonstration of binding of CRP with Protein A.

To exclude the interference of human IgG in CRP–Protein A binding, a control ELISA was carried out, which clearly

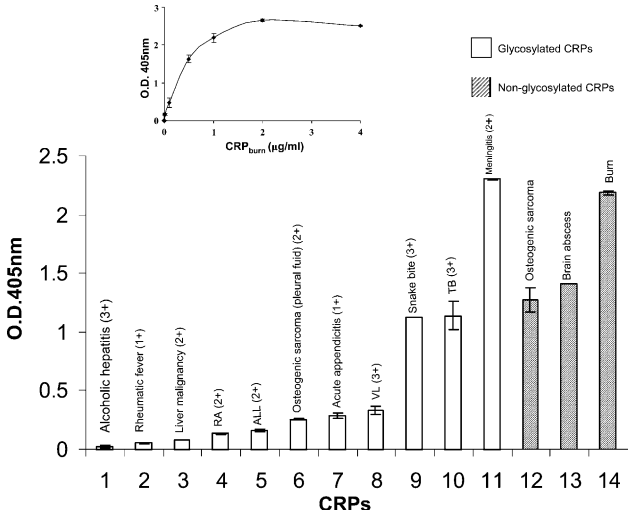

Fig. 3. Binding of Protein A with CRP as detected by ELISA. CRP (250 ng) coated wells were incubated with constant amount of HRP–Protein A and binding was detected by ABTS (Section 2.2) and extent of binding was compared. '+' denotes extent of glycosylation (Table 1) [11]. *Inset*. Different concentrations (0–4 μg/ml) of a CRP_{burn} was tested for Protein A binding.

showed that contaminating amount of IgG, if any, contributes very insignificantly to the Protein A binding as compared to CRP–Protein A binding (Fig. 2B).

3.3. Different molecular variants of CRP showed differential reactivity with Protein A

To assess the role of structural differences of CRP in Protein A binding, an ELISA was carried out using fixed amount of fourteen molecular variants of CRP purified from fourteen different clinical samples. All eleven glycosylated CRPs exhibited variable degree of Protein A binding (Fig. 3). An 18-fold higher binding was observed with $CRP_{meningitis}$ than that of CRP_{RA}. The binding decreased as follows: $CRP_{meningitis} \gg CRP_{TB} = CRP_{snakebite} \ggg CRP_{VL} = CRP_{acute\ appendicitis} > CRP_{osteogenic\ sarcoma\ (pleural\ fluid)} > CRP_{ALL} = CRP_{RA}$ again suggesting the contribution of structural variations induced in different molecular variants of CRP in Protein A binding. $CRP_{alcoholic\ hepatitis}$, $CRP_{rheumatic\ fever}$ and $CRP_{liver\ malignancy}$ showed minimal binding with Protein A at this concentration.

3.4. Binding of CRP with Protein A is not related to glycosylation

Although eight glycosylated CRPs (Fig. 3, columns 1–8) showed small amount of Protein A binding as compared to three non-glycosylated CRPs ($CRP_{osteogenic\ sarcoma}$, $CRP_{brain\ abscess}$ and CRP_{burn}), other two glycosylated CRPs (CRP_{TB} and $CRP_{snakebite}$) showed almost the same extent of Protein A binding as the non-glycosylated $CRP_{brain\ abscess}$ and $CRP_{osteogenic\ sarcoma}$. However, $CRP_{meningitis}$ (glycosylated) and CRP_{burn} (non-glycosylated) exhibited higher extent of Protein A binding. Thus, apparently, no correlation was observed between the extents of glycosylation with the extents of Protein A binding (Fig. 3).

3.5. Binding of Protein A with CRP is calcium independent

Although calcium plays a very important role in the binding of CRP with its classical ligand (PC), the extent of the binding of CRP with Protein A remained the same in the absence and presence of calcium, suggesting CRP–Protein A binding is independent of calcium and probably not through the PC binding site on CRP molecule. In contrast to Protein A-binding property, no significant difference in binding of these molecular variants with PC was observed. As expected, with increasing Ca^{2+} concentration, the PC-binding increased and reached a plateau at higher concentrations with a half maximal binding at 3 mM confirming the dependence of Ca^{2+} on PC-binding of all the molecular variants of CRP.

3.6. Protein A and PC binding epitopes on CRP are different

Inhibition studies on CRP–Protein A binding, using very high and wide range of concentrations of PC (50–150 mM), were carried out by incubating CRP with PC before and after coating on an ELISA plate. Alternatively, PC was added together with Protein A in CRP coated plate and the extent of binding of Protein A to CRP was compared with that in the absence of PC. In all these cases, the binding remained unaltered reflecting that PC and Protein A were not competing for the same site on CRP.

3.7. CRP binds to S. aureus Protein A

The increasing concentrations ($OD_{600\,nm} = 0.2$–0.7) of bacterial cells, known to contain high amount of Protein A on their cell surface, showed a steadily increased (between 50% and 100%) binding with ^{125}ICRPs. Both glycosylated ($CRP_{meningitis}$) and non-glycosylated ($CRP_{brain\ abscess}$) CRPs showed strong binding with these cells. CRPs pre-incubated with PC also showed more or less unaltered binding indicating that the PC moiety, present on the bacterial cells, is not interfering in this interaction reconfirming that PC and Protein A binding sites are different on CRP.

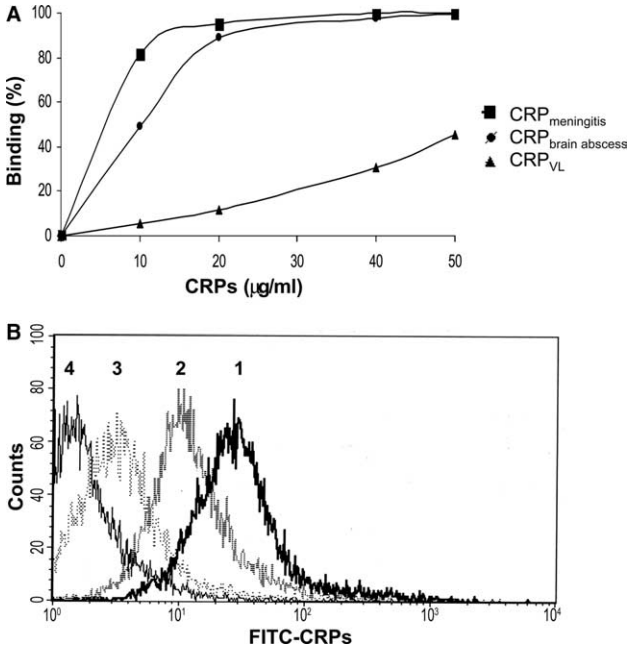

Fig. 4. Comparative analysis of binding of three CRPs with SpA. (A) A fixed number ($OD_{600\,nm} = 0.5$) of bacterial cells were incubated with different concentrations of (0–50 μg/ml) of FITC conjugated $CRP_{meningitis}$, $CRP_{brain\ abscess}$ and CRP_{VL} and analyzed by flow cytometry (Section 2.6). (B) A representative profile of flow cytometric analysis to compare the percentage binding of bacterial cells ($OD_{600\,nm} = 0.5$) to a fixed concentration (10 μg/ml) of FITC-$CRP_{meningitis}$ (1), $CRP_{brain\ abscess}$ (2) and CRP_{VL} (3), where (4) represents corresponding negative control.

3.8. FACS analysis confirmed the binding of CRP with SpA

The binding of FITC-CRP with bacterial cells was further corroborated by FACS analysis. Three representatives high ($CRP_{meningitis}$), medium ($CRP_{brain\ abscess}$) and low (CRP_{VL}) Protein A binding CRPs, as detected by ELISA (Section 3.3, Fig. 3), were selected. A wide variation in binding with these CRPs was observed with a fixed concentration of the bacteria (Fig. 4A). The amount of CRP needed for maximum (90%) binding differed, the concentration being 15 μg/ml for $CRP_{meningitis}$ and 25 μg/ml for $CRP_{brain\ abscess}$ whereas, CRP_{VL} did not attain to that point even at 50 μg/ml. No significant binding was observed below 1 μg/ml concentration. Maximum variation in binding was observed with 10 μg/ml CRPs (Fig. 4B), considered as early state of acute phase reaction, % of cells showing binding being 89%, 49% and 5% with $CRP_{meningitis}$ (1), $CRP_{brain\ abscess}$ (2) and CRP_{VL} (3), respectively, with respect to negative control (4).

The specificity of binding of CRP with SpA was demonstrated by two separate experiments. Initially, Protein A-incubated with either ^{125}ICRPs or FITC-CRPs were allowed to

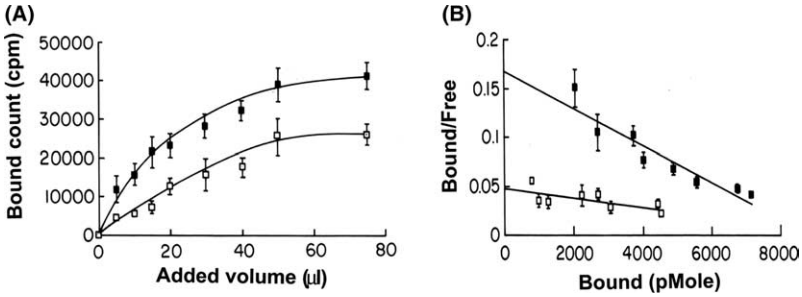

Fig. 5. Scatchard analysis of CRP–Protein A and CRP–PC binding. (A) Fixed amount of Protein A–Sepharose (□) or PC–Sepharose (■) (20 µl/tube of each) were separately incubated with increasing concentrations of $^{125}ICRP_{meningitis}$ (specific radioactivity 2.5×10^9 cpm/mg). Bound radioactivity was plotted against the volume (µl) of $^{125}ICRP$. (B) Scatchard plot, in which bound/free was plotted against the amounts of bound CRP. The apparent association constant was calculated from the slope of the Scatchard plot.

bind with bacteria and binding was monitored. A reduction of 45% and 30% binding of pre-incubated $^{125}ICRPs$ and FITC-CRPs with bacteria reflects its specificity towards Protein A.

3.9. Comparison of affinity constants between CRP–Protein A and CRP–PC binding

The plot of binding of ^{125}I-labeled CRP with Sepharose–Protein A or Sepharose–PC separately (Fig. 5A) demonstrated that the binding reached almost to saturation about 75 µl of added $^{125}ICRP$ in both the cases but to different levels. Apparent binding constants (K_a) of CRP with Protein A and PC, calculated from the slopes of Scatchard plots, (Fig. 5B) were found to be 4.9×10^6 and 1.9×10^7 M^{-1}, which correspond to dissociation constants (K_d) of 205 and 53 nM, respectively.

4. Discussion

The marked elevation of CRP levels following infection, tissue injury or a variety of inflammatory responses strongly suggests that CRP is actively involved in host defense mechanism [20]. Due to its extensive clinical use, for rapid 1000–3000-fold rise in serum concentration in an underlying inflammatory process, human CRP drew wide attention. In spite of in vitro demonstration of the involvement of CRP in an array of biological events like complement activation, opsonization, macrophage activation, selective deposition at the site of tissue damage and binding to several biological macromolecules [21], their precise physiological role remains to be elucidated.

The major achievements of the current investigation includes (i) purification, characterization and establishment of the induction of glycosylated molecular variants due to their structural variation of a panel of CRPs from several pathological conditions to introspect the CRP–Protein A interaction (Table 1, Fig. 1), (ii) demonstration of specific differential binding of Protein A (both as free and cell surface bound) to fourteen different glycosylated and non-glycosylated CRPs (Figs. 3 and 4), (iii) confirmation of no significant role of calcium and oligosaccharide moieties present on CRP in the interaction of CRP–Protein A binding (Figs. 3 and 4) and finally (iv) establishing two separate binding sites of Protein A and PC, thereby, demonstrating conclusively Protein A as a unique ligand for CRP.

Purified CRPs not only specifically bind to Protein A but also exhibit variable extents of binding as evidenced by several approaches (Figs. 3,4). Binding parameters using free Protein A and cell surface bound Protein A, as detected by ELISA (Fig. 3), FITC-CRP (Fig. 4) or ^{125}I-CRP (Fig. 5) convincingly demonstrated this specific interaction. Therefore, free or bound Protein A, either conjugated with Sepharose beads or cell surface, showed similar binding with CRP molecules indicating Protein A as a true ligand for CRP. The specificity of the interaction was again confirmed by inhibition of binding using CRP pre-incubated with saturable amount of free Protein A.

Notably, CRPs from different sources showed different extent of binding with Protein A (Fig. 3). CRP_{VL} showed small binding (5%) with SpA at 10 µg/ml concentration, whereas the binding was 49% and 89% with the same concentrations of $CRP_{brain\ abscess}$ and $CRP_{meningitis}$. However, at higher concentrations >25 µg/ml, SpA binding reaches to a plateau for $CRP_{brain\ abscess}$ and $CRP_{meningitis}$, while binding with CRP_{VL} was still much smaller ~30%. Therefore, it may be envisaged that different molecular variants of CRP possess variable capability of binding with bacteria through Protein A depending on their concentration in serum, severity of that particular disease and requirement for neutralization. Thus, it may be postulated that the CRP–Protein A interaction is a general phenomenon for all molecular variants of CRP and the binding epitope for Protein A is differentially expressed with variable capacity on different disease-induced CRPs.

The presence and nature of the attached oligosaccharides on glycoproteins is known to influence many biological functions. Notably, the interaction of CRP–Protein A is independent of induction of glycosylation on CRP (Figs. 3 and 4), indicating that the structural modification due to glycosylation have no impact on CRP–Protein A binding. These findings suggest that the binding site for Protein A and the glycosylation site are possibly present in two different regions on CRP molecule reflecting flexibility of CRP molecule to interact with various microbes. Incidentally, like CRP, carbohydrate moieties of IgG present in Fc region also do not contribute to SpA binding [22].

Comparison of the binding characteristics of CRP with Protein A and its universal ligand, PC, indicates that Ca^{2+} ion, an essential component for CRP–PC binding, is not required for CRP–Protein A interaction suggesting that the Protein A binding site does not undergo the calcium induced conformational change. Moreover, CRP–Protein A binding is not

modulated by the PC binding as demonstrated by ELISA, where the CRP–Protein A binding could not be inhibited by saturable amount of PC suggesting existence of completely two different regions on CRP and possibly these two sites remain accessible for both these ligands PC and Protein A. Earlier molecular modeling study clearly indicated that PC binding site and glycosylation site are present in two opposite sides of the CRP molecule [11]. It would be interesting to build molecular model of CRP–Protein A complex to identify the location of Protein A binding. These observations have far reaching application, as CRP, using its both arms (Protein A and PC binding sites), will be able to bind to infective molecules for their clearance. Although CRP has affinity for both PC and Protein A, it showed higher affinity for PC (Fig. 5) consistent with earlier widely known anti-streptococcal activity of CRP [23].

The interaction of CRP with SpA further confirms the presence of a novel ligand, the Protein A. This binding opens a new avenue for identifying the biological role of CRP, where CRP simultaneously may perform its protective role by binding to both PC and Protein A on infective organisms. These observations deserve attention for understanding the pathophysiological role of CRP in the recognition, clearance and destruction of infectious organisms by the immune system.

To the best of our knowledge, this is the first report that describes the unique ability of disease-induced molecular variants of human CRP to bind differentially with Protein A, an important cell surface component of microorganisms, thus playing its anti-infective and anti-microbial roles [3] through this new ligand for combating various microorganisms by opsonization and clearance.

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