Localization of Sites through which C-reactive Protein Binds and Activates Complement to Residues 14–26 and 76–92 of the Human Clq A Chain

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Summary

Studies were initiated to localize the C-reactive protein (CRP) binding site on the collagen-like region (CLR) of Clq. CRP bound preferentially to the A chain of reduced Clq, in contrast to aggregated immunoglobulin G (Agg-IgG), which reacted preferentially with the C chain. A group of Clq A chain peptides, including peptides identical to residues 81–97, 76–92, and 14–26, respectively, were synthesized from predicted binding regions. Peptide 76–92 contained two proximal lysine groups, and peptide 14–26 contained four proximal arginine groups. CRP-trimers and CRP-ligand complexes did not bind to immobilized peptide 81–97, but bound avidly to immobilized peptides 76–92 and 14–26. Agg-IgG did not bind to any of the peptides. Peptide 76–92 partially, and peptide 14–26 completely, inhibited binding of CRP to intact Clq. Peptide 14–26 also blocked C consumption initiated by CRP, but not by IgG. Replacement of the two prolines with alanines, or scrambling the order of the amino acids, resulted in loss of ability of peptide 14–26 to inhibit Clq binding and C activation by CRP, indicating a sequence specificity, and not a charge specificity alone, as the basis for the inhibitory activity of the peptide. Similar investigations with scrambled peptides showed a sequence specificity for the effects of peptide 76–92 as well. DNA and heparin inhibited binding of CRP trimers to intact Clq, as well as to each peptide 14–26 and 76–92, suggesting involvement of these regions in Clq-CLR binding reactions generally. Collectively, these data identify two cationic regions within residues 14–26 and 76–92 of the Clq A chain CLR as sites through which CRP binds and activates the classical C pathway, and suggest that these residues represent significant regions for Clq CLR binding reactions generally. To our knowledge, this represents the first delineation of sites on Clq through which binding and activation of the classical C pathway can occur.

C-reactive protein (CRP), a prototypic acute-phase reactant and member of the pentraxin family of proteins (2, 3), is known to activate the classical pathway of complement via an interaction with Clq (4, 5). Recently CRP-induced C activation was shown to occur via the collagen-like region (CLR) of Clq (6, 7), in contrast to IgG and IgM which activate C via the globular region (GR) of Clq (8). Other substances in addition to CRP which react with the Clq CLR include DNA (9–11), fibronectin (12), heparin (13).

Abbreviations used in this paper: Agg-IgG, heat-aggregated IgG; CLR, collagen-like region of Clq; CRP, C-reactive protein; DTT, dithiothreitol; GR, globular region of Clq; TBS, Tris-buffered saline; and VBS**, veronal-buffered saline with CaCl₂.

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react with both CLR and GR preparations, and hence have been presumed to react with a determinant on the CLR tail within amino acid residues 81–97 from the NH₂ terminus (11, 12). In additional preliminary experiments DNA, but not Agg-IgG, inhibited binding of CRP to Clq (1), consistent with the hypothesis that CRP binds to Clq via CLR residues 81–97. Collectively, these results suggested that CRP reacts with A chain residues 81–97, and further, that the CLR serves as a general site for Clq binding and C activation.

The present study was initiated to localize the CRP-binding site on Clq, and to further investigate C activation via the CLR. The results establish that CRP binds preferentially to the Clq A chain, but in the area of CLR residues 76–92 rather than 81–97, and in the area of CLR residues 14–26 as well. DNA and heparin also bind in both of these regions. CRP-trimmers, as well as CRP-ligand complexes, bind to these regions, and both regions seem to be involved in the reactivity of CRP with the C system. We believe these results provide the first delineation of Clq sites through which the classical C pathway can be activated.

Materials and Methods

Aggregated Human IgG (Agg-IgG). Human IgG (Gamastan) was purchased from Miles Laboratories (Elkhart, IN), and further purified by FPLC Superose 12 preparative column chromatography in the presence of 10 mM Tris-buffered saline (TBS). To prepare Agg-IgG, IgG (7.5 mg/ml) was heated at 63°C for 20 min immediately before use.

BSA-anti-BSA Complexes. BSA was purchased from Sigma Chemical Co. (St. Louis, MO), and rabbit anti-BSA was purchased from Calbiochem Corp. (San Diego, CA). BSA-anti-BSA complexes were prepared by mixing equal volumes of BSA (20 μg/ml) and anti-BSA (10 μg/ml).

DNA, Heparin, and Phosphorylcholine. Lyophilized double-stranded calf thymus DNA, heparin, and phosphorylcholine were obtained from Sigma Chemical Co.

CRP. CRP, CRP-trimmers, and CRP-protamine mixtures were prepared as previously described (6, 7), and characterized using anti-CRP mAb (18, 19).

Clq. Clq was isolated at 4°C from human plasma by modification of the method of Tenner et al. (20). Briefly, 5 mM EDTA plasma was applied to a Bio-Rex 70 column (Bio-Rad Laboratories, Richmond, CA), and eluted with a linear (0.08–0.3 M) NaCl gradient. The protein peak was pooled and further purified by gel filtration on a preparative Superose 12 FPLC column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The concentration of Clq was determined by its absorbance at 280 nm using an extinction coefficient (mg/ml) of 0.68 (21). The purity of the final Clq preparation was 99%, as analyzed by SDS-PAGE with silver staining (Bio-Rad Laboratories), under both unreduced and dithiothreitol (DTT)–reduced conditions. The purified protein was stored at −70°C.

Clq Peptides. Five authentic and four modified Clq A chain peptides were synthesized by Research Genetics (Huntsville, AL). Clq peptide 81–97 (P-G-S-P-G-N-I-K-D-Q-P-R-P-A-F-S-A) was synthesized based on the sequence presented in reference 22, and Clq peptides 78–92 (K-G-T-K-G-S-P-G-N-I-K-D-Q-P-R) and 76–92 (C)-G-I-K-G-T-K-G-S-P-G-N-I-K-D-Q-P-R) were synthesized at a later time based on the sequence presented in reference 23. Data presented in the latter reference demonstrated the presence of a lysine rather than a proline at position 81. Clq peptides 14–26 (A-G-R-P-G-R-G-R-G-L-P-G-L-K) and 4–26 (C-R-A-P-D-G-K-K-G-E-A-G-R-P-G-R-G-L-P-G-L-K) also were synthesized based on data in reference 23. Peptides similar to 76–92, with the proline at position 84 replaced with alanine (G-I-K-G-T-K-G-S-P-G-N-I-K-D-Q-P-R; 76–92/alanine), or with the amino acids scrambled (D-P-I-G-T-K-G-S-P-G-N-I-D-Q-P-R; 76–92/scrambled), and peptides similar to 14–26 but with the prolines at positions 17 and 23 replaced with alanine (A-G-R-A-G-R-G-R-G-L-K; 14–26/alanine), or with the amino acids scrambled (K-G-A-P-R-R-G-G-L-P-R; 14–26/scrambled) were synthesized to help evaluate the role of sequence and conformation, as well as charge, in the activities of the Clq A chain peptides.

Biotinylation. Proteins were biotinylated by dialyzing into 0.1 M sodium bicarbonate buffer (pH 9.0), and incubating with N-hydroxysuccinimide-long-chain-biotin (Pierce Chemical Co., Rockford, IL) at ratios of 8:1 (wt protein: wt biotin) for 4 h at room temperature. The mixtures were dialyzed twice on a magnetic stirrer against 4 liters of 10 mM TBS²⁺ at 4°C. DNA was biotinylated using photoactivatable biotin and a slight modification of the procedure from Pierce Chemical Co. Briefly, equal amounts of DNA and biotin were mixed in the dark and incubated in an ice bath for 15 min under long wave (350–370 nm) UV light. Equal amounts of 0.1 M Tris-HCl (pH 9.0) and 1-butanol were added, the sample was mixed and centrifuged, and after washing the lower phase once with butanol, equal amounts of 4.0 M NaCl and ethanol were added. The mixture was maintained overnight at −20°C, centrifuged, and the precipitated DNA was redissolved in 0.1 mM EDTA, pH 8.0, for use as a concentrated stock solution.

SDS-PAGE and Western Blots. SDS-PAGE was carried out using 13% polyacrylamide minislab gels (Bio-Rad Laboratories) and the buffer system described by Laemmli (24). Intact Clq and the separated Clq chains were transferred to nitrocellulose membranes using the Bio-Rad transblot system (25). The nitrocellulose paper was incubated with 1% BSA for 30 min at room temperature, and washed three times with veronal-buffered saline containing 2 mM CaCl₂, 0.05% nonionic detergent, and 0.01% Tween 20. Biotinylated CRP was added for 30 min at 37°C followed by washing, and reacted with streptavidin-peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA). Color was developed by 4-chloro-1-naphthol (Bio-Rad Laboratories).

Solid Phase Binding Assays. Wells of microtiter plates (C. A. Greiner and Sohne, Nürtingen, Germany) were coated overnight at room temperature with 50 μl peptide per well (100 μg/ml) and air-dried to the plate. After washing, the plates werebackcoated with BSA (100 μl of a 1% solution), and incubated at 37°C for 30 min. The plates were washed with 0.075 M PBS²⁺, and biotinylated protein was added (37°C, 30 min), followed by washing and the addition of streptavidin-peroxidase (Jackson ImmunoResearch Laboratories). The plates were incubated for another 30 min at 37°C, washed, and substrate (ABTS in 0.1 M sodium citrate at pH 4.0; Bio-Rad Laboratories) was applied. Reactions were developed at 37°C for 10–30 min, and the A₄₁₀ was read in a Titertek Multiskan MC plate reader (Flow Laboratories, Helsinki, Finland).

Complement Source. Human serum was obtained from healthy blood donors and stored at −70°C until required. C1 and C2 were prepared from guinea pig serum as described by Nelson et al. (26). C4-deficient guinea pig serum was the kind gift of Dr. Alexander P. Osman (Oak Ridge Laboratories, Knoxville, TN). Guinea pig serum diluted in GVB-EDTA was used as the source of terminal C (C3-C9) components.

Total Complement (CH₅₀) and C4 Consumption Assays. The
Results

Preferential Reactivity of CRP with the Clq A Chain. To test the relative reactivity of CRP with the separate Clq chains, Clq was reduced with DTT, subjected to SDS-PAGE in minislab gels, transferred to nitrocellulose paper, and reacted with biotinylated CRP-trimers. As shown in Fig. 1, CRP bound preferentially to the Clq A chain, although lesser binding to the B and C chains was seen in some experiments. By comparison, biotinylated Agg-IgG bound preferentially, although not exclusively, to the Clq C chain. This reactivity confirmed the previous demonstration of binding of CRP (and IgG) to linear rather than conformational determinants on Clq (6), suggesting that the reactive site should be definable by the use of synthetic Clq peptides, and indicated that the binding site for CRP was preferentially present on the Clq A chain.

Reactivity of CRP with Clq A Chain Peptide 76-92. Since previous experiments had suggested that CRP binds to COOH-terminal CLR residues retained as a "CLR-like tail" on GR preparations (6), inferred to involve residues 81-97 on one or more of the Clq chains (11, 12), Clq A chain peptide 81-97 was synthesized, and biotinylated CRP-trimers were reacted with the immobilized synthetic peptide (Fig. 2 A). However, no binding was observed. A second Clq A chain peptide (78-92) was synthesized incorporating an additional three NH2-terminal residues, including lysine at position 78, omitting the four COOH-terminal residues that limited solubility of the peptide, and placing a lysine rather than a proline in position 81, as recently clarified (23). This immobilized peptide reacted distinctly, but minimally, with the CRP-trimers. A third Clq A chain peptide (residues 76-92) involving two additional NH2-terminal residues and an NH2-terminal cysteine also was prepared, and this immobilized peptide showed significant binding of CRP-trimers (Fig. 2 A). CRP-protamine complexes bound comparably well as CRP-trimers to peptide 76-92, while Agg-IgG failed to bind to this peptide (Fig. 2 B). The binding was not due to the cysteine added to the NH2-terminus because iodoacetamide treatment of the peptide did not influence the binding activity. The binding of CRP-trimers to peptide 76-92 was inhibited by preincubation in the fluid phase with either intact Clq or peptide 76-92 (Fig. 3 B), although peptide 76-92 reacted less strongly, supporting the specificity of the reaction and indicating that the peptide in the fluid phase, like the immobilized peptide, reacts with CRP. Replacement of the proline at position 84 with alanine, or scrambling the order of the amino acids, resulted in loss of the ability of the immobilized peptide to inhibit binding of CRP-trimers to intact Clq (Fig. 4 B), indicating a sequence specificity, and not a charge specificity alone, as the basis for its reactivity. Neither phosphorylcholine, nor EDTA inhibited binding of CRP to peptide 76-92. Peptide 76-92 (but not peptides 78-92 or
Figure 3. Inhibition of binding of biotinylated CRP-trimers (0.5 μg/well) to wells coated with Clq A chain peptide 14-26 (A; 5 μg/well) by preincubation of CRP-trimers (37°C, 1 h) with increasing amounts (up to 100 μg/ml) of Clq (▲) or peptide 14-26 (○), but not peptide 76-92 (□); and to wells coated with peptide 76-92 (B; 5 μg/well) by preincubation with increasing amounts (up to 100 μg/ml) of Clq (▲), peptide 14-26 (○) and, to a lesser extent, peptide 76-92 (□).

81-97 also inhibited binding of CRP-trimers to intact Clq, although less strongly than intact Clq (Fig. 4 B), emphasizing a role for residues 76-92 in the binding of CRP by Clq, but suggesting that a second site might also be involved.

Reactivity of CRP with Clq A Chain Peptide 14-26. The presence of two lysine groups at the important NH2-terminal of peptide 76-92 suggested that cationic amino acids might be involved in CRP binding at the Clq CLR. Since the Clq A chain has cationic residues between amino acids 4-26 not present on the Clq B or C chains, Clq A chain peptides 14-26 (with arginine at positions 16, 19, 20, and 22, and lysine at position 26) and 4-26 (with an additional arginine at position 5 and lysines at positions 10 and 11) were synthesized and tested for reactivity with CRP. CRP-trimers (Fig. 2 A) bound strongly to peptide 14-26, as did CRP-protamine complexes (data not shown). Since binding to peptide 4-26 was no greater than binding to peptide 14-26 (data not shown), the smaller peptide was used in further studies.

Binding of CRP-trimers to immobilized peptide 14-26 was inhibited, and to comparable degrees, by both fluid phase peptide 14-26 and Clq (Fig. 3 A), but not by phosphorylcholine or EDTA. Further, peptide 14-26 also inhibited binding of CRP-trimers to immobilized peptide 76-92 comparably well as Clq and much more effectively than did fluid phase peptide 76-92, while peptide 76-92 did not inhibit the binding of CRP-trimers to peptide 14-26, indicating preferential binding of peptide 14-26 with CRP (Fig. 3 A). Most strikingly, peptide 14-26 completely inhibited binding of CRP-trimers to intact Clq, supporting the concept that this cationic region of the Clq A chain is a major site involved in binding CRP (Fig. 4 A). Replacement of the prolines at positions 17 and 23 with alanines, or scrambling the order of the amino acids, resulted in loss of the ability of comparable concentrations of peptide 14-26 to inhibit binding of CRP-trimers to intact Clq (Fig. 4 A), indicating a sequence specificity, and not a charge specificity alone, as the basis for its reactivity.

Effect of Peptides 14-26 and 76-92 upon C Activation by CRP. Peptides 76-92, 78-92, and 81-97 had no effect on C consumption initiated by CRP-protamine complexes, but peptide 14-26 induced dose-dependent inhibition of the depletion of C4 (Fig. 5 A) and total C hemolytic activities otherwise observed when CRP and protamine are added to dilute normal human serum. ~250 μg/ml peptide 14-26 was required for 50% inhibition of CRP-induced C4 consumption. This is much greater than the ~2 μg/ml peptide 14-26 required for 50% inhibition of binding to Clq (Fig. 4 A), and probably reflects the lesser amount of Clq (0.5 rather than ~11 μg/ml) and CRP (0.5 rather than 20 μg/ml), use of CRP trimers rather than CRP-ligand complexes, and absence of serum proteins, in the Clq binding assay. Replacement of the prolines at positions 17 and 23 with alanines, or scrambling the order of the amino acids, resulted in loss of the ability of the peptide 14-26 to inhibit C4 consumption by CRP-ligand complexes (Fig. 5 B), again indicating a sequence specificity, and not a charge specificity alone, as the bases for its reactivity. Peptide 14-26 had no effect on C4 consumption initiated by Agg-IgG or BSA-anti-BSA immune complexes, indicating that this peptide specifically inhibits CRP-initiated activation (or perhaps activation via the CLR generally) of the classical pathway.

Figure 4. Inhibition of binding of CRP trimers to intact Clq by Clq A chain peptides 14-26 (▲) and 76-92 (B), but not by peptides with the amino acids scrambled, or with the prolines replaced by alanine. Biotinylated CRP-trimers (0.5 μg/ml) were preincubated with an equal volume of test peptide (up to 25 μg/ml for peptide 14-26 and 100 μg/ml for peptide 76-92), or peptides with the amino acids scrambled (14-26/S and 76-92/S, respectively; □) or with the prolines replaced with alanines (14-26/A and 76-92/A, respectively; □) at room temperature for 120 min before addition to immobilized Clq (0.025 μg/well). Inhibition of binding by comparable amounts of intact Clq (▲) is shown for comparison.
Use of Sites Within A Chain Residues 14-26 and 76-92 for Binding of DNA and Heparin. To test whether DNA and heparin, which are known to react with the Clq CLR (9-11, 13) also bind to a site(s) contained within residues 14-26 and 76-92, biotinylated DNA and heparin were reacted with the several Clq peptides. Like CRP, DNA and heparin both bound strongly to peptides 76-92 and 14-26, and weakly to peptide 78-92, but failed to bind to peptide 81-97. Further, DNA and heparin both blocked binding of CRP-trimers (2 μg/well) to intact Clq (0.5 μg/well) (Fig. 6). Only 0.6 μg DNA/well and 0.5 μg heparin/well were required for 50% inhibition. Comparable amounts of DNA and heparin blocked binding of CRP-trimers to immobilized peptides 14-26 and 76-92 as well (data not shown). These data are consistent with the hypothesis that Clq A chain residues 76-92 and 14-26 contain generally available binding sites for substances reactive with the Clq CLR.

Discussion

Previous experiments had shown that CRP binds to the CLR of Clq near its attachment to the GR (6). In the present experiments, this binding was shown to occur preferentially to the Clq A chain, and by use of appropriate A chain peptides, at sites within two cationic regions contained within residues 14-26 and 76-92. To our knowledge, this represents the first delineation of sites on Clq through which binding and activation of the classical C pathway can occur. Although several residues on IgG (glutamic acid, lysine, and lysine in positions 318, 320, and 322, respectively) have been implicated in binding to the Clq GR (30), the exact Clq sequence involved has not yet been defined.

It earlier was thought likely that CRP binds to Clq residues 81-97, since these residues were thought to be present on standard preparations of both CLR and GR, with which CRP (6), DNA (11), and fibronectin (12) react. Peptide 81-97 was synthesized according to the amino acid sequence published by Reid in 1979 (22), but failed to react with CRP. Peptide 78-92 next was synthesized according to the genomic sequence newly reported by Sellar et al. (23), which defined residue 81 as lysine rather than proline. This peptide, which also contained an additional lysine at position 78, bound CRP weakly. Peptide 76-92 was prepared with glycine at position 76, isoleucine at position 77, and a NH2-terminal cysteine, and this peptide bound CRP strongly. These data are consistent with the hypothesis that lysines at positions 78 and 81 of the Clq A chain are involved in the binding of CRP, with the binding enhanced by the presence of an additional free NH2-terminal amino acid group on the peptide. The structure and reactivity of the Clq chains further support this hypothesis: the Clq B chain has only one lysine and the C chain lacks lysine residues at the homologous positions. The relatively poor binding of CRP-trimers to peptide 78-92, which contains an additional lysine at position 78, as well as lysine rather than proline at position 81, makes it unlikely that peptide 81-97 failed to bind CRP because of the incorrect placement of lysine rather than proline at position 81.

The inability of peptide 76-92 to completely inhibit CRP binding to Clq (Fig. 4B), as well as evidence for two heparin-binding sites on the Clq-CLR (13), suggested that a second binding site for CRP might be present on the Clq A chain. The binding of CRP to a cationic region within Clq A chain residues 76-92, along with the presence of another even more cationic region within residues 14-26 of the Clq A chain not present on the B or C chains, suggested that the second site might reside in this region, and this proved to be the case. CRP bound strongly to peptide 14-26 (which contains four arginine and one lysine residues) and to peptide 4-26 (which contains an additional arginine and two lysines) as well. Comparable binding was observed to both of the latter two peptides, suggesting that the CRP-binding site in this
region of the molecule resides within residues 14–26. Peptide 14–26 completely inhibited, and peptide 76–92 partially inhibited, binding of CRP-trimers to intact immobilized Clq, in further support of the concept that these two cationic regions on the Clq A chain both represent binding sites for CRP, with greater binding activity expressed by the region within residues 14–26. Inhibition of CRP-initiated C consumption by peptide 14–26 further supported this hypothesis. It also is plausible that peptides 14–26 and 76–92 inhibit by reacting with the same region(s) of CRP, since soluble peptide 14–26 was more effective than peptide 76–92 in inhibiting binding of CRP to immobilized peptide 76–92, as well as to itself and intact Clq. It is not yet clear whether the ability of peptide 14–26 but not peptide 76–92 to inhibit C activation by CRP, and the greater effectiveness of this peptide in inhibiting the binding of CRP to Clq, can be attributed to the preferential presence of arginine groups, to its greater total positive charge, or to its conformation. Whatever the basis, the present investigations point to a predominant importance for a site within residues 14–26 of the Clq A chain in C binding and activation by CRP, and suggest that perhaps this peptide or an appropriate analogue will prove to be effective in the modulation of CRP-initiated C activation in vivo. Studies with recombinant Clq should lead to definitive characterization of the Clq binding site(s) for CRP.

Previous studies with mAb and intact Clq or Clq-CLR fragments, rather than with Clq A chain peptides, indicated that a site in the region of residues 76–92 also is important to C activation by CRP (1, 6). Six cationic amino acids are present within residues 14–26 of the three chains that comprise each Clq subunit and five of these reside on the A chain. By contrast, nine cationic amino acids are present within residues 76–92 of the three chains that comprise each Clq subunit and only four of these reside on the A chain. Perhaps maximal binding to the latter region is dependent upon the cumulative effect of cationic residues on each of the Clq chains and not on the A chain only, and hence would better be seen with intact Clq or Clq-CLR than with peptides from any single Clq chain.

Two types of modified peptides were synthesized to test whether the reactivity of the cationic peptides 14–26 and 76–92 was based on sequence specificity, and not attributable to charge specificity alone. Generally, scrambling the order of the residues within the peptides, or simply replacing the prolines with alanine at positions 17 and 23 of peptide 14–26 or at position 84 of peptide 76–92 to remove the proline-glycine bends and change the peptide conformation, resulted in loss of the inhibitory activities of both of the peptides. This indicated that charge alone was insufficient, and that an appropriate amino acid sequence was needed, for the peptides to be reactive with CRP.

Heparin and DNA inhibited binding of CRP to both peptides, suggesting that these two anionic substances also bind to sites containing Clq A chain residues 14–26 and 76–92. Experiments involving chemical modification suggested that bacterial lipopolysaccharide might bind to the Clq B or C chain in the region of histidines at positions 44 or 58, respectively (14). However, it is not yet clear whether lipopolysaccharides or other substances that bind to the Clq CLR, including fibronectin (12), collagen (31), laminin (32), and fibrinogen (33) also bind in the region of residues 14–26 and 76–92, or whether other regions of the CLR are involved in binding of CRP, DNA and the other substances as well. C activation initiated by CRP which occurs via the CLR, like IgG-initiated C activation which occurs via the GR, results in consumption of large amounts of classical pathway components C1, C4, C2, and C3, as well as C-dependent opsonization and cytolysis (2, 3, 34–37). However, the mechanism of C1 activation via the CLR nonetheless may be different, e.g., it is yet to be established whether the C1 subcomponents are used in a similar way. Since many biologically significant agents including endotoxin, viruses, and mucopolysaccharides react directly with Clq (reviewed in reference 38), it will be of interest to further define C binding and activation reactions via the two regions of the Clq A chain CLR identified in the present report. To our knowledge, this represents the first delineation of sites on the Clq CLR through which binding and activation of the classical C pathway can occur.

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1378 Clq Binding Site for C-reactive Protein
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