DNA Binds and Activates Complement via Residues 14–26 of the Human C1q A Chain*

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The mechanism by which DNA activates the classical complement pathway was investigated, with emphasis upon the C1q binding sites involved. DNA bound to both the collagen-like and globular regions of C1q. Binding reactivity with DNA was retained after reduction/alkylation and sodium dodecyl sulfate treatment of C1q. DNA bound preferentially to the A chain of C1q. Binding sites for DNA were localized by using synthetic C1q A chain peptides to two cationic regions within residues 14–26 and 76–92, respectively. Peptides 14–26 and 76–92 avidly bound DNA in enzyme-linked immunosorbent and gel shift assays. Peptide 14–26 also precipitated with DNA and blocked its ability to bind C1q and activate C. Replacement of the two prolines with alamines or scrambling the order of the amino acids resulted in loss of ability of peptide 14–26 to inhibit C1q binding and complement activation by DNA; similar investigations showed a sequence specificity for peptide 76–92 as well. These experiments identify C1q A chain residues 14–26 as the major site, and residues 76–92 as a secondary site, through which DNA binds C1q and activates the classical complement pathway, and demonstrate that a peptide identical to residues 14–26 can modulate C1q binding and complement activation by DNA.

C1 subcomponent C1q is comprised of six identical subunits, each consisting of three distinct polypeptide chains (termed A, B, and C) each of approximately 225 amino acid residues (2). Each chain has a collagen-like region (CLR)1 and a globular region (GR) (3); the collagen-like triple helices begin close to the N terminus and continue to about residue 89, while the remaining 136 C-terminal residues are folded to form a globular head which serves as the immunoglobulin-binding domain (2–4). The entire amino acid sequence and structure of the genes encoding the chains of C1q have been established (2–4, 5). Dimers of the C1r and C1s subcomponents bind to the C1q CLR (6, 7) to form the C1 macromolecular complex. The classical complement pathway can be activated at the level of C1q by both immune complexes which react with the C1q GR and nonimmune substances which react with the C1q CLR (reviewed in Ref. 8).

DNA is a nonimmune activator of the classical complement pathway which has been demonstrated to interact with both the CLR and GR regions of C1q (9, 10, 11–13). Agnello et al. (14, 15) found that C1q precipitated with DNA, and pepsin-treated C1q retained this ability (16), implying that DNA reacts with the C1q CLR. However, DNA also has been reported to bind to the C1q GR (16) and to both the CLR and GR (10). In either case, the precise region(s) of C1q involved in DNA binding and DNA-initiated complement activation is yet to be defined. Binding to both the CLR and GR has suggested the DNA binds to residues near the C terminus of the CLR region (10), i.e., residues 81–97, found in both preparations (17), but this is yet to be established. C-reactive protein (CRP) recently was shown to bind preferentially to the C1q A chain CLR in two distinct regions located within residues 14–26 and 76–92, respectively, and to activate complement via these binding regions; preliminary evidence indicated that DNA binds to the same sites (18). The purpose of the present investigation was to localize the binding site(s) on C1q for DNA and to define the role of this binding in complement activation.

MATERIALS AND METHODS

Buffers—Veronal-buffered saline (0.15 M NaCl; VBST**) as well as veronal-buffered NaCl at 0.075 and 0.5 M NaCl, each containing 2 mM CaCl2 and 0.05% Tween 20, were prepared as previously described.

DNA—Lyophilized single and double stranded calf thymus DNA were obtained from Sigma. A soluble form of calf thymus DNA was obtained from Boehringer-Mannheim Biochemicals. Salmon sperm and Escherichia coli DNA were obtained from Calbiochem Co. A 5.3-kb DNA fragment (HindIII-digested pGKSS) was a kind gift from Dr. Yong-Bao Pan of Rush Medical College.

Preparation of C1q—C1q was isolated at 4 °C from human plasma by modification of the method of Tenner et al. (19). Briefly, 5 mM EDTA plasma was applied to a Bio-Rex 70 column (Bio-Rad) 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 fast-protein liquid chromatography column (Pharmacia LKB Biotechnology Inc.). The concentration of C1q was determined by its absorbance at 280 nm using an extinction coefficient (mg/ml) of 0.68 (20). The purity of the final C1q preparation was >99%, as analyzed by SDS-PAGE using silver staining under both unreduced and dithiothreitol-reduced conditions. The purified protein was stored at −70 °C.

Preparation of C1q GR—C1q was digested by collagenase (Form III, Advanced Biofactures Corp., Lynbrook, NY) as described by Paques et al. (21), and the digestion mixture was applied to an analytical Superose 12 fast-protein liquid chromatography column, equilibrated, and eluted with a buffer consisting of 0.1 M Tris and 0.2 M NaCl (pH 7.4). The third peak, which contained the globular region of C1q, was collected, snap-frozen, and maintained at −70 °C until
time of use. The concentration of GR was determined by its absorbance at 280 nm using an extinction coefficient (mg/ml) of 0.7 (22). The purity of the GR fractions was established by SDS-PAGE using silver stain for protein detection as well as enzyme-linked immunosorbent assays and Western blot analyses involving anti-Clq GR and CLR monoclonal antibodies.

Preparation of Clq CLR—Clq was digested with pepsin (Sigma) by minor modifications of the method of Reid et al. (24). Briefly, pepsin was incubated with Clq (1:30, w/w) for 20 h at 37 °C, and the CLR was isolated as the second peak obtained upon passage through a fast-protein liquid chromatography Superose 12 analytical column and stored at -70 °C until the time of use. The concentration of CLR was estimated by its absorbance at 275 nm using an extinction coefficient (mg/ml) of 0.21 (25). The purity of CLR fractions was established by SDS-PAGE using silver stain for protein detection as well as enzyme-linked immunosorbent assays and Western blot analyses involving anti-Clq GR monoclonal antibodies.

Clq Peptides—Authentic and modified Clq A chain peptides, as well as those prepared for sequencing, were synthesized by researchers at the enzymatic site of the Clq A chain peptides.

Biota-binding of DNA and Clq—DNA was biotinylated by photoactivatable biotin essentially according to the method of Pierce Chemical Co. Briefly, equal amounts of DNA and photoactivatable biotin were mixed in the dark and incubated 15 min in an ice bath under a long wavelength (350-370 nm) UV light. Equal amounts of 0.1 M Tris-HCl (pH 9.0) and 1-butanol were added, mixed, and centrifuged. The lower phase was washed with 1-butanol, and equal amounts of 0.05 M NaCl and ethanol were added and incubated overnight at -20 °C. The mixture was centrifuged, and the precipitated DNA was dissolved in 0.1 mM EDTA, pH 8.0. Biotinylated Clq was obtained by dialyzing Clq into 0.1 M sodium bicarbonate buffer (pH 9.0), and incubating with N-hydroxysuccinimide long-chain alkaline phosphatase (Promega, Madison, WI) at a ratio of 8:1 (w/w, protein:alkaline phosphatase) for 4 h at room temperature. The mixture was diluted with 2 liters of 10 mM Tris (pH 8.0), and centrifuged. The supernatant was measured at 412 nm.

Complementary Sources—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. (28). Clq-depleted serum was obtained from Sigma. C4-deficient guinea pig serum was the kind gift of Dr. Alexander P. Osmand (Oak Ridge National Laboratory, Knoxville, TN). Guinea pig serum diluted in veronal-buffered saline with gelatin and EDTA was used as the source of terminal complement (C3-9) components.

Total Complement (CH50) Consumption—The CH50 was determined by a modification (29) of the method of Mayer (30). A mixture of 0.1 ml of 1:10 human serum and 0.1 ml of test material (usually DNA) was incubated at 37 °C for 30 min at 37 °C. Two-fold dilutions were prepared, 0.1 ml erythrocytes sensitized with hemolysin (GIBCO; 1 × 106 sensitized erythrocytes/ml) were added, and the mixture was incubated for 30 min at 37 °C. Veronal-buffered saline with gelatin and EDTA (1.0 ml) was added, and, after centrifugation, the optical density of the supernatant was measured on a Bio-Rad spectrophotometer at 412 nm.

Hemolytic Component Assays—C1 (29), C4 (31), and C2 (32) hemolytic activities were measured exactly as described.

Data Presentation—Each experiment performed in duplicate and generally repeated three times. The error bars represent ± 1 S.D.

RESULTS

Complement Activation by DNA—Double-stranded calf thymus DNA preincubated in normal human serum at 37 °C for 30 min followed dose-dependent depletion of total complement and C4 hemolytic activities; approximately 50 μg/ml DNA was required for 50% consumption and 100 μg/ml DNA for complete consumption of C4 in 0.075 M saline. Significant although approximately 50% less consumption also was observed in normal saline. Comparable complement consumption was observed when double-stranded DNA, calf-thymus DNA, or when single-stranded calf thymus DNA, was used. C1 and C2 hemolytic activities also were consumed, indicating activation of the classical complement pathway at the level of C1 (data not shown).

Reactivity of DNA with Intact Clq and the Clq CLR and GR—The interaction of DNA with Clq was investigated since DNA is known to bind to and precipitate with Clq (14, 15). Biotinylated DNA bound to immobilized Clq, and biotinylated Clq bound to immobilized DNA in reactions which were calcium-independent and inversely proportional to the ion strength; binding was optimal at 0.075 M NaCl and decreased by approximately 30% in 0.15 M NaCl. DNA bound to the Clq CLR as well as the GR by direct enzyme-linked immunosorbent and Western blot binding assays. However, even though Agg-IgG binds preferentially to the Clq GR (23), it did not block binding of DNA to the GR (data not shown).

Preferential Binding of DNA to the Clq A Chain—DNA bound preferentially to the A chain of Clq when single-stranded calf thymus DNA was used. C1 and C2 hemolytic activities also were consumed, indicating activation of the classical complement pathway at the level of C1 (data not shown).*
subjected to SDS-PAGE in 13% polyacrylamide minislab gels, trans-

DNA. Color was developed with streptavidin-peroxidase and sub-

volumes of plasmid DNA reduced Clq stained for protein showing typical A

ferred to nitrocellulose paper, and reacted with 50 pg/ml biotinylated electrophoresis by peptides 14-26 and 76-92. In

strate (4-chloro-1-naphthol). Molecular mass standards are in

wells at room temperature, backcoating with BSA (100 µg/ml; 37 °C for 30 min), and washing, biotinylated DNA was added

with 50 pg/ml solution; 37 °C for 30 min), and washing, biotinylated DNA was added

(bottom) with 50 µg/ml peptide (up to 100 µg/ml) in the fluid phase at room temperature for 30 min prior to addition to immobilized Clq (0.5 µg/ml). Peptide 14-26 (20 µg/ml) inhibited about 50% binding of DNA to Clq, while scrambled peptide 14-26 (14-26/S), peptide 14-26 with the prolines replaced with alanines (14-26/A), and peptide 76-92 had no effect.

was greater than that observed with peptide 76-92. Binding was optimal at 0.075 M NaCl, with approximately 55–70% maximal binding observed at 0.15 M NaCl (Fig. 5). Migration of DNA in agarose gel was retarded by peptide 14-26 (Fig. 3A); significant migration retardation was observed with as little as 12.5 µg of peptide 14-26, and retardation increased

resides on the Clq A chain and that linear rather than conformational determinants on Clq are involved in Clq-DNA interaction.

Identification of a DNA Binding Region within Residues 76–92 of the Clq A Chain CLR—Previous investigations suggested that DNA binds to a site within residues 81–97 of at least one of the Clq chains (10), but preliminary studies indicated that this might involve residues 76–92 of the Clq A chain (18); the latter peptide has 2 lysine residues near its N terminus which are not present in the homologous regions of the Clq B or C chains, and it seemed plausible that DNA would bind to this cationic region (18). We tested this possibility directly by utilizing Clq A chain peptides 76–92 and 81–97. DNA bound to peptide 76–92, but not to peptide 81–97 (Fig. 2). Similarly, preincubation with peptide 76–92 but not 81–97 led to retardation of migration of DNA in agarose gels (Fig. 3A), emphasizing reactivity of DNA with these residues of Clq. However, preincubation of DNA with peptide 76–92 did not lead to inhibition of binding of DNA to Clq (Fig. 4), suggesting that an additional or different binding site is involved.

Identification of a Second DNA Binding Region within Residues 14–26 of the Clq A Chain CLR—Since the Clq A chain also contains a cationic region within residues 14–26 not present in the homologous regions of the Clq B or C chains, a peptide identical to this region was synthesized and was found to strongly bind to DNA (Fig. 2); indeed, the binding
as the amount of peptide was increased (Fig. 3B).

**Preferential Involvement of Residues 14-26 in Binding and Precipitation of Clq with DNA**—In contrast to peptides 76-92, peptide 14-26 strongly inhibited binding of DNA to Clq. Preincubation of DNA (20 μg/ml) in the fluid phase with 10 μg/ml or more of peptide 14-26 resulted in inhibition of the ability of DNA to bind to immobilized intact Clq (Fig. 4); as little as 25 μg/ml induced >90% inhibition of binding. Peptide 76-92 lacked this ability in amounts in excess of 100 μg/ml (Fig. 4). These observations are consistent with the hypothesis that A chain residues 14-26 represent the major Clq binding site for DNA.

Furthermore, like intact Clq, peptide 14-26 strongly precipitated with DNA. The line of precipitation which formed between DNA and peptide 14-26 upon double diffusion in agarose gels was comparable to the line of precipitation formed between DNA and intact Clq; the basis for the fusion of these lines is not clear (Fig. 6, left). By comparison, peptide 76-92, which also binds to DNA, did not precipitate with DNA (Fig. 6, left). Peptide 14-26 did not precipitate with multiple other proteins including IgG, CRP, SAP, fibronectin, and BSA (Fig. 6, right), indicating a specificity for the precipitation of peptide 14-26 with DNA. Collectively, these results point to residues 14-26 of the Clq A chain as the major DNA binding region of Clq.

**Inhibition of DNA-initiated Complement Consumption by Peptide 14-26**—Since DNA is an effective activator of the classical complement pathway, the ability of the Clq peptides to inhibit complement depletion was tested. Peptide 14-26 blocked DNA-induced C4 consumption (Fig. 7); as little as 0.2 mg/ml induced about 50% inhibition and 0.6 mg/ml induced <90% inhibition of C4 consumption in 0.075 M NaCl. By comparison, amounts of peptide 76-92 in excess of 1 mg/ml had no inhibitory effect at all. Peptide 14-26 showed comparable inhibition of DNA-induced C4 consumption in normal saline, with 55% inhibition observed with 0.5 mg/ml of the peptide (data not shown). Peptide 14-26 induced comparable inhibition of C1 consumption. However, peptide 14-26 had no effect upon complement consumption initiated by Agg-IgG or immune complexes (data not shown), consistent with the hypothesis that inhibition of DNA-induced complement consumption by peptide 14-26 occurs at the level of DNA binding to Clq.

**Evidence for Sequence and Conformation Specificity, and Not Charge Alone, for the Activity of Peptides 14-26 and 76-92**—Peptides 14-26 and 76-92 both are decidedly cationic, containing 5 and 4 cationic amino acids, respectively. A group of experiments were performed with modified peptides in order to determine whether their reactivity was based on charge alone or involved a sequence/conformation specificity. Replacement of the prolines in peptide 14-26 at positions 17 and 23 with alanines, or scrambling the order of the amino acids, resulted in loss of ability of peptide 14-26 to bind (data not shown) to or precipitate with DNA (Fig. 6, left), inhibit binding of DNA to intact Clq (Fig. 4) or to block DNA-induced complement activation (Fig. 7). These experiments point to a sequence specificity, and not a charge specificity alone, as the basis for the activities of the peptide.

**DISCUSSION**

DNA has been demonstrated to interact with Clq (9, 10, 14, 15) and activate C1 and the classical complement pathway (8, 11–13). In the present study, both double stranded and single stranded DNA as well as DNA from several species was found to activate the complement system. Previous investigators reported that DNA binds to the Clq CLR (9), the Clq GR (16), or to both regions of Clq (10), and binding of DNA to both the CLR and GR was confirmed in the present study. Agg-IgG, which binds to the Clq GR (22), did not block the binding of DNA to the GR, suggesting the GR binding capacity of DNA is attributable to the well appreciated (17, 24) residual CLR tail present in standard GR preparations and inferred to involve residues 81–97 (10, 17). Binding reactivity with DNA was retained after reduction/alkylation and SDS treatment of Clq, and DNA bound preferentially to the A chain in contrast to IgG which binds preferentially to the C chain (18).

These properties suggested that DNA binds to a linear sequence in the region of residues 81–97 of the Clq A chain, and peptides from this region were tested for reactivity with DNA. Peptide 76-92 bound strongly to DNA, while peptide 81–97 did not; neither peptide bound to Agg-IgG. These data implicate residues 76–92, particularly residues 76–81, of the Clq A chain in the binding of DNA and suggest that the 2 lysines at positions 78 and 81 of this cationic region are involved. However, Clq A chain peptide 76–92 failed to block the binding of DNA to intact Clq, perhaps either because the affinity of intact Clq is much higher than that of the peptide or because an additional or different binding site on Clq is involved.

A second positively charged sequence containing 4 arginines and 1 lysine is present at positions 14–26 of the Clq A chain and contrasts with the homologous regions of the B and C chains which contain no arginine groups at all. Peptide 14-
26 was synthesized and found to strongly react with DNA by direct binding and retardation of DNA migration as well as by precipitation with DNA. The basis for the ability of peptide 14–26 but not peptide 76–92 to precipitate with DNA may be attributable to its arginine-rich rather than lysine-rich characteristic, 2 rather than 1 cluster of cationic residues, or a greater binding affinity for DNA. Chemical modification of the Clq arginine residues with cyclohexanedione abolished >85%, while chemical modification of the Clq lysines with methyl acetoacetate abolished only 25%, of the reactivity of Clq with DNA (16), supporting the hypothesis that Clq arginine residues are more important than lysine residues in the Clq-DNA interaction. When peptide 14–26 was preincubated with DNA, it not only blocked the binding of DNA to immobilized intact Clq, but also inhibited DNA-induced complement activation; again, peptide 76–92 lacked both these properties. Collectively, these results indicate that, while Clq A chain sequences 14–26 and 76–92 both contain binding sites for DNA, the 14–26 sequence contains the major DNA binding sites within residues 14–26 and/or 76–92 to remove the proline-glycine bends, resulting in loss of activity of both peptides. This indicated that charge alone was insufficient and that an appropriate amino acid sequence and conformation were needed for the peptides to be optimally reactive with DNA.

Residues 14–26 and 76–92 both are located within the Clq CLR, to which Clr and Cls (2, 3, 6, 7), heparin (33), fibro-nectin (17), bacterial membranes (34), collagen (35), fibrinogen (36), platelets (37), serum amyloid P component (38), and tides, 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, resulted in loss of activity of both peptides. This indicated that charge alone was insufficient and that an appropriate amino acid sequence and conformation were needed for the peptides to be optimally reactive with DNA.

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