Equilibrium Binding Studies of Recombinant Anti-single-stranded DNA Fab

ROLE OF HEAVY CHAIN COMPLEMENTARITY-DETERMINING REGIONS*

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We previously isolated nucleic acid-binding antibody fragments (Fab) from bacteriophage display libraries representing the immunoglobulin repertoire of autoimmune mice to expedite the analysis of antibody-DNA recognition. In the present study, the binding properties of one such anti-DNA Fab, high affinity single-stranded (ss) DNA-binding Fab (DNA-1), were defined using equilibrium gel filtration and fluorescence titration. Results demonstrated that DNA-1 had a marked preference for oligo(dT) (100 nM dissociation constant) and required oligo(dT) > 5 nucleotides in length. A detailed analysis of the involvement of the individual heavy chain (H) complementarity-determining regions (CDR) ensued using previously constructed HCDR transplantation mutants between DNA-1 and low affinity ssDNA-binding Fab (D5), a Fab that binds poorly to DNA (Calcutt, M. J. Komissarov, A. A., Marchbank, M. T., and Deutscher, S. L. (1996) Gene (Amst.) 168, 9–14). Circular dichroism studies indicated that the wild type and mutant Fab studied were of similar overall secondary structure and may contain similar combining site shapes. The conversion of D5 to a high affinity oligo(dT)-binding Fab occurred only in the presence of DNA-1 HCDR3. Results with site-specific mutants in HCDR1 further suggested a role of residue 33 in interaction with nucleic acid. The results of these studies are compared with previously published data on DNA-antibody recognition.

The presence of circulating antibodies that recognize DNA or RNA is diagnostic of certain autoimmune diseases including systemic lupus erythematosus (1, 2). Anti-DNA antibodies are also produced in autoimmune mice and have facilitated the study of systemic lupus erythematosus and nucleic acid-protein interactions. The well developed hybridoma technology for monoclonal antibody production has made possible the production of numerous murine anti-DNA antibodies. Consequently, there is substantial amino acid sequence information on murine anti-DNA antibodies (3–5), although significantly less is known about the exact mechanism of binding and interactions between autoantibodies and DNA. Murine monoclonal anti-DNA antibodies exhibit a high frequency of basic and aromatic amino acid residues in the hypervariable or complementarity-determining regions (CDR) in the antigen binding fragment (Fab) (6, 7). Mutagenesis studies (8, 9) and three-dimensional structure analyses of two Fab-oligo(dT) complexes (10, 11) have substantiated a direct role of these residues in nucleic acid interaction.

In general, the heavy chain (H) is thought to contribute more than the light chain to antigen binding, especially through HCDR3 (12). HCDR transplantation studies of an anti-fluorescein single chain antibody (SCA) 4–4-20 and an anti-ssDNA SCA BV04–01 have analyzed the roles of individual HCDR in combining site formation (13). Data from the transplantation studies indicated that hybrids containing HCDR3 or HCDR1 of SCA BV04–01 bound oligo(dT). No binding occurred, however, when both HCDR1 and -3 of SCA BV04–01 were present in the context of HCDR2 of SCA BV04–01 (13, 14). While studies have demonstrated the importance of HCDR3 in binding nucleic acid (15, 16), the roles of HCDR1 and -2 in this interaction are less clear.

We previously isolated ssDNA-binding Fab from combinatorial bacteriophage display libraries derived from the immunoglobulin repertoire of an autoimmune MRL/lpr mouse (17). Immunological methods were used to characterize two of these Fab, in particular, a high affinity ssDNA-binding Fab (DNA-1) and a Fab (D5) that bound DNA poorly. DNA-1 and D5 were identical in genetic background and were very similar in amino acid sequence, suggesting the Fab may contain similar combining sites. All possible combinations of HCDR transplantation mutants (TM) were previously created between DNA-1 and D5 in order to ascertain the key domains involved in high affinity binding. Results of immunoprecipitation analyses with the HCDR TM suggested HCDR3 was most important in ssDNA binding (18).

In order to gain a better understanding of DNA-1-oligo(dT) interactions, we undertook a thorough examination of the equilibrium binding properties of highly purified DNA-1, D5, HCDR TM, and mutant Fab containing site-specific amino acid substitutions. Fluorescence spectroscopy, as well as equilibrium gel filtration, was used to characterize the binding of Fab to oligo(dT). The results demonstrated that DNA-1 had a marked preference for oligo(dT) of at least 15 nucleotides and bound with a 1:1 stoichiometry. The HCDR transplantation

1 The abbreviations used are: CDR, complementarity-determining region(s); Fab, antigen binding fragment(s); H, heavy chain; SCA, single chain antibody; 4–4-20, anti-fluorescein antibody; ssDNA, single-stranded DNA; BV04–01, anti-ssDNA antibody; DNA-1, high affinity ssDNA-binding Fab; D5, low affinity ssDNA-binding Fab; FR, framework region(s); TM, transplantation mutant(s) Fab; Vn, variable H domain; Cn, constant H domain; PCR, polymerase chain reaction; BSA, bovine serum albumin; dsDNA, double-stranded DNA.
Binding Properties of Anti-ssDNA Fab

Reagents—Gamma Bind Sepharose, Sephadex, oligo(dT)-Sepharose, a Mono-S column, and polynucleotides were purchased from Pharmacia Biotech Inc. The other oligonucleotides were synthesized by the University of Missouri DNA Core Facility, using an Applied Biosystems DNA Synthesizer Model 380B. All oligonucleotides were column-purified and quantitated spectrophotometrically (19). Antibodies were purchased from Pierce. [γ-32P]ATP (3000 Ci/mmol) was purchased from DuPont NEN. All other reagents were obtained from Sigma or Fisher, unless otherwise noted.

Fab Gene Isolation, Construction of HCDR Transplants, and Site-specific Mutagenesis—The DNA-1 and D5 genes were previously isolated from an expression library, derived from autoimmune MRL/lpr mice immunoglobulin G (IgG) cDNA, contained in plasmid pComb3 (17). HCDR transplants were constructed between DNA-1 and D5 as described previously (18). The chimeric Fd (V_{\text{H}} + C_{\text{H1}}) DNAs were subcloned into pComb3 containing DNA-1 light chain.

VSX, a derivative of the plasmid pBC containing a chloramphenicol-resistance gene (Stratagene) and lacking the V_{\text{S}} and Sd restriction enzyme sites, was used as an intermediate vector for mutagenesis and sequencing procedures. Site-directed mutagenesis was performed using splice overlap PCR (20). Typically, two fragments of DNA-1 or D5 H DNA in VSX were amplified in two separate reactions using standard PCR conditions, resulting in fragments overlapping by at least 10 nucleotides. Full-length Fd (V_{\text{H}} + C_{\text{H1}}) products were generated by annealing and extension of the two overlapping fragments and were amplified by 25 cycles of PCR following the addition of flanking T3 and T7 primers. The final products were purified, cut with Xhol (Boehringer Mannheim) and VspI (Life Technologies, Inc.) restriction enzymes, and ligated into similarly cut DNA-1- or D5-VSX plasmid. Having verified the desired mutation had been introduced, an Xhol-BamHI fragment containing the V_{\text{H}} region was subcloned into Xhol-BamHI cut DNA-1-pComb3 or D5-pComb3, thus replacing the wild type sequence. Using this two-step procedure, only a 140-nucleotide fragment derived from PCR was present in the final construct, thus reducing the amount of DNA sequencing necessary to check for mutations caused by Taq DNA polymerase (Perkin-Elmer) errors. The correct mutation was verified by DNA sequencing using a Sequenase 2.0 kit (U. S. Biochemical Corp.). The relevant H amino acid sequences of DNA-1, D5, TM, as well as site-specific mutants are shown in Fig. 1.

Bacterial Expression and Purification of Fab—Cells from a single colony of Fab-expressing Escherichia coli DH12S (Life Technologies, Inc.) were grown, treated, and disrupted as described previously (18). The lysate was clarified by centrifugation at 32,540 × g for 30 min, and the resulting supernatant was applied to a 10-ml goat IgG anti-mouse Fab affinity column that had been pre-equilibrated with 50 mM phosphate buffer, pH 7.0. The column was washed with 0.5 mM phosphate, 0.5 mM citrate buffer, pH 4.7, and the Fab were eluted with the same buffer at pH 2.8, immediately neutralized, and dialyzed against 50 mM Tris-HCl, pH 7.0. The dialyzed preparations were passed over a Mono-S cationic exchange column and eluted with a 0–250 mM NaCl gradient. Fractions from the different steps were analyzed by 10% SDS-polyacrylamide gel electrophoresis (21) and were stained with Coomassie Blue. Protein concentration was determined by the method of Bradford (22) and further verified by the calculation of extinction coefficients. UV absorption spectra were characterized on a Hitachi U 2000 spectrophotometer (λ apan).

Circular Dichroism—Circular dichroism (CD) was used to monitor the overall secondary structure of the purified Fab using an Aviv 62DS spectropolarimeter equipped with a temperature control and stirring unit. The spectra were collected from samples in 0.1-cm cuvettes at 25 °C in 50 mM phosphate buffer, pH 7.0. Protein concentration ranged from 0.2 to 0.8 mg/ml.

Equilibrium Gel Filtration—A direct assessment of the binding of oligo(dT) to Fab was determined using equilibrium gel filtration (23). Solutions of DNA-1 were preincubated for 30 min at 4 °C with a predetermined concentration of [32P]-labeled oligonucleotide (18) in 50 mM Tris-HCl buffer, pH 7.0, with 0.12 M NaCl plus BSA (0.2 mg/ml). Sephadex G-50 columns (3 ml) were equilibrated with two-column vol-
RESULTS

DNA-1 Purification—Spectral analysis of DNA-1 and D5 obtained by affinity chromatography (17) indicated that these preparations may contain nucleic acid. Therefore, an additional cation exchange fast protein liquid chromatography purification step was used in the present study to obtain highly purified Fab preparations. The Fab were purified to apparent homogeneity, as evidenced by SDS-polyacrylamide gel electrophoresis, and had a homogeneity, as evidenced by SDS-polyacrylamide gel electrophoresis step was used in the present study to obtain highly concentrated DNA-1. SDS-polyacrylamide gel electrophoresis analysis estimated from data of sedimentation analysis obtained by supplier.

Fluorescence Titration—The fluorescence titration experiments were carried out using a SLM 8100 spectrophotometer interfaced to a DELL/433 PC running SLM AINCO 8100 series 2 software. The titrations were performed at 20 °C with varying amount of nucleic acid added to a fixed Fab concentration (4–200 nM) in 2 ml of TBS. Protein fluorescent emission was measured as described by Kim et al. (24) with 292 nm excitation and 346 nm emission (1-cm cuvette path length). The excitation and emission bandwidths were set at 4 nm. Each reading was collected for 5–20 s after a 0.5–1 min equilibration. Values reported are an average of at least three independent measurements. All experiments were corrected for dilution and photobleaching.

Calculation of Binding Parameters—The equilibrium dissociation constant (K_d) values for DNA-1, D5, and the mutant Fab were determined from binding curves derived from titration or equilibrium gel filtration profiles, using a single binding site curve-fitting procedure in Kaleidagraph (Abelbeck Software).

Optimal Ligand Size—The optimal oligo(dt) length for DNA-1 binding was evaluated by both fluorescence titration and equilibrium gel filtration techniques. Equilibrium gel filtration was used as an alternative technique to directly monitor complex formation. As shown in Fig. 3, DNA-1 bound with the same affinity to oligo(dt) 15 nucleotides or greater in length, with a K_d of approximately 140 nM, although binding was observed with (dT)_{10}, as well. A similar ligand size was obtained for DNA-1 using equilibrium gel filtration (Table I).

Binding Stoichiometry and Reversibility—To evaluate the stoichiometry of binding of (dT)_{20}, (dT)_{15}, and (dT)_{10} fluorescence titration experiments were performed with DNA-1 concentrations in excess of the K_d value. Typical fluorescence titration curves for (dT)_{20} and (dT)_{15} are shown in Fig. 4A. The stoichiometry of binding was found to be 1 mol of oligonucleotide per mol of DNA-1. For comparison, the binding of ^32P-labeled (dT)_{20} (dT)_{15}, and (dT)_{10} to the same DNA-1 preparation was also measured using equilibrium gel filtration (Fig. 4B and Table I). Quantitation of bound oligo(dt), determined from the results of equilibrium gel filtration, yielded dissociation constants and stoichiometric values that were consistent with the binding parameters determined from fluorescence titration experiments (Table I).

The DNA-1-oligonucleotide complex was examined for ionic strength sensitivity using fluorescence titrations. The initial fluorescence signal was almost completely recovered with NaCl concentrations greater than 1 M, establishing that the changes in DNA-1 fluorescence observed upon the addition of oligo(dt) were the result of a reversible interaction (data not shown). The dissociation of the complex at high salt concentrations is also indicative that aggregation or precipitation had not occurred. The same effect probably led to elution of DNA-1 from oligo(dt)-Sepharose (mentioned previously).

Characterization of HCDR TM—All possible HCDR TM between DNA-1 and D5 (Fig. 1) were constructed previously (18), expressed in E. coli, and purified as described under “Experimental Procedures.” SDS-polyacrylamide gel electrophoresis analysis indicated the resulting purified Fab preparations were homogeneous (data not shown). Results of UV absorption spectroscopy demonstrated the absence of nucleic acid impurities. The CD spectra of purified DNA-1, D5, and HCDR TM were analyzed in order to verify the conservation of secondary structure of the Fab. Comparison of the CD spectra (Fig. 5) indicated very similar amplitudes and shapes for all Fab investigated, typical for proteins with β-sheet structure (25, 26). Therefore,
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from a combinatorial bacteriophage Fab display library generated from autoimmune sources (17). In the present study, both fluorescence quenching and equilibrium gel filtration techniques were employed to evaluate the binding parameters of DNA-1 and TM under solution equilibrium conditions.

Fluorescence titrations were performed with polynucleotides of different base composition to examine the specificity of DNA-1-ligand interactions. The results demonstrated that DNA-1 preferred poly(dT). A similar selectivity has also been observed for the ssDNA binding proteins P13 (27) and gsp (28), and it was proposed that the affinity of these interactions was inversely related to nucleotide stacking in the free homopolymer. Thus, the $K_d$ values of DNA-1 were less for poly(dT) and poly(U) presumably because they have the most unstacked structure.

Binding, as monitored by fluorescence quenching titration, indicated the optimal ligand for DNA-1 Fab interaction was approximately 15 nucleotides, although smaller oligo(dT) could bind with reduced affinity. DNA-1 bound to oligo(dT)$_n$ ($n \geq 15$) with an affinity of 100–150 nM and a stoichiometry of 1 mol of oligonucleotide per mol of Fab. As expected, there was a significant decrease in the affinity of DNA-1 for oligo(dT) in the presence of high salt concentrations. Thus, formation of the DNA-1-oligonucleotide complex was reversible and not a result of aggregation. The $K_d$ values and stoichiometry of the complex obtained by fluorescence measurements were verified by equilibrium gel filtration experiments. These results were similar to those reported for anti-ssDNA Fab BV04–01, which had been shown to contain a binding site size of 6–8 nucleotides and a $K_d$ of 450 nM for (dT)$_9$ (29). X-ray crystallographic data obtained for a complex of BV04–01 with (dT)$_3$ led to the conclusion that the antigen combining site was a shallow cleft (11). DNA-1 and BV04–01, while varying in CDR amino acid sequence, had similar binding properties for ligands of the same repetitive structure, perhaps reflecting similarly shaped combining sites.

HCDR TM were constructed between DNA-1 and D5, in order to examine the importance of HCDR in ssDNA-binding. These Fab were chosen for HCDR transplantation because they have different binding properties, identical genetic background, very similar sequence, and perhaps, similarly shaped combining sites. The suggestion that DNA-1 and D5 contain similarly shaped combining sites is supported by models of numerous anti-DNA Fab derived from x-ray crystal studies (30) and from molecular modeling of DNA-1 and D5. Immunoprecipitation methods were used previously to analyze the TM and results indicated numerous CDR combinations, particularly those including HCDR3 of DNA-1, allowed for immune complex precipitation. However, the TM did not show a strict specificity for oligo(dT) in that other nucleic acids including U1RNA were also recognized (18). That additional components in the immunoprecipitation reactions (i.e. anti-Fab antibody) may have acted to artificially stabilize or destabilize the TM-nucleic acid complexes remained to be determined.

In the present study, more direct methods of binding analysis, including fluorescence quenching and equilibrium gel filtration, were used to analyze the HCDR TM. Highly purified HCDR TM were monitored by CD spectroscopy prior to equilibrium binding analyses. Results indicated there were no significant differences in CD spectra, indicative of the conservation of the secondary structure of the Fab. HCDR3 of DNA-1 in the context of all other HCDR combinations of D5 or DNA-1 resulted in the ability of the Fab to bind oligo(dT) with a similar affinity as that of native DNA-1. The TM did not bind well to U1RNA, in contrast to results with immunoprecipitation binding assays (18). The HCDR1 and 2 of DNA-1 in the context of HCDR3 of D5 or DNA-1 did not drastically affect binding to oligo(dT), suggesting less involvement of these HCDRs in direct interaction. However, the transplantation of HCDR1 from D5 into DNA-1 was accompanied by more than a 50% increase in the maximal value of fluorescence quenching and was due to an additional Trp residue present in HCDR1 of D5. Taken together, the results of the transplantation experiments demonstrated a critical involvement of HCDR3 of DNA-1 in the direct binding of oligo(dT) and, furthermore, suggested the possible involvement of residue 33 of HCDR1 in complex formation.

These data are consistent with previous studies of HCDR3 grafting, which also illuminated the contribution of this region to Z-DNA (15) and dsDNA (16) binding. HCDR transplantation studies with an anti-fluorescein SCA 4–4–20 (pocket-shaped combining site) and the anti-ssDNA SCA BV04–01 (claw-shaped combining site) resulted in binding with the presence of any two HCDR from the same SCA, tandemly arranged. Transplantation of HCDR1 from SCA 4–4–20 to SCA BV04–01 led to significant changes in CD spectra of the mutants, reflecting possible changes in their secondary structure. These results, and subsequent studies of the stability of SCA 4–4–20 and SCA BV04–01 TM to denaturation (31), were explained from the point of view that there were drastic differences in the binding site shapes and charge distribution in the combining sites of the constructed TM.

Our results on HCDR transplantation were obtained with TM of similar sequence, secondary structure, and probable combining sites. Therefore, our data may be interpreted in terms of the retention of combining site conformation after CDR transplantation, suggesting the observed differences in oligo(dT) binding were probably due to the contribution of the

| Ligand | $K_d$ (nm) | $Q_{max}$ (%) | $K_d$ (nm) | $Q_{max}$ (%) |
|-------|-----------|--------------|-----------|--------------|
| (dT)$_{10}$ | 20,000 | ND | >20,000 | ND |
| (dT)$_{15}$ | >20,000 | ND | >20,000 | ND |
| (dT)$_{20}$ | >20,000 | ND | >20,000 | ND |

$Q_{max}$: maximal quenching expressed in % of initial Fab fluorescence.

$a$: Represents CDR from D5 Fab; $b$: Represents CDR from DNA-1 Fab.

$^c$: Value exceeded the limit of detection.

$^d$: Not determined.
individual HCDR in ligand interaction. X-ray crystallographic structural investigations are now in progress with liganded DNA-1 and should provide sufficient data to elucidate the combining site structure. A molecular model of DNA-1 has been constructed based on the crystal structure of the mouse monoclonal antibody R19.9, which contains very similar framework sequences and CDR lengths (32). Analysis of the model indicates that Tyr-97 and Arg-98 (Fig. 1) are at the apex of HCDR3 of DNA-1 and are likely to be most crucial for DNA binding. The importance of the individual amino acids of DNA-1 HCDR3 in ligand interaction, however, awaits future mutagenesis and binding studies.

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