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DNA Binding by the VH Domain of Anti-Z-DNA Antibody and Its Modulation by Association of the VL Domain

Yan Chen and B. David Stollar

mAb Z22 is a highly selective IgG anti-Z-DNA Ab from an immunized C57BL/6 mouse. Previous studies showed that heavy chain CDR3 amino acids are critical for Z-DNA binding by the single chain variable fragment (scFv) comprising both V region heavy chain (VH) and V region light chain (VL) of mAb Z22 and that the VH domain alone binds Z-DNA with an affinity similar to that of whole variable fragment (Fv). To determine whether Z-DNA binding by VH alone and by Fv involves identical complementarity determining region residues, we tested effects of single or multiple amino acid substitutions in recombinant VH, scFv, and associated VH-VL heterodimers. Each recombinant protein was a fusion protein with a B domain of Staphylococcal protein A (SPA). Z22VH-SPA alone was not highly selective; it bound strongly to other polynucleotides, particularly polypyrimidines, and ssDNA as well as to Z-DNA. In contrast, scFv-SPA or associated VH-VL dimers bound only to Z-DNA. VL-SPA domains bound weakly to Z-DNA; SPA alone did not bind. Introduction of multiple substitutions revealed that the third complementarity determining region of the heavy chain (CDR3H) was critical for both VH and scFv binding to Z-DNA. However, single substitutions that eliminated or markedly reduced Z-DNA binding by scFv instead caused a modest increase or no reduction in binding by VH alone. Association of VH-SPA with Z22VL-SPA restored both the effects of single substitutions and Z-DNA selectivity seen with Fv and intact Ab. Polypyrimidine and ssDNA binding by the isolated VH domain of immunization-induced anti-Z-DNA Ab resembles the activity of natural autoantibodies and suggests that VH-dependent binding to a ligand mimicked by polypyrimidines may play a role in B cell selection before immunization with Z-DNA.


A nt-DNA Abs are a hallmark of systemic lupus erythematosus and serve as markers for diagnosis and prognosis (1). Knowledge of how these Abs bind to various forms of nucleic acids (2) and how some of them cross-react with non-nucleic acid substances (3–5) helps us to understand the formation of immune complexes and their pathogenic disposition in various organs (6, 7). Analysis of anti-DNA Abs induced by immunization with nucleic acid-protein complexes also helps to evaluate potential mechanisms by which disease-related autoantibodies are formed (8) and how Ab protein recognizes nucleic acid structure (9).

Crystallographic analysis has provided important insights into heavy (H) and light (L) chain contributions to Ag binding and has revealed that all six complementarity determining region (CDR) residues of both H and L chains provide contacts for Ag binding by most Abs (10). The third CDR of the H chain (CDR3H) domain provides contact sites in all Abs and, in most cases, it provides more contacts than the other CDR loops (10). That is true for an anti-DNA autoantibody that has been crystallized with bound oligonucleotide (dT), (11), and is predicted to be true for other anti-nucleic acid Abs that have been crystallized in the unliganded form (12).

Crystal structures are not available for most Abs, but information about their important binding site amino acids can be inferred, with caution, from experiments involving directed mutagenesis. Many anti-DNA autoantibodies contain Arg residues at relatively high frequency in CDR sequences (13, 14) and mutagenesis studies have shown that these residues are often important for DNA binding (15). In many anti-DNA autoantibodies, the V region H chain (VH) plays a dominant role, determining nucleic acid binding even when combined with various V region L chains (VL) domains (16). One strong anti-dsDNA Ab was affinity labeled only in the H chain by oligonucleotide (17); and, in the cases of several anti-DNA Abs, the VH domain alone can bind to nucleic acid Ags (18–21). Recently we found that VH domains coded by cDNA from PBLs of healthy human adults (and therefore not known to be derived from anti-DNA Abs) could bind ssDNA and poly(dT) (22).

One of the DNA-binding VH domains is that of mAb Z22, obtained from a C57BL/6 mouse immunized with Z-DNA-MBSA complexes (20). mAb Z22 is an IgG mAb highly selective for Z-DNA. It binds to Z-DNA of varying base sequence, probably interacting in part with the phosphate backbone of the helix (23). Recombinant single chain variable fragment (scFv), containing both VH and VL domains of Z22, has the same affinity, selectivity, and Id as the parent Ab (20). Swapping of domains of rZ22 scFv with those of other IgGs revealed that a correct VH-VL combination is required for Ag binding within the context of Z22 variable fragment (Fv) (24) and that CDR3H residues play a critical role in that binding. Substitution of just one or a few CDR3H residues either eliminates Ag-binding by the Fv or modifies its selectivity to yield autoantibody-like polyreactivity with ssDNA and dsDNA as well as Z-DNA (25). Moreover, the Z22 VH domain alone can bind the Z-DNA form of poly(dG-dC) with approximately the same affinity.
as the scFv or Fab. Like the parent Ab, the VH domain does not bind the B form of poly(dG-dC) (20).

These findings raise the question of whether Z-DNA binding by the VH domain of Z22 is the same as or differs fundamentally from binding by the Fv domain. We first compared Ag-binding selectivity of VH and Fv domains. Then, to test whether Z22 CDR3H is a critical region for DNA binding in VH as it is in Fv, and whether the same amino acids are involved in DNA binding by both VH alone and VH within Fv, we compared the effects of several amino acid substitutions on binding by both VH and Fv domains. We discuss implications of natural autoantibody-like activity of the isolated VH domain.

Materials and Methods

Construction, expression, and purification of Z22 VH and substituted VH proteins

Bacterial plasmid vectors plg16 and plg20, designed for expression of scFv containing original or substituted VH and VL Z22 domains, and plg16VH and plg20VH (with only the VH domain), were reported previously (20, 24, 26). DNA fragments coding for VH domains with one to four substitutions on binding by both VH and Fv domains. We discuss implications of natural autoantibody-like activity of the isolated VH domain.

Transformation of Escherichia coli BL21(DE3)pLysE (27) with plg16- or plg20-based constructs of VH, mutant VH, scFv, and mutant scFv, identification of isopropylthiogalactoside-sensitive colonies, induction of recombinant protein synthesis, and purification of the products were performed as described previously (21, 24). Samples of 1.5 μg of purified recombinant proteins were subjected to SDS-PAGE with 2-ME for analysis of the size and purity of products (21). Gels were stained with Coomassie blue.

Ags for binding assays

Poly(dA), poly(dT), poly(dU), and poly(dG-dC) were purchased from Pharmacia (Piscataway, NJ). (dC)80 was synthesized by the Protein Resource Laboratory at Tufts University School of Medicine (Boston, MA). The Z-DNA form of poly(dG-dC) was prepared and stabilized by bromination as described (28) and dialyzed against PBS at 4°C overnight. Calf thymus dsDNA from Sigma (St. Louis, MO) was used as dsDNA and was boiled for 10 min and chilled on ice for preparation of ssDNA.

Poly nucleotide binding by recombinant scFv, modified scFv, VH, and modified VH proteins

Binding of recombinant proteins to various polynucleotides was tested by ELISA as described (29). Wells of UV-irradiated microtiter plates (Immulon I, Dynatech, Alexandria, VA) were coated with polynucleotides at a concentration of 2 μg/ml in PBS. Rabbit IgG, 1 μg/ml in PBS, was used to detect the SPA domain of recombinant protein. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN), diluted 1:6000, was used to detect bound rabbit IgG. All reaction volumes were 100 μl. A410 of 0.5 was chosen because it was near the endpoint but still in the linear portion of parallel titration curves. In competitive ELISA, Z22 VH was incubated with polynucleotides in solution for 30 min and the mixture was added to wells coated with Z-DNA or ssDNA. In all ELISA experiments, Ab fragments were incubated in Ag-coated wells for 1 h. Each Ab fragment concentration was tested in duplicate, and the values were compared to a standard curve. Relative binding activity, a measure of binding per unit protein concentration, was calculated based on the concentration required to reach an A410 of 0.5. The value of 0.5 was chosen because it was near the end-point but still in the linear portion of parallel titration curves. In competitive ELISA, Z22 VH was incubated with polynucleotides in solution for 30 min and the mixture was added to wells coated with Z-DNA or ssDNA. In all ELISA experiments, Ab fragments were incubated in Ag-coated wells for 1 h. Each Ab fragment concentration was tested in duplicate, and experiments were repeated two or three times, as indicated in figure legends.

Thrombin cleavage of plg20 VH-SPA fusion protein

A total of 200 μg of rVH-SPA fusion protein in 500 μl of PBS with 2.5 mM CaCl2 was digested with 3.5 μg of thrombin (17.5% W/W; Hematologic Technologies, Essex Junction, VT) at 25°C for 2 h. EDTA was added to a final concentration of 5 mM to stop the reaction. The mixture of
cleaved SPA and VH was mixed with 200 μl of IgG-Sepharose beads and rocked overnight at 4°C. On centrifugation, the VH was in the supernatant; the SPA fragment was bound to sedimented beads and was eluted from them with 0.1 M acetic acid, pH 3.4.

**Gel filtration of VH- and VH-Z-DNA complexes**

In a test of the VH-SPA domain size under nondenaturing conditions, 10 μg of recombinant protein in 1 ml of PBS was passed through a polyacrylamide P100 (Pharmacia) size-exclusion column (1.4 × 48 cm) equilibrated with PBS, pH 7.2. Protein was washed through the column with 80 ml of PBS, and fractions of 1 ml were collected. For detection of recombinant protein, 50-μl samples of each fraction were coated on wells of a microtiter plate, and the immobilized protein was detected through its SPA domain with rabbit IgG and alkaline phosphatase as in the ELISA (21). The column had been calibrated with samples of 500 μg of each of albumin (M, 66,000; Sigma), carbonic anhydrase (M, 29,000; Sigma) and cytochrome c (M, 12,400; Sigma).

To measure soluble complexes, we incubated an excess (100 μg) of Z-DNA with 25 μg of rVH-SPA in 1 ml of PBS for 1 h at room temperature and passed the mixture through the Sephacryl S100 size-exclusion column (1.4 × 48 cm). Fractions of 1 ml were collected, and recombinant protein was detected by ELISA with rabbit IgG and enzyme-conjugated anti-rabbit IgG as described above. The fraction of VH that bound to Z-DNA was calculated as the ratio of A_{410} readings at the void volume peak (VH complexed to Z-DNA) to the sum of the A_{410} readings for the void volume and the included peak (free VH).

**In vitro association of VH and VL**

A total of 400 μg of rVH-SPA and rVL-SPA fusion proteins were mixed and incubated at room temperature for 1 h. The mixture was dialyzed in PBS at 4°C overnight and applied to a Sephacryl S100 (3.5 × 56 cm) size-exclusion column. Then, 240 ml of PBS was collected in 5 ml fractions for the first 80 ml and 2-ml fractions for the following 160 ml. The A_{280} reading for each fraction provided the size distribution of protein in the VH plus VL mixture. Samples of 10 μl from several fractions were analyzed for VH and VL content by Western blotting. Rabbit IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG were used for detection of recombinant protein on the blots.

**Results**

**Expression and characterization of recombinant Abs**

In previous studies, soluble recombinant scFv and VH fragments of anti-Z-DNA Ab Z22 retained the affinity of the parental Fab (20). The Fab, scFv, and VH fragments all bound the Z-DNA but not the B-form of poly(dG-dC) (20). In the present study, DNA fragments coding for VH domains with directed substitutions (Table I) were cloned into plg16H- or plg20H-based vectors for the expression of VH-SPA domains alone. The plg20 and plg16 products differ only by the presence of a thrombin cleavage site between the Ig and SPA domains in the plg20 form. DNA sequencing of scFv and VH mutants confirmed that expected mutations were present and the correct reading frame was intact.

All scFv, scFv mutants, VH, and VH mutants were produced by transformed *E. coli* BL21(DE3)pLysE cells as soluble fusion proteins, each linked to one B domain of SPA. The SPA served as a tag for purification of recombinant proteins by IgG-Sepharose. Proteins were boiled in SDS and 2-ME buffer and separated on 15% SDS-PAGE. Marker proteins (left) are in a prestained protein ladder (LifeTechnologies, Grand Island, NY). The N99K, H3M18, and H3M34 VH proteins are slightly larger than the others because a larger PCR primer (including several bases of the C region) was used in the original cloning of this domain into the scFv (25).

To determine their size distribution under nondenaturing conditions, we passed recombinant proteins through a polyacrylamide P100 column calibrated with a set of standard proteins ranging from 12.4 to 66 kDa. The VH domain began to emerge at a position corresponding to a 20-kDa monomer, and more than 90% of the wild-type Z22 VH protein emerged in a large peak in a lower volume than that for cytochrome c (Fig. 24). No aggregated material was detected.

**Binding of Z22 VH to soluble Z-DNA**

The ability of VH protein to bind to Z-DNA in solution was tested by incubation of a mixture of 100 μg of Z-DNA (determined in preliminary experiments to be an excess) and 25 μg VH in 1 ml of PBS at room temperature for 1 h, followed by analysis with a Sephacryl S-100 column. Free VH-SPA protein was entirely in the included volume. Approximately 19% of the Z22 VH protein formed a stable complex with polynucleotide by this measurement, emerging at the void volume with Z-DNA (Fig. 3, A and B). The amount of protein in the complex was increased to 32% by incubation of the VH and DNA mixture at 37°C for 3 h before it was loaded onto the column. When VH protein with a S98A mutation
was incubated with 50 μg of Z-DNA, 23% of the protein emerged with the Z-DNA.

Binding of Z22 scFv and VH to polynucleotides

Fab or scFv of Ab Z22 bind selectively to Z-DNA, but not to dsDNA or ssDNA (20). In the present study, a high concentration of scFv did show weak binding to poly(dT) but not to several other synthetic polynucleotides. In contrast, Z22VH alone was much less selective. It showed strong binding to Z-DNA, poly(dT), poly(dC), and poly(dU), moderate binding to ssDNA, and weak binding to dsDNA. It did not bind to poly(dA) or the B-form poly(dG-dC) (Table II). In competitive ELISA, testing interactions in solution, Z-DNA was the most effective competitor among the soluble polynucleotides tested, whether Z-DNA or ssDNA was the immobilized Ag (Fig. 4).

VH domains from both pIg16 and pIg20 were expressed and tested for Ag binding. The thrombin site residues in pIg20 did not affect selectivity of polynucleotide binding (not shown). In a previous study, the VL-SPA domain alone did not bind to Z-DNA (20). A newly prepared sample used in the current experiments did bind the DNA when used at concentrations six times higher than those required with the VH-SPA domain. Even at the higher concentrations, the Z22 VL-SPA domain with F94L and F96W substitutions did not bind any of the polynucleotides tested; nor did the VL domain of a closely related anti-Z-DNA mAb, Z44, or that of an anti-guanylate mAb.

Table II. Relative polynucleotide binding activity of Z22 scFv and VH proteins

<table>
<thead>
<tr>
<th>Polynucleotides</th>
<th>Z22 scFv-SPA</th>
<th>Z22 VH-SPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-DNA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>dsDNA</td>
<td>–</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>ssDNA</td>
<td>–</td>
<td>0.53 ± 0.06</td>
</tr>
<tr>
<td>poly(dA)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>poly(dC)</td>
<td>–</td>
<td>0.72 ± 0.11</td>
</tr>
<tr>
<td>poly(dT)</td>
<td>0.002</td>
<td>0.89 ± 0.14</td>
</tr>
<tr>
<td>poly(dU)</td>
<td>–</td>
<td>0.79 ± 0.14</td>
</tr>
<tr>
<td>poly(dG-dC)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Reactions of various concentrations of recombinant Z22 scFv-SPA and Z22 VH-SPA were tested with polynucleotides by ELISA. Relative binding activity was based on the recombinant protein concentration required to yield an A410 of 0.5, normalized to the concentration required for reaction with Z-DNA (0.6 nM for Z22 scFv and 0.25 μM for Z22 VH). Values are the mean ± SD from three experiments. ELISA values were determined in duplicate in each experiment. Dashes indicate there was no reaction at the highest concentration tested (0.3 μM for Z22 scFv-SPA and 1 μM for Z22 VH-SPA).
Each protein.

Activity was based on the recombinant protein concentration required to yield an A

VH domains, were tested by ELISA with immobilized Z-DNA. Relative specific

tion tested (0.3 m

VH proteins a

and applied to a Sephacryl S-100 size-exclusion column, most of

major peak had equimolar amounts of VH and VL, indicating that

VH-VL heterodimer (Fig. 5

the protein emerged as one major peak corresponding in size to a

VH-only form showed low selectivity, like the unmodified Z22 VH (data not

showed). Two framework mutations, N28S (FR1) and I116T (FR4),

did not affect the binding activity of scFv and caused modest

reduction, respectively, in scFv binding of Z-DNA (25), N99K

effect on Fv binding than on VH binding activity. Whereas single

substitutions, VH binding activity was much higher than that of

polynucleotides (Fig. 7, A). Association with VL similarly increased the selectivity

C

erodimer bound only to Z-DNA and not to other polynucleotides

of a VH domain containing a single S98A substitution (Fig. 7, A)

), the associated Z22VH-Z22VL het-

). Thus, with the mutant proteins

the soluble recombinant V domains are able to assemble in solution into a stable 1:1 complex (Fig. 5B). The same experiments were also performed with the modified VH domains S98A and N99K. S98A VH associated with Z22 VL as effectively as did wild-type Z22 VH. However, N99K VH associated poorly with Z22 VL; only a small percentage of the protein mixture was in the heterodimer peak.

The isolated VH-VL heterodimer fractions were tested for binding to polynucleotides. Like Z22 Fab and scFv (Fig. 6, A and B), and unlike VH alone (Fig. 6D), the associated Z22VH-Z22VL heterodimer bound only to Z-DNA and not to other polynucleotides (Fig. 6C). Association with VL similarly increased the selectivity of a VH domain containing a single S98A substitution (Fig. 7, A and B). As was true for corresponding scFv products, the sp. act. (A

per unit protein) of S98AVH-Z22VL was lower than that of Z22VH-Z22VL. Isolated N99K VH-Z22VL heterodimer, like the N99K-containing scFv, did not bind to either Z-DNA or to other polynucleotides (Fig. 7, C and D). Thus, with the mutant proteins as well as with unmodified Z22 domains, association with VL

Figure 5. A. Gel filtration of a preincubated mixture of 400 µg Z22VH-SPA plus 400 µg VL-SPA protein mixture through a Sephacryl S-100 HR size-exclusion column (3.5 × 56 cm), monitored by A

VH-SPA (0.25 µM). Dashes indicate there was no reaction at the highest concentration tested (0.3 µM for Z22 scFv-SPA and 1 µM for Z22 VH-SPA). All ELISA values in this study were determined in duplicate in each experiment.

Average and standard deviation from three experiments.

Data for scFv from Ref. 20.

Values for scFv determined in this study are the average of two experiments for each protein.

Table III. Relative Z-DNA binding activity of modified Z-22 scFvs and VH proteins*  

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Z22 scFv-SPA (relative sp. act.)</th>
<th>Z22 VH-SPA (relative sp. act.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scFv/VH</td>
<td>1e</td>
<td>1d</td>
</tr>
<tr>
<td>Q95E</td>
<td>0.3e</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Q95D</td>
<td>0.3e</td>
<td>0.62 ± 0.22</td>
</tr>
<tr>
<td>Q95H</td>
<td>0.5f</td>
<td>2.4 ± 1.14</td>
</tr>
<tr>
<td>Y97F</td>
<td>0.37f</td>
<td>0.84 ± 0.21</td>
</tr>
<tr>
<td>S98A</td>
<td>0.08f</td>
<td>1.46 ± 0.4</td>
</tr>
<tr>
<td>N99Q</td>
<td>1.0f</td>
<td>0.89 ± 0.76</td>
</tr>
<tr>
<td>N99K</td>
<td>&lt;0.001f</td>
<td>2.43 ± 0.55</td>
</tr>
<tr>
<td>Y100F</td>
<td>1e</td>
<td>0.35 ± 0.16</td>
</tr>
<tr>
<td>H3M18</td>
<td>0.005f</td>
<td>1.39 ± 0.39</td>
</tr>
<tr>
<td>H3M34</td>
<td>0.08f</td>
<td>1.0 ± 0.27</td>
</tr>
<tr>
<td>CDR3HM1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDR3HM2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Reactions of various concentrations of recombinant proteins, as either scFv or VH domains, were tested by ELISA with immobilized Z-DNA. Relative specific activity was based on the recombinant protein concentration required to yield an A


c

b

H3M34 0.08f 1.0 ± 0.27

CDR3HM1 – –

CDR3HM2 – –

A

B

C

D

Extensive modification of the CDR3H segment, replacing four residues by Ala and two residues by Asp, eliminated polynucleotide binding activity of both the scFv and the VH domains (Table III), indicating that CDR3H contributes to binding by both proteins. Previous studies showed that a single N99K substitution in the scFv context totally eliminated Z-DNA binding, indicating the importance of N99 for Z22 activity. However, more than that Asn residue is required, as neither the scFv nor the VH with the mutant DAAANAAAMDY sequence bound to any of the polynucleotides. Substitution of three or four amino acids (H3M18 and H3M34), including N99, caused a marked loss of binding by Fv but no reduction in binding by VH (Table III).

Certain single amino acid substitutions also had a very different effect on Fv binding than on VH binding activity. Whereas single N99K or S98A substitutions caused total elimination or a 10-fold reduction, respectively, in scFv binding of Z-DNA (25), N99K caused a modest increase in polynucleotide binding by VH alone, and S98A caused no reduction (Table III). Therefore, with these substitutions, VH binding activity was much higher than that of scFv. The Y100F substitution caused a modest reduction in VH binding but not in Fv binding. Several other substituted domains retained high binding activity both in the form of VH alone and in the scFv (Table III). All of the substituted domains in the VH-only form showed low selectivity, like the unmodified Z22 VH (data not shown). Two framework mutations, N28S (FR1) and I116T (FR4), did not affect the binding activity of scFv and caused modest changes (less than twofold) in VH domain binding (data not shown).

Direct tests for modulation of polynucleotide binding by Z22 VL

When equal amounts of VH-SPA and VL-SPA were mixed in vitro and applied to a Sephacryl S-100 size-exclusion column, most of the protein emerged as one major peak corresponding in size to a VH-VL heterodimer (Fig. 5A). Western blotting showed that the major peak had equimolar amounts of VH and VL, indicating that
converted the polyreactive DNA-binding VH domain to a highly
selective or inactive Fv.

Discussion
Comparison of Fv and VH domains
Our experiments identified two major differences in nucleic acid
Ag binding by the VH alone and the Fv domain of anti-Z-DNA
mAb Z22. First, although the VH retains the ability to distinguish
between Z-DNA and B-DNA forms of poly(dG-dC), it is much
less selective than the scFv when assayed with other polynucle-
otides. Unlike the scFv, VH alone binds to immobilized or soluble
polypyrimidines and ssDNA as well as Z-DNA. Second, introduc-
tion of single and combined mutations revealed that the contacts
with Z-DNA are not precisely the same for the scFv and the VH
alone. The experiments also demonstrated that association of VL
with VH is able to modulate or veto the DNA binding potential of
the VH domain.

These results extend previous work in this laboratory with mAb
Z22 domains (20, 25). The earlier work had not measured reac-
tions of VH alone with polynucleotides other than Z-DNA or B-
DNA forms of poly(dG-dC) (20) and had measured the effects of
CDR mutations only in the context of the scFv (25). Consistent
with the previous studies (20), higher concentrations of VH than
scFv were required for an equivalent ELISA measurement of bind-
ing to Z-DNA immobilized on the solid phase (Tables I and II),
even though affinities measured by surface plasmon resonance

![FIGURE 6](image-url)

FIGURE 6. ELISA, comparing binding of polynucleotides by Z22Fab (1 μg/ml) (A), Z22scFv-SPA (10 μg/ml) (B), associated VH-SPA plus VL-SPA heterodimer (1 μg/ml) (C), and Z22VH-SPA (20 μg/ml) (D). Wells of UV-treated microtiter plates were coated with 2 μg/ml of Z-DNA (■), dsDNA (○), ssDNA (□), poly(dA) (△), poly(dC) (□□), poly(dT) (△△), poly(dU) (□□□), or poly(dG-dC) (◇). Rabbit anti-mouse Fab serum at a 1:10,000 dilution and AP-conjugated goat anti-rabbit IgG were used for detection of Z22 Fab.

![FIGURE 7](image-url)

FIGURE 7. VH-VL association. ELISA, comparing binding of polynucleotides by S98AVH-SPA (20 μg/ml) (A), S98AVH-SPA plus Z22VL-SPA (1 μg/ml) (B), N99KVH-SPA (20 μg/ml) (C), N99KVH-SPA plus Z22VL-SPA (1 μg/ml) (D). Wells were coated with Z-DNA (■), dsDNA (○), ssDNA (□), poly(dA) (△), poly(dC) (□□), poly(dT) (△△), poly(dU) (□□□), or poly(dG-dC) (◇). Rabbit IgG and AP-conjugated goat anti-rabbit IgG were used for detection.
were similar for the two domains (20). Competitive immunoassays were more concordant with the affinities, as similar concentrations of soluble Z-DNA (~0.1 μg/ml) were required for 50% inhibition of VH or scFv binding to immobilized Z-DNA (20); the same soluble Z-DNA concentration was inhibitory in the present study (Fig. 4). As discussed previously, the differences in noncompetitive and competitive ELISA may reflect concentrations of properly folded protein or Fv dimerization.

A different combination of CDR3H residues is required for Z-DNA binding by the VH domain alone and the scFv. Substitution of four CDR3H residues by alanine and one by aspartate completely eliminated binding by the VH alone, indicating that CDR3H is important for the VH alone, as it is for the scFv (20). However, although N99 is critically important in the Fv binding, it is not critical in the VH domain alone. Contributions from CDR1 and CDR2 to Ag binding by the VH domains are possible, as they were for isolated anti-oxazolone and anti-lysozyme VH domains (30). B cell superantigen-like interaction with framework residues (31) may also contribute. Substitutions in the CDR3H can also affect VH-VL interaction, as evidenced by the poor association of the N99K-containing VH with wild-type Z22VL.

A question arises whether the VH resembles a native V domain. The presence of a B domain of SPA in the fusion protein may favor correct folding (32). Other examples of isolated VH domains have features of native structure, identified by nuclear magnetic resonance spectroscopy (33) or x-ray crystallography (34). The Z22VH-SPA fusion protein had little or no aggregated material. About 75% of the VH and VL protein associated to form a functional 1:1 heterodimer with the same binding selectivity as the parent Fab, indicating that most of the VH was at least able to assume a native conformation. However, only part of a Z22VH sample formed a stable complex when mixed with an excess of Z-DNA. An increase in the active fraction following incubation of the VH protein at 37°C indicates that some VH conformations may be interchangeable between active and inactive forms. Another portion of the protein may not be activatable, perhaps as a result of exposure to low pH during elution from the affinity column.

The role of VH in Ag binding

The Ag-binding activities of separated H and L chains of various Abs were assessed soon after the separation of Ab chains was first achieved (35). In most of these early experiments, the separate chains retained little activity, but Ag binding was partially restored on recombination of the chains (36–39). The most complete recovery of activity occurred when homologous H and L chains were recombined, indicating that both chains contribute to forming an Ag-binding site. This conclusion was substantiated when it was recognized that both H and L chains could be affinity labeled by hapten (40), and it has been amply confirmed in many crystal structures of Ab complexes (10). However, in most complexes there are more H chain contacts than L chain contacts with Ag (10), and the CDR3H makes more contacts than other CDRs. This distribution of contact residues is consistent with the early experiments with separate chains, noted above, in which the H chain alone had more Ag binding activity than the isolated L chain, even if both were very much less active than intact IgG. A particularly high level of activity of a single chain occurred in the case of a polyclonal purified Ab to p-aminophenyl-β-lactoside, in which the H chain affinity for hapten was 87% of that measured with mildly reduced but still intact Ig (41). The high Ag binding potential of H chains is evident in camel serum Ab, a large fraction of which is comprised of H chain dimers (42). In recent years, several examples of Ag binding VH domains other than those of anti-DNA Abs have been identified, including lysozyme- or hemocyanin-binding VH domains isolated from immunized animals (43).

The H chain often plays a dominant role in Abs to DNA. The prominance of VH in determining DNA binding activity is a property of disease-related autoantibodies (16, 19, 21) as well as Abs induced by immunization with nucleic acids (20). In mice transgenic for the H chain of a disease-related anti-DsDNA autoantibody, the ability of that H chain to direct dsDNA binding, i.e., potentially harmful autoreactivity, led to either elimination of large numbers of B cells (44) or B cell anergy (45). Some B cells did escape this negative regulation through a mechanism of receptor editing (46), in which that H chain became associated with an L chain that vetoed DNA binding. Both the modulating activity of the L chain and its contribution to the binding of different Abs may determine the fate of developing B cells (47). In a lupus-prone mouse, B cells develop even if they express a DNA-binding Ab (48). Thus normal regulatory mechanisms monitor the autoreactive dsDNA binding activity of B cell products, and the H chain properties are particularly prominent in that autoreactivity. A breakdown of normal regulation permits expansion, class switching, mutation, and affinity maturation of anti-DNA Abs in lupus subjects.

A possible role for VH ligand binding activity in preimmunization B cell selection

Perhaps this close monitoring of DNA binding follows from the relatively frequent occurrence of anti-ssDNA reactivity in “natural autoantibodies” or products of quiescent but potentially autoreactive B cells in normal subjects (49, 50). The binding properties of these natural autoantibodies resemble those of the isolated VH domain of mAb Z22. For example, IgM anti-DNA autoantibodies are not as selective as immunization-induced Abs; they often bind poly(dT), and some of them bind Z-DNA (51). It is not known whether B cells making natural autoantibodies are direct precursors of cells that make disease-related autoantibodies or immunization-induced Abs, but poly(dT) binding is common not only among nonpathogenic IgM natural autoantibodies; it is also common among class-switched mutated IgG Abs in humans or mice with active disease (52–54). Representation of Z-DNA binding in the background of “natural autoantibody” may account for the fact that Z22 and another highly selective IgG mAb, Z44, have been formed with very few VH mutations from germline sequences (55, 56).

Polypyrimidine binding is a form of autoreactivity that was also noted with normal VH domains, such as those coded in cDNA libraries made from normal circulating B cells. As discussed previously, the differences in competitive and competitive ELISA may reflect concentrations of properly folded protein or Fv dimerization.

**References**


