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DNA Binding by the VH Domain of Anti-Z-DNA Antibody and Its Modulation by Association of the VL Domain[1]

Yan Chen and B. David Stollar[2]

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 product 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.   *The Journal of Immunology,* 1999, 162: 4663–4670.

Anti-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 deposition 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)[3] 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 $(dT)_3$ (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 Igs 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 rZ22 VH domain alone can bind the Z-DNA form of poly(dG-dC) with approximately the same affinity

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[3] Abbreviations used in this paper: H, heavy; L, light; CDR, complementarity determining region; CDR3H, third complementarity determining region of the heavy chain; Fv, variable fragment; scFv, single chain variable fragment, comprising both VH and VL; SPA, Staphylococcal protein A; VH, variable domain of the heavy chain; comprising V_H, D_H, and J_H segments; VL, variable domain of the light chain, comprising V_L and I_L segments.

0022-1767/99/$02.00

Table I. *CDR3H amino acid sequences of Z22 and modified domains*

Mutants	Sequences of VH-CDR3										
WT Z-22	Q	A	Y	S	N	Y	G	A	M	D	Y
CDR3HM1	D		A	A	D	A	A				
CDR3HM2	D		A	A		A	A				
Q95E	E										
Q95D	D										
Q95H	H										
Y97F			F								
S98A				A							
N99Q					Q						
N99K					K						
Y100F						F					
H3M18				Y		K		D			
H3M34				S	N	K		C			

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 pIg16 and pIg20, designed for expression of scFv containing original or substituted VH and VL Z22 domains, and pIg16VH and pIg20VH (with only the VH domain), were reported previously (20, 24, 26). DNA fragments coding for VH domains with one to four substitutions (25) were cut out of the scFv constructs by digestion with restriction enzymes *Xma*I and *Xba*I (New England Biolabs, Beverly, MA). The excised DNA fragments were gel purified and ligated into similarly digested and purified pIg16VH or pIg20VH vectors for expression of each VH domain fused to a B domain of Staphylococcal protein A (SPA) (Table I and Fig. 1).

More extensively substituted CDR3H regions were created with the Quick-Change site-directed mutagenesis kit (Strategene, La Jolla, CA). The CDR3H was modified by six simultaneous changes from the wild-type amino acids (QAYSNYGAMDY) to DAAADAAAMDY (CDR3HM1) or, by five changes with retention of the important N99, to DAAANAAAMDY (CDR3HM2). Next, 5–50 ng of Z22VH plasmid DNA was amplified with 125 ng of each oligonucleotide primer containing the desired mutations and 2.5 U of Pfu DNA polymerase (Stratagene) at 95°C for 30 s, 48°C for 1 min, and 68°C for 8 min for 18 cycles. The primers for the segment coding for six-amino acid substitutions were: forward, 5′-GTGAGAGACGCAGCAGCA GACGCAGCAGCAATGGACTACTGGG;reverse,5′-CCCAGTAGTCCAT TGCTGCTGCGTCTGCTGCGTCTCTCAC. Primers for the segment coding for 5-amino acid substitutions were: forward, 5′-GTGAGAGA CGCAGCAGCA<u>A</u>ACGCAGCAGCAATGGACTACTGGG;reverse,5′-CCC AGTAGTCCATTGCTGCTGCGT<u>T</u>TGCTGCTGCGTCTCTCAC. The single base differences between forward and reverse primers in the two sets is underlined. The PCR product was digested with *Dpn*I (New England Biolabs) to cleave the methylated, nonmutated parental plasmid DNA. The circular, nicked dsDNA carrying mutations was transformed into XL1-blue supercompetent cells and was repaired in vivo to form plasmid with desired mutations. Plasmid DNA was isolated using a Qiagen plasmid minipreparation kit (Chatsworth, CA). DNA sequencing was performed with dye terminator cycle sequencing, with the Ready Reaction kit (Abi Prism, Perkin-Elmer, Branchburg, NJ) and T7 primer. PCR was performed with a program of 95°C for 10 s, 50°C for 10 s, 60°C for 5 min for 25 cycles, and the PCR products were sequenced, confirming the presence of expected mutations and a correct reading frame. A T7 sequence was used as primer for sequencing: 5′-ATACGACTCACTAT AGGG.
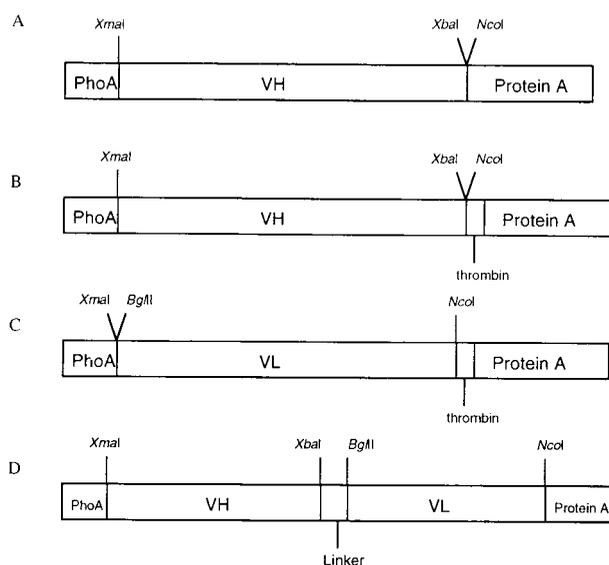
FIGURE 1.   Bacterial expression vectors for production of pIg16H, VH-SPA (*A*); pIg20H, VH-SPA with a thrombin cleavage site (*B*); pIg20L, VL-SPA with a thrombin cleavage site (*C*); and pIg16, scFv-SPA (*D*). The vectors are derived from pGEMEX (Promega, Madison, WI) as described (20, 24, 26).

Transformation of *Escherichia coli* BL21(DE3)pLysE (27) with pIg16- or pIg20-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.

Polynucleotide 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-treated 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. A_{410} was read after 1 h of color development after addition of substrate. Relative binding activity, a measure of binding per unit protein concentration, was calculated based on the concentration required to reach an A_{410} 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 pIg20 VH-SPA fusion protein

A total of 200 μg of rVH-SPA fusion protein in 500 μl of PBS with 2.5 mM CaCl$_2$ 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 phophatase as in the ELISA (21). The column had been calibrated with samples of 500 μg of each of albumin (M_r 66,000; Sigma), carbonic anhydrase (M_r 29,000; Sigma) and cytochrome c (M_r 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-form 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 pIg16H- or pIg20H-based vectors for the expression of VH-SPA domains alone. The pIg20 and pIg16 products differ only by the presence of a thrombin cleavage site between the Ig and SPA domains in the pIg20 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(DE)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 and detection of recombinant proteins by Western blot and ELISA assays. It was shown previously that the SPA in the fusion protein does not interfere with VH-VL association or Ag binding (20). In a further test in this study, SPA was cleaved from fusion protein. Isolated SPA did not bind to any of the polynucleotides tested (data not shown).

Yields of purified scFv or VH proteins varied from 0.3 to 2.2 mg/L of bacterial culture supernatant. SDS-PAGE and Coomassie blue staining confirmed that the recombinant fragments had the predicted sizes: 34 kDa for the scFv-SPA and 20 kDa for the VH-SPA (Fig. 2B). 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).
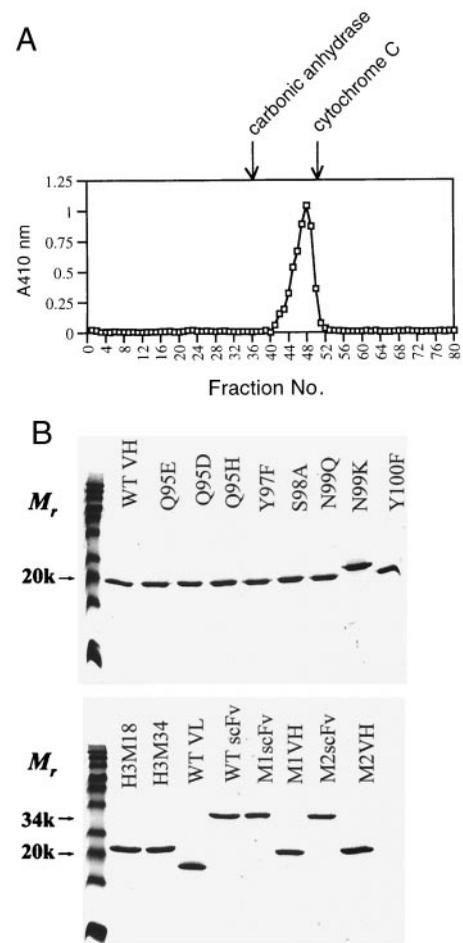

FIGURE 2. *A*, Gel filtration of 10 μg VH-SPA recombinant protein through a 1.4 × 48 cm column of polyacrylamide P100. VH-SPA was detected, through its SPA domain, by ELISA, with rabbit IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG. *B*, Coomassie blue staining of purified recombinant proteins. Each sample was 1.5 μg of protein purified 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).

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. 2A). 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

A

B

Fraction No.
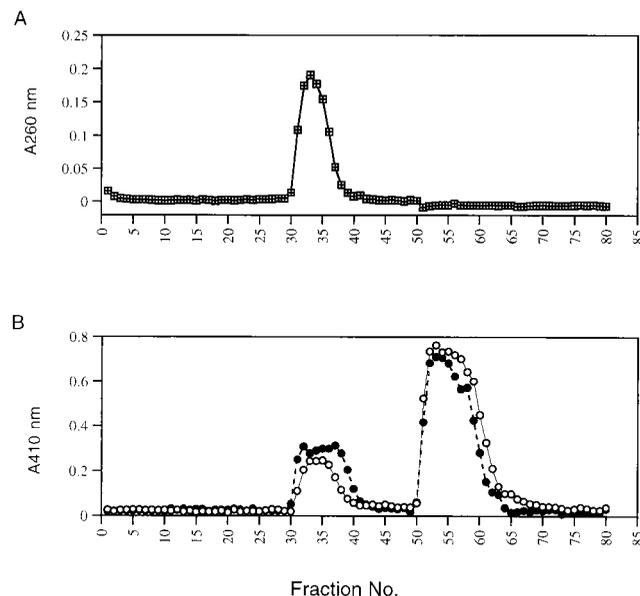
FIGURE 3. Gel filtration of 100 μg Z-DNA (*A*) and 100 μg Z-DNA plus 25 μg of freshly thawed (○) VH-SPA or VH-SPA that had been pre-incubated at 37°C for 3 h (●) (*B*). The mixture of Z-DNA and VH-SPA was incubated for 1 h at room temperature before being loaded onto a 1.4 × 48 cm column of Sephacryl S-100 HR. The Z-DNA, measured by A(260 nm), emerged at the void volume in *A*. VH-SPA was detected by ELISA.

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-

Table II. *Relative polynucleotide binding activity of Z22 scFv and VH proteins[a]*

Polynucleotides	Z22 scFv-SPA	Z22 VH-SPA
Z-DNA	1	1
dsDNA	–	<0.2
ssDNA	–	0.53 ± 0.06
poly(dA)	–	–
poly(dC)	–	0.72 ± 0.11
poly(dT)	0.002	0.89 ± 0.14
poly(dU)	–	0.79 ± 0.14
poly(dG–dC)	–	–

[a] 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 A$_{410}$ 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).

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.

FIGURE 4. Competitive ELISA for binding of soluble polynucleotides to recombinant proteins Z22VH-SPA (*A* and *B*) and N99KVH-SPA (*C* and *D*). For *A* and *C*, wells were coated with Z-DNA; for *B* and *D*, wells were coated with ssDNA. Soluble competitors were Z-DNA (□), dsDNA (◇), ssDNA (○), poly(dT) (◇), and poly(dG-dC) (⊞). A total of 10 μg/ml of recombinant protein was incubated with varying concentrations of polynucleotides for 30 min and added to the wells coated with 2 μg/ml of Z-DNA or ssDNA. Rabbit IgG and goat anti-rabbit IgG-AP were used for detection of bound protein.
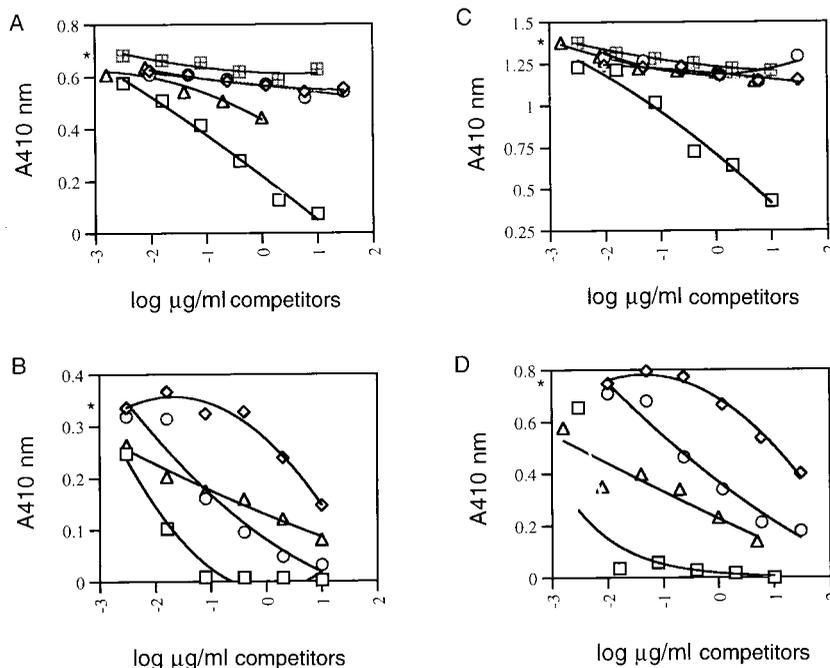
A

log μg/ml competitors

C

log μg/ml competitors

B

log μg/ml competitors

D

log μg/ml competitors

Table III. *Relative Z-DNA binding activity of modified Z-22 scFv and VH proteins*[a]

Mutants	Z22 scFv-SPA (relative sp. act.)		Z22 VH-SPA (relative sp. act.)[b]
scFv/VH	1[c]	1[d]	1
Q95E	0.3[c]		0.40 ± .04
Q95D	0.3[c]		0.62 ± 0.22
Q95H	0.5[c]		2.4 ± 1.14
Y97F	0.37[c]		0.84 ± 0.21
S98A	0.08[c]	0.06[d]	1.46 ± 0.4
N99Q	1.07[c]		0.89 ± 0.76
N99K	–[c]	<0.001[d]	2.43 ± 0.55
Y100F	1[c]		0.35 ± 0.16
H3M18	0.005[c]		1.39 ± 0.39
H3M34	0.08[c]	<0.001[d]	1.0 ± 0.27
CDR3HM1	–		–
CDR3HM2	–		–

[a] 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_{410} of 0.5, normalized to the concentration required for wild-type scFv-SPA (0.6 nM) or 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.
[b] Average and standard deviation from three experiments.
[c] Data for scFv from Ref. 20.
[d] Values for scFv determined in this study are the average of two experiments for each protein.

Binding of substituted CDR3H proteins to Z-DNA

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
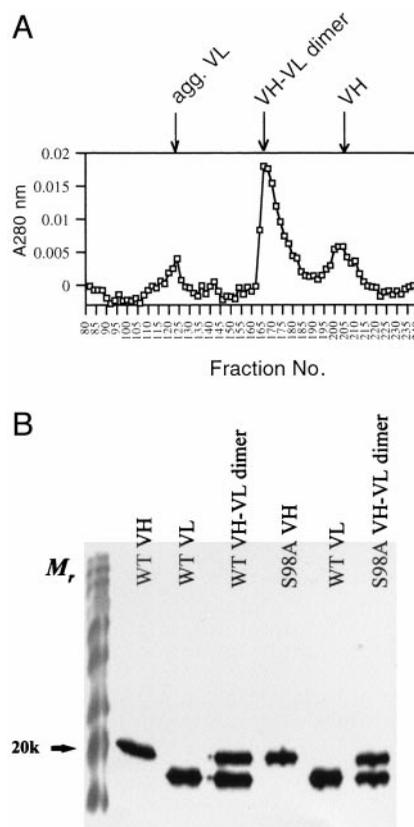

FIGURE 5. *A*, Gel filtration of a preincubated mixture of 400 μg Z22VH-SPA plus 400 μg VL-SPA protein mixture through a Sephacryl S-100 HR size-exclusion column (3.5 × 56 cm), monitored by A_{280}. A total of 400 μg of VH was mixed with 400 μg of VL and applied to the column. The first 80 ml was collected in 5-ml fractions, and the next 160 ml was collected in 2-ml fractions. Mixtures of the mutant N99KVH-SPA and S98AVH-SPA domains with Z22VL-SPA were analyzed with the same column. Contents of tubes at positions marked as VH, VH-VL, and aggregated VL (agg. VL) were determined by Western blotting. *B*, Western blot analysis of Z22VH-SPA plus Z22VL-SPA and S98AVH-SPA plus Z22VL-SPA from the size-exclusion column. Samples were taken for Western blotting from the fraction with the highest concentration at the volume corresponding to heterodimer peak. The blot was developed with rabbit IgG, AP-conjugated goat anti-rabbit IgG, and substrate (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate).

the soluble recombinant V domains are able to assemble in solution into a stable 1:1 complex (Fig. 5*B*). 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, N99KVH 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. 6*D*), the associated Z22VH-Z22VL heterodimer bound only to Z-DNA and not to other polynucleotides (Fig. 6*C*). 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_{410} per unit protein) of S98AVH-Z22VL was lower than that of Z22VH-Z22VL. Isolated N99KVH-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 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 ($\square$), dsDNA ($\diamond$), ssDNA ($\bigcirc$), poly(dA) ($\triangle$), poly(dC) ($\boxplus$), poly(dT) ($\diamondsuit$), poly(dU) ($\oplus$), or poly(dG-dC) ($\triangledown$). 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.
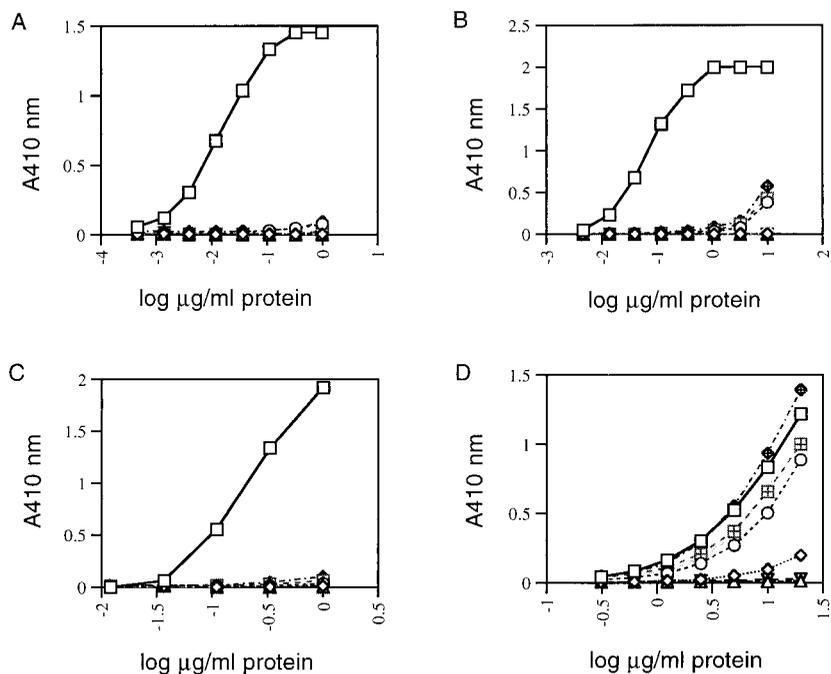
converted the polyreactive DNA-binding VH domain to a highly selective or inactive Fv.

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 polynucleotides. Unlike the scFv, VH alone binds to immobilized or soluble polypyrimidines and ssDNA as well as Z-DNA. Second, introduction 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 reactions 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 binding to Z-DNA immobilized on the solid phase (Tables I and II), even though affinities measured by surface plasmon resonance
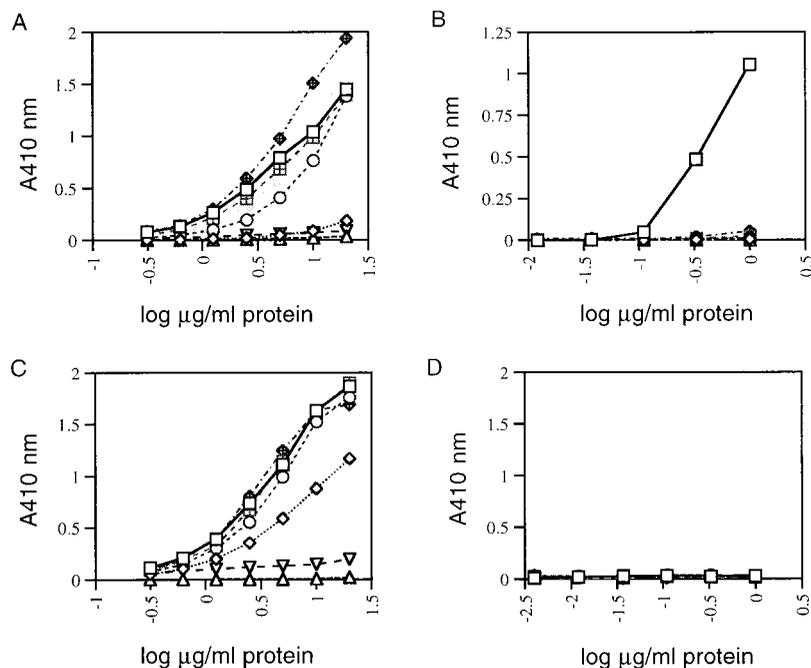
FIGURE 7. VH-VL association. ELISA, comparing binding of polynucleotides by S98AVH-SPA (20 μg/ml) (*A*), S98AVH-SPA plus Z22VL-SPA (1 μg/ml) (*B*), N99KVH-SPA (20 μg/ml) (*C*), N99KVH-SPA plus Z22VL-SPA (1 μg/ml) (*D*). Wells were coated with Z-DNA ($\square$), dsDNA ($\diamond$), ssDNA ($\bigcirc$), poly(dA) ($\triangle$), poly(dC) ($\boxplus$), poly(dT) ($\diamondsuit$), poly(dU) ($\oplus$), or poly(dG-dC) ($\triangledown$). 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 activable, 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 Ag-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 prominence 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 Ags 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 clones of cDNA libraries made from normal circulating B cells (19, 22). In these cases, it could not be known whether the VH domains were derived from natural anti-DNA Abs, but the frequency of poly(dT) binding was high (5 of 11) in a small sample of cDNA clones from a healthy young adult (22). We are now exploring the occurrence of such binding activity with VH domains cloned from cDNA libraries and single human neonatal (cord blood) B cells. A high frequency of polypyrimidine binding could reflect a functionally important role for this property in B cell development and selection before exposure to exogenous Ags. Structural features of polypyrimidines or Z-DNA may be, or may mimic, self-ligands that are important in positive as well as negative selection of B cells.

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