Specific recognition of a dsDNA sequence motif by an immunoglobulin V_H homodimer

HULIN JIN,¹ JORGE SEPÚLVEDA,¹ AND OSCAR R. BURRONE

International Centre for Genetic Engineering and Biotechnology, 34012-Trieste, Italy

(RECEIVED June 9, 2004; FINAL REVISION August 6, 2004; ACCEPTED August 14, 2004)

Abstract

Anti-DNA antibodies have the potential to be applied in vast fields of fundamental as well as medical research. They are found in autoimmune diseases, such as systemic lupus erythematosus. In most cases, anti-dsDNA antibodies do not present sequence specificity and are of low affinity. The dominant role of V_H domains in DNA recognition induced us to search for binders based on V_H dimers (VHD), previously reported to bind different protein antigens. We screened a phage displayed homo-VHD library against a 19-bp dsDNA sequence. A sequence-specific binder was selected, which recognizes the terminal located CTGC motif with a K_d of 250 nM. Association of the two identical V_H domains of the molecule was shown to be essential for binding.

Keywords: anti-DNA; VHD; phage display; antibodies

DNA-binding proteins recognize DNA through different domains, such as the helix-turn-helix, zinc finger, leucine zipper, and basic helix-loop-helix (Harrison 1991; Pabo and Sauer 1992). Also natural antibodies with DNA-binding activities have been found in the serum of healthy individuals and patients with autoimmune disorders. In normal individuals, antibodies are usually polyreactive, without DNA sequence specificity, and with low affinity for DNA (Naparstek et al. 1986; Stollar 1994; Barbas et al. 1995). Instead, anti-DNA antibodies with higher affinities are the hallmark of the autoimmune disorder systemic lupus erythematosus (SLE) syndrome (Barbas et al. 1995; Blatt and Glick 1999; Mandik-Nayak et al. 1999), and are also found in the autoimmune MRL/lpr mice (Naparstek et al. 1986), and are the major component of the intrathecal IgG response in patients with multiple sclerosis (MS) (Williamson et al. 2001).

On the basis of their stable structure and potent binding capabilities, antibodies are a convenient source for protein

engineering to produce molecules with designed binding specificity (McLane et al. 1995; LeBlanc et al. 1998). Numerous monoclonal anti-DNA antibodies derived from hybridoma and phage display technologies have been studied for this purpose (Komissarov et al. 1996, 1997). Investigations into the reactivity of these antibodies revealed that, in general, they are not sequence-specific. The antibodies can be classified as specific for ssDNA or dsDNA and, in certain cases, to recognize DNA motifs containing immunodominant epitopes, such as oligo(dT) and G/C-rich sequences (Stollar et al. 1986; Sanford and Stollar 1990; Herron et al. 1991; Barry and Lee 1993; Swanson et al. 1994, 1996; Blatt and Glick 1999). Thermodynamic studies have revealed that specific ssDNA binding is achieved depending on defined secondary structures, with a preference for thymine (Herron et al. 1991; Stevens and Glick 1999; Ackroyd et al. 2001). Interestingly, a high affinity sequence-specific anti-dsDNA monoclonal antibody was successfully generated to immunize mice with a protein–DNA complex (Cerutti et al. 2001). Generally, the V_H domain of anti-DNA autoantibodies, especially through the third CDR loop (H3), appears to play a dominant role in nucleic acid binding (Brigido et al. 1993; Radic et al. 1993; Barbas et al. 1995; Polymenis and Stollar 1995; Li et al. 2000; Tanner et al. 2001). Moreover, in some cases the V_H was able to maintain DNA-binding activity, even when combined with various V_L domains

Reprint requests to: Oscar R. Burrone, International Centre for Genetic Engineering and Biotechnology, Area Science Park, Padriciano 99, 34012-Trieste, Italy; e-mail: burrone@icgeb.org; fax: +39-040-226555.

¹These authors have contributed equally to this work.

Article published online ahead of print. Article and publication date are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.04921704>.

(Radic et al. 1991). On the other hand, fewer studies report a partial contribution of the L-chain (Brigido et al. 1993; Jang et al. 1998; O'Connor et al. 2001). However, the kinetic factors and molecular mechanisms governing anti-DNA/DNA binding and recognition, and the specificities of these antibodies are still poorly known (Stevens and Glick 1999; Ackroyd et al. 2001).

We have recently described antigen-specific binders based on dimerized immunoglobulin V_H domains, termed VHD, which can exist as homo- or hetero-VHD depending, respectively, on the association of two identical or two different V_Hs. These VHDs can be expressed in bacteria and mammalian cells in different formats, including single chain (sc) [V_H(1)-linker-V_H(2)], double chain (dc) [(V_H)₂], and IgG analogs having the V_L replaced by V_H (Jin et al. 2003; Sepúlveda et al. 2003).

In an attempt to investigate the possibility of obtaining sequence-specific DNA binders, we screened a library of homo-VHD displayed on filamentous phages. Here we report a selected homo-VHD binder that is capable of binding, with sequence specificity, to a terminally located dsDNA motif.

Results

Library screening

An interesting characteristic of homo-VHDs is the dimerization of a single V_H that creates a symmetrical binding surface that could potentially bind symmetrical antigens such as palindromic DNA sequences in dsDNA. To investigate this possibility, we performed a selection of VHD binders, using as target a 19-bp dsDNA (named dsPRK) that contained three 6-nucleotide long palindrome sequences (corresponding to PstI, EcoRI, and KpnI restriction enzyme sites) as well as a centred 10-bp long palindrome (Fig. 1). This was done to determine whether it was possible to select homo-VHDs specifically recognizing symmetrical structures within the dsDNA. For the selection of the phage-displayed homo-VHD library (Jin et al. 2003),

Figure 1. Sequences of the 19-bp long dsDNA (dsPRK), used as target for binder selection and of the three control dsDNAs (dsC1, dsC2, and dsC3). Palindromes are indicated.

the DNA plus strand was 3' end-labeled with biotin to facilitate immobilization to magnetic beads coated with streptavidin. Three control dsDNA sequences (dsC1, dsC2, and dsC3) were also designed, having the same C/G content as dsPRK, but with a scrambled sequence and no palindromes.

Binding of phages displaying VHDs to dsPRK was performed in solution and phage–DNA complexes were obtained by streptavidin-coated magnetic beads. After five rounds, only one specific clone termed VHD^{D8} was selected, and expressed in dc and sc formats (dcVHD^{D8} and scVHD^{D8}). As shown in Figure 2A, the scVHD^{D8} purified in a nickel affinity column showed a molecular weight of 30 kDa by SDS-PAGE, as expected for a single-chain construct. However, when analyzed by gel filtration, scVHD^{D8} eluted as a single peak of ~60 kDa, suggesting that it assembles into a diabody-type molecule. In contrast, in gel filtration, the dcVHD^{D8} showed an apparent molecular weight of 30 kDa, consistent with the association of two V_H domains (Fig. 2B).

Binding activity tested by ELISA revealed that, regardless of the format, VHD^{D8} specifically bound to dsPRK, but not to the control DNA dsC1, suggesting sequence-specific binding (Fig. 2C). As expected, binding to two control proteins, streptavidin and lysozyme, was also negative. The sequence of V_H^{D8} encodes a 116-amino-acid human V_H domain of the VH3 gene family (locus 3–23) with a relatively short CDR3 of 7 residues (Fig. 2D).

Binding of scVHD^{D8} to the target dsPRK at different concentrations was studied by ELISA using other dsDNA sequences as competitors. Only two dsDNA, namely dsPRK and ds(L/C), the 19-bp dsDNA containing the left 10-bp sequence derived from dsPRK fused to the right 9-bp from control dsC1, showed strong competition (Fig. 3). Conversely, ds(C/R), the 19-bp dsDNA containing the right 10-bp sequence from dsPRK fused to the left 9-bp from control dsC1, as well as the two single-stranded PRK (ssPRK), and controls dsC2 and dsC3 showed a largely reduced competitive activity. Two competitors, however, the minus strand of ssPRK and control dsC2, showed an ~10-fold reduced competition activity with respect to dsPRK, whereas the other competitors were between 60- and 80-fold less active. These data indicated that VHD^{D8} was a dsDNA binder with sequence specificity, located within the leftmost 10-bp of dsPRK. The interaction of scVHD^{D8} with the target dsDNA was also confirmed in a gel retardation assay, as shown in Figure 4. A shifted band of the protein–DNA complex was completely competed out by 10-fold excess of dsPRK but not of the negative control dsC1.

Mapping recognition sequence

To determine the DNA sequence recognized by scVHD^{D8}, we performed a competitive ELISA with dsDNA fragments

Figure 2. (A) SDS-PAGE (Coomassie blue staining) and size exclusion chromatography of purified scVHD^{D8}. (B) Size exclusion chromatography analysis of dcVHD^{D8}. Column fractions were analyzed by Western blot with anti-SV5 mAb and binding activity determined by ELISA on dsPRK-coated plates. (C) ELISA performed with both scVHD^{D8} and dcVHD^{D8} formats on plates coated with the indicated dsDNAs. Two negative control protein-coated plates (lysozyme and streptavidin) were also used. (D) Nucleotide and amino acid sequence of V_H^{D8} domain. CDRs are underlined.

(competitors), which contained from 10 to 1 nucleotide long stretches of dsPRK (in a background of dsC3) encompassing the dsPRK sequence from the 3' to the 5' ends (Fig. 5). Binding of scVHD^{D8} to immobilized dsPRK was not com-

peted out by oligonucleotides *a* to *i*, which contained 10-bp sequences of dsPRK up to the second 5' end nucleotide. On the other hand, oligonucleotides *j* to *p* containing dsPRK sequences, corresponding to the first 10-bp down to the first 4 bp at the 5' end, were as efficient as the original wild-type

Figure 3. Competitive ELISA binding of scVHD^{D8} to dsPRK with the indicated 19-bp dsDNAs, dsPRK, dsC2, dsC3, ds(L/C), and ds(C/R), and the ssDNA PRK (ssPRK1, plus strand; ssPRK2, minus strand).

Figure 4. Band-shift assay. Competition of [³²P]dsPRK binding to scVHD^{D8} with 10-fold excess of unlabeled dsPRK or dsC1, as indicated. Arrowheads indicate the DNA-protein complex.

Figure 5. Mapping the recognition motif. Inhibition of scVHD^{D8} binding to dsPRK in competitive ELISA by different dsDNA competitors (at 2 μ M) with the indicated sequences. The CTGC motif is squared. Sequences corresponding to dsPRK are shown in black and those corresponding to dsC3 in gray.

dsPRK in competing with scVHD^{D8} binding. Oligonucleotides with further truncation of the terminal 5' sequence of dsPRK (*q* to *s*) did not compete. These results indicated that the recognition motif is constituted by the 4-bp terminal sequence CTGC. Terminal location of the CTGC motif appears to be crucial, as mutants in which the same motif was internally shifted by one to four positions, did not compete (data not shown).

Determination of binding affinity

To determine the binding affinity of VHD^{D8} to dsDNA, we used the nickel column-purified scVHD^{D8}. We performed a filter-binding assay using [³²P] 5' end-labeled DNA and purified scVHD^{D8} protein incubated to equilibrium. The mixtures were then passed through a nitrocellulose membrane to retain only protein complexed DNA. Quantification of the retained radioactivity allowed estimation of the dissociation constant (K_d) to be of ~ 250 nM (255 ± 10 nM) (Fig. 6).

Dimerization of domain V_H^{D8} is required for dsDNA binding

As previously reported for VHDs with protein-binding activity (Jin et al. 2003; Sepúlveda et al. 2003), binding to the "CTGC" motif was dependent on dimerization of the V_H^{D8} domain. Different scVHD were constructed, in which one of the two V_H^{D8} (either in position 1 or position 2 of the sc) was replaced with several different irrelevant V_Hs. All these constructs contained V_Hs of varied sequences and were expressed as periplasmic proteins (Fig. 7B). When tested by

ELISA (Fig. 7C) or by the filter-binding assay (not shown) in the same conditions as the positive control scVHD^{D8}, none of the domain shuffled constructs showed any significant binding.

In addition, to rule out the possibility that DNA recognition was the consequence of independent binding by each V_H (avidity effect) we assayed two other different constructs: (1) a camelized version of the scVHD^{D8} (scVHD^{D8}-cml) in which mutation of the V_L/V_H interface residues G44, L45, and W47 of V_H into E, R, and G, respectively, avoids the formation of dimers, and (2) a single V_H version derived from the dcVHD^{D8} with the terminal cysteine deleted. These proteins did not show any significant binding, thus demonstrating that dimerization of the V_H^{D8} domain was essential for binding activity, with both domains contributing to the formation of the binding site (Fig. 7C).

Discussion

We have previously reported on the spontaneous association of V_H domains to form dimers with different protein antigen specificities, in both heterodimer and homodimer formats, which can be selected from appropriate phage display libraries (Jin et al. 2003; Sepúlveda et al. 2003).

Here we addressed whether homo-VHD could have dsDNA sequence-specific binding activity. There are several points that make the study of dsDNA binding by homo-VHDs of special interest. First, V_H domains are known to play a crucial role in antibody–DNA interactions. Antibodies that bind to DNA have been identified as an important component of the autoimmune disease systemic lupus erythematosus (Blatt and Glick 1999). However, it is known that these naturally occurring autoantibodies are not targeted to any particular DNA sequence (Stevens and Glick 1999). Importantly, their binding activity has been mainly

Figure 6. Filter binding assay of scVHD^{D8} to [³²P]-labeled DNA. The plot corresponds to the relative binding obtained at different concentrations of the purified protein binder.

Figure 7. (A) Schematic representation of the different constructs used. (B) Expression of constructs analyzed by Western immunoblot with anti-SV5 mAb. V_H¹ to V_H⁸ represent different randomly picked V_H sequences. *Bottom* panel was in nonreducing conditions. Open and filled arrowheads indicate monomeric and dimeric forms, respectively. The scVHD^{7.6} corresponds to an anti-lysozyme VHD, used as negative control. (C) ELISA binding of the indicated protein constructs, used at the same concentrations, in dsPRK-coated plates.

attributed to the CDR3 loop of the V_H domain. In contrast to most protein-binding antibodies (Radic et al. 1991), DNA-binding antibodies were also frequently found to bind to DNA regardless of their V_L partner (Barry and Lee 1993), as exemplified by the intensively studied V_H domain 3H9 (Radic et al. 1991; Guth et al. 2003).

Second, there have been very few reports on sequence-specific anti-dsDNA. So far, most of them showed relatively low affinities for synthetic homopolymers, such as poly[d(G-C)] (Stollar et al. 1986; Sanford and Stollar 1990; Blatt and Glick 1999). In one report, however, a high affinity sequence-specific mouse antibody was obtained by

immunization with a protein–dsDNA complex. The binding motif has not yet been clarified (Cerutti et al. 2001). Specific binding of a dsDNA sequence was also obtained by transplanting an α -helical DNA-binding domain from a helix-loop-helix transcription factor into an antibody V_H CDR3 loop (McLane et al. 1995; LeBlanc et al. 1998).

Third, the recognition of DNA by antibodies is probably different from that of natural DNA-binding domains (Ackroyd et al. 2001), which bind DNA at its surface conferring a substantial bent to the molecule, whereas antibody-binding sites normally display large flat and extended surfaces or deep cavities (Cerutti et al. 2001). However, many DNA-

binding proteins bind as homodimers and recognize operators containing symmetrically related half-sites (Simoncsits et al. 1999). Similarly, the newly introduced homo-VHD may present a symmetrical structure as a binding site, formed by the two identical V_H domains, which does not exist in natural V_L/V_H heterodimers.

Our target was a 19-bp dsDNA containing three different six-nucleotide palindromes and a central 10-nucleotide palindrome. It is known that for anti-dsDNA autoantibodies, binding encompass ~6 contiguous nucleotide pairs (Smeenk et al. 1996; Blatt and Glick 1999). Contrary to our expectations, we characterized the sequence specificity of VHD^{D8} to a nonpalindromic, 4-bp, terminally located CTGC motif.

The binding affinity of scVHD^{D8} ($K_d = 250$ nM) was found to be higher than that of the autoimmune monoclonal antibody Jel241 specific for both poly[d(A-T)] and native calf thymus DNA (Blatt and Glick 1999) (K_d in the micromolar range), but considerably lower than the one described by Cerutti et al. (2001) ($K_d = 0.73$ – 13.7 nM). In this respect it is worth mentioning that VHD^{D8} was derived from a relatively low diversity naïve library (diversity $\sim 5 \times 10^6$), which in the case of classic scFv, usually produce binders of relatively low affinities (Vaughan et al. 1996).

Through a series of domain shuffling and camelization experiments we demonstrated that binding to the CTGC motif was the consequence of V_H dimerization, a characteristic already shown for VHD binders specific for protein antigens (Jin et al. 2003; Sepúlveda et al. 2003).

It has been reported that the CDR3 of V_H domains play an essential role in DNA recognition by natural autoantibodies (Barbas et al. 1995; Komissarov et al. 1997). The presence of basic residues, mainly arginine within the V_H CDR3 loop, appear to be critical for the interaction with both double-stranded (ds) and single-stranded (ss) DNA (Brigido et al. 1993; Radic et al. 1993; Li et al. 2000; Tanner et al. 2001; Guth et al. 2003), in some cases forming hydrogen bonds with base paired guanine and cytidine groups, as well as with unpaired and base paired cytosine (van Es et al. 1991; Li et al. 2000). It was reported from a group of investigators that 16 to 21 monoclonal antibodies contained arginine (from one to four residues) within the V_H CDR3 (Shlomchik et al. 1990). In our case, V_H^{D8} has a single arginine within the 7-amino-acid CDR3 (G-R-H-P-I-D-Y), and one in CDR1. It would be interesting to determine whether either of these arginines play a role in VHD^{D8} sequence specificity. In the high affinity anti-DNA described by Cerutti et al. (2001), no arginine residue is present in V_H CDR3.

VHD could provide a new source of sequence-specific dsDNA binders. Because high-resolution structures of anti-dsDNA antibodies have not been yet reported (Blatt and Glick 1999), future work on the detailed crystal structural of scVHD^{D8}, or similar sequence-specific binders, could also

be of great value for the detailed understanding of V_H in dsDNA recognition.

Materials and methods

Bacterial strains

Phage infection was carried out in strain DH5 α F' (F'/endA1 hsdR17($r_k^-m_k^+$) supE44 thi-1 recA1 gyrA (Nal^r) relA1 D (lacZYA-argF) U169 deoR (F80dlac Δ (lacZ)M15)) (GIBCO-BRL). The nonsuppressor strain HB2151 (K12, ara Δ (lac-pro), thi/F'proA⁺B⁺, lackIqz Δ M15) was used for expression of soluble fragments.

Library and phage preparation

The homo-VHD library used was previously described (Jin et al. 2003). A single V_H domain is followed by the 11-amino-acid SV5 tag (Hanke et al. 1992), a cysteine residue, and the His 6 tag. Bacteria were cultured in 2 $\times$ YT, 100 μ g/mL ampicillin and 2% glucose, by shaking at 37°C for 2 h until OD₆₀₀ reached 0.5. Helper-phage was then added at a 20:1 ratio of phages-to-bacteria. The mixture was left at 37°C for 30 min, followed by 30 min of shaking at 100 rpm at 37°C. Bacteria were centrifuged and cultured overnight at 30°C in 2 $\times$ YT, 100 μ g/mL ampicillin, and 25 μ g/mL kanamycin. Phage particles were purified by two rounds of precipitation using PEG solution (20% polyethylene glycol 6000 [Fluka] and 2.5 M NaCl).

DNA-binding phage selection

Screening of homo-VHD phage library was performed in solution by incubating 10^{12} phages in 2% milk-PBS with target dsDNA (100 nM) for 2 h at room temperature. Bound phage/DNA complexes were recovered by immobilization through the 3' biotinylated (+ strand) dsDNA on 200 μ L of streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin) and pulled down by placing the mixture in a magnetic rack for 2 min. After washing with 0.1% Tween-20 in PBS, bound phages were eluted by adding 2 mL of DH5 α F' bacteria of OD₆₀₀ = 0.5 at 37°C for 30 min, and then plated.

Soluble homo-VHD protein expression

The HB2151 strain was used for expression of soluble homo-VHD. Bacteria were cultured by incubating at 37°C with shaking until OD₆₀₀ reached 0.7. Expression was induced by 0.5 mM of IPTG (isopropyl- β -D-thiogalactopyranoside) and incubated at 30°C in a shaker (200 rpm) for 4 h. The bacteria pellet was resuspended in PPB buffer (200 mg/mL sucrose, 1 mM EDTA, 30 mM Tris-HCl at pH 8.0) at 1/40 total volume for 20 min on ice. After spinning down cells, supernatant was collected, pellet resuspended in 5 mM MgSO₄ at 1/40 of total volume for 20 min on ice, and recentrifuged. Both supernatants were combined, dialyzed against PBS, and applied to a Ni-NTA (Ni⁺⁺-Nitriloacetic acid) agarose column (Qiagen). The column was washed with 10 mL of 35 mM Imidazole (Riedel-deHaën) in PBS, and protein eluted with two volumes of 250 mM imidazole in PBS. Quantification of recovered protein was carried out using Coomassie-plus protein assay reagent kit (Pierce).

Gel filtration

The FPLC (fast performance liquid chromatography) gel filtration was performed using a Superdex 75 HR 10/30 prepacked column (Pharmacia) and PBS buffer. Two hundred fifty microliters of Ni-NTA purified scVHD (~50 µg) was loaded and run at a flow rate of 0.5 mL/min. The column was calibrated with the LMW kit (Pharmacia) containing four different markers: ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), and albumin (67 kDa).

SDS-PAGE and Western blot

The PEG-precipitated phages or soluble VHD protein were run in 15% SDS-PAGE and transferred to Immobilon-P membrane (Millipore) for 2 h at 200 mA. The membrane was blocked with 4% milk, 0.1% Tween-20 in PBS for 2 h at room temperature, reacted with anti-SV5 mAb (Hanke et al. 1992) and developed with HRP-conjugated goat anti-mouse IgG(γ) (KPL) followed by ECL (enhanced chemiluminescence) reagents (Amersham Pharmacia Biotech).

ELISA

ELISA were performed using 96-well Maxisorb Nunc plates coated with streptavidin (Sigma, 0.5 µg/mL). The dsDNA target was obtained by annealing redundant minus-strand to the 3' biotinylated plus-strand oligonucleotide at the ratio of 1.5:1 (10 sec at 90°C followed by slow cooling for 5 h). DNA immobilization was carried out with 100 pmol of biotinylated dsDNA per well. The plate was blocked by 2% BSA-PBS. VHD protein (1 µg) or purified phage (1×10^{11} TU) were mixed with 50 µL of 2% milk PBS, added to the well and incubated for 1 h at room temperature. The assay was performed using anti-SV5 mAb, followed by HRP conjugated goat anti-mouse IgG(γ) (KPL) and developed with TMB (3,3',5,5'-Tetramethylbenzidine) (Pierce) by reading at 450 nM. For competitive ELISA, VHD samples (0.5 µM) were pre-mixed with a solution containing: competitive DNA, in 0.1% Tween 20 in PBS for 1 h at room temperature, before loading to the plate.

DNA labeling

DNA labeling was performed by 5' phosphorylation using [γ -³²P]ATP and T4 polynucleotide kinase (PNK, BioLabs) using standard protocols. To obtain labeled dsDNA, plus and minus strand oligonucleotides were quantified by UV spectrophotometer and annealed at 1:1 ratio before labeling.

Band-shift assay

Gel mobility shift assay was performed by incubating 1 µM VHD with [γ -³²P]ATP-labeled DNA in binding buffer (20 mM Tris-HCl [pH 7.5], 40 mM KCl, 5 mM MgCl₂, 100 µg/mL BSA) for 1.5 h at room temperature, loaded into a precooled native PAGE (16% in TBE buffer), and run at 200 V. The gel was dried and exposed for autoradiography.

Filter-binding assay

Nitrocellulose filter binding was performed using a dot blot apparatus, to retain antibody bound labeled-DNA on a nitrocellulose membrane. To reduce retention of free ssDNA, membrane was presoaked for 10 min in 0.4 M KOH and rinsed with H₂O (Milli-Q

grade). The VHD-dsDNA binding was performed in PBS buffer, containing 1 nM labeled dsDNA, different concentrations of VHD, 500 µg/mL BSA, 100 nM poly[d(A-T)], in a total volume of 80 µL for 1 h at 37°C. The mixture was then filtered on the membrane and immediately washed twice with PBS. All experiments were done in duplicate. Signals on the membrane were quantified using radioactivity measurement with an Instant Imager apparatus (Packard Corp.). The signal of VHD bound ([DNA_{bound}]) was plotted against the input concentration of VHD ([X_{total}]). When [DNA_{total}] << [X_{total}], it can be estimated that [X_{free}] ≈ [X_{total}], and [DNA_{bound}]/[DNA_{total}] ≈ 1, at the plateau of the plotted curve. The K_d is subsequently obtained from the [X_{total}]_{Y = 0.5} from the normalized curve plotted, [DNA_{bound}]/[DNA_{total}] versus [X_{total}] (Neogy et al. 1974; Wong and Lohman 1993; Arosio et al. 2002).

References

- Ackroyd, P.C., Cleary, J., and Glick, G.D. 2001. Thermodynamic basis for sequence-specific recognition of ssDNA by an autoantibody. *Biochemistry* **40**: 2911–2922.
- Arosio, D., Cui, S., Ortega, C., Chovanec, M., Di Marco, S., Baldini, G., Falaschi, A., and Vindigni, A. 2002. Studies on the mode of Ku interaction with DNA. *J. Biol. Chem.* **277**: 9741–9748.
- Barbas, S.M., Ditzel, H.J., Salonen, E.M., Yang, W.P., Silverman, G.J., and Burton, D.R. 1995. Human autoantibody recognition of DNA. *Proc. Natl. Acad. Sci.* **92**: 2529–2533.
- Barry, M.M. and Lee, J.S. 1993. Cloning and expression of an autoimmune DNA-binding single chain Fv. Only the heavy chain is required for binding. *Mol. Immunol.* **30**: 833–840.
- Blatt, N.B. and Glick, G.D. 1999. Anti-DNA autoantibodies and systemic lupus erythematosus. *Pharmacol. Ther.* **83**: 125–139.
- Brigido, M.M., Polymenis, M., and Stollar, B.D. 1993. Role of mouse VH10 and VL gene segments in the specific binding of antibody to Z-DNA, analyzed with recombinant single chain Fv molecules. *J. Immunol.* **150**: 469–479.
- Cerutti, M.L., Centeno, J.M., Goldbaum, F.A., and de Prat-Gay, G. 2001. Generation of sequence-specific, high affinity anti-DNA antibodies. *J. Biol. Chem.* **276**: 12769–12773.
- Guth, A.M., Zhang, X., Smith, D., Detanico, T., and Wysocki, L.J. 2003. Chromatin specificity of anti-double-stranded DNA antibodies and a role for arg residues in the third complementary-determining region of the heavy chain. *J. Immunol.* **171**: 6260–6266.
- Hanke, T., Szawlowski, P., and Randall, R.E. 1992. Construction of solid matrix-antibody-antigen complexes containing simian immunodeficiency virus p27 using tag-specific monoclonal antibody and tag-linked antigen. *J. Gen. Virol.* **73**: 653–660.
- Harrison, S.C. 1991. A structural taxonomy of DNA-binding domains. *Nature* **353**: 715–719.
- Herron, J.N., He, X.M., Ballard, D.W., Blier, P.R., Pace, P.E., Bothwell, A.L., Voss Jr., E.W. and Edmundson, A.B. 1991. An autoantibody to single-stranded DNA: Comparison of the three-dimensional structures of the unliganded Fab and a deoxynucleotide-Fab complex. *Proteins* **11**: 159–175.
- Jang, Y.J., Sanford, D., Chung, H.Y., Baek, S.Y., and Stollar, B.D. 1998. The structural basis for DNA binding by an anti-DNA autoantibody. *Mol. Immunol.* **35**: 1207–1217.
- Jin, H.L., Sepúlveda, J., and Burrone, O.R. 2003. Selection and characterisation of binders based on homo-dimerisation of immunoglobulin V_H domains. *FEBS Lett.* **554**: 323–329.
- Komissarov, A.A., Calcutt, M.J., Marchbank, M.T., Peletskaya, E.N., and Deutscher, S.L. 1996. Equilibrium binding studies of recombinant anti-single-stranded DNA Fab. *J. Biol. Chem.* **271**: 12241–12246.
- Komissarov, A.A., Marchbank, M.T., Calcutt, M.J., Quinn, T.P., and Deutscher, S.L. 1997. Site-specific mutagenesis of a recombinant anti-single-stranded DNA Fab. *J. Biol. Chem.* **272**: 26864–26870.
- LeBlanc, J.F., McLane, K.E., Parren, P.W.H.I., Burton, D.R., and Ghazal, P. 1998. Recognition properties of a sequence-specific DNA binding antibody. *Biochemistry* **37**: 6015–6022.
- Li, Z.D., Schettino, E.W., Padlan, E.A., Ikematsu, H., and Casali, P. 2000. Structure-function analysis of a lupus anti-DNA autoantibody: Central role of the heavy chain complementarity-determining region 3 Arg in binding of double- and single-stranded DNA. *Eur. J. Immunol.* **30**: 2015–2026.

- Mandik-Nayak, L., Seo, S.J., Sokol, C., Potts, K.M., Bui, A., and Erikson, J. 1999. MRL-lpr/lpr mice exhibit a defect in maintaining development arrest and follicular exclusion of anti-double-stranded DNA B cells. *J. Exp. Med.* **189**: 1799–1814.
- McLane, K.E., Burton, D.R., and Ghazal, P. 1995. Transplantation of a 17-amino acid α -helical DNA-binding domain into an antibody molecule confers sequence-dependent DNA recognition. *Proc. Natl. Acad. Sci.* **92**: 5214–5218.
- Naparstek, Y., Andre-Schwarz, J., Manser, T., Wysocki, L.J., Breitman, L., Stollar, B.D., Gefter, M., and Schwartz, R.S. 1986. A single germline VH gene segment of normal A/J mice encodes autoantibodies characteristic of system lupus erythematosus. *J. Exp. Med.* **164**: 614–626.
- Neogy, R.K., Chowdhury, K., and Kerr, I. 1974. Nitrocellulose filter retention method for studying drug–nucleic acid interactions. *Biochem. Biophys. Acta* **374**: 96–107.
- O'Connor, K.C., Nguyen, K., and Stollar, B.D. 2001. Recognition of DNA by VH and Fv domains of an IgG anti-poly(dC) antibody with a singly mutated VH domain. *J. Mol. Recognit.* **14**: 18–28.
- Pabo, C.O. and Sauer, R.T. 1992. Transcription factors: Structural families and principles of DNA recognition. *Annu. Rev. Biochem.* **61**: 1053–1095.
- Polymenis, M. and Stollar, B.D. 1995. Domain interactions and antigen binding of recombinant anti-Z-DNA antibody variable domains. The role of heavy and light chains measured by surface plasmon resonance. *J. Immunol.* **154**: 2198–2208.
- Radic, M.Z., Mascelli, M.A., Erikson, J., Shan, H., and Weigert, M. 1991. Ig H and L chain contributions to autoimmune specificities. *J. Immunol.* **146**: 176–182.
- Radic, M.Z., Mackle, J., Erikson, J., Mol, C., Anderson, W.F., and Weigert, M. 1993. Residues that mediate DNA binding of autoimmune antibodies. *J. Immunol.* **150**: 4966–4977.
- Sanford, D.G. and Stollar, B.D. 1990. Characterization of anti-Z-DNA antibody binding sites on Z-DNA by nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* **265**: 18608–18614.
- Sepúlveda, J., Jin, H.L., Sblattero, D., Bradbury, A., and Burrone, O.R. 2003. Binders based on dimerised immunoglobulin V_H domains. *J. Mol. Biol.* **333**: 355–365.
- Shlomchik, M., Mascelli, M., Shan, H., Radic, M.Z., Pisetsky, D., Marshak-Rothstein, A., and Weigert, M. 1990. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. *J. Exp. Med.* **171**: 265–292.
- Simoncsits, A., Tjornhammar, M.L., Wang, S., and Pongor, S. 1999. Isolation of altered specificity mutants of the single-chain 434 repressor that recognize asymmetric DNA sequences containing the TTAA and TTAC subsites. *Nucleic Acid Res.* **27**: 3474–3480.
- Smeenk, R.J.T., Berden, J.H.M., and Swaak, A.J.G. 1996. dsDNA Autoantibodies. In *Autoantibodies* (eds. J.B. Peter and Y. Shoenfeld), pp. 227–236. Elsevier Science B.V., Amsterdam.
- Stevens, S.Y. and Glick, G.D. 1999. Evidence for sequence-specific recognition of DNA by anti-single-stranded DNA autoantibodies. *Biochemistry* **38**: 560–568.
- Stollar, B.D. 1994. Molecular analysis of anti-DNA antibodies. *FASEB J.* **8**: 337–342.
- Stollar, B.D., Zon, G., and Pastor, R.W. 1986. A recognition site on synthetic helical oligonucleotides for monoclonal anti-native DNA autoantibody. *Proc. Natl. Acad. Sci.* **83**: 4469–4473.
- Swanson, P.C., Cooper, B.C., and Glick, G.D. 1994. High resolution epitope mapping of an anti-DNA autoantibody using model DNA ligands. *J. Immunol.* **152**: 2601–2612.
- Swanson, P.C., Yung, R.L., Blatt, N.B., Egan, M.A., Norris, J.M., Richardson, B.C., Johnson, K.J., and Glick, G.D. 1996. Ligand recognition by murine anti-DNA autoantibodies. *J. Clin. Invest.* **97**: 1748–1760.
- Tanner, J.J., Komissarov, A.A., and Deutscher, S.L. 2001. Crystal structure of an antigen-binding fragment bound to single-stranded DNA. *J. Mol. Biol.* **314**: 807–822.
- van Es, J.H., Gmelig Meyling, F.H., van de Akker, W.R., Aanstoot, H., Derksen, R.H., and Logtenberg, T. 1991. Somatic mutations in the variable regions of a human IgG anti-double-stranded DNA autoantibody suggest a role for antigen in the induction of systemic lupus erythematosus. *J. Exp. Med.* **173**: 461–470.
- Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J., and Johnson, K.S. 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat. Biotechnol.* **14**: 309–314.
- Williamson, R.A., Burgoon, M.P., Owens, G.P., Ghausi, O., Leclerc, E., Frime, L., Carlson, S., Corboy, J., Parren, P.W.H.I., Shanna, P.P., et al. 2001. Anti-DNA antibodies are a major component of the intrathecal B cell response in multiple sclerosis. *Proc. Natl. Acad. Sci.* **98**: 1793–1798.
- Wong, I. and Lohman, T.M. 1993. A double-filter method for nitrocellulose-filter binding: Application to protein–nucleic acid interactions. *Proc. Natl. Acad. Sci.* **90**: 5428–5432.