Selection and characterisation of binders based on homodimerisation of immunoglobulin V_H domains

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Abstract The antigen-binding surface of antibodies is formed by the heterodimerisation of the two variable domains of the light (V_L) and heavy (V_H) chains. We have previously described the spontaneous formation of V_H dimers (VHD) in both bacteria and mammalian cells. The self-association of a single domain produces a homo-VHD, in which the two identical V_H domains generate a unique symmetric surface for antigen binding that is never found in the normal V_L/V_H antibody binding site. We developed a phagemid vector for the construction of phage display libraries in which a cysteine residue, introduced at the C-terminus of the only V_H cloned, allowed display of homo-VHDs. Panning of the library on different proteins yielded antigen specific binders against lysozyme, glutathione *S*-transferase and streptavidin. A lysozyme specific homo-VHD was further characterised with an apparent affinity determined to be 216 ± 6.6 nM. Importantly, the results showed that its binding activity was fully dependent on the dimerisation of both identical V_H domains.

Key words: Antibody engineering; Heavy chain variable domain; Phage display; pIII

1. Introduction

The Fv is the smallest antigen-binding antibody fragment, inheriting both binding specificity and affinity from the intact full antibody and formed by the heterodimeric association of the two variable domains V_L and V_H of the light (L) and heavy (H) chain, respectively. The actual antibody-combining site (paratope) is formed by six complementarity-determining regions, three from each V region, also named L1, L2, L3 (from V_L) and H1, H2, H3 (from V_H) [1–4]. However, their contributions to the antigen-binding surface are not evenly distributed. Detailed crystal structure analysis of antigen–antibody complexes have revealed that V_H domains in general, and the H3 loop in particular, contribute more to the antigen-binding surface, and are responsible for most of the molecular interaction with the antigen [5,6], although each individual antibody is different with respect to the contribution of the different loops, and in some cases an extra framework loop may also contribute to antigen binding. In fact, it has been found in some cases that V_H is able to bind to the antigen even in the absence of its light chain partner [7–9]. In particular, the larger diversity and binding ability of V_H is the result of a greater variability in the length and sequence, and hence the shape, of the H3 loop, which is the consequence of the VDJ recombination occurring during lymphocyte ontogeny. Furthermore, conformational diversity within a single antibody, in which the H3 loop can adopt at least four different conformations has also recently been shown [10]. For these reasons V_H domains are excellent candidates for the construction of novel binders based on dimerisation.

We have recently shown that V_H domains can spontaneously assemble into dimers (VHDs) that can be efficiently expressed and secreted from both mammalian cells and bacteria [11]. However, an interesting aspect of V_H dimerisation resides in the fact that each V_H can, in principle, associate with itself producing homodimers. These homo-VHDs would have a symmetric surface for antigen binding, a structure that is never present in the classic V_L/V_H-based antibody molecules. We thus wanted to investigate whether we could derive antigen specific binders based on dimerisation of single V_H domains. Here we show the selection and characterisation of such binders, which were derived from a filamentous phage library constructed to display homo-double chain (dc) VHDs. In one case we demonstrated that binding was completely dependent on the participation of the two identical V_H domains.

2. Materials and methods

2.1. Bacterial strains

Phage infection and propagation was carried out in strain DH5αF′ (F′/*endA1 hsdR17*($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA* (*Nal*^{r}) *relA1 D* (*lacZYA-argF*) *U169 deoR* (F80*dlacΔ*(*lacZ*)*M15*)), purchased from Gibco BRL. The non-supressor strain HB2151 (K12, *ara Δ*(*lac-pro*), *thi*/*F′proA*$^+$*B*$^+$, lackIqz*ΔM15*) was used for expression of soluble fragments [12].

2.2. Library construction

The original phagemid pDAN3 [13] was modified, for display and expression of dc V_H homodimers (homo-dcVHD/pDAN3) by introducing a linker sequence (encoding Gly-Cys-Gly) between the SV5 and His_6 tags. The single V_H was cloned into the unique *Bss*HII/*Nhe*I restriction sites. Total RNA (extracted from peripheral blood lymphocytes from 40 healthy donors) used for the library construction was kindly provided by A. Bradbury [14]. First strand cDNA was synthesised by reverse transcription (RT) using random hexamers. To obtain the human V_H fragments, polymerase chain reaction (PCR) amplification was carried out with primers previously described

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by Sblattero et al. [15]. The final PCR products were digested with *Bss*HII and *Nhe*I and ligated into the homo-dcVHD/pDAN3 phagemid vector. Ligated DNA was electroporated into the *Escherichia Coli* strain DH5αF′.

2.3. Homo-scVHD construction

For the construction of homo-scVHD, specific V_H fragment was amplified by PCR reaction using appropriate primers to introduce *Bss*HII/*Sal*I restriction enzyme sites for position 1 and *Xho*I/*Nhe*I for position 2. Amplified fragments were digested by the corresponding restriction enzyme, and cloned into the pDAN3 phagemid vector.

2.4. Fingerprinting

V_H fragments were amplified by PCR with primers of M13-reverse (backward, 5′-aacagctatgaccatg-3′) and Fdseq (forward, 5′-gaattttctgtatgagg-3′). PCR products were subsequently digested by *Bst*NI at 60°C for 2–3 h. The fingerprinting pattern was obtained by analysing the digestion products in a 2% agarose gel electrophoresis.

2.5. Phage selection and preparation

Phage selection was performed using a homo-VHD library (5×10^6 cfu) on immobilised lysozyme (from hen egg white, Boehringer), glutathione *S*-transferase (GST, Sigma), and streptavidin (from *Streptomyces avidinii*, Sigma). The selection was performed as described [14], starting with approximately 10^{12} phage particles per cycle. We started with low-stringency washes, recovering from 10^6 to 10^8 phages and increased the stringency after the 3rd cycle of panning. In the case of the selection of lysozyme binders, more than 90% of phages recovered after the last cycle were corresponded to VHD[7.6]. DH5αF′ bacterial strain was used for the preparation of phages. The bacteria were freshly cultured in 2×YT, 100 μg ml^{-1} ampicillin and 2% glucose, by shaking at 37°C for approximately 2 h till OD_{600} reached 0.5. Helper phage was then added with a ratio of phages:bacteria between 10:1 and 20:1. The mixture was left at 37°C for 30 min standing with occasional agitation, followed by 30 min shaking at 100 rpm at 37°C. Bacteria were recovered by centrifugation and cultured overnight at 30°C in fresh 2×YT, 100 μg ml^{-1} ampicillin supplemented with 25μg ml^{-1} kanamycin. Phage particles in the supernatant were collected and purified by two rounds of precipitation using PEG solution (20% polyethylene glycol 6000, Fluka, 2.5 M NaCl).

2.6. Enzyme-linked immunosorbent assay (ELISA) and Western blot

Binding activities of selected binders were performed by ELISA in 96-well Maxisorb Nunc plates coated with different concentrations of antigens (0.1–1 μg ml^{-1}). When using PEG-precipitated phages or soluble expressed proteins, an anti-SV5 tag mouse monoclonal antibody was used as first antibody followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG(γ) (KPL) and developed with TMB kit (Pierce). Data was obtained reading at 450 nM. For Western blots, PEG-precipitated phages (10^9–10^{10} phages per lane) or soluble protein (20–50 ng per lane), were run in a 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequently transferred to Immobilon™-P transfer membrane (Millipore) for 2 h at 200 V or overnight at 50 mA. The membrane was blocked with 4% milk, 0.1% Tween 20 in phosphate-buffered saline (PBS) for at least 2 h at room temperature, reacted with anti-SV5 mAb and developed with HRP-conjugated goat anti-mouse IgG(γ) (KPL) followed by ECL reagents kit (Amersham Pharmacia Biotech).

2.7. Soluble homo-VHD protein expression and purification

The HB2151 bacterial strain was used for expression of soluble homo-VHD. Bacteria were freshly cultured by incubating at 37°C with shaking till OD_{600} reached 0.7. Expression was induced by adding IPTG (to 0.5 mM) and incubated at 30°C in a shaker (200 rpm) for at least 4 h. Bacteria pellet was resuspended in PPB buffer (200 mg ml^{-1} sucrose, 1 mM EDTA, 30mM Tris–HCl pH8.0) at 1/40 total volume for 20 min on ice, centrifuged and the supernatant collected. The cell pellet was resuspended in 5 mM $MgSO_4$ buffer at 1/40 of total volume, kept on ice for 20 min, and recentrifuged. The supernatant was collected, combined with the previous one, dialysed against PBS and applied to a Ni-NTA agarose column (Qiagen) for purification. The column was washed with 10 ml 35 mM imidazole (Riedel-deHaën) in PBS, and protein eluted with 2 ml 250 mM imidazole in PBS. Quantification of recovered purified proteins was carried out using Coomassie-plus protein assay reagent kit (Pierce).

2.8. Affinity determination

Determination of binding affinities was based on the ELISA method of Shigeo Katoh et al. [16]. In this method two 96-well microtitre plates (Maxisorb, Nunc) are used to determine the concentration of binder in solution at equilibrium with Ag adsorbed on the surface of the plates: one plate, P(E), is used for equilibration of the binder (VHD) at different known initial concentrations (C_0) (from 2×10^{-9} to 2×10^{-6} M), with the plate coated with a fixed amount of antigen (2 μg lysozyme per well); the second plate, P(M), coated with a lower amount of antigen (0.2 μg lysozyme per well) is used to determine the concentration of free binder (C) after incubation to equilibrium (2 h, at room temperature) in plate P(E). The signal of bound VHD in plate P(M) was developed using HRP-conjugated goat anti-mouse IgG(γ) (KPL) and the TMB kit (Pierce). The equation $(C_0-C)/C=-Kmn(C_0-C)+KnA_{max}/v$ is used for the affinity determination (where: n, antibody valence = 1 for scVHD; m, considered to be one in the case of protein antigens; v, volume of the binder in the well; A_{max}, number of total binding sites per well). The value of the association constant K is determined from the slope of the straight line obtained by plotting $(C_0-C)/C$ vs. (C_0-C).

3. Results

3.1. Design and construction of homo-dcVHD phage library

Following our demonstration that VHD are spontaneously assembled in both bacteria and mammalian cells [11], we wanted to investigate the ability of single V_H domains to assembly into homo-VHDs and to study their antigen-binding capacities. For this purpose we modified a previously reported vector for phage display of scFvs or scVHDs constructs [13] by introducing a cysteine residue between the SV5 and the His_6 tags, to facilitate disulphide bond formation in the bacterial periplasm (Fig. 1). We also adapted the restriction sites to accommodate a single V_H. In our strategy we took advantage of the presence of an amber codon separating the V_H/tag moiety from pIII. Since the efficiency of amber suppression (stop→Gln) in DH5αF′ bacteria is around 50%, two different products were expected: V_H/SV5/cys/His_6/pIII and V_H/SV5/cys/His_6. Dimerisation in the bacterial periplasmic fraction between these two V_H domains should produce a covalently disulphide bond-stabilised homo-VHD, which would be incorporated and displayed by the filamentous phage. In addition, dimerisation of the short product should generate a dcVHD

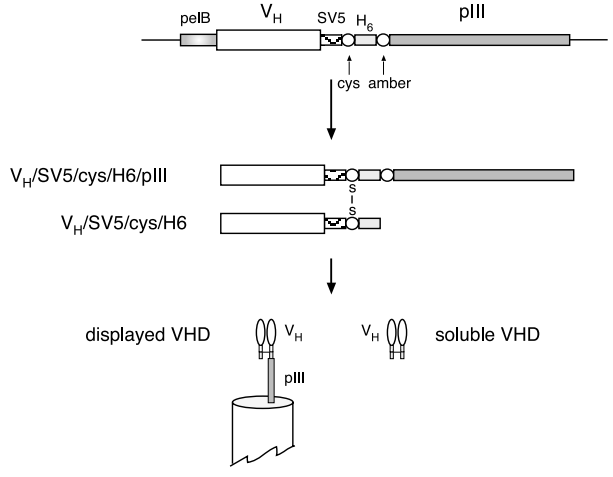

Fig. 1. Schematic representation of the phagemid constructed for the selection of homo-VHD. Two proteins can be produced, one of them fused to the pIII phage protein. Upon association in the periplasmic fraction, disulphide-bonded stabilised VHD can be phage displayed or produced as soluble dcVHD.

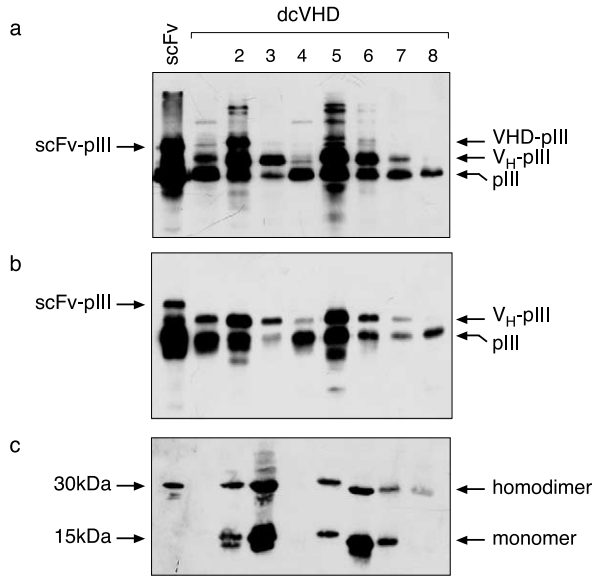

Fig. 2. Western blot analysis of randomly picked phage displayed VHDs, run under non-reducing and reducing conditions (a and b, respectively) and the corresponding soluble products (c) analysed in non-reducing conditions. Mouse anti-SV5 mAb was used in all three cases.

that could be directly recovered from the culture supernatant or bacterial periplasmic fraction. In order to select homo-VHD binders we constructed a phage display library with human V_H obtained by RT-PCR amplification of total RNA derived from PBLs of different healthy donors. The library obtained had a relatively small size of 5×10^6 total colonies. To study the display capability of homo-dcVHDs on the surface of the phage particle, we randomly picked colonies from the library for phage production. The purified phages were analysed by Western blot under reducing and non-reducing conditions.

As shown in Fig. 2a, two forms of the pIII fusion protein could be observed under non-reducing conditions in some, but not all, of randomly picked clones; the lower band representing display of the single V_H domain while the upper one corresponded to the covalently bound homo-VHD. As expected, under reducing conditions, only a band corresponding to the single V_H-pIII fusions was observed (Fig. 2b).

This result suggested that inter-chain disulphide bond-stabilised homo-dcVHDs fused to the pIII phage minor coat protein could be displayed on the surface of the phage particle. In addition, expression of soluble homo-dcVHD protein by different random clones was confirmed by Western blot analysis of periplasmic fractions, showing both the expected 30 kDa band of the dimer and the ~15 kDa of the monomer (Fig. 2c). However, in one case (clone 8), all the expressed V_H protein detected was stabilised by an inter-chain disulphide bond, though with a relatively lower level of expression.

3.2. Selection and characterisation of homo-dcVHDs

The constructed phage library was panned against different antigens to select homo-VHDs with antigen specific binding activity. We selected clones against lysozyme (dcVHD$^{7.6}$), GST (dcVHD^{G8}), and streptavidin (dcVHD^{S6}), which were expressed as dimers (Fig. 3a). Binding of the selected homo-dcVHDs to their corresponding antigens was studied by ELISA. Each homo-dcVHD showed specific interaction to the corresponding antigen (Fig. 3b). No binding activities were found for any homo-dcVHD in any of the two formats, to the other three control proteins. Randomly picked dcVHD were used as a negative control in ELISA.

Of the three binders, homo-VHD$^{7.6}$ appeared to have the strongest binding activity to its antigen, and was the only one

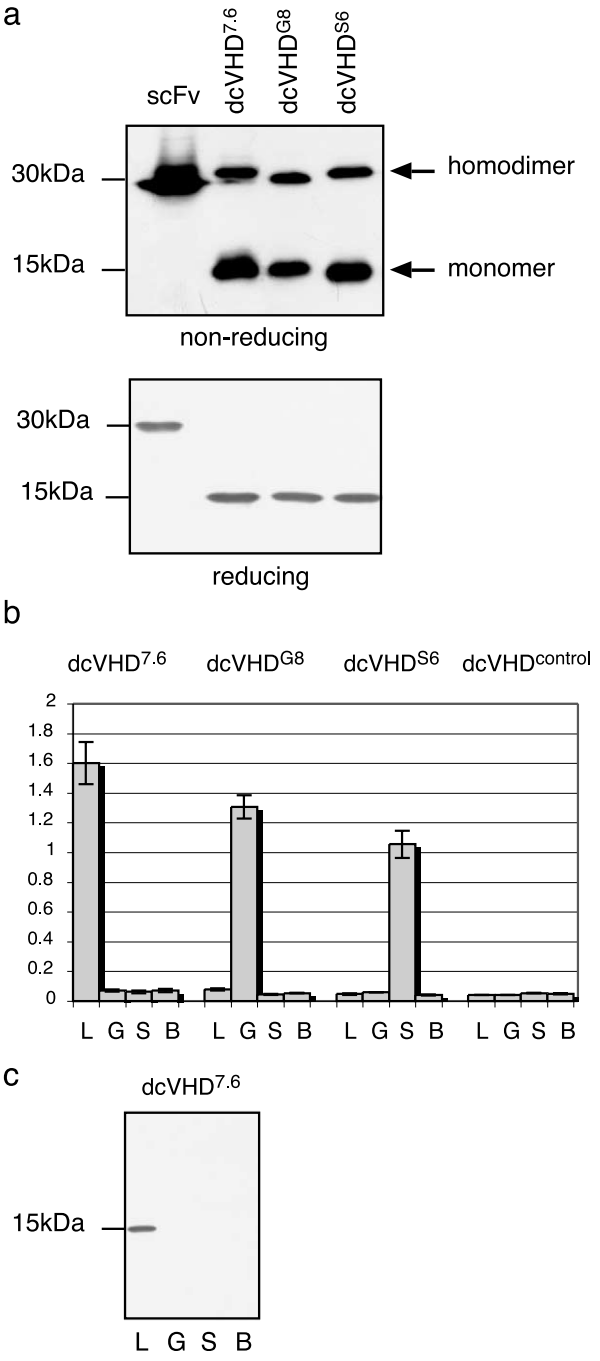

Fig. 3. Analysis of soluble fractions of selected VHDs in the dc format. a: VHDs were obtained from periplasmic fractions and analysed in reducing and non-reducing conditions, as indicated. b: ELISA of the three selected VHDs, reacted with plates coated with lysozyme (L), GST (G), streptavidin (S), and BSA (B). An irrelevant VHD was used as control. c: Western blot of the four different proteins (100 ng each) resolved in a 10% SDS–PAGE for membrane transfer, and reacted with dcVHD$^{7.6}$ (2 μg VHD in 10 ml PBS).

$V_H^{7.6}$

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1/1                                     31/11                                   61/21
GAG GTG CAG CTG GTG GAG ACT GGA GGA GGC TTG ATC CAG CCT GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT GGG TTC ACC GTC AGT
E   V   Q   L   V   E   T   G   G   G   L   I   Q   P   G   G   S   L   R   L   S   C   A   A   S   G   F   T   V   S
91/31                                   121/41                                  151/51
AGC AAC TAC ATG AGC TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTC TCA GTT ATT TAT AGC GGT GGT AGC ACA TAC TAC GCA
S   N   Y   M   S   W   V   R   Q   A   P   G   K   G   L   E   W   V   S   V   I   Y   S   G   G   S   T   Y   Y   A
181/61                                  211/71                                  241/81
GAC TCC GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTT CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG
D   S   V   K   G   R   F   T   I   S   R   D   N   S   K   N   T   L   Y   L   Q   M   N   S   L   R   A   E   D   T
271/91                                  301/101                                 331/111
GCC GTG TAT TAC TGT GCG AAG GGG GGG GAA CTG GAC CTC GAC TAC TAC TAC TAC GGT ATG GAC GTC TGG GGC CAA GGG ACC ACG GTC ACC
A   V   Y   Y   C   A   K   G   G   E   L   D   L   D   Y   Y   Y   Y   G   M   D   V   W   G   Q   G   T   T   V   T
361/121
GTC TCC TCA
V   S   S
```

V_H^{G8}

```
1/1                                     31/11                                   61/21
GAG GTG CAG CTG GTG GAG ACT GGG GGA GGC TTG GTC CAG CCT GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTT GGT
E   V   Q   L   V   E   T   G   G   G   L   V   Q   P   G   G   S   L   R   L   S   C   A   A   S   G   F   T   F   G
91/31                                   121/41                                  151/51
GAT TAT GCT CTG AGC TGG TTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTC TCA TCC ATT AGT AGT AGT AGT AGT TAC ATA TAC TAC
D   Y   A   L   S   W   F   R   Q   A   P   G   K   G   L   E   W   V   S   S   I   S   S   S   S   S   Y   I   Y   Y
181/61                                  211/71                                  241/81
GCA GAC TCA GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAC GCC AAG AAC TCA CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC
A   D   S   V   K   G   R   F   T   I   S   R   D   N   A   K   N   S   L   Y   L   Q   M   N   S   L   R   A   E   D
271/91                                  301/101                                 331/111
ACG GCT GTG TAT TAC TGT GCG AGA GAT TCG GCC TAT GAT AGT AGT GGT GAT GAT GCT TTT GAT ATC TGG GGC CAA GGG ACA ATG GTC ACC
T   A   V   Y   Y   C   A   R   D   S   A   Y   D   S   S   G   D   D   A   F   D   I   W   G   Q   G   T   M   V   T
361/121
GTC TCT TCA
V   S   S
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V_H^{S6}

```
1/1                                     31/11                                   61/21
CAG GTG CAG CTG CAG GAG TCG GGC CCA GGA CTG GTG AAG CCT TCG GAG ACC CTG TCC CTC ACC TGC ACT GTC TCT GGT GGC TCC ATC AGC
Q   V   Q   L   Q   E   S   G   P   G   L   V   K   P   S   E   T   L   S   L   T   C   T   V   S   G   G   S   I   S
91/31                                   121/41                                  151/51
AGT AGT AGT TAC TAC TGG GGC TGG ATC CGC CAG CCC CCA GGG AAG GGG CTG GAG TGG ATT GGG AGT ATC TAT TAT AGC GGG AGC ACC TAC
S   S   S   Y   Y   W   G   W   I   R   Q   P   P   G   K   G   L   E   W   I   G   S   I   Y   Y   S   G   S   T   Y
181/61                                  211/71                                  241/81
TAC AAC CCG TCC CTC AAG AGT CGA GTC ACT ATA TCA GTA GAC ACG TCC AAG AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACT GCC GCA
Y   N   P   S   L   K   S   R   V   T   I   S   V   D   T   S   K   N   Q   F   S   L   K   L   S   S   V   T   A   A
271/91                                  301/101                                 331/111
GAC ACG GCC GTG TAT TAC TGT GCG AGA GAC CGT GGC TAC GGT GGT AAC CCC TTT GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC
D   T   A   V   Y   Y   C   A   R   D   R   G   Y   G   G   N   P   F   D   Y   W   G   Q   G   T   L   V   T   V   S
361/121
TCA
S
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Fig. 4. DNA and deduced amino acid sequences of the V_H domains of selected VHDs. The sequences of the H3 loops are underlined.

able to recognise the antigen (lysozyme) in Western blot (Fig. 3c).

The V_H sequences of the selected clones (Fig. 4) correspond to V_H families VH3 and VH4 and have H3 of average lengths (15, 14 and 11 for $VHD^{7.6}$, VHD^{G8} and VHD^{S6}, respectively). However, V_Hs, from randomly picked clones, corresponding to other VH families were also found to dimerise (data not shown).

3.3. Apparent affinity binding of homo-scVHD$^{7.6}$

To measure the apparent affinity for lysozyme binding, soluble $VHD^{7.6}$ was expressed in the single chain (sc) format with a His_6 tag ($scVHD^{7.6}$). The $scVHD^{7.6}$ protein was purified on a nickel column and visualised in SDS–PAGE (Coomassie blue staining) as a single band of ~31 kDa (Fig. 5a).

ELISA [16] was used to measure the apparent binding affinity of homo-$scVHD^{7.6}$/lysozyme interaction. Fig. 5b shows the plot from which the apparent affinity, derived from the slope of the curve, produced a value of 216 ± 6.6 nM.

3.4. Binding of homo-VHD$^{7.6}$ is dependent on V_H dimerisation

We then wanted to address the important point of the relative contribution of either one or two of the V_H domains to the formation of the binding site. To confirm that the binding of the homo-VHD to antigen was dependent on the dimerisation of the two V_H domains, we expressed homo-$VHD^{7.6}$ in the sc format and designed a domain-shuffling experiment to study whether or not two $V_H^{7.6}$ were needed for binding. We therefore replaced one $V_H^{7.6}$ by several randomly picked, non-relevant V_L or V_H and expressed them to study their binding activities (Fig. 6a).

Clones of scFv (V_L-$V_H^{7.6}$) were constructed by subcloning different V_L fragments into position 1, whereas scVHDs were constructed with the non-relevant V_H either in position 1 (V_H-$V_H^{7.6}$) or position 2 ($V_H^{7.6}$-V_H). All these clones were characterised by *Bst*NI fingerprinting, and the expressed proteins analysed by Western blot (Fig. 6b,c).

Analysis of the binding activities of all clones (Fig. 6d) revealed that, contrary to the highly positive binding of the two controls homo-$VHD^{7.6}$, none of them was able to specif-

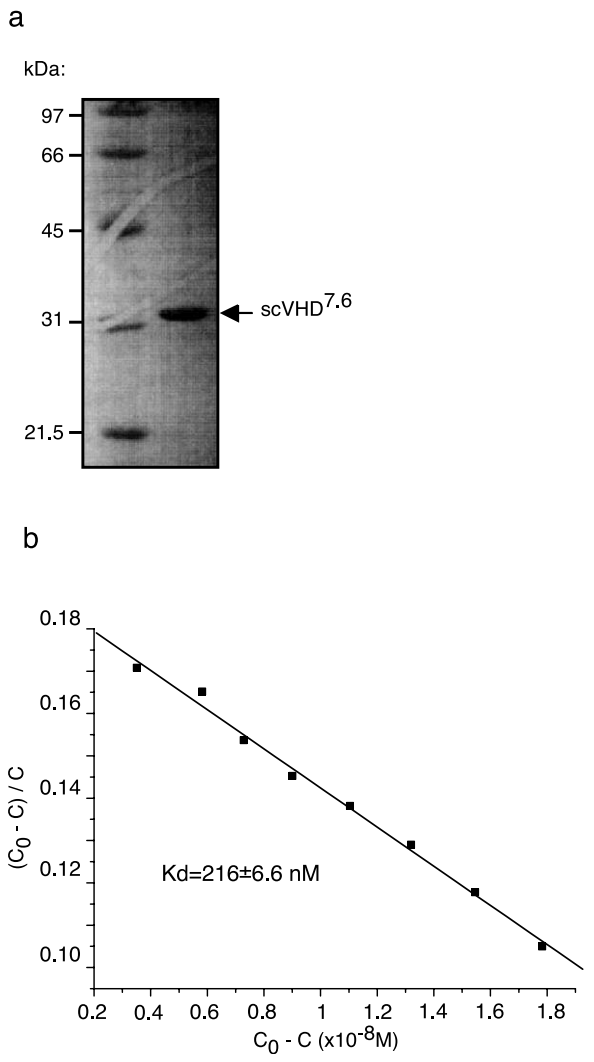

Fig. 5. Affinity determination of the scVHD$^{7.6}$/lysozyme binding. a: Coomassie blue staining of the purified material used to determine the apparent K_d. b: The value of 216 ± 6.6 nM was obtained from the slope of the straight line. C_0 and C correspond to the initial concentration of scVHD and the concentration of free scVHD in equilibrium, respectively.

ically recognise the lysozyme antigen, suggesting that the binding site was indeed formed by self-dimerisation of $V_H^{7.6}$.

To further exclude the possibility that lysozyme-binding activity of homo-VHD$^{7.6}$ was the consequence of independent binding by each single $V_H^{7.6}$ subunit within the molecule, we performed two additional experiments. First, $V_H^{7.6}$ was expressed as a single domain without the terminal cysteine. The expressed protein did not show any lysozyme-binding activity (Fig. 7a), suggesting that dissociation of the two chains in ELISA eliminated the binding site and therefore antigen binding. In the second type of experiment, we constructed a 'camelised' [17] sc version of homo-$V_H^{7.6}$ (scVHD$^{7.6}$-cml) in which FR2 residues in the interface, G44, L45 and W47, were mutated into E, R and G, respectively. As shown in Fig. 7b, this construct was well expressed but completely unable to bind antigen. Taken together, all these results confirmed the absolute requirement of the two dimerised identical $V_H^{7.6}$ domains for antigen binding.

4. Discussion

Detailed crystal structure analysis of antigen–antibody complexes have revealed that V_H domains in general, and the H3 loop in particular, contribute more to the antigen-binding surface, and are responsible for most of the interaction with the antigen [18,19].

The specific role of H3 in the antibody–antigen interaction has been supported by the fact that sequence diversity is highly concentrated in this loop [2,3]. It has been reported that the diversity of H3 loop is sufficient for most antibody specificities, permitting otherwise identical IgM molecules to distinguish between a variety of hapten and protein antigens [6]. It was also found in many cases that the V_H is able to bind to the antigen even in the absence of its light chain partner [7–9]. Furthermore, libraries of synthetic H3 loops inserted into a fixed scFv platform have been reported to be a source of antigen specific binders [20]. We have recently described that immunoglobulin V_H domains can associate into dimers with antigen-binding specificity [11]. VHDs can be formed by either two different V_H fragments (hetero-VHDs), or by two identical ones (homo-VHDs), as described in this report. Both of them, however, take advantage of the spontaneous ability of these fragments to associate between them as well as of the larger binding capacity and diversity of V_H in relation to V_L.

We assume the two V_H domains to interact through the same interface that associates with V_L and in the same relative orientation. This would be similar to the homodimerisation of L chains, which are known to dimerise through pairing of the two homologous domains (V_L/V_L and CL/CL) [21]. V_L dimers with binding activity have also been described [22]. The most conserved V_H residues involved in the V_L/V_H interface (like Gly^{44}, Leu^{45}, Glu^{46}, Trp^{47}, hydrophobic^{48}, in FR2 and Trp^{103}, Gly^{104}, Gly^{106} in FR4), which are in part of hydrophobic nature and participate in the large interface surface of 1000–1700 Å^2, could serve as a platform for V_H/V_H interaction [23,24].

To select homo-VHDs, we used a vector that allowed the formation of an inter-chain disulphide bond to obtain covalently stabilised V_H homodimers in the dc format, displayed through the minor coat protein pIII. The inter-chain disulphide bond was achieved by the introduction of a cysteine residue at the C-terminal to the V_H domain. This strategy is similar to the one applied for the display of Fab on the filamentous phage [12,25], although in our system, the two partners were generated from the same transcript. Phage display of homodimeric peptides fused to the phage major coat protein pVIII has also been recently reported [26].

We selected antigen specific binders that were expressed as soluble proteins in both sc and dc formats. In the case of the dc format, the relative amount of dimer formation was found to vary among different clones, possibly reflecting differences in the ability of dimerisation of the diverse V_H families. In fact, variability in the relative ability of heterodimerisation between V_L and V_H with different sequences has also been described [27]. Interestingly, the amino acid sequences of the three selected V_Hs have largely maintained the germline sequence of the corresponding VH gene segments (two of them from the family VH3 and one from the family VH4).

The measurement of the apparent relative affinity of the lysozyme specific VHD was around 200 nM. This affinity,

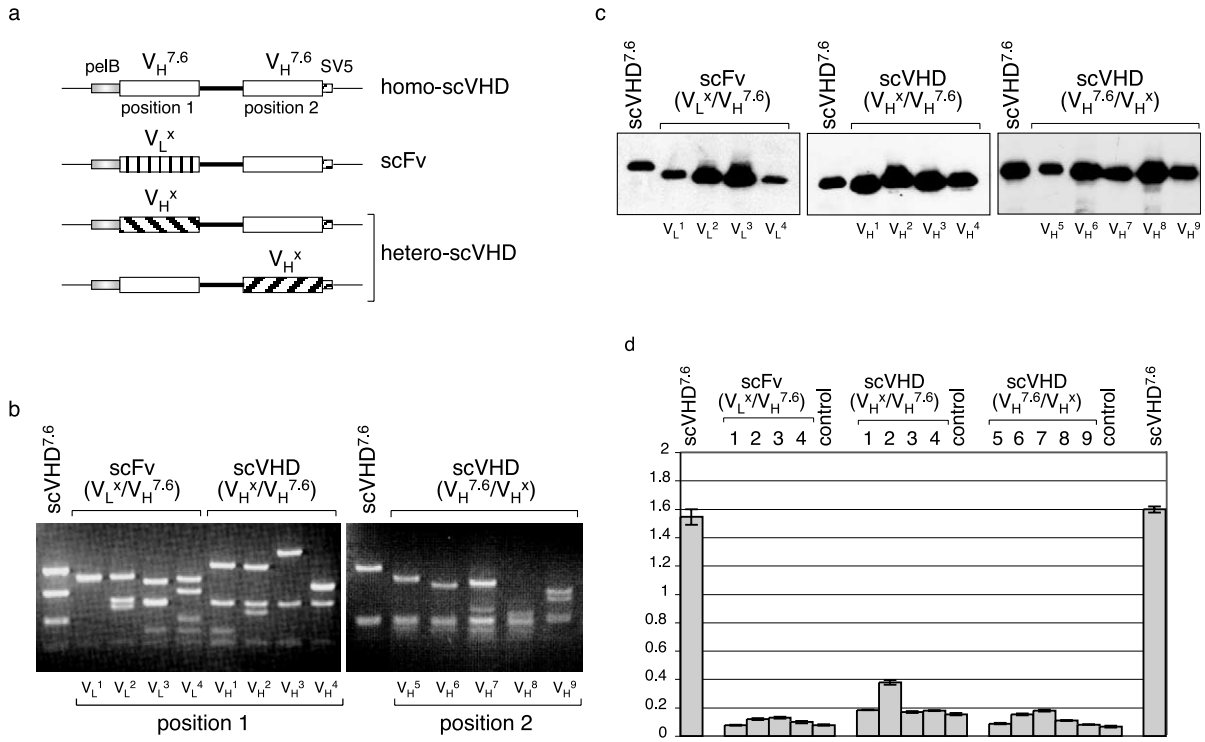

Fig. 6. Domain shuffling in scVHD$^{7.6}$. a: Randomly picked V domains were inserted in either position 1 (V_Ls or V_Hs) or position 2 (only V_Hs) of scVHD$^{7.6}$. Fingerprinting (b) and Western blot analysis (c) of the indicated clones. d: ELISA of individual clones on lysozyme-coated plates. Control corresponds to an irrelevant scVHD clone.

for a binder selected from the dcVHDs phage library with a diversity of 5×10^6 is actually promising. In fact, when scFv or Fab phage libraries contained a larger number (3×10^7) of clones, the average affinity of the selected antibodies was in the 10^{-5}–10^{-7} M range [28,29], while when selecting from libraries with $\sim10^{10}$ independent clones, the affinities could reach the 10^{-8}–10^{-9} M range [14,30–32]. In order to address the question of whether both V_H domains of the homo-VHD participate in the recognition of the antigen (lysozyme), we designed and performed experiments with three different types of constructs: (a) a domain-shuffling experiment using the sc format, in which one of the two $V_H^{7.6}$ was replaced by several irrelevant V_Hs or V_Ls, (b) a dc format in which the C-terminal cysteine was deleted, thus allowing also monomeric interac-

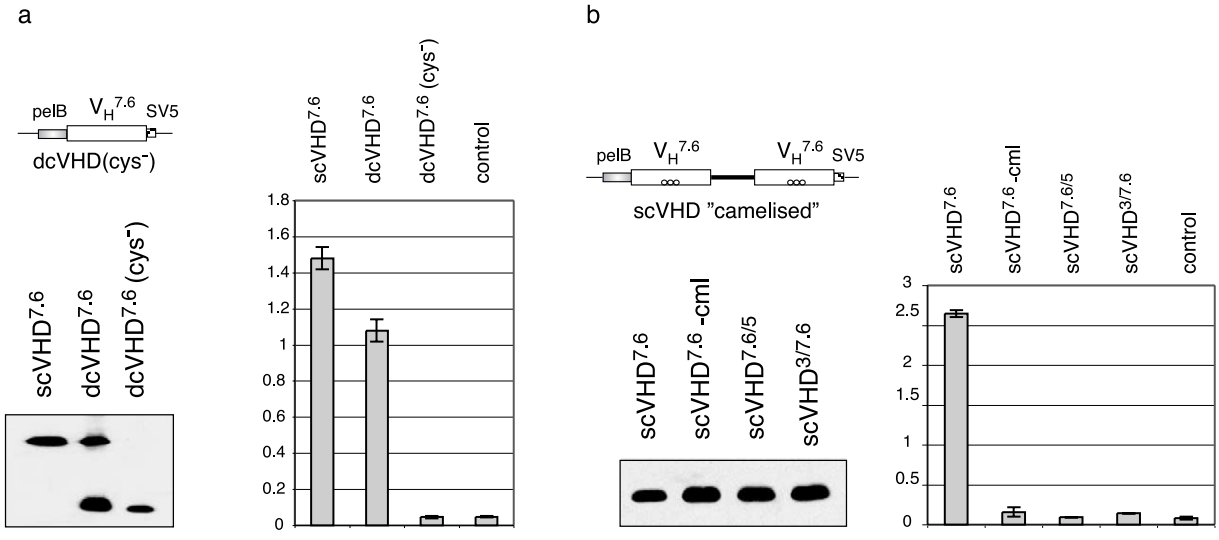

Fig. 7. Binding depends on homodimerisation of $V_H^{7.6}$. a: A dcVHD$^{7.6}$ construct with no cysteine was expressed (monomer in Western blot of non-reducing SDS–PAGE) and its binding activity determined by ELISA. Binding of the sc and dc versions of VHD$^{7.6}$, as well as an irrelevant scVHD, was also measured as controls. b: A 'camelised' version of the scVHD$^{7.6}$ (scVHD$^{7.6}$-cml) was constructed with three substitutions in FR2 to eliminate interaction between the two V_Hs. Binding activity was determined by ELISA including, as negative controls, two scVHD (scVHD$^{7.6/5}$ and scVHD$^{3/7.6}$) and, as positive control, the wild-type scVHD$^{7.6}$.

tions and (c) a 'camelised' sc format in which the two $V_H^{7.6}$ would not be associated, yet still linked, thus maintaining the possible bivalency [17].

If antigen binding were dependent on a single V_H domain, we would have expected binding activity in all three different cases. On the contrary, if only $V_H^{7.6}$-associated homodimers were active binders, we would not see any binding in all three cases, as it was indeed the case. Hence, we concluded that binding by $VHD^{7.6}$ was the consequence of an antigen-binding surface created by homodimerisation.

The work presented here demonstrated that V_H homodimers can be displayed and selected from filamentous phage libraries. The interesting dimeric structure formed by the association of two identical V_H domains with a symmetric antigen-binding site provides a novel platform for the construction of antigen specific binders that could serve as the basis for recognition of symmetric targets.

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