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Expression and secretion of repertoires of VH domains in *Escherichia coli*: isolation of antigen binding activities

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INTRODUCTION

The production of monoclonal antibodies using hybridoma technology (Köhler and Milstein, 1975) represents a landmark for the use of antibodies as reagents for medicine and biology. It is now possible to use recombinant DNA methods to isolate and genetically manipulate antibody genes, and the resulting immunoglobulins or immunoglobulin fragments can be efficiently expressed in mammalian or bacterial hosts (for a review, see Morrison et al., 1988).The immunoglobulin molecule consists of a string of domains, each domain consisting of about 100 amino acid residues. The constant domains carry the effector functions, such as complement mediated lysis and ADCC. The antigen binding site is fashioned by both heavy (VH) and light (VL; Vκ or Vλ) chain variable domains, as demonstrated by the solved crystallographic structures of antibody in association with antigen (Amit et al.,1986; Sheriff et al., 1987; Colman et al.,1987) or hapten (Satow et al, 1986). Variable domains have been pasted onto constant domains (Morrison et al.,1984; Boulianne et al., 1984; Neuberger et al.,1985) and hypervariable loops (CDRs) onto the underlying β-sheet framework of variable domains (Jones et al.,1986; Verhoeyen et al.,1988; Riechmann et al.,1988a). Grafting CDRs has been used to humanise rodent antibodies and one such antibody used in the successful treatment of 2 patients with non-Hodgkins lymphoma (Hale et al.,1988; Riechmann et al.,1988a).

As the variable domains of an antibody confer the affinity and specificity of binding, their isolation and characterisation are prerequisites for both structure-function analyses and medical applications. There is therefore a need for methods of rapidly generating and screening banks of variable domains for desired binding specificities. The expression and secretion of antibody variable domains using *E.coli* as a host represents a convenient way of cloning and analysing binding activities, and is generally much faster than the route using mammalian expression systems.

We have been analysing the interactions of the anti-lysozyme antibody D1.3 with antigen (Amit et al., 1986), and have expressed the VH and VL domains individually, or in association as an Fv fragment by secretion into the *E. coli* periplasm (Skerra and Plückthun, 1988). Both the Fv fragment and the VH domain can be purified. They both bind antigen with a high affinity. This observation led us to generate repertoires of VH domains for the expression of binding activities in *E. coli*. To generate these VH repertoires two approaches have been used; (1) rearranged VH genes have been cloned from antibody producing cells of immunised mice using the polymerase chain reaction (PCR), (2) the gene encoding the VH domain of the anti-lysozyme D1.3 antibody has been extensively mutated. For both repertoires, VH domains with desired binding specificities have been identified and, in some cases, purified and characterised kinetically.

METHODS

Vectors

For expression of the VH domain (VHD1.3) of the D1.3 antibody, the vector pSW1-VHD1.3 was constructed by cloning the VHD1.3 gene into a pUC19 vector (Yanisch-Perron et al., 1985) with a synthetic oligonucleotide encoding a pelB signal sequence (Better et al.,1988) (Fig. 1). For expression of both domains, the vector pSW1-VHD1.3-VKD1.3 was constructed by cloning the Vκ domain and pelB signal into pSW1. For cloning and expression of the VH repertoire, the vector pSW1-VHPOLY was built by cloning a restriction enzyme polylinker sequence to replace the body of the VHD1.3 gene. This vector was further adapted (pSW1-VHPOLY-TAG1) by ligating a synthetic oligonucleotide encoding a peptide tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn) from c-myc (Evan et al., 1985; Munro and Pelham, 1986), to the C-terminal end.

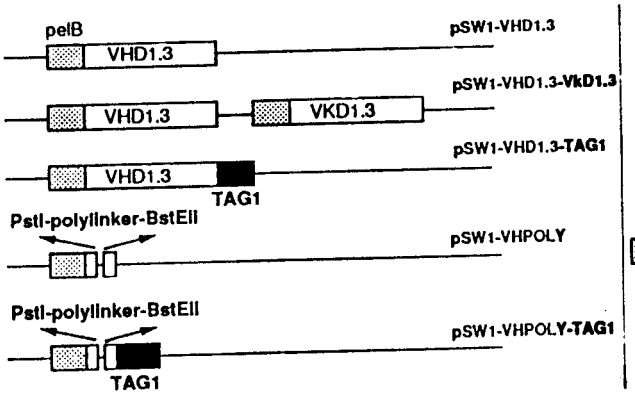

Figure 1. Expression vectors for secretion of immunoglobulin fragments from *E. coli*.

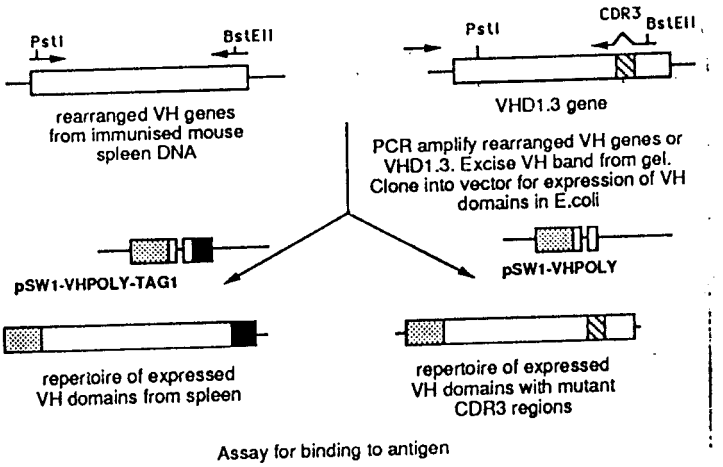

Figure 2. Strategy for the cloning of VH repertoires into *E. coli* expression vectors.

PCR amplification of mouse DNA (see Fig. 2)

Balb/c mice were hyperimmunised with hen egg-white lysozyme (100 µg antigen i.p. day 1 in complete Freunds adjuvant and 50 µg antigen i.v. day 35 in incomplete Freunds adjuvant, kill day 39) or similarly with keyhole limpet haemocyanin (KLH). DNA was prepared from the spleen (Maniatis et al., 1982) and the rearranged mouse VH genes were amplified using PCR (Saiki et al., 1985; Orlandi et al., 1989) using the primers VH1FOR-2 (5' TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC 3') and VH1BACK (5' AGG T(C/G)(C/A) A(G/A)C TGC AG(G/C) AGT C(T/A)GG 3') and conditions described in Ward et al., 1989.

PCR mutagenesis (see Fig. 2)

The VHD1.3 gene cloned into M13mp19 was amplified with a mutagenic primer based in CDR3 and a primer based in the M13 vector backbone (5' AAC AGC TAT GAC CAT G 3'). The mutagenic primer 5' GGA GAC GGT GAC CGT GGT CCC TTG GCC CCA GTA GTC AAG NNN NNN NNN NNN CTC TCT GGC 3' (where N can be any of the four bases T, C, G or A) hypermutates the central 4 residues of CDR3 (Arg-Asp-Tyr-Arg).

Cloning and expression of antigen binding activities

PCR amplified DNA was digested with PstI and BstEII (encoded within the amplification primers) and fractionated on an agarose gel. A band of about 350 bp was extracted and cloned into the M13VHPCR1 vector (Orlandi et al., 1989) for sequencing or into the pSW1-VHPOLY or pSW1-VHPOLY-TAG1 vector for expression. The recombinant plasmids were transformed into *E. coli* BMH71-18 (Gronenborn, 1976), colonies selected on TYE plates (Miller, 1972) with 1% glucose (GLU) and 100 µg/ml ampicillin (AMP), and toothpicked into 200 µl 2 x TY (Miller, 1972), AMP, GLU in wells of ELISA plates. Colonies were grown and induced as in Ward et al., 1989.

Western blotting

As in Towbin et al., 1979: supernatant (10 µl) from the cultures was subjected to SDS-PAGE (Laemmli, 1970) and the proteins transferred electrophoretically to nitrocellulose. The VH domains were detected via the peptide tag with the 9E10 antibody (Evan et al., 1985; Munro and Pelham, 1986), using horse radish peroxidase conjugated rabbit anti-mouse antibody and 4-chloro-1-naphthol as the peroxidase substrate.

ELISA

Wells of ELISA plates (Falcon) were coated with antigen in phosphate buffered saline (PBS) overnight (3 mg/ml lysozyme or 50 µg/ml KLH), then blocked with 2% skimmed-milk powder in PBS for 2 hr at 37 °C. Bacterial supernatants were screened for binding activities as in Ward et al., 1989. For competition ELISA, the binding of VHD1.3 tagged with the c-myc peptide (Fig. 1) was assayed in the presence of dilutions of untagged VH domain (VH1).

Purification of Fv and VH domains binding to lysozyme

500 ml cultures were grown and induced as above, and the supernatant passed through a 0.45 µm filter (Nalgene), then down a 5 ml column of lysozyme-Sepharose (Riechmann et al., 1988b). After washing with phosphate buffered saline (PBS), the Fv fragment or VH domains were eluted with 50 mM diethylamine, neutralised with 1/10 volume 1M Tris-HCl pH 7.4, and analysed by SDS-PAGE.

Affinity for lysozyme

The purified D1.3 Fv fragment and VH domains were titrated with lysozyme using fluorescence quench (Perkin Elmer LS-5B Luminescence Spectrometer) to determine their affinities of binding. The stoichiometry of binding of the VHD1.3 domain was measured by fluorescence quench titration (to yield the total number of lysozyme binding sites), and by amino acid hydrolysis (to yield the total amount of protein). The kinetics of lysozyme binding were determined by stopped-flow (HI Tech Stoped Flow SHU) at 20 °C under pseudo-first order conditions with binding sites in 5-10-fold excess over lysozyme . The number of binding sites was determined by fluorescence quench titration with lysozyme in excess (Ward et al.,1989).

RESULTS

Expression of D1.3 Fv and VH in *E. coli*

The Fv fragment of the D1.3 antibody was expressed in *E. coli* using the plasmid construction shown in Fig.1. Secreted Fv could be purified with a yield of about 10 mg/litre culture. SDS polyacrylamide gel electrophoresis of the pure Fv showed 2 bands of about 14 kDa. N-terminal sequence determination after transfer of the 2 separated bands onto PVDF membranes (Matsudaira, 1987; Fearnley et al., 1989), showed that the pelB leader had been cleaved correctly. The VH domain of the D1.3 antibody was also expressed and secreted using the construct lacking the D1.3 Vκ gene (Fig. 1), and could be purified using lysozyme sepharose chromatography. The yield of the VH domain was much lower than that of the Fv, suggesting that for the Fv, the Vκ may prevent VH aggregation and/or stabilise the folding.

Affinity measurements using fluorescence quench titrations (Perkin Elmer LS-5B luminescence spectrometer) and stopped flow kinetics were carried out with the purified Fv and VH (Table 1). The affinity of the Fv fragment (3 nM) is similar to that of the parent antibody (2 nM). The affinity of the VH domain for lysozyme was determined as < 40 nM by fluorescence quench and as 19 nM by stopped-flow. Thus the affinity of the VH domain is only approximately 10-fold weaker than the complete antibody. The stoichiometry of binding of the VH domain was determined as 1.2 mole of lysozyme per mole of VH, suggesting an equimolar complex.

Table 1.Binding affinities of immunoglobulin fragments for lysozyme

	Stoichiometry	Affinity nM	kon $M^{-1}s^{-1}$	koff s^{-1}	koff/kon nM
Fv-D1.3	n.d.	3	0.8×10^6	n.d.	n.d.
VHD1.3	1.2	<40	3.8×10^6	0.075	19
VH1	n.d.	<15	n.d.	n.d.	n.d.
VH3	n.d.	n.d.	2.9×10^6	0.036	12
VH8	n.d.	n.d.	3.3×10^6	0.088	27

Generation and sequence analysis of a VH domain repertoire from mouse spleen cells

The PCR was used to amplify rearranged heavy chain genes from spleen DNA of a mouse which had been hyperimmunised with hen egg lysozyme. Following gel purification, the amplified DNA was cloned into an M13 vector, and 48 clones sequenced. Each of the 48 clones were found to have unique CDR3 sequences, and VH genes corresponded to all but 2 of the mouse heavy chain gene families as defined in Kabat (Kabat et al., 1987). A summary of the D- and J-segments found in these 48 clones is given in Table 2.

Table 2. Usage of VH gene, D segment and J-region families in the VH repertoire

VH genes		D segments		J regions	
Family	Number	Family	Number	Family	Number
IA	4	SP2	14	JH1	3
IB	12	FL16	11	JH2	7
IIA	2	Q52	5	JH3	14
IIB	17			JH4	14
IIIA	3				
IIIB	8				
IIIC	1				
VA	1				

Characterisation of VH domain binding activities from the repertoire

Amplified DNA from a mouse immunised with lysozyme was ligated into an expression vector which incorporates a C-terminal tag to facilitate detection of expressed VH domains (Figs. 1 and 2), and used to transform *E. coli*. To analyse the expression of these amplified VH domains, 17 clones were grown and induced for expression, and culture supernatants fractionated on a polyacrylamide gel. Western blotting of this gel showed that 14 of these clones secreted VH domains of the expected size. Two thousand colonies were toothpicked in groups of 5 into wells of ELISA plates, and the supernatants tested for binding to lysozyme coated plates. Twenty one supernatants were shown to have lysozyme binding activity. As a control, the supernatants were tested for binding to keyhole limpet haemocyanin (KLH), and 2 supernatants identified with KLH binding activity. VH domains were also derived by amplification and cloning of VH genes from spleen DNA of a mouse which had been hyperimmunised with KLH. From 2000 clones analysed, 14 showed KLH binding activity in an ELISA, whereas a single clone had binding affinity for lysozyme.

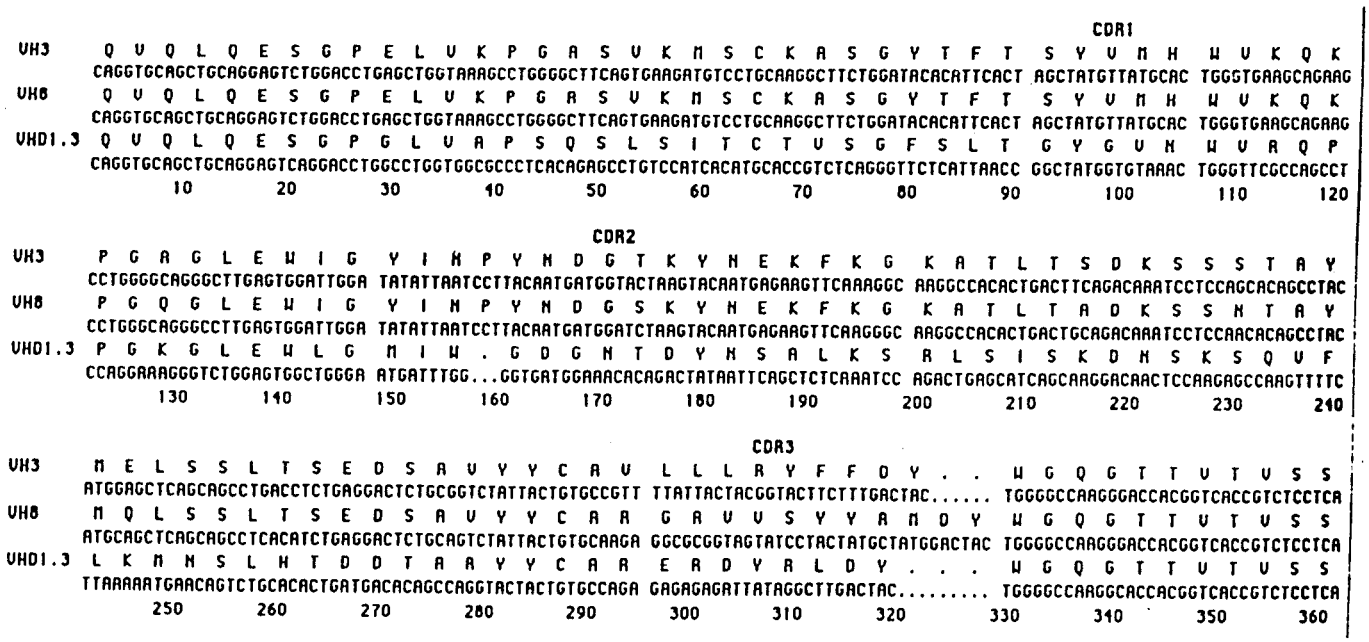

Figure 3. Nucleotide sequences of VH3, VH8 and VHD1.3 and encoded amino acid sequences.

Two of the genes corresponding to VH domains (VH3 and VH8) with anti-lysozyme affinities were sequenced, and both are different from VHD1.3 (Fig. 3). They belong to the same VH family (Kabat II) and D-segment family (FL16). The J-segments of the 2 domains are different (JH2 and

JH4), as are the CDR3s (Fig. 3). VH3 and VH8 were purified by affinity chromatography and their affinities measured using stopped flow kinetics and found to be 12 nM and 27 nM respectively (Table 1). The specificity of VH3, VH8 and the VHD1.3 domains was checked for binding to 4 other protein antigens (glyceraldehyde-3-phosphate dehydrogenase, KLH, insulin and cytochrome c), foetal calf serum, milk powder and microtitre plate plastic. No binding was observed.

In conclusion, it appears that VH domains can be rapidly isolated from mouse spleen DNA using PCR, and that 2 of these anti-lysozyme VH domains bind specifically with affinities of about 20 nM.

Mutagenesis of D1.3 VH to Generate a Repertoire

The PCR was used with a partially degenerate primer to randomly mutate the 4 central residues of the third CDR of the D1.3 VH. The amplified products were cloned and analysed by DNA sequencing. The mutant genes were subsequently ligated into the expression vector pSW1-VHPOLY and transformed into *E.coli*. Recombinant clones were toothpicked in pools of 5 into the wells of ELISA plates, grown up and induced for expression and secretion of VH domains. Culture supernatants from a total of 2000 clones were analysed for binding to lysozyme and KLH; 19 supernatants showed lysozyme binding activity and 4 had KLH binding activity.

To determine whether it is possible to generate VH domains of higher affinity than the parent domain (D1.3 in this case) using this mutagenesis approach, one of the mutant VH domains (VH1) which binds to lysozyme was selected for further analysis. VH1 was purified using affinity chromatography, and shown to compete with wild type D1.3 VH in a competition ELISA. Furthermore, fluorescence quench titrations indicate that this VH has an affinity which is less than 15 nM, and therefore significantly better than that of the parent VH. Sequence analysis shows that CDR3 of VH1 is completely different to that of D1.3, and the 4 central residues Arg-Asp-Tyr-Arg are replaced by Thr-Gln-Arg-Pro.

DISCUSSION

In previous work, separated heavy and light chains were identified with antigen (Fleischmann et al., 1963) or hapten binding activities (Utsumi and Karush, 1964), but the affinities were poor with no evidence for binding by single chains (Jaton et al., 1968) rather than dimers (Edmundson et al., 1984). Thus the observation that the VH domain of the D1.3 antibody binds lysozyme with high affinity in a 1:1 complex is novel.

In the D1.3 antibody, lysozyme makes extensive interactions to both domains. The VH domain makes 9 hydrogen bonding interactions, whereas the Vκ makes only 3. Binding of lysozyme buries about 300 A^2 of Vκ domain to solvent, and 400 A^2 of the VH domain (Amit et al., 1986; Chothia, unpublished results). Despite these interactions, the Vκ domain appears to make only a small net contribution to the energetics of binding. As the VH domain competes with the Fv fragment of the D1.3 antibody for lysozyme binding (not shown) it presumably binds antigen in a similar way as the antibody. It is possible, however, that the whole surface of interaction might reorientate slightly, perhaps by rocking on side chains to create a new set of contacts (Chothia et al., 1983), or that the loops of the VH domain could adjust to binding of antigen (Getzoff et al., 1987).

The report (Orlandi et al.,1989) in which PCR and 2 "universal"primers have been used to clone VH genes from cDNA of mouse hybridomas has now been extended to the cloning of a VH gene repertoire from mouse spleen DNA. Although from the analysis of the sequences of 48 VH domains the repertoire appears diverse, we cannot yet rule out the possibility of a systematic bias due to our choice of primers or hybridisation conditions.

The VH domains have been cloned and expressed in *E. coli*, and specific binding activities of high affinities (in the range of 20 nM) isolated. Immunisation increases the frequency of antigen specific clones, but is not essential. Thus PCR cloning of VH domains, followed by expression and secretion in *E. coli* may provide a convenient alternative to hybridoma technology. We are currently developing additional screening formats, using different vector systems, to facilitate the isolation of specific binding domains. In addition, it should be possible to reconstruct complete Fv fragments starting from a given VH domain, by screening a repertoire of Vκ domains for binding to the selected VH. These Vκs have been generated using PCR and Vκ specific primers with mouse spleen DNA.

The monomeric nature of the domain suggests that these molecules might be useful reagents for the blocking of canyon sites on viruses (Rossman et al., 1985); these canyon sites are too small to allow an Fv fragment to penetrate. Their small size should also faciltiate NMR analysis and high resolution epitope mapping.

An alternative route to the production of antigen specific VH domains is by random mutation of selected regions of an existing VH domain. For this approach, we have used PCR to randomly mutate the 4 central residues of the third hypervariable loop of the D1.3 VH. CDR3 was selected as it is the site of V-D-J recombination, and therefore the most variable region in both length and sequence of an antibody combining site. From a library of D1.3 derived VH domain, with 'randomised' CDR3s, most of the VH domains lose affinity for lysozyme; this is not surprising as CDR3 of this VH forms multiple contacts with lysozyme (Amit et al.,1986). A number of mutants have however been identified which retain high affinity for lysozyme, and one of these (VH1) binds with higher affinity than the parent VH domain. The CDR3 residues of this domain are completely different to those of D1.3 VH, and Pro99 (numbering as in Kabat et al., 1987) may kink the antigen binding loop. In addition, VH domains with affinity for KLH have also been identified from this D1.3 derived library; thus we believe that this approach may be extended to other binding activities to allow us to analyse the fine specificity of VH domains, and thus evaluate their utility in therapy.

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