Generating Binding Activities from *Escherichia coli* by Expression of a Repertoire of Immunoglobulin Variable Domains

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The immunoglobulin molecule consists of a string of domains, each consisting of about 100 amino acid residues. The antigen-binding site is fashioned by both heavy (V_H) and light (V_L; V_κ or V_λ) chain variable domains, as demonstrated by the solved crystallographic structures of antibody in association with antigen (Amit et al. 1986; Colman et al. 1987; Sheriff et al. 1987) or hapten (Satow et al. 1986). In the crystallographic structures of antibody-antigen complexes, the relative contributions of V_H and V_L domains to antigen binding appear to vary, although both domains make extensive interactions with the antigen. Light-chain dimers (Bence-Jones proteins) have also been crystallized, in which the two chains form a cavity that is able to bind to a single molecule of hapten (Edmundson et al. 1984).

We have been dissecting the interactions of the anti-lysozyme antibody, D1.3, with antigen (Amit et al. 1986) and have expressed the V_H and V_L domains individually, or in association as an Fv fragment by secretion into *Escherichia coli* periplasm (Skerra and Plückthun 1988). We find that both the Fv fragment and the V_H domain bind to antigen with a high affinity. This inspired us to generate a repertoire of V_H domains for the expression of binding activities in *E. coli*. Two approaches were used for the generation of a repertoire: (1) Residues in the third hypervariable region of a cloned V_H domain were mutated extensively, and (2) rearranged V_H genes were cloned from antibody-producing cells using the polymerase chain reaction (PCR) (Saiki et al. 1985; Orlandi et al. 1989). Each repertoire was cloned into vectors for expression of V_H domains in the periplasm of *E. coli* and screened for antigen-binding activity.

METHODS

Vectors. For expression of the V_H domain (V_HD1.3) of the D1.3 antibody, the vector pSW1-V_HD1.3 was built by cloning the 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-V_HD1.3-V_κD1.3 was built by cloning the V_κ domain and pelB signal into pSW1. For cloning and expression of the V_H repertoire, the vector pSW1-V_HPOLY was built by cloning a restriction enzyme polylinker sequence to replace the body of the V_HD1.3 gene. This vector was adapted further (pSW1-V_HPOLY-TAG1) by adding 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 carboxy-terminal end.

PCR amplification of mouse genomic DNA. BALB/c mice were hyperimmunized with hen egg white lysozyme (100 μg antigen intraperitoneally day 1 in complete Freund's adjuvant, and 50 μg antigen intravenously day 35 in incomplete Freund's adjuvant; kill day 39) or similarly with keyhole limpet hemocyanin (KLH). DNA was prepared from the spleen (Maniatis et al. 1982), and the rearranged mouse V_H genes were amplified using PCR (Saiki et al. 1985; Orlandi et al. 1989) using the primers V_H1FOR-2 (5'-TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC-3'. and V_H1BACK (5'-AGG T(C/G)(C/A) A(G/A)C TGC AG(G/C) AGT C(T/A)GG-3' (Fig. 2). The conditions for amplification were 50–200 ng DNA, 25 pmoles of each primer, 250 μM of each dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, and 100 μg/ml gelatin. The sample was overlaid with paraffin oil and heated to 95°C for 2 minutes (denature), to 65°C for 2 minutes (anneal), and to 72°C. The *Taq* polymerase (2 units Cetus) was added after the sample had reached the elongation temperature (72°C), and the reaction was continued for 2 minutes at 72°C. The sample was then subjected to an additional 29 rounds of temperature cycling, using the Techne PHC-1 programmable heating block.

PCR mutagenesis. The V_HD1.3 gene cloned into M13mp19 was amplified with a mutagenic primer based in complementarity-determining region (CDR) 3 and a primer based in the M13 vector backbone (Fig. 2). 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 is an equimolar mixture of T, C, G, and A) hypermutates the central four residues of CDR3 (Arg-Asp-Tyr-Arg). The PCR is the same as above, except a cycle of 95°C for 1.5 minutes, 25°C for 1.5 minutes, and 72°C for 3 minutes was used.

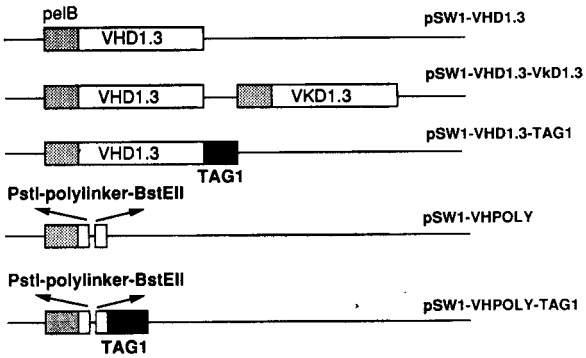

Figure 1. Expression vectors.

Cloning and expression of antigen-binding activities. PCR-amplified DNA was digested with *Pst*I and *Bst*EII (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-V_HPOLY or pSW1-V_HPOLY-TAG1 vector for expression. The recombinant plasmids were transfected into *E. coli* BMH71-18 (Gronenborn 1976), and colonies were selected on TYE plates (Miller 1972) with 1% glucose and 100 μg/ml ampicillin and toothpicked into 200 μl of 2 × TY (Miller 1972), ampicillin, and glucose in wells of enzyme-linked immunosorbent assay (ELISA) plates. Colonies were grown at 37°C for 16 hours. Cells were pelleted and resuspended in 200 μl of 2 × TY, ampicillin, and 1 mM inducer isopropyl-thiogalactoside (IPTG) and grown for an additional 16–24 hours. The cells were cooled and pelleted, and supernatants were screened for secretion of V_H domains (by Western blotting) or antigen-binding activity (by direct ELISA).

Western blotting was done according to the method of Towbin et al. (1979). Supernatant (10 μl) from the cultures was subjected to SDS-PAGE (Laemmli 1970), and the proteins were transferred electrophoretically to nitrocellulose. The V_H domains were detected via the peptide tag with the 9E10 antibody (Evan et al. 1985; Munro and Pelham 1986), using horseradish peroxidase-conjugated rabbit anti-mouse antibody and 4-chloro-1-naphthol as the peroxidase substrate.

Wells of ELISA plates (Falcon) were coated with antigen in 50 mM $NaHCO_3$, pH 9.6, overnight (3 mg/ml lysozyme or 50 μg/ml KLH) and blocked with 2% skimmed milk powder in PBS for 2 hours at 37°C. Bacterial supernatant was added and incubated at 37°C for 2 hours. V_HD1.3 domains were detected with rabbit polyclonal antiserum raised against the D1.3 Fv fragment, using peroxidase-conjugated goat anti-rabbit immunoglobulin. Tagged V_H domains were detected as described in Western blotting, except with 2,2'-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid) as the peroxidase substrate. Three washes of 0.05% Tween 20 in PBS were followed by three washes of PBS between each step (only PBS washes before addition of blocker or bacterial supernatants).

Purification of Fv and V_H domains binding to lysozyme. Cultures (500 ml) were grown and induced as above, and the supernatant was passed through a 0.45-μm filter (Nalgene) and down a 5-ml column of lysozyme-Sepharose (Riechmann et al. 1988). After washing with PBS, the Fv fragment or V_H domains were eluted with 50 mM diethylamine and analyzed by SDS-PAGE.

Affinity for lysozyme. The purified D1.3 Fv fragment and V_H domains were titrated with lysozyme using fluorescence quench (Perkin-Elmer LS 5B lumin-

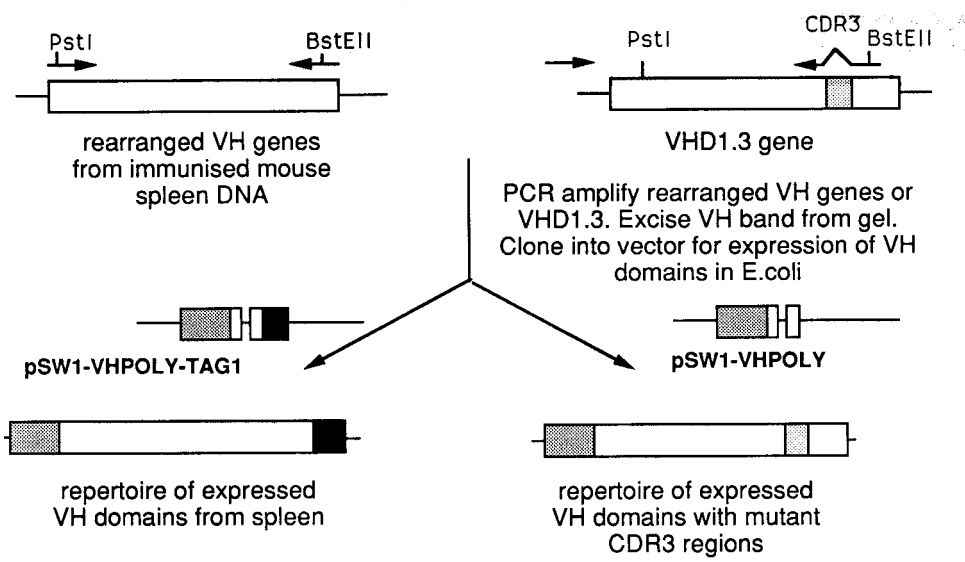

Figure 2. Scheme for cloning V_H gene repertoire.

Table 1. Binding Affinities of Immunoglobulin Fragments for Lysozyme

	Stoichiometry	Affinity (nM)	k_{on} $M^{-1}s^{-1}$	k_{off} s^{-1}	k_{off}/k_{on} nM
Fv-D1.3	n.d.	3	1.9×10^6	n.d.	n.d.
V_HD1.3	1.2	<40	3.8×10^6	0.075	19
V_H1	n.d.	<15	n.d.	n.d.	n.d.
V_H3	n.d.	n.d.	2.9×10^6	0.036	12
V_H8	n.d.	n.d.	3.3×10^6	0.088	27

n.d. indicates not determined. k_{on} = rate constant for association; units, moles^{-1} sec^{-1}; k_{off} = rate constant for dissociation; units, sec^{-1}.

escence spectrometer) to determine their affinities of binding (see Jones et al. 1986). The stoichiometry of binding of the V_HD1.3 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 experiments (HI Tech Stopped Flow SHU) at 20°C under pseudo-first-order conditions with binding sites in five- to tenfold excess over lysozyme (Levison et al. 1970). The number of binding sites was determined by fluorescence quench titration with lysozyme in excess.

RESULTS

Expression and Activity of the V_H Domain of D1.3 Antibody

The Fv fragment of the D1.3 antibody was purified on a lysozyme affinity column and analyzed by SDS-PAGE. Two bands of about M_r 14,000 were revealed, and the amino-terminal sequences were checked by gas-phase protein sequencing after elution of the bands onto PVDF membranes (Matsudaira 1987; Fearnley et al. 1989). The V_H domain was purified the same way, and the binding affinity of the Fv fragment and V_HD1.3 to lysozyme was determined by fluorescence quench titration (Table 1). The affinity of the Fv fragment (3 nM) is similar to that of the parent antibody (2 nM). The affinity of the V_H domain for lysozyme was determined as less than 40 nM by fluorescence quench and as 19 nM by stopped-flow. Thus, the affinity of the V_H domain is only tenfold weaker than the complete antibody. The stoichiometry of binding of the V_H domain was determined as 1.2 mole V_H per mole of lysozyme, suggesting an equimolar complex.

Repertoire of V_H Sequences from Antibody-producing Cells

M13 clones from the mouse V_H repertoire generated from spleen DNA of a mouse immunized with lysozyme were sequenced. The complete sequences of 48 V_H gene clones were determined (Fig. 3). Three families of D segments, four families of J segments, and all but two of the mouse V_H gene families (Kabat et al. 1987) were represented (Table 2). The different sequences of CDR3 marked each of the 48 clones as unique. Nine pseudogenes and 16 unproductive rearrangements were identified: Of the clones sequenced, 27 have open reading frames. V_H gene libraries have also been generated from mRNA of human peripheral blood lymphocytes (J. Marks, unpubl.).

Repertoire of Antigen-binding Activities from Antibody-producing Cells

Amplified DNA from a mouse immunized with lysozyme was cloned for expression into a vector that incorporates a carboxy-terminal tag to facilitate detection of expressed V_H domains (Figs. 1 and 2). Bacterial supernatants were analyzed by SDS-PAGE, followed by Western blotting, and bands of the expected size ($\sim M_r$ 14,000) were detected for 14 of 17 clones by probing with antibody directed against the tag. To screen for lysozyme-binding activities, about 2000 colonies were toothpicked in groups of five into wells of ELISA plates, and the supernatants were tested for binding to lysozyme-coated plates. Of the supernatants, 21 were shown to have lysozyme-binding activity. As a control, the supernatants were tested for binding to KLH, and two supernatants were identified with KLH-binding activity. A second expression library was

Table 2. Usage of V_H gene D-segment and J-region Families in the Repertoire

V_H genes		D segments		J regions	
family	number	family	number	family	number
IA	4	SP2	14	J_H1	3
IB	12	FL16	11	J_H2	7
IIA	2	Q52	5	J_H3	14
IIB	17			J_H4	14
IIIA	3				
IIIB	8				
IIIC	1				
VA	1				

	FR1	CDR_1	FR2	CDR_2	FR_3	CDR_3	
KABAT IA							
A07	PGLVKPSQSLSLTCSVTGYSIT	SGYYWN	WIRQFPGNKLEWMG	YISYDGSNNYNPSLKN	RISITRDTSKNQFFLKLNSVTTEDTATYYCAR	EGNWDGFAY	
A09	PGLVKPSQSLFLTCSITGFPIT	SGYYWI	WIRQSPGKPLEWMG	YITHSGETFYNPSLQS	PISITRETSKNQFFLQLNSVTTEDTAMYYCAG	DRDKLGPWFAY	
E03	PGLVKPSQSLSLTCSVTGYSIT	SGYYWN	WIRQFPGNKLEWMG	YISYDGSNNYNPSLKN	RISITRDTSKNQFFLQLNSVTTEDTATYYCAR	DSSGSMDY	
G01	PGLVKPSQSLSLTCSVTGYSIT	SGYYWN	WIRQFPGNKLEWMG	YISYDGSNNYNPSLKN	RISITRDTSKNQFFLKLNSVTTEDTATYYCAR	VSSGYESMDY	
KABAT IB							
A06	PVLVAPSQSLSITCAVSDFSLT	NYGVL	WVRQPPGKGLEWLG	VIWAGGITNYNSALMS	RLSISKDTSKSQVFLKMNSLQTDDTAVYYCAK	HGDSSGYFDY	
25G07	PGLVQPSQSLSITCTVSGFSLT	SYGVH	WVRQSPGKGLEWLG	VIWSGGSTDYNAAFIS	RLSISKDNSKSQVFFKMNSLQADDTAIYYCAR	NDGYY	
B03	PGLVAPSQSLSITCTVSGFSLT	SYGVD	WVRQPPGKGLEWLG	VIWGGGSTNYNSALMS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAK	LGRGYAMDY	
G03	PGLVQPSQSLSITCTVSGFSLT	SYGVH	WVRQSPGKGLEWLG	VIWSGGSTDYNAAFIS	RLSISKDNSKSQVFFKMNSLQADDTAIYYCAR	KRDYDYDRGYYYAMDY	
H09	PVLVAPPQSLSITCTVSGFSLT	SYGVH	WVRQPPGKGLEWLG	VIWAGGSTNYNSALMS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAI	YYDGSFFAY	
25C10	PGLVAPSQSLSITCTVSGFSLT	SYAIS	WVRQPPGKGLEWLG	VIWTGGGTNYNSALKS	RLSISKDNSKSQVFLKMNSLQTDDTARYYCAR	EGYYYFAY	
A12	PGLVAPSQSLSITCTVSGFSLT	SYAIS	WVRQPPGKGLEWLG	VIWTGGGTNYNSALKS	RLSISKDNSKSQVFLKMNSLQTDDTARYYCAR	IYYDGSSDYYAMDY	
A08	PGLVAPSQSLSITCTVSGFSLT	SYGVH	WVRQPPGKGLEW**	*****GSTTYNSALKS	*RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAR*	13 nt.	Ps.gene/Unproductive
25G08	PGLVAPSQSLSITCTVSGFSLT	SYDVD	WVRQSPGKGLEWLG	VIWGGGSTNYNSALKS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAR	21 nt.	Unproductive
A03	PGLVQPSQSLSITCTVSGFSLT	SYGVH	WVRQSPGKGLEWLG	VIWSGGSTDYNAAFIS	RLSISKDNSKSQVFFKMNSLQADDTAIYYCAR	28 nt.	Unproductive
C07	PVLVAPSQSLSITCTVSGFSLT	SYGVH	WVRQPPGKGLEWLG	VIWAGGSTNYNSALMS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAK	37 nt.	Unproductive
H04	PGLVAPSQSLSITCTVSGFSLT	SYGVD	WVRQSPGKGLEWLG	VIWGVGSTNYNSALKS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAS	32 nt.	Unproductive
KABAT IIA							
E04	PELVRPGVSVKISCKGSGYTFT	DYAMH	WVKQSHAKSLEWIG	VISTYYGDASYNQKFKD	KATMTVDKSSSTAYMELARLTSEDSAVYYCAR	40 nt.	Unproductive
H07	PELVRPGVSVKISCKGSGYTFT	DYAMH	WVKQSHAKSLEWIG	VISTYYGDASYNQKFKD	KATMTVDKSSSTAYMELARLTSEDSAVYYCAR	22 nt.	Unproductive
KABAT IIB							
A02	AELVMPGASVKLSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	EIDPSDSYTNYNQKFKG	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCVR	RGLTYAMDY	
B04	AELVKPGASVKMSCKASGYTFT	SYWIT	WVKQRPGQGLEWIG	DIYPGSGSTNYNEKFKS	KATLTVDTSSSTAYMQLSSLTSEDSAVYYCAR	YYSNYFDY	
C05	AELVKPGASVKLSCKASGYTFT	SYWMH	WVKQRPGRGLEWIG	RIDPNSGGTKYNEKFKS	KATLTVDKPSSTAYMQLSSLTSEDSAVYYCAR	PNWDHYYYGMDV	
C09	AELVKPGASLKLSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	EINPSNGGTNYDEKFKS	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCTL	LYYYAMDY	
D06	ASLVKPGASVKMSCKASGYTFT	SYWIT	WVKQRPGQGLEWIG	DIYPGSGSTNYNEKFKS	KATLTVDTSSSTAYMQLSSLTSEDSAVYYCAR	SSGYDY	
D08	PELVKPGASVKLSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	EINPSNGGTNYNEKFKS	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCTI	GAARATNAY	
E07	AELVRPGASVKLSCKASGYTFT	DYEMH	WVKQTPVHGLEWIG	AIDPETGGTAYNQKFKG	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCAR	GGFAY	
G08	PELVKPGASVKISCKASGYTFT	DYYIN	WVKQRPGQGLEWIG	WIYPGSGNTKYNEKFKG	KATLTVDTSSSTAYMQLSSLTSEDSAVYYCAR	SPMDY	
G10	AELVKPGASVKVSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	RIHPSDSDTNYNQKFKG	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCAI	EVPGGFYATDY	
25G09	AELVKPGASVKMSCKASGYTFT	TYPIE	WVKQNHGKSLEWIG	NFHPYNDDTKYNEKFKG	KATLTVEKSSSTVYLELSRLTSDDSAVYYCAR	MDYYGSSLWFAY	
F04	TELVKPGASVKLSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	NINPSNGGTNYNQKFKG	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCAK	TTVVAFDY	
H02	AELVKPGASVKLSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	NIDPSDSETHYNQKFKD	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCAR	KRDYSTYFDH	

H01	AELVMPGASVKLSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	EIDPSDSYTNYN*KVQG	*KATLTVDKSSSTAYMQLSSLTSEDSAVYYCAP*	*TGTEFAY*	Ps.gene
25C05	PELVRPGTSVKMSCKASGYTFF	NYWMK	WV*QRPGQGLEWIG	*QIFPASGSIYYNEMHKD*	*KAAWAVDTSSSTAYMQLSSLTSEDTAVYFCL**	24 nt.	Ps.gene/Unproductive
B01	AELVKPGASVKMSCKASGYTFT	SYWIT	WVKQRPGQGLEWIG	DIYPGSGSTNYNEKFKS	KATLTVDKPSDTAYMQLSSLTSEDSASYYCAR	9 nt.	Unproductive
B05	AELVRPGSSVKLSCKDSYFAFM	RHAMH	WVKQRPGHGLEWIG	SFTMYSDATEYSENFKG	KATLTANTSSSTAYMELSSLTSEDSAVYYCAR	23 nt.	Unproductive
B11	AELVKPGASVKMSCKASGYTFT	SYWIT	WVKQRPGQGLEWIG	DIYPGSGSTNYNEKFKS	KATLTVDTSSSTSYMQLSSLTSEDSAVYYCAR	15 nt.	Unproductive
KABAT III A							
25G05	GGLVQAWGSLSLSCAASGFTFT	DYYMS	WVRQPPGKALEWLG	FIRNKANGYTTEYSASVKG	RFTISRDNSQSILYLQMNALRAEDSATYYCAR	YMILGAMDY	
C16	GGLVQPGGSLSLSCAASGFTFT	DYYMN	WVRQPPGKALEWLA	LIRHKANGYTMEYSASVKG	RFTISRDNSQSILYLQMNALRAEDSATYYCAR	GYYYDGSYYAMDY	
B07	GGLVQPGGSLSLSCAASGFTFT	DYYMS	WVRQPPGKALEWLA	LIRNKANGYTTEYSASVKG	RFTISRDNSQSILYLQMNALRAEDSATYYCAR	23 nt.	Unproductive
KABAT III B							
G05	GGLVKPGGSLKLSCAASGFTFS	DYGMH	WVRQAPEKGLEWVA	YISSGSSTIYYADTVKG	RFTISRDNAKNTLFLQMTSLRSEDTAMYYCAR	AKFHLYFDY	
B12	GGLVQPGESLKLSCESNEYEFP	SHDMS	WVR*********VA	*AINSDGGSTYYPDTMER*	*RFIISRDNTKKTLYLQMSSLRSEDTALYYCAR*	*REGVVESRLDGDV*	Ps.gene
D04	GGLVQPGGSLRLSCAASGFTFS	SYAMS	WVA*APGKGLEWVS*	*AISGSGGSTYYADSVKG*	*RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAD*	*RGLHWFDP*	Ps.gene
D05	GGLVQPGGSLRLSCAASGFTFS	SYAMS	WVA*APGKGLEWVS*	*AISGSGGSTYYADSVKG*	*RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK*	*RNYGSSPFDY*	Ps.gene
F12	GGLVQPGESWKLSCVIQQ****	*****	*WVRQ*PEKRLELVA*	*AINSDGGSTYYPDTMER*	*RFIISRDNSKKTLYLQMSSLRSEDTALYYCAR*	*PPMMPSY*	Ps.gene
F06	GGLVQPGGSLRLSCAASGFTFS	SYAMS	WVA*APGKGLEWVS*	*AISGSGGSTYYADSAKG*	*RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK*	43 nt.	Ps.gene/Unproductive
D02	GGLVQPGESLKLSCESNEYVIP	*HDMS	*WVRQDSGE*LELVA*	*AINSDGGSTYYPDTMER*	*RFIISRDNTKKTLYLQMSSLRSEDTALYYCAR*	28 nt.	Ps.gene/Unproductive
F09	GDLVKPGGSLKLSCAASGFTFS	SYGMS	WVRQTPDKRLEWVA	TISSGGSYTYYPDSVKG	RFTISRDNAKNTLYLQMSSLKSEDTAMYYCAR	35 nt.	Unproductive
KABAT III C							
E06	GGLVQPGGSMKLSCAASGFTFS	DAWMD	WVRQSPEKGLEWVA	EIRNKANNHATYYAESVKG	RFTISRDDSKSRVYLQMNSLRAEDTGIYYCTG	30 nt.	Unproductive
KABAT V A							
C04	AELVKPGASVKLSCKASGYTFT	EYTIH	WVKQRSGQGLEWIG	WFYPGSGSIKYNEKFKD	KATLTADKSSSTVYMELSRLTSEDSAVYFCAR	HEDRDSSGYAMDY	

Figure 3. Encoded amino acid sequences of V_H domain repertoire arranged as Kabat V_H gene families (Kabat et al. 1987). For unproductive rearrangements (stop codons, frameshifts in CDR3), the total number of nucleotides (nt.) in CDR3 is indicated. For pseudogenes, the positions of frameshifts or stop codons are indicated by asterisks (*), and the sequences downstream from these positions are translated as if no frameshift were present and are in italics.

prepared from a mouse immunized with KLH and screened as above. Of the supernatants, 14 had KLH-binding activities, and two supernatants had lysozyme-binding activity.

Characterization of Two Lysozyme-binding Clones

Two of the clones (V_H3 and V_H8) with lysozyme-binding activities were sequenced (Fig. 4). They belong to the same V_H gene (Kabat IIB) families and D-segment families (FL16) but have different J segments (J_H2 and J_H4) (Kabat et al. 1987). There are only six amino acid differences between the (unrearranged) V_H genes, but the sequences of CDR3 are completely different. The V_H domains were purified, and affinities for lysozyme were determined (Table 1). The affinities, in the 20 nM range, are similar to those of the V_H domain of the D1.3 antibody.

Repertoire of Binding Activities by Mutagenesis

The central four residues of CDR3 of the V_HD1.3 domain were mutated extensively, using a partly degenerate primer and PCR. The amplified DNA was cloned into an M13 vector for sequencing and also into pSW1-V_HPOLY for expression and secretion of the mutated domains. Sequencing confirmed that CDR3 had been mutated extensively. Supernatants from the expression library were screened for secretion of lysozyme- and KLH-binding activities, and 2000 clones were screened, as above, for lysozyme- and KLH-binding activities; 19 supernatants were identified with lysozyme-binding activities, and 4 were identified with KLH-binding activities.

One clone (V_H1) with lysozyme-binding activity was characterized further. The domain was purified on a lysozyme affinity column and titrated by fluorescence quench, and used in a competition ELISA with the V_HD1.3 domain. The titration suggested a binding affinity of less than 15 nM (Table 1), and the V_H1 also competed effectively in ELISA with V_HD1.3 for binding to lysozyme. The V_H1 gene was sequenced and shown to be identical to the V_HD1.3 gene, except for the central residues of CDR3 (Arg-Asp-Tyr-Arg); these were replaced by Thr-Gln-Arg-Pro.

DISCUSSION

The secretion of associated V_H and V_κ domains of the D1.3 antibody from *E. coli* confirms previous work of Skerra and Plückthun (1988), although our levels of expression are higher (10 mg/liter of active fragment in the supernatant compared with 0.5 mg/liter). However, the isolated V_H domain of the D1.3 antibody is expressed at much lower levels, suggesting that the presence of the V_κ domain may help the folding of the V_H domain or prevent its aggregation.

Our finding that the V_H domain of the D1.3 antibody binds to lysozyme in an equimolar complex and has a good affinity for the antigen is a new observation. In previous work, separated heavy and light chains were identified with antigen-binding (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). However, although our data suggest a complex of one V_H domain with one molecule of lysozyme, they do not rule out a complex of two V_H domains with two molecules of lysozyme.

In the D1.3 antibody, lysozyme makes extensive interactions to both domains, including three hydrogen bonds to the V_κ domain and nine hydrogen bonds to the V_H domain. Binding of lysozyme buries about 300 $Å^2$ of V_κ domain to solvent, and 400 $Å^2$ of the V_H domain (Amit et al. 1986; C. Chothia, unpubl.). Despite these interactions, the V_κ domain appears to make only a small net contribution to the energetics of binding. The V_H domain presumably binds to lysozyme in a similar way as the antibody, although it is possible that the whole surface of interaction might reorientate slightly, presumably by rocking on side chains to create a new set of contacts (Chothia et al. 1983), or that the loops of the V_H domain could adjust to binding of antigen (Getzoff et al. 1987).

The finding that a diverse library of V_H genes can be cloned from the chromosomal DNA of mouse spleen using the PCR and two "universal" primers extends our previous results in which V_H genes, corresponding to different V_H families, were cloned from cDNAs of mouse hybridomas (Orlandi et al. 1989). Clearly, we can generate a diverse repertoire of V_H genes using PCR, but we cannot rule out a systematic bias due to our choice of primers or hybridization conditions.

Furthermore, from the library of V_H genes, we show that V_H domains can be derived with binding activities to lysozyme and KLH (and presumably other antigens). Prior immunization facilitates the isolation of these activities. The affinities of the V_H domains for lysozyme (20 nM) lie within the range expected for monoclonal antibodies for protein antigens; thus, PCR cloning of V_H domains from immunized spleen may offer an alternative to hybridoma technology. Although we have used plasmid vectors for secretion of the V_H domains and assayed bacterial supernatants for binding activities, a variety of other vectors and formats for screening antigenic activities should also be possible. For example, bacterial colonies, lytic plaques from λ vectors or nonlytic plaques from M13 vectors, could in principle be transferred to nitrocellulose and screened for binding to antigen directly.

Hypermutation of CDR3 of V_H domains also appears to be a promising way of generating antigen-binding activities. CDR3 was chosen because it is the most diverse portion of sequence in antibodies, derived by the joining of three genetic elements, V, D, and J, and would be expected to carry major determinants for binding to antigen. For example, in the D1.3 antibody, the three residues Arg-99, Asp-100, and Tyr-101 make six of the nine hydrogen bonds of the heavy chain (Amit et al. 1986) and, presumably, have similar roles

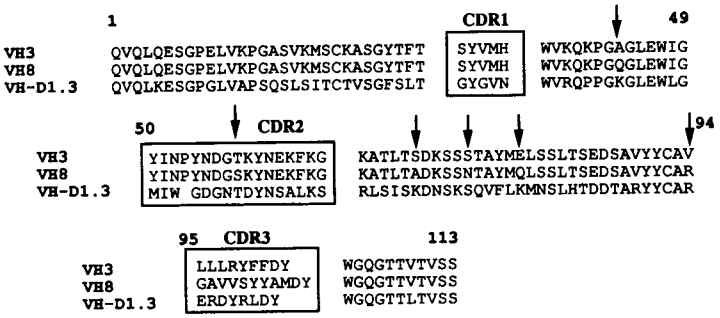

Figure 4. Sequences of two V_H domains with lysozyme-binding activities. The sequences of the V_H domains are aligned with that of the D1.3 antibody. Arrows indicate differences between unrearranged V_H3 and V_H8 (see text).

in the isolated V_H domain. When these residues are changed en bloc by PCR mutagenesis, the vast majority of mutants lose affinity for lysozyme. However, of 2000 colonies screened, 19 retain high affinity for lysozyme and 4 acquire the ability to bind to KLH. One of the mutants (V_H1), which binds to lysozyme, has a completely different amino acid sequence in CDR3 and has a slightly improved affinity compared with the parent V_H domain. The interaction of lysozyme with this region (and perhaps also with CDR1 and CDR2) is also likely to differ, as the main chain now incorporates a proline at residue 102.

V_H domains with binding activities can be generated in a matter of days without recourse to tissue culture and may also have other advantages over monoclonal antibodies. For example, the smaller molecule should penetrate tissues more readily, could permit the blocking of "canyon" sites on viruses (Rossman et al. 1985; Weis et al. 1988), and could allow epitope mapping at higher resolution. However, we also envisage that V_H domains with binding activities could also serve as the building blocks for making Fv fragments or complete antibodies. For example, V_H domains could be coexpressed with a repertoire of V_κ domains, derived by PCR amplification of V_κ genes (Orlandi et al. 1989) and screened for association of the domains and antigen binding.

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