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Making antibody fragments using phage display libraries

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TO by-pass hybridoma technology and animal immunization, we are trying to build antibodies in bacteria by mimicking features of immune selection[1]. Recently we used fd phage[2] to display antibody fragments fused to a minor coat protein[3,4], allowing enrichment of phage with antigen[3]. Using a random combinatorial library of the rearranged heavy (VH) and kappa (Vκ) light chains[5–8] from mice immune to the hapten 2-phenyloxazol-5-one (phOx), we have now displayed diverse libraries of antibody fragments on the surface of fd phage. After a single pass over a hapten affinity column, fd phage with a range of phOx binding activities were detected, at least one with high affinity (dissociation constant, $K_d = 10^{-8}$ M). A second pass enriched for the strong binders at the expense of the weak. The binders were encoded by *V* genes similar to those found in anti-phOx hybridomas but in promiscuous combinations (where the same *V* gene is found with several different partners). By combining a promiscuous *V*H or *V*κ gene with diverse repertoires of partners to create hierarchical libraries, we elicited many more pairings with strong binding activities. Phage display offers new ways of making antibodies from *V*-gene libraries, altering V-domain pairings and selecting for antibodies with good affinities.

We used the polymerase chain reaction (PCR)[9] to amplify the *V*H and *V*κ genes from the spleen messenger RNA of mice immunized with phOx, and also developed a 'PCR assembly' process[10] to link these genes together randomly for expression as single-chain Fv (scFv) fragments[11,12] (Fig. 1*a–c*). The assembled genes were cloned in a single step into the vector fdDOG1 (Fig. 1*e*) for display as a fusion with the fd gene III coat protein. This initial library of 2×10^5 clones seemed to be diverse (Fig. 1*d*), and sequencing revealed the presence of most VH groups[13] and Vκ subgroups[14] (data not shown). None of the 568 clones tested bound to phOx as detected by enzyme-linked immunosorbent assay (ELISA).

The library of phages was passed down a phOx affinity column (Table 1*a*), and eluted with hapten. Of the eluted clones, 13%

TABLE 1 Affinity selection of hapten-binding phage

	Precolumn	Clones binding to phOx* After first round	After second round	After third round
(*a*) Random combinatorial libraries				
phOx-immunized mice	0/568 (0%)	48/376 (13%)	175/188 (93%)	—
Unimmunized mice	—	—	0/388 (0%)	—
(*b*) Hierarchical libraries				
*V*H-B/*V*κ-*rep* library	6/190 (3%)	348/380 (92%)	—	—
*V*H-rep/*V*κ-d library	0/190 (0%)	23/380 (7%)	—	—
(*c*) Fractionation of VH-B/Vκ-d and VH-B/Vκ-b phage*				
Mixture of clones	88/1,896 (4.6%) (44/1,740 (2.5%)†)	55/95 (57.9%)	1,152/1,156 (99.7%)	1,296/1,299 (99.8%)

Selection of phage with hapten-binding activities from the random combinatorial and hierarchical libraries (*a* and *b*, respectively), and fractionation of clones with different affinities for phOx (*c*). For the random combinatorial libraries fdDOG1 RF was extensively digested with *Not*I and *Apa*LI, purified by electroelution[24] and 1 μg ligated to 0.5 μg (5 μg for the hierarchical libraries) of the assembled *scFv* genes in 1 ml with 8,000 units T4 DNA ligase (New England Biolabs) overnight at 16 °C. Purified ligation mix was electroporated in six aliquots into MC1061 cells[25] and plated on NZY medium[24] with 15 μg $\mathrm{ml^{-1}}$ tetracycline, in 243 × 243 mm dishes (Nunc); 90–95% of clones contained *scFv* genes by PCR screening (see legend to Fig. 1). Colonies were scraped into 50 ml 2 × TY medium[26] and shaken at 37 °C for 30 min. Liberated phage were precipitated twice with polyethylene glycol and resuspended to 10^{12} transducing units (TU) $\mathrm{ml^{-1}}$ in water (titred as in ref. 3). For affinity selection, a 1-ml column of phOx-BSA–Sepharose[27] M. Dreher and C. Milstein, unpublished results) was washed with 300 ml PBS, and 20 ml PBS containing 2% skimmed milk powder (MPBS). Phage (10^{12} TU) were loaded in 10 ml MPBS, washed with 10 ml MPBS and finally 200 ml PBS. The bound phage were eluted with 5 ml 1 mM 4-ε-amino-caproic acid methylene 2-phenyl-oxazol-5-one (phOx-CAP). About 10^6 TU eluted phage were amplified by infecting 1 ml log phase *E. coli* TG1 (ref. 28) and plating as above. For a further round of selection, colonies were scraped into 10 ml 2 × TY medium and then processed as above. For the hierarchical libraries, *V*H-B and *V*κ-d genes were individually recloned, then assembled with the *V*H or *V*κ repertoires. For the fractionation of clone VH-B/Vκ-d, 7×10^{10} TU phage in the ratio 20 VH-B/Vκ-b:1 VH-B/Vκ-d were loaded onto a phOx-BSA-Sepharose column in 10 ml MPBS and eluted as above. Eluted phage were used to reinfect *E. coli* TG1, and phage produced and harvested as before. About 10^{11} TU of phage were loaded onto a second affinity column and the process repeated to give a total of three column passes. Dilutions of eluted phage at each stage were plated in duplicate and probed separately[24] with oligonucleotides specific for *V*κ-b (5′-GAGCGGGTAACCACTGTACT) or Vκ-d (5′-GAATGGTATAGTACTACCCT).

* In (*c*), numbers refer to VH-B/Vκ-d.

† Numbers after three reinfections and cycles of growth. This control, omitting the column steps, confirms that a spurious growth or infectivity advantage was not responsible for the enrichment of clone VH-B/Vκ-d.

bound to phOx, and ranged from poor to strong binding in ELISA. We sequenced 23 of these hapten-binding clones and found eight different *V*H genes (A–H) in a variety of pairings with seven different *V*κ genes (a–g) (Fig. 2*a*). Most of the domains, such as VH-B and Vκ – d, were able to bind hapten with any of several partners[15]. The probability of finding multiple partners for a given chain should depend mainly on the inherent promiscuity of the chain and on the number of available partners and competing chains. Two other examples of promiscuous pairings have been noted in random combinatorial libraries made in λ phage[6,8], so this may prove to be a feature of small combinatorial libraries from immunized animals.

The sequences of the *V* genes were related to those seen in the secondary response to phOx, but with differences (Fig. 2*b*). Thus most phOx hybridomas from the secondary response use somatically mutated derivatives of three types of *V*κ genes, *V*κox1, '*V*κox-like' and *V*κ45.1 genes[16]. These can pair with *V*H genes from several groups, but *V*κox1 more commonly pairs with the *V*Hox1 gene (*V*H group 2; ref. 13). *V*κox1 genes are always, and *V*κox-like genes often, found in association with heavy chain genes (including *V*Hox1) that encode a short five-residue CDR3, with the sequence motif Asp-X-Gly-X-X (where X is any amino acid[16]), in which the central glycine creates a cavity for phOx (ref. 17). In our library, nearly all of the *V*H genes belonged to group 1, and most of the *V*κ genes were ox-like and associated with *V*H genes encoding a five-residue CDR3 motif Asp/Asn-X-Gly-X-X (Fig. 2*b*). *V*κox1 and *V*Hox1 were found only once (*V*κ – f and *V*H-E), and not in combination with each other: indeed *V*κ-f does not encode the Trp 91 involved in phOx binding[17] and was paired with a *V*H gene (*V*H-C) encoding a six-residue CDR3.

The promiscuity of the VH-B and Vκ-d domains prompted us to force further pairings, by assembling these genes with the entire repertoires of either *V*κ or *V*H genes from the same immunized mice. The resulting hierarchical libraries, (*V*H-B × *V*κ-*rep* and *V*H-*rep* × *V*κ-d), each with 4×10^7 members, were subjected to a round of selection and hapten-binding clones isolated (Table 1*b*). Most were strong binders by ELISA

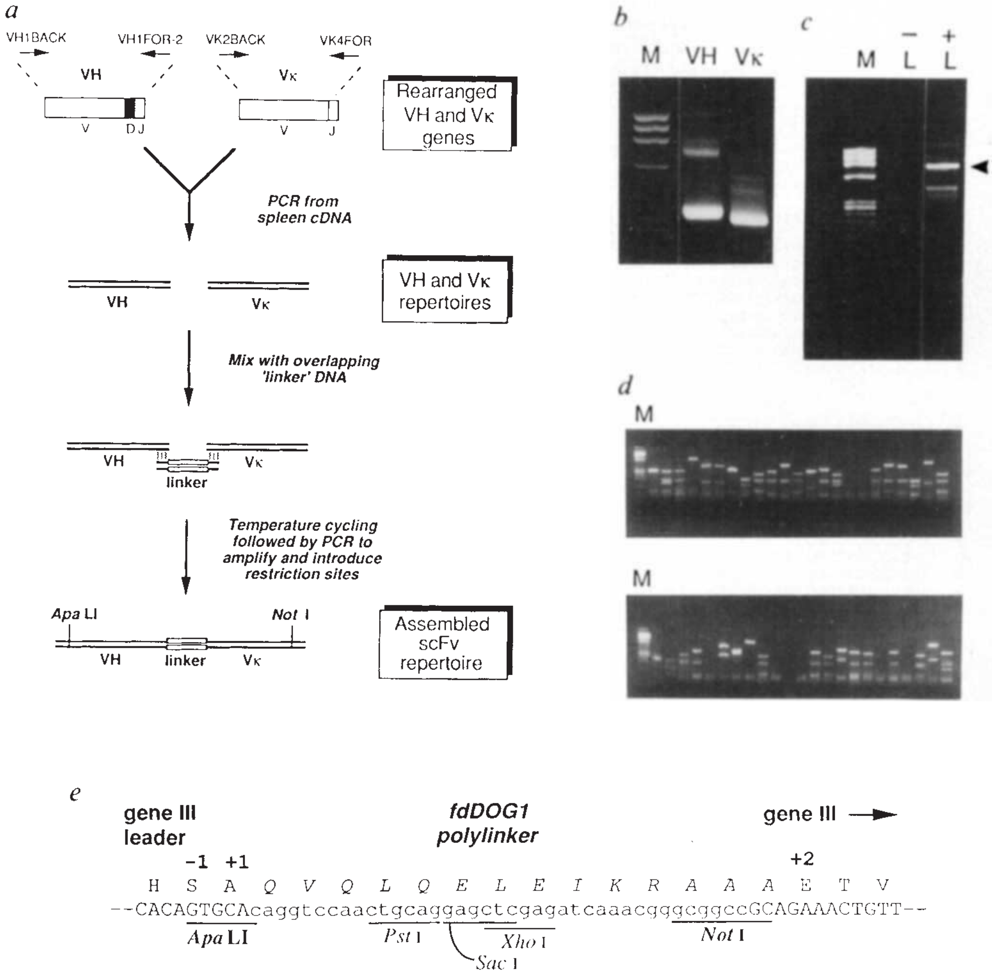
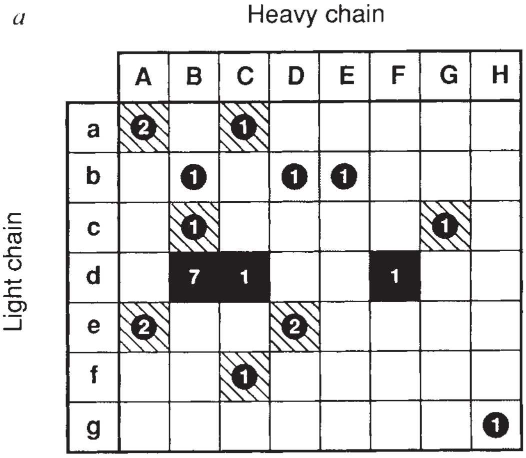


FIG. 1 PCR assembly of scFv library. *a*, *V*H and *V*κ genes are separately amplified, then mixed with a linker fragment that overlaps them both. The linker (93 base pairs) encodes the short peptide, $(Gly_4Ser)_3$, which links VH and Vκ in scFvs (ref. 11). Cycles of annealing–denaturation, followed by reamplification of the mixture, generate a random combinatorial cassette of *V*H and *V*κ genes joined in-frame for expression. *b*, *V*H and *V*κ gene repertoire PCR products from the immunized mice analysed by electrophoresis on agarose (1%) gel. *c*, PCR assembly of *scFv* gene repertoires with linker (+L) or without (–L); arrow indicates assembled repertoire. M is DNA marker ΦX174 replicative form DNA digested with *Hae*III. *d*, Diversity of library as seen by *Bst*NI fingerprinting of individual clones. *e*, Sequence of *fd* gene III around the signal peptide cleavage site in fdDOG1.

METHODS. For the random combinatorial libraries, cytoplasmic RNA was isolated[29] from the pooled spleens of either 5 male BALB/c mice boosted 8 weeks after primary immunization with phOx coupled to chicken serum albumin[27], or of 5 unimmunized mice. The cDNA was made with avian myoblastosis virus reverse transcriptase (Anglian Biotech)[30] and primers that straddle the junction between the variable and constant regions of γ heavy chains and κ light chains (C. Marks, unpublished data). *V*H and *V*κ repertoires were amplified from the cDNA with 25 cycles of PCR (94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min) using Vent polymerase (New England Biolabs) and the primers VH1BACK (ref. 19) and VH1FOR-2 (ref. 31) or the primers VK2BACK and VK4FOR. The linker DNA was similarly amplified from pSW2scD1.3 (ref. 3) using primers LINKFOR and LINKBACK (complementary to VK2BACK and VH1FOR-2 respectively). After gel purification, 1 μg each of the VH and Vκ products were mixed with 300 ng linker in a 25 μl PCR reaction mix without primers, and cycled 7 times (94 °C 2 min, 72 °C 4 min) to join the fragments, then amplified for 20 cycles (94 °C 1.5 min, 72 °C 2.5 min) using 25 pmol each VH1BACK and VK4FOR primers. Finally, the assembled products were gel-purified and reamplified with VH1BACK-ApaLI and VK4FOR-NotI ('tagged' versions of the original primers) to append restriction sites. Products (1–5 μg) were extensively digested with *Apa*LI and *Not*I for cloning into fdDOG1. Recombinant colonies were screened by PCR[32] with the primers VH1BACK and VK4FOR, followed by digestion with the frequently cutting enzyme *Bst*NI. Primers: VK2BACK 5′-GACATTGAGCTCACCCAGTCTCCA; VK4FOR, an equimolar mix of 5′-CCGTTTGATTTCCAGCTTGGTGCC, 5′-CCGTTTTATTTCCAGCTTGGTCCC, 5′-CCGTTTTATTTCCAACTTTGTCCC and 5′-CCGTTTCAGCTCCAGCTTGGTCCC; LINKFOR, 5′-TGGAGACTGGGTGAGCTCAATGTC; LINKBACK, 5′-GGGACCACGGTCACCGTCTCCTCA; VH1BACK-ApaLI, as VH1BACK (ref. 19) but with 5′-CATGACCACAGTGCAC added at the 5′ end; VK4FOR-NotI, as VK4FOR but with 5′-GAGTCATTCTGCGGCCGC similarly added (restriction sites underlined).



	A	B	C	D	E	F	G	H
a	▨ 2		▨ 1					
b		1		1	1			
c		▨ 1					▨ 1	
d		■ 7	■ 1			■ 1		
e	▨ 2			▨ 2				
f			▨ 1					
g								1

FIG. 2 *a*, Matrix of *V*H and *V*κ genes identified in phOx-binding clones selected from random combinatorial library. The number of clones found with each combination is shown. The binding to phOx–BSA, as judged by the ELISA signal, seemed to vary (marked by shading): no binding was seen to BSA alone. Optical density at 405 nm: 0.2–0.9, dotted box; 0.9–2.0, hatched box; >2.0, solid box. *b*, Encoded protein sequences of phOx-binding clones. Sequences of phOx-binding clones isolated (single-letter amino-acid code) after one round of selection of the random combinatorial library, with pairings as above, or the hierarchical library. Note that the first eight or seven residues, and the last nine or eleven residues, of the *V*κ or *V*H genes, respectively, are encoded by the PCR primers. Classifications into VH groups[13] and Vκ subgroups[14], and the position of residue 91 encoded by the *V*κ genes (#), are indicated. The relationship to genes from the hybridoma analysed secondary response to phOx (ref. 16) is also shown; all of the *V*κ genes are 'ox-like', apart from those marked * with an asterisk, which are *V*κox1, and the only example of VH group 2 (VH-E) is VHox1. The intensity of ELISA signals from the hierarchical libraries, corrected relative to the signal from control phage, are indicated: Optical density at 405 nm 0.9–2.0 (++), >2.0 (+++). Multiple isolations of sequences are marked, and sequences (*V*H-B and *V*κ-c) isolated from the random combinatorial library, and also the hierarchical libraries, are shown in italics. The VH-B/Vκ-d and VH-B/Vκ-c pairings gave similar signals (after correction of ELISA) when recovered from either combinatorial or hierarchical libraries.

METHODS. We screened for binding of the phage to hapten by ELISA: 96-well plates were coated with 10 μg ml^{-1} phOx–BSA[27] or 10 μg ml^{-1} BSA in PBS overnight at room temperature. Colonies of phage-transduced bacteria were inoculated into 200 μl 2×TY medium[26] with 12.5 μg ml^{-1} tetracycline in 96-well plates ('cell wells', Nuclon) and grown with shaking (300 r.p.m.) for 24 h at 37 °C. At this stage, cultures were saturated and phage titres were reproducible (10^{10} TU ml^{-1}). Phage supernatant (50 μl), mixed with 50 μl PBS containing 4% skimmed milk powder, was then added to the coated plates. Further details given in ref. 3. To sequence the clones, template was prepared[24] from the supernatants of 10-ml cultures grown for 24 h, and sequenced using the dideoxy method[33] and a Sequenase kit (USB), with primer LINKFOR for the *V*H genes and primer fdSEQ1 (5′-GAATTTTCTGTATGAGG) for the *V*κ genes.

b

VH sequences

From combinatorial library:

		CDR1		CDR2		CDR3			Group	ELISA signal
A	QVQLQQSGAELARPGASVKMSCKASGYTFT	SYTMH	WVKQRPGQGLEWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAN	RYGAY	WGQGTTVTVSS	x4	1	
B	QVKLQQSGAELAKPGASVKMSCKASGYTFT	RDWMH	WLKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	NYGLY	WGQGTTVTVSS	x9	1	
C	QVQLQQSGPELVKPGASVKMSCKASGYTFT	SYVMH	WVKQKPGQGLEWIG	YINPYNDGTKYNEKFKG	KATLTSDKSSSTAYMELSSLTSEDSAVYYCAI	YRSFPY	WGQGTTVTVSS	x3	1	
D	QVQLQQSGPELVKPGASVKISCKASGYSFT	GYFMN	WVKQSHGKSLEWIG	RINPYNGDTFYNQKFKG	KATLTVDKSSSTAHMELLSLTSEDSAVYYCVG	ITTRFAY	WGQGTTVTVSS	x3	1	(see Fig.2a)
E	QVQLQESGPGLVAPSQSLSITCTVSGFSLT	SYGVH	WVRQPPGKGLEWLG	VIWAGGSTNYNSALMS	RLSISKDNSKSQVFLKMNSLQTDDTAMYYCAR	DRGDY	WGQGTTVTVSS		2	
F	QVQLQQSGPELAKPGASVKMSCKASGYTFT	SYLMH	WVKQRPGQGLKWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	
G	QVKLQQSGAELVRPGASVKLSCKASGYTFT	RYLMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	EATLTADKSSNTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	
H	QVQLQQSGPELMKPGASVKISCKASGYSFS	RNYMH	WVKQSHGKSLEWIG	YIAPFNGGTTYNQKFKG	KATLTVDRSSSTAYMHLSSLTSEDSAVYYCAT	DYGRD	WGQGTTVTVSS		1	

From hierarchical library VH-rep × Vκ-d:

		CDR1		CDR2		CDR3			Group	ELISA signal
I	QVKLQQSGPELARPGVSVKMSCKASGYTFT	SYAMH	WVKQSQSKSLEWIG	VISTYNGNTNYNQKFKG	KATMTVDKSSSTAYMELARLTSEDSAIYYCAR	DYGDY	WGQGTTVTVSS		1	+++
J	QVKLQQSGAELARPGASVKMSCKASGYTFT	RYTMH	WVKQRPGQGLEWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DRGAY	WGQGTTVTVSS		1	+++
K	QVKLQQSGAELAKPGASVKMSCKASGYTFT	RDWMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	NYGLY	WGQGTTVTVSS	x3	1	+++
L	QVQLQQSGLELAKPGASVKMSCKASGYTFT	NYLMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS	x2	1	++
M	QVKLQQSGAELAKPGASVKMSCKASGYTFT	NYWMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSDDSAVYYCAR	DYGYF	WGQGTTVTVSS		1	+++
N	QVQLQQSGAELVKPGASVKLSCKTSGYTFT	SYTMN	WVKQRPGQGLEWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	++
O	QVQLQQSGAELAKPGASVKMSCEASGYTFT	SHLMH	WVKQRPGQGLEWIG	YINPRTGYTEYNQKFKD	KATFTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGAY	WGQGTTVTVSS		1	++
P	QVKLQQSGAELAKPGASVKMSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	++
Q	QVKLQQSGAELAKPGASVKMSCKATGYTFT	SYLMH	WVKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	+++
R	QVQLQQSGAELAKPGASVKMSCKASGYTFT	SYVMH	WVKQRPGQGLEWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	NYGIY	WGQGTTVTVSS		1	++
S	QVQLQQSGAELAKPGASVKMSCKASGYTFT	TFLMH	WLKQRPGQGLEWIG	YINPSTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS	x2	1	+++
T	QVKLQQSGAELARPGASVKMSCKASGYTFT	SYTMH	WVKQRPGQGLGWIG	YINPSSGYTNYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS	x6	1	+++
U	QVKLQQSGAELAKPGASVKMSCKASGYTFT	SYTMH	WVKQRPGQGLEWIG	YINPTTGYTEYNQKFKD	KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR	DYGYY	WGQGTTVTVSS		1	+++
B	*QVKLQQSGAELAKPGASVKMSCKASGYTFT*	*RDWMH*	*WLKQRPGQGLEWIG*	*YINPSTGYTEYNQKFKD*	*KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR*	*NYGLY*	*WGQGTTVTVSS*		1	+++

Vκ sequences

From combinatorial library:

		CDR1		CDR2		CDR3					
a	DIELTQSPSSLSASLGERVSLTC	RASQEISGYLS	WLQQKPDGSIKRLIY	AASTLES	GVPKRFSGSRSGSDYSLTISSLESEDFADYYC	LQYASYPT	FGAGTKLEIKRA	x3	V		
b	DIELTQSPAIMSASPGEKVTMTC	RASSSVSSSYLH	WYQQKSGASPKVWIY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGAGTKLEIKRA	x3	IV		
c	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPLT	FGAGTKLEIKRA	x2	IV		(see Fig.2a)
d	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WFQQKPGFSPKLLIS	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSTIPFT	FGSGTKLEIKRA	x9	IV		
e	DIELTQSPAIMSASPGEKVTITC	SASSSVNYMH	WFQQKPGTSPKLWIY	STSNLAS	GVPTRFSGSGSGTSYSLTISRMEAEDAATYYC	QQRSSYPPT	FGSGTKLEIKRA	x4	VI		
f	DIELTQSPAIMSAFPGEKVTMTC	SASSSVSYMN	WYQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQFSSNPLT	FGAGTKLELKRA		VI	*	
g	DIELTQSPAIMSASPGEKVTMTC	SASSSINYMH	WYQQKPGASPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	HQRNSYPWT	FGGGTKLEIKRA		VI		
						#					

From hierarchical library VH-B × Vκ-rep:

h	DIELTQSPAIMSASPGEKVTMTC	SASSSVSYMH	WYQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWSSNPLT	FGAGTKLEIKRA	x4	VI	*	+++
i	DIELTQSPAIMSASPGEKVTITC	SASSSVSYIH	WFQQKPGTSPKLWIY	STSNLAS	GVPARFSGSGSGTSYSLTISRMEAEDAATYYC	QQYHSYPLT	FGAGTKLEIKRA		VI		+++
j	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WFQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPLT	FGGGTKLEIKRA		IV		++
k	DIELTQSPTTMAASPGDMITITC	SATSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPPRFSGSGSGTSYSLTIGAMEAEDVATYYC	QQGSSIPYT	FGAGTKLEIKRA		IV		+++
l	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPYT	FGGGTKLEIKRA		IV		++
m	DIELTQSPTTMAASPGEKITITC	SASSSISSNHLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSGIPYT	FGGGTKLEIKRA		IV		+++
n	DIELTQSPTTMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPFT	FGGGTKLEIKRA		IV		++
o	DIELTQSPAIMAASPGEKITITC	SASSSISSNYLH	WYQQKPGFSPKLLIY	RTSNLAS	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	QQGSSIPYT	FGGGTKLEIKRA	x2	IV		+++
p	DIELTQSPAIMSASPGEKVTMTC	SASSSVSYMH	WYQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDVATYYC	QQWSSNPLT	FGAGTKLEIKRA	x2	VI	*	+++
q	DIELTQSPAIMSASPGDKVTLTC	SASSSVRYVN	WFQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWTSNPPT	FGGGTKLEIKRA		VI	*	+++
r	DIELTQSPAIMSASPGEKVTMTC	SASSSVSYMH	WYQQKSGTSPKRWIY	DTSKLAS	GVPARFSGSGSGTSYSLTISSMEAEDAATYYC	QQWSTNALT	FGAGTKLEIKRA		VI	*	++
s	DIELTQSPAIMSASPGEKVTMTC	RASSSVTSSYLN	WYQQKSGASPKLWVY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGAGTKLEIKRA		IV		+++
t	DIELTQSPAIMSASPGEKVTMTC	RASSSVSSSYLN	WYQQKSGASPKLWIY	STSNLAS	GVPARFSGSGSGTSYSLTISRMEAEDAATYYC	QQRSSYPLT	FGAGTKLEIKRA		IV		+++
u	DIELTQSPAIMSASPGEKVTMTC	RASSSVSSSYLH	WYQQKSGASPKLWIY	STSNLAS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGAGTKLEIKRA		IV		++
v	DIELTQSPAIMSASPGEKVTMTC	RASSSVSSSYLH	WFQQKSGASPKLWIY	STSNLPS	GVPARFSGSGSGTSYSLTISSVEAEDAATYYC	QQYSGYPLT	FGGGTKLEIKRA		IV		+++
c	*DIELTQSPTTMAASPGEKITITC*	*SASSSISSNYLH*	*WYQQKPGFSPKLLIY*	*RTSNLAS*	*GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC*	*QQGSSIPLT*	*FGAGTKLEIKRA*	*x3*	IV		++
						#					

(Fig. 2*b*). By sequencing 23 clones from each library, we identified 14 new partners for VH-B and 13 for Vκ-d; apart from VH-B and Vκ-c, none of the previous VH-B or Vκ-d partners (or indeed other partners) cloned and sequenced from the random combinatorial library was isolated again. These features are consistent with the much larger number of available partners (4×10^7) for the VH-B (or Vκ-d) domain, and the promiscuous nature of the domain. The *V*κ genes were mainly ox-like and the *V*H genes mainly group 1, but the only examples of *V*κox1 (Vκ-h, −p, −q and −r) encode Trp 91, and the VH-CDR3 motif Asp-X-Gly-X-X now predominates. Thus some features of the phOx hybridomas seem to emerge more strongly in the hierarchical library. The new partners differ from each other mainly by small alterations in the CDRs, indicating that much of the subtle diversity had not been tapped by the original random combinatorial library. More generally we find that a range of related antibodies can be made by keeping one of the partners fixed and varying the other, and this could be invaluable for fine tuning of antibody affinity and specificity.

To determine the range of antibody affinities for phOx, we recloned the combinations of VH-B with Vκ-b and Vκ-d (which gave weak and strong binding signals to phOx in ELISA) for secretion as soluble scFv fragments (Fig. 3, legend). Fluorescence quench titrations determined the K_d of VH-B/Vκ-d for phOx-GABA as 1.0×10^{-8} M (Fig. 3*a*), indicating that antibodies with affinities representative of the secondary response can be selected from phage display libraries. Indeed of anti-phOx hybridomas from the secondary response, only two (out of 11 characterized) secrete antibodies of a higher affinity than VH-B/Vκ-d (ref. 16). The K_d of VH-B/Vκ-b for phOx-GABA was determined as 1.8×10^{-5} M (Fig. 3*b*); thus phage bearing scFv fragments with weak affinities can also be selected with antigen, probably because of the avidity of the multiple antibody heads on the phage.

A second round of selection of the original, random combinatorial library from immune mice resulted in 93% of eluted clones binding phOx (Table 1*a*). Most of these clones were Vκ-d combinations, and bound strongly to phOx in ELISA (data not shown). Few weak binders were seen. This suggested that affinity chromatography had not only enriched for binders, but also for the best. To confirm this we mixed the phage VH-B/Vκ-d with a 20-fold excess of the phage VH-B/Vκ-b and subjected the mixture to rounds of selection: after only two rounds, essentially all the eluted phage were VH-B/Vκ-d (Table 1*c*).

We also constructed a random combinatorial library (2×10^6 members) from unimmunized mice, but found no phOx-binding clones after two rounds of selection (Table 1*a*). Immunization therefore seems to be necessary to create and/or enrich for VH or Vκ domains with at least some of the features required for hapten binding. With libraries of this size ($\sim10^6$ members), such domains need to be represented at a high frequency to reconstitute a binding site[1], and immunization ensures this by biasing the spleen lymphoid cell population heavily towards messenger RNA-rich blast cells making specific antibody (R. Hawkins and G.W., unpublished data). With larger libraries ($>10^7$) now accessible using selection[3] rather than screening[5-8], immunization may be unnecessary for the isolation of antibody fragments. It has been estimated that a library of 10^7 different antibodies will probably recognize >99% of epitopes with a dissociation constant of $\geqslant10^{-5}$ M (ref. 18), and we have shown here that we can recover antibody fragments with such affinities

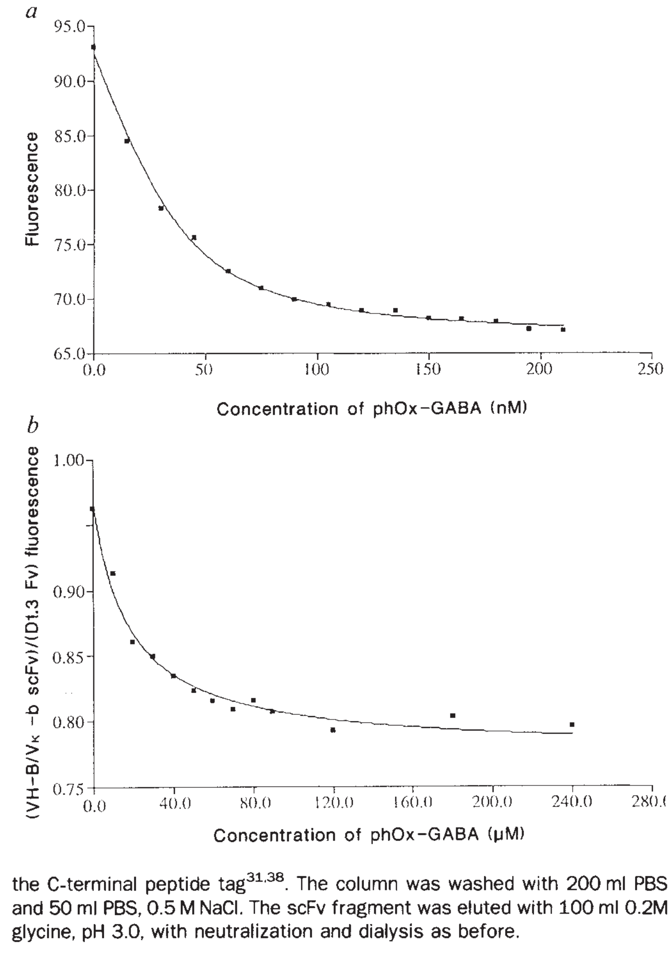


FIG. 3 Fluorescence quench titration of soluble scFv fragments. *a*, The K_d ($1.0\pm0.2\times10^{-8}$ M) for clone VH-B/Vκ-d was determined by fluorescence quench titration[34] of purified scFv (100 nM) with 4-γ-amino-butyric acid methylene 2-phenyl-oxazol-5-one (phOx-GABA). Excitation was at 280 nm, emission was monitored at 340 nm and the K_d calculated as in refs 35 and 36. All values were calculated with standard errors included. The K_d was determined to be $1.0\pm0.2\times10^{-8}$ M with 0.38 ± 0.05 binding sites per scFv molecule. *b*, For measurement of the K_d of the low affinity clone VH-B/Vκ-b, 2 μM purified scFv protein was titrated with phOx-GABA as above. But to minimize light absorption by the higher concentrations of phOx-GABA required, excitation was at 260 nm and emission was monitored at 304 nm. In addition, the fluorescence values were divided by those from a parallel titration of the lysozyme binding Fv fragment D1.3 (ref. 31). The K_d was calculated as described in refs 34 and 36 and determined to be $1.8\pm0.3\times10^{-5}$ M, with a fractional quench of 0.20 ± 0.01.

METHODS. Clones VH-B/Vκ-b and VH-B/Vκ-d were reamplified with VK4FOR-*Not*I and VH1BACK-*Sfi*I (5′-CATGCCATGACTCGCGGCCCAGCCGGCCATGG-CC(G/C)AGGT(C/G)(A/C)A(A/G)CTGCAG(C/G)AGTC(A/T)GG-3′), a primer that introduces an *Sfi*I site (underlined) at the 5′ end of the VH gene. *V*H-B/*V*k-d was cloned into the phagemid pJM1 (A.D.G. and J. Marks, unpublished results) as an *Sfi*I–*Not*I cassette, downstream of the pelB leader for periplasmic secretion[37], with a C-terminal peptide tag for detection[31,38], and under the control of a λP_L promoter[39]. Cultures (10 l) of *Escherichia coli* N4830-1 (ref. 40) harbouring each phagemid were induced[26] and supernatants precipitated with 50% ammonium sulphate. The resuspended precipitate was dialysed into PBS, 0.2 mM EDTA (PBSE), loaded onto a 1.5-ml column of phOx:Sepharose[41] and the column washed sequentially with 100 ml PBS; 100 ml 0.1 M Tris–HCl, 0.5 M NaCl pH 8.0; 10 ml 50 mM citrate, pH 5.0; 10 ml 50 mM citrate, pH4.0 and 20 ml 50 mM glycine, pH 3.0. The scFv fragment was eluted with 50 mM glycine, pH 2.0, neutralized with Tris base and dialysed against PBSE. VH-B/Vκ-b was cloned into a phagemid vector (A.D.G., unpublished results) based on pUC119 (ref. 42) encoding identical signal and tag sequences to pJM1, and expression induced at 30 °C in a 10-culture of *E. coli* TG1 (ref. 28) harbouring the phagemid, as in ref. 43. The low affinity of clone VH-B/Vκ-b made its purification on phOx-Sepharose impossible. Therefore after concentration by ultrafiltration (Filtron, Flowgen) the supernatant (100 ml of 600 ml) was loaded onto a 1-ml column of protein A–Sepharose coupled[44] to the monoclonal antibody 9E10 that recognizes the C-terminal peptide tag[31,38]. The column was washed with 200 ml PBS and 50 ml PBS, 0.5 M NaCl. The scFv fragment was eluted with 100 ml 0.2M glycine, pH 3.0, with neutralization and dialysis as before.

from phage display libraries. The antibody fragments could be rebuilt from their genes into complete antibodies, and expressed in myeloma cells if required, as described in ref. 19.

It may be possible to retain the original VH/Vκ pairings of the splenocytes, as in hybridoma technology. In principle, PCR assembly could be used to construct such 'natural' libraries, if the *V* genes from individual cells could be amplified and assembled in capsules. More immediately, affinity selection from combinatorial and hierarchical libraries promises an attractive route to high-affinity antibodies, in particular those from humans that are difficult to produce by hybridoma technology[1]. But the use of phage display libraries is not limited to antibodies: it offers a powerful and general method to change and refine the properties of any other protein[20] or peptides[21–23] that can be displayed on the phage surface. ☐

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Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene

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A CYCLIC AMP-stimulated choride conductance appears when the cystic fibrosis gene is expressed in non-epithelial cells by infection with recombinant viruses[1,2]. Cyclic AMP-stimulated conductance in this system is mediated by the same ohmic, low-conductance Cl^- channel as in human secretory epithelia[2–4], but control of this channel by phosphorylation has not been directly demonstrated. Here we report the appearance of the low-conductance Cl^- channel in Chinese hamster ovary cells after stable transfection with the cystic fibrosis gene. The channel is regulated on-cell by membrane-permeant analogues of cAMP and off-cell by protein kinases A and C and by alkaline phosphatase. These results are further evidence that the cystic fibrosis transmembrane regulator is a Cl^- channel which can be activated by specific phosphorylation events and inactivated by dephosphorylation; they reveal an unsuspected synergism between converging kinase regulatory pathways.

The coding sequence of the cystic fibrosis transmembrane regulator (CFTR) was cloned behind the metallothionein promoter of a plasmid that also contained a mutant dihydrofolate reductase gene, driven by the simian virus 40 early promoter (Fig. 1*a*). Stably transformed colonies were selected with methotrexate after calcium phosphate transfection of Chinese hamster ovary (CHO)-K1 cells. CFTR-expressing variants were chosen for further study on the basis of their capacity to produce a protein of the same apparent size as that present in T84 cell membranes in western blots probed with monoclonal antibodies against CFTR (Fig. 1*b*). CFTR protein was localized in a highly enriched plasma membrane vesicle fraction. In variants containing nearly the same amount of CFTR as T84 cells, cAMP-regulated chloride permeability, as monitored by ^{125}I efflux, was indistinguishable from that in T84 cells (Fig. 1*c*).

Patch-clamp recording was used to identify the channel responsible for cAMP-stimulated ^{125}I efflux. Channels became active in cell-attached patches after a lag of 69 ± 26 seconds when cells were exposed to membrane-permeant derivatives of cAMP; this was reversed by washing cAMP from the bath ($n = 7$, Fig. 2*a*). The channel was observed in 80% of all seals during cAMP stimulation (225/282) at an average density of between five and ten channels per patch. By contrast, it was recorded only once in 55 patches on unstimulated, CFTR-transfected cells and was never observed on cAMP-stimulated CHO cells that had been transfected with vector alone (0/31). Figure 2*b* shows that open probability was relatively independent of voltage, despite increased flickering at hyperpolarized potentials. Flickering was not observed using excised patches (see below), therefore these brief closures may reflect voltage-dependent, fast channel block by some anion in the cytosol. The current-voltage relationship rectified slightly in the outward direction during cell-attached recordings (Fig. 2*c*), but was linear ($r^2 = 0.9997$) when patches were excised and bathed symmetrically with 154 mM Cl^- (data not shown). In cell-attached patches the reversal potential (E_{rev}) was near the membrane potential (0.6 ± 0.3 mV applied potential) and the slope conductance at E_{rev} was 9.6 ± 0.5 pS ($n = 5$). The E_{rev} shifted to $+32.4 \pm 2.0$ mV when the pipette solution contained 110 mM sodium gluconate and

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