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VH SHUFFLING CAN BE USED TO CONVERT AN Fv FRAGMENT OF ANTI-HEN EGG LYSOZYME SPECIFICITY TO ONE THAT RECOGNIZES A T CELL RECEPTOR Vα

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Abstract—This study describes the isolation and characterization of Fv fragments that recognize a T cell receptor Vα (Vα1934.4). A VH gene repertoire from an immunized mouse was recombined with the anti-hen egg lysozyme (HEL) VκD1.3 gene as single chain (sc)Fvs, and an Fv with reasonable affinity for binding to Vα1934.4 isolated. The Fv (VH14/VκD1.3) does not bind to HEL, indicating that the heavy chain shuffling has converted an anti-HEL specificity to one that recognizes the unrelated Vα1934.4. The association constant for the Fv–Vα1934.4 interaction has been determined using surface plasmon resonance (SPR) and is 1.2×10^7 M^{-1}. Recombinant antibodies of reasonable affinity can therefore be generated by combining a VH library with a 'fixed' Vκ. To improve the affinity further, light chain shuffling has been used to generate an Fv (VH14/Vκ9) that has a 30-fold higher affinity for binding to Vα1934.4 than the parent (VH14/VκD1.3) Fv, and SPR measurements demonstrate that the affinity improvement is due to an increase in on-rate. Unexpectedly, Vκ9 differs from VκD1.3 by only two amino acids at positions 30 and 91 and, consistent with the change in binding affinity, both of these residues are located in CDRs.

Key words: bacteriophage display, VH, recombinant Fv, affinity, T cell receptor Vα.

INTRODUCTION

Monoclonal antibodies have extensive uses in biology and medicine, and recent developments in genetic engineering allow the production of designer antibodies with the effector functions and binding specificity of choice (reviewed in Winter and Milstein, 1991; Burton and Barbas, 1993). The development of bacterial systems for the expression of antibodies as secreted Fv, Fab fragments and single variable domains (Skerra and Plückthun, 1988; Better *et al.*, 1988; Ward *et al.*, 1989) has facilitated the rapid and efficient production of genetically manipulated antibodies. In particular, the isolation of antibodies with binding specificities that were difficult or impossible to isolate using hybridoma technology (Köhler and Milstein, 1975) is now possible using recombinant techniques. Libraries of VH and VL genes can be derived from antibody expressing cells using the polymerase chain reaction (PCR; Saiki *et al.*, 1988) and designed oligonucleotide primers (Orlandi *et al.*, 1989; Larrick *et al.*, 1989; Huse *et al.*, 1989). Alternatively, diverse semi-synthetic repertoires can be built from limited numbers of VH and VL genes using *in vitro* techniques (Hoogenboom and Winter, 1992; Barbas *et al.*, 1992). The antibody genes can be expressed as single chain Fvs (scFvs; Huston *et al.*, 1988; Bird *et al.*, 1988) or Fabs using bacteriophage display systems (McCafferty *et al.*, 1990; Kang *et al.*, 1991*a*; Breitling *et al.*, 1991; Chang *et al.*, 1991) and bacteriophage bearing the desired antigen binding activity selected by panning. It is now possible to generate antibody gene libraries that approach the size and diversity of the immune repertoire and to mimic the immune system by selection of bacteriophage bearing antigen binding specificities (Clackson *et al.*, 1991; Barbas *et al.*, 1991; Marks *et al.*, 1991; Burton *et al.*, 1991). Furthermore, the affinities of the selected antibodies can be improved by chain shuffling (Marks *et al.*, 1992) or random mutagenesis (Gram *et al.*, 1992; Hawkins *et al.*, 1992). Thus, the potential for the generation of antibodies of both murine and human origin is almost unlimited and, for example, circumvents many of the earlier problems (Carson and Freimark, 1986) associated with the production of human monoclonals.

In this paper, two closely related Fv fragments that recognize a T cell receptor (TCR), Vα (Ward, 1992) have been isolated in a two-step procedure. Both X-ray crystallography (Amit *et al.*, 1986; Padlan *et al.*, 1989; reviewed in Wilson and Stanfield, 1993) and chain shuffling experiments (Collet *et al.*, 1992; Zebedee *et al.*,

Abbreviations: CDR, complementarity-determining region; ELISA, enzyme-linked immunosorbent assay; HEL, hen egg lysozyme; IPTG, isopropyl-β-D-galactopyranoside; PCR, polymerase chain reaction; SPR, surface plasmon resonance; TCR, T cell receptor; Vα, alpha chain variable domain; VH, heavy chain variable domain; Vκ, kappa light chain variable domain; VL, light chain variable domain.

1992) indicate that the VH of an antibody frequently contributes a greater number of interactions upon binding to cognate antigen than the VL and in some cases, isolated VH fragments can have antigen binding activities of reasonable affinities (Ward *et al.*, 1989; Suter *et al.*, 1992). This suggests that by replacing the VH in an Fv/scFv fragment of known specificity with a VH library, in combination with the same Vκ, it might be possible to isolate Fvs of different specificities. In this study a VH gene library derived from a Vα-immunized mouse has been combined with the anti-hen egg lysozyme (HEL) VκD1.3 gene and an Fv isolated that binds to the Vα but no longer recognizes HEL. In this way a high affinity anti-HEL antibody has been converted to an anti-Vα specificity. SPR has been used to determine the affinity of the monovalent Fv fragment, and the affinity of this Fv for binding to the Vα has subsequently been improved 30-fold by light chain shuffling.

MATERIALS AND METHODS

Bacterial strains and vectors

Escherichia coli BMH 71-18 (Rüther *et al.*, 1981; K12, Δ(*lac-pro*), *sup*E, *thi*/$\bar{F}$, *pro*A$^+$B$^+$, *lacI*^{q}, *lacZ*ΔDM15), TG1 [(Gibson, 1984); K12, Δ(*lac-pro*), *sup*E, *thi*, *hsdD*5/$\bar{F}$*traD*36, *pro*A$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15] and HB2151 [K12, *ara*, Δ(*lac-pro*), *thi*/$\bar{F}$*pro*A$^+$B$^+$, *lacI*^{q}ZΔM15], and the cloning vectors pHEN1 (Hoogenboom *et al.*, 1991) and pUC19 or pUC119 (Viera and Messing, 1987) were used in this study. For display of scFvs on the surface of bacteriophage and the secretion of (sc)Fvs using the vector pHEN1, *E. coli* TG1 and HB2151 respectively, were used. For the expression of secreted (sc)Fvs or single variable domains (VH, Vκ) using plasmid derivatives of pUC19 or pUC119, *E. coli* BMH 71-18 was used as host.

Immunization of mice

Two BALB/c mice were immunized with purified Vα (designated Vα1934.4; Ward, 1992) derived from the 1934.4 T cell hybridoma (Wraith *et al.*, 1989) using previously described methodology (Ward *et al.*, 1989). The serum antibody titres were analysed using sera obtained from tail bleeds and ELISAs. The mouse which gave the higher titre was sacrificed for the isolation of immunoglobulin VH and Vκ genes.

Construction of VH and Vκ gene libraries

VHD1.3-sc-VκD1.3-myc/pHEN1 was constructed by amplifying the scFvD1.3 gene (McCafferty *et al.*, 1990) using the primers SfiVH1BACK (the same as VH1BACK with an appended *Sfi*I site) and NotIVκFOR-2 (see below) and the PCR (Saiki *et al.*, 1988). The PCR product was restricted with *Sfi*I and *Not*I, gel purified and ligated into *Sfi*I–*Not*I restricted pHEN1.

For the construction of the VH gene library, the VH genes were isolated using the primers VH1BACK and VH1FOR-2 in the PCR with cDNA derived from total spleen RNA (Ward *et al.*, 1989; Clackson *et al.*, 1991). The PCR products were restricted with *Pst*I and *Bst*EII and ligated into VHD1.3-sc-VκD1.3-myc/pHEN1 (Fig. 1).

For the cloning of a Vκ gene repertoire into VH14-sc-VκD1.3-myc/pHEN1, the Vκ genes were isolated from splenocyte derived cDNA using the PCR and the following primers: Vκ2 BACK, 5′ GAC ATT *GAG CTC* ACC CAG TCT CCA 3′; Vκ4FOR-1, 5′ CCG TTT TAT TTC CAR CTT KGT CCC 3′ where R = A/G and K = T/G; Vκ4FOR-2, 5′ CCG TTT SAK YTC CAG CTT GGT SCC 3′ where S = G/C, K = T/G and Y = T/C; VκFOR-1 and VκFOR-2 were used in equimolar amounts. *Not*I tagging primers: Vκ4NOTFOR-1, 5′ TTC TCG ACT T*GC GGC CGC* CCG TTT TAT TTC CAR CTT KGT CCC 3′; Vκ4NOTFOR-2, 5′ TTC TCG ACT T*GC GGC CGC* CCG TTT SAK YTC CAG CTT GGT SCC 3′.

These primers are slightly modified versions of those described by Clackson *et al.* (1991). The genes were restricted with *Sac*I (site in Vκ2BACK shown in italic) and *Not*I (sites in tagging primers shown in italic) and ligated into *Sac*I–*Not*I restricted VH14-sc-VκD1.3-myc/pHEN1 to replace the VκD1.3 gene (Fig. 1). For both VH and Vκ gene libraries, the diversity was analysed using dideoxynucleotide sequencing (Sanger *et al.*, 1977) and Sequenase (USB Biochemicals). For each library 20 clones were sequenced.

Panning of the libraries

Recombinant bacteriophage were produced and panned using essentially the method of Marks *et al.* (1991) with the following modifications. Cultures (50 ml) of recombinant clones were grown up to mid-exponential phase, infected with VCSM13 (Stratagene, La Jolla, CA) as described (Marks *et al.*, 1991) and at the time of kanamycin addition, isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.04 mM was added. Cultures were incubated for 15 hr at either 30°C or 37°C with shaking at 250 rpm. Phage particles were precipitated from the culture supernatant and initially panned for 30–60 min against an uncoated Nunc immulon tube that had been blocked with 2% milk powder/phosphate buffered saline (MPBS). Unbound phage were then used in a second round of panning using a Nunc immulon tube that had been coated overnight with 50 μg/ml Vα1934.4, rinsed with PBS and then blocked for 1–2 hr at room temperature with MPBS. Following incubation for 1–2 hr with agitation, the tube was washed 20 times with 0.1% Tween/PBS followed by 20 times with PBS. For the Vκ gene library, the tubes were also washed with 50 mM Tris–HCl pH 8.3 and 500 mM NaCl for 10 min prior to elution. Bound phage were eluted and used to infect exponentially growing TG1 or HB2151 cells as described (Marks *et al.*, 1991).

Expression of the anti-Vα scFvs and analyses of binding activities

Following 2–3 rounds of panning, HB2151 clones resulting from bacteriophage infection were grown up individually and induced for expression of secreted scFvs

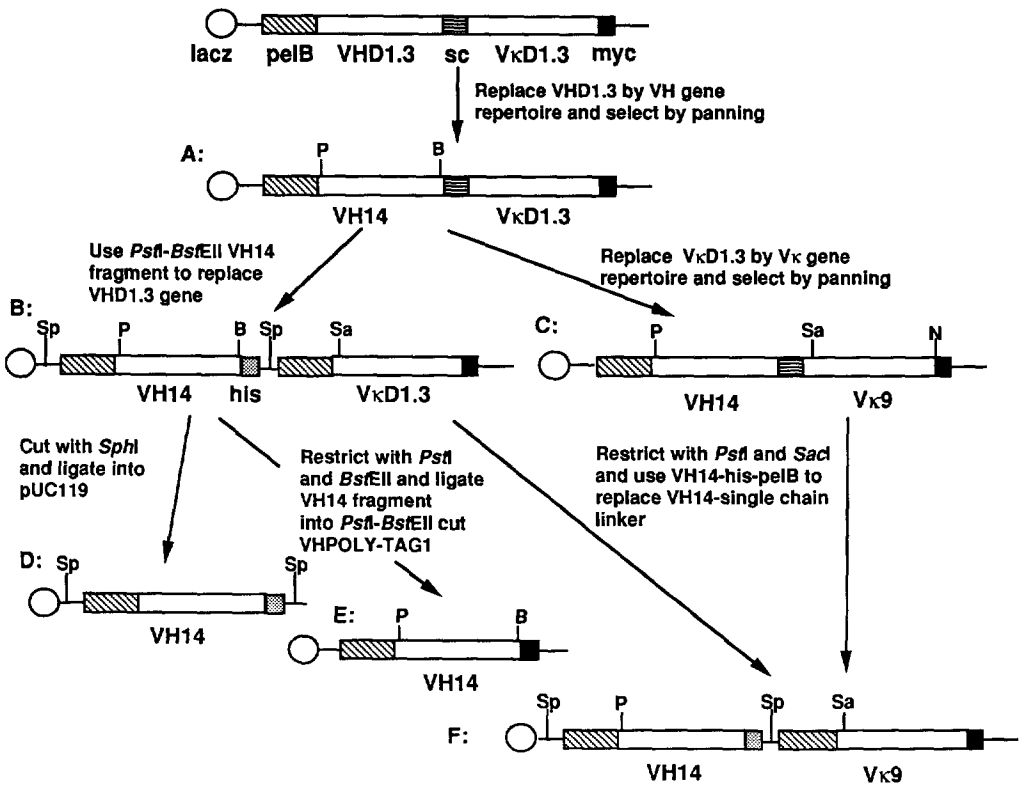

Fig. 1. Strategy for the construction of plasmids for the expression of VH14-containing Fvs or VH14 as a single domain. (A) VH14-sc-VκD1.3-myc/pHEN1; (B) VH14-hisVκD1.3-myc/pUC19; (C) VH14-sc-Vκ9-myc/pHEN1; (D) VH14-his/pUC119; (E) VH14-myc/pUC19 and (F) VH14-his: Vκ9-myc/pHEN1. Lacz, lacz promoter (lacz); pelB, pelB leader ; sc, single chain linker sequence (horizontal lines); myc, c-myc peptide tag (filled in boxes); his, his6 peptide tag (stippled boxes) and the VH and Vκ genes are represented by open boxes. Backbone vector sequences are indicated by single lines. For clarity, only the relevant restriction sites for each cloning step are shown and P, *Pst*; B, *Bst*EII; Sp, *Sph*I; Sa, *Sac*I; N, *Not*I.

using the following methodology. Cultures (1.5 ml) in 4 × TY plus 100 μg/ml ampicillin and 1% glucose were grown for 8 hr at 37°C. Cells were pelleted by centrifugation, washed once with 2 × TY and resuspended in 2 × TY plus 100 μg/ml ampicillin, 0.1 mM IPTG and 1 μg/ml leupeptin and grown for 16 hr at 30°C. Culture supernatants were analysed for the presence of scFvs with binding activity using 96-well plates coated with 50 μg/ml Vα1934.4 or 50 μg/ml Vα85.33 [derived from the 85.33 T cell hybridoma (Myers *et al.*, 1993) and expressed and purified in the same way as Vα1934.4 (Ward, 1992)]. All assays were carried out in duplicate and bound scFvs detected using the anti-c-myc tag antibody 9E10 (Evan *et al.*, 1985) followed by horseradish peroxidase-conjugated anti-mouse IgG (ICN Immunochemicals, Costa Mesa, CA) as described previously (Ward *et al.*, 1989).

Recloning of the VH and Vκ genes for expression as secreted scFvs and Fvs with his6 peptide tags

To purify VH14-sc-VκD1.3 (Fig. 1) using Ni^{2+}-NTA-agarose (Hochuli *et al.*, 1988), the VH and Vκ genes were recloned into scVαVβpelBHis (Ward, 1992) to replace the TCR genes using the following two-step cloning strategy: the major part of the VH14 gene was isolated as an *Nco*I–*Bst*EII fragment and then ligated into appropriately cut vector [the *Nco*I site is at the 3′ end of the pelB leader (Hoogenboom *et al.*, 1991) and the *Bst*EII site is shown in Fig. 1]. The resulting plasmid was restricted with *Bst*EII and a *Bst*EII fragment encoding the carboxy terminus of the VH gene, the single chain linker and the VκD1.3 gene (tailored by PCR with a 3′ *Bst*EII site) was ligated into the vector. The orientation of the *Bst*EII fragment was checked by PCR screening (Güssow and Clackson, 1989) and the *Bst*EII insert of correctly orientated clones was analysed by nucleotide sequencing (Sanger *et al.*, 1977) to eliminate the possibility of the presence of PCR errors.

To reclone VH14 into a vector designed for the expression of an Fv fragment comprising VH14 with a carboxy terminal his6 tag and VκD1.3 with a c-myc tag (Fig. 1), the gene encoding VH14 in VH14-sc-VκD1.3-myc/pHEN1 was restricted with *Pst*I and *Bst*EII and ligated into *Pst*I–*Bst*EII VHD1.3-his:VκD1.3-myc to replace the VHD1.3 gene. [VHD1.3-his:VκD1.3-myc was made by ligating the oligonucleotide duplex 5′ GTC ACC GTC TCC TCA CAT CAC CAT CAC CAT CAC TAA TAA 3′ and 3′G CAG AGG AGT GTA GTG GTA GTG GTA GTG ATT ATT CAG TG 5′ into *Bst*EII restricted VHD1.3-VκD1.3TAG1 (Ward *et al.*, 1989). The coding strand is indicated by underlining]. The VH14 derivative of this plasmid was designated VH14-his:VκD1.3-myc.

To construct a plasmid for the expression of VH14 and Vκ9 as a non-covalently associated Fv fragment, the strategy shown in Fig. 1 was used. Briefly, a *Pst*I–*Sac*I fragment encoding VH14 and the pelB leader linked to the 5′ end of the VκD1.3 gene (same as Vκ9 in this region) in VH14-his:VκD1.3-myc was used to replace VH14 and the single chain linker in VH14-sc-Vκ9-myc/pHEN1.

Construction of plasmids for the expression of single VH and Vκ domains

To express VκD1.3 and Vκ9 as single variable domains with c-myc tags, the vectors were cut with *Sph*I and religated (see Fig. 1 for location of *Sph*I sites). For the cloning of VH14 for expression with a his6 tag (Fig. 1), the *Sph*I fragment released by digestion was ligated into pUC119 and clones harbouring the desired orientation for expression driven by the lacz promoter were identified by PCR screening (Güssow and Clackson, 1989). To express VH14 with a c-myc tag (Fig. 1), a *Pst*I–*Bst*EII fragment encoding a major part of the VH14 gene was ligated into *Pst*I–*Bst*EII-restricted VHPOLYTAG1 (Ward *et al.*, 1989).

Purification of the expressed Fvs and VH14-his

Recombinant clones harbouring VH14-his:VκD1.3-myc, VH14-his (backbone vector pUC19 or pUC119) or VH14-his:Vκ9-myc (backbone vector pHEN1) were grown up and induced for expression as described previously for the purification of soluble TCR fragments with carboxy terminal his6 peptide tags (Ward, 1992). An additional wash with two column volumes of 10 mM imidazole was also included to remove non-specifically bound proteins. Protein concentrations were determined using the Pierce BCA reagent.

Expression of single VH and Vκ domains tagged with c-myc peptides

For analysis of VH14-myc, VκD1.3-myc and Vκ9-myc, recombinants were grown up, induced for expression and osmotic shock fractions dialysed against PBS as described previously (Ward, 1992). Immunoblotting (Towbin *et al.*, 1978) was used to estimate the amount of myc tagged protein in the osmotic shock fractions using the 9E10 antibody (Evan *et al.*, 1985) as described previously (Ward *et al.*, 1989).

Analyses of the binding activity of the anti-Vα Fvs and single variable domains using ELISAs

Purified Fvs (VH14-his:VκD1.3-myc and VH14-his:Vκ9-myc) or osmotic shock fractions (VH14-myc, VκD1.3-myc and Vκ9-myc) were analysed for binding specificity using ELISA. As a control, the anti-HEL Fv VHD1.3-his:VκD1.3-myc was also purified using Ni^{2+}-NTA-agarose and used in these assays. Plates were coated with 50 μg/ml Vα1934.4, 50 μg/ml Vα85.33 or 1 mg/ml HEL overnight and subsequently blocked with either MPBS or 3% BSA/PBS for 1–2 hr at room temperature. Fvs were added in PBS in two-fold serial dilutions (100 μl per well), and bound Fvs were detected using the 9E10 antibody and previously described methodology (Ward *et al.*, 1989). For ELISAs with single domains tagged with c-myc peptides, the estimated concentration (from immunoblots) used was 50–100 μg/ml (100 μl per well).

For competition ELISAs using VH14-his, Fvs were mixed with an 8–1024 molar excess of VH14-his and added to wells as above. To assess the level of non-specific inhibition by VH14-his, the anti-HEL Fv, VHD1.3-his:VκD1.3-myc, was mixed with VH14-his at the same concentrations and binding to HEL coated plates quantitated.

BIAcore measurements

For measurement of on- and off-rates for binding of the Fvs to Vα1934.4, SPR measurements (BIAcore, Pharmacia Biosensor; Karlsson *et al.*, 1991; Borrebaeck *et al.*, 1992) were carried out by Robertson Sensor Technologies (Ithaca, NY) using the following methodology: Vα1934.4 was immobilized on the carboxymethylated dextran surface of a gold-covered glass chip using the standard amine coupling procedure (Johnsson *et al.*, 1991). To test for non-specific binding of the Fvs to the chip, buffer was used in the immobilization procedure instead of Vα1934.4 and no non-specific binding was observed. For VH14-his:Vκ9-myc and VH14-his:VκD1.3-myc, concentrations of 1.2–2700 nM and 3.7–8100 nM, respectively were used. Complete dissociation of the Fv fragments from the chip surface occurred during a 10 min dissociation period and for this reason no regeneration buffer was used. The kinetic runs were designed such that there were several concentrations between 20 and 80% of Rmax (maximum amount of Fv that can theoretically bind to the Vα coated surface), and the running buffer was 10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl and 0.05% P20, pH 7.4. Fvs at different concentrations were injected in 30 μl volumes at a flow rate of 3 μl/min, and the dissociation rate was monitored for 10 min with a flow rate of 100 μl/min.

RESULTS

Construction and panning of a VH gene library

A library of VH genes in combination with VκD1.3 was assembled and expressed using the bacteriophage display vector pHEN1 (Fig. 1; Hoogenboom *et al.*, 1991). The library size was estimated to be 5×10^4 clones and was panned against the immunogen Vα1934.4. Following the third round of panning, 44 HB2151 clones that were infected with eluted phage were grown up and induced for expression. Culture supernatants were analysed and four positive clones producing scFvs that bound to Vα1934.4 were identified. The specificity of the clones was analysed using Vα85.33 (Myers *et al.*, 1993) as antigen, and no binding was observed. The Vα85.33 domain shares 32% amino acid sequence homology with Vα1934.4 (unpublished).

CDR1
*QVKLQQSG*AELVKPGASVKLSCKASGYTFTSYWMHWVKQRPGQGLEW

CDR2
IGEINPSNGRTNYNEKFKSKATLTVDKSSSTAYMRLSSLTSEDSAVYYC

CDR3
ARGSWFAYW*GQGTTVTVSS*

Fig. 2. Amino acid sequence of VH14. The sequences that are encoded by the PCR primers are indicated by italics, and the CDR residues by underlining.

Characterization of the selected Fvs

Nucleotide sequencing of the four positive clones indicated that they contained the same VH gene (designated VH14), and the amino acid sequence is shown in Fig. 2. The VH gene is derived from the IIB (V-region) and JH3 (J-segment) families (Kabat *et al.*, 1991). The unusually short CDR3 (six residues) makes it impossible to unambiguously determine which D-segment was used during V-D-J recombination. One of the clones, VH14-sc-VκD1.3, was chosen for further analysis. To allow purification on Ni^{2+}-NTA-agarose (Hochuli *et al.*, 1988) the clone was modified so that the c-myc tag was replaced by a his6 peptide tag (Ward, 1992). However, the yield of secreted scFv was low, and estimated to be approximately 100–200 μg/litre culture. Furthermore, scFvs have a tendency to aggregate as dimers and higher order multimers (Holliger *et al.*, 1993; Essig *et al.*, 1993) and although this property may be useful in increasing avidity, it introduces unwanted effects of bi- or multi-valency when using surface bound antigens to determine on- and off- rates. For these reasons, a second round of vector constructs were made (Fig. 1) to co-express VH14 and VκD1.3 as a non-covalently linked Fv fragment, as we have observed that the yields of secreted Fvs are frequently higher than those of scFvs (E.S.W. and S. Popov, unpublished data). The Fv fragment comprising VH14-his:VκD1.3-myc could be purified from *E. coli* cultures using Ni^{2+}-NTA-agarose in yields of 5–10 mg per litre of culture (Fig. 3). Immunoblotting using the 9E10 antibody indicates that VκD1.3-myc associates with VH14-his to produce heterodimers even under the conditions of washing the column with 10 mM imidazole (data not shown). Further indirect evidence that the protein purified from the column is heterodimeric, rather than a mixture of VH14-his and VH14-his/VκD1.3-myc heterodimers, has been obtained from binding studies described below using VH14 and VκD1.3 as single variable domains.

The binding specificity of VH14-his:VκD1.3-myc was analysed using ELISA, and was specific for Vα1934.4 (Fig. 4). Background binding was detected when HEL was used as antigen, indicating that the replacement of VHD1.3 by VH14 ablates the anti-HEL binding activity of this Fv. To determine whether the Fv was recognizing an epitope that included the his6 peptide, the reactivity of VH14-his:VκD1.3-myc was also tested against a single chain (sc)TCR comprising Vα1934.4 linked to Vβ1934.4 (Ward, 1992). In this scTCR, the Vα domain has no carboxy terminal his6 peptide. The reactivity was the same for Vα1934.4 as antigen (data not shown), demonstrating that VH14-his:VκD1.3 recognizes determinants located solely in the Vα. The affinity of the Fv for binding to Vα1934.4 has been determined using SPR (BIAcore), and the association constant (K_a) is 1.2×10^7 M^{-1} with on- and off-rates of 3.8×10^4 M^{-1} s^{-1} and 3.1×10^{-3} s^{-1}, respectively (Table 1).

Replacement of VκD1.3 by a Vκ gene library

In an attempt to further improve the affinity of the VH14/VκD.13 Fv for binding to Vα1934.4, a light chain shuffling experiment was carried out. A diverse Vκ repertoire was generated and ligated into VH14-sc-VκD1.3/pHEN1 to replace the VκD1.3 gene (Fig. 1). The resulting library (size estimated to be $1–2 \times 10^4$ clones) was panned in the same way as above, and ampicillin resistant HB2151 clones derived by infecting HB2151 cells with the phage eluates from the second round of panning were grown up and analysed for the production of scFvs that bound to Vα1934.4. Two out of 24 of the clones produced scFvs that bound to Vα1934.4 and the Vκs of these positives were designated Vκ3 and Vκ9.

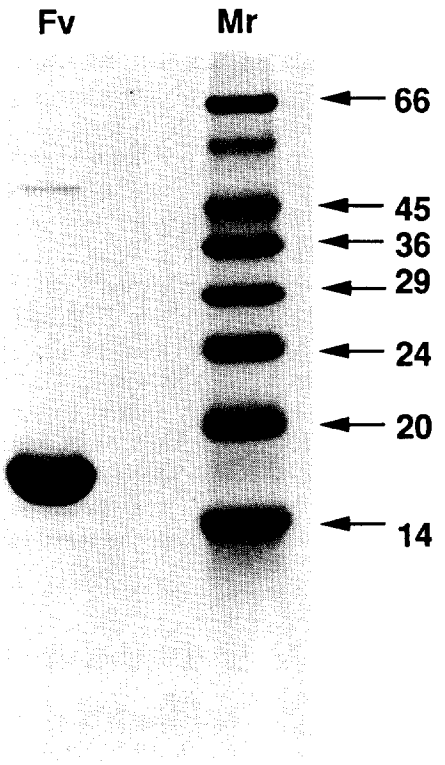

Fig. 3. SDS–polyacrylamide gel analysis (15%) of purified VH14-his:VκD1.3-myc (Fv). M_r = molecular weight standards with the sizes indicated in kilodaltons on the right margin.

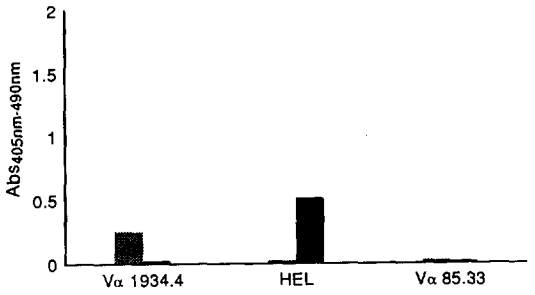

Fig. 4. Specificity of binding of VH14-his:VκD1.3-myc (stippled boxes) and VHD1.3-his: VκD1.3-myc (filled-in boxes) to Vα1934.4, HEL and Vα85.33. Absorbance values for Fvs at concentrations of 50 μg/ml (100 μl per well) are shown.

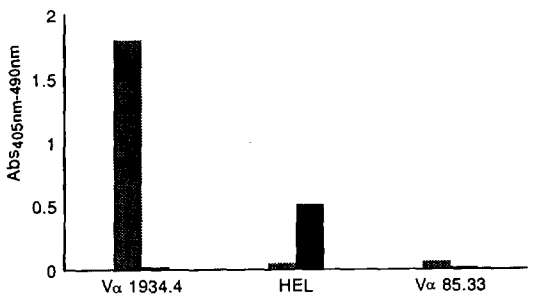

Fig. 6. Specificity of binding of VH14-his:Vκ9-myc (stippled boxes) and VHD1.3-his:VκD1.3-myc (filled in boxes) to Vα1934.4, HEL and Vα85.33. Absorbance values for Fvs at concentrations of 50 μg/ml (100 μl per well) are shown.

Characterization of the selected Fvs

Nucleotide sequencing of Vκ3 and Vκ9 indicated that Vκ3 had the same sequence as VκD1.3, but unexpectedly that Vκ9 was the same with the exception of two amino acid differences (His30 > Arg in CDR1, and Phe91 > Leu in CDR3). The nucleotide sequence of Vκ9 at the 3′ end was derived from Vκ4FOR-1 which is different to that of the corresponding region of VκD1.3, but the encoded amino acid sequences are the same (Fig. 5). Vκ3 is presumably a background clone, due to either PCR contamination or incomplete digestion of the vector, whereas the origin of Vκ9 is less clear (see Discussion).

The similarity of Vκ9 and VκD1.3 suggested that the expression of VH14-sc-Vκ9 (i.e. as a single chain Fv) would be low, and therefore the vector VH14-his:Vκ9-myc was constructed to express the Fv as a non-covalently associated heterodimer. Furthermore, this would allow the determination of the affinities of monovalent Fv fragments for binding to surface bound antigen. Recombinant clones harbouring VH14-his:Vκ9-myc were grown up and induced for expression and the Fv fragment purified using Ni^{2+}-NTA-agarose. The yields were approximately 5–10 mg/l of culture, and immunoblotting showed that Vκ9 associated quantitatively with VH14 to form heterodimers (data not shown). The specificity of VH14-his:Vκ9-myc was analysed using ELISAs (Fig. 6), and indicates that the Fv binds strongly to Vα1934.4, at background levels to HEL and at slightly higher levels than background to Vα85.33. The latter weak binding activity is probably due to the 32% amino acid sequence homology that is shared by the two Vαs.

The affinity of VH14-his:Vκ9-myc was measured using SPR (BIAcore), and the association constant, on- and off-rates are shown in Table 1 together with those for VH14-his:VκD1.3-myc. To check the reproducibility of the measurements, kinetic data were obtained for two independently prepared batches of VH14-his:Vκ9-myc, and both data sets are shown.

Binding activities of individual VH and Vκ domains

To assess whether the individual domains (VH14, VκD1.3 and Vκ9) had binding activity for Vα1934.4, these domains were expressed with c-myc peptide tags and osmotic shock fractions used in ELISAs. The amounts of myc-tagged protein were estimated from immunoblotting data. All three domains (and in particular, Vκ9 and VκD1.3) showed a relatively high level of non-specific binding to uncoated, blocked ELISA plates, and for VH14 and Vκ9, binding to lysozyme was also observed (Fig. 7). The binding to Vα1934.4 by the single domains was at a similar level to the non-specific binding, and of particular significance, VκD1.3 showed weak non-specific binding to Vα1934.4. In contrast, VH14-his:Vκ9-myc shows highly specific binding activity.

VH14-his was also expressed and purified as a single variable domain using Ni^{2+}-NTA-agarose (in yields of approximately 0.5 mg/l) and used in competition binding assays with VH14-his:VκD1.3-myc and VH14-his:Vκ9-myc. Consistent with the data obtained from the direct binding assays, no specific inhibition of binding was observed (data not shown).

DISCUSSION

In this paper, the isolation of two related Fv fragments that bind to a recombinant TCR Vα is described. The Fvs were isolated in two steps. Firstly, a VH gene library derived from an immunized mouse was cloned in combination with the somatically mutated VκD1.3 gene (Boulot *et al.*, 1990; Hawkins *et al.*, 1993). In association with VHD1.3, this Vκ forms an Fv that binds to HEL with

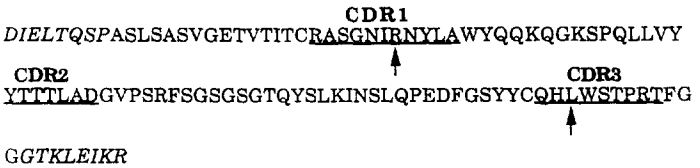

Fig. 5. Amino acid sequence of Vκ9. The sequences that are encoded by the PCR primers are indicated by italics and the CDR residues by underlining. Arg30 and Leu91 are indicated by arrows.

Table 1. Kinetic parameters of Fvs for binding to Vα1934.4

	VH14-his: VκD1.3-myc	VH14-his: Vκ9-myc[a] (a)	(b)
$k_{on}(M^{-1}\,s^{-1})$	3.8×10^4	1.8×10^6	1.5×10^6
$k_{off}(s^{-1})$	3.1×10^{-3}	4.4×10^{-3}	4.9×10^{-3}
$K_a(M^{-1})$	1.2×10^7	4.1×10^8	3.1×10^8

[a](a) and (b) are datasets for two independently prepared batches.

high affinity and specificity (Ward *et al.*, 1989). The recombinant scFvs were displayed on the surface of bacteriophage and a VH (VH14) was isolated that linked to VκD1.3 as a scFv fragment, binds to Vα1934.4. The VH14-sc-VκD1.3 scFv was secreted from *E. coli* cells at relatively low levels and to facilitate analysis was expressed and purified in high yields as a non-covalently associated Fv. We have frequently observed that scFvs are expressed at much lower levels than the corresponding non-covalently linked Fvs (S. Popov and E. S. W., unpublished data). The Fv appeared to be stably associated as a VH–VL heterodimer, and in this respect is similar to the FvD1.3 (Ward *et al.*, 1989) but in contrast to the McPC603 Fv fragment (Skerra and Plückthun, 1988). Furthermore, as scFvs tend to dimerize (Holliger *et al.*, 1993; Griffiths *et al.*, 1993) or form higher order multimers (Essig *et al.*, 1993), production of VH14/VκD1.3 as an Fv allowed the measurement of on- and off-rates to surface bound antigen as monovalent fragments in the absence of avidity effects due to bi- or multivalency. In addition, we have sometimes observed higher levels of non-specific binding for scFvs relative to the corresponding Fvs (E.S.W. and S. Popov, unpublished data) and the cause of this is not clear but may be due to aggregation and/or multivalency. For characterization of the binding affinity in the absence of potential artefacts due to dimerization/multimerization, it may therefore be preferable to analyse unlinked Fvs if the interaction of VH and VL domains appears to be stable, as in this study. For use *in vivo* however, it may be necessary to stabilize the association of the VH and VL domains (Cumber *et al.*, 1992) and this can be done, for example, by incorporating an additional -S—S- bridge (Glockshuber *et al.*, 1990) or by linking the VH and VL genes to CH1 and Cκ genes, respectively to build Fab fragments (Better *et al.*, 1988; Huse *et al.*, 1989).

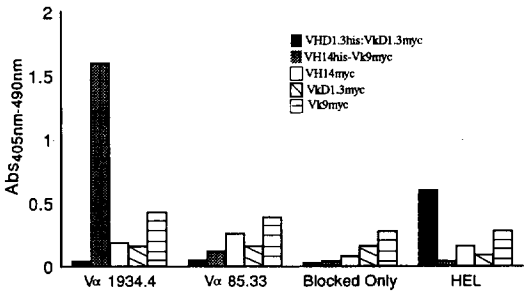

Fig. 7. Specificity of binding of VH14-myc, VκD1.3-myc and Vκ9-myc to Vα1934.4, Vα85.33, uncoated blocked plates and HEL. As controls, VH14-his: Vκ9-myc and VHD1.3-his:VκD1.3-myc were used at concentrations of 50 μg/ml.

The VH14/VκD1.3 Fv has no binding activity for HEL nor Vα85.33 which shares 32% homology with Vα1934.4. Replacement of VHD1.3 with VH14 has therefore ablated the binding activity of the FvD1.3 for HEL, which is not unexpected as the VH domain of the FvD1.3 makes extensive contacts with antigen (Amit *et al.*, 1986; Bhat *et al.*, 1990) and also has a relatively high binding affinity for HEL as an isolated VH domain (Ward *et al.*, 1989).

In an attempt to improve the affinity of the VH14/VκD1.3 Fv, Vκ genes from an immunized mouse were used to replace the VκD1.3 gene in a light chain shuffling experiment (Clackson *et al.*, 1991; Kang *et al.*, 1991*b*). Using this approach a Vκ was isolated which, in association with VH14, generates an Fv with 30-fold higher affinity for binding to Vα1934.4. This Vκ differs by two amino acids from VκD1.3 with His30 and Phe91 being replaced by arginine and leucine, respectively. Both these amino acids are located in CDRs, and in the structure of the FvD1.3:HEL complex the backbone O of Phe91 forms a hydrogen bond with Gln121 of HEL, whereas His30 makes no contacts with antigen (Amit *et al.*, 1986; Bhat *et al.*, 1990). The His30 > Arg30 and Phe91 > Leu91 changes may improve the affinity either by direct contact with Vα1934.4 or by altering the conformation of the CDR loops in such a way that neighbouring residues contact the Vα more favourably. In this respect, for the D1.3 antibody mutation of Ile29 which fits between two important contact residues and does not itself contact HEL (Amit *et al.*, 1986; Bhat *et al.*, 1990) improves the affinity several fold (Hawkins *et al.*, 1993). Furthermore, other studies have shown that somatic or *in vitro* mutations of amino acids that lie outside the antibody–antigen interface can significantly improve binding affinities (Sharon, 1990; Lavoie *et al.*, 1992; Riechmann *et al.*, 1988; Foote and Winter, 1992). Clearly, an improved understanding of the molecular basis for the increase in affinity of the VH14-his:Vκ9-myc Fv requires detailed structural analyses.

The observation that the 30-fold difference in affinity for the two Fvs is due to an increase in on-rate suggests that the replacement of His30 by Arg and/or Phe91 by Leu may have removed or decreased a structural constraint on the binding of the Fv to Vα1934.4, in a similar way to that described for affinity maturation involving repertoire shift of anti-2-phenyl-5-oxazolone antibodies (Foote and Milstein, 1991). There is little difference in the off-rates for the VκD1.3 and Vκ9 containing Fvs, suggesting that once bound, the interactions for both Fv–Vα pairs are thermodynamically similar. Furthermore, these data are in contrast to the

chain shuffling data of Marks and colleagues (Marks *et al.*, 1992), where the affinity increases were found to be due to decreases in off-rates.

As single variable domains, VH14, VκD1.3 and Vκ9 show weak binding to Vα1934.4 that is similar to the level of non-specific binding. In addition, the level of non-specific binding is much higher than that of the corresponding Fvs. This indicates that for both high affinity and specificity of binding, pairing of VH14 with either VκD1.3 or Vκ9 as an associated heterodimer is necessary. This also indicates that no significant dissociation of VH14 from VκD1.3 or Vκ9 occurs in solution, as ELISAs show the heterodimer to be highly specific.

Although no VκD1.3 genes were detected in the Vκ repertoire by nucleotide sequencing of 20 clones, the near identity of VκD1.3 and Vκ9 suggests that Vκ9 may be derived from PCR contamination with the VκD1.3 gene followed by two PCR errors. However, an error rate of approximately 1/160 bases is much higher than we usually observe under the PCR conditions that are used in our laboratory, suggesting that Vκ9 may be a *bona fide* Vκ expressed (and selected by immunization) in the repertoire of the immunized mouse. Regardless of the origin of Vκ9, the data indicate that a similar result could be achieved using a different strategy, namely by random mutagenesis of the VκD1.3 gene followed by selection in a similar way to that described by others (Gram *et al.*, 1992; Hawkins *et al.*, 1992). The observation in the light chain shuffling experiment that only one light chain was found which, together with VH14 generates a scFv with higher binding affinity than the parent VH14/VκD1.3 (sc)Fv raises questions as to the lack of promiscuity of light chain pairing for VH14. In addition to the analysis of 24 clones as described in the Results section, a further 24 HB2151 transfectants were analysed for expression of anti-Vα1934.4 activities. No light chains other than VκD1.3 or Vκ9 were isolated that, in combination with VH14, formed a scFv with the desired binding activity. Light and heavy chain promiscuity has been observed for a number of other hapten and protein antigens (Clackson *et al.*, 1991; Kang *et al.*, 1991*b*; Marks *et al.*, 1992; Collet *et al.*, 1992; Barbas *et al.*, 1992). For anti-2-phenyl-5-oxazolone (Clackson *et al.*, 1991) and anti-gp120 (Collet *et al.*, 1992) antibodies however, it was found that the promiscuity varied from one heavy or light chain to another. This suggests that by 'fixing' the heavy chain as in this study, the number of light chains that can pair to form a binding activity is determined. In addition, although the light chain library described in this study appeared to be diverse, the PCR primers do not bind to the 5′ ends of some Vκ genes (for example, many of the VκV family members do not have Pro at codon 8 which is encoded by the 3′ bases of Vκ2BACK; Kabat *et al.*, 1991) nor Vλ genes, and this, in addition to the relatively small library size ($1–2 \times 10^4$ clones), could account for the lack of light chain promiscuity that has been observed.

In summary, the data show that it is possible to recombine a VH gene library derived from a Vα-immunized mouse with a somatically mutated Vκ of specificity for HEL and to isolate an Fv that has reasonable binding affinity for the immunogen. This provides further evidence for the dominance of the VH in forming the antigen binding site of an immunoglobulin molecule. Furthermore, this approach offers a simple route for the isolation of specific antibodies as it avoids the need for randomly combining VH and VL genes as either scFvs or Fabs. The affinity of the Fv for binding to the Vα can be further improved by light chain shuffling, as has been shown previously for anti-hapten Fvs (Marks *et al.*, 1992) but not for protein antigens. Finally, the data suggest a high degree of plasticity for immune receptors, as a Vκ associated with two different VHs can show high affinity and specificity for binding to two unrelated protein antigens.

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