

Selection of complementary single-variable domains for building monoclonal antibodies to native proteins

Tomoyuki Tanaka and Terence H. Rabbitts*

Leeds Institute of Molecular Medicine, University of Leeds, St. James's University Hospital, Leeds LS9 7TF, UK

Received November 30, 2008; Revised January 18, 2009; Accepted January 20, 2009

ABSTRACT

Antibodies are now indispensable tools for all areas of cell biology and biotechnology as well as for diagnosis and therapy. Antigen-specific single immunoglobulin variable domains that bind to native antigens can be isolated and manipulated using yeast intracellular antibody capture technology but converting these to whole monoclonal antibody requires that complementary variable domains (VH or VL) bind to the same antigenic site. We describe a simple approach (CatcherAb) for specific isolation of such complementary single domains allowing the constitution of functional Fv, forming the basis of antigen-specific whole immunoglobulin and thus antibody production. We illustrate this approach by developing high-affinity Fv from single variable domains binding to RAS and LMO2 oncogenic proteins.

INTRODUCTION

The speed and versatility with which monoclonal antibodies (human or mouse) can be isolated has increased since the first Köhler–Milstein description (1), partly due to phage display technologies that have rendered isolation of single-chain Fv (scFv, comprising linked VH and VL segments) (2) or single V domains achievable (3,4). The importance of high-quality reagents in a number of cell and molecular biology applications (5) necessitates the development of convenient technologies that can be applied in basic research laboratories as tools but also for clinical use where mouse–human chimaeric monoclonal antibodies (6,7) or humanized versions (8) are making a major impact. A number of important laboratory techniques also require antibodies that can bind to native, intracellular proteins, such as flow cytometric detection of intracellular proteins, using cell permeabilization (9), and immunoprecipitation-based methods including

pull-downs, analysis of protein complexes and chromatin immunoprecipitation analysis.

Single-domain antibody (Dab) libraries have provided sources of antibody fragments that can be used as antibody reagents (10) or as intracellular antibody fragments (11). Furthermore, single domains that bind to native proteins can be preferentially isolated using *in vivo* library screening in the yeast intracellular antibody capture (IAC) technology (12,13), because the target protein is expressed inside the cell within a normal cellular environment. In addition, the method can detect previously silent epitopes that may, for instance, fall within small clefts in antigen targets.

Whilst the isolation of single-domain antibodies has been made simple by screening with phage display and yeast, obtaining compatible pairs of VH and VL segments that bind at the same epitopic region requires additional steps. Furthermore, scFv obtained by phage display do not always provide complementary binders to a single epitopic region because of the way that the scFv are built from separate VH and VL segments. Ideally, engineering an scFv from single domains for binding native proteins *in vivo* could involve screening libraries of V regions to select a single domain of choice followed by a second screen that depends on the co-location of VH and VL on the antigen surface. We describe such a method (CatcherAb) for drawing together diverse single domains (VH and VL) into a high-affinity Fv format that can form the basis of a complete antibody (which can have any tag required, be of any class required and of any species). The method involves the mandatory interaction of VH and VL domains in contact with native antigens as the basis for selection. We illustrate this with the selection and engineering of two distinct Fv that specifically bind to their antigenic target (namely oncogenic RAS or LMO2) at the same epitopic location. The final scFvs have nanomolar affinity.

MATERIALS AND METHODS

Plasmids

The yeast vectors, pBTM116-HRAS(G12V) and pVP16*, are described in detail elsewhere (13). pBTM116- δ LMO2

*To whom correspondence should be addressed. Tel: +44 113 343 8518; Fax: +44 113 343 8601; Email: thr@leeds.ac.uk

was constructed by cloning the N-terminal truncated *LMO2* cDNA (14) into EcoRI-SalI sites of pBTM116 vector. The pCatcher plasmid is described in detail in Supplementary Figure S1. The mammalian expression vectors, pM-HRAS(G12V) (Gal4-DBD fusion bait) and the pEFVP16 (VP16-AD fusion prey), are described elsewhere (13). pM- δ LMO2 was constructed by cloning the N-terminal truncated *LMO2* cDNA into EcoRI-SalI sites of the pM vector. pEF/myc/nuc/VH was constructed by sub-cloning the anti-RAS VH#6 (13) or anti-LMO2 VH#576 (TT and THR, unpublished results) into the NcoI and NotI sites of pEF/myc/nuc (Invitrogen). All constructs were sequenced to confirm in-frame fusion of the inserts with signal peptide or fusion partners.

Yeast single-domain VL library construction

Single-domain human VL libraries were constructed in the yeast prey vector pVP16*. For the initial library construction for step-one screens (Figure 1A), a mixture of VL DNA fragments were PCR amplified from the human scFv phage libraries I or J (15) as template with the primers SfiVLF and pHENSeqR (all primer sequences used in PCR are shown in Supplementary Table S1). The PCR products were sub-cloned into the SfiI-NotI sites of pVP16*. The constructed VL libraries are composed of a single framework for Vk1 and diversified at two residues in CDR2 and five residues in CDR3 of VL domain (Figure 1B). The size of the VL libraries was $\sim 3.3 \times 10^6$ (VL-I library from scFv phage library I) and 1.1×10^6 (VL-J from library J), respectively. The sequences of 10 randomly picked clones derived from both VL-I and VL-J were sequenced for quality control and two of the 10 clones in each library had in-frame translational stop codons or frame shifts.

For construction of the VL library for an optional second-round screening (Figure 1A, Step 1, optional), positive clones from initial screenings of the VL library were used for randomization of the CDR1 region by PCR mutagenesis as described elsewhere (16). For the anti-RAS screen, two PCRs were performed with the pairs of primers: sFvVP16F and VLCDR1R; or VP162R and rdmVLCDR1F. The amplified DNA fragments were purified and assembled using a second PCR reaction involving the primers sFvVP16F and VP162R (16). The randomizing CDR1 VL fragment was sub-cloned into the SfiI-NotI sites of pVP16*. The size of the second VL library for RAS screening was $\sim 1.2 \times 10^6$. Clones were randomly picked from the library, plasmids prepared and sequenced to verify the insert and randomization CDRs.

CatcherAb yeast screening of human VL Dab libraries

Screening of the human VL single-domain libraries was performed essentially according to the protocol of IAC technology as described (12,13). First, 2.5 μ g each of pBTM116 and pCatcher plasmid was transformed into the L40-ura3 yeast strain (Invitrogen) using lithium acetate transformation and the transformed yeast grown on plates lacking tryptophan (Trp) and uracil (Ura) to establish yeast L40-ura3 clones stably expressing LexA-antigen (Ag) and VH. Five hundred micrograms of a pVP16*-VL

library were transfected into the above yeast host and positive clones selected on plates lacking Trp, leucine (Leu), Ura and histidine (His). To identify human VL with highest affinity for binding to antigen in the presence of VH, yeast transformants were plated on selective medium containing progressively increased concentrations of 3-amino-1,2,4-triazole (3-AT) (Sigma) (3-AT inhibits *his3* activity in a dose-dependent manner). The plates were incubated at 30°C for 5–9 days (depending on the concentration of 3-AT). The clones that reached >3 mm in size were counted, and 40 clones were randomly selected from the plate containing the highest concentration of 3-AT. The VL-VP16 clones were rescued, re-transformed into yeast L40-ura3 expressing LexA-Ag bait with or without VH and assessed for growth on plates lacking His and for β -gal activation. Following these assays, the relevant human VL segments were sequenced.

ATF-2-VP16 (11), cRAF-RBD (RAS-binding domain of cRAF)-VP16, Y13-293 (scFv derived from a anti-RAS rat monoclonal antibody)-VP16 (17) and VH#6-VP16 were used as positive or negative controls for binding specificity.

Mammalian transfection assays

Chinese Hamster Ovary (CHO) cells and derived CHO-GFP reporter cells (11) were grown in α -minimal essential medium (Invitrogen) with 10% FCS, penicillin and streptomycin. To establish a CHO-GFP stable line expressing Gal4DBD-HRAS(G12V) bait, cells were co-transfected with pM-HRAS(G12V) plus SV40-neomycin (from pEF/myc/nuc) using lipofectamine (Invitrogen) and selected in medium containing 1 mg/ml G418 and 0.3 mg/ml hygromycin B for 1 week. One hygromycin–neomycin resistant clone (CHO-GFP-RAS clone 35) was chosen for further experiments. The cells were seeded in 6-well plates (2×10^5 /well) the day before transfection. One microgram of each plasmid (pEF-VLVP16 alone or pEF-VLVP16 plus pEF/myc/nuc/VH#6) was co-transfected and 48 h later, the cells were harvested, washed and resuspended in PBS. The GFP reporter fluorescence expression was measured with a FACSCalibur and the data were analyzed using FCSPress software.

Mammalian luciferase reporter assays were performed as described (11). pM-Ag bait (0.2 μ g), 0.2 μ g pEF-VLVP16 prey and 0.2 μ g pEF/myc/nuc/VH catcher plasmids were transiently co-transfected with the reporter plasmid 0.2 μ g pG5-Luc and 0.02 μ g pRL-CMV (Promega) into COS-7 cells in 12-well plates (4×10^4 /well) using lipofectamine. After 48 h, the cells were lysed and assayed by the dual-luciferase reporter system (Promega) in a luminometer.

Protein expression and purification

To co-express recombinant proteins in bacteria, VH#6, HRAS(G12V) and VL (VLI21 or VL#204), the bacterial tri-cistronic expression vector, pRK-HISTEV-VH-RAS-VL (diagrammatically shown Figure 3C) (18) was transformed into the C41(DE3) bacterial host, grown to an OD₆₀₀ of 0.6 and induced with IPTG (final 0.5 mM) at 37°C for 4 h. The proteins were extracted from bacteria

after sonication and French press disruption in extraction buffer (25 mM Na phosphate, pH 7.4, 300 mM NaCl and 20 mM imidazole) and proteins co-purified using His-Trap Ni-affinity columns (GE Healthcare) using gradient elution with 20–300 mM imidazole histidine analog. The purified proteins were analyzed by SDS-PAGE and visualized by staining Coomassie brilliant blue.

For purification of GST fusion protein, pGEX-HRAS(G12V) or pGEXHISTEV-LMO2 were transformed into C41(DE3). The GST-RAS purification was performed as described elsewhere (18). For GST-LMO2, cells were grown at 37°C to an OD₆₀₀ of 0.6, ZnSO₄ was added to a final concentration of 100 μM and protein induced with 0.5 mM IPTG at 16°C for 14 h. The soluble proteins were extracted, and the GST-LMO2 was bound to glutathione-sepharose. Soluble GST-LMO2 was eluted with 50 mM Tris-HCl pH 8.0, 10 mM reduced glutathione, 150 mM NaCl and 0.05 mM ZnSO₄.

For purification of anti-RAS scFv, the plasmid pRK-HISTEV-scFv was prepared by sub-cloning scFv into pRK172 as described elsewhere (11).

Affinity measurement by surface plasmon resonance

The binding kinetics of antibody fragments were measured using a BIAcore 2000 or 3000 (Biacore) as described (11). GST or GST-HRAS(G12V)-GTPγS or GST-LMO2 were captured on a CM5 sensor chip (Biacore) through immobilized anti-GST polyclonal antibody (GE Healthcare). Affinity measurements for anti-RAS or anti-LMO2 scFv were performed by injection of purified proteins with various concentrations (0.5 nM–1000 nM) in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂ and 0.005% Tween 20 or in HBS-EP buffer (Biacore). Evaluation and calculation of the binding parameters were carried out according to the BIA evaluation 2.1 software.

Immunoprecipitation

For immunoprecipitation assays with anti-RAS scFv, NIH 3T3 fibroblasts were maintained in DMEM containing 10% foetal calf serum (FCS). For serum stimulation of RAS expression, cells were washed with serum-free medium and incubated in DMEM without serum for 24 h before re-introduction of 10% calf serum. Cells were harvested at various times after serum treatment. For the assay with anti-LMO2 scFv, pEF-BOS-LMO2-myc (19) was transiently transfected into CHO cells. After 72 h, the cells were washed three times with ice-cold PBS and lysed in buffer-containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 1% NP-40 and 10% glycerol for 30 min on ice. Lysates were either used immediately or frozen in liquid nitrogen and stored at -70°C until use. For immunoprecipitations, the lysate was mixed with purified His-tagged anti-RAS scFv (VH#6-VL#204), anti-LMO2 scFv (VH#576-VL#826) or scFv (VH#576-VL#827) and Ni-NTA agarose beads (Qiagen) and mixed by rotation for 30 min at 4°C. The mixture was centrifuged briefly, the supernatant discarded and the agarose pellets washed at least five times with PBS. The beads were finally resuspended in SDS-PAGE buffer and the solubilized proteins separated by

SDS-PAGE and transferred to membranes by western blotting. The membranes were incubated with anti-pan RAS (Ab-3, Calbiochem) antibody for RAS protein detection or anti-myc (9E10, Sigma) antibody for LMO2 protein detection and the bound antibody visualized using peroxidase-conjugated anti-mouse IgG antibody and detected by ECL (GE Healthcare).

RESULTS

Outline of the CatcherAb method

The objective was to develop a screening procedure for isolation of single-domain intrabodies (i.e. variable domains of the heavy chain, VH, and variable domains of the light chain, VL) that bind to the same epitopic region on an antigen. The CatcherAb approach, schematically shown in Figure 1A, comprises selecting an antigen-specific VL single domain as a prey via enhanced binding to antigen bait, facilitated by tertiary interaction with an antigen-specific VH domain (Catcher) (Figure 1A, Step 1). The essence of the CatcherAb method is that the VL prey does not have sufficient affinity to be selected through binding to the antigen bait alone in the *in vivo* system and must be strengthened by the interaction with the antigen bait through the complementary VH.

Two VL-VP16AD yeast libraries served as the initial libraries for the Catcher method. These diverse libraries were transfected into yeast co-expressing a LexA-DBD-antigen bait and a Catcher VH segment (Supplementary Figure S1) and antigen-specific clones isolated using selective media. These clones comprise VL that bind to the same epitopic region on the bait as does the cognate Catcher VH. Low-affinity binders were eliminated by growth of the selected clones on histidine-minus medium with increasing concentrations of the histidine biosynthesis inhibitor, 3-AT. In some cases, the first VL clones were pooled and further CDR randomization carried out (Figure 1A, optional step) followed by a re-screen in the bait yeast strain with a Catcher VH. Finally, VL complementary to the Catcher VH was confirmed using a mammalian transfection assay (Figure 1A, Step 2). Figure 1B shows the amino-acid sequence of the VL libraries with the indicated CDR changes in the initial and second form.

Isolation of anti-RAS complementary VL

First-round yeast screens were carried out with the two VL iDab libraries in L40-ura3 expressing the bait LexA-HRAS(G12V) and VH#6 and 40 million transfected cells were selected on Trp, Leu, Ura and His-minus plates with increasing 3-AT concentrations. As expected, increasing 3-AT levels in the media severely affected the number of colonies and no colonies appeared on plates with 75 mM 3-AT or more (Figure 2A). Twenty clones were selected from the colonies growing on plates containing 20–50 mM 3-AT. Interaction of the bait and prey was confirmed by tested β-gal expression (using the recovering bait plasmid) and β-gal expression was found in nine clones derived from VL-I and 17 from VL-J. The diversity of the VL sequences was determined by sequencing (sequences shown in Supplementary Figure S2) and six clones were

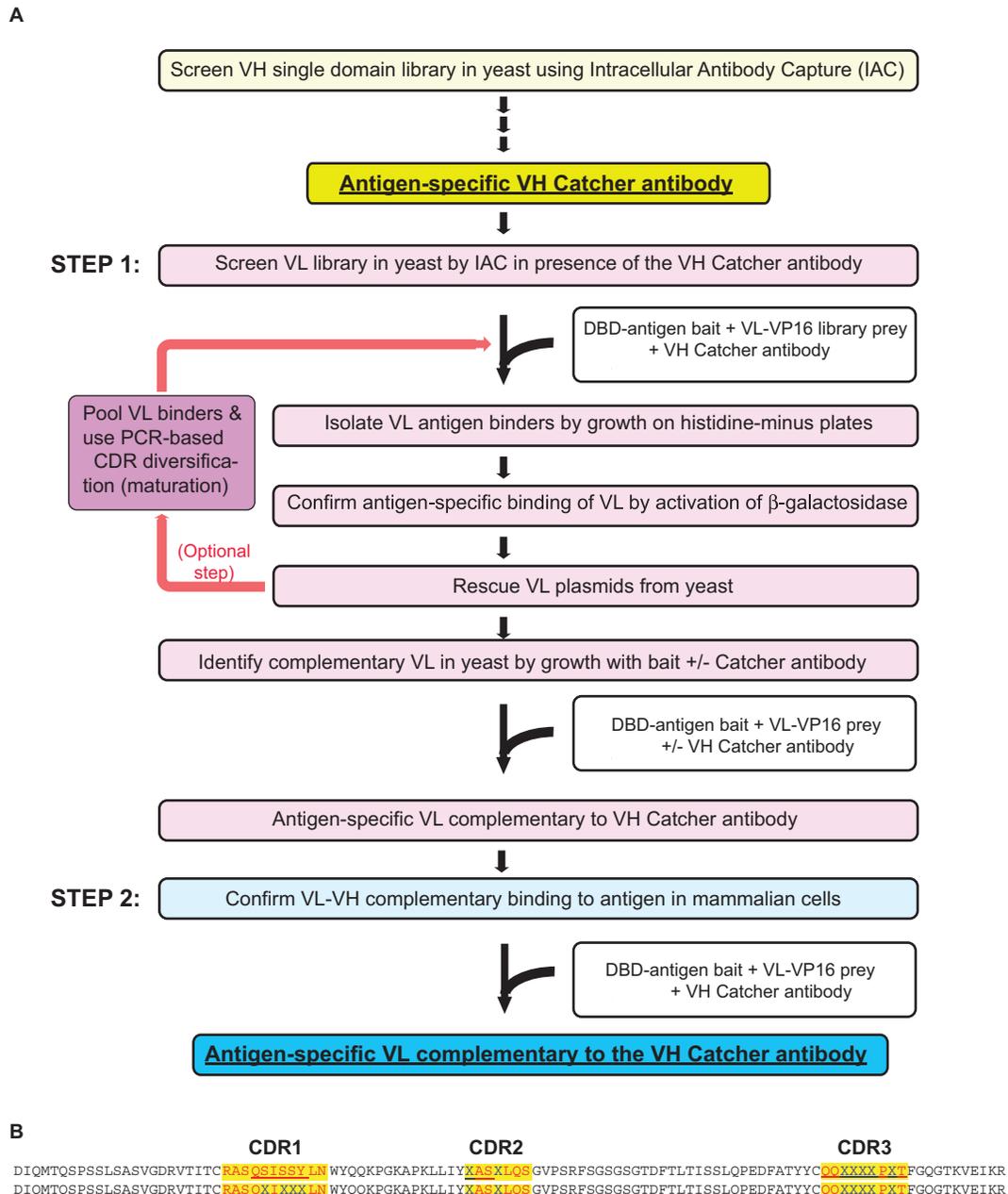


Figure 1. CatcherAb for selecting complementary single domains. The method relies on the isolation of antigen-specific immunoglobulin single domains dependent on simultaneous interaction of VH and VL with antigen. **(A)** A flow diagram of the CatcherAb screening method for selecting VL binders complementarily with VH. Initially, a single VH domains is selected with intracellular antibody capture methods (IAC) as the Catcher antibody. In Step 1, yeast expressing an antigen bait and the Catcher antibody are screened with a diverse VL prey library. Selected clones can (optional) be pooled, further randomized and rescreened in the Catcher system or taken directly into Step 2, where the VL-VH complementarity is confirmed. **(B)** Amino-acid sequences (single-letter code) of the two diversified human VL libraries (CDR regions highlighted). The VL library from the first screening (shown in the top line) was made from the synthetic human scFv phage libraries I or J (15) by PCR of the VL segments and cloning into pVP16* (13). The VL library for the second screening, where necessary (bottom line), was constructed from VL DNA of the CatcherAb selected clones from initial screening and diversified by PCR assembly (16) (diversification was accomplished for CDR1, four positions; CDR2, two positions; CDR3, five positions, indicated by X).

used for further study based on their growth in high 3-AT concentrations. These clones were pooled and the CDR1 randomized by PCR and fragment assembly (16) to generate a second-round VL-VP16* library of 1.2 million clones. This library was screened again by the CatcherAb method and 40 clones were sequenced (all 10

that grew at 150 mM 3-AT and 30 from growth at 100 mM 3-AT), and the sequences of 16 are shown Supplementary Figure S2. The variation in the ability of several first- and second-round VL clones to support growth in the yeast three hybrid (Y3H) assay in the absence of histidine is shown in Figure 2B. Whilst yeast expressing round one

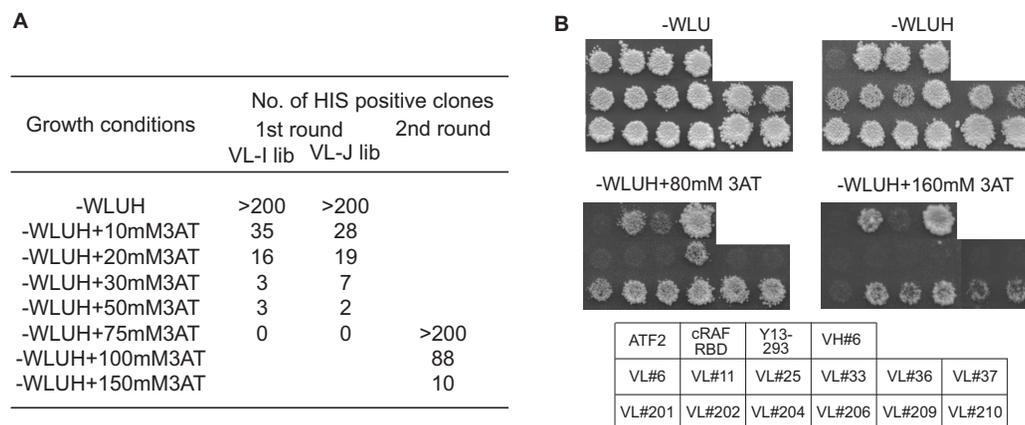


Figure 2. Isolation of complementary anti-RAS VL single domains by the CatcherAb method. (A) Two VL-VP16 libraries were screened with a RAS bait and the anti-RAS VH#6 CatcherAb and positive clones assessed by growth in the absence of Trp, Leu, Ura and His (-WLUH), with increasing concentrations of 3-AT. After the second round of selection, improved affinity of VL binding was achieved by PCR diversification using six of the first-round clones that grew on plates supplemented with between 30 or 50 mM 3-AT. The pooled library was re-screened in a second round and clones selected by growth in the absence of WLUH and with 75, 100 or 150 mM 3-AT. (B) Verification of complementary antigen binding was obtained by co-transfecting the LexA-HRAS(G12V) bait, pCatcher-VH#6 and VL-VP16 fusion vectors into yeast L40-ura3 cells. The transfected yeast was patched, grown on selective master plates (-WLU or -WLUH) and replicated on -WLUH plates supplemented with 80 or 160 mM 3-AT. The bottom panel shows the key for clone locations on the master plates. cRAF-RBD-VP16 (11), Y13-293-VP16 (17) and VH#6-VP16 (18) were used as positive controls and ATF-2-VP16 as negative control, respectively, for binding with the RAS bait.

VL#33 with RAS and VH#6 grew up to 80 mM 3-AT, none of the round one clones grew at 160 mM 3-AT. On the other hand, all six of the second-round VL clones supported growth at 80 mM 3-AT and three grew robustly under the strong selection of 160 mM 3-AT.

Anti-RAS scFv comprising of anti-RAS VH and complementary VL

The requirement for the VH-RAS interaction to achieve VL binding to antigen was confirmed using a mammalian three hybrid reporter assay using CHO-GFP-HRAS cells carrying a Gal4 DNA binding site-GFP reporter gene (11) and expressing Gal4DBD-HRAS(G12V) fusion antigen bait). No GFP activation occurred with any of the selected VL segments expressed as VL-VP16 fusions in these reporter cells (Figure 3A), whereas variable amounts of GFP fluorescence could be detected by flow cytometry when the reporter cells were co-transfected with VL-VP16 and the Catcher VH (Figure 3B), VL#204 showed the greatest stimulation. We conclude that the anti-RAS VL single domains only bind to antigen sufficiently when in the presence of VH#6 by the formation of a trimeric complex.

The physical association of VH, VL and antigen was verified by co-expressing recombinant RAS, anti-RAS VH#6 and VL#206 in the cytoplasm of bacteria using a tricistronic vector and purifying the complex directly by affinity chromatography via a histidine tag on the VH#6 segment (Figure 3C). All three recombinant proteins were synthesized in soluble form at 37°C and co-purified with the VH on nickel-agarose. The yields of the purified proteins were more than 4 mg/l of culture, showing that both VH and VL single domains are stable, fold properly and function in antigen binding inside cells under natural human body temperatures.

The VH#6 and VL#204 were formulated into an scFv molecule and *in vitro* affinity measurements determined by surface plasmon resonance (SPR). The equilibrium dissociation constant (K_d) was determined at 0.3 nM, which is 36 times better than a combination of VH#6 and non-relevant VLI21) (Figure 3D).

To evaluate the use of the anti-RAS antibody (scFv) derived from the CatcherAb screening in immunoprecipitations, the recombinant anti-RAS scFv antibody (comprising VH#6 and VL#204 with a linker peptide) was used to immunoprecipitate endogenous activated RAS expressed after serum-stimulation of fibroblasts. A time course of serum stimulation of NIH3T3 cells was carried out and the newly produced, activated RAS was immunoprecipitated with the scFv and separated by SDS-PAGE, followed by western detection using an anti-pan RAS antiserum (Figure 3E).

Complementary VH and VL anti-LMO2 single domains

The generation of effective and specific antibodies binding to native LMO2 has proven difficult. We applied the CatcherAb method to generate an anti-LMO2 scFv able to immunoprecipitate native LMO2 from mammalian cells. We have developed an anti-LMO2 VH using IAC selection (VH#576, TT and THR unpublished results), and this was used for our CatcherAb screening. After first-round screening, several yeast clones were isolated that could grow on plates with stringent 3-AT conditions (Figure 4A). These clones were compared by DNA sequence analysis and this showed that 20 different VL sequences had been selected (shown in Supplementary Figure S3).

In particular, it was noted that the CDR3 region showed great variability and, thus selected clones were screened directly in the mammalian luciferase reporter

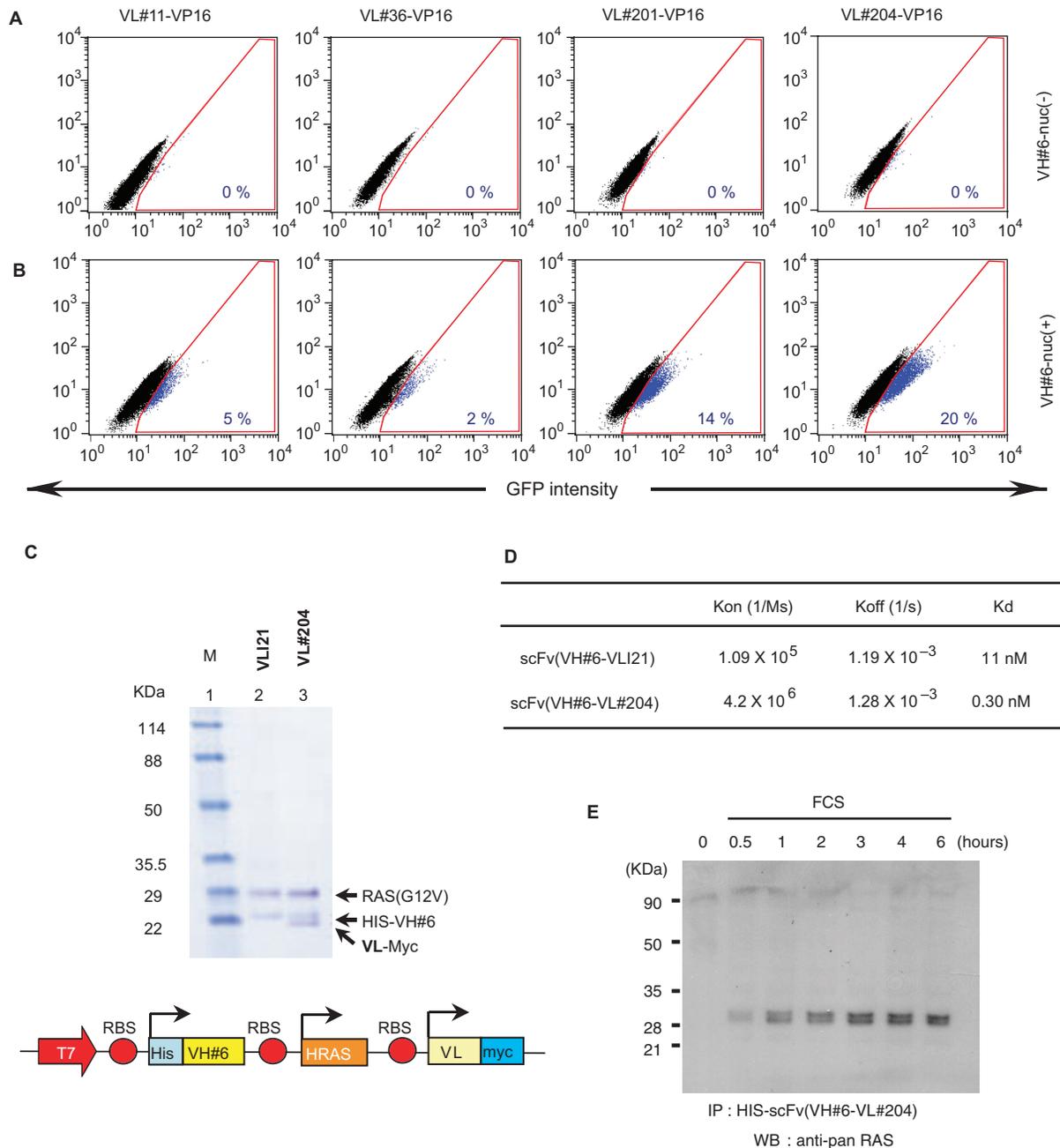


Figure 3. Interaction of RAS, VH and VL. (A and B) Flow cytometry of CHO-GFP reporter cells expressing the Gal4DBD-RAS bait and the indicated VL-VP16 prey only (A) or cells expressing the RAS bait, VL-VP16 prey plus the VH#6 Catcher antibody (B). The x-axis shows the fluorescence intensity and the percentages shown indicate the proportion of cells in the transfected populations expressing GFP. (C) Bacterial expression and co-purification of RAS, anti-RAS VL and anti-RAS VH. Diagram of the tri-cistronic vector, pRK-HISTEV-VH-RAS-VL, for co-expressing the anti-RAS VH#6, HRAS(G12V) antigen is shown (bottom). Expressed proteins (VH#6, HRAS and VL) were co-purified by Ni-affinity column using the His-tag on the VH#6 protein, fractionated on SDS-PAGE gels and stained with Coomassie Brilliant Blue. Lane 1: molecular weight markers; lane 2, purified proteins expressed from pRK-HISTEV-VH#6-RAS-VLI21; lane 3, purified proteins were expressed from pRK-HISTEV-VH#6-RAS-VL#204. (D) The binding affinities of engineered scFv were determined by SPR. (E) Detection of native GTP-bound RAS by the anti-RAS scFv (VH#6-VL#204). NIH3T3 cells were serum starved and treated with 10% FCS for the times indicated. Endogenous, activated RAS protein was immunoprecipitated (IP) from the cells with His-tagged scFv VH#6-VL#204 protein and bound RAS protein recovered on Ni-NTA agarose. Recovered RAS was subjected to SDS-PAGE, immunoblotted for detection with an anti-pan RAS antibody (Ab-3, Calbiochem).

assay. Seventeen of the CatcherAb-selected anti-LMO2 VL segments were tested in the assay in which the VL prey was co-expressed with an LMO2 bait and the anti-LMO2 VH#576 segment (Figure 4B) or with the anti-RAS VH#6 as a negative binding control (Figure 4C).

Nine of the anti-LMO2 VL showed direct binding to LMO2, because the luciferase activation was not dependent on co-expression of the anti-LMO2 Catcher VH and three VL segments activated the luciferase reporter in a Catcher VH#576-dependent fashion (Figure 4B).

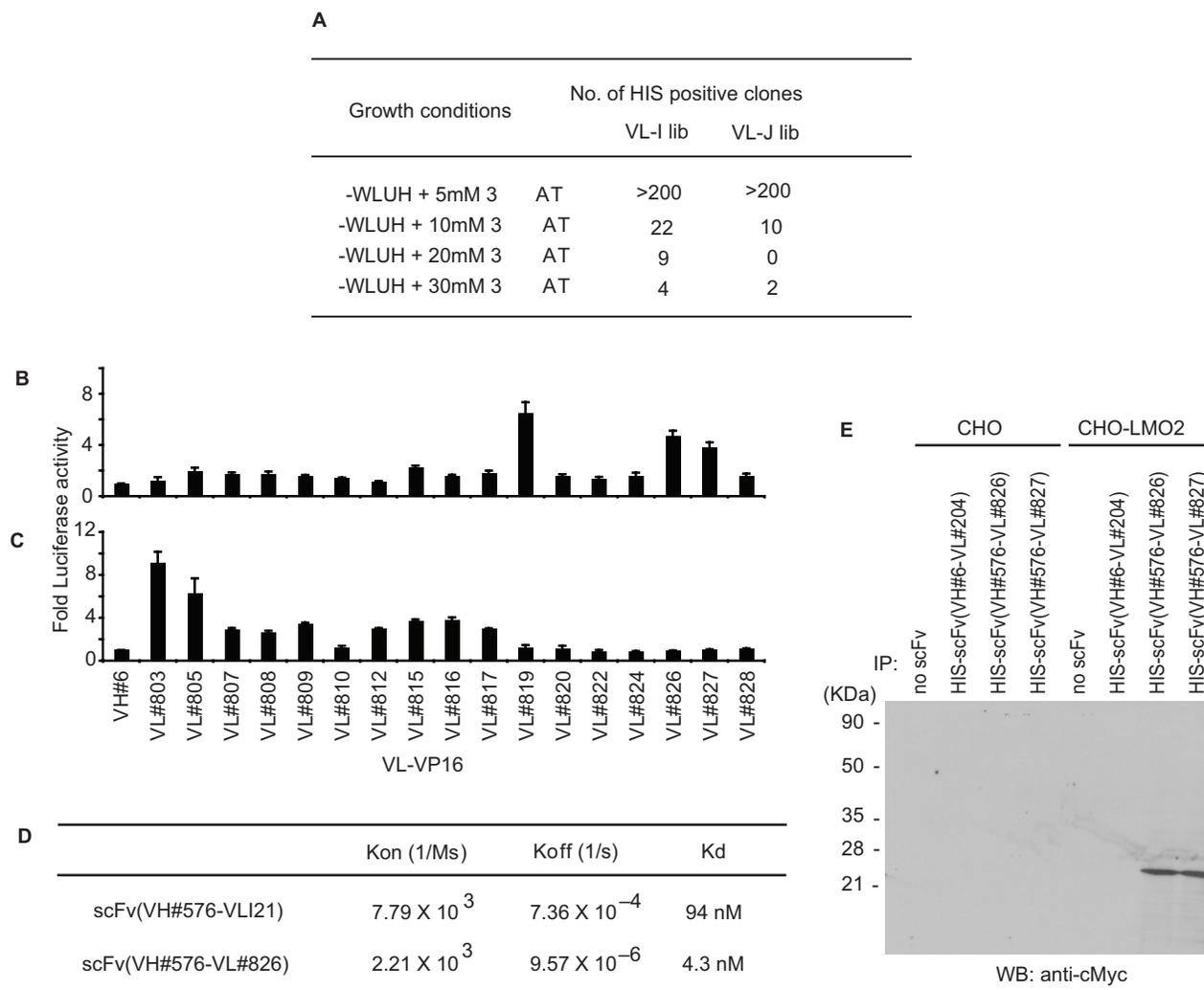


Figure 4. Anti-LMO2 VL complementary single domains. (A) VL-I and VL-J libraries were screened with an LMO2 bait and the anti-LMO2 VH576 CatcherAb and positive clones assessed on -WLUH plates, with increasing concentrations of 3-AT as indicated. (B and C) Luciferase reporter assays for anti-LMO2 VL binding. COS-7 cells were transiently transfected with plasmids expressing the individual prey VL-VP16 fusion proteins (shown on the x-axis) together with (B) the pM- δ LMO2 bait plus pEF/myc/nuc/VH#576 (anti-LMO2 catcher VH) or (C) the pM- δ LMO2 plus pEF/myc/nuc/VH#6 (anti-RAS catcher VH), together with luciferase reporters. Luciferase activities were measured after 48 h. Normalized fold firefly and renilla luciferase inductions are shown as the mean \pm SEM of four individual experiments. The luciferase activity after co-transfecting pM- δ LMO2 with pEF-VH#6-VP16 was used as negative control for normalization. (D) The affinities of engineered scFv binding with LMO2 were determined by surface plasmon resonance. (E) Immunoprecipitation of LMO2 protein using anti-LMO2 scFv (VH#576-VL#826) or scFv(VH#576-VL#827) recombinant proteins. CHO cells were transfected with plasmids expressing a myc-tagged LMO2 or were mock-transfected and lysed after 72 h. LMO2 protein was immunoprecipitated with HIS-tagged scFv and precipitated material recovered by binding to Ni-NTA agarose. Bound LMO2 protein was detected by immunoblotting with anti-myc monoclonal antibody (9E10).

VL#826 was engineered into an scFv with VH#576 and the binding affinity determined using SPR. The scFv (VH#576-VL#826) showed a K_d of 4.3 nM (Figure 4D) compared with a K_d of 94 nM for the VH#576 coupled to the non-relevant VLI21.

The ability of the anti-LMO2 scFv recombinant proteins (VH#576 with either VL#826 or with VL#827) to detect native LMO2 protein was determined in a biochemical application. Transient expression of LMO2, with a myc-epitope tag, was carried out in CHO cells. The protein from these cells was immuno-precipitated with either the VH#576-VL#826, VH#576-VL#827 anti-LMO2 scFv or the anti-RAS scFv as negative control. The recovered

LMO2 protein was identified by western detection of the myc-tag present on the LMO2 (Figure 4E).

DISCUSSION

The use of the IAC method to select single domains *in vivo* (11) is useful for the production of high affinity and functional intracellular antibody fragments, which can be used to target and validate disease target molecules [see, for example, ref. (18)]. Conversion of these antibody fragments into scFv (and also to full antibody) has many potential uses beyond intracellular antibody studies, including laboratory procedures that require binding to

native proteins such as flow cytometry. In order to facilitate this objective, we have described a simple method for selecting complementary single domains to existing, characterized iDabs.

The reagents produced with a combination of IAC and CatcherAb technologies recognize native protein antigens and can be engineered into scFv, which could in turn be made into whole antibodies if required. The interaction of single domains at the same location (epitopic region) on a target antigen can also be used to bring close together cargoes that might be required to activate other pathways within cells (20) or to serve as pro-drugs. The importance of monoclonal antibodies in modern biological research is apparent from the range of laboratory techniques that use antibodies corresponding to different species (mouse, rat, human, etc.). In addition, the importance in modern medicine is apparent with the successes of chimaeric mouse-human antibodies [e.g. anti-TNF infliximab (21) or anti-CD20 Rituximab (22)] or of humanized mouse monoclonal antibodies [e.g. anti-HER2 Trastuzumab (23)], and the potential cannot be over-stated. Simple methods that do not require animal immunizations, such as phage display, and that can be tailored to specific targets should increase the versatility and efficacy of these reagents. An additional advantage of the CatcherAb technique allows isolation of antibodies that bind to native protein antigens (thus fragments of extracellular proteins could be used as antigen in this approach). Further only requires the knowledge of the primary DNA sequence to engineer an *in vivo* expression clone to develop the antibody. In this way, proteins that cannot easily be made in recombinant, soluble form will be targetable by this approach giving a flexible platform from which to develop a range of antibodies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Dr E. Obayashi for plasmids pRS426 and pRK172 vectors and Dr I. Tomlinson for synthetic human antibody phage libraries I & J.

FUNDING

The work was partly funded by the Medical Research Council and by the Leeds Institute of Molecular Medicine (University of Leeds). Funding for open access charge: University of Leeds.

Conflict of interest statement. None declared.

REFERENCES

- Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **256**, 495–497.
- Winter, G., Griffiths, A.D., Hawkins, R.E. and Hoogenboom, H.R. (1994) Making antibodies by phage display technology. *Annu. Rev. Immunol.*, **12**, 433–455.
- Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T. and Winter, G. (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature*, **341**, 544–546.
- Holliger, P. and Hudson, P.J. (2005) Engineered antibody fragments and the rise of single domains. *Nat. Biotechnol.*, **23**, 1126–1136.
- Harlow, E. and Lane, D. (1999) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, New York, USA (CSHL Press).
- Morrison, S.L., Johnson, M.J., Herzenberg, L.A. and Oi, V.T. (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc. Natl Acad. Sci. USA*, **81**, 6851–6855.
- Neuberger, M.S., Williams, G.T., Mitchell, E.B., Jouhal, S.S., Flanagan, J.G. and Rabbitts, T.H. (1985) A hapten-specific chimaeric IgE antibody with human physiological effector function. *Nature*, **314**, 268–270.
- Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S. and Winter, G. (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature*, **321**, 522–525.
- Fleisher, T.A., Dorman, S.E., Anderson, J.A., Vail, M., Brown, M.R. and Holland, S.M. (1999) Detection of intracellular phosphorylated STAT-1 by flow cytometry. *Clin. Immunol.*, **90**, 425–430.
- Holt, L.J., Herring, C., Jespers, L.S., Woolven, B.P. and Tomlinson, I.M. (2003) Domain antibodies: proteins for therapy. *Trends Biotechnol.*, **21**, 484–490.
- Tanaka, T., Lobato, M.N. and Rabbitts, T.H. (2003) Single domain intracellular antibodies: a minimal fragment for direct *in vivo* selection of antigen-specific intrabodies. *J. Mol. Biol.*, **331**, 1109–1120.
- Tse, E., Lobato, M.N., Forster, A., Tanaka, T., Chung, G.T.Y. and Rabbitts, T.H. (2002) Intracellular antibody capture technology: application to selection of single chain Fv recognising the BCR-ABL oncogenic protein. *J. Mol. Biol.*, **317**, 85–94.
- Tanaka, T. and Rabbitts, T.H. (2003) Intrabodies based on intracellular capture frameworks that bind the RAS protein with high affinity and impair oncogenic transformation. *EMBO J.*, **22**, 1025–1035.
- Nam, C.H., Lobato, M.N., Appert, A., Drynan, L.F., Tanaka, T. and Rabbitts, T.H. (2008) An antibody inhibitor of the LMO2-protein complex blocks its normal and tumorigenic functions. *Oncogene*, **27**, 4962–4968.
- de Wildt, R.M., Mundy, C.R., Gorick, B.D. and Tomlinson, I.M. (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat. Biotechnol.*, **18**, 989–994.
- Tanaka, T., Chung, G.T.Y., Forster, A., Lobato, M.N. and Rabbitts, T.H. (2003) *De novo* production of diverse intracellular antibody libraries. *Nucleic Acids Res.*, **31**, e23.
- Visintin, M., Tse, E., Axelson, H., Rabbitts, T.H. and Cattaneo, A. (1999) Selection of antibodies for intracellular function using a two-hybrid *in vivo* system. *Proc. Natl Acad. Sci. USA*, **96**, 11723–11728.
- Tanaka, T., Williams, R.L. and Rabbitts, T.H. (2007) Tumour prevention by a single antibody domain inhibiting binding of signal transduction molecules to activated RAS. *EMBO J.*, **26**, 3250–3259.
- Warren, A.J., Colledge, W.H., Carlton, M.B.L., Evans, M.J., Smith, A.J.H. and Rabbitts, T.H. (1994) The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell*, **78**, 45–58.
- Tse, E. and Rabbitts, T.H. (2000) Intracellular antibody-caspase mediated cell killing: a novel approach for application in cancer therapy. *Proc. Natl Acad. Sci. USA*, **97**, 12266–12271.
- Nahar, I.K., Shojania, K., Marra, C.A., Alamgir, A.H. and Anis, A.H. (2003) Infliximab treatment of rheumatoid arthritis and Crohn's disease. *Ann. Pharmacother.*, **37**, 1256–1265.
- Reff, M.E., Carner, K., Chambers, K.S., Chinn, P.C., Leonard, J.E., Raab, R., Newman, R.A., Hanna, N. and Anderson, D.R. (1994) Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. *Blood*, **83**, 435–445.
- Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B., Henner, D., Wong, W.L., Rowland, A.M., Kotts, C., Carver, M.E. *et al.* (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc. Natl Acad. Sci. USA*, **89**, 4285–4289.