Design and Use of a Phage Display Library

HUMAN ANTIBODIES WITH SUBNANOMOLAR AFFINITY AGAINST A MARKER OF ANGIOGENESIS ELUTED FROM A TWO-DIMENSIONAL GEL

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We report the construction and the use of a phage display human antibody library (>3 × 10⁸ clones) based on principles of protein design. A large repertoire of functional antibodies with similar properties was produced by appending short variable complementarity-determining region 3 (CDR3) onto the two antibody germ line segments most frequently found in human antibodies. With this strategy we concentrated sequence diversity in regions of the antibody structure that are centrally located in the antigen binding site, while leaving residues in more peripheral positions available for further mutagenesis aimed at improving the affinity of the selected antibodies.

In addition, the library was tested by selecting antibodies against six biologically relevant antigens. Using only 0.3 μg of antigen eluted from a two-dimensional gel spot, we isolated binders specific for the ED-B domain of fibronectin, a marker of angiogenesis. These antibodies recognize the native antigen with affinities in the 10⁷–10⁸ M⁻¹ range, and perform well in immunosorbent assays, in two-dimensional Western blotting and in immunohistochemistry. The affinity of one anti-ED-B antibody was improved by 27-fold by combinatorially mutating six strategically selected residues in the heavy chain variable domain. A further 28-fold affinity improvement could be achieved by mutating residues 32 and 50 of the light chain. The resulting antibody, L19, bound to the ED-B domain of fibronectin with very high affinity (Kd = 54 pM), as determined by real-time interaction analysis with surface plasmon resonance detection, band shift analysis, and by competition experiments with electrochemiluminescent detection.

As the era of intensive gene discovery approaches its conclusion, major efforts are under way to develop systematic means for the discovery of gene function and regulation. Advanced methodologies in this field will be particularly useful if they also operate at the level of the genes’ functional products: the proteins. The efficient description of the protein phenotype in complex biological samples may allow unraveling of the protein composition of cells and tissues and analysis of diseases (as well as the effects of drugs and other xenobiotics) in terms of changes in protein composition (1).

Two-dimensional gel polyacrylamide electrophoresis (two-dimensional PAGE) is arguably one of the most powerful methodologies for the description of protein phenotypes (2, 3), because it can separate and quantitate thousands of individual proteins from complex biological samples. The potential of twodimensional PAGE would be extended if good quality affinity reagents (e.g. antibodies) could be produced against individual spots from gels. Such reagents could provide the structural information that is typically lost during treatment of biological samples needed for electrophoresis (total protein preparations are often obtained by lysing and homogenizing cells or tissues). The production of antibodies against gel spots would for example allow the microscopic analysis of the proteins in structurally intact samples (e.g. by immunofluorescence or immunohistochemistry) and the development of immunometric assays for the rapid quantitation of proteins in different specimens. The large number of potentially useful markers identified by two-dimensional PAGE analysis, however, greatly outstrips the capacity of conventional methods for monoclonal antibody production.

Phage antibody technology (4, 5) could in principle offer a solution, as the methodology is simple, inexpensive, and lends itself to simultaneous processing of several antigens. Relatively large amounts of pure native proteins have so far been used for the isolation of antibodies from naive phage libraries (5–10), although a few direct phage antibody selections on cells have been described (11–14). Recent advances in sample application (15) have allowed the application on two-dimensional gels of up to 1-ml samples, containing up to 10 mg of protein, without significant loss of resolution. As a consequence, microgram quantities of individual antigens are available in practice for antibody production. This poses stringent requirements on the quality of the antibody phage display library and on the selection strategy.

We have therefore aimed at producing a large functional antibody library, that could reliably yield good quality antibodies against relevant biological markers. Because for several biological applications high-affinity binders are needed, the phage library should be constructed in a way that allows the facile affinity maturation of antibodies of interest. We have also

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The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CDR3, complementarity-determining regions 3; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; CHAPS, 3-[3-cholamidopropyl]-dimethylammonium]-1-propanesulfonic acid.
aimed at developing a selection methodology that could work with minute amounts of antigen, such as proteins eluted from two-dimensional gel spots.

We describe the production of a robust antibody phage library (>300 million clones), constructed using principles of protein design. We successfully selected antibodies against six biologically relevant proteins, including the ED-B domain of fibronectin, a marker of angiogenesis (16–18), eluted from a two-dimensional gel spot. These antibodies performed well in biochemical applications such as immunosorbenent assays, two-dimensional Western blotting, and immunohistochemistry. Thanks to the modular design of the antibody library, one anti-ED-B antibody could easily be affinity-matured, yielding a binder with dissociation constant in the picomolar range (K_D = 54 pm).

**EXPERIMENTAL PROCEDURES**

**Library Construction and Cloning**—The antibody library was cloned using single VH (DP47; Ref. 19) and Vk (DP42; Ref. 20) germ line genes (see Fig. 1 for the cloning and amplification strategy). Antibody residues are numbered according to Ref. 34. The VH component of the library was created using partially degenerate primers (Fig. 1 and Table 1) in a PCR-based method to introduce random mutations at positions 95–98 in CDR3. The VL component of the library was generated in the same manner, by the introduction of random mutations at positions 91, 93, 94, and 96 of CDR3. PCR reactions were performed as described (6).

VH-VL scFv fragments were constructed by PCR assembly (Fig. 1; Ref. 21) from gel-purified VH and VL segments, double-digested with Ncol/NotI, and ligated into 15 μg of NotI/Ncol-digested pDN332 phagemid vector.

pDN332 is a derivative of phagemid pHEN1 (7), in which the sequence following the NotI site and the amber codon preceding the gene III has been replaced by the following sequence, coding for the D3SD2-FLAG-His_8 tag (22).

**SEQUENCE 1**

\[
\text{SEQUENCE 1}
\]

Transformations into TG1 Escherichia coli strain were performed according to Marks et al. (6), and phages were prepared according to standard protocols (9). Five clones were selected at random and sequenced to check for the absence of pervasive contamination. The percentage of clones that express folded antibodies was determined by immunoblot and dot-blot analysis using anti-FLAG M2 antibody (Eastman Kodak Co.) and anti-mouse horseradish peroxidase immunoglobulin (Amersham, Amersham, United Kingdom).

Phage display preparations were then subjected to one to four rounds of panning. After each round, phage were eluted with minute amounts of antigen, such as proteins eluted from two-dimensional gel spots.

Selections against human tenasin-C (25), human chorionic somatomammotropin (ScIvago Diagnostics, Siena, Italy), tetanus toxoid (kind gift of Prof. Dr. Cesare Montecucco, University of Padova, Italy), Factor VIII (ScIvago Diagnostics), and interferon γ (kind gift of Dr. Ger Ganschütz, Fraunhofer-IGB, Hannover, Germany) were performed in immunoassay (Maxisorp; Nunc, Roskilde, Denmark) coated with 25 μg/ml antigen as described (12).

Two-dimensional PAGE and Western Blotting—Two-dimensional electrophoresis was performed using a nonlinear immobilized pH gradient (range 3.5–10) in the first dimension with Immobiline strips (Pharmacia, Uppsala, Sweden). Approximately 45 mg of COLO-38 (26) and 1 μg of recombinant ED-B containing 7BS9 (16) were loaded onto the gel. COLO-35 cells were pelleted by low speed centrifugation and resuspended in 5 mM Tris base, 65 mM dithioerythritol. Recombinant 7BS9 was used instead of ED-B because it is easier to blot (16) by virtue of its larger size.

A SDS-PAGE 9–16.5% (w/v) acrylamide gradient was used in second dimension (27), followed by protein transfer onto nitrocellulose membrane and immunodetection. The membrane was blocked with 2% MPBS for 2 h at room temperature. E1 anti-ED-B culture supernatant (see “Preparation of scFv Fragments”) diluted 4:1 in 10% MPBS was then added (1.5 h incubation at room temperature). The membrane was washed twice for 5 min with 2% MPBS PBST and PBS only, then incubated with anti-FLAG monoclonal antibody (M2; Kodak) in 2% MPBS PBST and PBS only, then incubated with anti-FLAG monoclonal antibody (M2; Kodak) in 2% MPBS (0.5 μg/ml) for 1 h at room temperature, followed by horseradish peroxidase-conjugated anti-mouse IgGs (Jackson ImmunoResearch, West Grove, PA) in 2% MPBS (1:20,000 dilution). After washing as above, detection was obtained by ECL enhanced chemiluminescence (Amersham, Amersham, United Kingdom).

Biologically relevant proteins, including the ED-B domain of fibronectin, a marker of angiogenesis (16–18), eluted from a loading buffer, and eluted by fragmenting the gel piece and soaking in PBS overnight. Eluted protein was separated from the gel by centrifugation with a Millipore Ultrafree 0.45-μm microcentrifuge fil-tration device (Millipore, Yonezawa, Japan). The resulting solution was analyzed by gel electrophoresis to confirm protein purity after recovery and to quantitate ED-B concentration at standards to known concentration. Protein recovery using this methodology was approximately 50%.

**Antibody Affinity Maturation**—The gene of scFv(E1) was PCR-amplified with primers LMB1bis and DP47CDR1for (see Table I for primer sequences) to introduce random mutations at positions 51–53 in the CDR1 of the VH for numbering, see Refs. 28 and 34) and with primers DP47CDR1back and DP47CDR2for to randomly mutate positions 50, 52, and 54 in CDR2 of the VH. The remaining fragment of the scFv gene, covering the 3'-portion of the VH gene, the peptide linker, and the VL gene, was amplified with primers DP47CDR1back and JforNot (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min). The three resulting PCR products were gel-purified and assembled by PCR (21) with primers LMB1bis and JforNot (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min).

The resulting single PCR product was purified from the PCR mix, double-digested with NotI/Ncol, and ligated into NotI/Ncol-digested pDN332 vector. Approximately 9 μg of vector and 3 μg of insert were used in the ligation mix, which was purified by phenol extraction and ethanol precipitation, resuspended in 50 μl of sterile water, and electro- troporated in electrocompetent TG1 E. coli cells. The resulting affinity maturation library contained 4 × 10^6 clones.

Antibody-phage particles, produced as described (9), were used for a first round of selection on a B98-coated immunotube (16). The selected phages were used for a second round of panning performed with biotiylated ED-B, followed by capture with streptavidin-coated magnetic beads (Dynal, Oslo, Norway; see previous paragraph). After selection, approximately 25% of the clones were positive in soluble ELISA. From the candidates positive in ELISA, we further identified the one (H10; using an anti-FLAG M2 monoclonal antibody (Kodak) as detecting reagent. 32% of screened clones were positive in this assay, and the three of them that gave the strongest ELISA signal (E1, A2, and G4) were sequenced and further characterized.
Tables II and III) with lowest \( k_{\text{on}} \) by BIACore analysis (29).

The gene of scFv(H10) was PCR-amplified with primers LMB1bis and DPKC1D1for to introduce a random mutation at position 32 in CDR1 of the VL (for numbering, see Ref. 28) and with primers DPKC1D2back and DPKC1D2for to introduce a random mutation at position 50 in CDR2 of the VL. The remaining portion of the scFv gene was amplified with oligonucleotides DPKC2D2back and 7forNot (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min).

The three resulting products were assembled, digested, and cloned into pDN332 as described above for the mutagenesis of the heavy chain.

The resulting library was incubated with biotinylated ED-B in 3% bovine serum albumin for 30 min, followed by capture on a streptavidin-coated microtiter plate (Boehringer Mannheim GmbH, Mannheim, Germany) for 10 min. The phages were eluted with a 20 mM DTT solution (1,4-dithio-DL-threitol, Fluka) and used to infect exponentially growing TG1 cells.

Analysis of ED-B binding of supernatants from 96 colonies by ELISA and by BIACore allowed the identification of clone L19.

Affinity Measurements—Affinity measurements by BIACore were performed with purified antibodies as described (30). Band shift analysis was performed as described (22), using recombinant ED-B fluorescently labeled at the N-terminal extremity (16, 30) with the infrared fluorophore Cy5 (Amersham).

BIACore analysis does not always allow the accurate determination of kinetic parameters for slow dissociation reactions because of possible rebinding effects, base line instability, and long measurement times needed to ascertain that the dissociation phase follows a single exponential profile. We therefore performed measurements of the kinetic dissociation constant \( k_{\text{off}} \) by competition experiments (31). In brief, anti-ED-B antibodies (30 nM) were incubated with biotinylated ED-B (10 nM) for 10 min, in the presence of M2 anti-FLAG antibody (0.5 \( \mu \)g/ml) and polyclonal anti-mouse IgG (Sigma), which had previously been labeled with a ruthenium complex as described (32). To this solution, in parallel reactions, unbiotinylated ED-B (1 \( \mu \)l) was added at different times. Streptavidin-coated Dynabeads, diluted in Origen assay buffer (32), were then added (20 \( \mu \)l, 1 mg/ml) and the resulting mixtures analyzed with a Origen analyzer (IGEN Inc., Gaithersburg, MD). This instrument detects an electrochemiluminescent signal (ECL) that correlates with the amount of scFv fragment still bound to the biotinylated ED-B at the end of the competition reaction. Plot of the ECL signal versus competition time yields a profile that can be fitted with a single exponential with characteristic constant \( k_{\text{off}} \).

Preparation of scFv Fragments—Anti-ED-B antibody fragments were produced by inoculating a single fresh colony in 1 liter of 2YT medium as described previously in Fini et al. (33) and affinity-purified onto a streptavidin column (Pharmacia), which had been coupled with 10 mg of ED-B containing 7B89 recombinant protein (16). After loading, the column was washed with 50 ml of equilibration buffer (PBS, 1 mM EDTA, 0.5 M NaCl), and the resulting fragments were then eluted with triethylamine 100 mM, immediately neutralized with 1 M Hepes, pH 7, and dialyzed against PBS.

Immunohistochemistry—Immunostaining of sections of glioblastoma multiforme samples frozen in liquid nitrogen immediately after removal by surgical procedures was performed as described (16, 18). In short immunostaining was performed using M2-anti-FLAG antibody (Kodak), biotinylated anti-mouse polyclonal antibodies (Sigma), a streptavidin-biotin alkaline phosphatase complex staining kit (BioSpa, Milan, Italy), and naphthol-AS-MX-phosphate and fast red TR (Sigma). Immunostaining was performed using M2-anti-FLAG antibody and biotinylated ED-B at the end of the competition reaction. Plot of the ECL signal versus competition time yields a profile that can be fitted with a single exponential with characteristic constant \( k_{\text{off}} \).

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RESULTS

Design, Construction, and Use of the Synthetic Antibody Phage Display Library—Human antibodies are assembled from 51 different VH germ line genes and 70 different functional VL segments (40 Vk and 30 Vl; Refs. 94–36). However, one VH (DP47) and one Vk (DPK22) dominate the functional repertoire (37) and are well represented in binders isolated from synthetic phage libraries (10). Because our purpose was to generate a highly diverse library of functional antibodies with similar expression and performance, we opted to utilize only these two germ line gene segments, whereas randomizing four amino acid residues in the VL CDR3 (positions 91, 93, 94, and 96) and four residues in the VH CDR3 (positions 95–98; Fig. 1), in accordance with their role as common antigen contacts (38).

Like Hoogenboom and Winter (23) we did not randomize the structurally relevant and biologically conserved F-D-Y sequence at the end of the CDR3 VH (Fig. 1; Table I). Residues 92 and 95 of the VL CDR3 were kept constant (G and P, respectively), to allow for tight turn formation.

The antibodies were cloned in scFv configuration (39, 40) in a novel phagemid vector, pDN332, which appends at the C-terminal extremity of the recombinant antibody a described previously \( D_{\text{SD}}^{\text{d}} \)-FLAG-His\(_b\) versatile tag (22). Considering that eight amino acid positions were randomized, the potential diversity of the library is \( 20^8 = 2.56 \times 10^{12} \). In practice, because of limits in electroporation efficiency, our library consisted of \( > 3 \times 10^9 \) individual clones, tapping only a small fraction of the potential diversity. 88% of the clones expressed functional antibodies, as determined by Western blot and dot-blot analysis using anti-FLAG and protein A as detecting agents (see “Experimental Procedures”).

To test the functionality of the library, we panned it against a number of antigens. The sequences of selected antibody clones isolated from the library are reported in Table II. Expression of antibody clones in shaker flasks typically ranged between 5 and 50 mg/liter of bacterial culture.

Antibody Selection from a Two-dimensional PAGE Spot—One of the challenges of panning phage libraries using proteins from two-dimensional PAGE spots is that selections and screenings have to be performed with minute amounts of antigen (typically

FIG. 1. Designed antibody phage library. A, antibody fragments are displayed on phage as pIII fusion proteins, as schematically depicted. In the antibody binding site (antigen’s eye view), the Vl CDRs backbone is in yellow and the VH CDR backbone is in blue. Residues subject to random mutation are Vk CDR3 positions 91, 93, 94, and 96 (yellow) and VH CDR3 positions 95, 96, 97, and 98 (blue). The Cj atoms of these side chains are shown in darker colors. Also shown (in gray) are the residues of CDR1 and CDR2, which can be mutated to improve antibody affinity. Using the program RasMol (http://www.chemistry.ucsc.edu/wipke/teaching/rasmd.html), the structure of the scFv was modeled from protein data base file ligm (Brookhaven Protein Data Bank; http://www2.ebi.ac.uk/pcser/pdbdb.htm). B, PCR amplification and library cloning strategy. The DP47 and DPK22 germ line templates were modified (see “Experimental Procedures”) to generate mutations in the CDR3 regions. Genes are indicated as rectangles and CDRs as numbered boxes within the rectangle. The VH and the VL segments were then assembled and cloned in pDN332 phagemid vector. Primers used in the amplification and assembly are listed in Table I.
in the order of 1 μg of protein). Immobilization of the antigen on specialty resins or plastic for selections and ELISA assays (4, 6, 8–10) require substantially larger amounts of material.

Panning using biotinylated antigens (10, 24) appears to have several advantages. Performing selections in solution should favor the partial refolding of the antigen of interest. Our experience has shown that good quality libraries can be challenged with antigen concentrations in the 10 nM range and that streptavidin-coated beads can be used for efficient capture of antigen-bound phage particles. Three rounds of panning using a 1-ml volume and a 33-kDa antigen would therefore require:

\[3 \times 10^{-8} \times 3.3 \times 10^{3} \times 10^{-3} = 10^{-6}\text{grams (rounds of panning) \times (concentration) \times (molecular weight) \times (volume) = grams.}\]

In practice, larger amounts of starting material may be required, because of limitations in the efficiency of static elution of the antigen from the gel, biotinylation yield, and protein loss during electrophoretic separation. Biotinylated antigens can also be efficiently immobilized on streptavidin-coated microtiter plates for ELISA screening of individual colonies after selection.

To test the feasibility of the isolation of antibodies against two-dimensional PAGE spots, we chose as a model system a complete fibronectin type III repeat of 91 amino acid residues (the ED-B domain of fibronectin; Ref. 17): a biologically relevant antigen for which antibody performance could be evaluated with technically challenging applications. This protein domain is a marker of angiogenesis (16–18). Recombinant antibodies against the ED-B domain have been used to efficiently detect angiogenesis in cryostat sections of tumors and to target tumoral neovasculature in vivo upon intravenous injection (30).

Biotinylated ED-B was eluted from a two-dimensional PAGE spot and used at 10 nM concentration in three rounds of panning, with capture mediated by streptavidin-coated magnetic beads. 0.3 μg of antigen was used in total for the selection. The phage population from the third round of panning was used to infect nonsuppressor HB2151 cells as described (9), and bacterial supernatants obtained from single colonies were used to detect binding to ED-B by ELISA. 32% of tested clones were positive in this assay. The three clones giving the strongest ELISA signal (E1, A2, and G4; Table II) were sequenced and chosen for further characterization.

**Antibody Characterization and Performance**—ELISA assays were performed using biotinylated ED-B recovered from a gel spot, biotinylated ED-B that had not been denatured, ED-B linked to adjacent fibronectin domains (recombinant 7B89; Ref. 16), and a number of irrelevant antigens. Antibodies E1, A2, and G4 reacted strongly and specifically with all three ED-B containing proteins. This, together with the fact that the three recombinant antibodies could be purified from bacterial supernatants using an ED-B affinity column, strongly suggests that they recognize an epitope present in the native conformation of ED-B. No reaction was detected with fibronectin fragments that did not contain the ED-B domain (data not shown).

To test whether the antibodies isolated against a gel spot had a good affinity toward the native antigen, real-time interaction

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**TABLE I**

| Synthetic antibody library primers: |
|------------------------------------|
| VH primers                        |
| a DP47baNco                       |
| b CDR3for                         |
| c Vhpullth                        |
| d Jassm                           |
| e DPK22asm                        |
| f DPK3for                         |
| g JforNot                         |
| h pullth                          |

**Affinity maturation primers:**

| LMB1bis                           | 5′-GGG GCC CAG CCC GGC ATG GCC GAG-3′ |
| JforNot                           | 5′-TCA TTC TGC ACT TGC GGC CGG-3′    |
| DP47CDR1for                       | 5′-GAG CTC GGC GGA CCC AGC TCA TNN MN  |
| DP47CDR1back                      | 5′-ATG AGC TGG TGC CAG CAG GCT CC-3′ |
| DP47CDR2for                       | 5′-GTC TGG GTA TGA TGT GGG ACC MN  |
| DP47CDR2back                      | 5′-GCA TGG ACT TGG GGA CCC ATC CAG GAG-3′ |

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**TABLE II**

| Sequences of selected antibody clones |
|---------------------------------------|
| Relevant amino acid positions of antibody clones isolated from the designed synthetic libraries. Positions that are mutated in the primary antibody library are underlined. Residues in H10 and L19, mutated during the affinity maturation procedure, are in boldface. Single amino acid codes are used according to standard IUPAC nomenclature. The sequences of the reported clones have been deposited in the EBI data base. TN-C, human tenasin-C; HCS, human choricomic somatomedin; IFN-G, interferon γ, TeTox, tetanus toxoid. |

| Antigen | Clone | VH chain | VL chain |
|---------|-------|----------|----------|
| ED-B    | A2    | S Y A A I S G S G G L S I Y Y G N G W Y P W | G Y V G P H |
|         | G4    | S Y A A I S G G S S S F S F | Y Y G Y V P H |
|         | E1    | S Y A A I S G S F P F Y Y G T G R T P P  |
| H10     | S H O F S S I R G S S F P F Y Y G T G R T P P  |
| TN-C    | H7    | S Y A A I S G S P V V Y Y G T G R R P F  |
| HCS     | H1    | S Y A A I S G S E P E F Y Y G Y V P H  |
| Factor  | H1    | S Y A A I S G S T A R A Y Y G A L G R P  |
| VIII    | IFN-G | S Y A A I S G S R A P A Y Y G M G D S P T  |
|         | TeTox | S Y A A I S G S S L P L Y Y G W E K P L  |

| a Numbering is according to Ref. 34. |
High-affinity Antibodies from a Designed Human Antibody Library

Affinities of anti-ED-B scFv fragments

For the high-affinity binders H10 and L19, $k_{on}$ values from BIACore experiments are not sufficiently reliable due to effects of the negatively charged carboxylated solid dextran matrix; $K_D$ values are therefore calculated from $k_{off}$ measurements obtained by competition experiments (see “Experimental Procedures”). $k_{on}$, kinetic dissociation constant; $k_{off}$, dissociation constant. Values are accurate to $\pm 50\%$, on the basis of the precision of concentration determinations.

| Clone | $k_{on}$ | $k_{off}$ | $K_D$ |
|-------|----------|-----------|-------|
| A2    | $1.5 \times 10^{3}$ | $2.8 \times 10^{-3}$ | $1.9 \times 10^{-8}$ |
| G4    | $4.0 \times 10^{4}$ | $3.5 \times 10^{-3}$ | $8.7 \times 10^{-8}$ |
| E1    | $1.6 \times 10^{3}$ | $6.5 \times 10^{-3}$ | $4.1 \times 10^{-8}$ |
| H10   | $6.7 \times 10^{3}$ | $5.6 \times 10^{-3}$ | $9.9 \times 10^{-8}$ |
| L19   | $1.1 \times 10^{3}$ | $9.6 \times 10^{-5}$ | $6.0 \times 10^{-11}$ |

$^a$ Measured by competition with electrochemiluminescent detection.

$^b$ Measured on the BIACore.

$^c$ $K_D = k_{off}/k_{on}$.

Affinity Maturation of an Anti-ED-B Antibody—ScFv(E1)

was selected to test the possibility of improving its affinity with a limited number of mutations of CDR residues located at the periphery of the antigen binding site (Fig. 1A). We combinatorially mutated residues 31–33, 50, 52, and 54 of the antibody VH and displayed the corresponding repertoire on filamentous phage. These residues are found to frequently contact the antigen in the known three-dimensional structures of antibody-antigen complexes. The resulting repertoire of $4 \times 10^5$ clones was selected for binding to the ED-B domain of fibronectin. After two rounds of panning, and screening of 96 individual clones, an antibody with 27-fold improved affinity was isolated (H10; Tables II and III). Similarly to what others have observed with affinity-matured antibodies, the improved affinity was because of slower dissociation from the antigen, rather than by improved $k_{on}$ values (41–43). The antibody light chain is often thought to contribute less to the antigen binding affinity as supported by the fact that both natural and artificial antibodies devoid of light chain can still bind to the antigen (44, 45). For this reason we chose to randomize only two residues (32 and 50) of the VL domain, which are centrally located in the antigen binding site (Fig. 1A) and often found in three-dimensional structures to contact the antigen. The resulting library, containing 400 clones, was displayed on phage and selected for antigen binding. From analysis of the dissociation profiles using real-time interaction analysis with a BIACore instrument (29) and $k_{off}$ measurements by competition experiments with electrochemiluminescent detection (Fig. 4; see “Experimental Procedures”), a clone (L19) was identified that bound to the ED-B domain of fibronectin with a $K_D = 54$ pM (Tables II and III).

DISCUSSION

By incorporating principles of protein design, we have constructed a large repertoire of functional antibodies, which can further be improved using a general and rapid affinity maturation strategy.

The use of antibody phage libraries has shown that the most frequently used antibody germ line segments in human repertoires are often selected also from synthetic repertoires (10).
Furthermore, synthetic antibody repertoires constructed with a single germ line segment have reliably yielded good binders against a large variety of antigens (9, 16, 33, 46). For these reasons, we have used only the germ line segments DP47 and DPK22 (19, 20, 34–36) for the synthetic antibody library construction.

The choice to mutate only CDR3 loops in our repertoire was dictated by the following considerations. CDR3s of VH and VL are centrally located in the antigen binding site (Fig. 1), a geometric property paralleled by their role in determining the antigen-binding affinity and by the high diversity of CDR3 sequences observed in natural antibody repertoires. In all the three-dimensional structures of antibody-antigen complexes known so far, at least one residue of the CDR3 loops, but not necessarily of CDR1 and CDR2, is in contact with the antigen. In nature the length of CDR3 can vary from few residues to more than 20 residues (10). However, because very high affinity antibodies can be obtained with short CDR3 in the heavy chain (48), we opted for short CDR3s to limit the potential diversity of the library and reduce clone to clone variability. The short CDRs used in our library design may facilitate the modeling of antigen recognition by the antibody using a computational approach (49). Furthermore, the use of short CDR3s in VH and VL immunoglobulin domains is generally associated with better antibody stability to proteolysis and improved binding and bacterial expression (18, 21, 22).

The selection of antibodies from primary repertoires, followed by mutagenesis of CDRs and phage display selection, allows the isolation of binders with improved affinity (30, 33, 41, 42). The anti-ED-B scFv(L19), obtained by combinatorial

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2 P. M. Kirkham, D. Neri, and G. Winter, submitted for publication.

3 A. Pini, F. Viti, A. Santucci, B. Carnemolla, L. Zardi, P. Neri, and D. Neri, unpublished observations.
High-affinity Antibodies from a Designed Human Antibody Library

mutagenesis of judiciously selected CDR residues is one of the highest affinity antibodies isolated from phage display libraries. Thanks to the modular design of the antibody library consisting of a single germ line segment for both VH and VL, the maturation strategy did not rely on sequencing. The same degenerate oligonucleotide primers used for L19 should be applicable for the maturation of other antibodies isolated from the same library. Only few positions had to be randomized to achieve significant improvements in binding affinity. Previous work had shown that antibodies derived from natural repertoires required substantially more sequencing, mutagenesis, and cycles of panning of phage display libraries (41, 42) to achieve comparable levels of binding affinity. Unlike what has been described by other authors, we did not find that several cycles of stringent selections were necessary for the isolation of very high-affinity binders from antibody repertoires on phage (41, 42, 50). Our affinity maturation libraries, selected in parallel with different biopanning strategies, rapidly yielded improved binders when selected on antigen-coated solid supports, but not when selected with the stringent kinetic methodology of Low et al. (Ref. 50; data not shown).

Markers of angiogenesis are becoming increasingly popular for immunotherapy of cancer and other diseases associated with vascular proliferation (51). They are expressed in the majority of aggressive solid tumors and should be readily accessible to specific binders injected intravenously (51, 52). Occlusion of the neovascularature may result in tumor infarction and collapse (53, 54), and antibody affinity appears to play a role in determining tumor targeting efficiency (30). Experiments and collapse (53, 54), and antibody affinity appears to play a role in determining tumor targeting efficiency (30). Experiments and collapse (53, 54), and antibody affinity appears to play a role in determining tumor targeting efficiency (30).

The introduction of two-dimensional electrophoresis with immobilized pH gradients (for review, see Refs. 55 and 56) has produced significant improvements in the resolution, reproducibility, and amounts of proteins that can be used in two-dimensional PAGE. The use of minute amounts (<1 μg) of biotinylated antigens eluted from two-dimensional gels is sufficient for the isolation of recombinant antibodies from phage display libraries. These antibodies can recognize the antigen in the native conformation and have proved to be useful reagents in a number of immunochemical techniques, including ELISA, two-dimensional immunoblotting, and immunohistochemistry. Because selections with biotinylated antigens are performed in solution, partial protein renaturation may occur after elution from the gel. This was observed in the case of the ED-B domain of fibronectin. Whereas it should be possible to biotinylate the protein sample after electrophoretic separation, prebiotinylation is likely to be a more convenient approach. Suitable treatment of the biological sample may lead to the biotinylation of only a subset of total protein content (e.g. extracellular domains of membrane proteins). Reaction of standard biotinylating reagents with lysine residues, however, converts a positively charged side chain into an uncharged amide. It should be possible, however, to design biotinylating reagents that incorporate a positive charge (e.g. a tertiary amine) with a pk<sub>a</sub> similar to the one of lysine side chains. Such reagents should increase only minimally the molecular weight of the labeled protein, while not perturbing its isoelectric point.4

Additional advantages of raising phage antibodies against gel spots include the possibility of obtaining several affinity reagents against unpurified antigens contained in the protein mixture applied to the gel.

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