Cloning Murine Antibody V-genes with Non-degenerate Primers and Conversion to a Recombinant Antibody Format

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Monoclonal antibodies are produced in cultured hybridoma cell lines, but these cells tend to be unstable; it is therefore necessary to rescue the corresponding genetic information. Here we describe an improved method for the amplification of antibody variable gene (V-gene) information from murine hybridoma cells using a panel of specific, non-degenerate primers. This primer set allows sequences to be rescued from all murine V-genes, except the lambda light chain genes, which rarely contribute to murine immune diversity. We tested the primers against a range of antibodies and recovered specific amplification products in all cases. The heavy and light chain variable regions were subsequently joined by a two-step cloning strategy or by splice overlap extension PCR.

Introduction

FOR MANY YEARS, cell lines producing monoclonal antibodies (mAbs) have been established from hybridomas, which are generated by fusing murine B-cells to myeloma cells. The creation and cultivation of hybridoma cell lines is labor intensive, and the resulting cell lines are prone to microbial contamination and genetic instability, leading to unreliable production rates.^(1,2) These issues have been addressed by engineering single-chain fragment variable (scFv) antibodies, combining the variable regions of the antibody heavy and light chains (V_H and V_L) into a single polypeptide. In this context, V_H and V_L are connected by a flexible linker that allows them to fold into their native conformation and retain the antigen-binding specificity of the parent antibody. The advantages of scFv antibodies include their ease of expression in different heterologous systems, their ability to penetrate tissues more rapidly and to be taken up by cells more easily than full-size murine antibodies, and the lower risk of immunogenicity in humans.⁽³⁾ This allows them to be used in diverse research and diagnostic applications and makes them useful candidates for antibody-mediated therapy.

Currently, scFv constructs are prepared using a small set of highly degenerate primers to rescue the genetic information from the immunoglobulin V-genes.^(4,5) One major limitation of this procedure is the incorporation of incorrect sequence information in the primer-binding regions, which can result in the degradation of PCR products when using a proof-reading DNA polymerase.⁽⁶⁾ Such mutations can also reduce antibody binding affinity or inhibit antibody binding completely.⁽⁴⁾ Alternative procedures use RACE (rapid amplification of cDNA ends), in which the PCR is used to add a linker to the 5' end of the immunoglobulin heavy and light chain cDNAs, allowing amplification of the variable regions using one linker-specific primer and one constant region-specific primer.⁽⁷⁾ The exact composition of the variable regions can then be determined by sequencing, allowing specific primers to be designed that flank the variable regions precisely. In both cases, a further amplification step using a second primer set is necessary to add restriction sites and/or a linker for subsequent cloning procedures.

To address the limitations of the methods described above, we have developed an improved procedure that allows the rescue of V-gene sequences from murine hybridoma cells without degenerate primers and without a second amplification

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step. We designed a new primer set that amplifies nearly every published V_H and $V_{L(\kappa)}$ gene, but not $V_{L(\lambda)}$ genes because these rarely contribute to murine antibody diversity.⁽⁸⁾ The germ-line sequences for primer design were extracted from the NCBI IgBlast database, which combines the results from several research groups.^(8–11) We incorporated all 349 functional V-gene sequences from the heavy chain and all 98 from the kappa light chain, as well as four joining segment sequences from the heavy chain gene (J_H) and five from the kappa light chain gene (J_κ). The efficiency of these primers was demonstrated by rescuing V-gene information from different monoclonal antibodies recognizing structural proteins from hepatitis C virus (HCV) and breast cancer-related antigens (BCRAs).

Materials and Methods

Cell lines

Mouse hybridoma cell lines Sp2/mIL-6⁽¹²⁾ and Sp2/0-Ag14 were obtained from the ATCC (accession nos. CRL-2016 and CRL-1581; Manassas, VA). Fusions with spleen cells from immunized animals were prepared in previous studies, in which the animals were immunized with the recombinant HCV antigen Core or E2 or with BCRAs. HEK293T cells for recombinant protein expression were obtained from the ATCC (accession no. CRL-3216). Breast cancer cell line MDA-MB-468 was obtained from the ATCC (accession no. HTB-132), and the negative control cell line U937 was obtained from the DSMZ (accession no. ACC5; Braunschweig, Germany).

Primers and vectors

The primer set was designed based on the murine germ line V-gene sequences obtained from IgBlast (www.ncbi.nlm.nih.gov/projects/igblast/showGermline.cgi). The 3' primers have been added to GenBank with accession numbers FM993421 to FM993429, and the 5' primers with accession numbers FM993988 to FM994083. The sequences were aligned using vector NTI 10.1.1 (Invitrogen, Darmstadt, Germany). Oligonucleotide primers were synthesized and purified by HPLC (Invitrogen/Eurofins Genomics, Ebersberg, Germany). The intermediate vectors pKF-VH (GenBank accession no. FM991887) and pKF-VL (GenBank accession no. FM991888) were based on pUC19c, incorporating a new multiple cloning sites (MCS) synthesized by Eurofins Genomics. The final vector, pKF-SC (GenBank accession no. FM991889), was based on pHEN1.^(13,14) All cloning was carried out using *Escherichia coli* strain DH5 α , and prokaryotic expression was carried out in *E. coli* strains Xl1blue MRF' (Stratagene, Amsterdam, Netherlands) or Rosetta 2 (DE3) (Novagen, Darmstadt, Germany). Antibody constructs expressed in HEK293T cells were cloned in vectors based on pMS.⁽¹⁵⁾

RNA isolation and cDNA synthesis

RNA was isolated from hybridoma cells using the M&N NucleoSpin RNA II Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions, and its integrity was determined by agarose gel electrophoresis. First strand cDNA was synthesized with SuperScript[®] III reverse transcriptase (Invitrogen) using the following gene-specific

primers (Eurofins Genomics): Ig κ , 5'-AGT GGC CTC ACA GGT AT-3'; IgM, 5'-GGT ATT CAT CTG AAC CTT CAA G-3'; IgG, 5'-ACA GTC ACT GAG CTG C-3'. The cDNA was diluted 1:4.5 in water before V-gene amplification.

DNA amplification by PCR

V-gene sequences were amplified using the proof-reading Finnzymes Phusion High-Fidelity DNA Polymerase (NEB, Frankfurt, Germany) in a 50 μ L reaction volume containing 200 nM of each primer and 1 μ L of diluted cDNA. We used the Mastercycle gradient thermocycler (Eppendorf, Hamburg, Germany) with the following thermal cycling conditions: 98°C for 30 s, then 28 cycles at 98°C for 8 s, 57°C for 10 s, and 72°C for 15 s, followed by a final elongation step at 72°C for 5 min.

V-gene cloning

PCR products were separated on 1.2% (w/v) UltraPure Agarose gels (Invitrogen) and purified using the M&N NucleoSpin Extract II kit (Macherey Nagel). The intermediate vectors, digested with appropriate restriction endonucleases (NEB), were isolated from 0.8% (w/v) UltraPure Agarose gels by centrifugation.⁽¹⁶⁾ After a 1 h ligation using Quick T4 DNA Ligase (NEB), *E. coli* DH5 α cells were transformed by heat shock and cultivated to prepare plasmid DNA, which was isolated with the M&N plasmid NucleoSpin Plasmid kit (Macherey Nagel). Alternatively, the V-genes were inserted into pUC19c as blunt fragments prior to amplification by splice overlap extension (SOE)-PCR.

Sequencing was carried out on an ABI Prism[®] 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Verified V_H and V_L sequences were isolated from intermediate vectors and transferred to the scFv expression vector pKF-SC or assembled by SOE-PCR using primers previously described by Schäfer and colleagues⁽¹⁷⁾ before direct cloning in the expression vector pMS.⁽¹⁵⁾

Production of scFv in bacteria

Verified scFv coding sequences were isolated from pKF-SC by digestion with NcoI/EagI and inserted into a modified pET26b+ vector (Novagen) containing an additional sequence encoding a c-Myc tag to facilitate the detection of the recombinant protein. The antibodies were produced in *E. coli* strain Rosetta 2 (DE3) (Novagen) by cultivating the cells at 37°C until the OD₆₀₀ reached 0.6–0.8, then inducing them with 1 mM IPTG (final concentration) and incubating them at 28°C overnight. The medium was analyzed for secreted proteins by SDS-PAGE and Western blot, whereas the scFv was isolated under native conditions from the cell lysate by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany).

Production of scFv in HEK293T cells

Verified sequences for scFv antibodies recognizing BCRAs were inserted into vector pMS, which contains sequences encoding c-Myc and His₆ tags to facilitate the detection and purification of the recombinant protein. HEK293T cells were transfected using 5 μ L Rotifect (Carl Roth, Karlsruhe, Germany) and 2 μ g DNA, and protein production was analyzed 3 days later. The protein was purified under native conditions from the cell culture supernatant using Äkta HPLC system

(GE Healthcare/Amersham Pharmacia, Uppsala, Sweden) fitted with a Ni-NTA Superflow cartridge (Qiagen).

Analysis of scFv expression by SDS-PAGE and Western blot

Supernatants and cell lysates from bacteria expressing the scFv were denatured in Laemmli sample buffer and separated on a 12% polyacrylamide/SDS gel (160 V, 60 min).⁽¹⁸⁾ Proteins were blotted onto nitrocellulose membranes (350 mA, 70 min; Whatman, Schleicher & Schuell, Dassel, Germany) and blocked for 1 h at room temperature in PBS containing 2% (w/v) non-fat milk powder. The membranes were washed three times in PBS containing 0.1% Tween-20 (PBST) and incubated with an alkaline phosphatase (AP)-conjugated mouse anti-c-Myc antibody (anti-c-Myc-AP, diluted 1:5000; Sigma-Aldrich, St. Louis, MO,) for 1 h at room temperature. Following three extensive washing steps, specific binding was detected with NBT/BCIP reagent (Roth) for 10 min.

The scFv antibodies purified from the HEK293T cell culture supernatant were analyzed by SDS-PAGE and Western blot as described above. The nitrocellulose membranes were blocked for 1 h at room temperature with 1% (w/v) BSA in PBS and were visualized by incubating the membrane with a mouse anti-His₅ antibody (diluted 1:5000, Qiagen) for 1 h at room temperature, followed by an AP-conjugated goat anti-mouse IgG_{FC} (diluted 1:5000, Sigma-Aldrich), also for 1 h at room temperature, and then NBT/BCIP detection as above. Each incubation step was followed by three extensive washes with PBS-T.

Analysis of scFv binding activity by ELISA

High-binding microtiter plates (Greiner-Bio-One, Frickenhausen, Germany) were coated with 100 ng HCV antigen or 50 ng BCRA per well for 2 h in carbonate buffer (0.1 M Na₂CO₃/NaHCO₃, pH 9.6). After blocking for 30 min with PBS containing 2% (w/v) non-fat milk powder (for HCV antigens) or 1 h with PBS containing 1% (w/v) BSA (for BCRA), the expressed scFv (undiluted/diluted with PBS) was added to the plate and incubated for 2 h at 37°C for HCV and for 1 h at room temperature for BCRA. Bound scFv was detected by adding anti-c-Myc-AP (diluted 1:5000) for 1 h at room temperature. The enzyme reaction was visualized by adding para-nitrophenylphosphate (pNPP, Sigma-Aldrich) for 1 h and measuring the absorbance at 405 nm. After each incubation step, samples were washed extensively in PBS-T and all experiments were carried out in triplicate.

Analysis of anti-BCRA scFv binding by flow cytometry

The binding of purified scFv antibodies to a breast cancer cell line (MDA-MB-468) and a negative control cell line (U937) was analyzed by flow cytometry using a FACSCalibur (BD, Heidelberg, Germany) and WinMDI v2.9. We incubated 4×10^5 cells with 1 or 2 μ g of the scFv followed by 50 μ L of an anti-Myc antibody (produced in house, diluted 1:100) and 50 μ L of phycoerythrin (PE)-conjugated goat anti-mouse IgG (diluted 1:200, Dianova, Hamburg, Germany). All incubation steps were 30 min and were carried out in the dark on ice. The cells were washed between each step in a cell washer (Dade Serocent, Heraeus Sepatech, Osterode, Ger-

many) with PBS. Before analyzing the specific binding of the scFv to the cells, the cells were resuspended in 400 μ L PBS.

Results

In order to streamline the amplification of antibody V-gene sequences, and to avoid the use of degenerate and nested primers, we devised a strategy to amplify V-gene segments directly from murine hybridoma cDNA by one-step amplification with non-degenerate primers followed by a two-step scFv cloning procedure. We also devised an alternative strategy based on SOE-PCR.

The first stage of the strategy involved the collection of all functional murine germ-line V-gene sequences from the NCBI IgBlast database, allowing the first and last 20 nucleotides of each functional rearranged V-gene to be used for primer design. To take account of the sequence diversity upstream of each V-gene, the sequences were aligned and a genealogical tree was constructed for the V_H and V_L sequences. These data were used to arrange the sequences into 43 primer groups for V_H, and 53 primer groups for V_{L(K)}, differing in some cases at one or two positions but with no degenerate nucleotides. The complementary sequences were determined for reverse primers, and restriction endonuclease sites were added to the 5' end of each primer (PacI for V_H and EagI for V_L). Incorporation of the data for J-gene segments resulted in four additional primers for J_H and five for J_K. These primers covered almost all of the published sequences in NCBI IgBlast; even rare sequences were represented by a unique primer (Fig. 1).

We designed two intermediate vectors with the appropriate restriction endonuclease sites to achieve two-step scFv cloning. Synthetic polylinkers were inserted into the existing multiple cloning site of pUC19c to generate vectors pKF-VH and pKF-VL (Fig. 2A). The pKF-VH vector featured new SfiI, HpaI, and PacI sites to facilitate V_H cloning, whereas pKF-VL featured new AscI, HpaI, and EagI sites to facilitate V_L cloning. Directional cloning was achieved by linearizing pKF-VH with PacI and HpaI, and pKF-VL with EagI and HpaI, and using a semi-blunt end strategy to ligate the 5' end of each V-gene into the corresponding vector. Alternatively, V-genes used to generate scFv sequences by SOE-PCR were inserted as blunt fragments into pUC19c using the SmaI site.

For scFv assembly and expression, we designed a third vector based on the phagemid pHEN1, which we named pKF-SC (Fig. 2B). The V_H gene was excised from pKF-VH using SfiI and PacI, and inserted into pKF-SC, which had been linearized with the same enzymes. Similarly, the V_L gene was inserted using the AscI and EagI sites. This placed the V_H and V_L genes on either side of the (Gly₃Ser)₃ linker in the pKF-SC multiple cloning site. The vector also provided in-frame c-Myc and His₆ tags at the 3' end of the expression construct to facilitate affinity purification and immunodetection. The vector provided a choice of two scFv expression strategies: (1) on the surface of bacteriophage M13; or (2) as a soluble product exported to the bacterial periplasm. The cloning sites in the vector are also compatible with those of pET26b+ and pMS,⁽¹⁵⁾ which are optimized systems for prokaryotic and eukaryotic protein expression, respectively.

Following V_H and V_L gene amplification with the primer sets described above, the products were separated by agarose

Primer VH**VH 5' - Mix A**

5'PrimerVH-01 GAAGTGACGCTGGTGGAGT
 5'PrimerVH-02 GAAGTGACGCTGGTGGAGT
 5'PrimerVH-03 GAAGTGAACTGGTGGAGT
 5'PrimerVH-04 GAAGTGAACTGGTGGAGT
 5'PrimerVH-05 GAGGTGCAGCTGGTGGAGT
 5'PrimerVH-06 GAGGTGCAGCTGGTGGAGT
 5'PrimerVH-07 GAGGTGAAGCTGGTGGAGT
 5'PrimerVH-08 GAGGTGAAGCTGGTGGAGT

VH 5' - Mix B

5'PrimerVH-09 GAGGTGCAGCTGTAGAG
 5'PrimerVH-10 GAGGTGCAGCTGTAGAG

VH 5' - Mix C

5'PrimerVH-11 GAGGTGAAGCTCTCGAGT
 5'PrimerVH-12 GAAGTGAACTGTGAGAGT
 5'PrimerVH-13 GATGTGAAGCTGGAAGTGT
 5'PrimerVH-14 GAGGTGAAGCTGCATGAGA

VH 5' - Mix D

5'PrimerVH-15 GATGTGCAGCTTCAGGAGT
 5'PrimerVH-16 GATGTACAGCTTCAGGAGT
 5'PrimerVH-17 GAGGTGCAGCTTCAGGAGT
 5'PrimerVH-18 CAGATGCAGCTTCAGGAGT

VH 5' - Mix E

5'PrimerVH-19 CAGGTCCAGCTGCAGCAGT
 5'PrimerVH-20 GAGGTCCAGCTGCAGGAGT
 5'PrimerVH-21 CAGGTCCAGCTGAAGCAGT
 5'PrimerVH-22 GAGGTCCAGCTGAAGCAGT
 5'PrimerVH-23 CAGGTGCAGCTGCAGCAGT
 5'PrimerVH-24 CAGGTGCAGCTGCAGGAGT
 5'PrimerVH-25 GAGGTGCAGCTGAAGCAGT
 5'PrimerVH-26 GAGGTGCAGCTGAAGCAGT

VH 5' - Mix F

5'PrimerVH-27 CAGGTCCAGCTGCAGCAGT
 5'PrimerVH-28 GAGGTCCAGCTGCAGCAGT
 5'PrimerVH-29 CAGGTCCAGTTGCAACAGT
 5'PrimerVH-30 GAGGTCCAGTTGCAACAGT
 5'PrimerVH-31 GAGATCCAGTTGGTGCAGT
 5'PrimerVH-32 GAGATCCAGCTGGTGCAGT

VH 5' - Mix G

5'PrimerVH-33 CAGGTCTACTCTGAAAGAGT
 5'PrimerVH-34 CAGATTACTCTGAAAGAGT
 5'PrimerVH-35 CAGGCTTATCTACAGCAGT
 5'PrimerVH-36 CAGGTTACCTACAACAGT

5'PrimerVH-37 CAGGTTCAGCTGCAACAGT
 5'PrimerVH-38 GAGGTTCAGCTCCAGCAGT

VH 5' - Mix H

5'PrimerVH-39 CAGGTTCAGCTGCAGCAGT
 5'PrimerVH-40 GAGGTTCAGCTGCAGCAGT
 5'PrimerVH-41 CAGGTCCAGCTGCAGCAGT
 5'PrimerVH-42 GAGGTCCAGCTGCAGCAGT
 5'PrimerVH-43 CAGGTCCAAGCTGCAGCAGT

3' Primers JH

3'PrimerJH-01-PacI
 CGTTAAATTAAGTTTGAGGAGACGGTGACCGTGG
 3'PrimerJH-02-PacI
 CGTTAAATTAAGTTTGAGGAGACGTGTGAGAGTGG
 3'PrimerJH-03-PacI
 CGTTAAATTAAGTTTGAGGAGACAGTGACCCAGAG
 3'PrimerJH-04-PacI
 CGTTAAATTAAGTTTGAGGAGACGGTGACTGAGG

Primer VL**VL 5' - Mix 1**

5'PrimerVLkappa-01 GACATCCAGATGACCTCAGT
 5'PrimerVLkappa-02 GACATCCAGATGATTCAGTC
 5'PrimerVLkappa-03 GATGTCCAGATGATTCAGTC
 5'PrimerVLkappa-04 GATATCCAGATGACACAGAC
 5'PrimerVLkappa-05 GACATCCAGATGACACAGTC
 5'PrimerVLkappa-06 GACATCCAGATGACACAATC
 5'PrimerVLkappa-07 GACATCCAGATGACCCAGTC
 5'PrimerVLkappa-08 GACATCCAGATGAACCCAGTC
 5'PrimerVLkappa-09 GAAATCCAGATGACCCAGTC
 5'PrimerVLkappa-10 GACATCAAGATGACCCAGTC

VL 5' - Mix 2

5'PrimerVLkappa-11 GACATTCAGATGACCCAGTC
 5'PrimerVLkappa-12 GACATCCTGCTGACCCAGTC
 5'PrimerVLkappa-13 GACATCCTGATGACCCAAATC
 5'PrimerVLkappa-14 GACATCTTGCTGACTCAGTC
 5'PrimerVLkappa-15 GACATTTTGATGACCCAGTC
 5'PrimerVLkappa-16 GACATTTGTGATGACCCAGTC
 5'PrimerVLkappa-17 GACATTTGTGATGACTCAGTC
 5'PrimerVLkappa-18 GACATTTGTGATGACACAGTC
 5'PrimerVLkappa-19 GACATTTGTGATGTCACAGTC

VL 5' - Mix 3

5'PrimerVLkappa-20 AACATTATGATGACACAGTC
 5'PrimerVLkappa-21 GACATTTGTAATGACCCAAATC
 5'PrimerVLkappa-22 AACATTGTAATGACCCAAATC
 5'PrimerVLkappa-23 AACATTGTGCTGACCCAAATC
 5'PrimerVLkappa-24 GACATTTGTGCTGACCCAAATC

VL 5' - Mix 4

5'PrimerVLkappa-26 GATGTTGTGATGACCCAAAC
 5'PrimerVLkappa-27 GATGCTGTGATGACCCAAAC
 5'PrimerVLkappa-28 GATGTTTGTGATGACCCAAAC
 5'PrimerVLkappa-29 GATGTTTGTGATGACCCAAAC
 5'PrimerVLkappa-30 GATGTTTGTGATGACCCAAAC
 5'PrimerVLkappa-31 GATGTTTGTGATGACCCAAAC
 5'PrimerVLkappa-32 GATATTGTGATGACCCAGGC
 5'PrimerVLkappa-33 GATATTGTGATGACTCAGGC
 5'PrimerVLkappa-34 GATATTGTGATAACCCAGGA

VL 5' - Mix 5 (no conserved region)

5'PrimerVLkappa-25 AGTATTGTGATGACCCAGAC
 5'PrimerVLkappa-35 GAAACAACTGTGACCCAGTC
 5'PrimerVLkappa-36 ATCCACTGCAGATATTGTGA
 5'PrimerVLkappa-37 ACTGGAGAAACACACAGGC
 5'PrimerVLkappa-38 GATGTCCAGATAACCCAGTC

VL 5' - Mix 6

5'PrimerVLkappa-39 GACATTGTGCTGACACAGTC
 5'PrimerVLkappa-40 GACATTGTGCTAACCAGTC
 5'PrimerVLkappa-41 GATATTGTGCTAACCAGTC
 5'PrimerVLkappa-42 GACATTGTGCTACCCAAATC

VL 5' - Mix 7

5'PrimerVLkappa-43 GAAATTTGTGCTCAGTC
 5'PrimerVLkappa-44 GAAATTTGTGCTCAGTC
 5'PrimerVLkappa-45 GAAATTTGTGCTCAGTC
 5'PrimerVLkappa-46 GAAATTTGTGCTCAGTC
 5'PrimerVLkappa-47 GAAATTTGTGCTCAGTC
 5'PrimerVLkappa-48 GAAATTTGTGCTCAGTC

VL 5' - Mix 8

5'PrimerVLkappa-49 GAAATTTGTGCTCAGTC
 5'PrimerVLkappa-50 GAAATTTGTGCTCAGTC
 5'PrimerVLkappa-51 CAAATTTCTCAGTC
 5'PrimerVLkappa-52 CAAATTTCTCAGTC
 5'PrimerVLkappa-53 CAAATTTCTCAGTC

3' Primers Jkappa

3'PrimerJLkappa-01-EagI
 CGACGGCCGAACGTTTATTTCCAGCTTGG
 3'PrimerJLkappa-02-EagI
 CGACGGCCGAACGTTTATTTCCAGCTTGG
 3'PrimerJLkappa-03-EagI
 CGACGGCCGAACGTTTATTTCCAGCTTGG
 3'PrimerJLkappa-04-EagI
 CGACGGCCGAACGTTTATTTCCAGCTTGG
 3'PrimerJLkappa-05-EagI
 CGACGGCCGAACGTTTATTTCCAGCTTGG

FIG. 1. Nucleotide sequences of PCR primers used for V-gene amplification. The different 5' primers are arranged in eight possible groups representing the heavy and light chains, allowing the use of 96-well PCR plates (one column per 3' primer). Grouping of 3' primers is also possible to reduce the total number of reactions necessary for V-gene amplification.

gel electrophoresis and one amplification product was selected for each antibody chain (Fig. 3A, C). After purifying the corresponding bands from the gel, digesting with PacI/EagI and ligating into the intermediate vectors or pUC19c, bacterial transformation resulted in abundant colonies, which were selected for analysis by colony PCR and sequencing. Because the exact V-gene sequence was not known, it was important to exclude false positive gene amplification by controlling the sequence of several clones and confirming the immunoglobulin type using the Kabat rules.⁽¹⁹⁾ After this verification step, the V_H and V_L fragments were inserted into the final vector pKF-SC using a serial cloning procedure to obtain the full-length scFv expression construct (Fig. 3B).

The pKF-SC vector is suitable for small-scale scFv expression, but for higher yields it is necessary to use a dedi-

cated expression vector, such as pET26b+. The expression construct was transferred from pKF-SC to pET26+ using compatible restriction sites, and the recombinant vector was introduced into *E. coli* strain Rosetta2 (DE3). After overnight expression, small amounts of protein were detected in the medium (Fig. 4A), but larger amounts were detected after bacterial lysis. The purified scFv was used in a standard ELISA procedure, which showed that the scFv was able to bind its corresponding antigen (Fig. 4B).

Alternatively, the validated V-gene sequences were used to deduce new primers that integrated a linker region, facilitating the fusion of V_H and V_L by SOE-PCR (Fig. 3D). A 15-residue linker (Gly₄Ser)₃ was used for the V_H-V_L orientation, and a 20-residue (Gly₄Ser)₄ linker was used for the V_L-V_H orientation.^(17,20) By integrating the correct restriction endonuclease

FIG. 2. Vector maps of intermediate vectors pKF-VL and pKF-VH, and final vector pKF-SC. Expanded sections above the intermediate vectors (GenBank accession nos. FM991887/FM991888) show the new restriction endonuclease sites (boxes) introduced into the existing polylinker. The direction of the VH and VL inserts is shown as a small arrow within the polylinker. The expanded section above the final vector (GenBank accession no. FM991889) shows the components of the scFv expression construct (boxes) with restriction sites indicated. In all three vectors, additional open-reading frames are shown as arrows.

FIG. 3. V_H and V_L PCR products and cloned fragments. (A) PCR products from the V-genes of six representative MAbs separated by 1.2% (w/v) agarose gel electrophoresis for 35 min at 110 V. Lanes 1–8, V-gene amplification products from four IgMs directed against HCV E2; lanes 10–13, V-gene amplification products from two IgG1s directed against HCV Core; lane 9, 100-bp ladder (NEB). (B) Cloned heavy and light chains and corresponding assembled full-length scFv3625 directed against HCV E2 separated by 1.2% (w/v) agarose gel electrophoresis for 60 min at 80 V. Lane 1, VL product; lane 2, V_H product; lane 3, assembled scFv; lane 4, 2log ladder (NEB). (C) PCR products from the V-genes of five representative IgG1k antibodies directed against BCRA, separated by 2.0% (w/v) agarose gel electrophoresis for 50 min at 90 V. Lanes 1–10, V-gene amplification products; lane 11, 2log ladder (NEB). (D) PCR products from the V_H and V_L genes of one IgG1k antibody generated with SOE-PCR primers as well as the assembled PCR product (V_H - V_L orientation) separated by 2.0% (w/v) agarose gel electrophoresis for 60 min at 100 V. Lanes 1–2, V_H and V_L amplification products; lane 3, 2log ladder (NEB); lane 4, PCR product after SOE-PCR.

FIG. 4. Functional expression of an anti-HCV Core scFv in bacteria. (A) Coomassie stained SDS-polyacrylamide gel (12%) and corresponding Western blot showing the presence of the scFv in the bacterial supernatant after overnight expression. PSM, pre-stained protein marker (NEB). (B) ELISA to confirm that the expressed scFv binds to its corresponding HCV antigen. After blocking the coated HCV antigen with 2% (w/v) non-fat milk powder in PBS, scFv was added and detected with anti-c-Myc-AP antibody and pNPP. To demonstrate specific binding, each scFv was serially diluted to a maximum concentration of 200 ng per well.

FIG. 5. Functional expression of anti-BCRA scFv in HEK293T cells. (A) Coomassie stained SDS-polyacrylamide gel (12%) and corresponding Western blot showing the presence of the scFv (VH-VL orientation) in the elution fraction after Ni-NTA affinity chromatography. PSM, pre-stained protein marker (NEB). (B) ELISA to validate the specific binding of each scFv to its corresponding BCRA. After blocking the coated BCRA with 1% (w/v) BSA in PBS, each scFv was added and detected with anti-c-Myc-AP and pNPP. To demonstrate specific binding, each scFv was serially diluted to a maximum concentration of 200 ng per well.

recognition sites, it was possible to achieve direct cloning into the pMS or pET vector systems.

The BCRA-specific scFv antibodies were expressed in HEK293T cells and purified by Ni-NTA affinity chromatography. Protein enrichment was confirmed by SDS-PAGE and Western blot (Fig. 5A). The specific binding of each scFv to its antigen was validated by ELISA, and concentration-dependent binding was observed for both scFv variants (Fig. 5B). Furthermore, flow cytometry confirmed specific binding of the scFv to the breast cancer cell line MDA-MB-468, whereas there was no binding to the negative control cell line U937 (Fig. 6).

Discussion

In an attempt to simplify and streamline the hybridoma rescue procedure, we investigated the potential of families of gene-specific primers with near complete coverage of murine V-gene diversity and combined this with a simplified two-step cloning procedure to rescue scFv antibody sequences from hybridoma cDNA in three straightforward stages.

In the first stage, RNA was reverse-transcribed into cDNA using gene-specific primers binding specifically to the constant region (C1) of the heavy and light chain genes. Depending on

FIG. 6. Flow cytometry analysis of an anti-BCRA scFv to confirm binding to the surface of breast cancer cell line MDA-MB-468. Cells were incubated with 1 µg of a breast cancer-related positive control or with anti-BCRA scFv. GaM PE, cell line + GaM PE; pos. control, cell line + positive control + anti-myc + GaM PE; scFv V_H-V_L 1 µg, cell line + 1 µg anti-BCRA scFv + anti-myc + GaM PE; scFv V_H-V_L 2 µg, cell line + 2 µg anti-BCRA scFv + anti-myc + GaM PE.

the expected antibody isotype, different gene-specific primers were needed to convert the V-gene RNA into cDNA. This circumvented the need for oligo(dT) primers, which are non-specific and increase the likelihood of amplifying irrelevant products such as aberrant Igk pseudogenes or dynactin 2 (data not shown).

Small sets of highly degenerate primers produce much greater diversity than required.^(3–6,21–23) In contrast, our method ensures that almost all naturally occurring V_H and V_L sequences are covered by a total of 45–53 individually synthesized specific primers. For subsequent experiments, the primers can be pooled in equimolar amounts according to the experimental requirements (Fig. 1). The principle of our method was confirmed by successful V-gene amplification using cDNA from approximately 50 different IgMκ and IgG1κ MAb-producing hybridomas (Fig. 3). Cloning was simplified by the use of a directional strategy in which each PCR product was inserted into the intermediate vectors with a blunt 5'-end and a cohesive 3'-end. Because errors are likely to occur during PCR amplification, selected clones may contain deficient V_H or V_L sequences. Therefore the consensus sequence must be deduced from several verified sequences, which should also be checked against the Kabat rules.⁽¹⁹⁾ This mandatory step is conducted as early as possible in the procedure, because all clones in the intermediate vectors must be verified by sequencing and can be aligned to check for mismatches. The general sequencing of PCR products only reveals the position of different bases, whereas sequencing several clones indicates the probability of each base being represented at a certain position.

In contrast to other strategies,^(3,7,21–27) the use of intermediate vectors requires only one set of primers, which has several advantages: (1) there is a low error rate because only one round of PCR is required and a proof-reading polymerase can be used; (2) the method is inexpensive because it allows large numbers of V-genes to be rescued; (3) a universal primer set is sufficient for all amplifications; (4) it is not necessary to combine V_H and V_L directly because long-term storage is possible in the intermediate vectors; and (5) there are fewer manipulation steps and therefore less chance of error.

To circumvent the need for specific SOE-PCR primers for each scFv (especially when working with many different antibody V-genes), the final vector providing the scFv linker region was designed to incorporate V_H and V_L sequences using a cut-and-paste strategy (Fig. 4B). This also ensures in-frame fusion with the c-Myc and His₆ tags. The V_H-V_L orientation in the final vector allowed the introduction of a (Gly₃Ser)₃ linker, which is derived from the standard Gly₄Ser linker but allows the inclusion of restriction sites AscI and PacI and integrates additional amino acids, increasing the length from 15 to 20 residues between V_H and V_L. The length and amino acid composition of the linker affects scFv stability, solubility, and resistance to proteolytic cleavage.^(23,27,28)

We optimized our method so that rescued V_H and V_L sequences could be expressed robustly in the prokaryotic pET26b+ vector system in combination with *E. coli* strain Rosetta2 (DE3). The vector was modified by integrating a 3' c-Myc tag sequence, generating a C-terminal tag to facilitate immunological detection. The ability of the purified recombinant scFv to bind its cognate antigen is shown in Figure 5. The pKF-SC vector can also be used to express the scFv as a fusion with protein III on the surface of bacteriophage M13 in

combination with a suppressor strain such as *E. coli* XL1blue MRF' (Stratagene).

Alternatively, the primer set can be combined with a SOE-PCR strategy to create scFv sequences from isolated V_H and V_L genes, as shown for a BCRA-specific antibody. After cloning in the intermediate vector pUC19c, the sequences can be used to design new primers, which combine the V-genes with a linker. The SOE-PCR fusion strategy has two benefits for the generation of scFv antibodies against BCRA: (1) the scFv can be cloned directly into a eukaryotic or prokaryotic expression vector; and (2) the variable regions can be combined in either orientation. The V_H-V_L orientation usually generates scFv antibodies with better performance⁽²⁹⁾; this was borne out by our experiments (Figs. 5B, 6).

Conclusion

We have described an improved method for the preparation and expression of tagged scFv antibodies that allows the rapid and efficient amplification of V_H and V_L genes from a broad spectrum of antibodies (IgM and IgG1). Each screening for the correct primer combinations generated a small number of products, confirming the specificity of the primer set. This method should considerably simplify the process of rescuing immunoglobulin genes from hybridoma cell lines and will facilitate the transfer of this information to stable expression platforms for the production of large quantities of functional recombinant antibodies.

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Author Disclosure Statement

The authors declare no conflicts of interest in this work.

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