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A New Approach for Rapidly Reshaping Single-Chain Antibody *In Vitro* by Combining DNA Shuffling with Ribosome Display

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Antibody reshaping is an effective way to reduce the immunogenicity while maintaining or improving the affinity of murine antibodies. This paper describe a new *in vitro* approach for rapidly reshaping murine antibodies by combining DNA shuffling with ribosome display. With the new method, a reshaping anti–4-1BB single-chain antibody (scFv), Re-4B4-1 scFv, which bound to its antigen (4-1BB) specifically and strongly, was selected from a reshaping library. These results proved definitely the feasibility of the new designed approach for antibody reshaping.

Key words: antibody reshaping, DNA shuffling, ribosome display, single-chain antibody.

Abbreviations: AP, alkaline phosphatase; BSA, bovine serum albumin; bp, base pair(s); CDR, complementarity-determining region; cDNA, complementary DNA; ddH_2O, double distilled water; DNase, deoxyribonuclease; DNA, deoxyribonucleic acid; dNTP, deoxyribonucleoside triphosphate; DTT, dithiothreitol; *E. coli*, *Escherichia coli*; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FR, framework region; HAMA, human anti-murine antibody; HCDR1, the first CDR of heavy chain; HCDR2, the second CDR of heavy chain; HCDR3, the third CDR of heavy chain; HFR1, the first FR of heavy chain; HFR2, the second FR of heavy chain; HFR3, the third FR of heavy chain; HRP, horseradish peroxidase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid; IMGT, ImMunoGeneTics IgG, immunoglobulin G; LCDR1, the first CDR of light chain; LCDR2, second CDR of light chain; LCDR3 the third CDR of light chain; PCR, polymerase chain reaction; mAb, monoclonal antibody; mRNA, messenger ribonucleic acid; NTP, nucleotide triphosphate; RNase, ribonuclease; RT-PCR, reverse transcriptase-polymerase chain reaction; RNA, ribonucleic acid; RNasin, ribonuclease inhibitor; scFv, single-chain variable fragment or single chain antibody; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; tRNA, transfer RNA; VH, variable region of heavy chain; VL, variable region of light chain.

Since the foundation of hybridoma techniques in 1975 (*1*), thousands of rodent monoclonal antibodies (mAbs), especially murine mAbs, have been made, and many of them have been used in curing infectious diseases, autoimmune diseases or cancers. However, the curative effect of mAbs is limited by human anti-murine antibody (HAMA) responses (*2–4*) elicited *in vivo*. To make full use of these antibodies, it is necessary to reduce allogenous immunegenicity while maintaining or improving their affinity. Researchers have been trying to achieve this for many years with the strategies of antibody humanization. Generally there are three main types of humanized antibodies: chimeric antibodies (*5*), CDR (complementarity-determining region) grafting antibodies (*6*), and reshaped antibodies (*7*, *8*). Among them, reshaped antibodies were developed most recently and are considered near optimal. Briefly, the whole procedure of antibody reshaping is made up of two parts: CDR grafting and optimization of key FR (framework region) residues. In practice, the templates for CDR grafting are selected by homologous searching from databases of antibody sequences, such as IMGT (*9*), Kabat database (*10*), and V-base (*11*). Key FR residues or candidate key FR residues are identified according to the structure of parent antibodies or by sequence analysis. With the increase of candidate FR key residues, the method of site-directed mutation is apparently not satisfactory for optimization. Thus an *in vivo* screening method was designed to optimize more candidate FR key residues simultaneously (*12–17*), which is achieved by constructing a reshaped antibody library in advance by introducing random mutations at the sites of candidate key FR residues on the basis of CDR grafting and screening with phage display. Although several murine antibodies have been reshaped successfully by this method, unavoidable limitations still exist. A reshaping library has to be transfected into *E. coli*, which restricts both the capacity and diversity of the library and in turn affects successful selection of reshaped antibodies from it. Also, if we need to re-introduce mutations after each cycle of screening, switching between in *vitro* mutation and *in vivo* selection will be very laborious and time-consuming.

During the past 5 yr, *in vitro* selecting techniques, such as ribosome display (*18*, *19*) and mRNA display (*20–22*), have been developed that can be operated entirely *in vitro* by circumventing the step of transfection, and can be easily combined with *in vitro* mutation methods, such as error-prone PCR (*23*) or DNA shuffling (*24*). Subsequently, the advantages of these methods in reconstructing antibodies (*18*, *19*, *25–33*), enzymes (*34–36*) and even peptides (*37–39*) have been validated. Thus, the above limitations associated with *in vivo* screening of reshaped antibodies can in principle be circumvented by replacing phage display with *in vitro* selection techniques. Accord-

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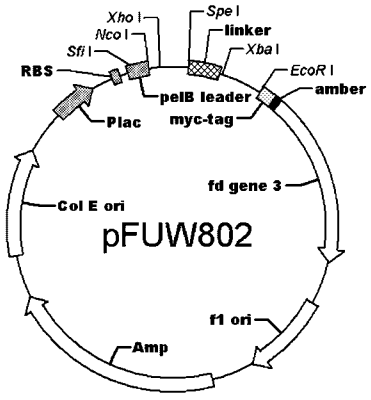

Fig. 1. **Map of pFUW802.**

ingly, a new *in vitro* screening method for reshaped antibodies that combines ribosome display and DNA shuffling was designed and a murine anti–4-1BB scFv was reshaped to test it.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Antibodies—E. coli HB2151 (Amersham Pharmacia Biotech.) was used for periplasmic expression of reshaped scFv in pFUW802, which was constructed in our laboratory (shown in Fig. 1). The plasmid contains an amber mutation between the multi-cloning site and gene 3 of fd phage, which endows it with dual functions: expression of recombinant protein in a non-inhibitory host for amber mutation, such as HB2151, and phage display of fusion protein in an inhibitory host such as TG1 or XL1-Blue.

Designing of Reshaping Anti–4-1BB scFv Library—The first step: By homologous searching in the Kabat database online (http://www.kabatdatabase.com) (*10*), we selected CDR grafting templates (human) for the VH (variable region of heavy chain) and for the VL (variable region of light chain) of murine parent antibody (4B4-1 mAb) (*40*). Then we carried out CDR grafting by replacing the FR sequence of VH or VL from murine parent antibody with that from human templates.

The second step: the human templates were sorted into the subgroups of their own in the Kabat database. The conservative FR residues within the species were picked out manually and made up of the consensus sequences of subgroups.

The third step: The FR residues that appeared in both murine sequences (murine parent sequence or consensus sequence of murine subgroup) and human ones (human templates or consensus sequence of human subgroup) were retained, while most of other FR residues were considered as candidate key FR residues to appear paratactically in the resulted reshaping VH and VL library as shown in Table 1(A) and (B). The remaining murine FR residues located in the middle of FRs or relatively far from the CDR region were retained to make the capacity

Table 1. **Reshaping library.**

(A) VH

Region	FR1	CDR1	FR2	CDR2
Re-vh	V S VK V S		R V M	
	QVQLQQPGAELVKPGASVKLSCKASGYTFT	SYWMH	WVKQRPGQGLEWIG	EINPGNGHTNYNEKFKS
M4b4-1-vh	QVQLQQPGAELVKPGASVKLSCKASGYTFS	SYWMH	WVKQRPGQVLEWIG	EINPGNGHTNYNEKFKS
hsubI-vh	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	SYAIS	WVRQAPGQGLEWMG	WINPYGNGDTNYAQKFQG
000040	QVQLVASGAEVNKPGASVKVSCKASGYTFS	SYGIS	WVRQAPGQGLEWMG	WISVYNGDTNYAQNLQG
000001	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	SYAMH	WVRQAPGQRLEWMG	WINAGNGNTKYSQKFQG
Region	FR3	CDR3	FR4	
Re-vh	RV I A T T R T			
	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCAR	SFTTARAFAY	WGQGTLVTVSS	
M4b4-1-vh	KATLTVDKSSSTAYMQLSSLTSEDSAVYYCAR	SFTTARAFAY	WGQGTLVTVSA	
hsubI-vh	RVTITADTSTSTAYMELSSLRSEDTAVYYCAR	APGYGSGGGCYRGDYFDY	WGQGTLVTVSS	
000040	RVTMTTDTSTSTAYMELRNLRSDDTAVYYCAR	APGYCSGGGCYRGDDY	WGQGTLVTVSS	
000001	RVTITRDTSASTAYMELSSLRSEDTAVYYCAR	AMILRIGHGQPQGY	WGEGTLVTVSS	

(B) VL

Region	FR1	CDR1	FR2	CDR2
Re-vl	P L S E AT		QA	
	DIVMTQSQATQSVTPGDRVSLSC	RASQTISDYLH	WYQQKSHESPRLLIK	YASQSIS
M4b4-1-vl	DIVMTQSQATQSVTPGDRVSLSC	RASQTISDYLH	WYQQKSHESPRLLIK	YASQSIS
hsubIII	EIVLTQSPGTLSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT
004983	EIVMTQSPATLSVSPGERVTLSC	RASQSVRVNLA	WYQQKPGQAPNFLIY	GSTTRAT
Region	FR3	CDR3	FR4	
Re-vl	D T FA		V	
	GIPSRFSGSGSGSDFTLSINSVEPEDVGVYYC	QDGHSFPPT	FGGGTKLEIK	
M4b4-1-vl	GIPSRFSGSGSGSDFTLSINSVEPEDVGVYYC	QDGHSFPPT	FGGGTKLEIK	
hsubIII	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGSSPPLT	FGQGTKVEIKRT	
004983	GIPARFSGSGSGTEFNFTISSLQSEDFAVYYC	QQYNNWPYT	FGQGTK	

hsubI-vh, hsubIII-vl: the consensus sequence of human subgroups; M4b4-1-vh, M4b4-1-vl: the murine sequence of anti–4-1BB VH, VL; Re-vh, Re-vl: the designed sequence of anti–4-1BB scFv reshaping library; 000040, 000001, 004983: the entry number of Kabat database of human template sequences; Paratactic residues: the candidate FR key residues.

Table 2. **Synthesized oligonucleotides for construction of reshaping library and preparation of ribosome display template.**

Oligonucleotides	Sequences (from 5′end to 3′end)
1	CCATGGACTACAAAGACCTC
2	CAGCTGACCGTGAGGTCTTTGTAGTCCAT
3	CTCGAGCAGGTTCAGCTGC(G)A(T)GCAAC(T)CGGGTGCGGAAC(G)TGG(A)T(A)GAAACCGGGTGCATCTG
4	TGAAGGTGTAACCGCTCGCTTTGCAAGACAG(C)TTTAACAGATGCAC CCG GTTTC
5	CGAGCGGTTACACCTTCAC(G)CTCTTACTGGATGCACTGGGTT
6	CCTG ACCCGGACGCTGT(A)T(C)T(G)AACCCAGTGCATCCAGTA
7	AGCGTCCGGGTCAGGG(T)TCTGGAATGGATC(G)GGTGAAATCAACCCGGG TAACGGCCAC
8	GCTTTTGAATTTTTCGTTGTAGTTGGTGTGGCCGTTACCCGGG
9	CGAAAAATTCAAAAGCA(C)A(G)A(T)GC(T)GACCC(A)TTACCGT(C)GGACAA(C)GTCTC(G)CAGACCGTACA
10	CAGGGAGCTCAGCTGCATGTA CGCGGTGCTG
11	GCAGCTGAGCCTGA(C)C(G)CAGCGAAGACAG(C)CGCTGTTTACTACTGCGC
12	CCCAGTAGGCGAACGCACGAGCGGTGGTGAAAGAACGTGCGCAGTAGTAA ACAGCG
13	GCGTTCGCCTACTGGGGTCAGGGCACCCTGGTGACCGTTTCCTCCACTAGT GGAGGC
14	TAGAGCTACCGCCACCCCCAGAACCACCACCGCCGCTCCCACCGCCTCCAC TAGTGGAG
15	GGTGGCGGAAGCTCTAGAGACATCGTTATGACCCAGAGCC
17	CCTGTCTTGCCGTGCGTCTCAGACCATCAGCGACTACCTGCACTGGTACC AACAGAAA
18	TGATCAGCAGACGCGGGGA(C)TTC(G)GTGGCTTTTCTGTTGGTACCAGTG
19	CCCGCGTCTGCTGATCAAATACGCATCTCAGTCTATCAGCGGTATCCCG
20	GGGTGAAGTCAGAACCGCTACCGGAACCAGAGAAACGGC(T)T(C)CGGGATACCGCTGATAGA
21	CGGTTCTGACTTCACCCTGAG(C)CATCAACAGCGTGGAACCGGAAGAC
22	GCGGGAAAGAGTGACCGTCCTGGCAGTAGTACACGC(G)CAAC(A)GTC TTCCGGTTCCACGC
23	CGGTCACTCTTTCCCGCCGACCTTCGGTGGTGGTACCAAA
24	ACCGAATTCTTTGATTTCCAG(C)TTTGGTACCACCACCGAA
T7-primer	ATACGAAATTAATACGACTCACTATAGGGAGACCACAACGG
T5te	CCGCACACCTTACTGGTGTGCGGATCACCAGTAGCACC

The bracketed letters represent the paratactic nucleotides of previous ones.

of the reshaping library not exceed the upper limitation for ribosome display.

The fourth step: The reshaping library was completed by linking the library sequence of VH and that of VL with a universal polypeptide linker (GGGGSGGGGSGGGGS), then back-translated into DNA format with the codes that *E. coli* uses most frequently (*41*). Thus paratactic residues at the sites of candidate key FR residues were transformed into the paratactic base pairs. Then the whole DNA format of the scFv reshaped library was split into 24 short single-chain complementary fragments (shown in Table 2), which would be reassembled by overlapping PCR.

Construction of Primary Anti–4-1BB scFv Reshaping Library by Overlapping PCR—The process of overlapping PCR is shown schematically in Fig. 2. For each PCR reaction, each fragment was added as template or primer. The PCR products were purified (if necessary) with an Agarose Gel DNA Purification Kit (Shanghai Huashun Company). Pfu polymerase and necessary reagents for PCR were purchased from Takara Biotech. (Dalian) Co. Ltd.

Step 1: Short fragments (from primer 1 to primer 24) were mixed in pairs and assembled as follows: 2 min at 94°C, then 5 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. Only two products ((*1*) and (*12*)) were purified in this step. Step 2: Non-purified products of step 1 were mixed in pairs directly (from (*2*) to (*11*)) and assembled under the same conditions as in step 1. All products were purified after this step. Step 3: The purified products of last two steps were used as templates in pairs and amplified with the outer fragments as primers: 2 min at 94°C, then 25 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C, final elongation at 72°C for 3 min. Step 4: The products of step 3 were used as templates in pairs and amplified with the outer fragments as primers under the same conditions as in step 3. Step 5: The VH and VL parts were used as templates in pairs and amplified with fragment 1 and 24 as primers under the same conditions as in step 3. At last, the primary anti–4-1BB reshaping scFv library is constructed.

Construction of Secondary scFv Reshaping Library by DNA Shuffling—DNA shuffling was performed according to Hansson and Mannervik (*42*) with some modification. DNase I was purchased from Promega. Firstly, 2 μg of primary library (DNA) dissolved in 20 μl of ddH_2O was mixed with 2.5 μl of buffer I (1 M Tris-HCl pH 7.5) and 2.5 μl of buffer II (200 mM $MnCl_2$). After equilibrating in a water bath at 15°C for 5 min, 25 μl of DNase I solution containing 1 unit of DNase I was added and four aliquots of 10 μl were removed after 30 s, 1 min, 2 min and 3 min. Then DNase I was immediately inactivated by mixing with 5 μl of buffer III (50 mM EDTA, 30% (w/v) glycerol). After electrophoresis with 2% (w/v) agarose gel, small fragments between 50 and 100 bp were collected by gel extraction and eluted in 30 μl of ddH_2O. Secondly, the small fragments were assembled without any primers. The reaction mix contained 10 μl of small fragments, 5 μl of 10× PCR buffer, 5 μl of dNTP (2 mM) and 2.5 unit of Pfu polymerase, and was supplemented with ddH_2O to a total volume of 50 μl. amplication was performed at 94°C for 4 min, then 40 cycles of 30 s at 94°C, 1 min at 55°C and 1 min 30 s at 72°C, with final elongation at 72°C for 3 min. The product was used as the template for next step

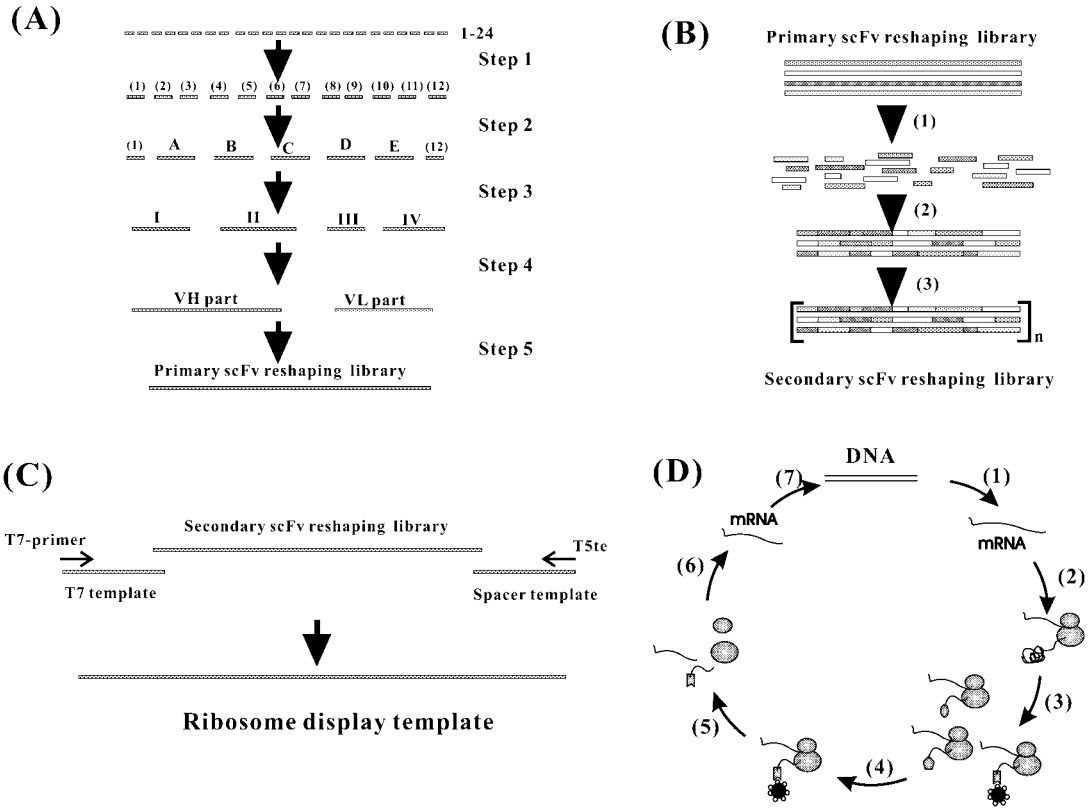

Fig. 2. **The schematic maps for the main steps in the new approach for reshaping scFv *in vitro*.** (A) Overlapping PCR for constructing the primary scFv reshaping Library. (B) DNA shuffling for constructing the secondary scFv reshaping library. (C) Assembly PCR for preparing the ribosome display template. (D) Ribosome display: (1) *in vitro* transcription, (2) *in vitro* translation, (3) affinity selection, (4) washing, (5) releasing mRNA from ribosome display complex, (6) mRNA purification, (7) RT-PCR.

directly without any purification. Finally, the full-length secondary anti–4-1BB scFv reshaping library (750 bp) was amplified with fragment 1 and fragment 24 (Table 2) as primers. PCR was performed as follows: 2 μl 10× PCR buffer, 1 unit of Pfu polymerase, 2 μl of dNTP (2 mM), 1 μl of each primer and 1 μl of template, supplemented with ddH_2O to a total volume of 20 μl. The mixture was treated at 94°C for 4 min, then 25 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, with final elongation at 72°C for 3 min.

Ribosome Display—Three cycles of ribosome display were carried out in turn to select reshaped anti–4-1BB scFv from the secondary reshaping library. Each cycle consisted of the steps of preparation of ribosome display template, *in vitro* transcription, *in vitro* translation, affinity selection and RT-PCR, which are described below.

Preparation of Ribosome Display Template by Assembly PCR—The ribosome display template containing all necessary elements was constructed by a single step of assembling PCR. The sequences of two primers (T7-primer and T5te) are listed in Table 2. Both T7 template and the spacer template (shown in Fig. 3) were synthesized in advance based on published sequences (*43*).

Assembling PCR mixture: 2 μl of 10× PCR buffer, 1 unit of Pfu polymerase, 2 μl of dNTP (2 mM), 1 μl of each primer and 1 μl of templates (T7 template, spacer tem-

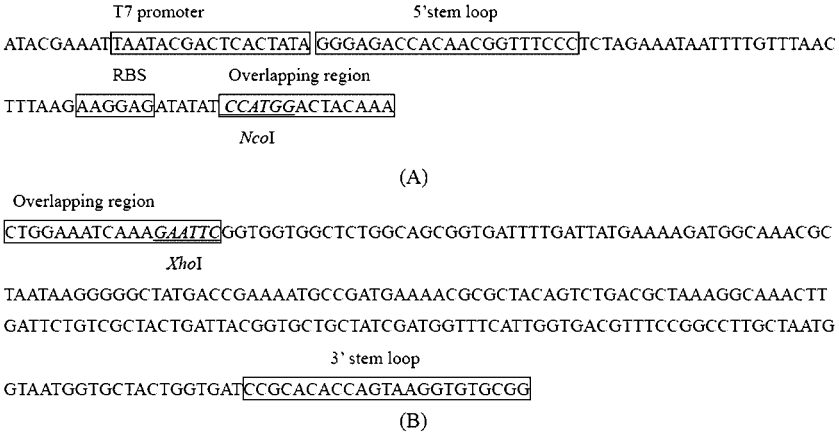

Fig. 3. **Maps of T7 template (A) and spacer template (B).**

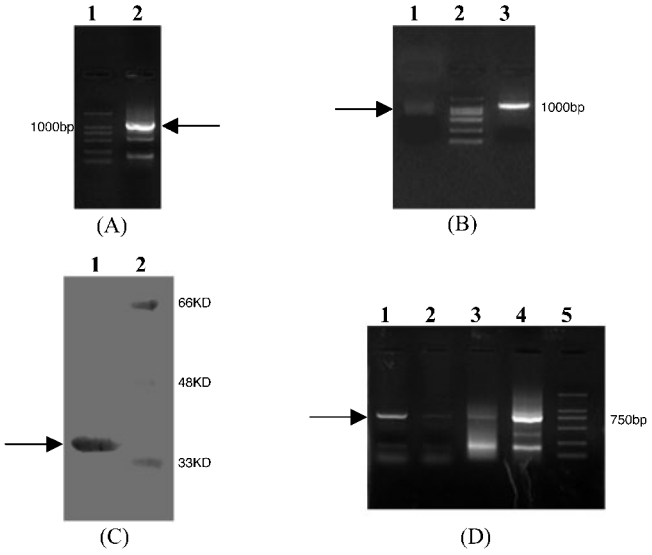

Fig. 4. **Monitoring of ribosome display.** (A) Preparation of ribosome display template by assembly PCR. Lane 1, DL2000 DNA marker. The full-length of ribosome display template is arrowed. (B) Identification of mRNA products from *in vitro* transcription. mRNA product is arrowed in lane 1; lane 2, DL2000 DNA marker; lane 3, transcription template (ribosome display template). (C) Identification of protein product of *in vitro* translation. The full-length protein products are arrowed in lane 1; lane 2, pre-stained protein marker (NEB). (D) Identification of RT-PCR products. Lane 1 is the positive control with ribosome display template as control. Lanes 2–4 are RT-PCR products for the first, the second and the third cycle respectively. Lane 5, DL2000 DNA marker.

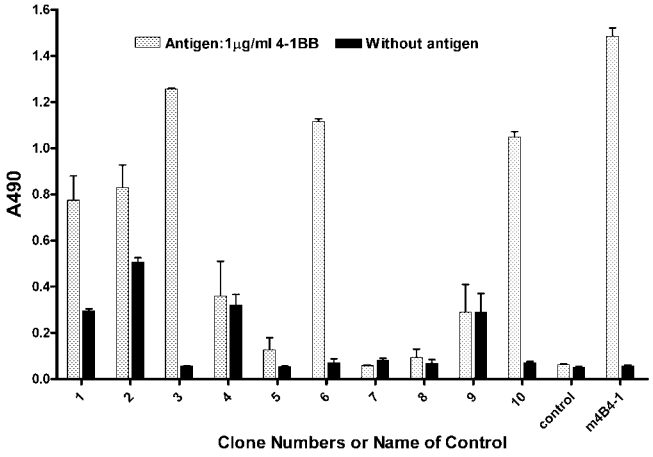

Fig. 5. **ELISA of clones selected by ribosome display.** Triplicate samples were assayed in the presence or absence of antigen. Empty vector expression was used as a negative control, while murine parent antibody (m4B4-1 scFv) expressed simultaneously was used as a positive control. A_{490} means the value of absorbance at 490 nm.

plate and secondary library). ddH_2O was added to a total volume of 20 μl. Assembly PCR conditions: 94°C for 4 min, then 25 cycles of 30 s at 94°C, 30 s at 40°C and 2 min 30 s at 72°C, with final elongation at 72°C for 3 min. PCR products of about 1,000 bp were purified by agrose gel electrophoresis and used as transcription templates for *in vitro* transcription.

In Vitro Transcription and In Vitro Translation—*In vitro* transcription was carried out according to Gurevich *et al*. (*44*) with some modification. *In vitro* transcription mixture conained 20 μg of ribosome display template (DNA), 30 μl of 5× T7 RNA polymerase buffer (1 M HEPES-KOH pH 7.6, 150 mM magnesium acetate, 10 mM spermidine, 0.2 mM DTT), 60 μl of DTT (100 mM), 120 unit of RNase inhibitor (Promega), 20 μl of NTP (50 mM, Promega), 300 unit of T7 RNA polymerase (Promega), supplemented with RNase-free ddH_2O to a total volume of 150 μl. The mixture was incubated at 37–39°C for 2 h to complete *in vitro* transcription. After adding an equal volume of ice-cold 6 M LiCl solution and keeping on ice for 20 min, mRNA product was selectively precipitated and used for further *in vitro* translation.

In vitro translation was carried out totally according to the protocols (*43*, *45*) from Plückthun's lab. *E. coli* S30 Extract System for Linear Templates was a product of Promega. To evaluate the result of *in vitro* translation, Transcend™ tRNA with biotinylated lysine (Promega) was added into the translation mixture. Thus, synthesized polypeptides were biotinylated during the process of *in vitro* translation. The products of translation were then identified by Western blotting with AP-strepavidin (Sigma) as visualizing reagent. SDS-PAGE under reductive condition and Western blotting were both carried out according to Molecular Cloning (*46*). As there were no stop codes in the ribosome display template and all operations after translation were kept on ice, the stable complex of mRNA-ribosome-protein can be used for affinity selection (*43*).

Affinity Selection—The r scFv eshaping library was screened by affinity selection in solution on 4-1BB (R&D), which was labeled with Sulfo-NHS-LC-Biotin (Pierce) according to the manufacturer's instructions. The bound complex was captured with streptavidin-coated magnetic beads (Promega). The whole procedure of affinity selection was carried out according to the protocols (*45*) from Plückthun's lab. finally, contaminated DNA templates were removed with a High Performance mRNA Purification Kit (Roche) to produce purified mRNA templates for RT-PCR.

RT-PCR—The purified mRNA was dissolved in 40 μl of RNase-free ddH_2O, immediately denatured at 70°C, and then chilled on ice. The mixture for synthesizing the first strand of cDNA contained 10 μl of 5× Stratascript first strand synthesis buffer, 10 μl of dNTP (2 mM each), 5 μl of DTT (100 mM), 50 unit of RNasin, 250 unit of Stratascript reverse transcriptase (Stratagene) and 1 μl of T5te as primer. ddH_2O was added to a total volume of 40 μl. by adding 10 μl of purified mRNA to the mixture and incubating at 42°C for 1 h, the first strand of cDNA was synthesized. Thus mRNA molecules coding binding-specific reshaped antibodies were transformed into single strand DNA molecules. Amplified with fragment 1 and 24 (Table 1) as primers, PCR products of the first two cycles of screening were then used to prepare ribosome display templates for next cycle under the same conditions as above, while that of the last cycle was cut with *NcoI* and *EcoRI* and ligated into pFUW802 (Fig. 1)

Periplasmic Expression of Enriched Clones—The ligation products were transfected into *E. coli* HB2151. The

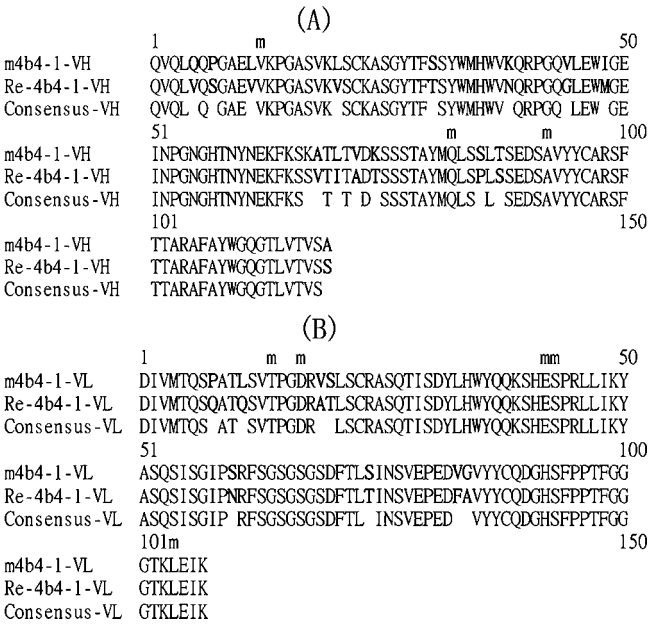

Fig. 6. **Sequence alignment of VH (shown in (A)) and VL (shown in (B)) of reshaped anti–4-1BB scFv (Re-4B4-1 scFv) with its murine parent antibody (m4B4-1 scFv).** Unpaired residues are thicked, while reserved murine residues are marked "m."

positive clones were picked out into 5 ml of SBA–1%G medium (35 g/liter tryptone, 20 g/liter yeast extract, 5 g/liter NaCl, 100 μg/ml ampicillin, and 1% (w/v) glucose, pH 7.5) and incubated at 30°C overnight with shaking. Then 50 μl of overnight culture was transferred into 5 ml of freshly prepared SBA-0.1%G medium (containing 0.1% glucose) and incubated at 30°C for about 3 h until A_{600} reached 0.9–1.0. IPTG (Takara Biotech. (Dalian) Co. Ltd.) was then added to 1 mM, and incubation was continued at 30°C for 20 h. Parent murine anti–4-1BB scFv (m4b4-1 scFv) constructed in our laboratory according to the published sequence (*40*) was expressed under the same conditions. Finally, periplasm fractions were prepared by osmotic shock (*47*) and used for ELISA directly.

ELISA of Positive Clones—With 4-1BB (R&D) as antigen (1 μg/ml), ELISA was performed routinely to identify the binding activity of the selected clones. Periplasm fractions were added in triplicate directly without any dilution. Anti–cmyc mAb (9E10 clone) (R&D) and HRP-conjugate of goat-anti-mouse IgG (R&D) were used as primary antibody and secondary antibody in tandem. The results of absorbance were detected at 490 nm. m4b4-1 scFv expressed simultaneously was analyzed as positive control.

Sequence and Structure Analysis—The positive clones identified by above ELISA were sent for sequencing in Shanghai Bioasia Biotech. Further, both parent murine antibody (m4b4-1 scFv) and the positive clone with the highest binding activity were modeled online (http://www.expasy.org) (*48*). The backbone structures returned were aligned with each other (http://cl.sdsc.edu) (*49*) to display the effect of sequence mutation on the structure. Free software, Swiss-Pdb Viewer 3.7, was downloaded from the website (http://www.expasy.org) and used to display the result of structure analysis.

RESULTS

Design of Anti–4-1BB scFv Reshaping Library—As shown in Table 1(A), (B), the primary library of VH and VL were designed separately and altogether 30 candidate key FR residues were found (17 in VH, 13 in VL). After back-translation, 30 paratactic amino acids were transformed into 38 paratactic nucleotides. Thus the novel capacity of the library would be 2^{30}–2^{38} (about 10^9–10^{12}), if all these sites were recombined randomly. In practice, nonsensical codes for non-designed amino acids or stop codes (TAA, TAG, TGA) would be produced at the sites of candidate key FR residues as the result of random recombination of paratactic nucleotides in the same codes. So designed mutants occupied only a part of the capacity. Considering random mutations introduced by adopting overlapping PCR and DNA shuffling during the courses of library construction, the capacity would be enlarged further. Such a large library exceeds the limits of all *in vivo* selection techniques. Therefore we adopted ribosome display (an *in vitro* selecting method) to screen it here, which could be used to screen a large library of up to 10^{14} recombinants (*18*, *43*).

Construction of Primary and Secondary Reshaping Libraries—The primary reshaping library was constructed by overlapping PCR. The products of every step of overlapping PCR were of specific size (data not shown) and the resulted primary library of 750 bp was purified with Agarose Gel DNA Purification Kit. In order to recombine paratactic candidate FR key residues profoundly, the secondary reshaping library was prepared by DNA shuffling (data not shown) and purified with Agarose Gel DNA Purification Kit.

Screening of Antibody Reshaping Library by Ribosome Display—A ribosome display template of about 1,000 bp (Fig. 4A) was prepared by assembling PCR and used as templates for *in vitro* transcription. The mRNA products of *in vitro* transcription were selectively precipitated by adding the same volume of 6 M LiCl, then identified with

Table 3. **Analysis of the mutaions in Re-4B4-1scFv.**

	Designs in the reshaping library		Mutations in Re-4B4-1 scFv		Causes of mutation
	Amino acid residues	Codes	Amino acid residues	Codes	
VH	K(R)38	A(C)A(G)A(T)	N38	AAT	Recombination within the paratactic code
	N(R)67	A(C)A(G)T(T)	S67	AGT	Recombination within the paratactic code
	S85	TCC	P85	CCC	Random mutation
	T(R)87	A(C)C(G)C	S87	AGC	Recombination within the paratactic code
VL	D(S)60	A(G)G(A)C	N60	AAC	Recombination within the paratactic code

The bracketed letters represent the paratactic nucleotides or residues of previous ones.

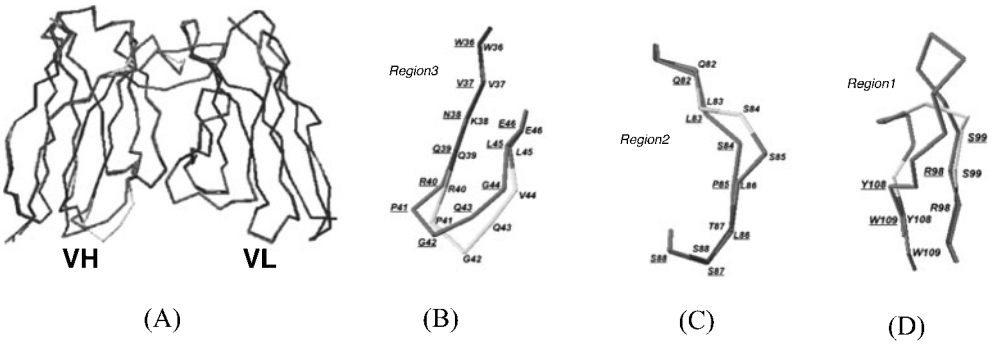

Fig. 7. **Structure alignment of Re-4B4-1 scFv with its murine parent antibody m4B4-1 scFv.** (A) Overall backbone alignment. (B), (C) and (D) show specific alignments for unpaired regions.

ordinary agrarose gel electrophoresis (Fig. 4B). Without designing any stop code in the ribosome display template, a stable antibody-mRNA-ribosome complex was produced after *in vitro* translation. Operating on ice at all times in further experiments, the complex was kept stable enough for affinity selection.

Further, the efficacy of the *in vitro* translation system was tested. By adding Transcend™ tRNA containing biotinylated lysine into the mixture for *in vitro* translation, synthesized polypeptides were biotinylated during the process of translation. Visualized with AP conjugate of avidin, a fusion protein containing intact scFv and spacer polypetides of about 36 kDa was detected by Western blotting (Fig. 4C).

Affinity selection was performed by incubating the complex of antibody-mRNA-ribosome with biotinylated 4-1BB in solution on ice, and the resultant complexes were then captured by strepavidin-coated magnetic beads. After washing the beads carefully, mRNA molecules coding specific-binding anti–4-1BB reshaped scFv were released and purified with a high performance mRNA purification kit. The purified mRNA products were reversed transcribed by RT-PCR. As shown in Fig. 4D, the output of the third cycle of RT-PCR was much higher than that of the first or second cycle, which suggests that enough enrichment of specific binding antibody-ribosome-mRNA complex is achieved after three cycles of screening. While the products of the first two cycles were used to prepare ribosome display templates for the next cycle, those of the third cycle were ligated into the plasmid pFUW802 by cutting with *Nco*I and *Eco*RI and transfected into *E. coli* HB2151 for further expression.

Identification of Reshaped Anti–4-1BB scFv by ELISA—The periplasm fractions of 10 transformed clones, as well as that of empty vector (pFUW802) expression and murine parent scFv (m4B4-1 scFv), were tested in triplicate by ELISA. The negative controls were tested in parallel in the absence of antigen (4-1BB). As shown in Fig. 5, seven positive clones were identified. Among them, three clones (N. 3, 6 and 10) bound to 4–1BB very strongly with a very low non-specific binding in the absence of antigen, while four clones (N. 1, 2, 4, and 9) bound to 4-1BB with lower specific binding and comparatively higher non-specific binding.

Sequence Analysis—Sequences of the above 10 clones were analyzed in detail, but the result were disparate that only the conclusion is depicted below. The three most strongly positive clones (NO. 3, 6 and 10) were found to derive from the same parent clone, thereinafter named Re-4B4-1. Sequence segments were found to be absent from either VH or VL in the four less strongly positive ones (N. 1, 2, 4, and 9), although no shift of open reading frame existed therein. For three negative clones, no antibody-coding segments had been inserted into the plasmids, which may be the product of self-ligation of the pre-cut plasmid (pFUW802).

As shown in Fig. 6 (A) and (B), 23 unpaired residues were found by aligning VH and VL of Re-4B4-1 with these murine parent sequences (m4B4-1 scFv) by comparison with Table 1 (A) and (B), it was found that 18 sites selected human residues (VH: V5, S7, V11, V20, T30, G44, M48, V68, I70, A72, T74; VL: P8, L11, A19, T20, T74, F83, A84) and 5 sites mutated (VH: N38, S67, P85, S87; VL: N60). Additionally, 8 sites selected murine ones (VH: V12, M76, S91; VL: T14, D17, E42, S43, L104), which are labeled "m" in the alignment (Fig. 6 (A) and (B)). As shown in Table 3, four designed mutations appearing at designed sites (VH: N38, S67, S87; VL: N60) were caused by recombination within paratactically synthesized codes, while the sole mutation at a non-designed site (P85) was caused by random mutation of a single nucleotide (from T to C).

Thus with about 60% of candidate FR key residues fixed with human ones and maintaining parent-binding specificity, Re-4B4-1 was selected from pre-designed reshaping library with our new approach. The whole procedure of antibody reshaping, including designing and constructing reshaping library and screening it with ribosome display, was completed within 1 mo, which is much more rapid than other site-directed mutation or *in vivo* screening methods for antibody reshaping.

Structure Analysis—Both Re-4B4-1 scFv and m4B4-1 scFv were modeled online separately. The backbone structures were then aligned with each other. As shown in Fig. 7 (A), there were three obviously unpaired regions between VH of the two antibodies, while VL matched each other closely. Combined with the results of sequence analysis, it was found that region 1 covered whole HCDR3 (the third CDR of heavy chain) while region 2 and region 3 were located in HFR3 (the third FR of heavy chain) and HFR2 (the second FR of heavy chain) respectively. Also no mutation was found in region 1, while two mutations (P85, S87) appeared in region 2, and another mutation (N38) appeared in region 3. As it is generally accepted that CDR loops play an important role in antigen binding (*50*, *51*), we tried to find remote effect of region 2 and region 3 on region 1 (unpaired HCDR3). As

shown in Fig. 7 (B), (C) and (D), the mutation of N38 did not affect the adjacent structure of region 3, but two other mutations, P85 and S87, resulted in a shift of one residue downstream into region 3 (unpaired HCDR3), which affected the conformation of HCDR3 loop remotely.

In summary, the backbone structure of Re-4B4-1 matched that of its murine parent antibody m-4B4-1 scFv as a whole, which favored the maintenance of its parent binding specificity. The remote effect of mutations in HFR2 on HCDR3 may affect it binding intensity to 4-1BB, which needs to be validated in further experiments.

DISCUSSION

In vivo screening for reshaping antibodies was shown to have advantages over conventional site-directed mutagenesis-based method (*12–17*). However, the quantity of candidate FR residues contained in a reshaping library is restricted by the screening limit of phage display. And switching between *in vitro* mutation (such as error-prone PCR (*23*) or DNA shuffling (*24*)) and *in vivo* selection (phage display) by transfecting the library into *E. coli* is both laborious and time-consuming, and the capacity of reshaping library is reduced at every at every switch.

Here, we designed a new and rapid *in vitro* screening approach for antibody reshaping by combining ribosome display and DNA shuffling, and validated the method by selecting reshaped anti–4-1BB scFv from a designed reshaping library. Firstly, the anti–4-1BB scFv reshaping library was designed by paratactic synthesizing of murine residues and human ones in the sites of candidate FR key residues. Secondly, primary and secondary reshaping libraries were prepared by overlapping PCR and DNA shuffling respectively. finally, after three cycles of ribosome display, a reshaped anti–4-1BB scFv, Re-4B4-1 was successfully selected which had parent binding specificity, and of which about 60% of candidate FR key residues were replaced with human ones.

The reshaping templates were selected from the Kabat database (*52*) by homology searching, which has been used widely in antibody reshaping (*10*, *16*). As there was more than one human template for original murine antibody sequence, we sorted the template sequences into subgroups according to the Kabat database and displayed the consensus sequences of both VH subgroups and VL subgroups, which are made up of the most conservative residues at each sites in the same subgroups. The consensus sequences were used as templates for CDR grafting and selection of candidate FR key residues. The procedure has been adopted previously to design a reshaping library for selecting a reshaped single-domain antibody with *in vivo* screening approach (*16*). However in selecting a reshaped single-chain antibody, ribosome display, an *in vitro* selection technique, has to be used to exceed the limit of the extremely high capacity of the library.

In practice, all experiments until expression were performed entirely *in vitro*. The primary reshaping library was constructed by paratactic synthesis and overlapping PCR, and this was used in preparing the secondary library by DNA shuffling. After that, the template of ribosome display was prepared by a single step of assembly PCR, which differents from such published methods as two-step PCR or *in vitro* ligation (*43*). Other steps of ribosome display, including *in vitro* transcription, in vitro translation and affinity selection, were carried out according to the protocol (*28*, *43*) from Plückthun's laboratory with some modification. The enriched products of the *in vitro* screening were ligated into pFUW802 and transfected into *E. coli* HB2151 for periplasm expression, soluble products of which were used in ELISA without further purification.

To test the output of *in vitro* translation, Transcend™ tRNA containing biotinylated lysine was added into the mix of translation. Thus the product of translation is labeled with biotin without any additional steps and can be tested by ordinary Western blotting visualized with AP-avidin. The method is considered almost as sensitive as radioactive assay and is safer (*53*).

To test the optimization of candidate FR key residues in the selected reshaped antibody, the sequence of positive clone, Re-4B4-1, was analyzed in detail. It was found that most of designed paratactic sites the were fixed with human residues, and eight sites were fixed with murine ones, while four mutations took place at designed paratactic sites and one mutation took place at a non-designed site (P85). Thus there was a change of 23 residues between Re-4B4-1 scFv and m4B4-1 scFv, while the original binding specificity to 4-1BB was maintained.

The antigen-binding site of an antibody is made up of six CDR loops (HDR1, HCDR2, HCDR3, LCDR1, LCDR2, LCDR3) (*50*), of which HCDR3 is most important (*51*). The FR key residues affected the binding activity indirectly (*54*). By further backbone-structure alignment, only three unpaired regions were found between two antibodies (Re-4B4-1 scFv and m4B4-1 scFv), all of which are located in VH. Combined with the results of sequence analysis, the first unpaired region (Region 1) covered whole HCDR3 without any mutations, while the other two unpaired regions were located in HFR2 (Region 3) and HFR3 (Region 2). Two mutations in Region 2 resulted in the shift of one residue downstream into the HCDR3, while there was no remote effect of the mutation in region3. Thus the structure of Re-4B4-1 scFv match that of its murine parent antibody, m4B4-1 scFv, on the whole, and the change of the HCDR3 loop caused by mutations in HFR3 might affect the intensity of binding activity. In further experiments, optimization of these mutations might help to improve the affinity of the reshaped antibody.

Although the binding affinity and potential agonistic activity of the selected antibody, Re-4B4-1 scFv, and any new antigenicity possibly introduced by non-designed mutations or structure changes need to be validated by further experiments, it can be concluded that the new approach for antibody reshaping is rational in principle and feasible in practice. In summary, there are three main advantages to this new approach. (*1*) With ribosome display as a selection technique, more candidate FR key residues can be selected to design an extremely large library for successful reshaping, which is important for reshaping murine scFv. (*2*) With almost all steps completed *in vitro*, it is much more rapid than *in vivo* screening strategies (*12–17*), say nothing of site-directed mutagenesis-based methods (*7*, *8*). (*3*) With soluble-periplasm expression method, many enriched clones can be tested

by ELISA at one time. Thus, this approach provides us a new and powerful tool to carry out antibody reshaping and make full use of existing resources of valuable rodent antibodies.

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