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ORIGINAL ARTICLE

Construction of an Artificially Randomized IgNAR Phage Display Library: Screening of Variable Regions that Bind to Hen Egg White Lysozyme

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Abstract To develop a multi-antigen-specific immunoglobulin new antigen receptor (IgNAR) variable (V) region phage display library, CDR3 in the V region of IgNAR from banded houndshark (*Triakis scyllium*) was artificially randomized, and clones specific for hen egg white lysozyme (HEL) were obtained by the biopanning method. The nucleotide sequence of CDR3 in the V region was randomly rearranged by PCR. Randomized CDR3-containing segments of the V region were ligated into T7 phage vector to construct a phage display library and resulted in a phage titer of 3.7×10^7 PFU/ml. Forty clones that contained randomized CDR3 inserts were sequenced and shown to have different nucleotide sequences. The HEL-specific clones were screened by biopanning using HEL-coated ELISA plates. After six rounds of screening, nine clones were identified as HEL-specific, eight of which showed a strong affinity to HEL in ELISA compared to a negative control (i.e., empty phage clone). The deduced amino acid sequences of CDR3 from the HEL-specific phage clones fell into four types (I–IV): type I contains a single cysteine residue and type II–IV contain two cysteine residues. These results indicated that the artificially randomized IgNAR library is useful for the rapid isolation of antigen-specific IgNAR V region without immunization of target antigen and showed that it is possible to isolate an antigen-specific IgNAR V region from this library.

Keywords Banded houndshark (*Triakis scyllium*) · New antigen receptor (IgNAR) · Complementarity determining region (CDR) 3 · Phage display library · Hen egg white lysozyme (HEL)

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Introduction

Phage display is a unique technique that enables polypeptides with desired properties to be extracted from a large collection of variants. This technique was used in antibody engineering to screen a large repertoire of variable regions, each on the surface of an individual phage, for specific antibodies against a particular antigen (Aitken 2009). The main advantages of phage display are that (1) the constructed library contains more than 10^7 phage clones, each of which displays a different antibody variable region; (2) each phage clone displays only one type of variable region; and (3) the phage genomic DNA encodes the gene of the displayed molecule. So far, several types of antibody libraries have been constructed, including naïve libraries, synthetic libraries, and immunized libraries. Cartilaginous fishes (sharks, rays, and skates) have an immunoglobulin new antigen receptor (IgNAR), which is a homodimer of two heavy chains, each comprising a single antigen-binding variable (V) region and five constant (C) region (Dooley and Flajnik 2006). The V region contains a relatively short complementarity determining region (CDR) 1 loop and a longer CDR3 loop that are highly variable and critical for antigen binding. In the IgNAR V

region, the classical CDR2 loop is replaced with hypervariable region (HV) 2 and HV4 (Stanfield et al. 2004). In addition, the IgNAR V region is highly diversified by somatic hypermutations (Greenberg et al. 1995; Diaz et al. 1998).

For these reasons, the IgNAR V regions from nurse shark (*Ginglymostoma cirratum*), wobbegong shark (*Orectolobus maculatus*), spiny dogfish (*Squalus acanthias*), and smooth dogfish (*Mustelus canis*) have been used for the construction of phage display libraries (Roux et al. 1998; Nuttall et al. 2001; Dooley et al. 2003; Liu et al. 2007). Dooley and colleagues constructed an immunized library using cDNA prepared from peripheral blood leukocytes, which were extracted from a nurse shark immunized with hen egg white lysozyme (HEL). However, this method requires a longer time (6 months) to obtain a sufficiently high titer of HEL-specific IgNAR (Dooley et al. 2003; Dooley and Flajnik 2005). Moreover, the immunized library is only useful for finding antibodies against the immunized antigen, while IgNAR V regions possessing high affinity to an antigen can be obtained through affinity maturation. HEL is commonly used as a model antigen because of its antigenicity, low cost, and commercial availability (Dooley et al. 2003; Muyldermans et al. 2001). Toxins, proteases, pathogenic fungus, and cancer markers have also been used as antigens to obtain therapeutically valuable IgNAR V regions (Nuttall et al. 2002; Saerens et al. 2005; Dolk et al. 2005). On the other hand, Liu and colleagues constructed a naïve antibody library in which the IgNAR V region was obtained from the blood of a non-immunized dogfish (Liu et al. 2007).

To avoid the long immunization time and to obtain a comprehensive library containing the diversified CDR3 region in the IgNAR V region, we constructed in this study an artificially randomized CDR3–IgNAR synthetic phage display library from banded houndshark (*Triakis scyllium*). HEL was used as the target antigen for phage clone screening. The sequences of the randomized IgNAR V region specifically bound to HEL were determined.

Materials and Methods

Randomization of Shark IgNAR V Region Fragments and Construction of Phage Library

cDNA prepared from peripheral blood leukocytes (PBLs) of banded houndshark was used as a template for amplification of the IgNAR V region as described previously (Honda et al. 2010). The nucleotide sequences of the primers used for PCR are listed in Table 1. The first PCR amplification was performed in 25 μl reaction mixtures: 0.4 μM of FR/*Eco*RI/F and FR/R1 primers, 0.5 μl of PBL cDNA template, 5 μl of 5× reaction buffer, 0.2 mM dNTP, and 1.25 U Expand HiFi enzyme (Roche, Mannheim, Germany). The PCR conditions were as follows: 94 °C for 3 min, then 35 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by 5 min at 72 °C. After gel purification of the first PCR products using QIAquick gel extraction kit (Qiagen, Hilden, Germany), the second PCR was performed on the products from the first PCR using CDR3/R2/16aa primer in order to amplify products that will include the oligonucleotide sequences to randomize and to avoid the stop codon within the CDR3. The second PCR was performed using the same conditions as the first PCR except for an annealing temperature of 55 °C. After the second gel purification, the third PCR was performed to add the restriction enzyme site *Hin*dIII using FR/R3/*Hin*dIII primer and using the same PCR conditions as the first PCR. After the third gel purification, the purified PCR products were digested with restriction enzymes *Eco*RI and *Hin*dIII (Thermo Fisher Science, Waltham, MA, USA) for cloning into T7Select 10-3b vector (Merck KGaK, Darmstadt, Germany). The ligated vectors were used for packaging and amplifying the phage particles according to the manufacturer's instructions. The supernatants of the culture medium were stored as phage solution at −80 °C until use.

To determine the phage number, 100 μl of serial diluted phage solution was added to 250 μl of inoculated host strain *Escherichia coli* BLT5403 (OD_{600}=1.0) and then mixed

Table 1 Nucleotide sequences of the primers used for PCR

Name	Nucleotide sequence (5′→3′)	Purpose
FR/*Eco*RI/F	TGCGAATTCTGCACATGTCGATCAAACA	1st PCR, 2nd PCR and 3 rd PCR
FR/R1	CAGTAAAAGGTGACACTGTC	1st PCR
CDR3/R2/16aa	CACAGTCAMGGTGCCAGYCCCATCATRGCAMNNM NNMNNMNNMNNMNNGCAMNNMNNMNNMNNM NNMNNMNNMNNMNNAGCCKTGCAGTAAAATGT GACACTGTC	2nd PCR
FR/R3/*Hin*dIII	TTTTTTTTTAAGCTTATTCACAGTCAMGGTGCCAGY CCCATCAT	3 rd PCR
T7SelectUP	GGAGCTGTCGTATTCCAGTC	Sequencing
T7SelectDOWN	AACCCCTCAAGACCCGTTTA	Sequencing

with 3 ml of top agar and spread on an LB plate containing 50 μg/ml carbenicillin. After the plate was incubated for 3 h at 37 °C, plaques were counted to calculate the phage titer. To sequence the phage clone expressing IgNAR V region, single plaques were picked up from plates and used for PCR amplification as described in manufacture's instruction. The nucleotide and deduced amino acid sequences were aligned using ClustalX software.

Screening of Anti-HEL Phage Clones

To screen for anti-HEL phage clones, the biopanning method recommended by the T7Select system was used. Briefly, purified HEL (Sigma-Aldrich, MO, USA) was diluted with carbonate buffer (Sigma), and 100 μl of HEL solution (3 mg/ml) was applied to the wells of ELISA plate (Corning, Corning, NY, USA). After incubation at 4 °C overnight, the wells were washed with 1× TBS (10 mM Tris–HCl, 150 mM NaCl, pH 8.0) and blocked by solution (5 % skim milk containing 1× TBS) for 1 h at 37 °C. After washing five times with 1× TBS, 100 μl of phage solution was added to the wells and incubated at room temperature for 90 min. After washing five times with 0.05 % Tween 20 containing 1× TBS (TBST), 200 μl of elution buffer (1 % SDS containing 1× TBS) was applied to elute the binding phage and incubated at room temperature for 30 min. The eluted phage solution was transferred to 1.5 ml tubes and used for next round of screening. The 8 μl of eluted phage solution was added to 2 ml of inoculated BLT5403 (OD_{600}=0.5) and cultured at 37 °C for 2–3 h. After centrifugation at 13,400×*g* for 3 min, the supernatants were used for next round of screening. These steps were repeated six times.

ELISA

For the titration of anti-HEL phage, 100 μl of serial diluted HEL solutions was applied on ELISA plate and incubated at 4 °C overnight. After washing and blocking, the 100 μl of phage solution (10^6 PFU/ml) was applied to the wells and incubated at room temperature for 90 min. After washing with TBST, anti-T7 phage fiber mouse monoclonal antibody diluted to 1:2,000 (Merck, Darmstadt, Germany) was applied and incubated at room temperature for 90 min. After washing, 1:4,000 diluted HRP conjugated anti-mouse IgG goat antibody (KRL, Gaithersburg, MD, USA) was applied and incubated at room temperature for 90 min. After wash, 150 μl of developing buffer containing 42 mM 3,3′,5,5′-tetramethylbenzidine and 1 % H_2O_2 was applied to evaluate the HRP activity. After 30–60 min, 50 μl of stop solution (1 M H_2SO_4) was applied to the well, and the optical density

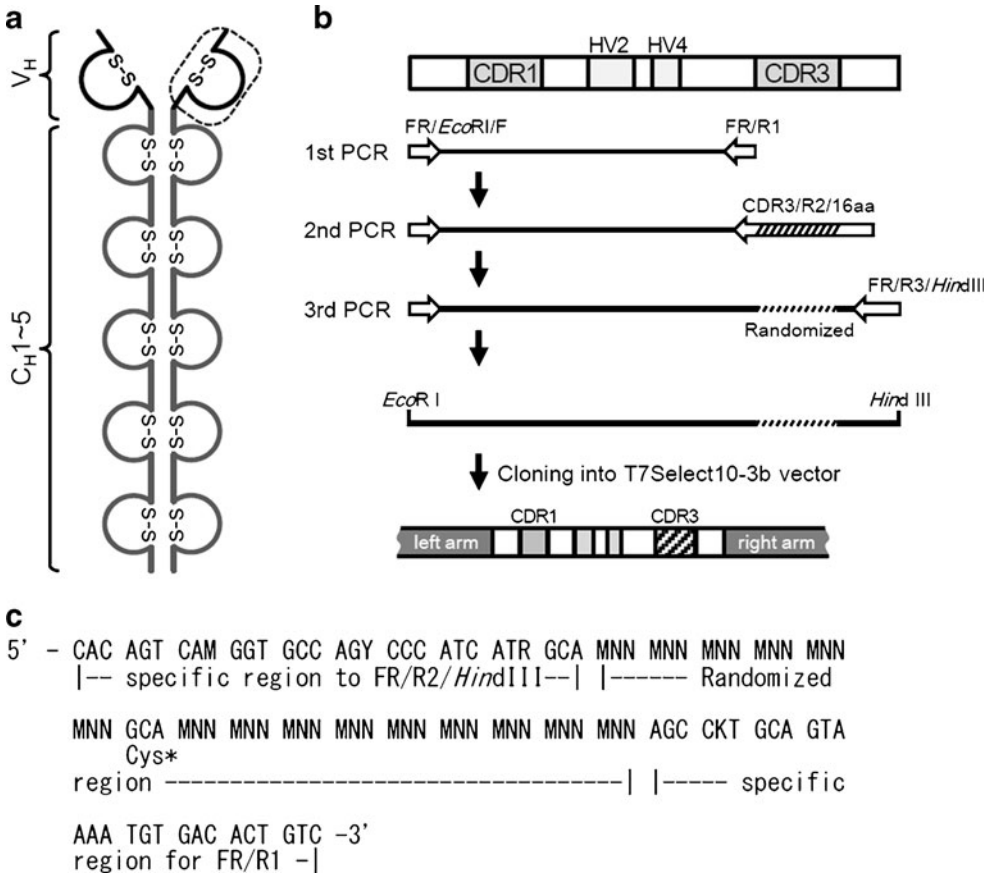

Fig. 1 Strategy of randomized IgNAR V region library construction by PCR. **a** Schematic drawing of monomeric IgNAR molecule. The disulfide bonds within V and C domains are shown by (*s-s*). Randomized V region is indicated by *broken line*. **b** Construction of the randomized IgNAR V region library. First, the FR fragment was amplified from banded houndshark shark PBL cDNA. The first PCR product was used for randomization of CDR3. The second PCR product introduced a cloning site and was digested with restriction enzymes to ligate it into the phage vector. *Vertical arrows* indicate the primer position. The sequences of primers are shown in Table 1. **c** Sequence of CDR3/R2/16aa primer used in second PCR for randomization of CDR3

(OD) was read at 450 nm using a microtiter plate reader. HEL-specific mouse monoclonal antibody #sc-73295 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted to 1:1000 was used as a positive control.

Results and Discussion

Several mutagenesis protocols have been used to evaluate the variety of the V region (Casson and Manser 1995; Shao et al. 2007; Kobayashi et al. 2008). In this study, we constructed an artificially randomized IgNAR V region to evaluate the variation of CDR3 in banded houndshark. As illustrated in Fig. 1b, randomly synthesized CDR3 oligomers were added to the tail of the first PCR products with PCR. The CDR3 (i.e., the length and conserved cysteine residues) used to design the randomized primer was obtained from previously determined banded houndshark IgNAR cDNA sequences (Honda et al. 2010), and the oligonucleotide encoding 16 residues of CDR3 region and fixed cysteine residue was designed as shown in Fig. 1c. The third PCR products were ligated into the C-terminal end of gene 10B (capsid protein) of the T7Select 10-3b vector and were expressed as a 10B–IgNAR V region fusion proteins on the phage surface. The ligation mixture was packaged with T7 phage lysate in vitro and then amplified with *E. coli* strain BLT5403 to obtain the library. To estimate how many phages encode the functional randomized IgNAR V region in this library, a cell lysate containing the phages was subjected to a plaque assay. The total phage titer was 3.7×10^7 PFU/ml, and the nucleotide sequences of 48 clones randomly picked up from the library were determined.

Forty of these clones (83 %) contained the expected insert size with completely random sequences in the CDR3 region (Fig. 2). The randomized portion of 17 of these clones (35 %) was in-frame with the rest of the CDR3, corresponding to a functional phage titer of 1.3×10^7 PFU/ml. This proportion of in-frame clones was a little lower than that of the nurse shark IgNAR library (42 %) which was determined using colony counting to be approximately 2×10^6 members (Dooley et al. 2003). The alignment (Fig. 2) showed that the TGC codons in the middle of the randomized CDR3, which encodes a cysteine residue, were conserved in all sequences, while gaps and mutations were found in the FR region. The gaps may be caused by secondary structures

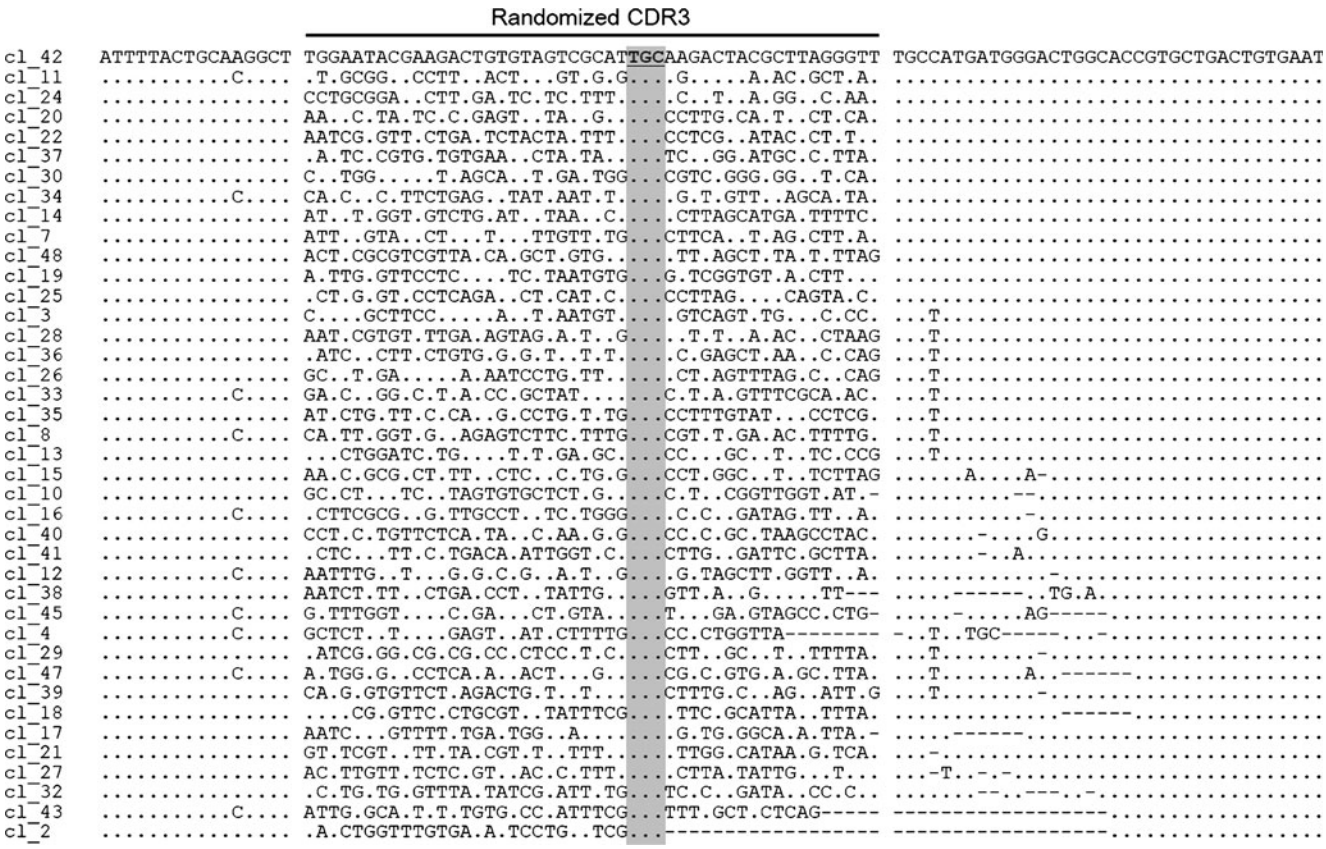

Fig. 2 Alignment of partial nucleotide sequences of the randomized IgNAR V region. *Horizontal bar* indicates the CDR3 region. Position of fixed cysteine residue in the CDR3 is shaded *gray*

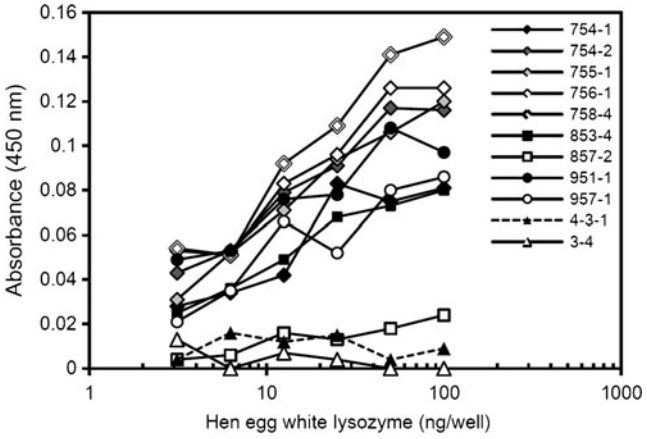

Fig. 3 Identification of HEL-specific monoclonal phage clones by ELISA. Eight of nine clones obtained by panning (all but 957–1) showed increased binding with increasing HEL concentration. A non-specific clone (4-3-1) has a normal IgNAR V region; 3–4 is another control that lacks an IgNAR V region. Data show the means of duplicated wells

formed by the CDR3/R2/16aa primer which synthesized 105 nucleotides, while the mutation in the FR regions probably came from cDNA templates.

To screen the phage clones for specificity to HEL, the library was subjected to four rounds of panning with 200 ng/well of HEL and then to two rounds with 20 ng/well of HEL. After the four rounds of panning, a total of 120 single phage plaques were separately picked up from the fourth stage phage plates. The nucleotide sequences of the IgNAR V region were determined by PCR amplification and sequencing, and the dominant sequences of the V region were also confirmed. Only in-frame clones were subjected to the fifth round of panning. After six rounds of panning, nine clones remained as HEL-specific phage clones.

The specificity of phage clones to HEL was confirmed by ELISA. The negative phage clones 4-3-1 and 3–4 did not show specificity to HEL (Fig. 3) while the positive control #sc-73295 showed high absorbance (OD_{450}=1.0) to 3 ng/well of HEL (data not shown). The positive phage clones showed five times lower absorbance than the positive control. All anti-HEL phage clones except for the 857–2 clone showed a higher absorbance than the negative controls which increased in a dose-dependent manner (Fig. 3). Thus, eight of the nine clones were specific for HEL. Although HEL-specific IgNAR V region clone (HEL-5A7) has been reported in nurse shark (Dooley et al. 2003), it was difficult to compare between the

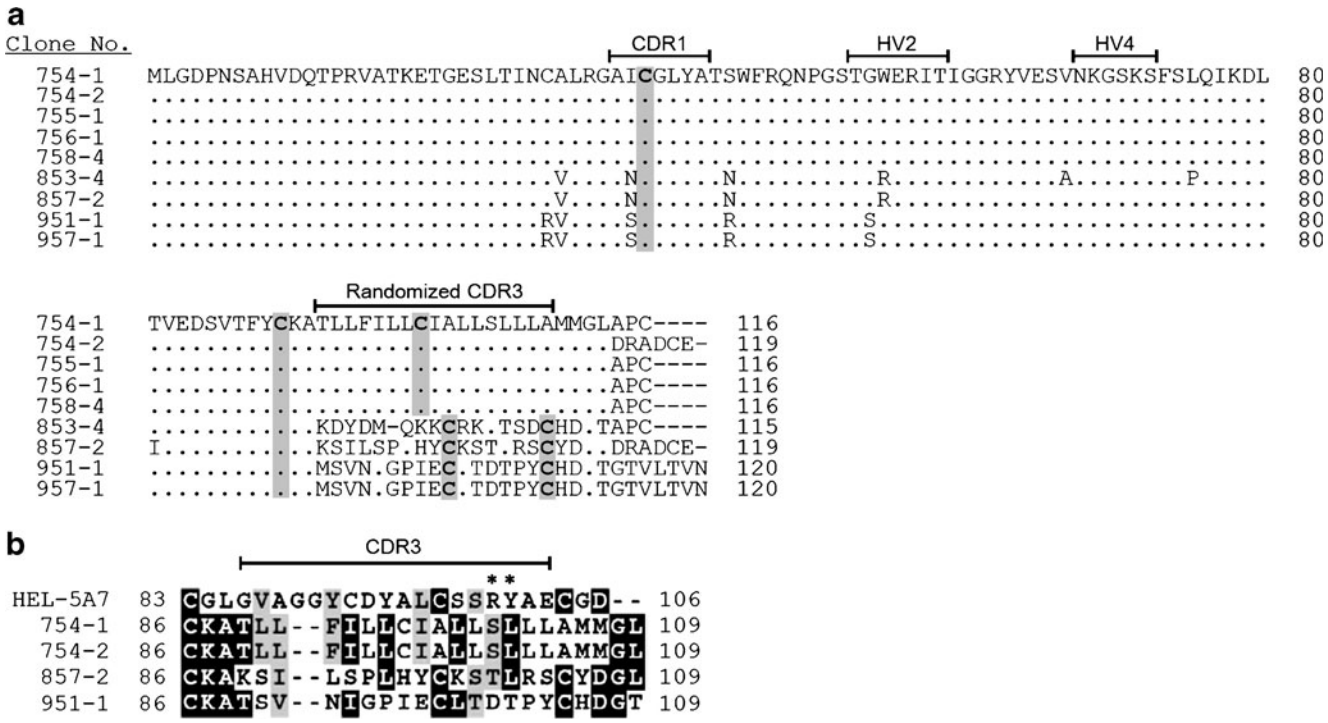

Fig. 4 Comparison of HEL-specific phage clone sequences. **a** Deduced amino acid sequences of HEL-specific phage clones (754–1, 754–2, 755–1, 756–1, 758–4, 853–4, 857–2, 951–1, and 957–1). Conserved cysteine residues are *bolded* and shaded *gray*. Identical amino acid residues and gap are shown *with dots* and *dashes*, respectively. The position of CDR1, CDR3, HV2, and HV4 are indicated. **b** Comparison of CDR3s isolated from HEL-specific IgNAR V regions. Four clones (754–1, 754–2, 857–2, and 951–1) were identified in this study, and HEL-5A7 clone was identified in a previous study (Dooley et al. 2003). Identical amino acids in the alignment are shaded *black* and similar amino acids are shaded *gray*. Amino acid residues Arg^{100} and Tyr^{101} in the HEL-5A7 clone are indicated with asterisks. Gaps are shown with *dashes*

affinities of HEL-5A7 and the eight clones isolated in this study because of the different ELISA systems that were used. Therefore, it is necessary to produce recombinant proteins of HEL-specific IgNAR V regions for affinity analysis

The IgNAR V regions of HEL-specific phage clones were sequenced. In the alignment of the deduced amino acid sequences (Fig. 4a), clones 754–1, 754–2, 755–1, 756–1, and 758–4 shared identical amino acids not only in the randomized CDR3 region but also in the CDR1, HV2, and HV4 regions. Although several C-terminal amino acid residues of 754–2 were similar to those in 857–2, the total C-terminal sequences did not completely correspond to each other; this is due to gaps in the nucleotide sequences of the CDR3 and FR regions, which can be detected in several clones (Fig. 2). The CDR3 regions of these five clones contained single cysteine residues at position 96 (Cys^{96}). The sequences of 951–1 and 957–1 were completely identical and included two cysteine residues at positions 98 (Cys^{98}) and 105 (Cys^{105}) in CDR3; 853–4 and 857–2 were different from other clones, and also encoded two cysteine residues Cys^{98} and Cys^{105} in CDR3, similar to 951–1 and 957–1. Interestingly, Cys^{98} and Cys^{105} of 857–2 and 951–1 corresponded to Cys^{97} and Cys^{104} of HEL-5A7 (Fig. 4b). Crystal structure analysis of HEL-5A7 and HEL (Stanfield et al. 2004) revealed that Cys^{97} and Cys^{104} form a disulfide bond in the CDR3 loop, and that Arg^{100} and Tyr^{101} are deeply buried in the HEL active site. However, Arg^{100} and Tyr^{101} are not located in CDR3 loop of 857–2 and 951–1. These differences in the CDR3 sequence might influence the degree of their affinity.

To evaluate the specificity or affinity to the synthetic V region, antibody genes can be redesigned by random mutagenesis based on error-prone PCR or point mutagenesis of CDR codons. Kobayashi and colleagues (2008) constructed a monoclonal antibody against estradiol-17β (E_2). By selective mutation of the single-chain Fv fragment (scFv) of this antibody, they were able to increase its affinity to E_2 threefold. Similarly, random mutagenesis of two amino acid residues in the CDR2 region of an antibody against *p*-azophenylarsonate (Ars) CDR2 increased its affinity to Ars (Casson and Manser 1995).

In conclusion, we constructed a shark IgNAR phage display library, in which CDR3 was artificially randomized by PCR and presented on the surface of T7 phage. Eight phage clones were identified by ELISA showing a HEL-specific IgNAR V region. The deduced amino acid sequences of CDR3 had either one or two cysteine residues. However, none of them were similar to the previously reported nurse shark HEL-5A7 clone. This research may lead to the synthesis of IgNAR V regions that recognize viral and bacterial surface antigens and may thus be useful for neutralizing pathogen infections.

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