Synthesis of Epitope-Targeting Antibody Based on Native Protein–Protein Interactions for Ftsz Filamentation Suppressor

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Abstract

Phage display and biopanning is a powerful tool for generating binding molecules for a specific target. However, the selection process based on binding affinity provides no assurance for the antibody's affinity to the target epitope. In this study, we propose a molecular-evolution approach guided by native protein–protein interactions to generate epitope-targeting antibodies. The binding-site sequence in a native protein was grafted into a complementarity-determining region (CDR) in the antibody, and a nonrelated CDR loop (in the grafted antibody) was randomized by phage display techniques. In this construction of antibodies by integrating graft and evolution technology (CANIGET method), suitable grafting of the functional sequence weakly functionalized the antibody, and the molecular-evolution approach enhanced the binding function to inhibit the native protein–protein interactions. Antibody fragments with an affinity for filamenting temperature-sensitive mutant Z (FtsZ) were constructed and completely inhibited the polymerization of FtsZ. Consequently, the expression of these fragments drastically decreased the cell division rate. We demonstrate the potential of the CANIGET method with the use of native protein–protein interactions for steady epitope-specific evolutionary engineering.

Introduction

Antibodies with high-target specificity and affinity are an important part of modern therapeutic and diagnostic approaches¹. To create an antibody against a specific target, in vitro selection from a large library of variants has been performed. A library of antibody fragments prepared from the immune system or by means of artificial gene synthesis are displayed on phages², yeast³, or ribosomes⁴, and the antibody variants with affinity for a specific target are selected based on target binding. Recent biological techniques enabled the generation of a library with more than 10^{10} variants⁵. Thus, the probability of generating high-affinity antibodies is increasing. However, the target-binding selection of antibodies from the library does not always lead to the identification of an antibody that can bind to an aiming epitope on target⁶.

In this study, we propose an approach to steadily create epitope-targeting antibodies by utilizing native protein–protein interactions. Previously, we proposed the construction of antibodies based on the integration of the graft and evolution technology (CANIGET) method to build unique antibody fragments with specificity to inorganic material surfaces^{7,8}. A material-binding peptide was grafted in complementarity-determining regions (CDRs) in the antibody fragment (peptide grafting process) without structural instability, and a nonrelated CDR loop in the peptide-grafted fragment was randomized to screen for high-affinity antibodies (evolution process). In this method, the grafting of a functional peptide sequence can lead to the binding of the grafted antibody fragment on the epitope of the applied peptide, and the affinity is enhanced by generating a binding function on the nonrelated CDR by means of an in vitro selection approach. Therefore, the grafting process can guide the evolution of the antibody to the amino acid sequence space wherein the variants have affinity for the target epitope.

Herein, the CANIGET method was applied to create biologically functional epitope-targeting antibodies. Neutralization, that is, the inhibition of a particular native protein–protein interaction, is a therapeutic approach with antibodies. If a mimic approach of native interaction on an antibody can be constructed, epitope-targeting antibodies can be easily designed. In this study, we focused on the creation of epitope-targeting antibodies against the filamenting temperature-sensitive mutant Z (FtsZ). The emergence of bacterial resistance to currently available antibiotics emphasizes the urgent need for antibiotics with new antibacterial mechanisms. Cell division is an important and common process in all living organisms. Therefore, specific inhibition of cell division in bacteria has the potential to be an antibiotic with a new antibacterial mechanism. FtsZ is an important protein for bacterial cell division^{9,10}. During the bacterial cell division, FtsZ self-assembles into tubulin-like protofilaments by a head-to-tail association with guanosine triphosphate (GTP), and forms a contractile ring-like structure known as the Z-ring on the cytoplasmic membrane^{11,12}. Subsequently, when GTP is

dephosphorylated by the GTPase activity of the T7 loop in the FtsZ molecule, the polymer formed by the FtsZ-Guanosine diphosphate (GDP) complex changes to a curved shape¹³. Finally, the Z-ring contracts and the cell divides at the midpoint following FtsZ depolymerization¹⁴. Therefore, interference of the interaction between the T7loop of FtsZ and GTP bound to another FtsZ results in the inhibition of cell division associated with depolymerization^{15,16,17}.

In this study, we generated antibiotic antibodies with inhibitory activity for the polymerization of FtsZ with the use of the CAnIGET method (Fig. 1). Suitable grafting of the T7loop in a CDR of a camelid variable heavy-chain domain of a variable heavy homodimer (VHH) antibody functionalized the grafted VHH, and the local randomization of the VHH generated the inhibition activity for cell division. The use of native protein–protein interactions in the CAnIGET method indicates the direction to the target's functional sequence space. We demonstrate the potential of the CAnIGET method for epitope-specific evolutionary engineering.

Results

Grafting T7 loop to CDRs in VHH

FtsZ polymerization is performed by the interaction between the T7 loop in FtsZ and another FtsZ. Therefore, inhibition of the T7loop binding can lead to the inhibition of cell division. To interfere with the interaction between the T7loop and another FtsZ, in this study, we attempted to create a molecule that overcomes competitive inhibition with FtsZ by enhancing the affinity of VHH grafted with T7loop by evolutionary molecular engineering. First, parent VHH (cAbCII10, VHH fragments of camel anti-BclII b-lactamase antibody) were genetically engineered by grafting T7loops in CDR1, CDR2, and CDR3. The amino acids involved in the interaction of the T7 loop with other FtsZ were determined by crystal structural analyses. The I201, A202, E206, N208, and D210 residues of the T7loop interact with other FtsZ monomers, and N208, D210, and D213 residues are related to the GTPase activity. To retain the function of the T7loop, we designed it to contain as many amino acid residues as possible. Furthermore, given that the T7 loop contains a part of the helix at both ends, and the CDR in the scaffold VHH does not have a helix, we designed the T7 loop of the three different lengths to reduce the structural distortion. Specifically, loop A: loop with N-terminal and C-terminal helices, loop B: loop with only N-terminal helix left, or loop C: loop with both removed (interaction residues I201 and A202 are also removed). The location for grafting to the CDR was also carefully chosen, while the structure of the T7loop was maintained. The differences in the distance between the ends of T7loop A, B, and C predicted from the three-dimensional structure analysis was 10 Å, 5.3 Å, and 8.8 Å. We chose the length of the replacement CDR, which would be the same end distance of these loops. In loop A: GGSEYSYSTF (endo-to-endo distance of 13.1 Å), SGGSEYSYSTF (12.8 Å) in CDR1, and YFM RLP (9.9 Å) in CDR3, in loop B: ASMGG (5.2 Å) and SMGG (5.5 Å) in CDR2, or in loop C: RGYFMRLPSSHNF (8.3 Å) and RGYFMRLPSSHNF (8.6 Å) in CDR3 were grafted (Table 1). As a result, seven variants, namely, VHH_{A11}, VHH_{A12}, VHH_{A33}, VHH_{B21}, VHH_{B22}, VHH_{C31}, and VHH_{C32}, were constructed. These variants were expressed in *Escherichia coli* (*E. coli*), purified to homogeneity, and were subsequently used in the following experiment.

Characterization of grafted VHH variants

Changes in the secondary structure of VHH variants constructed by T7 loop grafting were evaluated with the use of circular dichroism spectrometry (Fig. S1). None of the mutants yielded remarkable structural differences from the parent VHH. Instead, they yielded a typical immunoglobulin fold structure pattern. In VHH_{A11} and VHH_{A12}, the molar ellipticity at approximately 200 nm decreased slightly, thus indicating that the random structures increased by loop grafting. The binding affinity of VHH variants for FtsZ was evaluated with the use of the enzyme-linked immunosorbent assay (ELISA) (Fig. 2). All variants had an increased binding affinity to the parent. In particular, VHH_{B22} bound most strongly to FtsZ in all the variants. The binding affinity for FtsZ was measured using surface plasmon resonance (SPR)

for parent and VHH_{B22} in detail (Fig. 3). The parent could not detect the binding affinity for FtsZ, whereas VHH_{B22} showed binding affinity when $K_d = 4.3$.

Random mutagenesis of another CDR in VHH_{B22} variants and screening of variants with phage display

Furthermore, we tried to improve the affinity of the obtained VHH_{B22} with the use of an evolutionary engineering approach based on the phage display method. The VHH_{B22} gene was inserted into the phagemid vector to display VHH_{B22} to the pIII of M13 filamentous phage, and random mutations of CDR1 or CDR3 in VHH_{B22} were performed by overlap-extension polymerase chain reaction (PCR) (Table 2). A convenient NNK degenerate codon that can cover all 20 amino acids was employed for random mutation using primers, as shown in Table S1. Because the CDR1 and CDR3 libraries have 17 and 20 residues, respectively, the theoretical sequence space is estimated to be contain 20^{17} (1.3×10^{22}) and 20^{20} (1.0×10^{26}). The actual magnitudes of the obtained phage libraries were 4.1×10^5 colony forming units. Therefore, it is shown that this library is a condition that does not meet the theoretical sequence space of the mutant. This phage was subjected to biopanning while the concentration of the target FtsZ was gradually reduced from round to round. After four rounds of biopanning, the eluted phages were infected with *E. coli*, and the formed 186 colonies were monocloned and cultured. Recombinant phages in the supernatant were analyzed by phage ELISA to evaluate the binding affinity of FtsZ (Fig. S2a and b).

Consequently, signal improvements were observed in 174 out of 186 clones in the CDR1 library and in 184 clones out of 186 in the CDR2 library, despite not satisfying the theoretical mutation sequence space. Sixteen clones were selected from each library and sequenced. In particular, the VHH_{B22-35} sequence occupied nine out of 16 clones of the CDR3 library (Table S2). As a result, three types of sequences from CDR1 and seven sequences from CDR3 were identified (Table S2). These genes were transferred to an *E. coli* expression vector, and the protein was purified (Fig. S3). Finally, only four mutants (VHH_{B22-3}, VHH_{B22-23}, VHH_{B22-35}, and VHH_{B22-97}) were functionally expressed in *E. coli*. One mutant (VHH_{B22-42}) interacted unspecifically with the Size exclusion chromatography (SEC) column, and the other four mutants formed soluble aggregates.

Characterization of evolved VHH variants

ELISA analysis was performed to analyze the binding affinity for FtsZ of the purified VHH_{B22-3}, VHH_{B22-23}, VHH_{B22-35}, and VHH_{B22-97} (Fig. 4). All variants showed enhanced activity compared with the parent VHH and VHH_{B22}. In particular, VHH_{B22-23} and VHH_{B22-97} showed significantly enhanced activities. Subsequently, VHH_{B22-3} and VHH_{B22-35} showed enhanced activity. To eliminate the possibility that the apparent affinity was increased by becoming a multimer and to calculate the K_D value precisely, SPR analysis was performed with a small amount of immobilized FtsZ (Fig. 5, Table S3). Given that VHH_{B22-35} showed the highest binding affinity ($K_D = 0.042$ mM), it was found that VHH_{B22-35} had the highest binding activity. Therefore, we investigated whether VHH_{B22-35} could inhibit the polymerization of FtsZ (Fig. 6a, b and c). In the presence of GTP and MgCl₂, where FtsZ polymerization occurred, the parent VHH, VHH_{B22}, and VHH_{B22-35} were mixed with FtsZ and reacted for 30 min. After centrifugation, the soluble (Fig. 6a) and insoluble fractions (Fig. 6b) were analyzed with the use of polyacrylamide gel electrophoresis (SDS-PAGE). When FtsZ and parent VHH were mixed, the polymer was approximately half polymerized, whereas when VHH_{B22} was mixed with FtsZ, the polymer was approximately one-fourth. At VHH_{B22-35}, the polymer completely disappeared; this indicated that a strongly inhibited VHH variant could be obtained (Fig. 6c). In addition, *E. coli* growth in isopropyl β -D-thiogalactopyranoside (IPTG)-induced conditions was observed (Fig. 6d). *E. coli* growth was not delayed when parent VHH was mixed, whereas it was significantly delayed when VHH_{B22} was added. At VHH_{B22-35}, it completely inhibited growth.

Discussion

In general, it is not easy to acquire novel functions by normal directed evolution, such as error-prone PCR from a nonspecific binding scaffold^{18,19,20} compared with the improvement of the binding affinity with affinity maturation. This is because the sequence space obtained by random mutagenesis is enormous²¹. If a mutation is introduced in the VHH_{B22} loop by normal random mutagenesis techniques such as error-prone PCR, the sequence space reaches 20^{37} , thus indicating that we cannot easily obtain the best mutants. For example, even if an antibody showing binding ability is acquired from a vast sequence space with the use of effective techniques, such as phage display and biopanning, derived antibodies often do not have the desired therapeutic effect²². This result indicates that the antibody is climbing a mountain in the sequence space with a high-binding affinity. As a result, an antibody with a nonspecific strong affinity is obtained. A method is thus desired to obtain an antibody that climbs a mountain in the sequence space of the binding affinity and that retains the specific binding to an effective epitope.

In this study, the CANIGET method was employed, which was developed previously for the antibodyzation of metal-binding peptides. This study is the first report of an improvement in the affinity for biomolecules with the use of the CANIGET method. We first grafted a functional T7-loop to bind the epitope to limit the sequence space by giving it directivity, and randomized other loops based on the use of the NNK codon to improve the binding affinity by exerting a multivalent effect by means of the CANIGET method.

In the grafting step shown in Fig. 2, the binding affinity was demonstrated in all conditions and was significantly different owing to the combination of the length of the loop and the grafting site. This finding indicated that this grafting process was sensitive. In general, CDR3 is considered to be the most likely involved loop in VHH binding affinity because of its long length and flexibility; and only CDR3 of the three CDR loops has been often engineered, as reported previously^{23,24}. Hattori *et al.* also reported that CDR1 and CDR3 of VHH can be used for grafting peptide loops; however, peptide grafting to CDR2 led to structural disruptions⁸. Surprisingly, in this study, peptide loop grafting to CDR2 in VHH was the most functional, despite the use of the same scaffold. In a previous study, the length and structure of the peptide loop were not considered in detail, but this study may have succeeded in considering the difference in the end distance of the loop. This result proved that CDR2 is also a promising candidate for peptide loop grafting. However, according to detailed observations, no clear rule of grafting pattern was found owing to differences in binding affinities between the same grafting peptide loops and the same CDRs. In addition, the increase in the small end-distances of the A, B, and C loops to CDR increased the binding affinity. Our consideration of these results is that some adjustment of the end distance is effective for successful grafting; however, if the difference in the end distance is small, not only this distance but also other factors, such as the angle and twist may also be involved. This result indicates that fine tuning must be experimentally demonstrated by trial and error after the adjustment of the end distances of the loops.

In the evolutionary step, phage display and panning from a library with a magnitude of 10^5 were performed with the use of the obtained VHH_{B22}. Furthermore, the sequence space could be compressed up to 186 clones, and a mutant with enhanced function could be obtained in a single step with the use of the CANIGET method. Almost all variants yielded positive clones (94% of randomized clones in CDR1 and 99% of randomized clones in CDR3), thus justifying the successful construction of the library. The percentage of positive clones by the CANIGET method was higher than that of the general evolution technique with the use of the phage display^{25,26}. Four clones of the selected 10 independent clones (3 clones randomized for CDR1 and 7 clones randomized for CDR3) were successfully prepared as monomers in *E. coli*. These variants were randomized for CDR3. The other 5 clones (3 clones randomized for CDR1 and 2 clones randomized for CDR3) formed soluble aggregates in *E. coli*. This result indicated that at least the CDR1 mutation tended to aggregate VHH. Residual one clone was removed by us to bound unspecifically to the resin in SEC. The binding affinity was improved for all selected variants when the function of the purified mutant was examined. In particular, VHH_{B22-35} was bound to FtsZ with a K_D of 0.047 μ M. Montecinos-Franjola *et al.* and Sossong *et al.* reported K_D of 9 and 0.15 μ M in dimer dissociation constant of FtsZ^{27,28}. These results indicated that VHH_{B22-35} are promising as a competitive inhibitor

of FtsZ polymerization. Moreover, chemical FtsZ inhibitors were reported previously and showed K_D of μM order.^{16,15} The results appear to be that the multivalent effect of the two loops increased the binding affinity. In the inhibition test, parent VHH did not inhibit the growth of *E. coli*, whereas VHH_{B22} partially inhibited and VHH_{B22-35} completely inhibited growth. Under IPTG-induced conditions, the transformants showed considerable growth inhibition with an increase in inhibitory activity, indicating that the VHH variant has potential as an antibiotic reagent.

In conclusion, we have shown how the careful grafting and random mutation of the functional T7 loop of FtsZ with the use of the CAnIGET method can efficiently obtain variants with high-binding affinity while maintaining specific binding to the epitope. In the process, we succeeded to achieve active grafting by considering the end-to-end distances of the loop; however, we could not find any rules for loop grafting in detail. Thus, fine tuning is required. It is necessary to accumulate information through basic research to extract more effective grafting loop design rules. Regarding the evolutionary process, we have effectively built a library and variants with significantly increased binding affinities and inhibition effects. Antibiotics using proteins are expected to be expensive, but we believe that there is a possibility that they can be produced inexpensively by self-amplifying proteins using plasmids and phages.

Methods

Grafting T7 loop in FtsZ to CDR in VHH

The deoxyribonucleic acid (DNA) sequence encoding the VHH fragments of camel anti-BclI b-lactamase antibody (cAcBCII10)²⁹ was synthesized as a template for mutagenesis. Three lengths of the T7 loop (loop A: IAVSGEVNLD FAD, loop B: IAVSGEVNLD, or loop C: VSGEVNLD) in FtsZ were grafted to CDR1, CDR2, and CDR3 in the VHH. CDRs were determined according to the AbM definition. The combination of the loop and CDR was determined by the closeness of the end-to-end distance between the T7 and CDR loops (Table 1). The DNA sequence of each mutant was constructed by overlap-extension PCR with the primers listed in Table S1. These PCR fragments were digested with Nco I and Sac II and ligated into pRA vectors that were predigested at the same restriction sites to generate the grafted VHH expression vectors.

Preparation of VHH fragments

E. coli BL21 (DE3) transformants harboring the expression plasmid with the gene for the parent VHH fragments and its variants were cultured in a 2 × yeast extract tryptone medium containing 100 mg/mL of ampicillin at 28 °C at 160 revolutions per minute (rpm). Protein expression was induced with the addition of 1 mM IPTG. The soluble fraction was extracted from the harvested cells by sonication in 50 mM Tris-HCl buffer (pH: 8.0) which contained 200 mM NaCl and was purified by immobilized metal affinity chromatography (IMAC, Ni Sepharose FF, Cytiva, Tokyo, Japan) and size-exclusion chromatography (SEC, Hi-Load 26/600 Superdex 200 pg, Cytiva, Tokyo, Japan) to obtain pure VHHs. Purified VHHs were analyzed for molecular weight and degree of purification by SDS-PAGE and secondary structure by CD spectrometry.

ELISA

The binding affinity of variants to FtsZ was evaluated with the use of ELISA. First, 50 mL of FtsZ from *Staphylococcus aureus* (100 mg/ml) was immobilized in a well plate in phosphate-buffered saline (PBS). After washing three times with PBS, the wells were blocked with 150 mL of PBS which contained 0.5% bovine serum albumin (PBS-B) as a blocking buffer for 1 h at 20–25°C. After washing with PBS, which contained 0.05% Tween20 (PBS-T), 0.1, 0.3, 1, and 3 mM of purified VHH variants were added and incubated in PBS-T for 30 min at temperatures in the range of 20–25°C. After washing three times with PBS-T, the wells were incubated with *horseradish peroxidase* modified FLAG tag antibody (ANTI-FLAG M2 Peroxidase, Sigma-Aldrich, Japan) in PBS which contained 0.5% bovine serum albumin and 0.05%

Tween20 (PBS-BT) and incubated for 40 min at 20–25°C. After the wells were washed three times, 50 mL of TMB solution (1-Step™ Ultra TMB-ELISA Substrate Solution, Thermo Fisher Scientific, Japan) was added for coloration. Binding affinity was determined by measuring the absorbance at 450 nm.

Construction of a random phage library and selection of VHHs with high-binding affinity for FtsZ

A phage library with variants that had high-binding affinity for FtsZ was constructed by randomizing the CDRs (CDR1 or CDR3) of VHH_{B22}. A gene encoding the VHH_{B22} library with a randomized sequence in the CDR1 or CDR3 loop was synthesized by overlap-extension PCR using primers, as shown in Table S1, and a VHH_{B22} expression plasmid as a template. NNK degenerated codons that covered all amino acids, and one stop codon was used for random mutagenesis³⁰. The PCR fragments were ligated in the pSIII phagemid vector with the use of the SfiI site, which was designed by the deletion of 172 amino acids in the N-terminus and fusion of the V5tag in the C terminus of the pIII protein with the use of the phagemid vector pTZ-PsFv2³¹, to display the variants on the pIII protein in filamentous bacteriophage M13. FtsZ concentrations of 1.67 mg/mL (1st round), 0.33 mg/mL (2nd round), 0.067 mg/mL (3rd round), and 0.013 mg/mL (4th round) were immobilized on MAXISORP immune-tube (Thermo Fisher Scientific, MA, USA) in a 3 mL of PBS (pH: 7.4) at 4°C. After washing five times with PBS, PBS containing 3% skim milk was added to the tube and incubated for 30 min at 20–25°C for blocking. After washing five times with PBS, 50–100 mL of VHH displayed the phage library (approximately 4×10^5 phages) which was mixed with FtsZ in PBS which contained 3% skim milk, 0.02% HSA, and 0.02% BSA, and was incubated for 90 min at 20–25°C at 6 rpm with shaking. After washing ten times with PBS (which contained Tween 20), the bound variants were eluted with 2 mL of 200 mM Gly-HCl (pH: 2.2). The eluate was neutralized by the addition of 1 mL of 1 M Tris HCl (pH: 8.0). Phages were amplified by infection with *E. coli* (TG-1 strain). The panning procedure was repeated four times. A total of 186 clones were chosen randomly from the 4th round, and the elute phages were analyzed by phage ELISA.

Screening of variants by monoclonal phage ELISA

The binding affinity of VHH variants to FtsZ was evaluated with the use of ELISA. First, 50 mL of 100 mg/ml FtsZ was immobilized in a well plate in PBS. After three washes with PBS, the wells were blocked with 150 mL of blocking buffer (PBS contained 3% skim milk for 30 h at 20–25°C). After washing with PBS, monoclonal phages with variants were added and incubated in PBS (which contained 0.05% Tween20) for 40 min at 20–25°C. After three washes with PBS-T, the wells were incubated with horseradish peroxidase (HRP)-modified anti-M13 phage antibody in PBS (which contained 0.05% Tween20) and 0.5% BSA and incubated for 40 min at 20–25°C. The wells were washed three times with PBS-T. Subsequently, 50 mL of the TMB (3,3',5,5'-tetramethylbenzidine) reagent was added, and coloration was detected by measuring the absorbance at 450 nm. The amino acid sequence of the top 16 clones for each CDR was determined with the use of an ABI 3130xl Sequencer. The selected VHH genes were inserted into the NcoI–SacII restriction sites in the pRA5 vector for expression in *E. coli*.

SPR

The binding affinity between FtsZ and VHH variants was determined using a SPR instrument Biacore T200 (Cytiva, Tokyo, Japan). FtsZ was immobilized on an S-series CM5 sensor chip by amine coupling (up to 345.1 resonance units) in PBS that contained 0.005% Tween 20 (pH: 7.4). A dilution series (15.6–20000 nM) of VHH variants was applied on the chip at 25°C with the use of a flow rate of 30 mL/min. Gly-HCl (pH: 1.5) was used as the regenerated solution. The data were normalized by subtracting the response of a blocked blank cell from the collected values. BIA evaluation software (Cytiva, Tokyo, Japan) was used to analyze the data.

Inhibition assay for FtsZ polymerization

When FtsZ forms a complex with GTP, it binds to another FtsZ via the T7loop and forms a huge homopolymer by head-to-tail polymerization. To evaluate the inhibition of polymerization of FtsZ by variants, 0, 1, 5, and 10 mM of VHH variants were mixed with 10 mM FtsZ in 50 mM MES-NaOH buffer (pH: 6.5) (which contained 50 mM KCl) at 30°C and at 300 rpm for 30 min with shaking. Subsequently, 2 mM GTP was added to the mixture and shaken for 1–2 min. Subsequently, 10 mM MgCl₂ was added to the tube and shaken at 30°C at 300 rpm for 30 min. After centrifugation at 15000 × g for 30 min, the supernatant (unpolymerized FtsZ) and the precipitate (polymerized FtsZ) were analyzed by SDS-PAGE. Band intensity was calculated with the use of the ImageQuant software in LAS 4000 (Cytiva, Tokyo, Japan).

Growth of VHH variants with expressed *E. coli* strain

E. coli BL21(DE3) was transformed with the use of 10 ng of parent VHH, VHH_{B22}, and VHH_{B22-35} expression plasmids with the use of the calcium chloride method. Transformed cells were spread on LB plates that contained final 1 mM IPTG. The formation of colonies was visually observed.

Declarations

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Author Contributions

H.N. and M.U. designed the study.; H.N., M.U. T.I. and S.K. wrote the manuscript.; T.K., A.T. and T.Y. performed the experiment and analyzed the data.; T.M. and Y.T. participated in the discussion of the results.

Competing Interests

Unpaid membership in a government or non-governmental organization

Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to this article.

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Tables

Table 1 Grafting of T7loop to each complementarity-determining region (CDR) considering end-to-end distances

Label	Loops to Graft	Loops to be replaced in VHH scaffold			Tm/°C
	T7 loop	CDR1	CDR2	CDR3	
Parent (cAcBCII10)	-	-	-	-	76.6
Variable heavy homodimer (VHH _{A11})	DAFDLNVEGSVAI (10.0Å)	GGSEYSYSTF (13.1Å)	-	-	67.0
VHH _{A12}	DAFDLNVEGSVAI (10.0Å)	SGGSEYSYSTF (12.8Å)	-	-	71.1
VHH _{A33}	DAFDLNVEGSVAI (10.0Å)	-	-	YFMRLP (9.9Å)	72.9
VHH _{B21}	DLNVEGSVAI (5.3Å)	-	ASMGG (5.2Å)	-	73.4
VHH _{B22}	DLNVEGSVAI (5.3Å)	-	SMGG (5.5Å)	-	68.9
VHH _{C31}	DLNVEGSV (8.8Å)	-	-	RGYFMRLPSSHNF (8.3Å)	69.1
VHH _{C32}	DLNVEGSV (8.8Å)	-	-	RGYFMRLPSSHNFR (8.6Å)	70.1

Table 2 Randomization of another CDR in phage display

Label	CDR1	CDR2	CDR3
Parent (cAcBCII10)	²⁴ ASGGSEYSYSTFSLGWF ^{35C}	⁴⁸ VAAIASMGGLTYA ⁶⁰	¹⁰² AAVRGYFMRLPSSHNFYWG ¹¹⁷
VHH _{B22}	²⁴ ASGGSEYSYSTFSLGWF ^{35C}	⁴⁸ VAAIAIAVSGEVNLDLTYYA ⁶⁰	¹⁰² AAVRGYFMRLPSSHNFYWG ¹¹⁷
CDR1 library	²⁴ XXXXXXXXXXXXXXXXXXXX ^{35C}	⁴⁸ VAAIAIAVSGEVNLDLTYYA ⁶⁰	¹⁰² AAVRGYFMRLPSSHNFYWG ¹¹⁷
CDR3 library	²⁴ ASGGSEYSYSTFSLGWF ^{35C}	⁴⁸ VAAIAIAVSGEVNLDLTYYA ⁶⁰	¹⁰² XXXXXXXXXXXXXXXXXXXX ¹¹⁷

X indicates a randomized sequence.

Italics show the T7 loop inserted into VHHB22.

The underline shows the CDR defined by AbM.

Figures

Figure 1

Figure 1

Image of evolutionally design of antibody in this study.

Figure 2

Figure 2

Binding affinity of grafted VHH variants to FtsZ by ELISA. 3 μ M (black bar) or 1 μ M (gray bar) of grafted VHH variants were used for ELISA.

Figure 3

Figure 3

Surface plasmon resonance analysis of interactions between the parent (a) or selected VHHB22 (b) and FtsZ. FtsZ was immobilized on the sensor chip by amine coupling up to 345.1 resonance units. The dilution series (625–20000 nM) of the VHH variants was applied on the chip at 25°C at a flow rate of 30 μ L min⁻¹.

Figure 4

Figure 4

Binding affinity of evolved VHH variants to FtsZ by enzyme-linked immunosorbent assay (ELISA).

Figure 5

Figure 5

SPR analysis of interactions among VHHB22-3 (a), VHHB22-23 (b), VHHB22-35 (c) or VHHB22-97 (d) obtained by evolutionally step and FtsZ. FtsZ was immobilized on the sensor chip by amine coupling up to 345.1 resonance units. The dilution series (15.625–1000 nM) of VHH variants was applied on the chip at 25°C at a flow rate 30 $\mu\text{L min}^{-1}$.

Figure 6

Figure 6

Inhibition analysis of FtsZ polymerization with VHH variants. Concentrations equal to 0, 1, 5, and 10 μM of the parent, VHHB22, or VHHB22-35 were mixed with 10 μM of FtsZ. After centrifugation at $15000 \times g$ for 30 min, the supernatant (a) and precipitate (b) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). M is a low molecular weight marker. Arrows show the molecular weights of FtsZ and VHH variants. The band intensity of the insoluble fraction of FtsZ in SDS-PAGE was analyzed with an image quant (c) and was plotted for parent concentrations in the range of 0–10 μM (open circles), VHH4 (open triangles), and VHH variants (open squares). Growth of *E. coli* transformants with variants based on the isopropyl β -D-thiogalactopyranoside (IPTG) induction condition (d).

Supplementary Files

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