Synthesis of epitope-targeting nanobody based on native protein–protein interactions for FtsZ filamentation suppressor

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Abstract

Phage display and biopanning are powerful tools for generating binding molecules for a specific target. However, the selection process based only 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 nanobody, and a nonrelated CDR loop (in the grafted nanobody) was randomized to create a phage display library. In this construction of nanobodies by integrating graft and evolution technology (CAnIGET method), suitable grafting of the functional sequence added functionality to the nanobody, and the molecular-evolution approach enhanced the binding function to inhibit the native protein–protein interactions. To apply for biological tool with growth screening, model nanobodies with an affinity for filamenting temperature-sensitive mutant Z (FtsZ) from Staphylococcus aureus were constructed and completely inhibited the polymerization of FtsZ as a function. Consequently, the expression of these nanobodies 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.

KEYWORDS
directed evolution, FtsZ, grafting, nanobody, phage display

1 | INTRODUCTION

Antibodies with high-target specificity and affinity are an important part of modern therapeutic and diagnostic approaches.[1] To discover a new 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 is displayed on phages,[2] yeast,[3] or ribosomes,[4] and the antibody variants with binding affinity for a specific target are selected based on target binding. Recent biological techniques enabled the generation of a library with more than 10¹⁰ variants.[5] Thus, the probability of generating high-affinity antibodies is increasing. Despite the fact that antibodies with the desired therapeutic effect have been reported using target-binding affinity selection utilizing phage display...
methods, it is occasionally possible to acquire antibody clones with binding affinity but no therapeutically useful effect.\(^6\) In this study, we propose an approach to steadily create epitope-targeting antibodies by molecular evolution accompanied by molecular recognition of native protein–protein interactions. Previously, our group proposed the Construction of Antibodies based on the Integration of the Graft and Evolution Technology (CAAnIGET) method to build unique antibody fragments, with specificity to inorganic material surfaces using variable domain of heavy chain of heavy-chain antibodies (VHH), so-called nanobodies, that have simple structure that is easy to engineer.\(^7,8\) A material-binding peptide was grafted in complementarity-determining regions (CDRs) in the nanobody (peptide grafting process) without structural destabilization, and a nonrelated CDR loop in the peptide-grafted fragment was randomized to screen for high-affinity nanobodies (evolution process). In this method, the grafting of a functional peptide sequence can lead to the binding of the grafted nanobody on the epitope of the applied peptide, and the affinity is considerably enhanced (20–63 times increase for \(K_0\)) by enhancing 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 nanobody to the amino acid sequence space wherein the variants have affinity for the target epitope. However, the success of this technique has not yet extended to biomolecules other than inorganic substances.

Herein, the CAAnIGET method was applied to create biologically functional epitope-targeting nanobodies. Neutralization, that is, the inhibition of a particular native protein–protein interaction, is a common therapeutic function of 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 VHHs against the filamenting temperature-sensitive mutant Z (FtsZ). Prokaryotic tubulin homolog, FtsZ is an important protein for bacterial cell division.\(^9,10\) During 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 T7loop in the FtsZ molecule, the polymer formed by the FtsZ-Guanosine-diphosphate (GDP) complex changes to a curved shape.\(^13\) Finally, the Z-ring contracts and the cell divides at the midpoint following FtsZ depolymerization.\(^14\) 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.\(^15–17\) Cell division is an important and common process in all living organisms. Therefore, specific inhibition molecule of cell division has the potential as a biological tool for growth screening on broad organism species. The X-ray crystal structure of FtsZ has been resolved.\(^18\) Therefore, detailed information about the 3D structure for grafting is available. This is a good model for demonstrating the T7loop graft process in the CAAnIGET method.

In this study, we constructed evolutionarily a VHH with inhibitory activity for the polymerization of FtsZ from Staphylococcus (S.) aureus with CAAnIGET method (Figure 1). This study is the first to report an improvement in the affinity of biomolecules with the use of the CAAnIGET method. Suitable grafting of the T7loop in FtsZ in a CDR of the functionalized grafted VHH and the followed local randomization of the VHH enhanced the inhibition activity for cell division. The use of native protein–protein interactions in the CAAnIGET method indicates the direction to the target’s functional sequence space.

## 2 | METHODS

### 2.1 | Grafting T7loop in FtsZ to CDR in VHH

The deoxyribonucleic acid (DNA) sequence encoding the VHH fragments of camel anti-Bcl \(\beta\)-lactamase antibody (cAcBCII10)\(^19\) with FLAG tag and His tag at C terminus was synthesized as a template for mutagenesis. Three lengths of the T7loop (loop A: IAVSGEVNLDFADS, loop B: IAVSGEVNLDFAD, loop C: VSGEVNLDFAD) in FtsZ were grafted to CDR1, CDR2, and CDR3 in the VHH. CDRs were determined according to the AbM definition.\(^20\) The combination of the loop and CDR was determined by 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.

### 2.2 | Preparation of VHH fragments

*Escherichia coli* (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 \(\times\) yeast extract tryptone medium containing 100 \(\mu\)g/mL of ampicillin at 28°C at 160 revolutions per minute (rpm). After incubation for 8–14 h until the OD\(_{600}\) reaches 0.4–0.8, 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-\(\text{HCl}\) 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 purity by SDS-PAGE and secondary structure by CD spectrometry.

### 2.3 | ELISA

The binding affinity of variants to FtsZ was evaluated with the use of an ELISA. First, 50 \(\mu\)L of FtsZ from *S. aureus* (100 \(\mu\)g/mL) was immobilized in a well plate in phosphate-buffered saline (PBS) for 1 h at room temperature. After three washes with PBS, the wells were blocked

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with 150 µL 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 µM 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 (HRP) 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 µL of 3,3′,5,5′-tetramethylbenzidine solution (1-Step™ Ultra TMB-ELISA Substrate Solution, Thermo Fisher Scientific, Japan) was added to wells and these wells were incubated for 10 min at room temperature. Finally, 50 µL of 2-M H₂SO₄ was added to stop the enzyme reaction. The absorbance at 450 nm was measured to determine the binding affinity.

### 2.4 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 VHHB₂₂. A gene encoding the VHHB₂₂ 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 VHHB₂₂ expression plasmid as a template. NNK degenerated codons that covered all amino acids, and one-stop codon was used for random mutagenesis. The PCR fragments were ligated with the use of SfiI sites in the pSIII phagemid vector, 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 phagemid vector, to display the variants on the pIII protein in filamentous bacteriophage M13. FtsZ
concentrations of 1.67 μg/mL (1st round), 0.33 μg/mL (2nd round), 0.067 μg/mL (3rd round), and 0.013 μg/mL (4th round) were added on MAXISORP immune-tube for immobilization (Thermo Fisher Scientific, MA, USA) in 3 mL of PBS (pH: 7.4) at 4°C for 1 h. 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 μL of the VHH-displayed phage library (approximately 4 × 10⁵ phages) was added to the FtsZ-coated immune-tube 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 10 times with PBS, which contained 0.05% (w/v) 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). The 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 eluted phages were analyzed by phage ELISA.

2.5 | Screening of variants by monoclonal phage ELISA

The binding affinity of VHH variants to FtsZ was evaluated using ELISA. First, 50 μL of 100 μg/mL FtsZ was immobilized in a well plate in PBS for 1 h at room temperature. After three washes with PBS, the wells were blocked with 150 μL of blocking buffer (PBS containing 3% skim milk for 30 min 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 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. Fifty microliters of the TMB reagent were added to react to HRP enzymes. After incubation for 10 min at room temperature, 50 μL of 2-M H₂SO₄ was added to stop the enzyme reaction. The absorbance at 450 nm of the solution was measured. Coloration was detected by measuring the absorbance at 450 nm. Anti-V5tag antibody (ThermoFisher, MA5-15253) was also replaced and immobilized in well plates, and the rate of VHH presentation per phage was analyzed by ELISA. 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.

2.6 | 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 (15.6–20,000 nM) of VHH variants was applied on the chip at 25°C with a flow rate of 30 μL/min. Gly-HCl (pH: 1.5) was used as the regenerating 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.

2.7 | 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 μM of VHH variants were mixed with 10 μM 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 15,000 × g for 30 min, the supernatant (unpolymerized FtsZ) and the precipitate (polymerized FtsZ) were analyzed by SDS-PAGE. Band intensity was calculated using the ImageQuant software in LAS 4000 (Cytiva, Tokyo, Japan).

2.8 | Growth of VHH variants with expressed E. coli strain

E. coli BL21(DE3) was transformed with the use of 10 ng of parent VHH, VHHB22, and VHHB22-35 expression plasmids. Competent cells were prepared with the calcium chloride method. Transformed cells were spread on LB plates containing 100 μg/mL Ampicillin. The formation of colonies was observed visually.

3 | RESULTS

3.1 | Growth of T7loop to CDRs in VHH

FtsZ polymerization is performed by the interaction between the T7loop 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 an FtsZ polymerization inhibitor by enhancing the affinity of VHH grafted with T7loop by evolutionary molecular engineering. First, parent VHH (cAbCII10, VHH fragments of camel anti-BcII β-lactamase antibody) were genetically engineered by grafting T7loops in CDR1, CDR2, and CDR3. The amino acids involved in the interaction of the T7loop with other FtsZ were determined by analyses of crystal structure (PDB ID: 3VO8). The I201, A202, E206, 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 T7loop contains a part of the helix at both ends, and the CDR in the scaffold VHH does not have a helix, we designed the T7loop of the
three different lengths to reduce the structural distortion (Table 1 and Figure S1). Specifically, loop A: a loop with N-terminal and C-terminal helices; loop B: a loop with only the N-terminal helix left; or loop C: a 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 3D structure analysis were 10, 5.3, and 8.8 Å, respectively. We chose the length of the replacement CDR, which would be the similar end distance of these loops (Table 1 and Figure S1). In loop A: GGSEYSYSTF (endo-to-endo distance of 13.1 Å), SGGSEYSYSTF (12.8 Å) in CDR1, and YFMRLP (9.9 Å) in CDR3, in loop B: ASMGGL (5.2 Å) and SMGG (5.5 Å) in CDR2, or in loop C: RGYFMRLPSSHNFR (8.3 Å) and RGYFMRLPSSHNFR (8.6 Å) in CDR3 were grafted. As a result, seven variants, namely, VHH_A11, VHH_A12, VHH_A23, VHH_B21, VHH_B22, VHH_C31, and VHH_C32, were constructed. These variants were expressed in E. coli, purified to homogeneity, and subsequently used in the following experiment.

3.2 Characterization of grafted VHH variants

Changes in the secondary structure of VHH variants constructed by T7loop grafting were evaluated with the use of circular dichroism spectrometry (Figure S2). None of the mutants yielded remarkable structural differences from the parent VHH. In VHH_A11 and VHH_A12, the molar ellipticity at approximately 200 nm decreased slightly, thus indicating that the random structures were increased by loop grafting. The binding affinity of VHH variants for FtsZ was evaluated with the use of the enzyme-linked immunosorbent assay (ELISA) (Figure 2). All variants had an increased binding affinity for FtsZ compared to the parent. In particular, of all variants, VHH_B22 bound most strongly.

The binding affinity for FtsZ was measured using surface plasmon resonance (SPR) for parent and VHH_B22 in detail (Figure 3). The parent had no detectable binding affinity for FtsZ, whereas VHH_B22 showed a binding affinity with Kd = 4.3 µM.

3.3 Random mutagenesis of another CDR in VHH_B22 variants and screening of variants with phage display

Furthermore, we tried to improve the binding 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 fused 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. The magnitudes of the obtained phage libraries were 4.1 × 10^5 colony-forming units for CDR1 and 8.4 × 10^5 colony-forming units for CDR3. These phage (input of 1.5 × 10^9 – 1.5 × 10^8 for CDR1 and 1.1 × 10^9 – 1.1 × 10^7 for CDR3) 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 each of the 186 colonies that were formed from the CDR1 library (17aa) and the CDR3 library (20aa) were cultured as single clones, respectively. Recombinant phages in the supernatant were analyzed by phage ELISA to evaluate the binding
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 µL min⁻¹. N.D.: Not detected.

Table 2: Randomization of another CDR in phage display.

| Label                | CDR1               | CDR2               | CDR3               |
|----------------------|--------------------|--------------------|--------------------|
| Parent (cAcBCII10)   | 24 ASGGSEYSTFSGLWF³⁵C | 48 VAIAASMGGLTYYA¹⁰ | 10²AAVRGYFMRLPSHNSFRYWG¹¹⁷ |
| VHHB22               | 24 ASGGSEYSTFSGLWF³⁵C | 48 VAIAAVSGEVNLDTYYA¹⁰ | 10²AAVRGYFMRLPSHNSFRYWG¹¹⁷ |
| CDR1 library         | 24 XXXXXXXXXX      | 48 VAIAAVSGEVNLDTYYA¹⁰ | 10²AAVRGYFMRLPSHNSFRYWG¹¹⁷ |
| CDR3 library         | 24 ASGGSEYSTFSGLWF³⁵C | 48 VAIAAVSGEVNLDTYYA¹⁰ | 10²XXX XXX XXX XXX XXXXXXX |

Note: X indicates a randomized sequence. Italics show the T7 loop inserted into VHHB22. The underline shows the CDR defined by AbM.

The binding to immobilized FtsZ was normalized by dividing the ELISA response for binding to FtsZ by the ELISA response to anti-V5tag antibody, related to the display level of VHH on phage. Surprisingly, signal improvements were observed in 174 out of 186 clones in the CDR1 library and in 184 out of 186 clones in the CDR3 library, despite the size of the libraries was not large enough to cover the theoretical mutational sequence space. Three clones (VHHB22-c1-4, VHHB22-c1-13, and VHHB22-c1-12a) for CDR1 and 17 clones (VHHB22-3, VHHB22-23, VHHB22-35, VHHB22-45, VHHB22-47, VHHB22-50, VHHB22-58, VHHB22-74, VHHB22-97, VHHB22-100, VHHB22-113, VHHB22-125, VHHB22-132, VHHB22-133, VHHB22-178, and VHHB22-183) for CDR3 were selected from each library and sequenced. As a result, three types of sequences from CDR1 and seven sequences from CDR3 were identified as sequences without in/del. (Table S2). In particular, the “FPKARIVLGTSLSPYGL” and “CPFLTCRRRNTHIFYTPPC” sequences were harbored in nine (VHHB22-35, VHHB22-50, VHHB22-58, VHHB22-74, VHHB22-113, VHHB22-125, VHHB22-132, VHHB22-133, VHHB22-178, and VHHB22-183) and two (VHHB22-3 and VHHB22-47) out of the 17 clones in the CDR3 library (Table S2). Another eight kinds of clones were unique, and VHHB22-132 was a contamination of VHHB22-c1-13 in the CDR1 library.

A total of 10 independent clone genes were transferred to an E. coli expression vector, and the protein was purified (Figure S4). Finally, four mutants (VHHB22-3, VHHB22-23, VHHB22-35, and VHHB22-97) were able to be purified as nonaggregated monomeric proteins (Figure S5). We conducted the following experiments with the monomer fraction of these four mutants.

3.4 Characterization of evolved VHH variants

ELISA analysis was performed to analyze the binding affinity for FtsZ of the purified VHHB22-3, VHHB22-23, VHHB22-35, and VHHB22-97 (Figure 4A,B). All variants showed enhanced activity compared with the parent VHH and VHHB22. In particular, VHHB22-23 and VHHB22-97 showed significantly enhanced activities. These variants, with the exception of VHHB22-3, showed no binding to skim milk (Figure 4B),
presumably binding specifically to FtsZ. Subsequently, VHH_{B22-3} and VHH_{B22-35} showed enhanced activity. Moreover, SPR analysis was performed with a small amount of immobilized FtsZ (Figure 5 and Table S3). VHH_{B22-35} showed the highest binding affinity ($K_D = 0.042 \mu M$). Therefore, we investigated whether VHH_{B22-35} could inhibit the polymerization of FtsZ (Figure 6A–C). In the presence of GTP and MgCl$_2$, 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 (Figure 6A) and insoluble fractions (Figure 6B) were analyzed with the use of polyacrylamide gel electrophoresis (SDS-PAGE). When FtsZ and parent VHH were mixed, the polymer was approximately half-formed from band intensity, whereas when VHH$_{B22}$ was mixed with FtsZ, the polymer was decreased. At VHH$_{B22-35}$, the polymer completely disappeared; this indicated that a strongly inhibiting VHH variant could be obtained (Figure 6C). In addition, E. coli growth in isopropyl $\beta$-D-thiogalactopyranoside (IPTG)-induced conditions was observed (Figure 6D). E. coli growth was not delayed when parent VHH was added to the reaction mixture, whereas it was significantly delayed when VHH$_{B22}$ was added. The VHH$_{B22-35}$ completely inhibited E. coli growth.

4 | 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 compared with the improvement of the binding affinity with affinity maturation by biopanning. This is because the sequence space obtained by random mutagenesis is enormous. If a mutation is introduced in the VHH$_{B22}$ loop by commonly used mutagenesis techniques such as error-prone PCR, the sequence space reaches $2^{37}$, 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, the derived antibodies often do not have the desired therapeutic effect. For example, Chen et al. selected two mutants, 2E1 and 3B1, that can bind to RSF proteins using Phage display. It was concluded that 2E1 succeeded in obtaining an antibody having neutralizing activity and could be used as a therapeutic antibody, while 3B1 showed no neutralizing activity. This data indicate the need for strategies that keep specific binding of antibodies to valid epitopes.
In this study, the CANIGET method was employed. The method is the construction of nanobodies by integrating graft of CDR and evolution technology. We have experienced that the grafting of peptide to CDR does not improve binding affinity compared to peptide alone (data not shown). The advantage of the method is that randomizing other CDRs after grafting can improve the binding affinity by 20–63 times compared to peptide alone and CDR-grafted nanobody. We first grafted a functional T7loop to bind the epitope to assign it with desired epitope binding to limit the sequence space, and then randomized other loops based on the use of the NNK codon to improve binding affinity with the additive binding effect of two loops. In the grafting step shown in Figure 2, the binding affinity was demonstrated in all tested clones. All clones were successfully grafted as active form and were different in binding affinity owing to the combination of the length of the loop and the grafting site. 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.[27,28] 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 disruptions.[8] 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 the 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 $10^5$ independent clones were performed with the use of the obtained VHH$_{B22}$. The library could perform even better with a larger number of independent clones. However, contrary to expectations, a mutant with enhanced function could be obtained. After affinity selection, nearly all selected variants were shown to be strong antigen binders (94% of randomized clones in CDR1 and 99% of randomized clones in CDR3), thus justifying the successful construction of the library, indicating that we made a hot library containing enriched high-functioning mutants. Four variants, all with randomized CDR3, were successfully expressed in *E. coli*. The other five clones (three clones randomized for CDR1 and two clones randomized for CDR3) formed mostly soluble aggregates in *E. coli*. This result indicated that at least the CDR1 mutation tended to aggregate VHH. The remaining clone was not studied further because it 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.
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 15,000 × 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 the 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), VHHB22 (open triangles), and/or VHHB22-35 (opened squares). Growth of E. coli transformants with variants based on the isopropyl β-D-thiogalactopyranoside (IPTG) induction condition (D).

µM. Montecinos-Franjola et al. and Sossong et al. reported K_D of 9 and 0.15 µM in the dimer dissociation constant of FtsZ.[29,30] These results indicated that VHH B22-35 is promising as a competitive inhibitor of FtsZ polymerization similar to chemical FtsZ inhibitors K_D of µM order reported previously.[15,16] In the inhibition test, parent VHH did not inhibit the growth of E. coli, whereas VHHB22 partially inhibited and VHHB22-35 completely inhibited growth. Transformant growth correlated with increased binding activity of VHH to FtsZ, indicating that the VHH variants have potential as a growth control molecule. In conclusion, we have shown how the careful grafting and random mutation of the functional T7loop 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 in achieving active grafting by considering the end-to-end distances of the loop; however, we could not find any detailed rules for loop grafting. Thus, fine-tuning is required in each experiment. It is necessary to accumulate information through basic research to extract more effective grafting loop design rules. Regarding the evolutionary process, we had effectively built variants with significantly increased binding affinities and inhibition effects from our small library. The obtained growth control proteins can be contributed in a variety of biological screening systems.

**AUTHOR CONTRIBUTIONS**

Conceptualization: Hikaru Nakazawa, Yoshikazu Tanaka, and Mitsuo Umetsu. Data curation: Hikaru Nakazawa, Taiji Katsuki, and Mitsuo Umetsu. Formal analysis: Hikaru Nakazawa, Taiji Katsuki, Tomoyuki Ito, and Mitsuo Umetsu. Funding acquisition: Hikaru Nakazawa, Yoshikazu Tanaka, and Mitsuo Umetsu. Investigation: Hikaru Nakazawa, Taiji Katsuki, Atsushi Tsugita, Takeshi Yokoyama, and Mitsuo Umetsu. Visualization: Hikaru Nakazawa. Writing—original draft: Hikaru Nakazawa, Takashi Matsui, Tomoyuki Ito, Sakiya Kawada, and Mitsuo Umetsu. Writing—review and editing: Hikaru Nakazawa, Takashi Matsui, Tomoyuki Ito, Sakiya Kawada, and Mitsuo Umetsu.
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CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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