Preparation of Functional Single-Chain Antibodies against Bioactive Gibberellins by Utilizing Randomly Mutagenized Phage-Display Libraries

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Received November 22, 2004; Accepted December 9, 2004

Screening randomly mutagenized proteins displayed on a phage surface by biopanning is a powerful strategy to obtain evolved clones with improved properties such as higher stability and functionality. We utilized this method to overcome the problem that functional single-chain antibodies against active gibberellins, a class of plant hormones, cannot be prepared by some of the conventional methods. Single-chain antibody libraries with random mutations were constructed from two independent anti-bioactive gibberellin monoclonal antibody lines in a phagemid vector, so that the mutated scFvs were expressed in a phage-displayed form upon helper phage infection. From both libraries, scFv clones with binding activity to GA₄ were successfully obtained by successive rounds of biopanning against BSA-GA₄, the original immunogen. The results are highly suggestive that this approach might be a general solution when a single-chain antibody does not show binding activity. We found further that a ribosomal frameshift to complement a nonsense mutation frequently occurred in an amber suppressor strain of E. coli TG1, resulting in the display of a functional antibody, while such a nonsense mutant failed to produce a soluble antibody in a non-amber suppressor strain. This result explains at least partly why single-chain antibodies are sometimes functional only in a phage-displayed form, not in a soluble form.

Key words: single-chain Fv; gibberellin; phage-display library; random mutagenesis

A single-chain Fv (scFv) is a well-known recombinant form of antibody: the variable domains of both the heavy chain (V₉) and the light chain (Vₐ) are connected with a flexible linker peptide, resulting in the minimum unit of an Fv fragment. Since scFv is composed of a single peptide, the production of scFv in such other organisms as E. coli and plants does not require the assembly of V₉ and Vₐ, this not being true for the production of other forms of antibodies such as the full antibody, Fab or minibody composed of multiple peptides. On the other hand, an scFv is sometimes less likely to give antigen-binding activity than the other forms of recombinant antibodies because of the structural constraint caused by linking V₉ and Vₐ with a linker peptide.

In the course of our immunomodulational study on giving new properties to plants by expressing antibodies, we have prepared functional scFvs against GA₄, the precursor of an active gibberellin,1) the chlorpropham herbicide,2,3) and others4) in both E. coli and plants using the antibody genes from monoclonal antibody-producing hybridoma cells. But we were not able to obtain functional recombinant antibodies against active gibberellins; scFvs prepared from two independent monoclonal lines (8/E9 and 21/D13) did not show binding activity to GA₄, an active gibberellin. We overcame this problem by preparing randomly mutagenized scFv phage-display libraries and selecting the mutant clones with binding affinity to GA₄ by panning.

Phage display is a technique for displaying foreign peptides or proteins on the surface of a bacteriophage by expressing them as a fusion product with a coating protein. Since the sequence information is enclosed in the virion, affinity selection of the phages by the binding characteristics of displayed proteins to target molecules (panning) allows rapid identification of the protein sequences. This method has been applied to produce naive synthetic antibody libraries with wide diversity, from which it is possible to select phages displaying antibodies with affinity to the immobilized antigen by applying the panning procedure. In the present study, phage-display scFv libraries of two independent anti-GA₄ antibodies having random mutations incorporated by error-prone PCR were constructed, and the clones with binding activity to GA₄ were obtained by panning against GA₄ conjugated with BSA, the original immunogen for raising 8/E9 and 21/D13 antibodies. Since functional scFvs were obtained for both anti-GA₄ antibodies, this method can be applied as a general solution to the problem of an scFv not showing binding activity. On the other hand, some of the selected clones functioning in a phage-displayed form did not show binding activity when expressed as soluble scFvs. This
result is not peculiar to this study, but is a general problem when preparing scFvs with the phage-display system. We discuss this aspect based on the sequencing results.

**Materials and Methods**

Establishing the hybridoma cell line producing 21/D13 monoclonal antibodies. The immunogen, BSA-conjugated GA₄, was prepared according to the method of Nakajima et al.⁵ Preparation of the hybridoma clones producing the anti-immunogen antibody and the Ig fraction from the ascites fluid followed our previously reported methods.⁶

Radioimmunoassay for determining the recognition specificity of the 21/D13 antibody. A radioimmunoassay to measure the cross-reactivity of different GAs was performed by the method reported by Nakajima et al.,⁶ except that ³H-labeled 16,17-dihydroGA₄ (4.55 TBq mmol⁻¹) was used as a tracer instead of tritiated GA₄.

Cloning of gene fragments encoding VH and VL of 21/D13. A cDNA mixture was prepared by the conventional method from hybridoma cells. Amplification of the VH and VL fragments was performed with the following degenerated primers: VH-sense, 5'-gctctgcaacacgggcccagccggcc(SR)atgtgcagccatttctcctcgagct-3; VH-antisense, 5'-ccttcagttct(ApAlI)agacattgagcttcacctctgctca-3; VL-sense, cattgactcgag(XhoI)gacaggaggtgacgttgctctgccccc-3; VL-antisense, a mixture of the four primers, 5'-ggtctcctggcagcgcg(NotI)ccgttgtatttccaggttgctccc-3, 5'-ggtctcctggcagcgcg(NotI)ccgttgtatttccaggttgctccc-3, 5'-ggtctcctggcagcgcg(NotI)ccgttgtatttccaggttgctccc-3 and 5'-ggtctcctggcagcgcg(NotI)ccgttgtatttccaggttgctccc-3'. Each fragment digested with the restriction enzymes indicated in the primer sequences was cloned in the corresponding restriction sites of the phagemid vector, pHEN2 (generously provided by Dr. G. Winter, MRC). Preparation of the scFv genes of the 8/E9 antibody and anti-GA₂₄ antibody were as described by Suzuki et al.⁵ and Shimada et al. respectively.¹

Construction of the randomly mutagenized scFv phage-display libraries. The scFv genes with random mutations were constructed in pHEN2. These genes were amplified under conditions to incorporate errors,⁸ using 7 mM MgCl₂, 0.5 mM MnCl₂, 0.2 mM dATP, 0.2 mM dGTP, 1.0 mM dTTP, 1.0 mM dCTP, 0.5 μl of AmpliTaq polymerase (Amersham Biosciences, NJ), and a 10-fmol template vector in a total volume of 50 μl, while PCR was performed under standard conditions to obtain scFvs with no mutations, using 1.5 mM MgCl₂, 0.2 mM dNTPs, and no MnCl₂. The respective sense and antisense primer sequences were 5'-ggcccatgg(NcoI)agtgagaagcttgagtcggt-3' and 5'-tgggcgcgcg(NotI)ccgtttaatctccacctgtgcc-3'. The amplified fragments were ligated with pGEM-T Easy and transformed into DH 10B by electroporation. Plasmids were prepared from the culture of >10⁶ independent colonies and digested with NcoI and NotI (partially digested for the 8/E9 and anti-GA₂₄ scFv genes) before the excised scFv fragments were ligated into the same sites of pHEN2 and introduced into the TG1 cells by electroporation.

Panning procedure and cloning of monoclonal scFvs with antigen-binding activity. The TG1 cells containing each construct were incubated in a 2 × TY medium containing 1% glucose and ampicillin (100 μg ml⁻¹) at 37°C until the OD₆₀₀ value reached 0.5, to 10 ml of which solution helper phage M13K07 was infected in the ratio of 1:20. The cells were pelleted at 3,300 g for 10 min and re-suspended in 50 ml of the 2 × TY medium containing ampicillin (100 μg ml⁻¹) and kanamycin (25 μg ml⁻¹) before being incubated overnight at 30°C. The culture was centrifuged at 10,800 g for 10 min, and the resulting supernatant was mixed with 1/5 volume of a PEG solution (20% (w/v) PEG6000 and 2.5 mM NaCl). After incubating on ice for 1 h, the phages were precipitated by centrifuging at 10,800 g for 10 min. The pellet was re-suspended in 2 ml of PBS and centrifuged at 11,600 g for 10 min to remove the remaining bacterial debris. An immunotube (Nunc) was coated overnight with a BSA-GA₄ conjugate in 50 mM NaHCO₃ (pH 9.6) at the concentration indicated in “Results”. After blocking the surface of the tube with 2% (w/v) skim milk in PBS, 10¹² colony-forming units (cfu, about 1 ml of the above-mentioned phage preparation) of the phage solution in 4 ml PBS containing 2% skim milk was added to the immunotube, which was incubated at ambient temperature for 2 h. The tube was extensively washed with PBS containing 0.1% (v/v) Tween-20 and then with PBS. The bound phages were eluted by incubation with 7.18M triethylamine for 10 min, and the solution was neutralized with a half volume of a PEG solution (20% (w/v) PEG6000 and 2.5 mM NaCl). After centrifuging on ice for 1 h, the phages were precipitated by centrifuging at 10,800 g for 10 min. The pellet was re-suspended in 2 ml of PBS and centrifuged at 11,600 g for 10 min to remove the remaining bacterial debris. An immunotube (Nunc) was coated overnight with a BSA-GA₄ conjugate in 50 mM NaHCO₃ (pH 9.6) at the concentration indicated in “Results”. After blocking the surface of the tube with 2% (w/v) skim milk in PBS, 10¹² colony-forming units (cfu, about 1 ml of the above-mentioned phage preparation) of the phage solution in 4 ml PBS containing 2% skim milk was added to the immunotube, which was incubated at ambient temperature for 2 h. The tube was extensively washed with PBS containing 0.1% (v/v) Tween-20 and then with PBS. The bound phages were eluted by incubation with 7.18M triethylamine for 10 min, and the solution was neutralized with a half volume of 1 M Tris–HCl (pH 7.4). The eluted phages were infected with the exponentially growing TG1 cells, plated onto a TYE solid medium containing 1% (w/v) glucose and ampicillin (100 μg ml⁻¹), and incubated overnight at 30°C. The colonies were recovered in the 2 × TY medium and used for phage preparation in the next round of panning, as described above. Independent colonies were picked and liquid-cultured in the same way to produce the monoclonal phage-displayed antibody.

Preparation of soluble scFvs. The phage clones (10⁷ cfu) were infected with 200 μl of the exponentially growing HB2151 cells and incubated on TYE plates containing 1% (w/v) glucose and ampicillin (100 μg ml⁻¹) at 37°C. The independent colony was inoculated into 2 × TY containing 1% (w/v) glucose and ampicillin (100 μg ml⁻¹) and then cultured overnight. This overnight culture (100 μl) was incubated in 10 ml of 2 × TY containing 1% (w/v) glucose and ampicillin.
(100 \mu g \text{ml}^{-1}) at 37 ^\circ \text{C} until the OD_{500} value reached 0.9. The culture was centrifuged at 3,300 \text{g} for 15 \text{min}, and the pellet was re-suspended in 10 \text{ml} of 2 \times \text{TY} containing ampicillin (100 \mu g \text{ml}^{-1}) and 1 \text{mM IPTG}, and then cultured overnight at 30 ^\circ \text{C}. The culture medium was recovered by centrifugation at 3,300 \text{g} for 15 \text{min} as a soluble scFv preparation.

**ELISA analysis.** Each well of ELISA plates was coated overnight with 100 \mu l of a solution of the BSA-GA4 conjugate in NaHCO3 (50 mM, pH 9.6) at the concentration indicated in “Results”. After blocking the surface of the wells with 200 \mu l of 2% (w/v) skim milk in PBS, 100 \mu l of the phage solution ten times diluted with 2% (w/v) skim milk in PBS was added, and the plates were incubated for 1 h. An excess of GA4 (0.2 mM) was mixed with the phages before the reaction with the coated antigen for competitive ELISA. The wells were washed with PBS containing 0.1% (v/v) Tween-20 and then with PBS alone, and the bound phages were detected with the horseradish peroxidase (HRP)-conjugated anti-M13 antibody (5,000 times diluted with PBS, Amersham Biosciences, NJ) and subsequent reaction by adding 100 \mu l of a substrate solution [100 mM sodium acetate (pH 6) containing 3,3’5,5’-tetramethylbenzidine (100 \mu g \text{ml}^{-1}) and hydrogen peroxide (0.6% (v/v))]. The enzyme reaction was stopped by adding 50 \mu l of 1 M H$_2$SO$_4$. Peroxidase activity was evaluated by calculating the difference in absorbance (A) at 630 nm and 450 nm (A$_{630}$–A$_{450}$). To measure the binding activity of soluble scFvs, bound scFv was detected by incubation with the anti-c-myc antibody (100 \mu l of 9E10 mouse ascites 1,000 times diluted with PBS) for 1 h, followed by incubation with the HRP-conjugated anti-mouse antibody (a 100-\mu l solution 5,000 times diluted with PBS, Amersham) as a secondary antibody for 1 h.

**Western blotting analysis of scFvs.** The phage solution or culture medium containing soluble scFvs (the amounts are indicated in Fig. 6) was applied to SDS–PAGE. The proteins were electrotransferred onto a nitrocellulose membrane, and a c-myc tag fused to scFv or scFv-gIIIp was detected with the 9E10 antibody (an anti-c-myc tag antibody) and HRP-conjugated anti-mouse antibody, as for ELISA. HRP activity was detected with an ECL Plus western-blotting detection system (Amersham) and by exposure to X-ray films.

**Results**

**Characterization of the 21/D13 antibody**

Since we were unsuccessful in producing a functional 8/E9-scFv, although we could prepare functional scFvs for some other monoclonal antibody lines, we reasoned that this failure was attributable to the intrinsic characteristics of the 8/E9 antibody. Another monoclonal antibody line was therefore established. The antibody obtained, 21/D13, showed equally high affinity in a radioimmunoassay to GA4 and GA1, both of which have a common structure apart from one hydroxyl group difference on the D ring (Fig. 1). On the other hand, the antibody showed no significant cross reactivity (<0.01% of GA4) to GA9 and GA20, which lack the 3\beta-hydroxyl group on the A ring, or to GA13, which has no lactone ring bridging the two carbons on the A ring. GA3, which has an olefin group on the A ring, also showed low affinity (0.4% of GA4) to the antibody. These results...
indicate that the antibody specifically recognized the structure of the A ring. Since the immunogen had been prepared by modifying the exo-methylene group on the D ring residing on the opposite side of the A ring in the GA molecule to be linked to BSA, this result was in accordance with our expectations. The binding affinity of the 21/D13 antibody was similar to that of 8/E9, apart from 8/E9 showing relatively high affinity to GA$_3$ (17% of GA$_4$). Taking into account that GA$_3$ is a physiologically active GA comparable to GA$_4$ and GA$_1$ in many bioassay systems, perhaps 8/E9 was mimicking the gibberellin receptor better than 21/D13.

Screening functional scFvs by panning

In spite of efforts to obtain functional recombinant antibodies involving the use of such different production systems as phage-display scFv fused to gIII protein and GST-fused scFv by a conventional E. coli expression system, no significant binding activity was apparent for the 8/E9 and 21/D13 antibodies (data not shown). Unpublished information, including personal communications, indicated that this kind of failure is not rare but is generally encountered without any rational interpretation, so we applied an "evolutionary engineering method" to overcome the problem. Although this method has been used for obtaining antibodies with higher affinity or higher stability than the original clone, it has not been applied for the purpose in question in this study. Taking advantage of a panning procedure that allows screening of a library composed of $>10^{12}$ clones, we constructed scFv phage-display libraries with random point mutations incorporating error-prone PCR in the scFv region for 8/E9 and 21/D13 scFvs. A library with errors was also constructed with anti-GA$_{24}$ scFv as a negative control to monitor the enrichment of phages during the panning procedure. Since the N-terminal sequences of mature V$_H$ fragments of the 8/E9, 21/D13 and anti-GA$_{24}$ antibodies are similar, the N-terminal sequences of scFvs were adjusted to be identical using the same PCR primer (Fig. 2, and a

**Fig. 2.** Sequences of 8/E9- and 21/D13-scFvs. (A) 8/E9-scFv sequence constructed in this study. (B) 21/D13-scFv sequence constructed in this study. The complementarity-determining regions are double-underlined, the linker peptides are shown in italics, the underlined sequences are the annealing sites of the PCR primers to amplify the scFv genes, and arrow heads indicate the signal peptide cleavage sites predicted by the pSORT program.
The scFv gene was inserted in-frame to a stretch of histidine residues, an amber stop codon, and a gIII coating protein. In the amber-suppressor strain of *E. coli* like TG1, the scFv is produced as a fusion product of gIII protein, while soluble scFv is expressed only with the His/c-myc-tag in a non-amber-suppressor strain like HB2151.

**Table 1.** The Fluctuation of Titers after Successive Panning Steps

<table>
<thead>
<tr>
<th>conc. of coating antigen (µg/ml)</th>
<th>8/E9</th>
<th>21/D13</th>
<th>anti-GA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st 50</td>
<td>3.2 × 10^4</td>
<td>5.3 × 10^4</td>
<td>1.2 × 10^5</td>
</tr>
<tr>
<td>2nd 50</td>
<td>2.8 × 10^7</td>
<td>7.4 × 10^7</td>
<td>5.1 × 10^7</td>
</tr>
<tr>
<td>3rd 1</td>
<td>1.6 × 10^8</td>
<td>3.3 × 10^8</td>
<td></td>
</tr>
<tr>
<td>4th 1</td>
<td>6.7 × 10^8</td>
<td>4.1 × 10^7</td>
<td></td>
</tr>
<tr>
<td>5th 1</td>
<td>4.2 × 10^9</td>
<td>1.3 × 10^7</td>
<td></td>
</tr>
<tr>
<td>2nd^0.1</td>
<td>0.1</td>
<td>7.8 × 10^7</td>
<td>1.2 × 10^8</td>
</tr>
<tr>
<td>3rd^0.01</td>
<td>0.01</td>
<td>1.2 × 10^7</td>
<td>1.1 × 10^8</td>
</tr>
<tr>
<td>4th^0.001</td>
<td>0.001</td>
<td>6.8 × 10^6</td>
<td>4.7 × 10^7</td>
</tr>
</tbody>
</table>

The possibility that the scFvs on the enriched phages did not recognize the GA moiety but other parts of the BSA-GA₄ conjugate instead, the amplified phages were panned against GA₄ conjugated with KLH (5th panning). The titers were increased by another order for 8/E9 and remained at the same level for 21/D13, strongly suggesting that a substantial population of the enriched scFv clones recognized the GA moiety. Since a change in concentration of the coating antigen from 50 µg ml⁻¹ to 1 µg ml⁻¹ did not give any dramatic change in the titer, the amount of antigen was decreased stepwise to a much lower concentration. This procedure of using a lower concentration of antigen for panning of a randomly mutagenized scFv library has been successfully adapted to obtain scFv with higher affinity, and is known as “affinity maturation”.[10] The amplified phages after the first panning, which should include multiple clones, were panned against BSA-GA₄ coated at 0.1 µg ml⁻¹ for the second panning (designated the 2nd^0.1 panning). For the third and fourth panning steps (respectively designated the 3rd^0.01 and 4th^0.001 panning), 0.01 and 0.001 µg ml⁻¹ of BAS-GA₄ were used for coating the polystyrene tubes. Even with the lowest concentration, significantly more phages were recovered (>10ª) than with the first panning (Table 1).

**Identification of monoclonal scFvs**

To investigate whether the clones after the 4th^0.001 panning had higher affinity than those obtained after the other panning steps, the binding affinity was roughly compared by ELISA among 8/E9-phages after the 3rd, 4th, 5th, 2nd^0.1, 3rd^0.01, and 4th^0.001 pannings, as well as phages displaying 8/E9 scFv with no mutations (Fig. 4). The phages from all the panning steps, except for those displaying non-error scFvs, showed affinity to BSA-GA₄, which was competed by an excess of free GA₄. The result, however, unexpectedly showed no significant difference in the amount of phages captured by BSA-GA₄ regardless of its concentration for coating. Further characterization of the phages obtained was made by selecting independent monoclonal phages, and scFvs were expressed as phage-displayed scFvs and free soluble scFvs. After 96 clones had been examined subsequent to the 2nd, 5th, and 4th^0.001 pannings, more than 80% of the phage-displayed scFvs showed binding to BSA-GA₄, which was again competed by excess GA₄. There was no significant difference in the percentages of positive clones from the three panning
steps. Some of the scFvs which showed binding in the phage-displayed form were expressed as soluble scFv in the HB2151 E. coli host, a non-amber suppressor strain. ScFv is supposed to be expressed only as a fusion product with c-myc tag and His-tag sequences (Fig. 3). The result is shown in Fig. 5 and in Tables 2 and 3. In respect of the 8/E9 scFvs, seven (2nd-1, 2nd-5, 4th-0.001-B6, 4th-0.001-E5, 4th-0.001-F6, 4th-0.001-G1, and 4th-0.001-G6) out of the eight examined clones showed binding to BSA-GA₄ (Table 2), while non of the twelve 21/D13 clones showed any binding (Table 3).

Characterization of each monoclonal scFv
To identify the sequence variation and also to understand why some clones showed binding in both phage-displayed and soluble forms, while others showed binding in only the phage-displayed form, the scFv sequences of some of the clones were determined. There

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Fig. 4. ELISA Analysis of Polyclonal 8/E9-scFvs after Various Steps of Panning.
The ELISA plate was coated with various concentrations (indicated in the figure) of GA₄-BSA, and the amounts of phages bound to the antigen were measured by the anti-M13 antibody labeled with HRP as a rough evaluation of affinity.

Fig. 5. ELISA Analysis of Monoclonal scFvs.
The selected monoclonal scFvs were produced in both a phage-displayed form and a soluble form. The ELISA plate was coated with GA₄-BSA (1 µg ml⁻¹), and phage-displayed scFvs or soluble scFvs were reacted with the coated antigen. The bound scFvs were detected by anti-M13 or anti-c-myc antibodies.
were some variations in clones after the 2nd and 5th pannings, but all six sequenced clones after the 4th panning were identical for 8/E9 (Table 2), while four out of six 21/D13 clones after the 4th panning were identical, and two showed different mutations (Table 3). Surprisingly, there was a nonsense (frameshift) mutation caused by deletion of the 5th adenine in some of the clones, although they showed binding activity to BSA-GA4 in the phage-displayed form: 3 out of 22 clones sequenced for 8/E9, and 11 out of 12 clones for 21/D13. The clones which showed no binding when expressed in HB2151 corresponded to these clones with the nonsense mutation. All the selected clones showed at least one amino acid substitution, in addition to the mutation at the 5th adenine causing the frameshift mutation or the E2G substitution. Most of the additional mutations were not in the complementarity-determining region (CDR) residues, but instead in the framework residues.

**Western blotting analysis of scFvs**

Western blotting analysis was performed to investigate whether scFv-gIIIp fusion proteins were produced and incorporated in the phage particles (Fig. 6A, Table 2). The 8/E9-clone with the frameshift mutation, 5th-A5, showed clear bands at the size of scFv-gIIIp, while the clone with no mutation showed a very faint band. The 21/D13 clones with the frameshift mutation, 2nd-6, 4th0.001-A12, and 4th0.001-B8, likewise showed the presence of scFv-gIIIp fusion proteins in a virion, as well as the clone with no mutation. When soluble scFvs were induced in non-suppressor strain HB2151, the clone with the nonsense mutation did not produce scFv at a detectable level, while other clones without the frameshift mutation gave clear bands at the size of scFv, except for those scFvs with no mutation of 8/E9 and 21/D13 which respectively did not give any detectable band or gave a clear band with a larger molecular size than the others (Fig. 6B and Table 3). This result shows that the frameshift mutation was complemented in the TG1 cell to be able to express the scFv-gIIIp fusion protein in the phage-displayed form by a mechanism that did not work in HB2151 cells and resulted in a failure of scFv production. Since all the clones which showed binding activity as a phage-displayed scFv contained one base deletion or base substitution to guanine at the 5th adenine respectively, causing a frameshift or E2G substitution, this position was thought to be one of the keys to binding activity. Hence we introduced the same substitution from adenine to guanine into the original sequence of scFv to see whether only this mutation could confer binding activity. Both 8/E9 and 21/D13 with the E2G substitution showed the presence of scFv-gIIIp in the phage virion and binding activity in the form of phage display (Fig. 5 and 6A). On the other hand, these clones did not show binding activity when produced as soluble scFvs in HB2151, although the production of proteins of the correct size were detected (Fig. 6B). This result indicates that, although the point mutation of E2G was important, it was not alone sufficient to confer binding activity on the soluble scFv.
We further introduced the same mutation to 21/D13-4th0:001-D11, which showed binding activity when expressed in the phage-display form but no scFv production in HB2151 due to the frameshift mutation, to confirm whether the scFv would become functional if resulting from in-frame production. The soluble scFv was detected by western blotting analysis and found to be functional as expected (Fig. 5 and Fig. 6B).

Surprisingly, the overall results from the western analyses indicate that the level of the fusion protein was higher in the clones with the frameshift mutation than in those without it.

Discussion

Although there are numerous reports on the preparation of scFvs, the conventional method for preparing them sometimes results in non-functional scFvs. Since the non-functional scFvs, including clones with the E2G substitution, were detected in the soluble fraction in this study, the non-desirable results are not a matter of the stability of scFvs in the production system involving E. coli. In our work, scFv of neither 8/E9 nor 21/D13 produced by certain conventional expression systems showed antigen-binding activity. The fact that there were both successful and unsuccessful preparations of functional scFvs, even by similar constructions, caused us to speculate that a minor modification to a non-functional scFv could give a functional scFv. A rational approach to modify scFv sequences was not available, so we utilized the "in vitro evolution" approach, involving screening scFv variants with improved binding activity from a randomly mutagenized scFv library.

We further introduced the same mutation to 21/D13, which showed binding activity when expressed in the phage-display form but no scFv production in HB2151 due to the frameshift mutation. The soluble scFv was detected from frameshifted clones(4th0:001-D11), which showed binding activity when expressed in HB2151.

Table 3. Summary of Mutations in Mutagenized 21/D13-scFv Genes and Their Binding Activities

<table>
<thead>
<tr>
<th>mutations</th>
<th>binding activity</th>
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<tbody>
<tr>
<td>A5</td>
<td>A40</td>
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<tr>
<td>4th0:001-A12</td>
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<tr>
<td>4th0:001-B8</td>
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<td>4th0:001-G7</td>
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</tr>
<tr>
<td>non-Er(E2G)</td>
<td>DEL</td>
</tr>
<tr>
<td>4th0:001-D11(E2G)</td>
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</tr>
</tbody>
</table>

Fig. 6. Western Blotting Analysis of Soluble and Phage-Displayed scFvs.
(A) The phage-displayed scFvs (equivalent to 5 × 10⁸ cfu) were separated by SDS–PAGE and detected with the anti-c-myc tag antibody. Loading of approximately equal amounts was confirmed by Ponceau S staining of the blotted membrane (data not shown). (B) Five microliters of a culture medium containing the soluble scFv was separated by SDS–PAGE and detected with the anti-c-myc antibody.
it is highly likely that it would be effective as a general method for obtaining functional scFvs when those with no mutations are not functional.

Besides getting functional scFvs, we expected to be able to select clones with better binding by panning against an antigen coated at a relatively low concentration, since this method has been established as “affinity maturation" to obtain clones with higher affinity. Although the apparent affinity from an ELISA analysis was not significantly increased by this procedure, there must have been a kind of selection pressure, because all the 8/E9 clones after the 4th analysis was not significantly increased by this procedure to obtain clones with higher affinity. The advantage for the clones to be amplified by successive panning steps might be slightly higher affinity to the antigen, or a higher infection rate to the E. coli host compared to the other clones. Another round of random mutagenesis of the clones selected in this study and panning selection might give clones with clearly higher affinity.

The sequence analysis of the scFvs showed that the mutation on the 5th adenine was conserved in all the clones, which had affinity to GA4 in a phage-displayed form. This result strongly suggests that the mutation of the 2nd amino acid (E) was the key to binding activity for both the 8/E9 and 21/D13 antibodies. Since all the clones which showed binding to the antigen in a phage-displayed form contained not only this mutation, but also other amino acid substitutions, scFvs with only the E2G substitution were prepared. Both 8/E9- and 21/D13-E2G-scFvs showed antigen-binding activity when expressed in the phage-displayed form. The E2G mutation was not attributable to a conformational change in the scFv protein, because the position was just downstream of the signal peptide encoded in the pHEN2 vector, and the amino acid at this position was assumed to be included in the cleaved signal peptide as predicted by the pSORT program (http://psort.nibb.ac.jp/). The result therefore suggests that the E2G mutation affected the efficiency of signal cleavage. This speculation is supported by the results of the western blotting analysis showing that the amount of the scFv-gIIIp fusion protein in phages was much higher in the E2G clones than in the non-error clones (Fig. 6), which was also the case in the soluble scFv production of 8/E9 clones. On the other hand, soluble scFvs of 21/D13 were equally detected, but with different molecular sizes, between non-error clones and those with the E2G substitution. The larger size might have been due to unsuccessful signal cleavage, but it remains to be explained why the scFv was secreted into the medium without signal cleavage.

A frameshift mutation was detected in many clones, especially in the 21/D13 clones that showed antigen binding in the phage-displayed form. The results of the western blotting analysis to detect the c-myc tag showed that the clones with the frameshift mutation produced an scFv-gIIIp fusion protein of the correct size and frame, indicating that a complementary mechanism worked in the TG1 cell, probably involving ribosomal slippage. Ribosomal slippage causing a −1 shift is necessary to correct the frame of one base deletion. The major elements of −1 frameshifting consist of a slippery sequence, by which the ribosome slips a single nucleotide in the upstream direction, and a stimulatory RNA structure such as a stem-loop or pseudoknot which induces a ribosomal pause. Since the stop codon “TGA” appears immediately after the deletion site in the frameshifted mutant genes, the −1 shift must take place upstream of this codon. If the signal peptide encoded in the pHEN2 vector must be intact to be functional, the possible sequence of ribosomal slippage is restricted to the four-base stretch of guanine in -ATGGGTTGA-. Although this sequence does not match the typical slippery sites consisting of a heptamer sequence of the form XXXYYYY, a stretch of four or more single-nucleotide repeats sometimes functions as a slippery sequence. The result that the amount of scFv-gIIIp incorporated in phages was higher in the clones with the frameshift mutation than in those with the correct frame suggests not only that ribosomal slippage takes place at very high frequency, but also that the frameshifting has an unknown advantage for effective translation compared to in-frame translation of the non-deletion clones. Although a detailed discussion requires determining the protein sequences, it can be concluded that this kind of frameshift event is one of the reasons scFv clones functional in a phage-displayed form are sometimes non-functional in a soluble form.

The binding activity of the selected clones was not necessarily attributable to the E2G mutation, because the clones with only this mutation were not functional as soluble scFvs. The non-Er(E2G) proteins which had only the E2G mutation and did not show binding in the soluble form, and the other scFv proteins which had other mutations in addition to the E2G mutation and showed binding in the soluble form were equally detected in the western blotting analysis (Fig. 6b), indicating that their differential binding activity can be explained not by the different protein levels, but rather by the conformational difference conferred by mutations other than E2G. The reason scFvs with only the E2G mutation were functional solely in the phage-displayed form and not in the soluble form is not clear. The form of the fusion protein with gIIIp and/or assembly into a phage virion as a coating protein might have caused a conformational restriction to the scFv proteins on becoming functional. The crystal structure of the GA4-liganded 8/E9 Fab has indicated that the GA4 molecule interacted only with the CDR residues of Vh. It is apparent that the binding activity was recovered in the mutated scFvs not by any change to the binding sites of scFvs, but instead by a change of their frameworks. Daugherty et al. have also reported that the clones with higher affinity to the antigen were obtained from mutations to the framework residues. The results indicate that a mutation distant from the binding site...
exerted a profound effect on the binding characteristics. This further indicates that an improvement in functional affinity resulted from diverse and poorly predictable mechanisms, thus confirming the effectiveness of the random mutagenesis approach.

References


