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Light-chain residue 95 is critical for antigen binding and multispecificity of monoclonal antibody G2

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ABSTRACT

The monoclonal antibody, G2, specifically binds to the immunogen peptide derived from the chicken prion protein, Pep18mer, and two chicken proteins derived peptides, Pep8 and Pep395; G2 binds with equal affinity to Pep18mer. The amino acid sequences of the three peptides are completely different, and so the recognition mechanism of G2 is unique and interesting. We generated a single-chain Fv (scFv) antibody of G2, and demonstrated its correct folding with an antigen binding function similar to intact G2 antibody. We also generated a Pro containing mutant of G2 scFv at residue 95 of the light chain, and analyzed its antigen binding using a surface plasmon biosensor. The mutant lost its binding ability to Pep18mer, but remained those to Pep8 and Pep395. The results clearly indicate residue 95 as being critical for multispecific antigen binding of G2 at the site generated from the junctional diversity introduced at the joints between the V and J gene segments.

1. Introduction

Antibodies recognize various types of antigens at antigen-combining sites, which are constructed mainly from amino acid residues in complementarity determining regions (CDRs). Antibody diversity is generated by four main processes: 1) combinatorial diversity of heavy- and light-chain variable regions (VH and VL, respectively), 2) junctional diversity introduced at the joints between the gene segments, 3) combinatorial diversity of the possible different combinations of VH and VL, 4) somatic hypermutation mutated into the rearranged variable regions [1]. Each antibody specifically recognizes the particular immunized antigen. In contrast to this general phenomenon, we recently found that monoclonal antibody, G2, obtained by immunization of BALB/c mice with chicken prion protein (ChPrP), binds to the peptide derived from ChPrP, Pep18mer, and to peptides derived from two other chicken proteins, Pep8 and Pep395 [2,3]. The amino acid sequences of Pep8 and Pep395 are completely different from that of Pep18mer (Fig. 1A). These peptides were found during the study of the reaction of G2 with chicken brain using a complementary synthesized DNA library. We also previously demonstrated that the G2 binding affinities to Pep18mer and Pep8 are similar and high; the equilibrium association constant, K_a, determined using a surface plasmon resonance (SPR) biosensor, are 3.4×10^7 M^{-1} and 6.3×10^7 M^{-1}, respectively [2]. The SPR experiments also revealed higher association and dissociation rate constants, k_{on} and k_{off}, of Pep18mer than Pep8, indicating a different recognition mechanism of G2 to the two peptides. Some residues of G2 are considered to be somatically mutated (Fig. 2B). Whether somatic hypermutations introduced to increase binding affinity induce a change in specificity indicative of cross-reactivity to antigens remains controversial [4,5]. Oda and Azuma recently reported the increased affinity resulting from tuning of the complementary nature of the antibody combining sites to the structure of the immunizing antigen, (4-hydroxy-3-nitrophenyl)acetyl (NP) [6].

In this study, we produced a single-chain Fv (scFv) antibody of G2, and analyzed its structural, physical, and functional properties. One advantage in generating scFv antibodies is that the specific site of scFv can be changed by site-directed mutagenesis, and its effects on structure and function can be analyzed [7]. G2 lacks the residue at VL95 (κ-VL) (Fig. 1B), while the corresponding residue is Pro in most other antibodies [8]. Accordingly, we also produced a scFv of a G2 mutant containing Pro (G2VL95P) and compared the structural

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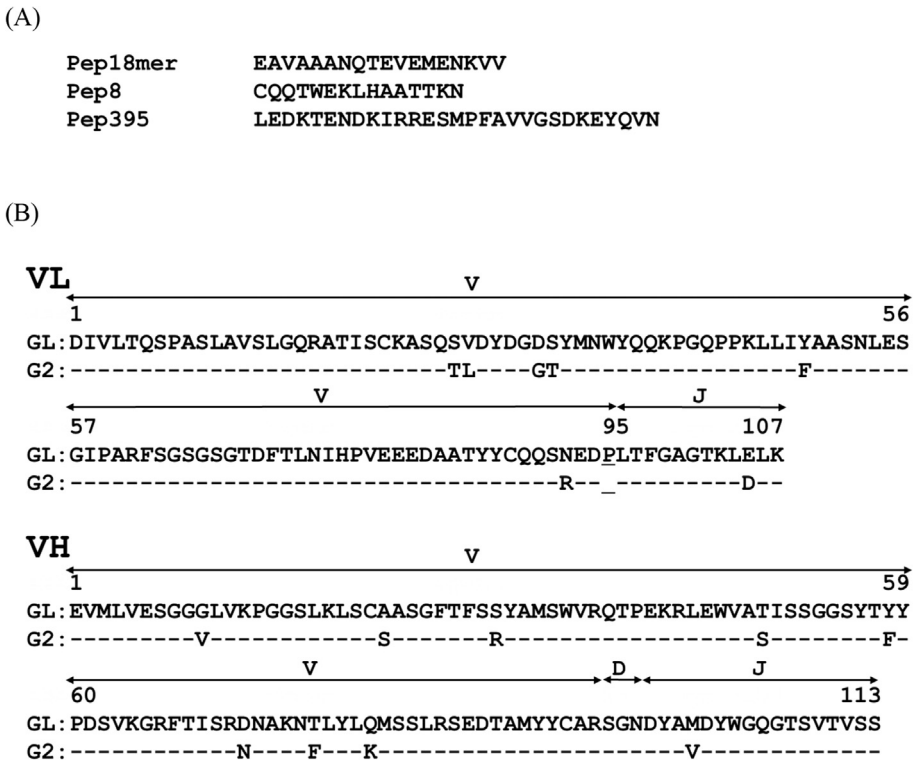

Fig. 1. Amino acid sequences of antigen peptides (A) and variable region of G2 antibody in comparison with those of germline-type antibody (B). The sequence of germline-type antibody (GL) was predicted from the alignment program, IgBlast [12]. The arrows indicate the regions corresponding to V, D, and J genes in VL and VH. The underlined site corresponds to the residue at VL95. The amino acid residues are numbered according to the consensus nomenclature of Kabat et al. [8].

and functional properties with those of G2 itself. The results clearly showed that the missing residue at VL95 is critical for the multispecificity of G2.

2. Materials and methods

2.1. Expression and purification of scFvs

Genes encoding the G2 scFv (-VL-$(G_4S)_3$ linker-VH-) were amplified by PCR using the template encoding VL and VH domains of G2 with following primers: VL-*Nco*I-sense: 5′-AAAACCATGGACATTGTACTGACCCAA-3′, VL-GS-antisense: 5′-ACCAGAGCCGCCGCCGCCGCTACCACCACCACCTTTCAGGTCCAGCTT-3′, VH-GS-sense: 5′-AGCGGCGGCGGCGGCTCTGGTGGTGGTGGATCCGAAGTGATGCTGGTG-3′, VH-*Eco*RI-antisense: 5′-AAAAGAATTCTCATCATGAGGAGACGGTGACTG-3’. The amplified gene fragment was inserted into plasmid pET-28a. The plasmids of G2 and G2VL95P, constructed by site-directed mutagenesis, were transformed into *E. coli* BL21 (DE3) codon plus, and the transformed cells were cultured in LB medium at 37 °C. When the culture reached an A_{600} of about 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. Cells were cultured for a further 12 h at 22 °C. The harvested cells were suspended in phosphate buffered saline (PBS), pH 7.4, and lysed by sonication. The insoluble fraction was collected by centrifugation and resuspended in washing buffer, 50 mM Tris-HCl (pH 9.0) containing 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100. This washing process was performed three times, and then, the sample was washed a further three times using washing buffer without Triton X-100 to remove the surfactant. Finally, the pellet was solubilized in solubilization buffer, 50 mM Tris-HCl (pH 9.0) containing 8 M urea, 150 mM NaCl, 1 mM EDTA, and 10 mM DTT. The solubilized scFv was refolded by stepwise dialysis. The urea concentration was decreased stepwise from 8 M to 0 M, via 2, 1 M. As additives, 0.6 mM glutathione (oxidized form), 1.2 mM glutathione (reduced form) and 0.4 M L-arginine were added at the 1 M urea step. Following dialysis, the solution was centrifuged to remove minor precipitation. Finally, the scFv was purified using an antigen column, in which glutathione S-transferase (GST)-tagged Pep8 with was immobilized on the resin (NHS-activated Sepharose 4; GE Healthcare). Captured scFv on the column was eluted with 10 mM Gly-HCl (pH 2.7) and neutralized immediately with 2 M Tris-HCl (pH 9.0). The concentration of scFv was calculated with molar absorption coefficient of $4.18 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm.

2.2. Expression and purification of antigen peptides with GST-tag

Genes encoding the antigen peptides were amplified by PCR using following primers: Pep18mer-*Spe*I-sence: 5′-AAAAACTAGTGAAGCTGTTGCAGCAGCAAATCAAACTGAGGTCGAG-3′, Pep18mer-*Xho*I-antisence: 5′-TTTTCTCGAGTCAGACAACCTTGTTTTCCATCTCGACCTCAGTTTGAT-3′, Pep8-*Spe*I-sence: 5′-AAAAACTAGTTGTCAGCAGACTTGGGAAAAGCTACATGCAGCGAC-3′, Pep8-*Xho*I-antisence: 5′-AAAACTCGAGTCAGTTCTTCGTAGTCGCTGCATGTAGCTTTTC-3′, Pep395-*Spe*I-sence: 5′-AAAAACTAGTCTGGAGGATAAAACAGAAAACGACAAAATCAGGAGGGAAAGCATGCCCTTTGCA-3′, Pep395-*Xho*I-antisence: 5′-TTTTCTCGAGTCAATTCACTTGGTACTCCTTGTCACTGCCCACTACTGCAAAGGGCATGCTTTCCCT-3’. The amplified gene fragment was inserted into plasmid pET-49b. The plasmids were transformed into *E. coli* BL21 (DE3) codon plus, and the transformed cells were cultured in LB medium at 37 °C. When the culture reached an A_{600} of about 0.6, IPTG was added to a final concentration of 0.1 mM. Cells were cultured for a further 12 h at 22 °C. The harvested cells were suspended in PBS (pH 7.4), and

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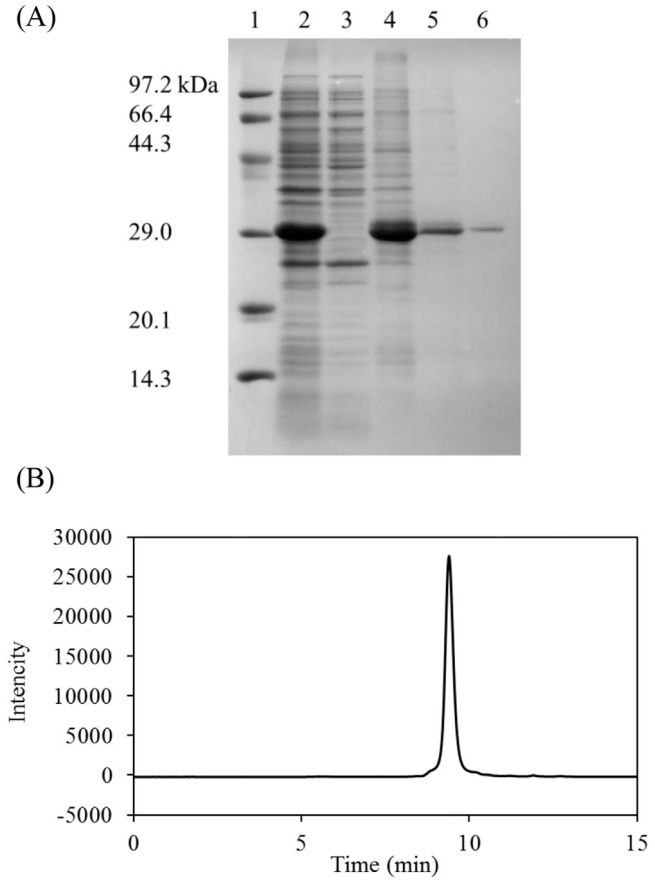

Fig. 2. SDS-PAGE and gel-filtration chromatography analyses. (A) 15% SDS-PAGE gel showing fractions from each purification step; lane 1: protein molecular weight marker. 2: after sonication, 3: soluble fraction; supernatant of sonicated suspension, 4: solubilization of insoluble fraction, 5: collected fraction after stepwise dialysis, 6: eluate of antigen column using 10 mM Gly-HCl (pH 2.7). (B) Gel-filtration chromatography analyses. A solution of 0.5 mg/ml G2 scFv was applied, and eluted at 9.4 min. The retention times of BSA, OVA, and HEL were 8.1, 8.5, and 10.2 min, respectively.

lysed by sonication. The soluble fraction was applied to glutathione column filled with resin (Glutathione Sepharose 4 Fast Flow; GE Healthcare). Captured antigen peptides with GST-tag on the column were eluted with PBS (pH 7.4) contains 30 mM glutathione (reduced form).

2.3. Size-exclusion chromatography

Gel-filtration HPLC was performed on a COSMOSIL 5Diol-300-II column (7.5 mm × 30 cm, Nacalai). HPLC was performed with PBS (pH 7.4) at the flow rate of 1.0 ml/min at room temperature. The eluate was monitored at 280 nm. The following proteins were used as standards to calibrate the column; bovine serum albumin (66 kDa), ovalbumin (45 kDa), and hen egg lysozyme (14 kDa).

2.4. Circular dichroism (CD) measurements

Far-UV CD spectra were measured in PBS (pH 7.4) at 20 °C on a Jasco J-825 spectropolarimeter. The scFv concentration was 0.04 mg/ml. CD spectra were obtained under the following condition: optical path length of 1 cm, scan rate of 20 nm/min, response time of 1 s, and bandwidth of 1 nm.

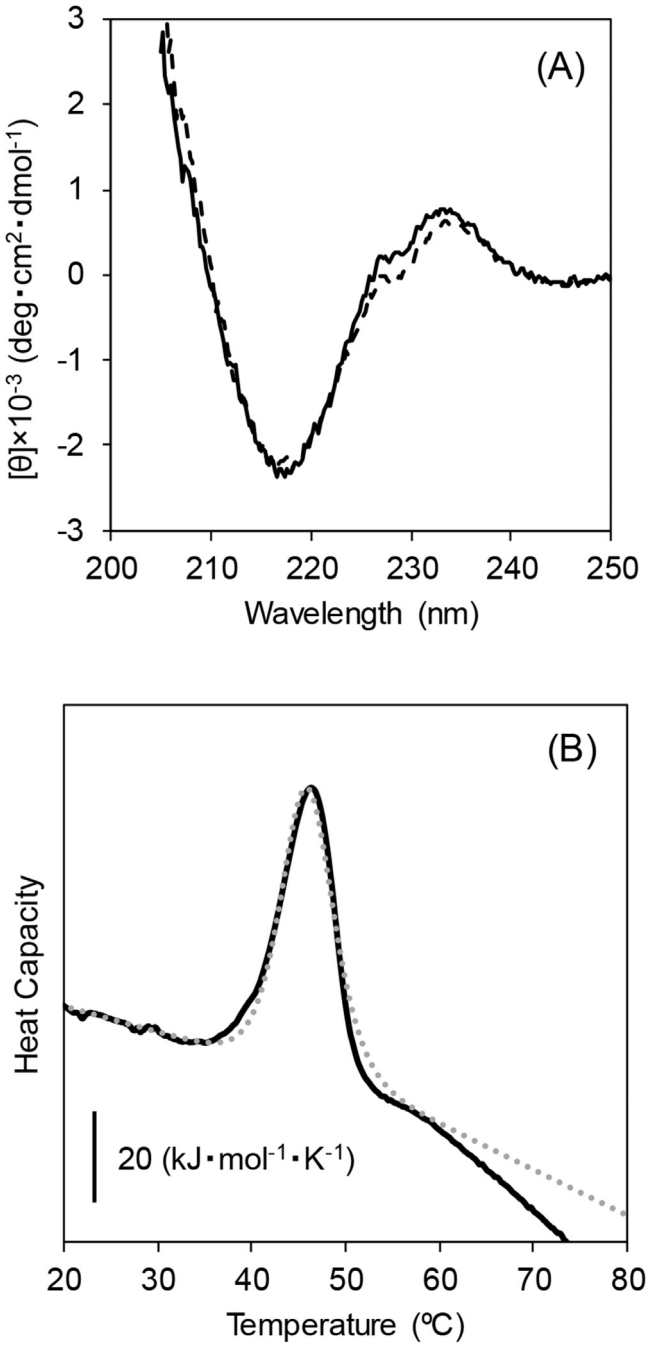

Fig. 3. Secondary structure and thermal stability analyses. (A) Far-UV CD spectra of G2 scFv (solid line) and G2VL95P scFv (broken line). (B) DSC curve of G2 scFv (solid line) with fitting curve (dotted line).

2.5. Differential scanning calorimetry (DSC) measurements

Thermal stability of scFv was analyzed using VP-Capillary DSC (Malvern). Solution of scFv at 1.0 mg/ml in PBS (pH 7.4) was heated from 10 to 90 °C at a scan rate of 1.0 °C/min. The data were analyzed using Origin 7.0 supplied by the manufacture, in order to subtract the baseline from heat capacity data and convert to molar heat capacity. The fitting analysis to obtain the denaturation temperatures (T_d) were performed by the least-squares methods on Origin 5.0 software, as described previously [9].

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2.6. SPR measurements

Antigen-antibody interactions were analyzed using SPR biosensor (Biacore T200, GE Healthcare). Antigen peptides with GST-tag, Pep18mer, Pep8, and Pep395, were immobilized on the dextran surface of a CM5 sensor chip (GE Healthcare) with amine coupling method. The resultant immobilization of Pep18mer, Pep8, and Pep395 were 280 RU, 110 RU, and 140 RU, respectively. The scFv antibodies were diluted at various concentrations in PBS containing 0.005% Tween 20 (running buffer) and the association of each solution was recorded at rate of 20 μl/min for 180 s. The dissociation of the complexes was measured by injecting running buffer alone for 180 s, followed by injecting of 3 M Guanidine-HCl containing 1 M acetate for 45 s as regeneration. The experiments were performed at 25 °C and collected data were analyzed using the BIAevaluation 3.1 software. Obtained curves were fitted to a 1:1 binding model to determine the kinetic rate constants, k_{on} and k_{off}. The K_a was calculated from the two rate constants.

3. Results

In the design of G2 scFv, VL and VH regions were connected by a linker region comprising three repeats of the peptide, GGGGS. The protein was expressed in the insoluble fraction, and solubilized using 8 M urea. Refolding was carried out by stepwise dilution of urea, as described in the "Materials and Methods" section. Most of the scFv could be solubilized, and only a little precipitation was observed during the refolding steps. Results of SDS-PAGE analyses for each purification step are shown in Fig. 2A. The final step of purification using an antigen column indicated that ~3.5% of the solubilized protein was correctly refolded and possessed antigen binding ability. To analyze the oligomeric state of scFv, gel-filtration chromatography experiments were carried out (Fig. 2B), indicating that the purified G2 scFv exists in a monomeric state.

The secondary structures of G2 scFv and its mutant, G2VL95P scFv, were analyzed using CD spectroscopy (Fig. 3A). The results indicated that both scFvs folded well into typical β-sheet rich structures. Heat capacity curves of G2 and G2VL95P scFvs analyzed at a heating rate of 1 °C/min are depicted in Fig. 3B. Thermal unfolding was irreversible. The T_d values of G2 scFv and G2VL95P scFv were 45.8 °C and 45.1 °C, respectively.

The antigen binding abilities of G2 and G2VL95P scFvs were analyzed using a SPR biosensor (Fig. 4). Binding to antigen peptides with GST-tag, Pep18mer, Pep8, and Pep395, immobilized on the sensor-chip was analyzed and the binding kinetics were determined (Table 1). No binding to immobilized GST was observed (data

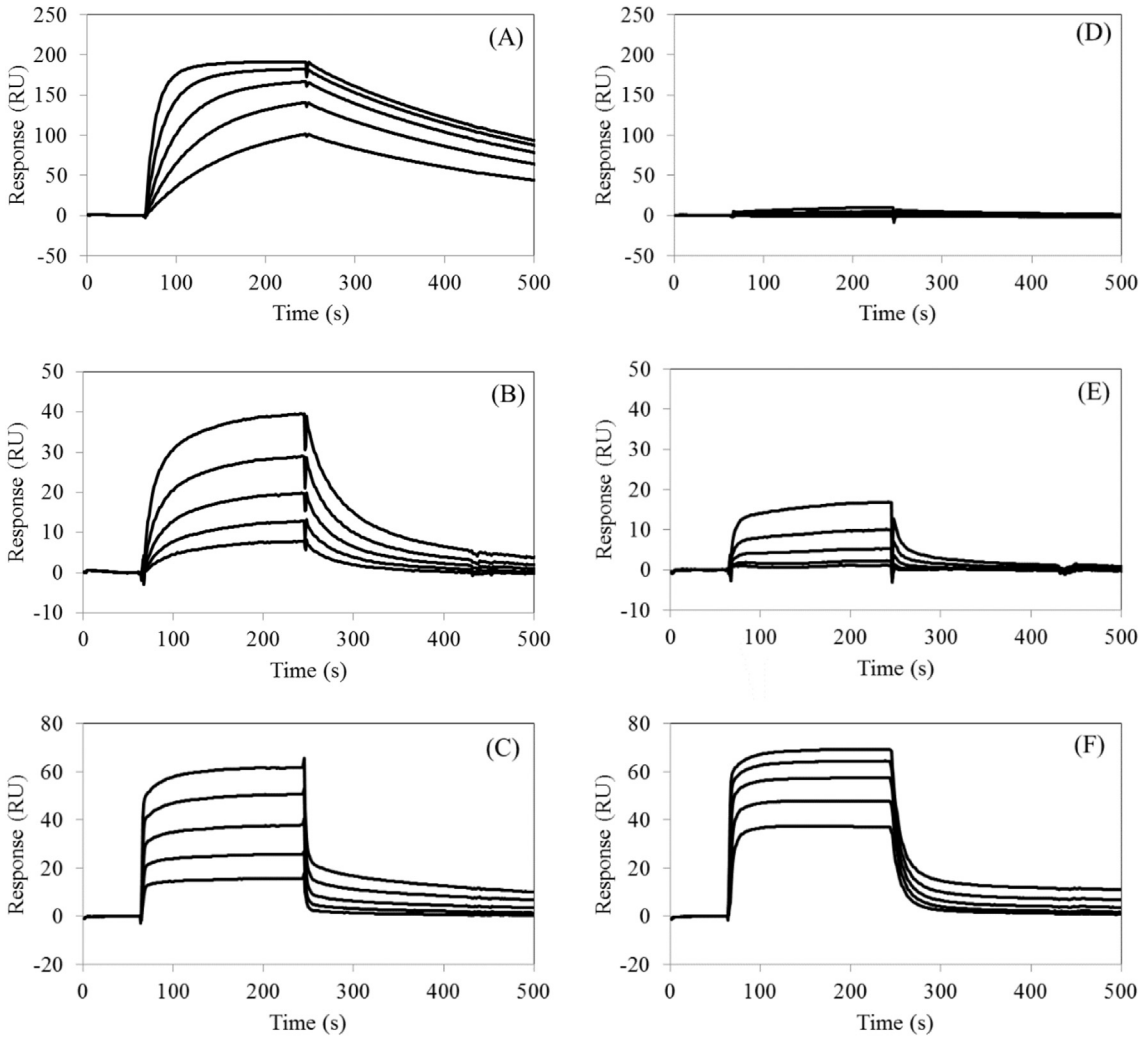

Fig. 4. SPR sensorgrams for antigen binding of G2 (A–C) and G2VL95P (D–F) scFvs. The antigen peptides with GST-tag, Pep18mer (A, D), Pep8 (B, E), and Pep395 (C, F), were immobilized on a CM5 sensorchip and the scFv (100, 200, 400, 800, and 1600 nM) was flowed over the chip surface at rate of 20 μl/min for 3 min, followed by 3 min dissociation.

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Table 1
Kinetic parameters of binding of G2scFv antibodies to immobilized antigens with GST-tag.

	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_a (M^{-1})[a]
G2 scFv[b]			
Pep18mer	5.41 (±0.13) × 10^4	2.96 (±0.06) × 10^{-3}	1.83 × 10^7
Pep8	2.28 (±0.26) × 10^4	1.52 (±0.10) × 10^{-2}	1.50 × 10^6
Pep395	1.62 (±0.01) × 10^5	6.52 (±0.32) × 10^{-2}	2.48 × 10^6
G2VL95P scFv[b]			
Pep18mer	n.d.[c]	n.d.[c]	n.d.[c]
Pep8	1.09 (±0.10) × 10^4	6.64 (±0.67) × 10^{-2}	1.68 × 10^5
Pep395	5.28 (±0.12) × 10^5	5.26 (±0.32) × 10^{-2}	1.00 × 10^7

[a] The K_a are calculated from the two rate constants: $K_a = k_{on}/k_{off}$.
[b] The k_{on} and k_{off} are the average of two experiments for protein concentrations from 100 to 1600 nM.
[c] Not determined due to the little increase in RU.

not shown). The binding kinetics of G2 scFv to Pep18mer was comparable to the previously reported kinetics of the G2 monoclonal antibody [2], indicating the correct refolding of G2 scFv into a functional structure. The binding affinity to Pep8 was lower than that of Pep18mer, due to the decreased k_{on} and increased k_{off} values. The binding affinity to Pep395 was also lower than that of Pep18mer, due to the increased k_{off} value, which was partially compensated by the increased k_{on} value. It should be noted that the introduction of Pro at VL95 eliminated the binding to Pep18mer, immunized antigen, while binding to Pep8 and Pep395 was retained. In comparison with G2 scFv, the binding affinity of G2VL95P to Pep8 was decreased, due to the decreased k_{on} and increased k_{off} values. In contrast, the binding affinity of G2VL95P to Pep395 was higher than that of G2 scFv, mainly due to the increased k_{on} value.

4. Discussion

In this paper, we first tested the validity of using scFv. SPR experiments showed that the antigen binding kinetics of G2 scFv to immobilized Pep18mer were similar to those of the Pep18mer binding to immobilized G2 monoclonal antibody [2]. Together with the results of CD and DSC, the SPR results suggest that a peptide linking VH and VL in scFv has little effect on the structure and function, and the scFv can fold correctly. The CD and DSC experiments indicated that the introduction of Pro at VL95 had little effect on the global structure of scFv (Fig. 3). In the present binding experiments, because the antigen peptides were immobilized to the sensor-chip by amine-coupling, Lys residues of peptides might affect scFv binding. In order to reduce the artificial effects from immobilization, peptides with a GST-tag were used, since most were immobilized via the Lys residues in GST. Despite the experimental design, the reduced binding affinity of G2 scFv to Pep8 in comparison with that of G2 monoclonal antibody reported previously might be due to the immobilization effects [2].

VL95 is located at the bottom of the CDR3 loop, and is considered to be generated from the junctional diversity introduced at the joints between the V and J gene segments (Fig. 1B). In this process, Pro is introduced at the residue 95 of κ-VL in most antibodies [10], while the residue is missing in G2 antibody. Antibody lacking VL95 would have an advantage to bind specifically to the immunogen, ChPrP. The present study clearly shows that Pro introduction abrogates the binding ability to Pep18mer derived from ChPrP (Fig. 4 and Table 1). Since Pro restricts the dihedral angle, the location of Pro at VL95 could stabilize the orientation of the CDR3 loop. The lack of Pro could increase the fluctuation of this loop, possibly allowing binding to Pep18mer, in addition to Pep8 and Pep395. Using antibodies against 2,4-dinitrophenyl antigen, Azuma et al. showed that the residue at the V-J boundary of the λ-VL has a pronounced effect on antigen binding [11]. As discussed in the paper, junctional amino acid variations derived from gene segment assembly on the tertiary structure of the variable region of the antibody would generate antigen-binding diversity in antibodies. To the best of our knowledge, this is the first study that demonstrates the critical role of the residue at the V-J boundary of the VL of anti-peptide antibody.

The binding kinetics of G2 scFv to Pep18mer is different from its binding kinetics to other peptides, indicating that the recognition mechanisms of G2 to respective peptides are different. For example, the k_{on} values are in order of Pep395, Pep18mer, and Pep8 (Table 1). The larger k_{on} values might be a result of the larger contribution of long-range electrostatic interactions. The significant result that the G2 mutant with Pro at VL95 lost its ability to bind to Pep18mer suggests that the CDR3 loop of VL or its fluctuation is critical for peptide recognition. In contrast, the loop might have little contribution in the binding to Pep395.

In conclusion, we generated scFvs of G2 and its mutant and analyzed its structural, physical, and functional properties by biophysical methods, showing that the lack of residue at VL95 has a critical role in the specific binding of G2 to its immunogen, Pep18mer. G2 that had no residue at VL95 could bind to Pep18mer, in addition to the other peptides, Pep8 and Pep395, with high affinity. The lack of Pro at VL95 would increase the flexibility of the CDR loop, resulting in the ability of multispecific antigen binding.

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