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Identification and characterization of a multispecific monoclonal antibody G2 against chicken prion protein

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Abstract: We previously generated a monoclonal antibody (mAb), G2, by immunizing mice with Residues 174–247 of the chicken prion protein (ChPrP^C). In this study, we found that G2 possessed an extremely unusual characteristic for a mAb; in particular, it could react with at least three proteins other than ChPrP^C, the original antigenic protein. We immunoscreened a complementary DNA library from chicken brain DNA and found three proteins (SEPT3, ATP6V1C1, and C6H10orf76) that reacts with G2. There were no regions of amino acid sequence similarity between ChPrP^C and SEPT3, ATP6V1C1, or C6H10orf76. We selected ATP6V1C1 as a representative of the three proteins and identified the epitope within ATP6V1C1 that reacts with G2. The amino acid sequence of the G2 epitope within ATP6V1C1 (Pep8) was not related to the G2 epitope within ChPrP^C (Pep18mer). However, enzyme-linked immunosorbent assay, surface plasmon resonance (SPR), and isothermal titration calorimetry (ITC) experiments indicated that these two peptides have similar binding affinity for G2. The apparent K_D values of Pep18mer and Pep8 obtained from SPR experiments were 2.9 $\times$ 10^{-8} and 1.6 $\times$ 10^{-8} *M*, respectively. Antibody inhibition test using each peptide indicated that the binding sites of the two different peptides overlapped each other. We observed that these two peptides substantially differed in several binding characteristics. Based on the SPR experiments, the association and dissociation rate constants of Pep18mer were higher than those of Pep8.

Abbreviations: Ab, antibody; Ag, antigen; ChPrP^C, chicken PrP^C; mAb, monoclonal antibody; ITC, isothermal titration calorimetry; IFA, indirect immunofluorescence assay; k_d, dissociation rate constant; k_a, association rate constant; K_D, equilibrium dissociation constant; PNGF, *N*-glycosidase F; PrP, prion protein; PrP^C, cellular form of PrP; PrP^{Sc}, scrapie form of PrP; rChPrP, recombinant ChPrP; RU, response unit; RAMFc, rabbit anti-(mouse Fc) IgG; SPR, surface plasmon resonance; TBST, Tris-buffered saline with 0.05% Tween 20.

Additional Supporting Information may be found in the online version of this article.

Yuji O. Kamatari and Shinri Ohta contributed equally to this work.

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A clear difference was also observed in ITC experiments. These differences may be explained by G2 adopting different binding conformations and undergoing different binding pathways.

Keywords: antigen recognition mechanism; multispecificity of antibodies; prion protein; surface plasmon resonance; isothermal titration calorimetry

Introduction

Transmissible spongiform encephalopathies (TSEs), collectively known as prion disease, are fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease of humans, scrapie of sheep and goats, bovine spongiform encephalopathy of cattle, transmissible mink encephalopathy of minks, and chronic wasting disease of mule deer and elks.[1] These disorders are characterized by the generation of an abnormally misfolded isoform of the cellular prion protein (PrP^C), PrP^{Sc}, which represents the major component of infectious prion particles.[2]

The prion protein gene (*PRNP*) is conserved in most mammalian species.[3,4] The first nonmammalian PrP gene was identified in chicken in 1991.[5] The chicken and mammalian prion protein genes have approximately 55% nucleic acid homology, allowing for conservative substitutions, including the N-terminal repeats of PrP.[5,6] Considerable evidence indicates that the biochemical properties of chicken PrP^C ($ChPrP^C$) are similar to those of mammalian PrP^C as follows. Chicken and mammalian PrP^C molecules share the same structural domains including a series of proline- and glycine-rich peptide repeats, two hydrophobic segments, and a pair of cysteine residues bracketing *N*-glycosylation sites. In addition, both prion proteins are anchored to the plasma membrane via a glycosyl–phosphatidylinositol anchor at their carboxyl terminus and have similar thermodynamic properties.[6–9] $ChPrP^C$ mRNA is shown to be widely distributed in the central nervous system by *in situ* hybridization.[7] Because PrP^{Sc} acts as a template for the conversion from PrP^C to PrP^{Sc}, the presence of PrP^C is essential for the establishment and further development of prion disease.[10] Detailed investigations about the localization of $ChPrP^C$ in chicken neural cells have been limited because of the lack of specific antibodies directed against $ChPrP^C$ epitopes.[7] Therefore, recombinant ChPrP (rChPrP) was produced in bacteria, and several mouse monoclonal antibodies (mAbs) against rChPrP were isolated.[11]

BALB/C mice were immunized with rChPrP protein, and four anti-ChPrP mAbs—D8-10A, D8-3D, 10G-8, and G2—were isolated.[11] The mAbs D8-10A, D8-3D, and 10G-8 were obtained by immunization with rChPrP Residues 25–247, and mAb G2 was obtained by immunization with rChPrP Residues 174–247. Western blot analysis of chicken brain lysate with each anti-ChPrP antibodies detected several bands specific for ChPrP.[11] To characterize the localization of PrP^C in chicken cells, chicken neural cells were analyzed using an indirect immunofluorescence assay (IFA) with several mAbs. The nuclei in the cells were intensely stained with G2, but the other mAbs did not react with nuclei in the cells.

We further investigated whether G2 reacts with the nuclei fraction isolated from chicken neural cell lysate. G2 appears to react with some proteins in the nuclei fraction and also ChPrP in the membrane fraction, suggesting that G2 cross-reacted with the other proteins in addition to ChPrP immunized antigen. Therefore, we further investigated the biological reaction between chicken brain and G2. Moreover, we synthesized a complementary DNA (cDNA) library from chicken brain and used this library to identify the proteins reacting with G2. As a result, G2 appears to be a unique mAb that recognizes multiple and distinct epitopes, and therefore, has multispecificity; G2 recognizes at least three chicken antigens (SEPT3, ATP6V1C1, and C6H10orf76) other than $ChPrP^C$. In addition to biological assays, we characterized the biophysical interactions between G2 and each of the two epitopes, the epitope on ATP6V1C1 and $ChPrP^C$ in detail.

In general, antibody (Ab)-antigen (Ag) interactions are extremely specific and Ab can only bind one Ag. However, a few Abs can bind more than one Ag specifically. G2 seems to be classified into such multispecific Ab. It is suggested that the multispecificity helps to increase the diversity of Ab repertoire,[12] confer an advantage to pathogen-specific antibodies[13,14] and have advantages for therapeutic application.[15] However, the detailed studies on the multispecific antibodies are still limited and the mechanism of the multispecificity is not understood. Therefore, G2 is a useful mAb for studying the multispecificity of Abs. Moreover, G2 is unique, because it is a naturally occurring multispecific Ab and can bind two different peptides each with high affinity. To understand the multispecificity of G2, we used surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) to examine the kinetics and thermodynamics of the binding between G2 and each epitope, respectively. We observed that the binding characteristics of these two peptides are considerably different, although two peptides have the similar binding constant.

Results

G2 recognizes multiple proteins

To determine whether G2 recognizes $ChPrP^C$, chicken brain lysate was subjected to Western blot

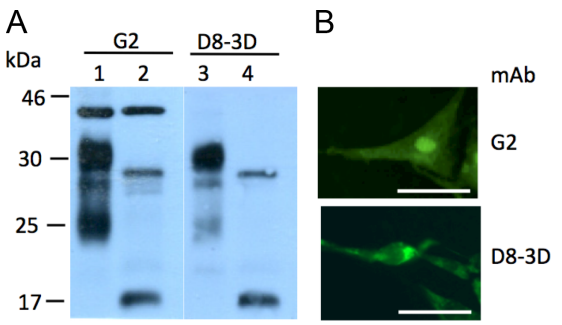

Figure 1. Western blot analysis of chicken brain homogenate with G2 or D8-3D (A) and indirect immunofluorescence assay with each mAb (B). (A) Chicken brain homogenates were loaded in Lanes 1 and 3. Chicken brain homogenates treated with *N*-glycosidase F were loaded in Lanes 2 and 4. Western blot analysis was performed with G2 (Lanes 1 and 2) and with D8-3D (Lanes 3 and 4). (B) Chicken neural cells were subjected to IFA with G2 or D8-3D. The nuclei of the chicken neural cells were intensely stained with G2 but not with D8-3D. All images were taken at 400× magnification. The scale bar presents 50 μm.

	Peptide Name	Amino acid sequence	O.D. value
Chicken Prion peptides	Pep18mer	212 EAVAAANQTEVEMENKVV 229	0.883±0.016
	PepF13	EAVAAANQTEVEM	0.640±0.029
	PepR13	ANQTEVEMENKVV	0.056±0.003
	Pep11-1	AVAAANQTEVE	0.056±0.001
	Pep11-2	EAVAAANQTEV	0.140±0.004
	Pep9mer	AVAAANQTE	0.063±0.002
	Pep6mer	AAANQT	0.060±0.003
Chicken ATP6V1C1 peptides	Pep7	13 KTCQQTWEKLHAATT	0.127±0.006
	Pep8	CQQTWEKLHAATTKN	1.336±0.027
	Pep9	QTWEKLHAATTKNNN	0.785±0.006
	Pep10	WEKLHAATTKNNNLS 35	0.692±0.017
	Pep11	KLHAATTKNNNLSTN	0.059±0.001

Figure 2. Amino acid sequences recognized by G2 in ELISA tests.

analysis with mAbs, G2, or D8-3D [Fig. 1(A)]. When Western blot analysis was performed with G2, three major bands were observed; one at approximately 42 kDa, another at 33 kDa, and the third at 25 kDa [Fig. 1(A), Lane 1]. When the *N*-glycosidase F (PNGF)-treated brain lysate was examined using Western blot analysis with G2, three bands—42 kDa, 30 kDa, and 17 kDa—were detected [Fig. 1(A), Lane 2]. However, when chicken brain lysate was examined by Western blot analysis using D8-3D, two bands at 33 and 25 kDa were detected; however, a 42 kDa band was not evident [Fig. 1(A), Lane 3]. The band at 42 kDa is the sole difference between the Western blot analysis with G2 and that with D8-3D. Furthermore, the 42 kDa band detected with G2 was not affected by PNGF treatment. Based on these Western blots, G2 recognized a 42 kDa protein in addition to two forms of $ChPrP^C$: the full length 33 kDa form of $ChPrP^C$ and a truncated 25 kDa form.

To investigate the localization of the proteins recognized by G2, chicken neural cells were subjected to indirect IFA with G2 or D8-3D. The nuclei of chicken neural cells were intensely stained with G2 but not with D8-3D [Fig. 1(B)]. However, D8-3D signal was clearly evident on the membranes and endoplasmic regions of these cells. Based on these results, G2 cross-reacted with other proteins in addition to $ChPrP^C$.

Identification of epitopes recognized by G2

Based on the pepspot analysis previously conducted,[11] the amino acid sequence recognized by G2 is AAANQT. However, the amino acid sequence AAANQT was not strongly recognized with G2 in enzyme-linked immunosorbent assay [ELISA; optical density (OD) value; 0.060 ± 0.003, Fig. 2]. To more precisely determine the amino acid sequences recognized by G2, several peptides from the ChPrP region that includes the AAANQT sequence were synthesized and subsequently examined for each of their interactions between G2 in ELISA (Fig. 2). Of the seven peptides tested using ELISA, Pep18mer had the highest OD value (0.883 ± 0.016), and PepF13 had the next highest (0.644 ± 0.029). These findings indicated that the epitope recognized by G2 comprises more amino acid sequences than the six amino acids (AAANQT).

Inhibition tests with the peptides

Pep18mer was used in Ab inhibition tests. Chicken brain lysate and PNGF-treated lysate were incubated with G2 or with G2 + Pep18mer and subsequently subjected to Western blot analysis [Fig. 3(A)]. With a short exposure time (30 s), none of the bands recognized by G2 were evident in the sample treated with G2 and Pep18mer, but they were evident in that treated with G2 alone [Fig. 3(A), Lanes 1, 2, 3, and 4]. However, with a longer exposure time (10 min), all three bands [42 kDa, 33 kDa, and 25 kDa in Fig. 3(A), Lane 7; 42, 30, and 17 kDa in Fig. 3A, Lane 8] were evident in both samples. Based on these findings, G2 recognizes the 42 kDa protein as well as $ChPrP^C$.

Identification of the non-$ChPrP^C$ proteins recognized by G2

A cDNA library was constructed from mRNA isolated from the chicken brain. The chicken brain cDNA library, which contained approximately 1×10^6 distinct phage clones, was screened with G2. The 16 clones were ultimately confirmed to positively interact with G2 and were subjected to *in vitro* excision to isolate the corresponding plasmid DNA clones.

Of the 16 plasmids, 11 encoded *PRNP* and the other five clones encoded genes other than *PRNP*. To estimate the coding genes, the DNA of 5 plasmids

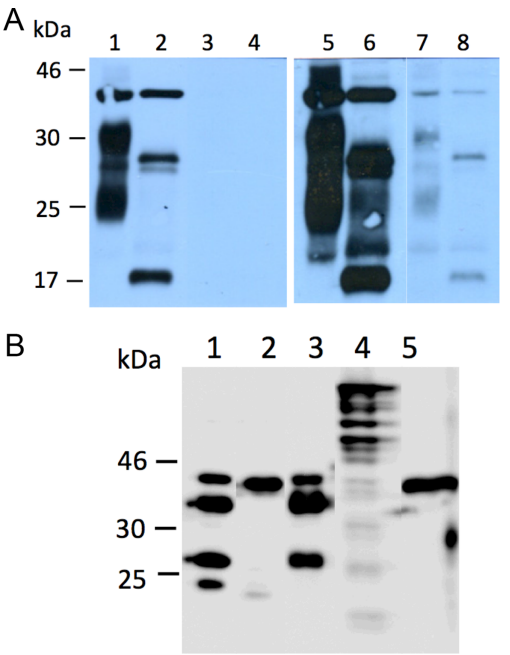

Figure 3. A. Inhibition of the reaction between G2 and the recognition peptide (Pep18mer). Chicken brain homogenates were loaded in Lanes 1, 3, 5, and 7, and chicken brain homogenates treated with PNGF were loaded in Lanes 2, 4, 6, and 8. Western blot analysis was performed with G2. Exposure time to films during the detection in the Western blot analysis was 30 s for Lanes 1, 2, 3, and 4. The time was 10 min for Lanes 5, 6, 7, and 8. Molecular sizes are indicated on the left. B. Detection of proteins expressed in *E. coli* BL21 cells using Western blot analysis with G2. Proteins expressed in five clones (G22, G6, F1, H4, or I6) of *E. coli* BL21 transformed with plasmid DNA were examined for a reaction with G2. Molecular sizes are indicated on the left. Lane 1, G22 clone; Lane 2, G6 clone; Lane 3, F1 clone; Lane 4, H4 clone; Lane 5, I6 clone.

was transformed into *Escherichia coli* BL21 cells to express their protein, and subsequently, the reaction with G2 was analyzed by Western blot. Based on these Western blots, each of the five plasmids (G22, G6, F1, H4, and I6) encoded a protein that reacted with G2 [Fig. 3(B)].

Each of the five clones (G22, G6, F1, H4, and I6) was subjected to DNA sequence analysis. Based on the sequence analysis, clone G22 had the same DNA sequences as clone F1, and G6 had the same DNA sequences as clone I6 (Table I). To identify the proteins encoded by each clone, the predicted amino acid sequences were used from a BLAST search of GenBank. The candidate genes identified in the BLAST search and the homology between the candidate genes and the respective clones are shown in Table I. Two clones, G22 and F1, were each predicted to encode a protein with homology to the SEPT3 protein. The clones, G6 and I6, were both predicted to encode proteins with homology to the ATP6V1C1 protein. The molecular weight of ATP6V1C1 is 42 kDa, which corresponds to 42 kDa band in Figure 1(A). H4 was predicted to encode a protein with homology to the C6H10orf76 protein. A homology search was conducted to determine whether the proteins encoded by the five different clones shared any amino acid sequence similarities with each other or with Pep18mer peptide recognized by G2. However, no common amino acid sequence was evident in this homology search.

Epitope analysis and the reaction between G2 and ATP6V1C1

Among 5 proteins reacted with G2, chicken ATP6V1C1 gene corresponding to clones G6 and I6 was selected and examined for the reaction region recognized by G2. First, to identify the amino acid region that reacted with G2, the G6 DNA sequences that encoded ATP6V1C1 were divided into two portions; the first half (Region A) encoded amino acids 1–202 and the second half (Region B) encoded amino acids 174–382. Each region was inserted into the expression vector pRSET-B and the encoded protein was expressed in BL21 cells. The recombinant Region A and B proteins were examined on Western blot with G2. Based on these blots, the Region A protein reacted with G2 but the Region B protein did not. To more precisely identify the G2 epitope within the Region A protein, this protein was divided into Region A1 encoding the Residues 1–120 and Region A2 encoding the Residues 92–202. The Region A1 protein, but not the A2 protein, appeared to react with G2. The A1 region was then divided into Region A1-1 encoding the Residues 1–74 and Region A1-2 encoding the Residues 52–120. Based on this series of cloning and expression experiments, G2 appeared to react with an epitope within the 74 amino acids of the N-terminal (Region A1-1) of ATP6V1C1 protein encoded by G6.

To more precisely identify the epitope within ATP6V1C1 that was recognized by mAb G2, 30

Table I. *Characterization of cDNA Inserts in Positive Clones*

			Homology (%)	
Clone (bp)	Matched candidate	Accession No. (bp)	DNA	Amino acid
G22, F1 (1152 bp)	SEPT3	XM_425473 (2146)	99	99
G6, I6 (1743 bp)	ATP6V1C1	XM_418370 (1827)	99	100
H4 (2153 bp)	C6H10orf76	XM_421719 (3130)	99	99

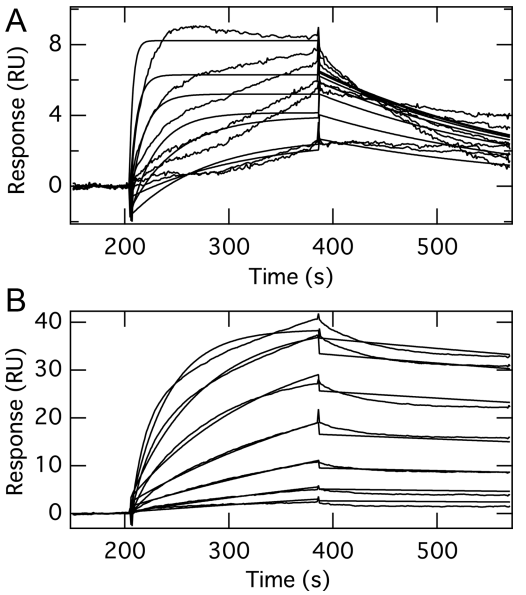

Figure 4. SPR measurements for interaction between G2 and peptides. SPR response for the binding of Pep18mer (A) or Pep8 (B) to G2 at pH 7.4 and 25°C. The peptide concentrations were 25, 50, 100, 200, 400, 800, and 1600 n*M* (from bottom to top). The fitting curves were drawn assuming a 1:1 binding model to obtain the association rate constant k_a, dissociation rate constant k_d, and dissociation constant K_D.

peptides representing regions within the Residues 1–74 of ATP6V1C1 were commercially synthesized. These 30 peptides were used in ELISAs to identify peptides that react with G2. Each peptide comprised 15 amino acids, and the 30 peptides were a series of related peptides that represented two-amino acid shifts from one spot to the next. Four peptides (Pep7, Pep8, Pep9, and Pep10) could react with G2 in an ELISA (Fig. 2). Among these four peptides, Pep8 (CQQTWEKLHAATTKN) showed the highest OD value in ELISA, suggesting that this amino acid sequence is the epitope site recognized by G2.

Purification of G2 and reaction profile with several peptides

To characterize the recognition properties of G2 with each of the several peptides, we cultured the hybridoma clone producing G2 in Hybridoma-SFM without fetal bovine serum (FBS) and purified G2 with protein A sepharose. Highly concentrated G2 (1.1 mg mL^{-1}) in PBS was obtained and used in all subsequent experiments. ELISA analysis for the interaction between the serially diluted G2 and each peptide is shown in Supporting Information Figure S1. In the ELISA tests, Pep8 from ATP6V1C1 and Pep18mer peptide from $ChPrP^C$ showed almost the same OD values with low dilution of G2, and the curve with high dilutions of G2 were similar for these two peptides (Supporting Information Fig. S1). These results indicate that G2 recognized two epitopes, Pep18mer of $ChPrP^C$ and Pep8 of ATP6V1C1, with extremely similar affinities.

Table II. *Thermodynamic and Kinetic Parameters of Peptide–G2 Complexes Determined From SPR Analyses*

Peptide name	K_D (M)	k_a (M^{-1} s^{-1})	k_d (s^{-1})
Pep18mer	2.88×10^{-8}	2.16×10^{5}	6.23×10^{-3}
Pep8	1.59×10^{-8}	4.05×10^{4}	6.37×10^{-4}

Analysis of G2 binding kinetics with peptides using SPR

To obtain more exact information on binding kinetics and binding affinity of the Ab-epitope pairs, we used the SPR method. Figures 4(A,B) show the SPR response during the binding experiments for Pep18mer and Pep8, respectively. Binding of Pep18mer to G2 rapidly proceeded with an association rate constant (k_a) of 2.2×10^5 M^{-1} s^{-1} (Table II). This binding was followed by a rapid dissociation process with a dissociation rate constant (k_d) of 6.2×10^{-3} s^{-1}, from which the dissociation constant K_D 2.9×10^{-8} M was calculated. In contrast, the binding of Pep8 to G2 was relatively slow with a k_a of 4.1×10^4 M^{-1} s^{-1}, which was 5.3 times slower than Pep18mer–G2 binding; moreover, binding of Pep8 to G2 was followed by a relatively slow dissociation with a k_d of 6.4×10^{-4} s^{-1}, which was 9.8 times slower than Pep18mer–G2 dissociation. Despite the considerably different k_a and k_d values, K_D values for Pep18mer–G2 and Pep8–G2 were similar, 2.9×10^{-8} and 1.6×10^{-8} M, respectively. The similar K_D values for these two peptide–Ab complexes were also evident from the ELISA (Supporting Information Fig. S1) and ITC experiments described subsequently.

Binding thermodynamics of G2 and peptide complexes using ITC

To determine the binding energetics of these peptide–Ab complexes, the interaction between each peptide and G2 was analyzed using ITC. Figures 5(A,B) show the binding isotherm and plotted titration curve, respectively, for binding of Pep18mer to G2 at 25°C. Figures 5(C,D) represent the binding isotherm and plotted titration curve, respectively, for the binding of Pep8 to G2. The thermodynamic parameters are summarized in Table III. As mentioned above, the K_D values for Pep18mer–G2 and Pep8–G2 complexes were similar, that is, 1.6×10^{-7} and 3.2×10^{-7} M, respectively. However, the contributions to K_D from the enthalpic (ΔH) and entropic (ΔS) terms for Pep18mer–G2 complexes were not similar to those for Pep8–G2 complexes. The ΔH for Pep18mer–G2 complexes was -11.3 kcal mol^{-1} and ΔS was -6.7 cal mol^{-1} deg^{-1}. In contrast, the ΔH

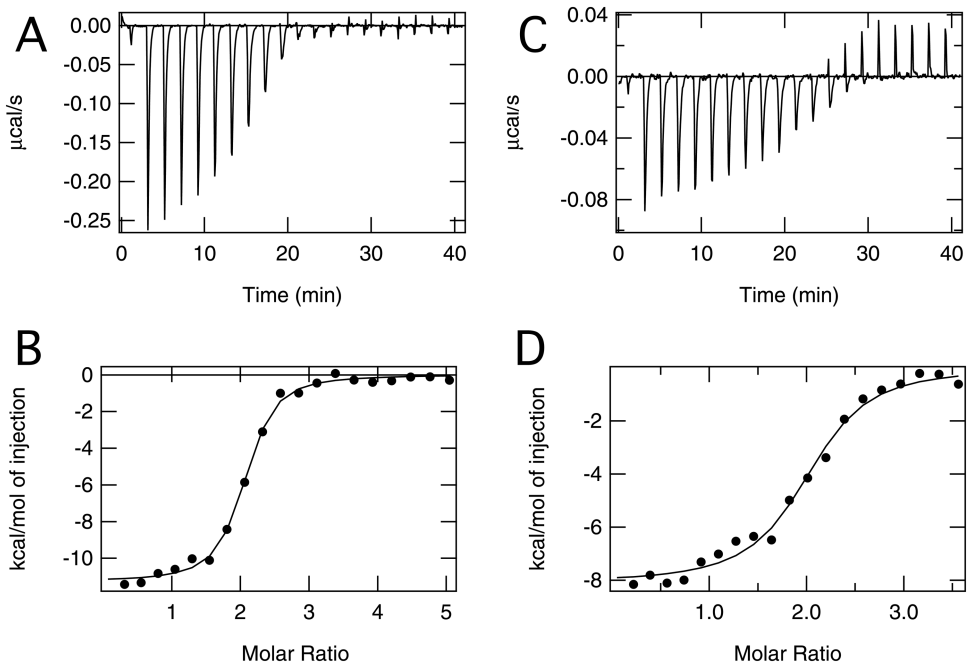

Figure 5. ITC measurements for the interactions between G2 and each peptide. The binding isotherm (A and C) and plotted titration curve (B and D) for the binding of Pep18mer (A and B) or Pep8 (C and D) to G2 at pH 7.4 and 25°C. The fitting curves were drawn assuming a 1:1 binding model to obtain the binding enthalpy (ΔH), entropy (ΔS), and the dissociation constant (K_D).

for Pep8–G2 complexes was −7.9 kcal mol^{-1} and ΔS was 3.2 cal mol^{-1} deg^{-1}, which had the opposite sign of Pep18mer binding ΔS.

Discussion

G2 recognizes several proteins

G2 was generated by immunizing mice with rChPrP Residues 174–247, and the epitope within ChPrP^C recognized by G2 was identified as AAANQT by a Pepspot analysis.[11] However, results of Western blot analyses and IFAs using chicken neural cells indicated that G2 recognized several proteins other than ChPrP^C. Of the seven mAbs (D8-10A, D8-D3, 6, 13, 14, 26, and G2) generated by immunization with rChPrP^C, five mAbs (D8-10A, D8-3D, 6, 26, and G2) recognized amino acid sequences near the AAANQT residues in ChPrP^C.[10] Of these five mAbs, only G2 possessed an extremely unique characteristic of multi-reactions of proteins other than ChPrP^C. Based on ELISA experiments, the actual amino acid sequences recognized by G2 appeared to be EAVAAANQTEVEMENKVV (Pep18mer) rather than AAANQT (Pep6mer), which was identified by a Pepspot analysis.

Table III. *Thermodynamic and Kinetic Parameters of Peptide–G2 Complexes Determined From ITC Analyses*

Peptide name	K_D (M)	ΔH (cal mol^{-1})	ΔS (cal mol^{-1} deg^{-1})	N
Pep18mer	1.63×10^{-7}	−11,300	−6.74	1.98
Pep8	3.24×10^{-7}	−7,890	3.23	2.02

In an immune screen of a chicken brain cDNA expression library, we identified three proteins (SEPT3, ATP6V1C1, and C6H10orf76) that interact with G2. However, there was no amino acid homology between ChPrP^C (the protein used for immunization) and any of the three proteins. From these three non-ChPrP^C G2-reactive proteins, we selected ATP6V1C1 as a representative. We used this representative to identify the G2-reactive epitope within ATP6V1C1 (Fig. 2). The G2-reactive epitope within ATP6V1C1 was CQQTWEKLHAATTKN; this amino acid sequence is not related to the G2-reactive epitope within ChPrP^C (Pep18mer) at the level of amino acid sequence. Although, the G2-reactive epitope within each of the other proteins (SEPT3 and C6H10orf76) was not identified in this study, G2 recognizes more than two completely different epitope sequences. We have to note that this recognition specificity of G2 is not influenced by repeat of cloning step nor purification level of G2. Moreover, single-chain variable fragment of G2 also recognizes these two different epitope sequences (unpublished data).

G2 can bind different amino acid sequences with similar affinities

Pep18mer peptide, EAVAAANQTEVEMENKVV, from ChPrP was observed to have the highest affinity to G2 from epitope analysis (Fig. 2), and it was confirmed that it can inhibit ChPrP binding to G2 (Fig. 3). Of the 30 ATP6V1C1-derived peptides, Pep8, CQQTWEKLHAATTKN, was recognized by G2 with the highest binding affinity (Fig. 2). We

could not identify any amino acid sequence similarity between these two peptides, but these peptides had similar binding affinity to G2, which was shown by ELISA (Supporting Information Fig. S1), SPR (Fig. 4), and ITC (Fig. 5) experiments. For example, the K_D values of Pep18mer and Pep8 obtained from SPR were 2.9×10^{-8} and 1.6×10^{-8} *M*, respectively.

Ab inhibition test with Pep18mer showed that it could inhibit the binding between ATP6V1C1 and G2 as well as that between ChPrP molecules and G2. Similarly, the Ab inhibition test with Pep8 showed that Pep8 also inhibits the binding between ChPrP and G2 as well as that between ATP6V1C1 and G2. These findings also indicate that the binding sites of these two peptides overlap each other.

Multispecific antibody G2

In general, Ab–Ag interactions are extremely specific, and the large Ag-receptor repertories are due to variation in the amino acid sequence at the Ag-binding site.[16] However, a few Abs can also bind more than one Ag specifically.[12–15,17–20] Antibody G2 seems to be in this category. This phenomenon is called multispecificity, polyspecificity, multireactivity, or polyreactivity. It is suggested that multispecificity helps to increase the diversity of Ab repertoire,[12] confer an advantage to pathogen-specific antibodies,[13,14] and have advantages for therapeutic application.[15] The affinity of polyreactive Abs for their different ligands is generally lower ($K_D = 10^{-3}-10^{-7}$ *M*) than that of monoreactive Abs for their cognate ligand ($K_D = 10^{-7}-10^{-11}$ *M*).[13] However, G2 is a unique Ab in terms of the ability of the binding to two different peptides, each with relatively high affinity ($K_D = 10^{-8}$ *M*).

G2 may adopt different binding conformations through different binding pathways

G2 can bind short peptides (18 residues) with high affinity ($K_D = 10^{-8}$ *M*). In general, such short peptides do not have any fixed tertiary structure, which is supported by the NMR experiment (Supporting Information Fig. S2). The NMR spectrum of Pep18mer is extremely similar to that simulated from random coil chemical shifts,[21] and this indicates that Pep18mer does not have any fixed tertiary structure. Therefore, G2 seems to recognize a linear or sequential epitope.

To understand the mechanisms by which G2 specifically recognizes the different sequential epitopes, we used SPR and ITC to examine the mAb–peptide binding kinetics and thermodynamics, respectively. We observed that the binding characteristics of these two peptides were considerably different. The SPR experiments showed Pep18mer to have faster association and dissociation rate constants than those of Pep8 (Fig. 4 and Table II). Moreover, the ITC experiments showed that the binding of Pep18mer to G2 was enthalpy-driven (Table III). In contrast, ΔS for the binding of Pep8 to G2, unlike that of Pep18mer, contributes to binding because the sign of ΔS was positive. The difference in characteristics between these two peptides may be explained by different interactions involved in binding. Electrostatic interactions, which are long range compared with other interactions and which accelerate binding speed, may contribute to binding between G2 and Pep18mer. Deletion of the N-terminal residues of Pep18mer, EAVAA, (compare OD values for Pep18mer and PepR13 in Fig. 2) or the C-terminal residues of F13, EM, (compare OD values for PepF13 and Pep11-2 in Fig. 2) dramatically decreased the binding affinity. Therefore, the core region of Pep18mer is EAVAANQTEVEM, and it contains three glutamic acid residues that are negatively charged at pH 7.2. These negative charges of Pep18mer seem to contribute to the electrostatic interactions between G2 and Pep18mer. In contrast, hydrophobic hydration of G2 in its free (unbound) state may be lost by the binding of Pep8 to G2. This loss of hydration may cause the favorable entropic contribution to the Pep8–G2 binding. Deletion of the N-terminal residues of Pep10, WE, (compare OD values for Pep10 and Pep11 in Fig. 2), or the C-terminal residues of Pep8, KN, (compare OD values for Pep8 and Pep7 in Fig. 2) dramatically decreased the binding affinity. Therefore, the core region of Pep8 is WEKLHAATTKN. The total charge of this core region is positive at pH 7.2, which is opposite to that of Pep18mer. This core region contains several hydrophobic amino acid residues, including tryptophan. The hydrophobic amino acid residues in Pep8 may interact with the hydrophobic residues of G2 and contribute to the loss of hydrophobic hydration, which is supported by the results of favorable entropy changes (Table III). The large differences in the characteristics of these two binding processes indicate that this single antibody (G2) adopted different binding conformations throughout some different binding pathways and could, therefore, bind to different peptides.

Putative mechanism of the multispecificity of G2

Protein structure is inherently dynamic so that proteins transiently populate low-lying excited states or sparsely populated higher-energy states, characterized by short lifetimes and higher free-energies relative to the predominant ground state.[22–29] The low-lying excited states sometimes have different binding affinities for a substrate and acquire new function, because of the different structures between the ground and excited states.[25,29] The multispecificity of Abs may be explained by such conformational diversity of protein. An Ab in such a low-lying excited state may take a different conformation from

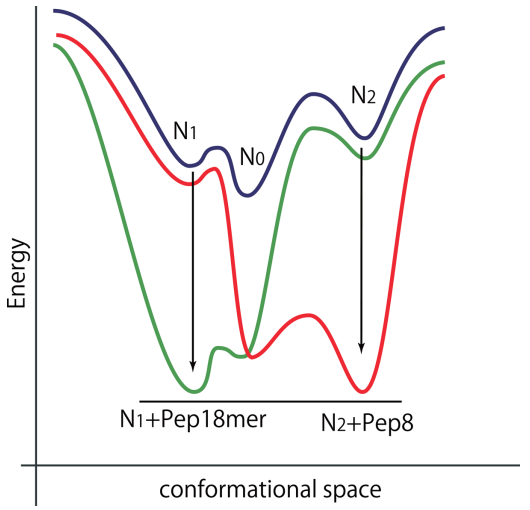

Figure 6. A schematic view of the energy landscape of G2 for Pep18mer or Pep8 binding. For Pep18mer binding, protein can reside on one of two energy landscapes: ligand-unbound (blue) or Pep18mer-bound (green) landscapes. For Pep8 binding, protein can reside on one of two energy landscapes: ligand-unbound (blue) or Pep8-bound (red) landscapes. Protein can jump between two landscapes by the ligand binding/unbinding. G2 at ground state (N_0) bind to Pep18mer with a small conformational change to N_1. G2 at N_0 also bind to Pep8 with a significant conformational change to N_2.

its ground state to have the ability to bind a different Ag than the one recognized by the ground state of the Ab. Although the population of the low-lying excited state is small, the ground and higher-energy states are rapidly exchanging. Therefore, a sufficient population of Ab molecules in the low-lying excited state and a sufficient stability of the complex may cause an Ab to have multispecificity in Ag recognition. One possible model for the multispecific recognition of G2 is shown in Figure 6. G2 at the ground state (N_0) may bind Pep18mer with small conformational changes to N_1, which may be relatively fast (Fig. 4 and Table II). In contrast, G2 at the low-lying excited state N_2 may be assumed to bind to Pep8. A population shift from N_0 to N_2 may be necessary for G2 binding to Pep8. Thus, it could be explained that the binding of Pep8 is relatively slow as shown by the SPR experiments (Fig. 4 and Table II). Similar binding affinity of G2 to Pep18mer and Pep8 is shown by the similar energy levels of two bound states (N_1 + Pep18mer and N_2 + Pep8).

G2 is a multispecific Ab with a unique characteristic. Therefore, further studies on G2 regarding the energy landscape of G2 in a wide range of conformational states (including the ground state N_0, two bound states, and the low-lying excited states, N_1 and N_2) will be useful in understanding mechanisms of protein structure–function relationships and Ab multispecificity.

Materials and methods

Primary chicken neural cells and tissue culture

Primary chicken neural cells were prepared from brain of 11-days-old chicken embryos using a SUMITOMO Nerve-Cell culture system kit (MB-X0802, SUMITOMO Bakelite, Tokyo, Japan). The hybridoma G2 clone producing G2 (Isotype G1) was usually cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% FBS and penicillin/streptomycin at 37°C in humidified 5% CO_2 or cultured in Hybrydoma-SFM (Gibco, Grand Island, NY), and the G2 was purified with protein A sepharose.

Indirect IFA

The chicken neural cells were plated at low density on cell culture slides and cultured for 3 days prior to immunostaining. The cells were incubated for 1 h at 4°C with G2 or D8-3D[11] and diluted with 1% FBS in PBS. Cells were washed 4 times with PBS and then incubated for 1 h at 4°C with anti-mouse IgG antibody conjugated with Alexa488 (Invitrogen, Carlsbad, CA). Cells were washed 5 times with PBS and then mounted in 50% glycerol in PBS. Each preparation was examined under a fluorescent microscope, ECLIPSE 80i (Nikon, Tokyo, Japan).

Peptide synthesis and ELISA

Several commercially synthesized peptides were purchased from SIGMA Life Science (Sapporo, Japan). ELISA was used for *in vitro* reaction between synthetic peptides and G2 as described by Ishiguro *et al.*[11]

Western blot analysis

Protein samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were transferred to PVDF membrane and react with G2.[11]

Preparation of brain homogenate and PNGF treatment

Chicken brain tissue was lysed in 6% sarkosyl, 0.5 m*M* DTT, 0.5 m*M* $MgCl_2$, 2 m*M* phenylmethylsulfonyl fluoride, and 20 m*M* Tris-HCl (pH 7.5) with a homogenizer. Each brain homogenate was then treated with PNGF (Roche Diagnostics, Basel, Switzerland) to remove sugar chains from $ChPrP^C$.

Construction of the cDNA library

Construction of the cDNA library was performed with a ZAP-cDNA Synthesis Kit and a ZAP-cDNA Gigapac III Gold Cloning Kit (Stratagene, La Jolla, CA). Briefly, the mRNA (5 μg) was reverse transcribed to first-strand cDNA in the presence of AccuScript reverse transcriptase and oligo(dT) linker-primer; DNA polymerase I and a poly(A) primer were used for second-strand cDNA synthesis. After *Eco*RI adapter ligation and digestion with *Xho*I, the cDNA fragments were size fractionated with

Sepharose CL-2B columns. Then, cDNAs were ligated into Uni-ZAP XR vector (Stratagene).

Screening the Ags identified with the G2 and in vivo *excision*

Immunoscreening of the proteins encoded by the cDNA in the phage library was performed with the *E. coli* XL1-blue host strain. Phage solution of 5 μL was added to a tube containing 100 μL of host XL1-Blue cells and incubated at 37°C for 15 min to allow the adsorption on bacterial cells. LB/$MgSO_4$ top agar was added to the mixture, poured onto LB/$MgSO_4$ agar plates, and incubated at 37°C for 4 h. Then, nitrocellulose membranes (Whatman, Kent, UK), which had been saturated with 10 m*M* IPTG and then dried, were applied to the agar plates and incubated for 3.5 h at 37°C. The membranes were stripped from the plates, washed in Tris-buffered saline with 0.05% Tween 20 (TBST) and blocked with 5% skim milk in TBST. Membranes were then incubated with a primary G2 in 1% skim milk in TBST for 1 h at room temperature. The membranes were washed with TBST and then incubated with appropriate secondary antibody conjugated with horseradish peroxidase (GE Healthcare). Detection of positive plaques was performed with an ECL Plus Western blotting detection system and X-ray film (FUJIFILM). In this first screen, positive plaques were identified by aligning the membrane with the agar plate. The ExAssist/SOLR system (Stratagene) was used according to the manufacture's instructions for *in vivo* excision of the phage identified in the screen.

Isolation of plasmid DNA and expression of protein

For each phage clone, a single colony of SOLR cell containing plasmid clones was selected and inoculated to 5 mL of LB broth. The QIAprep Spin Miniprep Kit (Qiagen, Tokyo, Japan) was used according to the manufacturer's instructions to extract plasmid DNA [pBluescript SK(−) with inserts] from each of these cultures. *E. coli* BL21 cells (DE3, Invitrogen) were transformed with plasmids with inserts and plated on LB-ampicillin plates. To investigate the reaction of G2 with each protein samples, Western blot analysis with G2 was performed as described above.

Construction of recombinant expression plasmids

To construct recombinant expression plasmids based on pRSET-B (Invitrogen), the target genes were amplified by polymerase chain reaction using a forward primer with a *Bam*HI site and a reverse primer with an *Eco*RI site. Each recombinant plasmid construct was transfected into BL21 cells, and protein expression was inducted with IPTG.

Sequencing and sequence analysis

Sequencing of the plasmid DNA was performed using vector-specific forward and reverse primers, and several primers (Supporting Information Table S1) on an automated ABI Prism DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). The BLAST DNA database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) was searched to identify gene candidate corresponding to the DNA sequences isolated in screens of the phage library.

SPR measurements

Real-time Ag–mAb interactions were measured with the Biacore biosensor system, Biacore T200 (GE Healthcare UK LTD, Buckinghamshire, UK). Each mAb was captured through rabbit anti-mouse Fc (RAMFc) Ab covalently linked to CM5 sensor chips, and Ags at various concentrations in 10 m*M* HEPES buffer (pH 7.4) containing 0.15 *M* NaCl and 0.005% Tween 20 were applied over the chip surface at 25°C, as described previously.[30]

ITC measurements

Interactions between G2 and the peptides were analyzed with the MicroCal iTC_{200} system (GE Healthcare UK LTD, Buckinghamshire, UK). The reference cell was filled with water, and the sample cell was filled with 7.3 μ*M* G2 in PBS at pH 7.2. The syringe was loaded with 170 μ*M* peptide in the same buffer with that in the sample cell. Each binding isotherm was obtained from 20 injections of the peptide into the G2 at 25°C. Full materials and methods are described in the Supporting Information.

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