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Carbohydrate recognition by pentadecapeptide ligands for a series of sialylated oligosaccharides

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ABSTRACT

Sialyloligosaccharides of glycoproteins and glycosphingolipids play important roles in biological events on cell membranes. GT1b is a ganglioside having a trisialyloligosaccharide and is a receptor for tetanus toxin. In the present study, pentadecapeptide ligands for GT1b were obtained by phage display selection from a random peptide library with the use of a GT1b monolayer. The artificial pentadecapeptides had high affinity for GT1b which tended to increase depending on the number of sialic acids in sialyloligosaccharides. Arg, Ser, and hydrophobic amino acids were found in a consensus motif and may contribute to carbohydrate recognition. The consensus motif of the GT1b-binding peptides was different from that of GM1-, GM2-, GM3-, or GD1a-binding peptides. Peptide ligands for GT1b should be investigated for trisialyloligosaccharide functions and the development of therapeutic agents against trisialyloligosaccharide-related diseases.

1. Introduction

Sialic acid is a family of 9-carbon acidic sugars and provides a negative charge on the cell surface.[1] The most representative sialic acid is *N*-acetylneuraminic acid (Neu5Ac) often found at the end of sugar residues of glycosphingolipids, *N*-glycans, and *O*-glycans. Sialylglycoconjugates are involved in cell–cell communication, the innate immune system, and pathogenic mechanisms of disease.[1] To investigate biological roles of sialylglycoconjugates, sialic acid-binding molecules such as bacterial toxins, plant lectins, and anti-glycan antibodies have been employed.[2] However, since the choice of useful sialic acid-binding proteins is limited, sugar-binding molecules have been designed. A galactose-binding lectin was transformed to a sialic acid-binding lectin by in vitro selection using ribosome display system.[3] A mannose/*N*-acetylglucosamine-binding lectin was selected from a lectin library by a phage display selection.[4] Such in vitro techniques are one of the most effective ways to obtain sugar-binding molecules from randomized libraries.

Gangliosides are sialic acid-containing glycosphingolipids involved in many important cellular functions depending on sugar structure. We have attempted to identify ganglioside-binding peptides by a random library selection method using phage-display technology, and succeeded in the selection of monosialoganglioside GM1- and GM3-binding peptides.[5–7] In our previous papers, GM1-binding peptides had affinity for GM1 with a dissociation constant (K_d) of 1.2 μM and inhibited the binding of cholera toxin to GM1 with a IC_{50} value of 1.0 μM.[5,8] Furthermore, GM3-binding peptides inhibited the infection of Mardin-Darby canine kidney cells by the influenza virus with IC_{50} values in the order of μM.[7] GM1 and GM3 have only one sialic acid in each sugar portion. In the present study, a ganglioside, GT1b, having three sialic acids was employed to obtain the peptide sequences that bind to trisialyloligosaccharides. GT1b is a receptor for a tetanus toxin (TeNT),[9] myelin-associated glycoprotein (siglec-4),[10] and botulinum neurotoxin.[11] GT1b is also known to relate to brain tumor metastasis.[12] Two kinds of peptides that bind to GT1b were identified, and Arg, Ser, and hydrophobic amino acids were found in a consensus motif. The amino acids in the motif were considered to contribute to the carbohydrate recognition. The kinetic parameters of the peptide-oligosaccharide determined by surface plasmon resonance analyses showed that these peptides have higher affinity for GT1b than monosialogangliosides.

2. Results

2.1. Selection of sialyloligosaccharide-binding peptides

A phage library displaying a random 15-mer peptide was used to identify peptides that had affinity for sialyloligosaccharides as described previously.[5] The trisialoganglioside GT1b was employed in each affinity selection (Fig. 1A). With this method, a ganglioside monolayer was used to avoid unexpected adsorption of phages to the lipid portion (Fig. 1B). After five rounds of selection, eleven individual phage clones were isolated and their binding affinity

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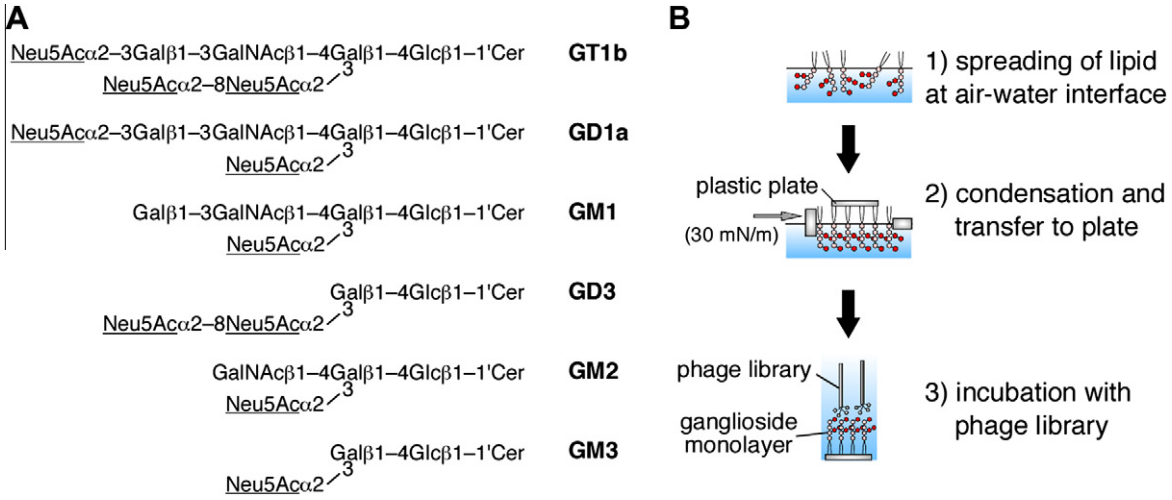

Figure 1. Preparation of the ganglioside monolayer for affinity selection. (A) Structure of gangliosides. Sialic acid is underlined. (B) Immobilization of the ganglioside monolayer. A ganglioside in $CHCl_3$/MeOH was spread at the air-water interface with a Langmuir trough (step 1). The resulting ganglioside monolayer was compressed until the surface pressure as 30 mN m^{-1}, then transferred horizontally to one side of a plastic plate (step 2). The ganglioside-immobilized plate was incubated with a phage library (step 3).

was determined by phage ELISA at 1 nM (Table 1). Two clones, GT1b/c03 and GT1b/c10, showed higher binding (1.8 or more of relative amount) to GT1b than control phages. Furthermore, to compare the amino acid alignments between mono-, di- and trisialoganglioside-binding peptides, affinity selection against GM2 and GD1a was also carried out. It was found that ten and three phage clones showed high affinity for GM2 and GD1a, respectively (Table 1). Ten GM2-binding peptides had new alignments, whereas two GD1a-binding peptides, DFRRLPGAFWQLRQP (GD1a/c01) and GWWYKGRARPVSAVA (GD1a/c03), were identical with the GM1-binding peptides in our previous study.[5]

2.2. Binding of phage clones to sialic acid-containing oligosaccharides

To evaluate the binding affinity of the peptides selected against sialyloligosaccharides, the amounts of GT1b-binding phage clones bound to various gangliosides were determined by phage ELISA. The amounts of phage clones, GT1b/c03 and GT1b/c10, significantly increased with the phage concentrations, compared to the primary phage library as a control (Fig. 2A). GT1b/c03 and GT1b/c10 also bound to monosialoganglioside (GM3) and disialogangliosides (GD1a and GD3) (Fig. 2B). These clones also showed affinity for multiple sialic acid-containing oligosaccharides.

2.3. Kinetic analyses of the interaction of synthetic peptides with oligosaccharides by the SPR method

To evaluate kinetic parameters of the peptide-sugar interactions, the affinity of synthetic peptides for ganglioside was determined for surface plasmon resonance (SPR) measurements. Two GT1b-binding peptides, GT1b/c03 and GT1b/c10, were chemically synthesized. The terminal carboxylate group of all peptides was amidated to block the influence of the negative charge. Kinetic parameters of binding to gangliosides were determined to characterize the specificity and affinity of synthetic peptides. A monolayer of the trisialoganglioside GT1b was immobilized onto the gold surface of a sensor chip, and the SPR response induced by the injection of peptide was measured. Figure 3A is a typical sensorgram of a peptide binding to the immobilized GT1b monolayer. When 10 μM of GT1b/c03 and GT1b/c10 was injected over GT1b, the increase in response at 180 s (R_{max}) was 150 and 272 resonance units (RU), respectively, whereas a control peptide, the N-terminal sequence of the coat protein III of phages (AEGDDPAKAAFDSLQ-NH_2), did not bind to GT1b (R_{max} = 30 RU).

The binding behavior of the peptides toward five gangliosides (GT1b, GD1a, GD3, GM1, and GM3), LacCer and Cer as a control was measured (Fig. 3B and Table 2). The dissociation constant (K_d) was calculated from the association rate constant (k_1) and

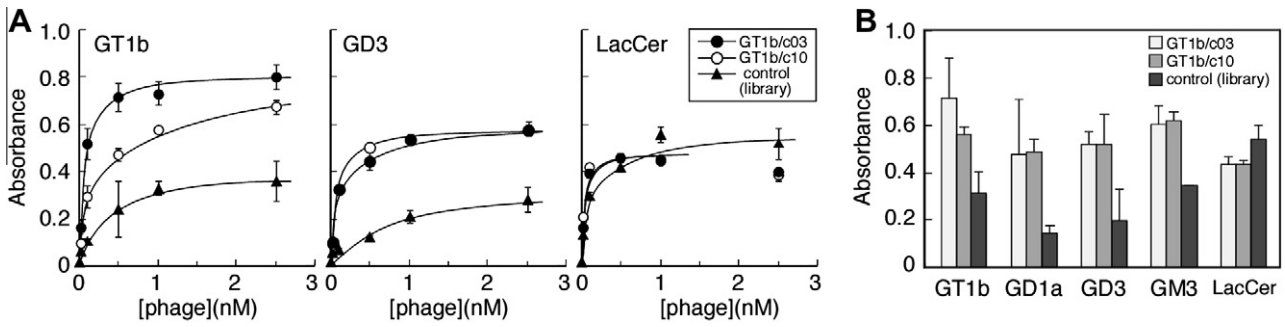

Figure 2. Binding affinity of phage clones for gangliosides determined by phage ELISA. (A) Amounts of phage clones bound to the ganglioside monolayer. The binding of phages (ΔA at 492 nm) is plotted against phage concentrations in the range of 0.01–2.5 nM. (B) Amount of phage (ΔA at 492 nm) bound to the ganglioside monolayer at 1 nM. The primary library was used as a control. Each plot is the mean of triplicate determination. The standard deviation is indicated by an error bar.

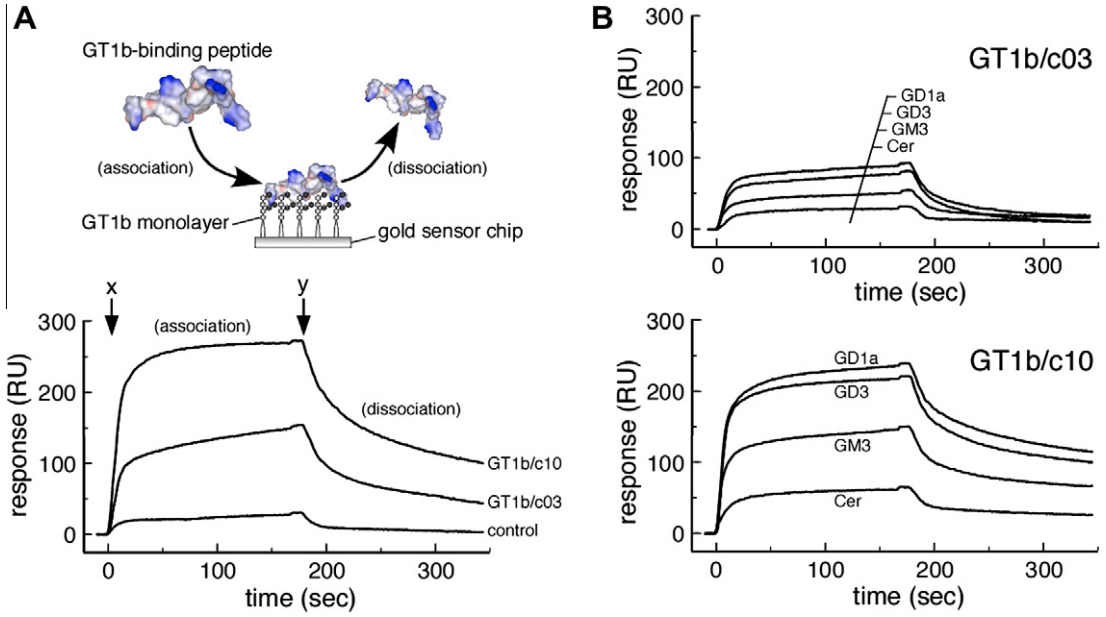

Figure 3. Binding affinity of peptides for gangliosides determined by SPR method. (A) Sensorgrams of the binding of GT1b/c03 and GT1b/c10 peptides to the GT1b monolayer. After an injection of peptide (arrow x), the GT1b-immobilized tip was allowed to incubate with the peptide at 10 μM for 180 s (association phase). The tip was rinsed with TBS for 180 s (dissociation phase). Arrow y is the time of replacement of the peptide solution by TBS. (B) Typical sensorgrams of the binding of GT1b/c03 and GT1b/c10 peptides to gangliosides. Sensorgrams of peptides against GD1a, GD3, and GM3 are shown. Cer was used as a control.

Table 1
Deduced amino acid sequences of phage isolated after five (GT1b) or four (GM2 and GD1a) rounds of affinity selection with ganglioside monolayer

Code	Amino acid sequence[a]	Frequency[b]	Relative amount[c]
GT1b/c03	RIRALFGRSPVPCCV	2/24	2.3
GT1b/c10	MRRPAPWLASRLMRP	1/24	1.8
GT1b/c02	GRLRYTSHSARIQRV	2/24	1.3
GT1b/c05	RSALRCLARVESCRQ	1/24	1.2
GT1b/c07	ARHQRFLSSIQRAPF	1/24	1.1
GT1b/c11	ALVRHPGARLRSFTA	1/24	1.0
GT1b/c09	ARFRHSTKSAQFVPL	1/24	1.0
GT1b/c08	GRLRPAKNHSVTVRR	1/24	0.89
GT1b/c06	PRRHGFSPSVRAVLP	1/24	0.76
GT1b/c01	RVPPRYHAKISPMVK	13/24	0.70
GT1b/c04	RRPHSSHSHVSRFTS	1/24	0.70
library	XXXXXXXXXXXXXXX	—	1.0
GM2/c10	GHRPRFSGSFVASRA	1/18	3.5
GM2/c07	VNRALPARWELWYPR	1/18	3.2
GM2/c06	HGLNAHLRPRPFLAR	1/18	3.2
GM2/c02	RNWNWPLRARVLSDA	1/18	2.9
GM2/c04	GRFHARPRTSSVWSF	1/18	2.8
GM2/c01	GTYYKRFGHSIPLVG	4/18	2.7
GM2/c08	PRRHRFSPSVRAVLP	2/18	2.4
GM2/c05	PRRGHFDESRFVHAV	1/18	2.2
GM2/c17	SSFSSGFVNWHRFAA	1/18	2.1
GM2/c13	YWPIDHKRVPLSRLV	1/18	2.1
GM2/c16	ASRSRFTSHWKRRTI	1/18	1.4
GM2/c20	HRTQLNRLRSHRSVV	1/18	1.4
GM2/c12	GGRFPHARDRVSFSR	2/18	1.2
fd	—	—	1.0
GD1a/c01	DFRRLPGAFWQLRQP	4/20	2.1
GD1a/c03	GWWYKGRARPVSAVA	15/20	2.1
GD1a/c18	RWGALLRGGAAALFQ	1/20	1.8
fd	—	—	1.0

[a] X, random amino acid.
[b] The number of isolated phage clones.
[c] $\Delta A/\Delta A_{control}$ ratio determined by phage ELISA at 1 nM. Control phage is primary library (for GT1b) or wild type fd (for GM2 and GD1a).

dissociation rate constant (k_{-1}) determined from the sensorgram according to Eqs. (1)–(3) (see Section 5). The K_d values of GT1b/c03 and GT1b/c10 for the GT1b monolayer were 0.97 μM and 0.64 μM, respectively. The carboxy-terminal domain of the heavy chain of TeNT forms a TeNT-GT1b complex with a K_d value of 0.17 μM.[13] GT1b/c03 and GT1b/c10 have high affinity for GT1b as well as TeNT. Expectedly, a neutral glycosphingolipid (LacCer) and sphingolipid (Cer) were not recognized by either peptide (K_d >10 μM). Sialic acid was an essential residue for the binding of the peptides.

The affinity of GT1b/c03 peptide for GT1b, GD1a, and GM1 (K_d, 0.97–1.3 μM) was about 10-times that for LacCer and Cer (15–24 μM). The GT1b/c03 peptide was found to have a high affinity for gangliosides with the pentasaccharide Gal-GalNAc-(Neu5Ac)-Gal-Glc (Fig. 4A). On the other hand, the GT1b/c10 peptide showed high affinity for GT1b and GD1a (K_d, 0.50–0.64 μM). The affinity of GT1b/c10 for GD3 and GM3 suggested that the terminal Neu5Ac-Gal was essential for the binding (Fig. 4A). In addition, the R_{max} value of GT1b/c10 for the trisialoganglioside GT1b (272 RU) was higher than that for the monosialoganglioside GM3 (146 RU).

Kinetic parameters such as k_1 and k_{-1} values are summarized in Table 2. For both GT1b/c03 and GT1b/c10, the k_{-1} values for gangliosides (2.7×10^{-3}–17×10^{-3} s^{-1}) tended to decrease as the number of sialic acids increased (Fig. 4B). In these cases, sialic acid is considered to contribute to the suppression of the dissociation of the peptides from gangliosides. Although the K_d value of GT1b/c03 for GD1a (1.3 μM) was the same as that for GM1 (1.3 μM), about a fivefold difference in the k_1 (or k_{-1}) value was found between GD1a (10×10^3 $M^{-1}s^{-1}$) and GM1 (2.1×10^3 $M^{-1}s^{-1}$).

2.4. Amino acid composition of GT1b-binding peptides

To discuss the influence of the number of sialic acids on the binding specificity of peptides, the amino acid composition of sialyloligosaccharide-binding peptide sequences was analyzed. Figure 5 shows appearance frequency for the peptides listed in Tables 1 and 3. Appearance frequency, an average of the frequency of an amino acid found in the pentadecapeptide, was calculated as follows:

Appearance frequency = sum of amino acid found in peptides/total number of peptides

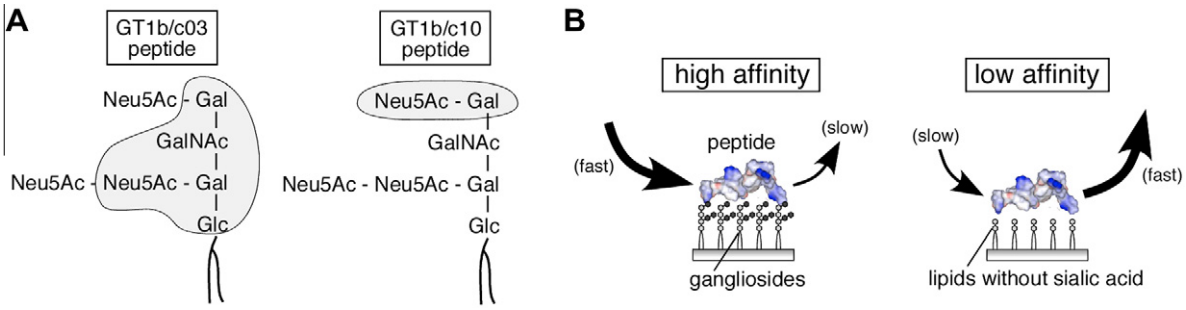

Figure 4. Comparison of the affinity of GT1b/c03 and GT1b/c10 peptides for gangliosides. (A) Schematic representations of GT1b and the portion of GT1b essential for the binding (shaded portion). (B) Schematic representation of the binding of peptides to gangliosides. The k_1 value was higher for gangliosides than lipids, and the k_{-1} value was lower.

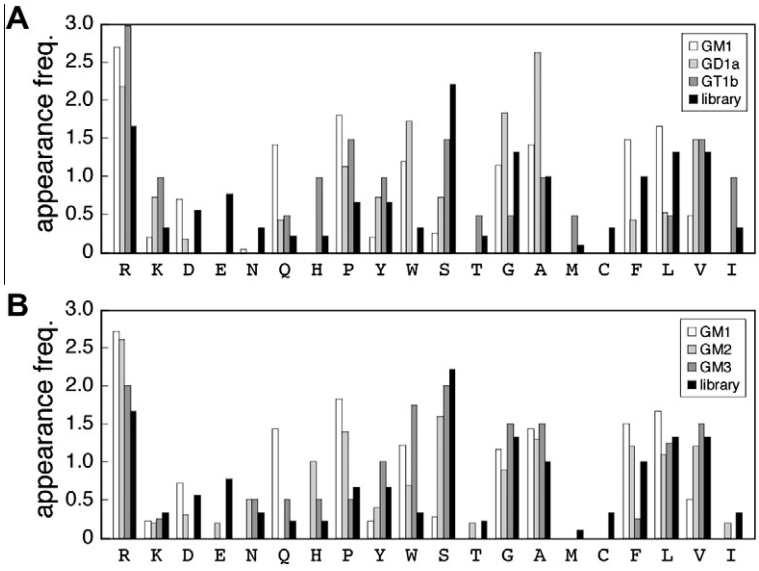

Figure 5. Apparent frequency of amino acids found in ganglioside-binding peptides. Composition of amino acids in peptide sequences listed in Tables 1 and 3. The apparent frequency indicates the average number in 15-amino acids. (A) GM1-, GD1a-, and GT1b-binding peptides. (B) GM1-, GM2-, and GM3-binding peptides.

Table 2
Binding affinity of GT1b-binding peptides for gangliosides determined by SPR

Lipid	Sialic acid[a]	GT1b/c03 (H-RIRALFGRSPVPCCV-NH_2)				GT1b/c10 (H-MRRPAPWLASRLMRP-NH_2)			
		R_{max} (RU)	k_1 (10^3 $M^{-1}s^{-1}$)	k_{-1} (10^{-3} s^{-1})	K_d (μM)	R_{max} (RU)	k_1 (10^3 $M^{-1}s^{-1}$)	k_{-1} (10^{-3} s^{-1})	K_d (μM)
GT1b	3	150	6.0	5.8	0.97	272	7.2	4.6	0.64
GD1a	2	90	10	13	1.3	236	7.0	3.5	0.50
GD3	2	78	3.0	10	3.3	218	11	13	1.2
GM1	1	115	2.1	2.7	1.3	155	4.9	11	2.2
GM3	1	50	4.5	17	3.8	146	10	15	1.5
LacCer	0	34	1.5	23	15	85	1.3	15	12
Cer	0	29	1.6	38	24	64	3.0	32	11

[a] Number of sialic acids in the lipid.

The appearance frequencies of amino acids in GM1-, GD1a-, and GT1b-binding peptides are summarized in Figure 5A. In these peptides, Arg, Pro, and Ala were consistently found at high frequency (appearance frequency of 1.0 or more), which means that a pentadecapeptide contains two arginines, one proline, and one alanine (or two alanines for GD1a-binidng peptides) on average. The appearance frequency of Lys, Tyr, Ser, and Val increased with the number of sialic acids in gangliosides, whereas that of Asp, Glu, and Cys decreased. Furthermore, it was found that Arg, Gly, and hydrophobic amino acids (Ala, Pro, and Trp) appeared at high frequency in the GM1-, GM2-, GM3-, GD1a-, and GT1b-binding peptides, especially Arg, Pro, and Trp in monosialoganglioside (GM1, GM2, or GM3)-binding peptides (Fig. 5B).[5,7] These amino acids were also found in the consensus motifs described in the next section.

2.5. Consensus motif

The consensus motif in the ganglioside-binding peptides was predicted based on a multiple sequence alignment algorithm using the CLUSTALW program.[14] GT1b-binding peptides showed a motif, RxxAx(F/W)xxSx(V/L) (Table 3). This motif was different from

Table 3
Consensus motifs of ganglioside-binding peptides predicted with the CLUSTALW program

Code	Amino acid sequence
GM1/c01[a]	DF**R**R**L**PGA**F**WQLRQ**P**
GM1/c03[a]	VW**R**L**L**APP**F**SNRLL**P**
motif	(F/W)**R**x**L**xxx**F**x(Q/N)xxx**P**
GM2/c10	**G**HRPRFS**G**SFVASRA
GM2/c01	**G**TYYKRF**G**HSIPLVG
motif	**G**xxx(R/K)xx**G**xx(V/I)
GM2/c07	V**N**RAL**P**A**R**WF**L**WYPR
GM2/c02	R**N**WNW**P**L**R**ARVLSDA
motif	**N**xxx**P**x**R**xx(L/V)
GM3/c01[b]	GW**W**YKGR**A**R**P**VS**A**VA
GM3/c03[b]	RAV**W**RHSV**A**T**P**SHS**V**
motif 1	**W**x(K/H)xx**A**x**P**xx(A/S)**V**
GM3/c15[b]	L**WR**P**V**L**F**HSAVRALG
GM3/c30[b]	**WR**GV**F**GDRWLGSQP
motif 2	**WR**x**V**x**F**
GD1a/c03	G**W**WYKG**R**ARPVSAVA
GD1a/c18	R**W**GALL**R**GGAAALFQ
motif	**W**xxxx**R**xxxx(S/A)
GT1b/c03	**R**IR**A**LFGR**S**P**V**PCCV
GT1b/c10	M**R**RP**A**P**W**LA**S**R**L**MRP
motif	**R**xx**A**x(F/W)xx**S**x(V/L)

[a] Adapted from reference.[5]
[b] Adapted from reference.[7]

GM1- and GM3-binding motifs identified previously, (F/W)RxLxxxFx(Q/N)xxxP, Wx(K/H)xxAxPxx(A/S)V, and WRxVxF.[5,7] In the case of GM2-binding peptides, to define the distances of amino acid sequences, a rooted Neighbor-Joining tree was drawn before CLUSTALW was used (Supplementary data, Fig. S1).[15] The representative consensus motifs among each peptide are summarized in Table 3 and Fig. S2 (Supplementary data). One or two consensus motifs was found among each peptide series, but identical motifs were not found.

3. Discussion

Previously, we have identified monosialoganglioside (GM1 or GM3)-binding peptides, and reported their affinity for glycolipids[8] and inhibitory activities against the cholera toxin[8] or influenza virus.[7] In the present study, to expand the peptides range of applications, phage-display selection was improved to identify peptide ligands that have affinity for oligosaccharides carrying multiple sialic acids. We compared the consensus motif for mono-, di-, and trisialoganglioside-binding peptides, and investigated the influence of the number of sialic acids on the peptide sequence and binding affinity.

To clarify the specificity of the GT1b-binding peptides, the affinity for six glycosphingolipids was investigated by the SPR method. The GT1b/c03 and GT1b/c10 pentadecapeptides showed a higher affinity for GT1b than other glycolipids (Table 2). The kinetic parameters indicated the high affinity for GT1b to be attributable to a fast association rate ($k_1 = 6.0 \times 10^3\ M^{-1}s^{-1}$) and a slow dissociation rate ($k_{-1} = 5.8 \times 10^{-3}\ s^{-1}$) (Fig. 4B).

To compare sequence similarity with protein sequences in databases, BLAST and FASTA algorithms were used.[16,17] The GT1b-binding peptides obtained in the present study showed no homology to any GT1b-binding molecules such as TeNT,[9] botulinum neurotoxin,[11] myelin-associated glycoprotein,[10] and synthetic Tet1 peptide.[18] The TeNT-GT1b complex has been analyzed using X-ray crystallography (Protein Data Bank code, 1FV2).[19,20] This structural data demonstrated that two sites on the sugar portion, Gal4–GalNAc3 and Neu5Ac7–Neu5Ac6 groups in GT1b, provide the key interaction for the recognition. Gal4–GalNAc3 is recognized by hydrogen bonds (Asp1222 and His1271) and hydrophobic interaction (Trp1289). Neu5Ac7–Neu5Ac6 is recognized by hydrogen bonds (Asp1147, Asp1214, Asn1216, and Tyr1229) and a salt bridge between Arg1226 and the carboxylate anion of a terminal sialic acid. Asp, His, Trp, Asn, Tyr, and Arg are essential amino acids for the carbohydrate recognition in the GT1b–TeNT complex. The consensus motif of the GT1b-binding peptides, RxxAx(F/W)xxSx(V/L), also had Trp, Arg, hydrophobic amino acids (Ala, Phe, Val, and Leu), and a hydrophilic amino acid (Ser) (Table 3), therefore these amino acids were considered to be involved in the carbohydrate recognition. This is due to the fact that protein–carbohydrate interaction mainly

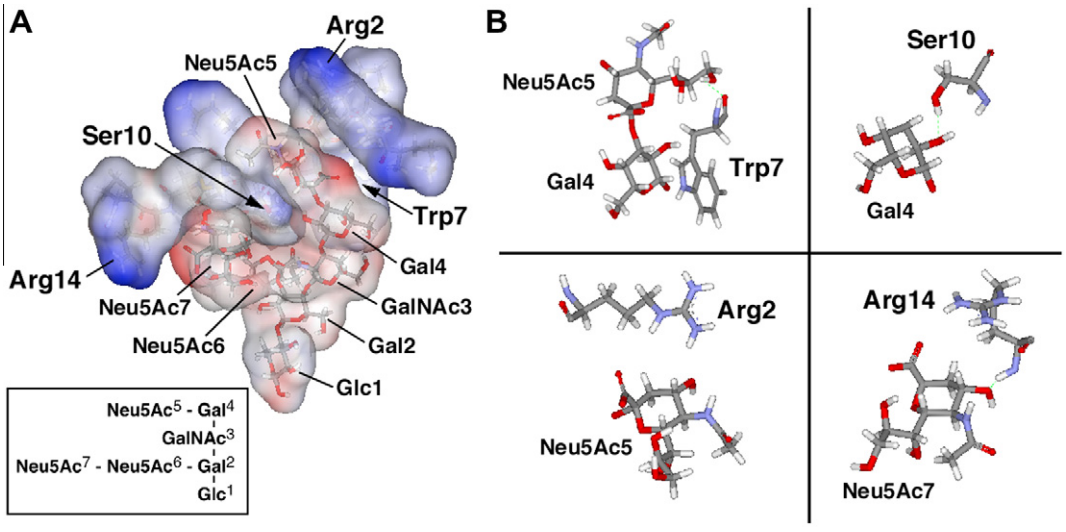

Figure 6. Deduced model of interaction between the GT1b/c10 peptide and GT1b heptasaccharide. (A) Superimposed image of the peptide on the heptasaccharide shown with a stick and molecular surface representation. Peptide conformation was obtained by molecular dynamics simulation, and a side chain of Trp7 of the peptide was put close to a B-face of Gal4 of the heptasaccharide, within 10 Å. The conformation of the GT1b heptasaccharide was obtained from Protein Data Bank entry 1FV2. (B) Models of carbohydrate recognition in the peptide-heptasaccharide complex. Deduced hydrogen bonds are shown with green dotted lines.

occurs with the combination of a hydrogen bond (or electrostatic interaction) and hydrophobic interaction.[2,21,22]

The molecular modeling of the GT1b/c10–GT1b heptasaccharide indicated that the stacking of Trp7 with Gal4 is a key interaction in the complex.[2] Figure 6 shows the structure when a side chain of Trp7 of the peptide is close to a B-face of Gal4 of the heptasaccharide, within 10 Å. In this structure, an indole ring and a carboxyl group of Trp7 were suggested to form a hydrophobic interaction with Gal4 and a hydrogen bond with 9-OH of Neu5Ac5, respectively. A hydrogen bond between a side chain of Ser10 and 2-OH of Gal4 was also suggested. In addition, two arginines, Arg2 and Arg14, were arranged near Neu5Ac5 and Neu5Ac7, respectively, where the distances between carbon atoms of the guanidium group of Arg and carboxyl group of Neu5Ac were 8–9 Å (Fig. 6B). This modeling suggested that Arg2, Trp7, and Ser10 in the motif are responsible for the binding.

The consensus motif and the appearance frequency of amino acids of the ganglioside-binding peptides were analyzed to understand the molecular mechanism of peptide-sugar interaction. In the consensus motif of GT1b-binding peptides, RxxAx (F/W)xxSx (V/L), one Arg, five hydrophobic amino acids (Ala, Phe, Trp, Val, and Leu), and Ser was found. In GM1-, GM2-, and GM3-binding peptides, a cationic amino acid (Arg or Lys) and hydrophobic amino acids (Phe, Trp, Leu, Pro, Ala, and Val) were found in the motifs predicted by the CLUSTALW program (Table 3).[6–8] These consensus motifs had only one Arg (or Lys) (Table 3), and the number of cationic amino acids in the motif did not depend on the number of sialic acid residues in target gangliosides. Sialic acid has one carboxylate anion at neutral pH, therefore electrostatic interaction with Arg and Lys may be expected as well as the sialic acid-recognition in siglec.[23] These results provide useful information to design tailored peptides that bind to sialyloligosaccharide.

4. Conclusion

We identified novel pentadecapeptides that bind to the trisialyl ganglioside GT1b using phage display selection. The consensus motif of the GT1b-binding peptides had one cationic amino acid, one hydrophilic amino acid, and multiple hydrophobic amino acids. Carbohydrate recognition in which the amino acids in the motif were considered to be involved was suggested by the combination of hydrophobic interaction and hydrogen bonds as well as glycan-binding proteins. A kinetic analysis of the peptide-glycosphingolipid interaction indicated that these peptides had higher affinity for GT1b than monosialogangliosides. Sialic acid-containing glycans are involved in the onset of various diseases, therefore these peptides could be suitable for the detection of disease-related glycans and as candidates for peptide drugs.

5. Materials and methods

5.1. Materials

Ceramide (Cer), Neu5Acα2–3Galβ1–4Glcβ1–1′Cer (GM3), Galβ1–3GalNAcβ1–4(Neu5Acα2–3)Galβ1–4Glcβ1–1′Cer (GM1), and Neu5Acα2–3Galβ1–GalNAcβ1–4(Neu5Acα2–8Neu5Acα2–3)Galβ1–4Glcβ1–1′Cer (GT1b) were purchased from Sigma. Ganglioside GalNAcβ1–4(Neu5Acα2–3)Galβ1–4Glcβ1–1′Cer (GM2) and Neu5Acα2–3Galβ1–GalNAcβ1–4(Neu5Acα2–3)Galβ1–4Glcβ1–1′Cer (GD1a) was kindly provided by Professor Kawanishi (Kitasato University, Japan). Neu5Acα2–8Neu5Acα2–3Galβ1–4Glcβ1–1′Cer (GD3) and Galβ1–4Glcβ1–1′Cer (LacCer) were obtained from Snow Brand Milk Products (Japan). The phage-displayed random pentadecapeptide library (10^8 diversity) was obtained as described previously.[24] A wild-type phage (fd phage) prepared from the vector fUSE5 was used as a control.

The pentadecapeptide amides (peptide-NH_2) were purchased from Sigma Genosys (Japan). The purity (>99%) and expected structure were verified by reversed-phase high-performance liquid chromatography and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry, respectively.

5.2. Ganglioside monolayer

To promote interaction between the phage library and gangliosides, a lipid monolayer prepared at the air-water interface was immobilized onto a solid support (Fig. 1B). A lipid solution (chloroform/methanol = 2:1 or 4:1, v/v) containing ganglioside GM2, GD1a, or GT1b (ca. 0.5 mg/mL) was spread on MilliQ or tris-buffered saline (TBS)(50 mM tris–HCl, 150 mM NaCl, pH 7.5) in a Teflon-coated Langmuir trough (USI Co., Japan). The sub-phase was maintained at 25 °C. The surface-pressure (π–A) isotherm was monitored with a Wilhelmy plate. The lipid monolayer was compressed at a constant rate (10 cm^2 min^{-1}), and transferred horizontally to one side of a plastic plate (diameter 13 mm) (code 174950, Nalge Nunc International) at a surface pressure of 30 mN m^{-1}. The lipid-immobilized plate was moved to a 24-well plate filled with 0.5 ml/well of TBS until used.

5.3. Affinity selection

Typical affinity selection with a ganglioside monolayer was performed as follows: 200 μL of phage library solution containing 5.0×10^{10} transducing units (TU) was incubated with the GT1b-immobilized side of the plastic plate for 30 min. The GT1b-plate was washed three times with 0.5 mL of TBS, and 0.4 mL of elution buffer (0.1 M glycine-HCl at pH 2.2) was added to elute GT1b-bound phages for 15 min. The eluate was neutralized with 20 μL of 1 M tris–HCl buffer (pH 9.1) and the buffer was replaced with TBS by using a Centricon-30 concentrator (code 4209, amicon). Ten micro liters of the resulting solution was used for titering and the remaining phages were amplified with host cells (*Escherichia coli* K91Kan) in 20 mL of Luria broth. The amplified phages were purified with a polyethyleneglycol/NaCl solution and redissolved in TBS. This process was repeated five times, thereby resulting in the enrichment of the GT1b-binding phages. Then twenty four phage clones were isolated, amplified, and purified. Single strand DNA of each isolated clone was purified with a QIAprep Spin M13 kit (QIAGEN) and used as a template for sequencing the terminal of the protein III.

5.4. Phage enzyme-linked immunosorbent assay (phage ELISA)

A 24-well plate was blocked with 1% bovine serum albumin (BSA)/TBS in advance, and the BSA solution was removed. Lipid-immobilized plastic plates prepared as described previously were incubated with phage solution (0.01–2.5 nM in 200 μL of TBS) for 30 min. The other side of each plate was blocked with 0.5% BSA/TBS and washed twice with 0.5% BSA/TBS. The bound phages were incubated with a 1:1000 (v/v) dilution of anti-fd bacteriophage antibody (code B7786, Sigma) for 1 h, and the wells were washed three times with TBS. A 1:1000 (v/v) dilution of peroxidase-conjugated anti-rabbit IgG antibody (code A8275, Sigma) was added and incubation continued for 1 h. The color was developed using *o*-phenylenediamine in citrate-phosphate buffer at pH 5.0, and detected at 492 nm. Each experiment was carried out in triplicate. Increases in absorbance (ΔA) at 492 nm showed simple saturation curves against phage concentrations (Fig. 2A). The relative amount of phage ($\Delta A/\Delta A_{control}$) was estimated at a phage concentration of

1 nM, with the wild-type fd phage (or primary library) used as a control (Table 1).

5.5. Surface plasmon resonance (SPR)

A lipid monolayer was compressed and transferred horizontally to the gold surface of a sensor tip (SIA Kit Au)(code BR-1004-05, Biacore International). The lipid-immobilized gold tip was docked with BIAcore X and rinsed with TBS at a constant flow rate of 10 μL/min for 30–40 min prior to use. The synthetic peptide (10 μM) was injected at a flow rate of 1 μL/min for 3 min (association), and the tip was rinsed with TBS for 3 min (dissociation). The sensor tip surface was regenerated, and the bound peptide was removed by injection of 4 M $MgCl_2$ at a flow rate of 10 μL/min for 8 min. Buffers and all samples were filtered at 0.22 μm and degassed.

In this system, a change in the surface protein concentration is followed by a change in resonance units (RUs).[25] The sensorgrams were analyzed by a BIAevaluation software (version 3.0) with a global fitting model (1:1 binding model). In this binding model, homogeneous interaction is given by

$$A + B \rightleftarrows AB \tag{1}$$

$$\mathrm{d[AB]/dt} = \mathrm{k_1[A][B]} - \mathrm{k_{-1}[AB]} \tag{2}$$

where A is defined to be the analyte (peptide), B is the ligand immobilized to the sensor tip (ganglioside or other lipid), k_1 (M^{-1} s^{-1}) is the association rate constant, and k_{-1} (s^{-1}) is the dissociation rate constant. Eq. (2) can be expressed in terms of the SPR signal as

$$\mathrm{dR_t/dt} = \mathrm{k_1 C R_{max}} - (\mathrm{k_1 C} + \mathrm{k_{-1}})\mathrm{R} \tag{3}$$

where R_t (RU) is the SPR signal at time t, R_{max} (RU) is the maximum binding capacity of the analyte, and C (M) is the analyte concentration injected. Finally, the dissociation constant K_d is defined as k_{-1}/k_1.

5.6. Molecular modeling

Molecular modeling was performed using Discovery Studio 2.1 software (Accelrys, Inc.). The peptide amide of GT1b/c10 was built using a DS Biopolymer module, where the main chains of 5–12 residues are taken as the helical structure, according to a prediction of secondary structure using the Chou and Fasman method. Simple minimization was performed using the CHARMm force field. After the minimization, molecular dynamics were performed using the Standard Dynamics Cascade (298 K, equilibration: 100,000 steps, time step 0.001 ps). The final structure was used for the drawing shown in Figure 6. Crystallographic coordinates of the X-ray structure of the GT1b heptasaccharide were obtained from the Protein Data Bank (1FV2). The GT1b/c10 peptide and GT1b heptasaccharide were superimposed.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.025.

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