Selection of ganglioside GM1-binding peptides by using a phage library

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Abstract Ganglioside Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1'Cer (GM1)-binding peptides were obtained from a phage-displayed pentadecapeptide library by an affinity selection. The selection processes were in situ-monitored by a quartz-crystal microbalance method, on which a ganglioside GM1 monolayer was transferred. After five rounds of biopanning, the DNA sequencing of 18 selected phages showed that only three individual clones were selected. The peptide sequences of the random region were found to be DFRRLPGAFWQLRQP, GWWYKGRARPVSAVA and VWRLLAPPFSNRLLP. Binding constants of these phage clones to the GM1 monolayer were 10^{10} M^{-1}. Three synthetic pentadecapeptides inhibited the binding of cholera toxin B subunit to the GM1 monolayer with an IC_{50} of 24, 13 and 1.0 μM, respectively. These peptides will be useful for searching functional roles of ganglioside GM1.

Key words: Ganglioside; Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1'Cer; Carbohydrate recognition; Monolayer; Phage-displayed peptide library; Quartz-crystal microbalance

1. Introduction

Carbohydrate-protein interaction is an important process in cell-cell recognition [1]. Thus, it is expected that the peptides which inhibit carbohydrate-protein interaction will have great potential in the prevention and therapy of various diseases. A phage display technique has been developed by Smith et al. to discover target-specific proteins (peptides) [2]. By using the phage display technique, Deng et al. selected lipopolysaccharide-binding antibody [3]. Yamamoto et al. selected protein having affinity with mucin from a cDNA library [4]. Gangliosides were also known as receptors for virus [5] and toxin [6], cell-cell adhesion molecules [7] and modulators of signal transduction [8–10]. Recently, Taki et al. developed glycosphingolipid mimicking peptides and observed their function as inhibitor for tumor metastasis [11,12]. In this study, we attempted to obtain amino acid sequences which recognize the oligosaccharide of gangliosides. There are no reports about glycolipid-binding peptides so far.

We carried out affinity selection, so-called 'biopanning', with monosialoganglioside galactose (Gal)β1→3*N*-acetylgalactosamineβ1→4(*N*-acetylneuraminic acid (NeuAc)α2→3)Galβ1→4glucoseβ1→1'ceramide (Cer) (GM1), known as a receptor for cholera toxin B subunit (CTB) [13]. We have tried to select GM1-binding peptides by using a GM1-adsorbed solid support. Though we obtained 60 individual phage clones, those phages showed no consensus peptide sequences (unpublished results). These results are considered to be caused by the various orientation of ganglioside. Accordingly, we reconsidered the strategy of biopanning and tried to employ an ordered ganglioside monolayer. Ganglioside monolayers can be prepared at an air-water interface as shown in Fig. 1. Glycolipid monolayers have been employed to investigate the recognition function of glycolipid with lectin [14,15] and influenza virus [16,17]. Hydrophilic carbohydrate portions of glycolipids at the monolayer are exposed to the water phase. Therefore, phages in the water phase will interact only with the carbohydrate portion, but not with the hydrophobic Cer. Such a monolayer is expected to be suitable for the selection of peptides which recognize oligosaccharide of gangliosides presented in biomembranes.

2. Materials and methods

2.1. Reagents

The pentadecamer (15-mer) random peptide library displayed on a filamentous phage (fd phage) and *Escherichia coli* K91Kan were obtained as previously described [18]. GM1 was obtained from Prof. Kawanishi of the Ogata Institute. GM1 was dissolved in chloroform/methanol (2:1) and stored below −20°C. Bovine serum albumin (BSA) (fraction V), anti-fd bacteriophage (rabbit IgG), peroxidase-conjugated anti-rabbit IgG and peroxidase-conjugated CTB were purchased from Sigma (St. Louis, MO, USA).

2.2. Biopanning and in situ monitoring

Experimental procedures were shown in Fig. 1. A mixed solvent of chloroform and methanol (2:1) containing GM1 was spread on Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) in a Teflon-coated trough (USI, Fukuoka, Japan). A quartz-crystal microbalance (QCM, 9 MHz) was attached horizontally to the GM1 monolayer at the surface pressure of 30 mN/m (0.67 nm^2/molecule, 0.39 μg/cm^2) [15]. The QCM was transferred to a plastic tube filled with 1 ml TBS. The phage library ($3–74\times10^{10}$ transducing units (TU), see Table 1) was added to the buffer. The frequency decrease of QCM (mass increase) corresponding to the binding of phages to the GM1 monolayer was followed with time. After the incubation for 12–15 min at 20°C, the QCM was picked up from the buffer and was washed three times with 50 μl TBS. The phages bound to the GM1 monolayer were eluted for 15 min with 0.1 N Gly-HCl buffer (pH 2.2). The eluate was neutralized with 1 M Tris-HCl buffer (pH 9.1) and treated with Centricon-30 (Amicon). The obtained phage clones were amplified by

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Abbreviations: Cer, ceramide; Gal, galactose; NeuAc, *N*-acetylneuraminic acid; GM1, Galβ1→3GalNAcβ1→4(NeuAcα2→3)-Galβ1→4Glcβ1→1'Cer; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; TBS, Tris-buffered saline; QCM, quartz-crystal microbalance; TU, transducing units; CTB, cholera toxin B subunit

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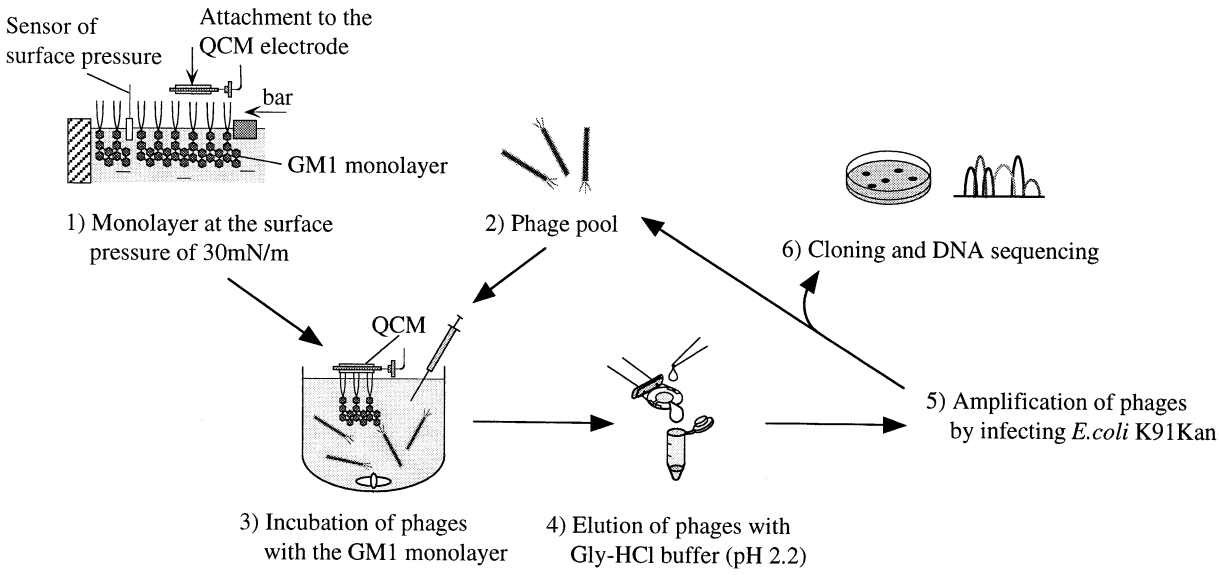

Fig. 1. Experimental procedures of biopanning.

infecting them to host bacterial cells (*E. coli* K91Kan). Such a biopanning process was repeated five times. After five cycles of biopanning, the selected phage clones were isolated and subjected to DNA sequencing according to the literature [11,12].

2.3. Assay for GM1-phage interaction by an enzyme-linked immunosorbent assay (ELISA)

The selected phage clones were amplified and purified by precipitation with polyethylene glycol 6000 (nacalai tesque, Japan). Virion concentrations were determined by measuring the absorbance at 260 nm. Plastic plates (13.5 mm diameter, cell desk, Sumitomo Bakelite, Japan) were attached horizontally to the GM1 monolayer. The phage clones (10^{-11}–10^{-8} M in 200 μl TBS) were incubated for 30 min at room temperature with the GM1 monolayer-transferred plastic plates. The plates were washed three times with TBS and transferred into a 24 well plate (Falcon). The plates were blocked with 1% BSA/TBS for 2 h at room temperature. The phages bound to GM1 monolayers were detected by the secondary antibody method (primary antibody, anti-fd bacteriophage; secondary antibody, peroxidase-conjugated anti-rabbit IgG). Orthophenylenediamine was used as color producing reagent and the color development was determined at 492 nm by a microplate reader (Multiskan MS, Labsystems, Germany).

2.4. Binding inhibition assay

The GM1 monolayer-transferred plastic plates were blocked with 1% BSA/TBS for 4 h at room temperature or overnight at 4°C, then washed with TBS. Horseradish peroxidase-conjugated CTB (1:5000 dilution in 200 μl TBS containing 0.5% BSA) and synthetic peptides (10^{-7}–10^{-3} M) were incubated with the GM1 plates overnight at 4°C in a 24 well plate. The amount of CTB bound to GM1 monolayers was determined by developing substrates as described above.

3. Results

3.1. Selection of GM1-binding peptides from a phage-displayed peptide library

The GM1 monolayer-transferred QCM was used to select GM1-recognizable phage clones. Binding of phages to the GM1 monolayer was monitored by frequency changes of QCM, which were responsible for the amount of phages bound to GM1 (Fig. 1). Frequency changes were saturated within 15 min after the addition of phages at each round. These results indicate that affinity selection completed within 15 min and that the amount of phages having affinity for GM1 increased with every round (Fig. 2). The recovery and enrichment of the phage library at each round of biopanning were determined and are shown in Table 1. The values of recovery and enrichment increased with proceeding of biopanning. These results resembled the results of QCM. At the fifth round, the values of recovery and enrichment were less than those at the fourth round. This may be caused by the addition of excess phage. The number of output at the fifth round was more than that at the fourth round. Thus, the fifth round of biopanning is considered to be successful. Sequence analysis of 18 clones obtained at the fifth round indicated that only three different clones having DFRRLPGAFWQLRQP (13/18, 72%, phage 1), GWWYKGRARPVSAVA (4/18, 22%, phage 2) and VWRLLAPPFSNRLLP (1/18, 6%, phage 3) were selected. The sequences DFRRLPGAFWQLRQP and

Table 1
Results of biopanning with the GM1 monolayer

Round (th)	Input ($\times 10^{10}$)[a]	Output ($\times 10^{5}$)[a]	Recovery[b] ($\times 10^{-6}$)	Enrichment (per round)	Enrichment (total)
1	6.1	2.4	3.9	–	1
2	5.5	13	24	6.2	6.2
3	4.8	30	63	2.6	16
4	3.0	130	430	6.8	110
5	74	590	80	0.19	21

[a]TU, transducing units.
[b]Output/input.

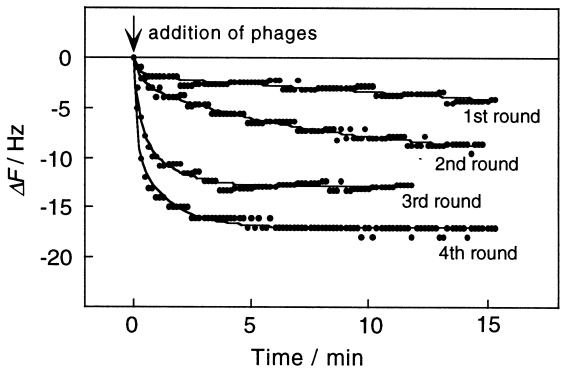

Fig. 2. In situ monitoring of the biopanning process by the QCM method. The arrow indicates the time at which the phage pool was injected into the buffer solution.

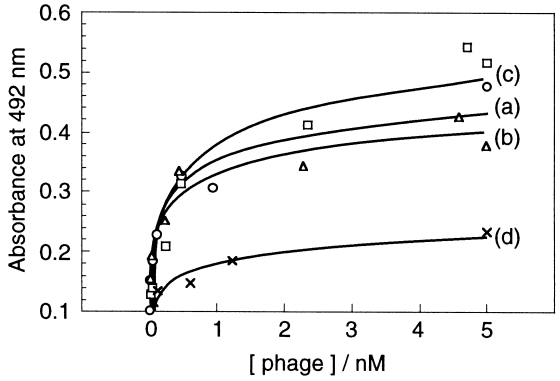

Fig. 3. Binding assay of selected phage clones to the GM1 monolayer. Phage clones were incubated with the GM1 monolayer-transferred plastic plates in TBS at room temperature for 30 min. After washing, bound phages were detected by a sandwich assay using an anti-fd phage antibody and an anti-rabbit IgG-peroxidase conjugate antibody. (a) Phage 1, (b) phage 2, (c) phage 3, (d) control phage (displaying LGRAGQSYPSFARGL). Each plot is the mean of triplicate.

VWRLLAPPFSNRLLP share a motif RxLPxxFxxxRxP (Table 2).

3.2. *Affinity of phage clones and synthetic peptides for GM1 monolayers*

Binding affinities of the three phage clones at the fifth round for GM1 monolayers were analyzed by the ELISA method (Fig. 3). The phage clones significantly bound to the GM1 monolayer depending on the concentration of phage, while the control clone (LGRAGQSYPSFARGL) hardly bounded. The dissociation constants, K_d values, were determined to be 0.2 nM for phage 1, 0.5 nM for phage 2 and 0.2 nM for phage 3. The selected phage clones have a high affinity to the GM1 monolayer in the order of 10^{-10} M. Since K_d values for GM1-CTB-binding were reported to be 10^{-8}–10^{-12} M [19–22], the binding abilities of the selected phages are found to be comparable to those of CTB.

The peptides, DFRRLPGAFWQLRQP (peptide 1), GWWYKGRARPVSAVA (peptide 2) and VWRLLAPPFSNRLLP (peptide 3), were chemically synthesized and binding affinities of these peptides for GM1 were evaluated by an inhibition assay. Fig. 4 shows the amount of CTB bound to the GM1 monolayer in the presence of peptides. These three peptides inhibited the binding of CTB to the GM1 monolayer. The IC_{50} values of synthetic peptides were determined to be 24, 13 and 1.0 μM for peptide 1, 2 and 3, respectively. Peptide 3 showed the highest inhibition for GM1-CTB-binding.

4. Discussion

In this study, we improved the method of biopanning to obtain ganglioside-binding peptides. Gangliosides in cells are localized in the plasma membrane [23]. Thus, it is important to display gangliosides that like the cell membrane for the selection of ganglioside-binding peptides. Our first attempts at selecting peptides specific to ganglioside (GM1) randomly adsorbed on a polyvinylidene difluoride membrane were unsuccessful, because more than 60 clones obtained had no consensus sequences and did not show significant binding to GM1 (unpublished results). From these results, we experienced the importance of orientation of the lipid molecules. Thus, we employed ganglioside-containing monolayers for biopanning. It is expected that the monolayer is a suitable condition to obtain peptides which bind to the oligosaccharide portion of a ganglioside.

In the present study, we employed a QCM method to monitor the binding of a phage library to the GM1 monolayer. A QCM system has been used to analyze the interaction of glycosphingolipids with proteins [14,15] and virus [16,17]. Fig. 1 showed that QCM can also detect the binding of filamentous phages employed in this study. Frequency changes of QCM showed that binding of the phage library to the GM1 mono-

Table 2
Deduced amino acid sequences of peptide displayed on the phage after the fifth round of biopanning

Clone name	Sequence[a]	Frequency[b]
Phage 1	DF**R**R**LP**GA**F**WQL**R**Q**P**	13/18
Phage 2	GWWYKGRARPVSAVA	4/18
Phage 3	VW**R**L**LA**P**P**F**S**N**R**LL**P**	1/18

[a]Amino acid sequences deduced from the DNA sequence. Consensus sequences are denoted by bold letters.
[b]Frequency is the number of sequences among the total phage clones isolated.

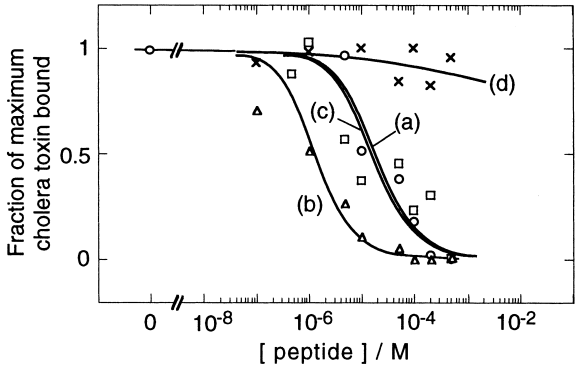

Fig. 4. Inhibition of synthetic peptides for CTB-binding to GM1 monolayers. (a) peptide 1, DFRRLPGAFWQLRQP; (b) peptide 2, GWWYKGRARPVSAVA; (c) peptide 3, VWRLLAPPFSNRLLP; (d) control peptide, AREYGTRFSLTGGYR. Each plot is the mean of triplicate.

layer was completed within 15 min and we could obtain the timing to go to the next operation. Malmborg et al. reported about direct monitoring of the panning process by surface plasmon resonance [24]. The present result indicated that QCM is also useful for detection of the panning process as well as surface plasmon resonance.

Inhibition studies for CTB-GM1 interaction showed that a synthetic peptide 3 has the highest activity, which is 24- and 13-fold higher than peptide 1 and peptide 2, respectively. Then, binding amounts of the synthetic peptides to the GM1 monolayers were estimated by the QCM method (27 MHz, 1 Hz = 0.31 ng/cm^2). The maximum binding amount of peptide 3 was 88 ng/cm^2, while those of the other two peptides were 17–27 ng/cm^2 (data not shown). These results suggest that the high inhibition activity of peptide 3 was caused by the large binding amount of it to GM1.

Previous studies on the carbohydrate-protein interaction suggest that certain amino acids play important roles on the interaction with carbohydrate [25,26]. For example: (a) arginine interacts with the carbonyl group of a sialic acid through electrostatic interaction for coat protein of murine polyoma virus [27], (b) phenylalanine interacts with the hydrophobic face of a galactopyranose ring for *Erythrina corallodendron* lectin [28] and (c) proline contributes to bending of peptide [29]. A RxLPxxFxxxRxP motif found in peptide 1 and peptide 3 contains two arginine (R), two proline (P) and a phenylalanine (F). Therefore, the RxLPxxFxxxRxP motif is expected to recognize Gal and NeuAc. In fact, the selected three peptide sequences bound to GM1 having Gal and NeuAc (Figs. 3 and 4). X-ray analyses for CTB-GM1 pentasaccharide complex were carried out by Merrit et al. and Zang et al. [30,31] and showed that several amino acids such as Glu-11, Tyr-12, His-13, Glu-51, Gln-56, Gln-61, Trp-88, Asn-90 and Lys-91 are correlated with CTB-GM1 interaction. No homology was observed between the GM1-binding site of CTB and the selected three peptides. But five carbohydrate-associable amino acids, Gln (Q), Tyr (Y), Asn (N), Trp (W) and Lys (K), were observed in the sequences of peptides 1–3.

In conclusion, the biopanning with the ganglioside monolayer was successful in selecting ganglioside-binding peptides. Ganglioside-peptide interaction is considered to depend on the orientation of the ganglioside. The technique of a phage-displayed peptide library combined with the lipid monolayer will be a strong tool for searching peptides which specifically bind to glycolipids in a biological membrane. Furthermore, the obtained peptides will be useful probes for investigating roles of glycolipids in the cell membrane.

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