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A Peptide–Glycolipid Interaction Probed by Retroinverso Peptide Analogues

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Cell surface glycolipids are implicated in the formation of lipid rafts and membrane microdomains, where they interact with protein receptors to mediate a variety of cellular processes such as cell–cell recognition, cell adhesion, and membrane signaling. Studies of glycolipid function at the local membrane structures have not been straightforward to date, because the locally clustered structures are labile and their protein binding affinities tend to be weak. While specific glycolipid-binding proteins have been employed as molecular probes for detecting lipid rafts, small peptides may be more suitable for probing glycolipids at the cell surface due to their small size as well as their ease of synthetic preparation and functionalization. Here we report an application of the retroinverso approach as a rapid method to obtain novel glycolipid-binding D-peptide sequences. We have prepared analogues of two known GM1-binding peptides by replacing L-amino acids with D-amino acids, followed by inverting the sequences and characterized their conformational propensity and glycolipid binding properties. Circular dichroism (CD) spectroscopic analysis indicated that all the peptide sequences interacted with GM1 under a micellar condition. We found, by a microplate-based competitive glycolipid binding assay, that one of the retroinverso D-peptide analogues, peptide 3, also binds GM1 as the parent L-peptide 1. These results suggested that in this glycolipid–peptide interaction, the positioning of the side chain functionalities of the peptide is important, while the peptide backbone polarity is not. Glycolipid binding retroinverso D-peptides should be useful for the design of new peptide-based probes for investigating the biological role of cell surface glycolipids.

Key words retroinverso peptide; glycolipid; peptide–glycolipid interaction; GM1; lipid raft

Glycolipids have been found as integral components of lipid rafts and membrane microdomains, where their binding interactions with proteins mediate many important cellular processes such as cell–cell recognition, cell adhesion, and membrane signaling.^{1–3} They also serve as receptors for many viruses and pathogens, as well as for amyloid peptides.^{4–6} However, studies of glycolipid function at the cell surface have traditionally been difficult because their locally clustered structures tend to be labile and their protein binding affinities are often weak. In addition, glycolipids are capable of multiple modes of interaction, which complicates the analysis of their binding partners: hydrophobic contacts with proteins at their

lipid tails in the membrane leaflets, for example, and hydrophilic interactions at their carbohydrate head groups facing the extracellular surface. Therefore, the development of suitable chemical tools to analyze the dynamic properties and interactions of glycolipids at the cell surface should be valuable for understanding their biological roles.

Protein-based probes, such as lectins, antibodies and glycolipid binding toxins, are most commonly employed for imaging the cell surface localization of glycolipids.^{7,8} One well-studied example is cholera toxin subunit B (CTB), a high affinity binder of a ganglioside GM1 enriched in lipid rafts, which is often utilized as a marker. While these approaches have been effective, the large molecular size of proteins in comparison to those of glycolipids may disrupt membrane surface structures, or induce artificial clustering of glycolipids. Moreover, protein-based probes are not suitable for studying the intracellular distribution of glycolipids in live cells. In this respect, peptides, which are smaller in size compared to proteins, would be favorable for directly detecting glycolipids based on their ease of synthetic preparation and functionalization. Matsubara *et al.* identified 15-residued peptide sequences through an *in vitro* selection of a phage displayed peptide library against a GM1 monolayer.⁹ Kraut and colleagues have developed a fluorescently labeled peptide probe based on a 25-residued glycolipid binding domain of amyloid β peptides for monitoring of glycolipid trafficking pathways.¹⁰ However, such successful precedence remains scarce in the literature. We are particularly interested in exploring the utility of peptides as chemical probes, which can be used at the cell surface to detect glycolipids by specific binding interaction at their carbohydrate head group.

Here we report an application of the retroinverso peptide approach^{11,12} as a facile method to obtain glycolipid-binding peptide sequences, which is potentially useful for studying cell surface glycolipids. By using GM1 as a model glycolipid, its cognate binding peptides and the corresponding retroinverso peptide analogues with a reversed sequence and the inverted chirality at each amino acid residue were prepared and analyzed for their glycolipid binding properties. One of the retroinverso D-peptide analogues bound to GM1 as the parent L-peptide 1. Since retroinverso peptides possess a reversed order of peptide sequence, these results indicated that in this glycolipid–peptide interaction, the positioning of the side chain functionalities of the peptide is important, while the peptide backbone polarity is not.^{11,12}

We chose to study a set of 15-amino acid peptide sequences (1–2), which have been relatively well characterized for their binding behavior toward a clustered surface of GM1 (Fig. 1a). We reasoned that employing these peptides is a good starting point in developing suitable glycolipid binding probes because the 15-mer peptides (molecular weight of *ca.* 1800 Da) are considerably smaller in comparison with lipid-binding proteins such as CTB (molecular weight of *ca.* 12 kDa). One

Fig. 1. (a) Structures of Peptides 1–4 and a Glycolipid, GM1

Putative GM1 binding residues are highlighted in red in peptides 1–4, and the double Pro residues in peptide 1 and 3 are highlighted in green. (b) Relationships among the topological peptide isomers: a parent L-peptide, mirror image D-peptide analogue, retro L-peptide analogue and a retroinverso D-peptide analogue.

of these peptide sequences (**1**) was identified previously by the Matsubara group from a phage displayed peptide library against a GM1 monolayer.⁹⁾ Peptide **1** was shown to interact with a GM1 monolayer at $K_d=1.2\mu\text{M}$.¹³⁾ A conformational analysis of peptide **1** by NMR has been reported by Nishimura and colleagues¹⁴⁾ They revealed that the free peptide has a bent structure, owing to a rigid turn imposed by the Pro7-Pro8 residues, with no defined secondary structure. The addition of GM1 in a micellar form dramatically induced stable α - and β -helical turns, with the double proline turn maintained, in which Trp2 residue was proposed to be an essential residue in the binding. In another structural study by Siebert *et al.*, the binding interaction between **1** and the pentasaccharide moiety of GM1 was analyzed using NMR and computational modeling, in which a lack of ligand-induced secondary structure formation for **1** was noted, and Arg3 and Arg12 were identified as key contacting residues to the sialic acid residue of GM1 in two separate sets of binding modes.¹⁵⁾ Peptide **2**, which is a triple alanine (Ala) mutant of **1** (Ala7, Ala8, Ala16), was subsequently found to be a slightly higher affinity binder toward a GM1 monolayer than **1**, based on alanine-scanning experiments to determine critical amino acid residues within peptide **1** for the GM1 binding.¹³⁾

Peptides **3–4** represent the retroinverso peptide analogues of **1–2**, respectively. Retroinverso peptides are one of the topological peptide isomers of bioactive L-peptides, which share amino acid composition but differ in the order of their sequences and/or chirality of amino acids.^{11,12)} They have been exploited to mimic or to probe the role of side chain functionalities in the bioactivity of interest. In a retroinverso peptide analogue, the sequence of the parent L-peptide is reversed and the chirality of amino acid residues are inverted by replacing L-amino acids with D-amino acid residues (Fig. 1b). When aligned to the parent L-peptide sequence in the opposite direction of the peptide backbone (the C-terminus to the N-terminus as opposed to the N-terminus end to the C-terminus), a retroinverso peptide can display side chain functionalities in a similar fashion to those of the parent sequence. Based on these structural features, retroinverso peptides can conveniently provide topological analogues of any short peptide sequence when the molecular recognition event between the peptide and its receptor involves only the side chain interactions.^{16–20)}

We are interested in exploring the utility of retroinverso D-peptide analogues as novel glycolipid-binding probes based on their synthetic accessibility, their facile synthetic modification, and their potential resistance toward proteases, which would be useful in the study of glycolipids in live cell settings. Retroinverso peptides also provide a convenient way to quickly test whether a specific interaction of the peptide backbone or its polarity are critical in glycolipid binding. Peptides **1–4** were designed as end capped sequences with their N-terminus acetylated and their C-terminus amidated, respectively, to eliminate the end charges and to enhance the intrinsic propensity to form a secondary structure.²¹⁾ All the peptides were synthesized by the solid-phase method using 9-fluoromethylcarboxy (Fmoc)-Rink resin and Fmoc chemistry. The coupling steps were performed using HATU/HOAT as coupling agents. Global deprotection of side chain protecting groups, and simultaneous cleavage off the resin, were carried out following a published protocol to give the crude products,²²⁾ which were then purified by preparative scale reverse-phase HPLC to

yield the desired peptide sequences.

The solution properties of the glycolipid binding peptides and their retroinverso peptide analogues were first evaluated by far-UV circular dichroism (CD) spectroscopy. As expected of short peptides, none of the peptides showed CD curves indicative of distinct secondary structures in phosphate buffer (Fig. 2). The CD spectra for peptide **1** weakly indicated the presence of β -turn conformations, which may be stabilized by double proline residues in the middle of its sequence. Thus, the peptide conformations are largely disordered in the absence of the target glycolipid. To probe the intrinsic propensity of these peptides to form secondary structures, we measured the spectra of the peptides in 2,2,2-trifluoroethanol (TFE), a known secondary structure stabilizing solvent²³⁾ (Fig. 3). The spectra of peptides at 90% TFE-phosphate buffer displayed modest α -helical propensities to a variable degree. While the spectra for peptide **1** indicated a mixture of turns, those of peptide **2** showed a distinct α -helical propensity, with a maximum at 192nm and minimum at around 208nm and 220nm, which may be due to Ala residues, a known helix former. Peptides **3–4** both exhibited weak helical propensity by the addition of TFE, which is characterized by the appearance of minima at around 192nm and maxima at 205nm. Due to their D-amino acid composition, the CD peaks for peptide **3–4** appeared in the opposite signs to those of helical L-peptides, which corresponded to the formation of left-handed turns as opposed to right-handed turns for L-peptides (Fig. 4).

We next evaluated if the conformation of the peptides become stabilized in the presence of GM1 by CD spectroscopy. In all of these CD experiments, GM1 was used at $100\mu\text{M}$, which is above its critical micellar concentration ($\text{CMC}=3.32\mu\text{M}$),²⁴⁾ and was thus expected to exist as micelles. As documented for peptide **1**,¹⁴⁾ if a peptide can interact with the glycolipid specifically, stabilization of a particular conformation should be observed. When peptide **1** ($25\mu\text{M}$) in phosphate buffered saline (PBS) was incubated with GM1, it showed an enhanced formation of turn conformations, whereas peptide **2** underwent a more drastic conformational change, with an induction of the maxima at 208 and 220nm, which are characteristic to a right-handed α -helical conformation. The retroinverso peptide **3** responded to the presence of GM1 with a pronounced structural transition with the disappearance of a maximum at 200nm, the appearance of a minimum at 195nm, and two additional maxima at 208nm and 225nm. Peptide **4** also underwent a conformational change, indicating the formation of an α -helical conformation. All of the peptide analogues of **1** showed conformational stabilization in the presence of GM1, which suggested that the binding interactions occurred between the peptides and the glycolipid, presumably at the sugar head group at the micellar surface. In a micellar form, GM1 is presented such that the hydrophobic lipid tails are buried inside and the hydrophilic sugar head group is facing the solution. It is expected that the sugar head groups are densely packed at the micellar surface area. As a consequence, anionic carboxylate groups are presented in a multivalent fashion, forming a strongly anionic surface. Since peptides **1–4** contain two arginine (Arg) residues, thus are cationic peptides, they can bind to multiple molecules of GM1 simultaneously or, alternately, through ionic interactions.

In order to assess the glycolipid binding abilities of the peptides, we employed a microplate-based competitive bind-

Fig. 2. CD Spectra of Peptides 1–4 at 25 μ M in Phosphate Buffer (pH 7.0)

Fig. 3. CD Spectra of Peptides 1–4 at 25 μ M in 90% TFE–Phosphate Buffer

ing assay which has been described in the literature as a facile assay method to rapidly screen inhibitors of cholera toxin, a well-studied high affinity GM1 binding protein.^{25,26)} The binding affinity of each peptide is therefore measured as inhibitory activity against the interaction between GM1 and CTB. Thus, the glycolipid binding abilities of the peptides were reported as IC_{50} values. CTB is a known lipid raft marker that binds monomeric GM1 with micromolar affinity,²⁷⁾ while its apparent binding capacity is amplified to nanomolar affinity²⁸⁾ when GM1 is presented in a densely clustered format by virtue of the multivalent effect. In our assay, GM1 was presented in low density on a microplate by its spontaneous absorption from a dilute stock solution (25 ng/mL) in order to avoid nonspecific surface binding of peptides to the acidic residue of GM1. The results showed that only peptide 1 and its retroinverso peptide 3 inhibited the GM1–CTB interaction, although their overall potency is rather weak (IC_{50} =245 μ M for 1 and 125 μ M for 3). Interestingly, retroinverso peptide analogue 3 exhibited slightly enhanced activity in comparison to its parent peptide 1. The reversal and inversion of the peptide sequence of 1 apparently did not matter in GM1 binding. On the other hand, it raises a possibility that peptide 3 can adopt a conformation in which the side chains of important contacting residues (Trp2, Arg3 and Arg12) are oriented to efficiently engage in the GM1 binding. Although the glycolipid binding affinity of the retroinverso peptide 3 was modest, it is expected to provide a new peptide sequence potentially useful as a chemical probe to study the target glycolipids on cell surfaces where glycolipids exist in high local concentrations.

On the other hand, peptides 2 and 4 displayed no inhibitory activities in this assay. These results are in contrast to a previous report where both peptide 1 and 2 were found to effectively inhibit CTB binding to the GM1 monolayer.⁹⁾ This may be explained by differences in the way GM1 was presented. Peptide 1 can bind to both monomeric GM1 and clustered GM1 (GM1 in a high density monolayer format), while peptide 2 can only bind to clustered GM1. Therefore, how GM1 is locally displayed presumably has a large impact on the ability of the GM1 binding peptides to engage in mo-

lecular recognition. Our finding is in accord with two previous studies, in which two different binding modes were described for the interaction between peptide 1 and GM1 presented in different formats. In one mode, one molecule of peptide 1 was calculated to bind approximately two molecules of GM1, which was presented with a high density monolayer format.⁹⁾ In another mode, evidence for the formation of a 1:1 complex was provided by electrospray ionization (ESI)-MS from a solution of 1 and the GM1 pentasaccharide at a 5:1 ratio.¹⁵⁾ It is conceivable that under our assay condition, the peptide–glycolipid interaction was not assisted by a multivalent presentation of GM1 to compete against CTB. The CD studies of peptides 1 and 2 showed that 2 responded to the presence of TFE or GM1 with a greater degree of spectral change than 1 (Figs. 1, 2). This observation indicated that 1 may possess a more preorganized conformation compared to 2, which can be stabilized by the double proline turns in the middle of the peptide sequence. Preorganization may play a role in the glycolipid binding property of 1, which may be less affected by the presentation of GM1 compared to 2. The preorganized peptide conformation may also be an important factor in the corresponding retroinverso peptide 3, which makes it active against GM1, whereas the retroinverso peptide 4 derived from peptide 2 was not.

In conclusion, we have employed known GM1 binding peptides and their retroinverso D-peptides to evaluate if they share similar glycolipid binding properties in order to rapidly obtain glycolipid binding peptide sequences, which would be potentially useful for investigating the cell surface glycolipids. All the peptides 1–4, induced helical conformations in the presence of micellar GM1, as monitored by CD spectroscopy, which indicated that they interacted with GM1 at the solvent-exposed carbohydrate head group moiety. In contrast, when GM1 was presented in low density in a microplate based competitive glycolipid binding assay against CTB, only peptide 1 and its retroinverso peptide 3 were found to bind GM1. Based on a reversed order of peptide sequence in retroinverso peptide 3 compared to peptide 1, these results indicated that neither the polarity or the direct interactions of the peptide

Fig. 4. CD Spectra of Peptides 1–4 at $25\mu\text{M}$ in the Absence and Presence of GM1 ($100\mu\text{M}$) in Phosphate Buffer (pH 7.0)

backbone play an important role in GM1 binding. Since the affinity of retroinverso peptide **3** is modest, one approach to further increase its affinity while minimizing the molecular size would be to replace the peptide backbone with a suitable scaffold, which can present the required side chain functionalities. The utility of the glycolipid binding retroinverso peptide will be explored in a live cell setting in future studies.

1. Experimental

All chemical reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan TCI and Sigma-Aldrich, St. Louis, MO, U.S.A., and were used as supplied unless otherwise noted. Solid-phase peptide synthesis (SPPS) was performed on a PSS-8 peptide synthesizer (Shimadzu Science, Kyoto, Japan). Positive ion ESI-TOF-mass spectra were acquired using a Bruker micrOTOF QII mass spectrometer (Bruker Daltonics, U.S.A.). Preparative scale and analytical HPLC experiments were performed on JASCO LC-2000 Plus system equipped with a UV detection unit, UV-2075 Plus (JASCO Co., Japan). CD spectra were recorded on a JASCO J-720 CD spectrometer (JASCO Co.).

2. Synthesis of Peptides (1–4)

Peptides were synthesized on Fmoc-Rink resin (Novabiochem) by an automated peptide synthesizer using Fmoc chem-

istry and HATU/HOAT as coupling agents. Global deprotection of side chain protecting groups, simultaneous cleavage off the resin, and precipitation of the crude products were carried out following a published protocol.²²⁾ The crude mixture was purified by preparative scale reverse-phase HPLC (JASCO) using a $10.0\times 25.0\text{ mm}$ ($5\mu\text{M}$) Waters Xbridge Prep C18 column at a flow rate of 4 mL/min (typically 40–50% MeCN–0.1% trifluoroacetic acid (TFA)/ H_2O –0.1%TFA gradient) with UV detection at 254 nm. The purity of the peptides was determined by analytical scale HPLC (JASCO) using a $4.6\times 25.0\text{ mm}$ ($5\mu\text{M}$) Waters Xbridge C18 column at a flow rate of 1 mL/min (5–95% MeCN–0.1%TFA/ H_2O –0.1%TFA gradient) with UV detection at 254 nm. The desired peptides were identified by ESI-TOF MS; Peptide **1**, high resolution (HR)-MS Calcd for $\text{C}_{88}\text{H}_{140}\text{N}_{24}\text{O}_{18}$ $[\text{M}+2\text{H}]^{2+}$: 910.5383. Found: 910.5390; Peptide **2**, HR-MS Calcd for $\text{C}_{82}\text{H}_{134}\text{N}_{24}\text{O}_{18}$ $[\text{M}+2\text{H}]^{2+}$: 871.5148. Found: 871.5128; Peptide **3**, HR-MS Calcd for $\text{C}_{88}\text{H}_{140}\text{N}_{24}\text{O}_{18}$ $[\text{M}+2\text{H}]^{2+}$: 910.5383. Found: 910.5347; Peptide **4**, HR-MS Calcd for $\text{C}_{82}\text{H}_{134}\text{N}_{24}\text{O}_{18}$ $[\text{M}+2\text{H}]^{2+}$: 871.5148. Found: 871.5123.

3. CD Spectroscopic Analysis of the Peptides

CD spectra were recorded on a JASCO J-720 spectrometer equipped with thermoelectric control of the cell jacket temperature, and a 0.1 cm path length cuvette at 10°C and 20°C . Each peptide sample solution at a final concentration of $25\mu\text{M}$ in

10 mM phosphate buffer (pH 7.2) was incubated in the absence or presence of TFE in varied amounts (0–90% v/v) or GM1 at a final concentration of 100 μ M in 10 mM phosphate buffer (pH 7.2). For each sample, three scans of data spanning from 190 nm to 240 nm in a step size of 0.2 nm with 4 s response time at each wavelength were collected.

4. Microplate-Based Competitive Binding Assay

The assay was adopted from previously reported procedures.²⁶⁾ Fifty microliter of a 13 μ g/mL solution of GM1 in ethanol was transferred to wells in a 96-well polypropylene microtiter (Polysorp Immuno™ Plate, Nunc) and allowed to air dry at room temperature for 12 h. Unattached gangliosides were removed by washing the wells twice with PBST (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4, 0.05% Tween-20). Additional binding sites on the plate surface were blocked by incubating the wells with 300 μ L of a binding buffer (SuperBlock® Blocking Buffer in PBS, Pierce) three times. The plate was incubated for 1 h with 25 μ L per well of the peptide solution at an indicated concentration in a binding buffer, which was then incubated with 25 μ L of a solution of horseradish peroxidase-conjugated CTB in a binding buffer at a final concentration of 25 μ g/mL. Incubation steps were carried out by shaking on a microplate shaker (N-704, Nissin Rika, Japan). After 1 h, the wells were washed 5 times with 200 μ L of PBST, and a peroxidase substrate TMB solution mixed with 30% hydrogen peroxide solution was added (ImmunoPure® TMB Substrate Kit, Pierce, U.S.A.). After 5 min, the reaction was quenched by 50% acetic acid solution in 1 N sulfuric acid solution. All the steps in this assay were performed at room temperature. Experiments were performed in triplicate to determine IC₅₀ values, unless noted otherwise. The amounts of viable cells were determined by measuring the UV absorbance at 490 nm on Micro Plate Reader Model 550 (Biorad, U.S.A.). The IC₅₀ values were obtained by non-linear regression curve-fitting to Hill's equation using the Prism program (GraphPad, U.S.A.).

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Conflict of Interest The author declares no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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