Preparation of peptides which mimic glycosphingolipids by using phage peptide library and their modulation on β-galactosidase activity

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Abstract We describe the use of a phage-displayed random pentadecamer peptide library for searching glycosphingolipid mimicking peptides. Two phage clones (AD-1 and AD-2) were selected by biopanning using monoclonal antibody AD117m, directed to lactotetraosylceramide (Le₄Cer). The amino acid sequences of the selected clones showed high homology (VPPXFXXXY) in 9-mer. Three phage clones were selected by using monoclonal antibody H11, directed to neolactotetraosylceramide (nLe₄Cer), the linkage isomer of Le₄Cer, and the displayed amino acid sequences were compared. One of these peptides showed the same amino acid sequence as that of AD-2 except for one amino acid substitution. Pentadecamer, 9-mer and point mutated 9-mer peptides were synthesized on the basis of the displayed amino acid sequences. Binding activity of the peptides to the monoclonal antibodies or Ricitus communis lectin showed that 9-mer peptides are enough to mimic the epitope carbohydrate structure. Furthermore, six of the synthesized peptides inhibited Jack bean β-galactosidase activity towards nLe₄Cer at a high concentration of the enzyme, whereas at lower enzyme concentrations some peptides showed potent activation of the enzyme activity. This is the first report of carbohydrate mimicking peptides which modulate glycosidase activity.

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Key words: Phage-displayed random peptide library; Glyco-replica peptide; Glycosidase inhibitor

1. Introduction

The carbohydrate moiety of glycosphingolipids has important roles in cell recognition by viruses and microorganisms [1–3], cell adhesion on inflammation and tumor metastasis [4–7], and cell differentiation [8,9]. To clarify the functional role of glycophospholipids, much experimental information is needed. There are some problems in obtaining ligand sugar chains; i.e. it is difficult to prepare glycosphingolipids from minute samples within a short time, purification of glycosphingolipids is complicated, and synthesizing glycosphingolipids needs specialized skills and expensive apparatus.

Recently, epitopes which are recognized by monoclonal antibodies have been determined by using a phage-displayed random peptide library [10–13]. Some papers indicate the phage library is available for preparation of peptides which mimic carbohydrate structures by using monoclonal antibodies directed to sugar chains [14–16].

We used a phage-displayed 15-mer random peptide library [17] and two monoclonal antibodies which recognize respectively lactotetraosylceramide (Le₄Cer) and its isomer glycosphingolipid neolactotetraosylceramide (nLe₄Cer) for preparation of peptides which mimic carbohydrate moieties of two glycosphingolipids. We selected phage clones having specific affinity against individual antibodies and investigated the binding of antibodies and a lectin to synthesized 15-mer and 9-mer peptides. In order to examine if the peptides actually mimic the biological function of glycosphingolipids, we also studied the modulation properties of the peptides on β-galactosidase activity.

2. Materials and methods

2.1. Phage-displayed random peptide library

The pentadecamer random peptide library displayed on a filamentous phage (fd phage) surface protein (pIII) was constructed as described previously [17]. The library has 2.5 × 10⁸ recombinants.

2.2. Selection of peptides which mimic the carbohydrate moiety of Le₄Cer and nLe₄Cer (biopanning)

Biopanning was done against anti-Le₄Cer monoclonal antibody (AD117m) and anti-nLe₄Cer monoclonal antibody (H11). 400 µg of antibody in 200 µl of 50 mM NaHCO₃, pH 8.8 was biotinylated by incubation with 210 µg of sulfo-NHS-biotin (Pierce, Rockford, IL) overnight at 4°C. Each biotinylated antibody was adsorbed onto a streptavidin-coated polystyrene Petri dish (10 µg/35 mm), and the antibody-coated dishes were reacted with the monoclonal antibody-coated Petri dish again. After three cycles of biopanning the selected phage clones were subcloned into Escherichia coli K91Kan. The amplified phage clones were amplified by infecting them to host bacterial cells (Escherichia coli K91Kan). The obtained phage clones were amplified by incubating with 210 µg of sulfo-NHS-biotin (Pierce, Rockford, IL) overnight at 4°C. Each biotinylated antibody was adsorbed onto a streptavidin-coated polystyrene Petri dish (10 µg/35 mm), and the antibody-coated dishes were reacted with the monoclonal antibody-coated Petri dish again. After three cycles of biopanning the selected phage clones were subjected to DNA sequence analysis.

2.3. DNA sequence

Phage from supernatants of positive colonies were precipitated with polyethylene glycol (PEG 6000), and their single strand DNA was prepared by phenol extraction. The DNA sequence of the positive phage clones obtained from biopanning was determined by dideoxy-nucleotide chain termination. An oligonucleotide 5’-TAAACAAGATGTTTCTGACCAGTA was used as an antisense primer. Each amino acid sequence was deduced from the DNA sequence.
2.5. Binding of monoclonal antibody and lectin to synthesized peptides

Monoclonal antibody (AD117m, 1 μg) was incubated with selected phage clones or synthetic peptides indicated in Table 2 overnight at 4°C. After this, the mixture was transferred to a Lc4Cer-coated polystyrene 96-well microtiter plate and left overnight at 4°C. After washing the plate with PBS several times, bound AD117m was determined with peroxidase-conjugated anti-mouse IgM antibody. The bound antibody was monitored by peroxidase activity, with o-phenylenediamine used as substrate, and the color developed was determined by a microtiter plate reader at 490 nm.

2.4. Inhibition of antibody binding by selected phage clones and synthetic peptides

Pentadecamer and 9-mer peptides indicated in Table 2 were synthesized (Peptide institute, Inc., Osaka) and their specificity for binding to monoclonal antibodies and Ricinus communis lectin, which recognizes the non-reducing terminal β-galactose of carbohydrate, was determined. Various amounts of individual peptides were coated on the bottom of a Multiscreen Immobilon-P Filtration plate (MAIP N45, Millipore, Molsheim, France). 1 μg of monoclonal antibodies or peroxidase-conjugated lectin was added in each well in 50 μl of PBS and left on the plate overnight at 4°C. After washing the plate with PBS, the bound antibodies or lectin were monitored by peroxidase activity with the Konica Immunostaining kit (Konica Co., Tokyo). Peroxidase-conjugated anti-mouse IgM was used as second antibody.

2.6. Effect of peptides on β-galactosidase activity

Jack bean β-galactosidase was incubated in the presence of various amounts of synthetic peptides in 20 μl of 0.1 M acetate buffer, pH 4.5 at 37°C using Lc4Cer as a substrate. After the incubation, the reaction mixture was air dried, the residue was dissolved in 10 μl of chloroform:methanol (2:1, v/v), and subjected to TLC. Chloroform:methanol:0.2% CaCl2 (60:30:8, v/v) was used as developing solvent mixture. After this, glycosphingolipids were made visible by spraying the plate with orcinol reagent. The enzyme activity was determined by monitoring the incubation product (Lc4Cer) with a densitograph (ATTO Co., Ltd., Tokyo).

3. Results and discussion

To develop a new approach to clarify the functional roles of glycoconjugates, we tried to prepare peptides which mimic the carbohydrate structure of glycosphingolipids using a phage-displayed random peptide library. The library is a vast mixture of filamentous phage clones each displaying a 15-mer peptide on a virion surface protein (pill) [17]. The phage were reacted with biotinylated monoclonal antibody AD117m directed to Lc4Cer, then placed on a streptavidin-coated plastic surface, thereby specific binding of the antibody-reactive phage to the plastic surface through the biotin-streptavidin bridge took place [10]. Free phage clones were washed away and bound clones were recovered in acid solution, and allowed to infect E. coli K91Kan. After repeating this biopanning three times, two phage clones were obtained. They were named AD-1 and AD-2, respectively. These two clones showed a potent inhibitory effect on the binding of the monoclonal antibody to Lc4Cer coated on a 96-well microtiter plate (Fig. 1). The peptide sequences expressed on each pIII protein of individual phage clones were determined by sequencing the DNA encoding the inserted region into the phage DNA. The deduced amino acid sequences of AD-1 and AD-2 are shown in Table 1. Nonamers of these two 15-mer peptides show significant homology.

Lc4Cer is a linkage isomer of paragloboside (nLc4Cer). The sugar sequences of these two glycosphingolipids are the same. The non-reducing terminal of β-Gal is linked at the 3 position of the penultimate GlcNAc in Lc4Cer; on the other hand, the Gal in nLc4Cer is linked to the GlcNAc of lactotriaosylceramide (Lc4Cer) through 1→4 linkage. To compare the amino acid sequences of Lc4Cer mimicking peptides with those of nLc4Cer, we selected phage clones using the monoclonal antibody H11, directed to nLc4Cer [18]. Three phage clones, named H11-1, -2, and -3, were obtained and their amino acid sequences, determined from DNA sequencing of the pIII protein, are shown in Table 1. Interestingly, the peptide sequence of AD-2 is almost the same as that of H11-2. Only one amino acid, isoleucine, is replaced by tyrosine in H11-2. But the other two peptide sequences are completely different from those of AD-1 and AD-2. On the basis of these amino acid sequences of the selected phage, we synthesized the peptides listed in Table 2. Three kinds of 9-mer peptides showed binding activity to the antibody indicating that peptide 9-mer is enough to mimic the carbohydrate structure of antigen glycosphingolipid. In Table 2, the amounts of individual peptide required for 50% inhibition of the binding of antibody AD117m to immobilized Lc4Cer on a microtiter plate are shown. For the inhibition of the binding, large amounts of peptides are required in each case compared to those for the binding experiment. This phenomenon seems to be similar to inhibition by epitope oligosaccharide. For the inhibition of antigen-antibody reaction, epitope oligosaccharide is required in a more than 1000-fold concentration compared with that of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Amino acid sequences (deduced from the DNA sequences) of 15-mer peptides expressed on pIII protein from selected phage clones by biopanning with anti-Lc4Cer antibody (AD117m) and anti-nLc4Cer antibody (H11)</th>
</tr>
</thead>
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<tr>
<td><strong>Consensus</strong></td>
<td>VFPFXXXYY</td>
</tr>
<tr>
<td>AD-1</td>
<td>RNVPEPNVDYYIAF</td>
</tr>
<tr>
<td>AD-2</td>
<td>(\text{NVPFPITLACGVR})</td>
</tr>
<tr>
<td>H11-1</td>
<td>RNVPEPNVDYYIAF</td>
</tr>
<tr>
<td>H11-2</td>
<td>RNVPEPNVDYYIAF</td>
</tr>
<tr>
<td>H11-3</td>
<td>ARFKEFLGDSRVSASH</td>
</tr>
</tbody>
</table>

AD-1 and -2 are peptides obtained from selected phage clones by biopanning with AD117m. H11-1, -2, and -3 are peptides obtained from selected phage clones by biopanning with H11. Consensus amino acids are underlined.

Fig. 1. Inhibition of antibody binding by selected phage clones, AD-1 and AD-2. Monoclonal antibody AD117m was incubated with serially diluted phage solutions at 4°C overnight. The mixture was transferred to a Lc4Cer-coated microtiter plate and left at 4°C overnight. After washing the plate with PBS, bound monoclonal antibody was determined with peroxidase-conjugated anti-mouse IgM antibody. Phage concentration was determined by measuring the absorbance at 264 nm.
Fig. 2. Inhibition of Jack bean β-galactosidase activity by the peptides. A: Incubation mixture contained 5 μg of nLC₄Cer, 10 mU of Jack bean β-galactosidase, and 50 μg of peptide in 20 μl of 0.1 M acetate buffer, pH 4.5. Incubation was done at 37°C for 2 h. Aliquots of the mixture was subjected to TLC and glycosphingolipids were made visible by spraying the plate with orcinol reagent. The enzyme activity was determined by measuring the incubation product (LC₃Cer) with a densitometer (Densitograph, ATTO Co., Ltd., Tokyo). B: Dose-dependent inhibition of Jack bean β-galactosidase by a nLC₄Cer replica peptide, H11-1-15.

Table 2

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>AD117m (μg)a</th>
<th>H11 (μg)a</th>
<th>IH₅₀ (AD117m) (μg/ml)</th>
<th>R. communis lectin (μg)a</th>
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</thead>
<tbody>
<tr>
<td>AD-2-9a</td>
<td>VPPFRTLMY</td>
<td>2</td>
<td>540</td>
<td>2</td>
</tr>
<tr>
<td>AD-2-9b</td>
<td>VPPCFRTLMY</td>
<td>8</td>
<td>150</td>
<td>nd</td>
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<tr>
<td>AD-2-9c</td>
<td>VPPFTLMY</td>
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<tr>
<td>AD-2-9d</td>
<td>VPPAFTLMY</td>
<td>nd</td>
<td>1800</td>
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<tr>
<td>AD-2-9e</td>
<td>VPPIFTLMY</td>
<td>nd</td>
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<td>8</td>
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<tr>
<td>AD-1-9</td>
<td>VPPIPNDVY</td>
<td>nd</td>
<td>1600</td>
<td>nd</td>
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<tr>
<td>AD-1-15</td>
<td>RNVPFTTNDVYVIAF</td>
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<td>0.5</td>
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<tr>
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<td>VPPIPNDV</td>
<td>nd</td>
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<td>nd</td>
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<tr>
<td>H11-2-15</td>
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<td>0.25</td>
<td>0.5</td>
<td>330</td>
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<tr>
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<td>FRGFHYHTGRWHLR</td>
<td>nd</td>
<td>1600</td>
<td>nd</td>
</tr>
<tr>
<td>H11-3-15</td>
<td>ARFPKELGRSRSARAH</td>
<td>nd</td>
<td>&gt;5000</td>
<td>nd</td>
</tr>
</tbody>
</table>

*aAmount of peptide required for binding of antibody and lectin. nd, not detected.
IH₅₀ (AD117m) is the amount of each peptide required for 50% inhibition of the binding between LC₄Cer and AD117m.
activation effect on the enzyme activity as shown in Fig. 3. When the enzyme concentration was less than 5 mU/20 μl, no incubation product was detected, but large amounts of Lc3Cer and Gal were produced by the addition of HI 1-3-15, AD1-15, AD2-9b, or AD2-9c peptide.

Although the mechanisms of activation and inhibition are difficult to clarify at the present time, an excess amount of the peptide seems to compete for the substrate binding site with nLc4Cer resulting in inhibition, and suitable amounts of substrate mimetic peptides may rather activate the enzyme to react to the micelle form of the substrate. It is well known that sufficient substrate is necessary to stimulate the enzyme reaction, and the addition of substrate mimetic peptide could make such a circumstance.

The activation mechanism of H11-3-15 showed no inhibitory effect at any incubation conditions used. The activation manner looks like an effector which binds to the activator site of the allostereic enzyme. Although we have no evidence that this enzyme is an allostereic enzyme, this kind of activator seems to be useful for future study of the enzyme regulation mechanism and also for a possible application to the therapy of glycosphingolipid storage disease. The present paper em-
phasizes the following items. (1) Possible preparation of peptides which mimic carbohydrate structure using phage-displayed random peptide library. (2) The functional roles of carbohydrate can be replaced by carbohydrate mimetic peptides. (3) Unexpected physiological activities such as inhibition or activation of enzymes could be searched for using carbohydrate mimetic peptides. A new approach for searching for functional roles of glycoconjugates will be opened by the present method. We want to propose using the term glyco-replica peptide for carbohydrate mimetic peptides.

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References