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Preparation of a mannose-6-phosphate glycan microarray through fluorescent derivatization, phosphorylation, and immobilization of natural high-mannose N-glycans and its application in ligand identification of P-type lectins

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Abstract

Glycan microarrays prepared by immobilization of amino-functionalized glycans on NHS-activated glass slides have been successfully used to study protein-glycan interactions. Fluorescently tagged glycans with an amino functional group can be prepared from natural glycans released from glycoproteins. These tagged glycans can be enzymatically modified with various glycosyltransferases, phosphotransferases, sulfotransferases, etc. to quickly expand the size and diversity of the tagged glycan libraries (TGLs). The TGLs, presented in the format of microarrays, provide a convenient platform for identifying the glycan ligands of glycan-binding proteins (GBPs). The chapter provides the background to prepare a defined glycan microarray and uses as an example glycans generated as phosphodiester and phosphomonoesters of high-mannose type *N*-glycans. The method describes the preparation of high-mannose type glycan-AEAB conjugates (GAEABs), the purification of their phosphodiester, and the subsequent mild acid hydrolysis to obtain corresponding phosphomonoesters. These GAEABs are covalently printed as a phosphorylated glycan microarray and used for analysis of the glycan ligand specificities of P-type lectins, such as the mannose-6-phosphate receptors (Man-6-P receptors or MPRs).

Keywords

Glycan microarray; glycan derivatization; phosphorylation; mannose-6-phosphate; Man-6-P receptor; P-type lectin

1. Introduction

Historically, the identification and characterization of protein-glycan interactions were determined using hapten inhibition of agglutination assays or hapten inhibition of binding detected in ELISA-like formats. These assays were sequential (one glycan hapten at a time), labor-intensive, and required large amounts of samples, which were difficult to obtain. The development of glycan microarrays (1–4), which is an extension of gene microarray technologies for genomics and protein microarray technologies of proteomics, has transformed the study of protein-glycan interactions into a micro-scale assay that can analyze hundreds of glycans simultaneously for their ability to bind proteins. The repertoire of glycans encompasses compounds that are either chemo/enzymatically synthesized (5, 6) or isolated from natural sources (7–9) and are able to be covalently (5, 10, 11) or non-covalently (12, 13) immobilized to an activated glass surface. This method can be used to test the binding specificity of any potential glycan binding protein (GBP) that can be detected fluorescently, including lectins, antibodies, viruses, and receptors.

The high mannose-type phosphorylated *N*-glycan array (14) is an example of a defined glycan array generated from naturally occurring glycans. In this example, glycans released by PNGase F digestion of bovine ribonuclease B and soybean agglutinin are purified and labeled with a bi-functional fluorescent linker; subsequently, the isomeric structures are separated and phosphorylated using recombinant form of the UDP-GlcNAc:glycoprotein *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) (15). The resulting phosphodiester are separated by HPLC, purified, structurally identified, and subsequently converted to their corresponding phosphomonoesters by mild acid hydrolysis as described previously (14). Thus, a library of fluorescently labeled *N*-linked high mannose-type *N*-glycans and their phosphodiester and phosphomonoesters was available for printing a phosphorylated *N*-glycan array. Prior to this array, there were no formats available for studying large panels of phosphomonoesters and phosphodiester of high-mannose *N*-glycans, due to the lack of methods for their chemical synthesis. The availability of a large panel of phosphomonoesters and phosphodiester of high-mannose *N*-glycans permitted the investigation of the detailed specificity of P-type lectins, notably the cation-dependent and cation-independent mannose-6-phosphate receptors (Man-6-P receptors or MPRs), which are critical for the intracellular delivery of hydrolytic enzymes to the lysosome (16–19). Although the specificity of MPRs for phosphomonoesters and phosphodiester had been observed (20), the array described here provides a platform for studying these important interactions (14, 21) and is available as a general platform for studies on P-type lectins.

2. Materials

2.1. Preparation of functional fluorescent high-mannose glycan conjugates

1. Ribonuclease B (RNaseB) (Sigma-Aldrich)
2. Soybean Agglutinin (SBA) (prepared as described previously) (22)
3. PNGase F (New England Biolabs), including Glycoprotein Denaturing Buffer (10X), G7 Reaction Buffer (10X), and NP-40 (10%)

4. 2-(N-aminoethyl)amino benzamide (AEAB) (9)
5. C18 Sep-Pak column (Millipore, 500 mg and 2 g)
6. Carboxylated SPE columns (Alltech, 150 mg, 300 mg and 1g)
7. Conjugation solvent: 7/3 (v/v) Dimethyl sulfoxide (DMSO)/Acetic acid (AcOH)
8. Methanol
9. Sodium cyanoborohydride (NaCNBH₃) (Sigma-Aldrich, 95%)
10. Trifluoroacetic acid (TFA) (Fisher Scientific, HPLC grade)
11. MilliQ water (dH₂O)
12. Analytical HPLC system with UV and Fluorescence detections (Shimadzu)
13. Semipreparative normal-phase (NP) HPLC column (250 × 9.2 mm, Agilent)
14. Hypercarb HPLC column (PGC-HPLC column, 150 × 4.6 mm, Thermo Scientific)
15. Hypercarb guard column (20 × 4 mm, Thermo Scientific)
16. Centri-vap (Labconco) at room temperature
17. Sodium azide (NaN₃)
18. Centrifuge filter (Nylon 0.2 μm, Costar)
19. MALDI-TOF/TOF (Bruker UltraflexII)
20. Matrix solution: 5 mg/mL 2,5-dihydroxybenzoic acid in 50% acetonitrile with 0.1% TFA
21. Solution A: 50% acetonitrile in 0.1% TFA
22. HPLC solvent A: Acetonitrile (Fisher Scientific, HPLC grade)
23. HPLC solvent B: 0.25 M ammonium acetate, pH 4.5
24. HPLC solvent C: water
25. HPLC solvent D: 1% TFA in water
26. Normal phase HPLC linear gradient: 0min: 80% A, 4% B, 16% C; 90min: 10% A, 50% B, 40% C

2.2. Synthesis of GlcNAc-6-phospho-GAEABs and 6-phospho-GAEABs

1. Fluorescent conjugates of purified isomers of high-mannose glycans were converted to their corresponding phosphodiester using a recombinant GlcNAc-6-phosphotransferase (20) and kindly provided by Stuart Kornfeld and Marielle Boonen (Washington University, St. Louis, MO).
2. Hydrochloric acid (HCl)
3. Acetonitrile
4. Trifluoroacetic acid (TFA)

5. LNnT-AEAB (prepared as described previously from LNnT) (9)
6. Hypercarb HPLC column ((PGC-HPLC column, 150 × 4.6 mm, Thermo Scientific)
7. Hypercarb guard column (20 × 4 mm, Thermo Scientific)
8. Phosphorylation buffer: 0.1 M Tris, pH 7.5, 0.1 M MgCl₂, and 0.1 M MnCl₂
9. Hypercarb (PGC) HPLC linear gradient: 0min: 15% A, 75% C, 10% D; 30min: 45% A, 45% C, 10% D

2.3. Printing microarrays

1. MALDI-TOF/TOF (Bruker UltraflexII)
2. Piezorray non-contact printer (Perkin-Elmer)
3. ProScanArray Scanner (Perkin-Elmer)
4. Printing buffer (2X): 600 mM sodium phosphate buffer, pH 8.5
5. NHS-activated microarray glass slides (Schott North America)
6. Biotin-hydrazine (Sigma-Aldrich)
7. Blocking buffer: 50 mM ethanolamine in 0.1 M Tris buffer, pH 9.0
8. 384 well plate, V-shape (Biorad)

2.4. Interrogation of Phosphorylated Glycan Microarray with CD-MPR and CI-MPRs

1. CI-MPR and CD-MPR and antibodies for their detection were kindly provided by Nancy Dahms (Medical College of Wisconsin, Milwaukee, WI)
2. Assay buffer: 50 mM imidazole, pH 6.5, 150mM NaCl, 10mM MnCl₂
3. Binding buffer: Assay buffer containing 1% bovine serum albumin (BSA) and 0.05% Tween 20
4. 16-Chamber adapter (Grace Biolabs)
5. Coplin jars
6. Biotinylated lectins Concanavalin A (ConA) and *Ricinus communis* agglutinin-I (RCA-I) (Vector labs)
7. Cyanine5 (Cy5)-streptavidin (Zymed)
8. Rabbit polyclonal antibodies specific for CD-MPR (B3.5) and CI-MPR (B14.5) (Generated in rabbits immunized with corresponding MPRs purified from bovine liver)
9. Alexa Fluor 488-Goat anti-rabbit IgG (Invitrogen)

3. Methods

3.1. Preparation of functional fluorescent high-mannose glycan conjugates from Ribonuclease B

1. Dissolve RNaseB (100 mg) in 5 mL 1x Glycoprotein Denaturing Buffer and boil for 10min at 100°C. To the cooled solution, add 1 mL of 10% NP40, 1 mL of 10X G7 buffer and 1 mL of 0.2 % sodium azide solution. Then add PNGase F (30 µL, 500,000 units/mL) and 2 mL water. Digest the glycoprotein at 37°C for 3 days in a stoppered tube to prevent loss of water. (NOTE 1)
2. Precondition C18 Sep-pak cartridges by washing with 1 column volume (c.v.) of methanol followed by 2 c.v. of dH₂O; Precondition Carbograph cartridges by washing with 1 column volume (c.v.) Solution A followed by 3 c.v. dH₂O.
3. The PNGase F digested solution is boiled for 5min, passed through a 2g C18 Sep-Pak cartridge and washed with dH₂O. (NOTE 2) The flow-through and 2 c.v. water wash are pooled and applied on a 1g Carbograph cartridge, which is subsequently washed with 6 c.v. dH₂O. Free glycans are then eluted from the Carbograph cartridge with 3 c.v. of Solution A. The eluent is dried by Centri-vap prior to fluorescent derivatization.
4. For fluorescent derivatization, prepare a solution of AEAB by adding 88 mg AEAB to 1 mL Conjugation solvent; vortex the mixture for 10min. This solution can be stored at -20°C for future use. Prepare fresh sodium cyanoborohydride solution by adding 64 mg NaCNBH₃ to 1 mL Conjugation solvent; vortex for 2min.
5. To the dried free glycans, add 500 µL AEAB solution and an equal volume of NaCNBH₃ solution. Vortex the mixture for 1min and incubate at 65°C for 2h. Add acetonitrile (10 mL) to precipitate the glycans. Cool the mixture at -20°C for 30min.
6. Centrifuge the mixture at 5000xg for 5min. Discard the supernatant. Dry the pellet in a Centri-vap for 5min and reconstitute it in 500 µL dH₂O. The solution is then centrifuge-filtered and ready for normal phase (NP) HPLC purification.
7. A linear gradient (0 min: 80% A, 4% B, 16% C; 90min: 10% A, 50% B, 40% C) is applied for the semi-preparative NP-HPLC separation of GAEABs from RNaseB. Five well-separated peaks are collected, which corresponds to Man5-AEAB, Man6-AEAB, Man7-AEAB (isomers), Man8-AEAB (isomers), and Man9-AEAB respectively. Fractions are dried in a Centri-vap.
8. Resuspend the dried fractions in dH₂O and pass them individually through a preconditioned carbograph (use 1 g carbograph for ~10 mg glycan-AEAB conjugates) for desalting as described in step 3. The eluent is lyophilized. Quantify

¹In this protocol, PNGase F digestion is applied directly to denatured ribonuclease B. For other glycoproteins, especially when larger amounts of glycoprotein are needed, the glycoproteins can be reduced and alkylated, then trypsinized. The dialyzed tryptic peptides are digested with PNGase F, after which the C18-carbograph purification procedure can be applied. This procedure increases the efficiency of PNGase F.

²The capacity of C18 Sep-pak and carbograph varies towards various glycans. Typically, for 100 mg glycoprotein starting material, a 2 g C18 cartridge and a 1g carbograph cartridge are used to obtain the free reducing glycans.

these fractions based on their UV 330nm absorption and fluorescence (Ex 330nm/Em 420nm); they are then ready for enzymatic phosphorylation.

9. Characterize the desalted fractions in step 8 using MALDI-TOF. Spot 0.5 μL Matrix solution and then 0.5 μL sample. Run MALDI-TOF using reflective positive mode.

3.2. Preparation of GlcNAc-6-phospho-GAEABs and 6-phospho-GAEABs

1. Incubate the purified GAEABs (0.25 to 0.5 μmol) separately at 37°C with 0.5 μg of purified GlcNAc-phosphotransferase (20), and 1 μmol of UDP-GlcNAc in 20 μL of Phosphorylation buffer. The reactions are stopped by freezing and lyophilizing after 48 h incubation.
2. The crude products are reconstituted into 100 μL dH_2O and centrifuged. The clear supernatant is injected into porous graphitized carbon (PGC)-HPLC for separation. (NOTE 3). Apply a linear gradient (0min: 15% A, 75% C, 10% D; 30min: 45% A, 45% C, 10% D) (Fig. 1). The mono- and di-phosphodiester eluted later than the starting GAEABs. Collect individual fluorescent peaks, which are then analyzed by MALDI-TOF and dried in Centri-vap. Structures are assigned according to the molecular weights and the HPLC behavior.
3. A portion of each fraction of phosphodiester is converted to its corresponding phosphomonoester by mild acid hydrolysis (0.01 M HCl, 1h, 100°C). After heating in 10 mM HCl (100 μL) at 100°C for 1 h, the solution is cooled down and injected into PGC-HPLC for purification. (NOTE 4)
4. The AEAB derivatives of the phosphomonoesters, as well as the starting non-phosphorylated and phosphodiester are quantified based on their UV absorbance relative to LNnT-AEAB standards and reconstitute to 200 μM in dH_2O and stored frozen until use. (NOTE 5)

3.3. Printing microarray of high-mannose glycan AEAB derivatives and corresponding phosphomonoesters and phosphodiester

1. We use a non-contact Piezarray printer (Piezarray, PerkinElmer) to print the microarrays.
2. For each sample, 5 μL of the 200 μM solution described above is mixed with 5 μL 2X printing buffer and loaded into a 384 well source plate with V-shape wells. The plate is fitted onto a metal adaptor for controlling the temperature during printing and set up securely in the printer.
3. According to the printer software, input the sample names, IDs, and positions. The printing pattern is programmed using the software according to manufacturer's

³PGC-HPLC (Hypercarb) is a well-suited reverse phase system for the purification of phosphomonoesters and phosphodiester. It has a wide pH range (0–14) and high capacity. The fractions collected can be dried and directly used for printing.

⁴Phosphodiester are relatively unstable toward acidic conditions, therefore need to be handled more carefully. However, the phosphomonoesters are fairly stable.

⁵Extreme caution should be used to avoid contamination of buffers or glassware with alkaline phosphatase that might come into contact with phosphorylated glycans.

instructions, including subarray number and sample replicate number. In this case, a microarray includes 14 identical subarrays. Each subarray incorporates 8 GAEABs, 8 phosphodiesteres and 8 phosphomonoesters along with 2 control glycans in 6 replicates. The structures and chart IDs are shown in Table 1. The printer software generates a GAL file based on the input, which is used by the Scanner software in the image processing step.

4. Bring NHS-activated slides from -20°C to room temperature in vacuum desiccators before opening the package. Then open the package, set a number of NHS-slides on the printing platform.
5. Tune the four tips of the printer so that ideal morphology and size dispersion are reached according to manufacturer's instructions. Start the printing process.
6. Perform pin washing step to avoid carry over contamination from different samples. Pins are washed by pushing out sample with 2 cycles of water washes. The outside of the pins are washed with flowing water. Pins are then submerged in 1% Tween-20 in printing buffer and then sonicated. Two more full round of wash cycles with water are performed again.
7. After the printing is finished, the slides are set in a box in a 55°C water bath without direct water contact for 1 h. The water bath serves as a high humidity chamber.
8. The slides are washed with 0.05% Tween-20 in PBS, then dH_2O , and then blocked with Blocking buffer for 1 h, washed again with 0.05% Tween-20 in PBS and subsequently dried by centrifugation and stored desiccated at -20°C until use.

3.4. Interrogation of Phosphorylated Glycan Microarray with CD-MPR and CI-MPRs

1. The binding assay on the microarray includes binding, washing, and scanning steps. The proteins interact and specifically bind to their ligands printed on the microarray during the binding step. Nonspecific bound proteins and excess reagents are washed away in the washing step. The fluorescence-based detection is carried out using the microarray scanner in the last step to give a fluorescence image. In this experiment, biotinylated ConA and RCA I are used as controls to validate printing of the microarray, since they bind to specific types of glycans on the array. RCA-I binds to terminal $\text{Gal}\beta 1,4\text{-GlcNAc}$, therefore it should only bind to LNnT-AEAB on the array; ConA binds to the tri-mannosyl core of N-glycans, therefore it should bind to all glycans printed on the array except LNnT-AEAB, which is not an N-glycan.
2. The bound lectins are detected by a secondary incubation with Cy5-streptavidin. The CD-MPR and CI-MPRs, on the other hand, are detected with specific rabbit polyclonal antibodies followed by Alexa Fluor488-labeled goat anti-rabbit IgG.
3. Remove slide(s) from desiccators and label slide as necessary on the top of the slide.
4. Do not write/touch on the slide where the glycans are printed.

5. Hydrate the slide by placing in a glass Coplin staining jar containing 100 mL of assay buffer for 5 min. (NOTE 6)
6. For multi-panel experiments on a single slide, affix the multi-chamber adaptor on the slide to separate a single slide into 14 chambers sealed from each other during the assay. This allows for simultaneous multiple assays on up to 14 identical subarrays on the same slide. Apply sample (for example, biotinylated lectin, 50–100 μ L) into each chamber and incubate the slide on a shaker at 60 rpm for 1 h. If some subarrays on one slide are not used, save the slide at -20°C for use at a later time.
7. For evaluation of printing efficiency, dissolve biotinylated lectins (ConA and RCA-I) in binding buffer (assay buffer containing 1% bovine serum albumin and 0.05% Tween 20) at appropriate concentrations. Add the lectin solutions (50–100 μ L) to separate chambers and incubate for 1 h at room temperature. Wash the chambers three times with the following buffers: (a) assay buffer containing 0.05% Tween 20 and (b) assay buffer. Detect bound lectins by a secondary incubation with 1 $\mu\text{g/mL}$ streptavidin labeled with Cy5 and subsequently washed as described above. To remove salts prior to drying, wash the chambers three times with dH_2O .
8. For assay with MPRs, apply proteins at the indicated concentrations in 70 μ L of binding buffer to glycan arrays separated by the 16-chamber adapter, incubated for 1 h at room temperature, and processed as described above. After washing, apply 70 μ L of 1:250 dilutions of rabbit polyclonal antibodies specific for CD-MPR and CI-MPR in binding buffer to appropriate subarrays and incubated for 1 h at room temperature. To detect MPRs, wash the subarrays as described, and add 70 μ L of goat anti-rabbit IgG labeled with Alexa Fluor488 to each subarray and incubated for 1 h at room temperature. After washing, air dry the slides.
9. Scan slide with a fluorescent scanner at the appropriate wavelengths. For Alexa Fluor488-labeled secondary antibodies, scan with 488 nm lamp, and to detect Cy5-streptavidin, scan with a 633 nm lamp. Save each image separately.
10. Process the image with Scanarray quantitation software to generate an excel worksheet as the raw data. Histograms with X-axis representing glycan chart IDs and Y-axis representing relative fluorescent units are generated for each protein sample. The binding of plant lectins and MPRs are shown in Fig. 2 and Fig. 3 respectively.

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⁶We have also used Tris buffers and MES buffers at pH 6.5 which is the pH optimum for ligand binding by the MPRs. Binding of the CD-MPR is influenced by divalent cations, with highest binding affinities observed in the presence of MnCl_2 , whereas the binding affinity of the CI-MPR is not significantly affected by divalent cations.

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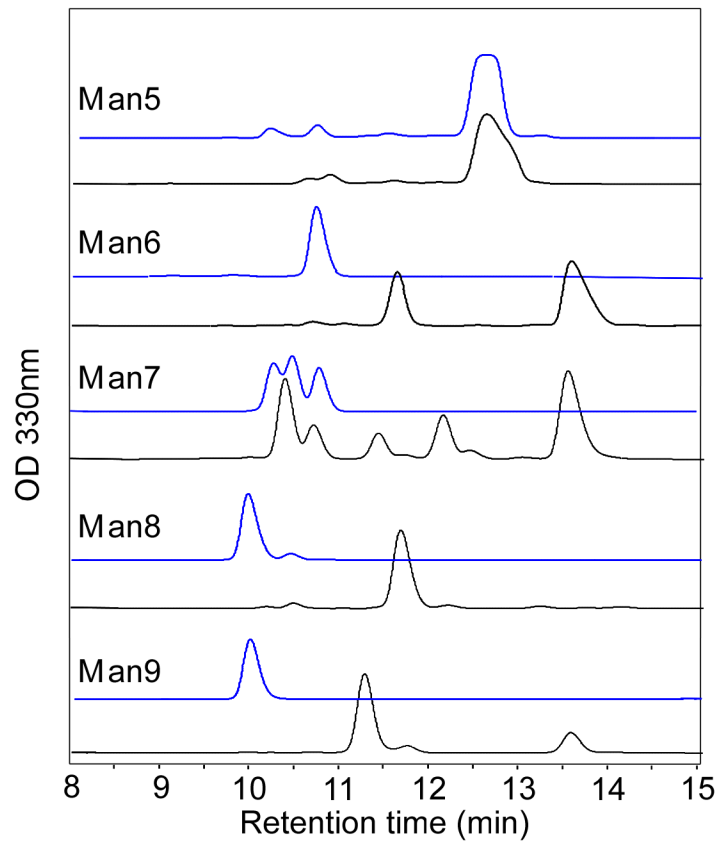


Fig. 1. Purification of phosphodiester products of AEAB-derivatized glycans (GAEABs) by HPLC on PGC-HPLC. The GAEABs prepared from Man5-9 released from RNase B are converted to phosphodiester products by GlcNAc phosphotransferase as described in the text. The starting material (upper profiles) and the corresponding products of the phosphotransferase reactions (lower profiles) were applied to PGC-HPLC, and eluted with a gradient of acetonitrile and dH₂O.

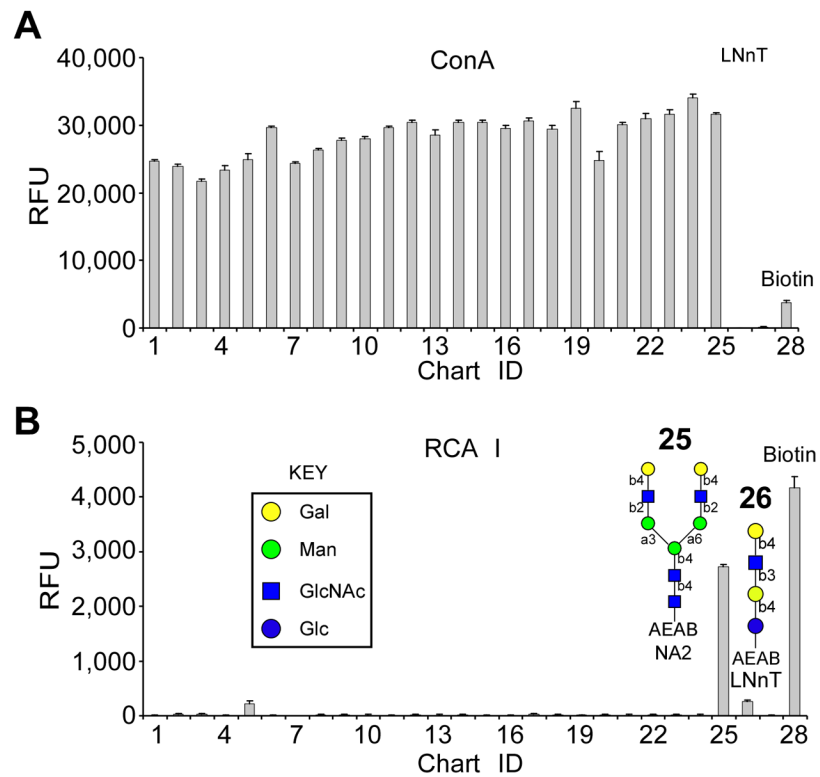


Fig. 2. Binding of plant lectins to the phosphorylated high-mannose glycan microarray. The glycan microarray was printed as described in the text and the individual glycans are identified by their glycan number as indicated in Table 1. A, biotinylated ConA (0.5 $\mu\text{g/ml}$) detected with 5 $\mu\text{g/ml}$ of Cy5-labeled streptavidin; B, biotinylated RCA-I (0.1 $\mu\text{g/ml}$) detected with 5 $\mu\text{g/ml}$ of Cy5-labeled streptavidin. Error bars indicate \pm 1 Standard Deviation.

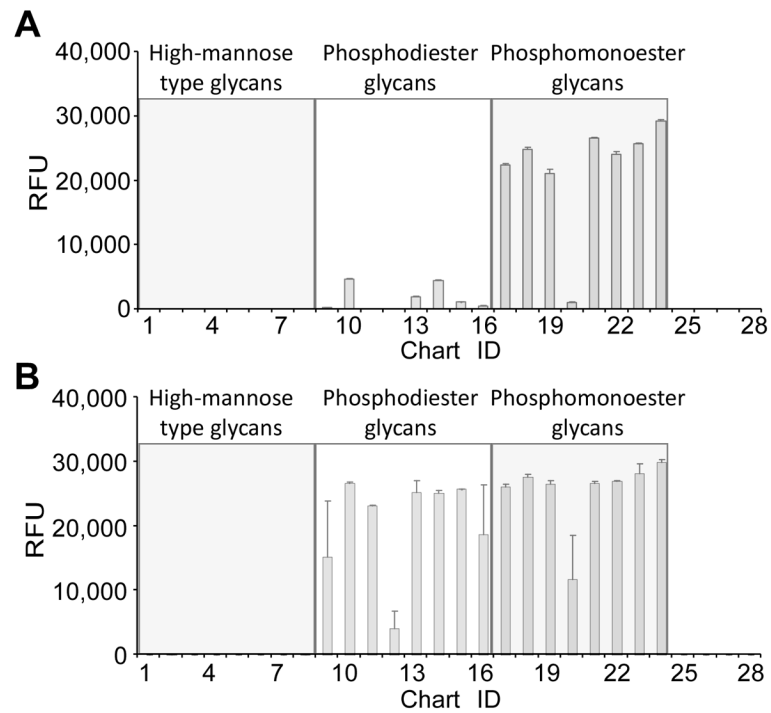
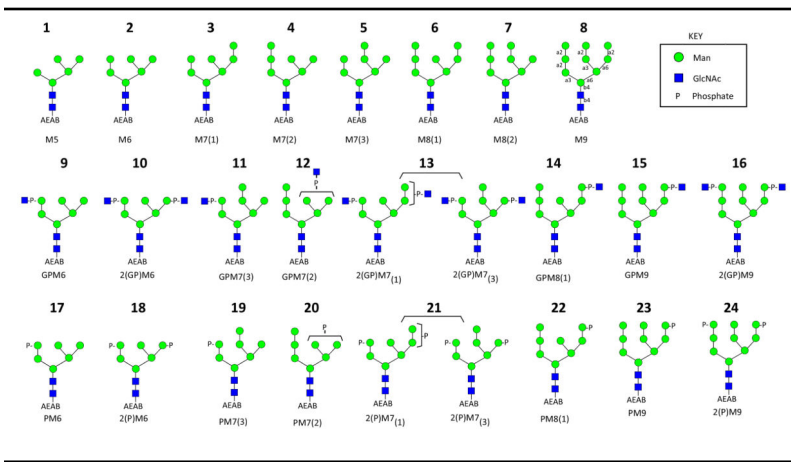


Fig. 3. Binding of CD-MPR and CI-MPR to the phosphorylated high-mannose glycan microarray. A, CD-MPR (50 $\mu\text{g}/\text{mL}$) detected with rabbit antibody B3.5 (1:250) and Alexa Fluor488-labeled goat anti-rabbit IgG (5 $\mu\text{g}/\text{mL}$); B, CI-MPR (50 $\mu\text{g}/\text{mL}$) detected with rabbit antibody B14.5 (1:250) and Alexa Fluor488-labeled goat anti-rabbit IgG (5 $\mu\text{g}/\text{mL}$).

Table 1

The chart IDs of the individual glycans printed on the microarray.



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