Glycan Microarray Analysis of P-type Lectins Reveals Distinct Phosphomannose Glycan Recognition*

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The specificity of the cation-independent and -dependent mannose 6-phosphate receptors (CI-MPR and CD-MPR) for high mannose-type N-glycans of defined structure containing zero, one, or two Man-P-GlcNAc phosphodiester or Man-6-P residues was determined by analysis on a phosphorylated glycan microarray. Amine-activated glycans were covalently printed on N-hydroxysuccinimide-activated glass slides and interrogated with different concentrations of recombinant CD-MPR or soluble CI-MPR. Neither receptor bound to non-phosphorylated glycans. The CD-MPR bound weakly or undetectably to the phosphodiester derivatives, but strongly to the phosphomonoester-containing glycans with the exception of a single Man7GlcNAc2-R isomer that contained a single Man-6-P residue. By contrast, the CI-MPR bound with high affinity to glycans containing either phospho-mono- or -diesters although, like the CD-MPR, it differentially recognized isomers of phosphorylated Man7GlcNAc2-R. This differential recognition of phosphorylated glycans by the CI- and CD-MPRs has implications for understanding the biosynthesis and targeting of lysosomal hydrolases.

The cation-independent and -dependent mannose 6-phosphate receptors (CI-MPR and CD-MPR) (P-type lectins) bind mannose 6-phosphate-containing high mannose-type N-glycans that are commonly found in most lysosomal hydrolases (1), and participate in a complex shuttling mechanism for delivering these enzymes to the lysosome (1–4). The key determinant for recognition of lysosomal hydrolases by the CI- and CD-MPR is the presence of mannose 6-phosphate (Man-6-P) on select mannose residues of the high mannose-type N-glycans. This determinant is generated through the action of the enzyme UDP-N-acetylgalactosamine:lysosomal enzyme N-acetylgalactosamine-1-phosphotransferase (GlcNAc-phosphotransferase; IUBMB accession EC 2.7.8.17) (5–7). This enzyme recognizes protein structural features on the lysosomal hydrolases as they traffic through the Golgi apparatus and adds GlcNAc-P residues to the donor UDP-GlcNAc to select mannose residues within N-glycans of lysosomal hydrolases (8–12). These Man-P-GlcNAc phosphodiester-containing glycans may be subsequently hydrolyzed by the N-acetylgalactosamine-1-phosphodiester α-N-acetylgalactosaminidase (uncovering enzyme) (IUBMB accession number EC 3.1.4.45) to generate Man-6-P phosphomonoesters (13, 14).

This sequential process of N-glycan modification may relate to the existence of two different MPRs that appear to have different specificity and modes of interactions (15). The 300-kDa CI-MPR contains 15 homologous, contiguous MRH (mannose 6-phosphate receptor homology) domains, three of which, domains 3, 5, and 9, contain a Man-6-P binding site (16–18). By contrast, the CD-MPR is a homodimer of 46-kDa subunits with one Man-6-P binding site per polypeptide (19). Although the structures of the extracytoplasmic region of the CD-MPR and domains 1–3 of the CI-MPR have been solved (20–22), understanding the detailed binding specificity of the carbohydrate binding domains has been a challenge. The difficulty in defining the specific interactions of the P-type lectins with glycans containing Man-6-P or Man-P-GlcNAc has been due to the lack of defined glycan structures that can be used for direct binding or inhibition studies. Recently, domain 5 of the CI-MPR was shown to prefer Man-P-GlcNAc phosphodiester, whereas domains 3 and 9 favored Man-6-P monoesters (22). These studies utilized recombinant α-glucosidase that was generated by treatment with recombinant GlcNAc-phosphotransferase alone or in combination with recombinant uncovering enzyme and binding was determined by surface plasmon resonance (22). However, this study did not provide information about binding of the complete extracytoplasmic domains of both MPRs, nor did it identify the contributions of the underlying high-mannose structures or the influence of the particular mannose residues that were phosphorylated. Also, these results are at variance with earlier studies showing that only glycans...
containing phosphomonoesters appeared to interact with immobilized, purified CI-MPR from bovine liver (23).

Sorting out the binding specificities of the different domains of the MPRs would be greatly facilitated by an analysis of their affinity for N-glycan structures containing the various combinations of Man-P-GlcNAc and Man-6-P residues that can potentially be generated on lysosomal hydrolases in vivo. Microarrays of immobilized glycans have been extremely useful in determining the detailed specificity of glycan binding proteins (24–27). To date, however, none of the available glycan microarrays contain a variety of phosphorylated derivatives of high mannose-type N-glycans. Here we have exploited a novel fluorescent, bifunctional linker (28), 2-amino-N-(2-aminoethyl)-benzamide (AEAB), to generate a library of fluorescence labeled high mannose-type N-glycans prepared from bovine ribonuclease B and soybean agglutinin. These glycans were purified, characterized, and then enzymatically modified to contain Man-P-GlcNAc phosphodiester and action of recombinant GlcNAc-phosphotransferase. A portion of each glycan was treated with mild acid to generate a panel of glycans containing Man-6-P phosphomonoesters. The phosphorylated glycans were used to prepare a covalent, printed glycan microarray (phosphorylated glycan microarray) using direct printing on N-hydroxysuccinimide-activated glass slides. Introduction of this microarray with the CD-MPR and CI-MPR indicated distinct differences in the preferences of these receptors for Man-P-GlcNAc phosphodiester versus Man-6-P phosphomonoesters, as well as the underlying glycan structure. Thus, the phosphorylated glycan microarray provides novel information concerning the glycan preferences by these P-type lectins, and suggests the possibility that individual domains of the CI-MPR may exhibit differences in glycan recognition.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals unless specified otherwise were from Sigma and used without further purification. HPLC solvents were purchased from Fisher. The bifunctional, fluorescent linker AEAB was prepared as described previously (28). PNGase F was purchased from New England Biolabs (Ipswitch, MA). An Ultraflex II MALDI-TOF/TOF system from Bruker Daltonics (Billerica, MA) was used for MALDI-TOF MS analysis of glycans and their derivatives. All MS data were collected using reflective and positive mode. Glycan microarrays were printed using a Piezorray Printer (PerkinElmer Life sciences), and a ProScanArray Scanner (PerkinElmer) equipped with 4 lasers was used for fluorescence analysis of protein binding to the arrays. N-Hydroxysuccinimide-activated microscope slides for printing microarrays were from Schott North America, Inc. (Louisville, KY). Bovine ribonuclease B (RNase B) was purchased from Sigma. Soybean agglutinin was purified from soybean as described previously (29). Streptavidin-Cy5 was from Zymed Laboratories Inc. (San Francisco, CA), and Alexa 488-labeled anti-rabbit IgG was from Invitrogen. Recombinant GlcNAc-phosphotransferase was kindly provided by William Canfield (Genzyme Corp.).

**Preparation of AEAB Derivatives of High Mannose-type N-Glycans**—The series of free, reducing high mannose-type N-glycans Man₉GlcNAc₂ (M5) up to Man₉GlcNAc₂ (M8), used for these studies were obtained by PNGase F digestion of bovine RNase B, and free, reducing Man₉GlcNAc₂ was obtained from soybean agglutinin as previously described (29). The released glycans were derivatized with AEAB (28), and the fluorescent glycans-AEAB derivatives (GAEABs) were separated and purified by HPLC carried out on a Shimadzu HPLC CBM-20A system coupled to a UV detector SPD-20A and a fluorescence detector RF-10Axl. UV absorption at 330 nm or fluorescence at 330 nm excitation and 420 nm emission was used to detect and quantify GAEABs using LNN-T-AEAB as a standard. For normal phase separation we used a 250 × 4.6-mm Zorbax NH₂ column (Agilent Technologies, Santa Clara, CA) with a mobile phase of acetonitrile, water, and 250 mM ammonium acetate (pH 4.5). The concentration of water increased from 16 to 40% and the concentration of ammonium acetate buffer increased from 4 to 50% over 60 min. We used porous graphitized carbon (PGC) columns (150 × 4.6 mm, Thermo Fisher Scientific) with acetonitrile and water with 0.1% trifluoroacetic acid as a mobile phase for the second dimension or final purification. The elution was initiated with 15% acetonitrile and increased from 15 to 35% over 20 min. Purified GAEABs and their phosphorylated derivatives were re-chromatographed on the PGC column, collected, analyzed by MALDI-TOF, and identified by the masses of their molecular ions.

**Synthesis of GlcNAc-6-phospho-GAEABs**—The purified GAEABs (0.25 to 0.5 µmol) of Man₉GlcNAc₂ (M5-AEAB), Man₉GlcNAc₂ (M6-AEAB), Man₉GlcNAc₂ (M9-AEAB), and isomers of Man₉GlcNAc₂ (M7-AEAB) and Man₉GlcNAc₂ (M8-AEAB) were incubated separately for 48 h at 37 °C with 0.5 µg of purified UDP-N-acetylgalactosamine-1-phosphotransferase (GlcNAc-phosphotransferase), prepared as described by Chavez et al. (22), and 1 µmol of UDP-GlcNAc in 0.02 ml of 0.1 m Tris (pH 7.5), 0.1 m MgCl₂, and 0.1 m MnCl₂. The reactions were stopped by freezing and lyophilizing the reaction mixtures. The products of the GlcNAc-phosphotransferase reactions were separated from the starting material and fractionated into their corresponding mono- and di-phosphodiester by HPLC on a PGC column as shown in Fig. 1. The phosphorylated GAEABs were identified by their chromatographic behavior and by the masses of their molecular ions as determined by MALDI-TOF analysis of the pooled fractions eluted from the columns. A portion of each fraction of phosphodiester was converted to its corresponding phosphomonoester by mild acid hydrolysis (0.01 m HCl, 1 h, 100 °C). The AEAB derivatives of the phosphomonoesters, as well as the starting non-phosphorylated and phosphodiester, were purified by HPLC on PGC, quantified based on their UV absorbance relative to LNN-T-AEAB standards, adjusted to 200 µm in distilled water, and stored frozen until use.

**Printing AEAB Derivatives and Their Corresponding Phosphomonoesters and Phosphodiester**—Aliquots (0.01 ml) of the AEAB derivatives of the non-phosphorylated high mannose glycans used as the starting material and the phosphomonoesters and phosphodiester derived from these glycans were mixed with an equal volume of 600 mM sodium phosphate buffer (pH 8.5) to make up a final concentration of 100 µm glycans in 300 mM sodium phosphate buffer and placed in wells of a 16 × 24 source plate on the Piezorray Printer. Non-contact
printing of 1/2 nanoliter per spot within 10% variation (intratip) and blocking was carried out as previously described (28). Each of the AEAB-derivatized glycans and a biotin control (0.5 μM) was printed in replicates of 6 in a subarray layout design using the Piezarray software. Sixteen subarrays were printed on each slide and arranged to correspond to a 16-chamber adaptor that was applied to the slide to separate the subarrays into 16 chambers sealed from each other during the microarray assay.

Purification of Soluble CI-MPR and CD-MPR—A soluble form of the CI-MPR was purified from fetal bovine serum by phosphomannan affinity chromatography as described previously (16). The CD-MPRHis construct (residues 1–154 containing only a single N-glycosylation site at position 81 plus a C-terminal His6 tag) in the pGAPZαA expression vector was expressed into Pichia pastoris yeast and purified from the medium using nickel-nitrilotriacetic acid affinity chromatography as described previously (30), except that an additional chromatography step using the anion exchange resin, DE-52 (Whatman), was performed. This recombinant CD-MPRHis protein was shown to bind lysosomal enzymes with a similar high affinity (Kd = 1.5 nm) as the full-length receptor (30).

Interrogation of Phosphorylated Glycan Microarray with CD-MPR and CI-MPR—To analyze proteins bound to the glycan array, the slides were fitted with the 16-chamber adapter to separate the subarray into individual wells for analysis. The subarrays were rehydrated for 5 min in assay buffer (50 mM imidazole, pH 6.5, 150 mM NaCl, 10 mM MnCl2). For evaluation of printing efficiency, biotinylated lectins (ConA and RCA-1) were dissolved in binding buffer (assay buffer containing 1% bovine serum albumin and 0.05% Tween 20) and (488 nm (excitation) and 519 nm (emission)) as described above. To remove salts prior to drying, the chambers were washed three times with 70% ethanol and dried in a laminar flow hood. After drying, the slides were scanned with a microarray scanner equipped with 4 lasers covering an excitation range from 488 to 637 nm. The scanned images were analyzed with the ScanArray Express software and the data are reported as average relative fluorescence units (RFU) per spot where the highest and lowest values for the 6 replicates are removed before averaging the remaining 4 values. For Cy5 fluorescence, 649 nm (excitation) and 670 nm (emission) were used. All images obtained from the scanner are in grayscale and colored for easy discrimination.

MPRs at the indicated concentrations were applied in 70 μl 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, 70 μl of 1:250 dilutions of rabbit polyclonal antibodies specific for CD-MPR and CI-MPR in binding buffer were applied to appropriate subarrays and incubated for 1 h at room temperature. To detect MPRs, the subarrays were washed as described, and 70 μl of goat antirabbit IgG labeled with Alexa 488 were added to each subarray and incubated for 1 h at room temperature. After washing, the slides were air dried and scanned with a microarray scanner for Alexa 488 fluorescence using 488 nm (excitation) and 519 nm (emission) as described above. CD-MPR was detected on the array using antibody B3.5, which was generated in rabbits immunized with CD-MPR purified from bovine liver. CI-MPR was detected on the array using antibody B14.5, which was generated in rabbits immunized with CI-MPR purified from bovine liver. To determine the relative affinities of MPRs for their respective phosphorylated glycan ligands, the concentrations of MPR in the assays were varied from 0.01 to 50 μg/ml. Neither of the polyclonal antibodies bound to glycans on the array as determined by omitting the MPR in the assay (data not shown). Antibody B3.5 (anti-CD-MPR) showed some cross-reactivity with CI-MPR (data not shown); however, this did not affect the assays because the antibodies were used with purified MPRs and antibody 14.5 (anti-CI-MPR) showed no cross-reactivity with CD-MPR.

RESULTS

Purification of the Phosphodiesters of High Mannose-type N-Glycans—To prepare a phosphorylated glycan microarray, we first isolated high mannose-type N-glycans. N-Glycans (Manα2GlcNAc2-Manα6GlcNAc2) were released from bovine RNase B and soybean agglutinin by treatment with PNGase F. Although RNase B has a single N-glycosylation site at Asn-34 that contains a spectrum of ManαGlcNAc2 to Manα6GlcNAc2 (31–33), soybean agglutinin has a single N-glycan that is primarily ManαGlcNAc2 (34). The released N-glycans were labeled by reductive amination with the fluorescent derivative (AEAB) (28). The GAEAb were purified by size chromatography to yield ManαGlcNAc2 (M5), ManαGlcNAc2 (M6), and ManαGlcNAc2 (M9), and mixtures of isomers of ManαGlcNAc2 (M7) and ManαGlcNAc2 (M8). Because the preparation of defined phosphorylated glycans has not been described previously, and the characterization of such glycans is key to understanding the molecular recognition by the MPRs, a careful study was undertaken to synthesize, isolate, and characterize all glycans used in this study.

These isolated species of ManαGlcNAc2-ManαGlcNAc2 (M5–M9) were incubated with recombinant GlcNAc-phosphotransferase in the presence of UDP-GlcNAc. The phosphodiester-containing glycans in the reaction mixture were separated by HPLC on a column of PGC. The elution profiles of the reaction products relative to the starting materials are shown in Fig. 1. The major peak in the M5-AEAB fraction used as a substrate for the GlcNAc-phosphotransferase reaction eluted between 12.5 and 13.0 min (Fig. 1A). This fraction contained a single molecular ion by MALDI-TOF analysis with an observed m/z = 1420.7, which is consistent with a value of 1420.5 calculated for ManαGlcNAc2-AEAB [M + Na]+. The starting material from the M5-AEAB fraction was relatively pure with minor contaminants comprised of M6-AEAB (m/z = 1582.8) and possibly one of the isomers of M5-AEAB eluting between 10 and 11 min that was recently described (35). The HPLC profile of the reaction products indicated that the major canonical structure M5-AEAB was unchanged by the GlcNAc-phosphotransferase reaction, which is consistent with M5 being a very poor substrate for the enzyme (36).
The HPLC profiles of the fractions containing M6-AEAB (Fig. 1B) and M9-AEAB (Fig. 1E) indicated the presence of only single peaks in each case, which contained single molecular ions with m/z values of 1582.8 and 2069.1, respectively. These data are consistent with calculated m/z values for Man₆GlcNAc₂-AEAB [M + Na]+ and Man₉GlcNAc₂-AEAB [M + Na]+ of 1582.6 and 2068.7, respectively. Upon incubation with the GlcNAc-phosphotransferase, M6-AEAB was converted to two distinct AEAB derivatives of m/z = 1887.7 and 2193.0 (Fig. 1B). These molecular ions were consistent with the calculated values of 1887.6 and 2192.6 for (GlcNAc-P)₆-Man₆GlcNAc₂-AEAB (GPM6) [M + 2Na]+ and (GlcNAc-P)₉-Man₆GlcNAc₂-AEAB [2(GP)M₆] [M + 3Na]+, respectively (Fig. 1B), indicating complete conversion of the M6-AEAB to mono- and diphosphodiesters of almost equal proportions. Similarly, M9-AEAB was converted to two distinct AEAB derivatives of m/z = 2374.1 and 2680.0 (Fig. 1E). These molecular ions were consistent with the calculated values of 2373.8 and 2678.8 for monophosphodiester (GlcNAc-P)₉-Man₉GlcNAc₂-AEAB (GPM9) [M + 2Na]+ and diphosphodiester (GlcNAc-P)₂-Man₉GlcNAc₂-AEAB [2(GP)M₉] [M + 3Na]+, respectively (Fig. 1E), indicating complete conversion of the M9-AEAB to the corresponding monophosphodiester as the major product, with the diphosphodiester being a minor product. The minor peak eluting at 11.7 min was not characterized due to the small quantity available.
The major peak in the M8-AEAB fraction used as a substrate for the GlcNAc-phosphotransferase reaction eluted at about 10 min (Fig. 1D), with a minor peak with the same mass (m/z = 1907.0) eluting at 10.5 min indicating that the M8-AEAB was primarily a single isomer with a mass consistent with the calculated value of 1906.7 for \( \text{Man}_9\text{GlcNAc}_2\text{-AEAB} \ [M + Na]^+ \). The GlcNAc-phosphotransferase reaction product of the major isomer was a single peak with an observed m/z = 2212.0 that is close to the calculated value of 2211.7 for \( \text{GlcNAc-P}\)-\( \text{Man}_n\text{GlcNAc}_2\text{-AEAB} \) (GPM8) \([M + 2Na]^+\) and indicated that the M8-AEAB was completely converted to the monophosphodiester with no subsequent conversion to the diposphodiester (Fig. 1D).

The HPLC profile of the M7-AEAB fraction clearly indicated the presence of at least 3 isomers of M7 designated M7(1), M7(2), and M7(3) with m/z values of 1744.9 or 1723.0, which are consistent with calculated values of 1744.6 and 1722.7 for \( \text{Man}_n\text{GlcNAc}_2\text{-AEAB} \ [M + Na]^+ \) and \( \text{Man}_n\text{GlcNAc}_2\text{-AEAB} \ [M + H]^+ \), respectively (Fig. 1C). Based on the comparison of the chromatographic profiles of the starting M7-AEAB fraction and the corresponding products, the M7(1) isomer appeared to be efficiently converted to phosphodiester, whereas M7(2) was apparently an extremely poor substrate; and M7(3) was incompletely converted to phosphodiester. The peaks at 11.4 and 12.2 min, designated as GPM7(3) and GPM7(2), both had an observed m/z = 2050.0 indicating that they were isomers with a composition consistent with \( \text{GlcNAc-P}\)-\( \text{Man}_n\text{GlcNAc}_2\text{-AEAB} \ [M + 2Na]^+ \), which has a calculated m/z = 2049.7. A single diposphodiester peak (m/z = 2355.0) was observed, and its molecular mass was consistent with a composition of \( \text{GlcNAc-P}_2\)-\( \text{Man}_n\text{GlcNAc}_2\text{-AEAB} \ [2\text{GP}]\text{M7} \ [M + 3Na]^+ \) with a calculated m/z = 2354.7. The phosphodiester-containing glycans can be converted quantitatively to Man-6-P monoesters-containing glycans by mild acid treatment.

**Designation of Glycans in the Library**—The glycan structures and controls are designated by glycan numbers that correspond to the structures identified in Table 1. The non-phosphorylated derivatives of the AEAB conjugated high mannose-type N-glycans were assigned glycans 1–8. These numbers correspond to M5-AEAB (glycan 1), which is the canonical structure eluting at 12.6 min in the upper profile of Fig. 1A; M6-AEAB (glycan 2) eluting at 10.7 min in the upper profile of Fig. 1B; three isomers of M7-AEAB (glycans 3–5) eluting between 10 and 11 min and designated M7(1), M7(2), and M7(3), in the upper profile of Fig. 1C; two isomers of M8-AEAB (glycans 6 and 7) eluting at 10 and 10.5 min and designated M8(1) and M8(2) in the upper profile of Fig. 1D, and M9-AEAB (glycan 8) eluting at 10 min in the upper profile of Fig. 1E. Phosphorylated derivatives of GAEABs generated from M6, M8, and M9 glycans represent single isomers of their most common or canonical structures, which were described recently (35) and shown in Table 1.

The Man-P-GlcNAc phosphodiester of the AEAB-conjugated high mannose-type N-glycans were assigned glycans 9–16. These numbers correspond to the monophosphodiester GPM6-AEAB (11.7 min, lower profile, Fig. 1B) and the diposphodiester 2(GP)M6-AEAB (13.7 min, lower profile, Fig. 1B) assigned glycans 9 and 10, respectively; two monophosphodiesters that are isomers of GPM7-AEAB eluting at 11.4 and 12.2 min (lower profile, Fig. 1C), designated GPM7(3) and GPM7(2) and assigned as glycans 11 and 12, respectively; the diposphodiester mixture of 2(GP)M7-AEAB (glycan 13) eluting at 13.6 min in the lower profile (Fig. 1C); the single isomer of the mono-phosphodiester GPM8-AEAB (glycan 14) eluting at 11.7 min in the lower profile (Fig. 1D); and the monophosphodiester GPM9-AEAB (glycan 15) and the di-phosphodiester 2(GP)M9-AEAB (glycan 16) eluting at 11.2 min and 13.6 min, respectively, in the lower profile (Fig. 1E).

The phosphomonoesters of all the phosphodiester glycans were generated by mild acid hydrolysis of the diesters, and were assigned glycans 17–24. Controls, including AEAB-derivatized N-linked, desialylated biantenary complex oligosaccharide terminated with Galβ1-4GlcNAc (NA2-AEAB), LNNt-AEAB, printing buffer alone and biotin, were assigned glycans 25–28, respectively.

To define the parent structures of the phosphorylated isomers of M7-AEAB, we treated the three phosphomonoesters generated by mild acid hydrolysis of phosphodiesters, GPM7(3), GPM7(2), and 2(GP)M7 (Fig. 1C, lower profile) with alkaline phosphatase and analyzed the dephosphorylated samples by HPLC on a PGC column together with re-purified M7 isomers as shown in Fig. 2, A–H. The co-elution results confirmed that GPM7(3) (glycan 11) is generated from M7(3) (glycan 5), GPM7(2) (glycan 12) is generated from M7(2) (glycan 4), and 2(GP)M7 (glycan 13) is generated from M7(1) (glycan 3)/M7(3) (glycan 5) with a ratio of 4/1 (Fig. 2). This finding is supported by the HPLC profile of the product mixture in Fig. 1C that shows M7(1) (glycan 3) is an excellent acceptor for the GlcNAc-phosphotransferase and was quantitatively converted to the major component of 2(GP)M7 (glycan 13). By contrast, M7(2) (glycan 4) is a very poor acceptor and was only partially converted to GPM7(2) (glycan 12). M7(3) is an intermediate acceptor and was also only partly converted to GPM7(11) (glycan 11) and makes up a minor component of 2(GP)M7 (glycan 13).

It is known that the difference among the three M7 isomers derived during glycoprotein processing relates to the branch of the M6 structure containing the outermost α1,2-linked mannose. MS/MS analysis of M7(1), M7(2), and M7(3) was carried out and clear fragmentation patterns are shown in Fig. 3A. Although it is difficult to discriminate isomers M7(1) and M7(3) from each other by MS/MS because they generate identical fragment ions at m/z = 1074.2 and 1398.5 (Fig. 3A), M7(2) showed a strong and unique fragment ion (m/z 1236.5) corresponding to the structure shown in Fig. 3A, middle panel.

The structures assigned for M7(1) and M7(3) in the top and bottom panels of Fig. 3A are based on their activities as substrates for recombinant GlcNAc-phosphotransferase. The phosphorylation of high mannose-type N-glycans by this enzyme and the structures before and after phosphorylation have been studied extensively (19–21). The relative preferences of individual mannose residues as acceptors for recombinant GlcNAc-phosphotransferase have been deduced from metabolically radiolabeled phosphorylated high mannose-type glycans, and these studies suggested that among the three M7 isomers, the M7(1) should be the best acceptor because mannose
residues D, E, F, and I (Fig. 3) have all been identified as sites of phosphorylation in metabolite radiolabeling studies where residue I is phosphorylated in the highest abundance (23). Thus, assigning the structure of M7(1), as shown in Fig. 3A, top panel, is consistent with the observation that M7(1) was completely converted to diphosphodiesters by the GlcNAc-phosphotransferase. Therefore M7(3) was assigned the structure shown in the lower panel of Fig. 3A. In this structure, residues D and F are sites of phosphorylation, whereas H has not been observed as a phosphorylated residue; notably, the phosphorylation of residue E when H is present was not observed in metabolically radiolabeled phosphorylated high mannose-type N-glycans (23). The expected intermediate activity of M7(3) as an acceptor is consistent with the fact that it was partially phosphorylated to phospho-mono and -diesters.

We then determined the phosphorylation pattern of the three phosphomonoesters of the M7 isomers based on MS/MS data and previous findings of the relative substrate activity of residues D, E, F, and I (Fig. 3) have all been identified as sites of phosphorylation in metabolite radiolabeling studies where residue I is phosphorylated in the highest abundance (23). Thus, assigning the structure of M7(1), as shown in Fig. 3A, top panel, is consistent with the observation that M7(1) was completely converted to diphosphodiesters by the GlcNAc-phosphotransferase. Therefore M7(3) was assigned the structure shown in the lower panel of Fig. 3A. In this structure, residues D and F are sites of phosphorylation, whereas H has not been observed as a phosphorylated residue; notably, the phosphorylation of residue E when H is present was not observed in metabolically radiolabeled phosphorylated high mannose-type N-glycans (23). The expected intermediate activity of M7(3) as an acceptor is consistent with the fact that it was partially phosphorylated to phospho-mono and -diesters.

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We then determined the phosphorylation pattern of the three phosphomonoesters of the M7 isomers based on MS/MS data and previous findings of the relative substrate activity of residues D, E, F, and I (Fig. 3) have all been identified as sites of phosphorylation in metabolite radiolabeling studies where residue I is phosphorylated in the highest abundance (23). Thus, assigning the structure of M7(1), as shown in Fig. 3A, top panel, is consistent with the observation that M7(1) was completely converted to diphosphodiesters by the GlcNAc-phosphotransferase. Therefore M7(3) was assigned the structure shown in the lower panel of Fig. 3A. In this structure, residues D and F are sites of phosphorylation, whereas H has not been observed as a phosphorylated residue; notably, the phosphorylation of residue E when H is present was not observed in metabolically radiolabeled phosphorylated high mannose-type N-glycans (23). The expected intermediate activity of M7(3) as an acceptor is consistent with the fact that it was partially phosphorylated to phospho-mono and -diesters.
Glycan Microarray Analysis of P-type Lectins

the mannose residues. For GPM7(3) (glycan 19, Fig. 3B, top panel), fragment ion m/z = 243.0 indicated a terminal phosphorylated mannose residue and fragment ions m/z = 1154.4 indicated that the phosphate is on the Manα1–2Manα1–3Manβ1–4 branch (D-B-A). This unambiguously placed the phosphate on residue D. The fact that fragment ion m/z 1154.4 is far more abundant than fragment ion m/z 1398.5 (Fig. 3B, top panel) is consistent with the fragmentation pattern observed with M7(1) and M7(3), in Fig. 3A, where the fragment ion generated from between mannose residues A–C (m/z 1074.2) is more abundant than from between mannose residues A–B (m/z 1398.5). The MS/MS analysis of GPM7(3) (glycan 20, Fig. 3B, middle panel) confirmed that it was derived from M7(3), as fragment ions (m/z = 1236.6 and m/z = 1316.6) are characteristic for this monophosphorylated structure. Based on the much higher abundance of fragment ions m/z 1236.6 than that of fragment ions m/z 1316.6, the phosphate site should be on either E or F (Fig. 3B, middle panel). This is also consistent with previous findings that the existence of residue Gly greatly inhibits the substitution at D (23).

2(GP)M7 (glycan 21, Fig. 3B, bottom panel) is a mixture of two diphospho derivatives generated from a mixture of M7(1) (major) and M7(3) (minor), as determined by chromatographic analysis of the dephosphorylated compounds as described above. The fragment ion (m/z = 1154.4) indicated one phosphate at residue D. Another fragment ion (m/z = 1478.1) indicated that the other phosphate is at other residues. For the diphospho glycan with an M7(1) backbone (major), the other phosphate could occur on E, F, or I with I being the most probable. For the diphospho glycan with an M7(3) backbone, residues D and F are phosphorylated, because E and H were not previously observed as phosphorylation sites when they co-exist in a glycan. The proposed structures of the glycans printed on the phosphorylated glycan microarray are summarized in Table 1 and identified by glycan number, as indicated in Figs. 4–6.

Interrogation of the Phosphorylated Glycan Microarray with Lectins and MPRs—To evaluate the utility of the phosphorylated glycan microarray, we first interrogated it with ConA, a plant lectin that binds all high mannose-type N-glycans, as well as complex-type biantennary N-glycans (37–39). As shown in Fig. 4A, 0.5 μg/ml of biotinylated ConA bound to all of the array targets with almost equal intensity, except for the control glycan LNnT-AEAB (glycan 26), which would not be expected to bind ConA, and the phosphate-buffered saline control.

We initiated the binding analyses of both the recombinant CD-MPR and the soluble, fetal bovine serum CI-MPR at 50 μg/ml. Binding of the CD-MPR was detected by rabbit antibody B3.5 (1:250) followed by Alexa 488-labeled goat anti-rabbit IgG, whereas binding of CI-MPR was detected by rabbit antibody B14.5 followed by the goat anti-rabbit IgG. The results of bind-
ing of the CD- and CI-MPRs are shown in Fig. 4, C and D, respectively. Neither MPR bound to the non-phosphorylated glycans (glycans 1–8). The CD-MPR bound very weakly to some glycans containing two Man-P-GlcNAc phosphodiesters (glycans 10, 13, and 14 at 50 μg/ml) and strongly to all of the phosphomonoester-containing glycans except PM7(2) (glycan 20). The CI-MPR bound to all of the phosphodiesters and monoesters except for the phosphorylated GPM7(2) (glycan 12) and PM7(2) (glycan 20), which was also not bound by the CD-MPR. These data are consistent with previous observations that the CI-MPR, but not the CD-MPR, has high affinity for both Man-P-GlcNAc phosphodiester bonds or Man-6-P phosphomonoesters in high mannose-type N-glycans (22).

Because the recombinant CD-MPR was His-tagged, we also explored its interaction with the microarray at 50 μg/ml using anti-His to detect binding, which has been successfully used in prior studies for His-tagged proteins in glycan microarray analyses (42). Detection of binding using anti-His showed similar results to those shown in Fig. 4, and demonstrated that the nature of detection with either antibody to the CD-MPR did not significantly affect the outcome of the experiment (data not shown).

In testing specificity of the interaction, we found that inclusion of 2 mM Man-6-P in the binding reaction on the slides using 50 μg/ml of either CD- or CI-MPR quantitatively inhibited binding, whereas inclusion of 2 mM UDP-GlcNAc had no effect (data not shown). These results demonstrate that the phosphorylated glycan microarray presents a valid format in which to explore molecular recognition of phosphorylated glycans.
Concentration-dependent Binding of CD-MPR and CI-MPR to Phosphorylated Glycan Microarrays—To determine whether the CD-MPR had differential specificity among the seven phosphomonoester-containing glycans that it bound (Fig. 4C), assays were performed using different concentrations of the receptor over the range of 0.1 to 50 μg/ml (Fig. 5). This type of concentration-dependent binding provides a relative binding isotherm by which to compare the apparent affinities of different glycans on the same surface. The glycans with two phosphorylated derivatives were clearly bound better than those with a single phosphorylated derivative, with the highest apparent affinity binding occurring with the mixture of 2(P)M7(1) and 2(P)M7(3) (glycan 21). The binding data in Figs. 4C and 5 were used to estimate the CD-MPR concentrations to achieve one-half maximum binding for each of the seven phosphorylated derivatives that were bound. From these data estimates of apparent molar dissociation constants were generated based on a size of 46 kDa for the CD-MPR and summarized in Table 2. The highest apparent binding affinity of the CD-MPR was displayed toward glycan 21 that has an apparent $K_d$ of 15.6 nM, whereas the weakest binding was displayed toward glycan 19 with an apparent $K_d$ of 611 nM. Thus, the CD-MPR displays a 40-fold difference in binding among different glycans with Man-6-P phosphomonoester. In addition, it is evident that the CD-MPR has weak, but detectable binding to glycans with Man-P-GlcNAc phosphodiesters (glycans 10 and 13–15), as shown also in Fig. 4C.

The concentration dependence of binding of the CI-MPR was also analyzed on the phosphorylated glycan microarray at concentrations of 0.01 to 10 μg/ml, and the data are summarized in Fig. 6. The CI-MPR exhibited high affinity binding to both phosphomonoester and phosphodiesters-containing glycans, with the exception of the phosphodiesters of M7-AEAB derivatives GPM7(3) and GPM7(2) (glycans 11 and 12) and the phosphomonoester PM7(2) (glycan 20). The apparent affinities of the CI-MPR for the phosphorylated glycans were estimated...
by determining the CI-MPR concentration at “one-half maximum binding” for each of the glycan derivatives that was bound, using an estimated size of the soluble CI-MPR of 520 kDa. Overall, the apparent molar dissociation constants of the CI-MPR for all glycans were in the \( K_d \) range of 0.06 to 0.6 nM except for glycans 12 and 20, which were poorly bound. Thus, the CI-MPR and CD-MPR show distinct differences in recognition and affinity of phosphorylated glycans in the microarray.

**DISCUSSION**

During biosynthesis, the high mannose-type N-glycans of many lysosomal hydrolases are modified in the Golgi apparatus by the GlcNAc-phosphotransferase and the donor UDP-GlcNAc to contain various amounts of Man-P-GlcNAc phosphodiesters (8, 43). These modifications are susceptible to conversion to Man-6-P phosphomonoesters by the action of the \( \alpha-N \)-acetylglucosaminidase (uncovering enzyme). The discovery of this process suggested that the two-step pathway of biosynthesis was needed to generate the Man-6-P phosphomonoesters recognized by CI- and CD-MPRs to...
facilitate movement of lysosomal hydrolases to their final destination in lysosomes.

However, there has been some uncertainty in published studies as to whether the MPRs can differentially recognize glycans with phospho-mono or -diesters. An earlier study showed that glycans with two Man-P-GlcNAc phosphodiester did not bind to immobilized CI-MPR (23), whereas equilibrium dialysis studies showed that the CI-MPR bound weakly to \[^{3}H\text{GlcNAc-}\alpha-P\text{-Man-R-Me, but had }\sim15\text{-fold higher affinity for }[^{3}H]\text{Man-6-P (19). More recent studies explored this question using surface plasmon resonance and recombinant lysosomal enzyme acid }\alpha\text{-glycosidase containing high mannose-type }N\text{-glycans with either Man-6-P phosphomonoesters or Man-P-GlcNAc phosphodiester. The results showed that recombinant domain 5 (Dom5) of the CI-MPR bound Man-P-GlcNAc phosphodiester with }\sim14\text{-to }18\text{-fold higher affinity over binding to Man-6-P phosphomonoester (22). However, those studies utilized preparations of acid }\alpha\text{-glycosidase modified to contain primarily high mannose-type }N\text{-glycans with an average of 8.35 Man residues per glycan, and variable amounts of Man-P-GlcNAc or Man-6-P. Therefore, it was not possible to assess whether differences in either glycan structures, numbers of phosphorylated glycans, degree, and position of phosphorylation, or their presentation on different lysosomal enzymes could affect their interactions with the MPRs.}

To explore this in more detail and gain information about the ability of each MPR and each domain to recognize phosphorylated glycans, it was necessary to generate a defined phosphorylated glycan microarray. This microarray was prepared using purified high mannose-type }N\text{-glycans (M5–M9) modified to contain 1 or 2 Man-P-GlcNAc phosphodiester or Man-6-P phosphomonoester. The advantages of this microarray approach is that only small amounts (micrograms) of glycan are needed, and each glycan is structurally defined and immobilized at similar concentrations on solid slide surfaces. This allows interrogation of extremely minute amounts (ng) of glycans with microquantities of MPRs, and promotes studies of concentration-dependent binding of the receptors. A limitation of this microarray is that only certain types of high mannose-type }N\text{-glycans that arise naturally through biosynthesis, as found on RNase B and soybean agglutinin, are available. Nevertheless, because of the conservation of }N\text{-glycan processing in animals it is likely that the }N\text{-glycans of RNase B represent those likely to occur on lysosomal hydrolases. This conclusion is supported by previous studies of glycan structures in purified lysosomal enzymes (23, 44, 45). An additional limitation is that only glycans efficiently modified by recombinant GlcNAc-phosphotransferase can be studied, but again the types of glycans modified in our study reflect those observed on purified lysosomal enzymes (23, 44–46).}

Our results show that both the CI- and CD-MPRs with full-length extracytoplasmic domains interact with all types of high mannose-type }N\text{-glycans (M6–M9) containing Man-6-P phosphomonoester, but did not detectably bind to non-phosphorylated glycans. A contrasting activity was observed for the different MPRs, where the CI-MPR, but not the CD-MPR, was found to bind high mannose-type }N\text{-glycans (M6–M9) containing GlcNAc-6-P phosphodiester. Interestingly, the CD-MPR was also able to bind selected glycans with GlcNAc-6-P phosphodiester, however, the affinity was estimated to be extremely weak, because binding was only detected at the highest concentration tested (50 }\mu\text{g/ml).}

We also observed that the CD-MPR bound to all of the M7 isomers containing a single Man-6-P phosphomonoester (glycans 19 and 21), with one exception (glycan 20). By contrast, the CI-MPR was able to bind glycan 20, but it did so with a lower affinity than it bound to other glycans with Man-6-P phosphomonoester. It also showed reduced binding to the same underlying glycan structure (glycan 12) containing a single Man-P-GlcNAc phosphodiester. This M7 isomer is also a weak acceptor for the GlcNAc-phosphotransferase relative to other glycans. It will be interesting in future studies to define in more detail the specificity and kinetics of the GlcNAc-phosphotransferase for individual high mannose-type }N\text{-glycans both in solution and expressed on a lysosomal enzyme. The observation that recombinant GlcNAc-phosphotransferase did not act on M5 glycans is consistent with its requirement for Manα2Man-R sequences and the observed lack of phosphorylated M5 glycans on biosynthetic lysosomal enzymes (23, 44).}

The CD-MPR bound relatively better to all glycans with two Man-6-P phosphomonoesters, such as glycans 18, 21, and 24, and some glycans with one Man-6-P phosphomonoester, such as glycans 22 and 23, but bound less well to other glycans with a single Man-6-P phosphomonoester, such as glycans 17 and 19. These results indicate that there may be specific structural features of the glycans that regulate receptor affinity, such as conformation or accessibility, independent of their number of Man-6-P phosphomonoesters. This stands in contrast to the CI-MPR, which showed little difference in affinity to any of the glycans with either phospho-mono or -diesters, except for Man-7 isomer glycans 12 and 20. These differences could be due to specific differences between the MPRs in glycan binding domains or it could reflect the overlapping, but differential recognition of glycans, within the context of the entire extracytoplasmic domain of the CI-MPR. To address these possibilities, independent studies on the binding specific of each domain for the CI-MPR compared with the CD-MPR are provided in Ref. 51.

Direct measurements of binding of Man-P-GlcNAc-methyl phosphodiester to the CI-MPR indicated that the binding affinity was relatively weak and in the }K_d\text{ range of }\sim0.1 \text{ nM (16), which was }\sim10\text{-to }15\text{-fold poorer than toward Man-6-P (19). Solution binding using surface plasmon resonance measurements showed that domain 5 (Dom5His) of the CI-MPR bound to recombinant acid }\alpha\text{-glycosidase expressing Man-P-GlcNAc phosphodiester in the }K_d\text{ range of }\sim18 \text{ }\mu\text{M (22). We estimated the apparent }K_d\text{ of the CI-MPR by analyzing its binding in a concentration-dependent fashion to the microarray and found that binding to glycans containing either phospho-mono or -diester was in the }K_d\text{ range }0.06\text{ to }0.6 \text{ nM. A more careful analysis of the potential differences in binding of different domain constructs is provided in Ref. 51. Thus, it is clear from these studies that high mannose-type }N\text{-glycans with Man-P-GlcNAc phosphodiester are recognized by the CI-MPR with relatively high affinity when present on large high mannose-type }N\text{-glycans.}
Although CI-MPR can recognize both the Man-P-GlcNAc phosphodiesters and Man-6-P phosphomonoester, it is uncertain whether recognition of Man-P-GlcNAc phosphodiesters is biologically important during lysosomal enzyme targeting. It is interesting to speculate that the ability of the CI-MPR to bind Man-P-GlcNAc phosphodiesters may be important to overall efficiency of lysosomal enzyme targeting and uptake. This is consistent with recent studies in mice generated to lack the α-N-acetylgalacosaminidase, where the phenotype is milder than seen for mice deficient in the GlcNAc phosphotransferase (47). Unlike the CD-MPR, a fraction of the CI-MPR is found in plasma membranes, where it can mediate the uptake of lysosomal enzymes into an early endocytic compartment, and after dissociation of ligands, the CI-MPR recycles through the endocytic recycling compartment back to the plasma membrane (3). By contrast, the CD-MPR is not important in surface internalization and clearance (48). The accumulating evidence here and in recent papers showing that the CI-MPR can bind to glycans with Man-P-GlcNAc phosphodiesters might provide new insights into the complex biosynthesis of lysosomal hydrolases involving both the GlcNAc phosphotransferase and the α-N-acetylgalacosaminidase. It is interesting to speculate that for those lysosomal enzymes that may be modified by the GlcNAc phosphotransferase but avoid modification by the α-N-acetylgalacosaminidase, binding of the CI-MPR to such phosphodiesters may provide a level of quality control to limit loss of the enzymes through the secretory pathway, where typically 5–20% of lysosomal hydrolases may be secreted (9). In addition, this could provide a mechanism for uptake and internalization of such enzymes with phosphodiesterases that may be secreted by neighboring cells (12).

The interactions of the P-type lectins with glycoproteins containing either Man-P-GlcNAc or Man-6-P derivatives and the availability of defined phosphorylated glycan microarray is of interest beyond lysosomal targeting. Non-lysosomal glycoproteins can also acquire these modifications. For example, the CI-MPR also binds to HFE, which is a class Ia histocompatibility protein, through its phosphorylated mannose residues on recombinant HFE expressed in the human cell line 293T (49). Interestingly, HFE bound to transferrin receptor 1 was prevented from binding CI-MPR, indicating lack of exposure of the Man-6-P signal in the HFE, but it is not yet known whether binding of HFE to CI-MPR is mediated by phospho-mono- or -diesters. The mannose 6-phosphate receptor homology domain (50) is found in other proteins, such as human OS-9 (51), but their potential interactions with phosphorylated glycans have not been defined. The availability of this defined phosphorylated glycan microarray could be useful in further defining specificities of such mannose 6-phosphate receptor homology-containing proteins, as well as further refining the specificities of the CD- and CI-MPRs.

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