Identification of a Low Affinity Mannose 6-Phosphate-binding Site in Domain 5 of the Cation-independent Mannose 6-Phosphate Receptor*

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The 300-kDa cation-independent mannose 6-phosphate receptor (CI-MPR) and the 46-kDa cation-dependent MPR (CD-MPR) are type I integral membrane glycoproteins that play a critical role in the intracellular delivery of newly synthesized mannose 6-phosphate (Man-6-P)-containing acid hydrolases to the lysosome. The extracytoplasmic region of the CI-MPR contains 15 contiguous domains, and the two high affinity (1 nM) Man-6-P-binding sites have been mapped to domains 1–3 and 9, with essential residues localized to domains 3 and 9. Domain 5 of the CI-MPR exhibits significant sequence homology to domains 3 and 9 as well as to the CD-MPR. A structure-based sequence alignment was performed that predicts that domain 5 contains the four conserved key residues (Gln, Arg, Glu, and Tyr) identified as essential for carbohydrate recognition by the CD-MPR and domains 3 and 9 of the CI-MPR, but lacks two cysteine residues predicted to form a disulfide bond within the binding pocket. To determine whether domain 5 harbors a carbohydrate-binding site, a construct that encodes domain 5 alone (Dom5His) was expressed in Pichia pastoris. Microarray analysis using 30 different oligosaccharides demonstrated that Dom5His bound specifically to a Man-6-P-containing oligosaccharide (pentamannosyl 6-phosphate). Frontal affinity chromatography showed that the affinity of Dom5His for Man-6-P was ~300-fold lower (K_d = 5.3 mM) than that observed for domains 1–3 and 9. The interaction affinity for the lysosomal enzyme β-glucuronidase was also much lower (K_d = 54 μM) as determined by surface plasmon resonance analysis. Taken together, these results demonstrate that the CI-MPR contains a third Man-6-P recognition site that is located in domain 5 and that exhibits lower affinity than the carbohydrate-binding sites present in domains 1–3 and 9.

The P-type lectins, the 300-kDa cation-independent mannose 6-phosphate receptor (CI-MPR)1 and the 46-kDa cation-dependent MPR (CD-MPR), play a key role in the formation of lysosomes in higher eukaryotes. The MPRs function in the intracellular routing of acid hydrolases that bear mannose 6-phosphate (Man-6-P) residues on their N-linked oligosaccharide chains by delivering their cargo from the trans-Golgi network to endosomal compartments, thereby diverting these soluble enzymes from the secretory pathway (1). Although both receptors exhibit similar trafficking itineraries, traveling between intracellular compartments and the plasma membrane to carry out multiple rounds of protein transport, only the CI-MPR functions at the cell surface in the binding and internalization of exogenous ligands (2, 3). In addition to lysosomal enzymes, a number of other proteins have been identified that contain Man-6-P. This growing list of extracellular ligands includes granzymes A and B (4), transforming growth factor-β precursor (5), proliferin (6), renin precursor (7), leukemia inhibitory factor (8), and herpes simplex virus glycoprotein D (9). Furthermore, several studies indicate that interaction with cell-surface CI-MPRs modulates the function and/or activity of these extracellular ligands, suggesting a role for the CI-MPR in various processes such as apoptosis, cell migration, cell growth, and viral entry (10).

The MPRs are type I transmembrane glycoproteins. Biochemical (11, 12) and structural (13) studies support the existence of the CD-MPR as a stable homodimer. The CI-MPR is also capable of forming oligomeric structures that are most likely dimers, and the binding of a lysosomal enzyme appears to stabilize the oligomeric state (14). The CI-MPR contains a large extracellular region consisting of 15 tandemly repeating units with an average size of 147 amino acids. Sequence analysis of each of the 15 domains of the CI-MPR demonstrated significant acidic amino acid identity to each other (16–38%) and to the ~150-residue extracytoplasmic region of the CD-MPR (14–28%) (15). In contrast to the CD-MPR, which binds one molecule of Man-6-P-polypeptide (16), the CI-MPR contains two high affinity (nanomolar) Man-6-P-binding sites, which have been mapped to domains 1–3 and 9 (17–19), with essential residues localized to domains 3 and 9 (20, 21). The CI-MPR, unlike the CD-MPR, also interacts with a number of non-Man-6-P-containing molecules that include insulin-like growth fac-
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tor II, plasminogen, the urokinase-type plasminogen activator receptor, and retinoic acid. Insulin-like growth factor II binds to domain 11 of the CI-MPR, and sequences in domain 13 have been shown to enhance the binding affinity by ~10-fold (22–24). Plasminogen and the urokinase-type plasminogen activator receptor bind to regions distinct from the Man-6-P- and insulin-like growth factor II-binding sites, and expression of truncated CI-MPR constructs has localized the plasminogen- and urokinase-type plasminogen activator receptor-binding sites to the N-terminal half of domain 1 (25). Limited information is available on the retinoic acid-binding site. Insulin-like growth factor II and Man-6-P do not inhibit retinoic acid binding, and studies indicate that the ~40-kDa C-terminal region of the receptor is essential for this interaction (26). Although domains 1, 3, 9, 11, and 13 have been assigned ligand binding functions, the role (i.e. ligand binding and/or structural) the remaining domains play in receptor activity is not known.

Previous sequence analyses of the 15 repeating domains of the CI-MPR by Lobel et al. (15) led to the observation that the two high affinity Man-6-P-binding sites (i.e. domains 3 and 9) of the CI-MPR exhibit significant sequence homology to each other and to the extracytoplasmic region of the CD-MPR, suggesting that the conserved sequences may be indicative of their common function of phosphomannosyl recognition. Interestingly, this study also revealed that domain 5 exhibits a comparable level of sequence identity to the CD-MPR as observed for domains 3 and 9. Our crystal structures of the CD-MPR (13, 27) and domains 1–3 of the CI-MPR (28, 29) plus mutagenesis studies (21, 30, 31) have identified conserved residues that are essential for Man-6-P binding. To evaluate the possibility that domain 5 binds carbohydrate, we performed a structure-based sequence alignment to compare domain 5 with the CD-MPR and domains 3 and 9 of the CI-MPR (see Fig. 1). The alignment reveals that domain 5 contains the four key residues (corresponding to Gin-66, Arg-111, Glu-133, and Tyr-143) that are conserved in the CD-MPR and in domains 3 and 9 of all CI-MPRs sequenced to date and that have been demonstrated to be essential for high affinity Man-6-P binding. However, this sequence alignment reveals that domain 5 lacks two cysteine residues that, based on the structures of the CD-MPR (13, 27) and domains 1–3 of the CI-MPR (28, 29), are predicted to form a disulfide bond that is critical for the formation of a Man-6-P-binding pocket. Thus, it is unclear from the alignment analysis whether domain 5 harbors a Man-6-P-binding site.

To directly demonstrate whether domain 5 of the CI-MPR contains a Man-6-P recognition site, a construct encoding domain 5 alone was generated and assayed for its ability to bind carbohydrate ligands. Although preliminary equilibrium binding studies using a lysosomal enzyme, β-glucuronidase, did not detect specific binding up to a ligand concentration of 250 nM, an oligosaccharide-based microarray (32, 33) demonstrated that domain 5 specifically interacts with pentamannosyl-6-phosphate. This critical observation led us to further probe the carbohydrate binding activity of this region of the CI-MPR by pentamannosyl phosphate-agarose affinity chromatography and surface plasmon resonance (SPR) analyses. Taken together, this study has identified a third Man-6-P recognition site in the CI-MPR that is localized to domain 5 and that exhibits a significantly lower affinity (~300-fold) for Man-6-P compared with domains 1–3 and 9 of the receptor.

EXPERIMENTAL PROCEDURES

Materials—The following materials and reagents were obtained commercially as indicated: *Pichia pastoris* wild-type strain X-33, *P. pastoris* expression vector pGAPZaA, Zeocin, and T4 DNA ligase (Invitrogen); BioMix DNA polymerase (Bioline); restriction endonucleases (New England Biolabs Inc.); GeneMate plasmid DNA miniprep kits (ISC BioExpress); Benchmark prestained and unstained protein markers (Invitrogen); endo-β-N-acetylglucosaminidase H (endo H) (Roche Applied Science); α-aminooxylation-agarose gel filtration standards, glucose-6-phosphate, Man-6-P, and mannose (Sigma); 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP), SwellGel bulk cobalt chelated discs, and SuperSignal West Pico chemiluminescent substrate (Pierce); polyvinylidene difluoride (Millipore Corp.); 3,5-dimethoxy-4-hydroxycinnamic acid (Aero Organics); and carrier-free Na218O (Amer sham Biosciences). Phosphomannan from *Hansenula holstii* was a kind gift from Dr. M. E. Slodki (Northern Regional Research Center, Peoria, IL). MITX3.2 cells expressing human β-glucuronidase were generously provided by Dr. W. Sly (St. Louis University School of Medicine, St. Louis, MO).

Generation of Truncated CI-MPR Constructs—The cDNA construct encoding extracytoplasmic domains 1–15 of the bovine CI-MPR was used as a template to generate a construct encoding domain 5 alone followed by a C-terminal His tag (Dom5His). Briefly, the sequences encoding domain 5 residues 584–725 followed by six histidine residues (CAC) and a stop codon (TGA) were amplified by PCR and subcloned into the P. pastoris expression vector pGAPZaA, which utilizes a constitutive promoter, in-frame with the Zuccharomycetes cerevisiae α-factor signal sequence. The Dom5His construct (residues 1184–1327 followed by a His6 tag) was subcloned into the pGAPZαA vector as described previously (19). DNA sequencing (Protein and Nucleic Acid Core Facility, Medical College of Wisconsin) confirmed the predicted sequences.

Expression and Purification of Dom5His and Dom9His Constructs—The cDNA constructs were linearized with BspHI and transformed into *P. pastoris* by electroporation, and Zoccin-resistant transformants were selected as described previously (34). Positive clones were inoculated in liquid medium containing 1% yeast extract, 2% peptone, and 2% dextrose, and cultures were harvested after 5 days of growth at 30 °C. Following removal of the cells by centrifugation, the medium was dialyzed against binding buffer (20 mM Tris and 500 mM NaCl, pH 8.0) (Dom5His) or binding buffer containing 10 mM imidazole (Dom9His). The dialyzed medium was passed over a cobalt-agarose resin, washed with binding buffer containing 10 mM imidazole, and then eluted with binding buffer containing 100 mM imidazole. Proteins were concentrated by filtration using a 0.5-mI Millipore Ultrafree spin column containing a Biomax membrane with a 5-kDa nominal molecular mass cutoff. The Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard was used to estimate protein yields.

*Endo H Digestion*—Purified Dom5His was incubated with *Endo H* in buffer containing 100 mM sodium citrate, pH 6.0, 0.075% SDS, and 10 mM β-mercaptoethanol at 37 °C. The samples were resolved by SDS-PAGE and detected by silver staining as described by the manufacturer (Bio-Rad).

*N-terminal Amino Acid Sequencing*—Purified Dom5His was subjected to N-terminal amino acid sequence analysis (Protein and Nucleic Acid Core Facility, Medical College of Wisconsin). Fifteen cycles of Edman degradation were performed, and the phenylthiohydantoin-derivatives were separated by reverse-phase high performance liquid chromatography.

Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS)—Purified Dom5His was mixed with a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid. The sample/matrix mixture was spotted on a MALDI target plate and analyzed on a Voyager DE-PRO MALDI mass spectrometer (PerSeptive Biosystems) as described previously (34).

*Gel Filtration Chromatography*—Purified Dom5His (300 µg) was chromatographed on a Superdex-75 fast protein liquid chromatography column (1 × 30 cm; Amersham Biotech) equilibrated in buffer containing 50 mM MES, 150 mM NaCl, and 5 mM β-glycerophosphate, pH 6.5 (5.0 ml) were collected at a flow rate of 0.5 ml/min. The elution profile was monitored at 280 nm. The column was calibrated with the following standards: bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa).

Chemical Cross-linking—Purified Dom5His (500 ng) was incubated in buffer containing 50 mM sodium phosphate, 100 mM NaCl, pH 7.4, and 1 mM DTSSP (a homobifunctional cross-linker) for 1 h at 25 °C. The reaction was quenched by the addition of glycine to 100 mM. The samples were resolved by SDS-PAGE followed by Western blotting as described previously (35), except that the proteins were detected using an anti-tetrahistidine monoclonal antibody (QIAGEN Inc.) followed by horseradish peroxidase-linked goat anti-mouse antibody (Amersham Biosciences).

Oligosaccharide Microarray Probed with Dom5His and Dom9His—An oligosaccharide array was prepared essentially as described (32). Briefly, oligosaccharides were converted to neoglycolipids by conjugation to 1,2-dihexadecyl-sn-glycerol-3-phosphothanolamine. Neoglyco-
lipids and glycolipids (10 mg/ml in chloroform/methanol/water, 25:25:8 by volume) were applied as 2-mm bands onto nitrocellulose-coated glass slides (FAST slides, Schleicher & Schuell) by jet spray with a Linomat IV sample applicator (Camag, Switzerland). FAST slides with lipid-linked saccharides were immersed at room temperature for 45 min in blocker casein solution, pH 7.4 (Fierce), and then overlaid for 2 h with Dom5His (500 mg/ml) or Dom9His (100 mg/ml) in the same solution. The slides were washed with phosphate-buffered saline, pH 8.8, and then overlaid for 1 h with mouse anti-tetrahistidine monoclonal antibody to 1 mg/ml in blocker casein solution, pH 7.4. After washing with phosphate-buffered saline, pH 7.4, the slide was overlaid for 1 h with a 1:100 dilution of goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (Dako Corp.) in blocker casein solution. The slides were washed with phosphate-buffered saline, and antibody binding was detected by nickel-enhanced 3,3'-diaminobenzidine substrate kit (Vector Laboratories). The image was captured using a digital camera and processed using Corel Photo-Paint software.

Pentamannosyl Phosphate-Agarose Affinity Chromatography and Acidic pH Dissociation—Purified Dom5His (4.5 mg) was subjected to pentamannosyl phosphate-agarose affinity chromatography (1 ml of resin at a flow rate of 2.5 ml/h by loading the protein onto the column (0.8 x 4 cm, with a 8-ml reservoir) in 3 ml of column buffer (50 mM imidazole, 150 mM NaCl, 5 mM β-glycerophosphate, and 10 mM MnCl₂, pH 6.5). The column was washed with 4 ml of column buffer and then eluted with 1.5 ml of 10 mM Man-6-P in column buffer. Fractions (0.5 ml) were collected starting at the beginning of sample application. In other experiments, the protein was loaded and washed in the presence of 10 mM Man-6-P, 10 mM glucose 6-phosphate, or 10 mM mannose. For acidic pH dissociation experiments, the protein was loaded and washed with acidic pH 3.5 buffer (50 mM sodium acetate, 150 mM NaCl, 5 mM β-glycerophosphate, and 10 mM MnCl₂, pH 3.5) followed by elution in acidic pH 3.5 buffer containing 10 mM Man-6-P. Fractions were precipitated with 10% (v/v) trifluoroacetic acid and subjected to SDS-PAGE, and the proteins were detected by silver staining as described above. The gels were analyzed by densitometry to quantify the amount of protein in each lane using Alphalager Version 2200 software.

Frontal Affinity Chromatography—A single Bio-Rad Econo-Pac polypropylene column (1.5 x 12 cm, with 15-ml reservoir) was packed with pentamannosyl phosphate-agarose resin (bed volume of 5.3 ml) and used for all frontal chromatographic operations performed at 4°C. The column was equilibrated with column buffer, and a solution of Dom5His (0.54 μM) prepared in the same buffer was applied to the column continuously at a flow rate of 4 ml/h. Equal volume fractions were collected starting at the beginning of sample application, and the concentration of protein in each fraction was determined spectrophotometrically at 280 nm. Multiple runs were performed on the same column in the presence of increasing concentrations of Man-6-P after pre-equilibrating the column with buffer containing the appropriate concentration of Man-6-P. The resin was regenerated by washing with column buffer containing 10 mM Man-6-P followed by acidic pH 3.5 buffer. In the frontal analysis (36), a solution of an analyte, A (Dom5His), is continuously applied to an immobilized low affinity ligand (pentamannosyl phosphate-agarose resin) that specifically interacts with the analyte. A dynamic state of equilibrium is attained once the amount of applied analyte exceeds the retaining capacity of the column and the concentration of the eluted analyte becomes equal to that of the applied analyte. The elution curve of the analyte is thus composed of a front and a plateau, and the elution volume (V) of the front can be determined accurately using Equation 1,

\[ V = \frac{\sum |A|}{|A|} \alpha + n a \]  

(Eq. 1)

where n is the number of a certain fraction at the plateau, α is the volume of one fraction, |A| is the concentration of fraction i, and |A₀| is the injection applied to the column. The elution volume of the analyte reaches its maximum limit, Vₘ, when |A| approaches zero; Vᵢ is the elution volume of the analyte in the presence of saturating amounts of soluble competitive inhibitor, 1 (Man-6-P), where the specific interaction of the analyte with the immobilized ligand is completely suppressed. The presence of a soluble ligand (1) therefore decreases the elution volume from V₀ to Vᵢ (Vᵢ is the elution volume in the elution volume of 1); and under conditions satisfying the requirement of |I| > |A₀|, the amount of adsorbed analyte decreases from |A₀|(V₀ - Vᵢ) to |A₀|(Vᵢ - Vᵢ), and these parameters are related to the dissociation constant (Kₒ) of the inhibitor by Equation 2.

\[ Vᵢ = V₀ + Kᵢ \left( \frac{V₀ - Vᵢ}{|I|} \right) \]  

(Eq. 2)
To accurately determine the extent of N-glycosylation, purified Dom5His was subjected to MALDI-TOF-MS analysis (Fig. 3). The m/z values representing the spectral peaks were deduced from the mean ± S.E. of three independent spectra. The highest intensity peak at m/z 17,283 ± 5 (Fig. 3) is consistent with the predicted mass (17,288 Da) of the mature unglycosylated form of Dom5His containing four extra residues of the α-factor (Fig. 3, EAEADom5His). The peak at m/z 17,083 ± 5 (Fig. 3) corresponds to the predicted mass (17,088 Da) of the mature unglycosylated form of Dom5His containing two extra residues of the α-factor (Fig. 3, EADom5His). Thus, the differential N-terminal processing of Dom5His was confirmed by both N-terminal sequencing and mass spectrometric analysis. Furthermore, the multiple peaks between m/z 19,147 ± 5 and 20,442 ± 5 (Fig. 3) are consistent with the glycosylated forms of Dom5His with an N-glycan mass adding up to a maximum of 3359 Da.

Oligomeric Structure of Dom5His—The CI-MPR has been shown to associate into oligomeric complexes (14, 41, 42). To determine whether Dom5His can form oligomers, the purified protein was passed over a Superdex-75 fast protein liquid chromatography column. The protein eluted as a broad peak (Kav = 0.33–0.51) with a molecular mass ranging from 45 to 17 kDa, indicating the existence of both dimeric and monomeric forms of Dom5His (Fig. 4A). As another approach to assess oligomerization, Dom5His was incubated with the homobifunctional cross-linking agent DTSSP (Fig. 4B). In the presence of DTSSP, a portion (∼30–40%) of the receptor migrated with an apparent molecular mass of 35–50 kDa (Fig. 4B). In addition, the three broad low abundance peaks at m/z 34,481 ± 10, 36,771 ± 10, and 38,891 ± 10 (Fig. 3) are consistent with dimeric complexes of Dom5His, as oligomeric forms are known to resolve in a MALDI-TOF mass spectra of intact proteins. Taken together, these results demonstrate the ability of Dom5His to form dimers.

Man-6-P Binding Property of Dom5His—Our initial attempts to assess phosphomannosyl binding using iodinated β-glucuronidase, an assay that can readily detect interactions in the low micromolar range (19), failed to detect specific binding to Dom5His up to a ligand concentration of 250 nM (data not shown). To further explore the possibility that Dom5His binds Man-6-P or other carbohydrates, we took a more sensitive
approach using an oligosaccharide microarray by which interactions with numerous carbohydrate sequences can be tested simultaneously. When an array of 30 different oligosaccharides (Table I) linked to lipid was overlaid with purified Dom5His, we observed specific binding to pentamannosyl 6-phosphate (Fig. 5, left panel, spot F1), as with Dom9His (right panel, spot F1), which was used as a positive control for the microarray experiment. We detected no appreciable binding of either Dom5His (Fig. 5, left panel, spot A1) or Dom9His (right panel, spot A1) to the arrayed high-mannose N-glycans and to the other glycans, indicating specific recognition of the Man-6-P moiety.

Pentamannosyl Phosphate-Agarose Affinity Chromatography of Dom5His—To further probe the interactions of Dom5His with carbohydrates, pentamannosyl phosphate-agarose affinity chromatography was performed. In initial experiments, no significant interaction was observed between Dom5His and the resin when the flow rate was controlled simply by gravity (110 ml/h) (data not shown). This is in contrast to results obtained for Dom9His, in which all of Dom9His bound to the column and could be specifically eluted with Man-6-P (19). However, when the flow rate was reduced 10-fold to 2.5 ml/h, Dom5His was significantly retarded on the pentamannosyl phosphate-agarose resin in the absence of any soluble ligand, with the majority of the protein eluting as a sharp peak in the wash fractions, followed by a smaller but prominent peak (11% of the total protein) upon elution with Man-6-P (Fig. 6A). In contrast, inclusion of 10 mM Man-6-P (Fig. 6B) in the buffer caused a dramatic change in the elution profile, with Dom5His exhibiting limited interaction with the resin (58% in wash fractions), compared with that observed in the absence of Man-6-P (96% in wash and elution fractions) (Fig. 6A). To investigate the specificity of interaction of Dom5His with the pentamannosyl phosphate-coupled resin, chromatography was repeated in the presence of 10 mM glucose 6-phosphate (Fig. 6C) or 10 mM mannose (Fig. 6D). The elution pattern in the presence of glucose 6-phosphate (Fig. 6C) was almost identical to that run in the absence of soluble ligand (Fig. 6, compare A and C), indicating no significant interaction of Dom5His with glucose 6-phosphate. In the presence of mannose (Fig. 6D), however, there was a slight but significant alteration in the elution profile, with a higher percentage being observed in the load fractions. Taken together, the results from pentamannosyl phosphate-agarose affinity chromatography analysis of Dom5His support the re-
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Table I
Designations and locations in Fig. 5 of 30 oligosaccharides investigated

| Location | Oligosaccharide* | Type | Sequence |
|----------|-----------------|------|----------|
| A1       | N-Glycan, Man,   | NGL  | Man6-6Mano-3Mano-6Mano-3Manoβ-4GlcNAcβ-4GlcNAc |
| A2       | N-Glycan, biantennary | NGL  | SAα-6Galβ-4GlcNAcβ-2Manβ-6(SAα-6Galβ-4GlcNAcβ-2Manβ-3Mano-4GlcNAcβ-4GlcNAc |
| A3       | O-Glycan, fucosyl | NGL  | Fucβ2HexHexNAcβ (bovine submaxillary mucin glycan fraction N6) |
| A4       | O-Glycan, SA-fucosyl | NGL  | SA-FucHexHexNAcβ (bovine submaxillary mucin glycan fraction A6) |
| B1       | O-Glycan, sialyl | NGL  | SAHexNAcβ (bovine submaxillary mucin glycan fraction A4) |
| B2       | LNT              | NGL  | Galβ-3GlcNAcβ-3Galβ-4Glc |
| B3       | LNnT             | NGL  | Galβ-4GlcNAcβ-3Galβ-4Glc |
| B4       | H (LNFP I)       | NGL  | Fucα2Galβ-3GlcNAcβ-3Galβ-4Glc |
| C1       | Leβ* (LNFP II)   | NGL  | Galβ-4(Fucα-3GlcNAcβ-3Galβ-4Glc |
| C2       | Leβ* (LNFP III)  | NGL  | Galβ-4(Fucα-3GlcNAcβ-3Galβ-4Glc |
| C3       | Leβ* (LNDFH I)   | NGL  | Fucα2Galβ-4(Fucα-3GlcNAcβ-3Galβ-4Glc |
| C4       | Leβ* (LNDFH II)  | NGL  | Fucα2Galβ-4(Fucα-3GlcNAcβ-3Galβ-4Glc |
| D1       | 3′-SA-Leβ-5      | NGL  | SAα-3Galβ-4(Fucα-4GlcNAcβ-3Galβ-4Glc |
| D2       | 3′-SA-Leβ-5      | NGL  | SAα-3Galβ-4(Fucα-3GlcNAcβ-3Galβ-4Glc |
| D3       | 3′-S-Leβ-5       | NGL  | Galβ3β-3Fucα-4GlcNAcβ-3Galβ-4Glc |
| D4       | 3′-S-Leβ-5       | NGL  | Galβ3β-4(Fucα-3GlcNAcβ-3Galβ-4Glc |
| E1       | 3′-S-Leβ-5       | NGL  | Galβ3β-4(Fucα-3GlcNAcβ-3Galβ-4Glc |
| E2       | 6′-3′-SA-Leβ-5   | NGL  | SAα-3Galβ-4(Fucα-4GlcNAcβ-3Galβ-4Glc |
| E3       | 6′-S-3′-SA-Leβ-5 | GL   | SAα-3Galβ-3β-3Fucα-4GlcNAcβ-3Galβ-4Glc |
| E4       | HNK-1            | GL   | GlcUA3β-3β-3GlcNAcβ-3Galβ-4Glc |
| F1       | Man, P           | NGL  | Man6Pα-3Mano-3Mano-3Mano-2Man |
| F2       | CSA 2-mer        | NGL  | HexUAα-3GlcNAc(4S) |
| F3       | CSA 14-mer       | NGL  | HexUAα-3GlcNAc(4S)-4GlcUAβ-3GlcNAc(4S) |
| F4       | CSA 2-mer        | NGL  | HexUAα-3GlcNAc(4S) |
| G1       | CSA 14-mer       | NGL  | HexUAα-3GlcNAc(4S)-4IdoUAα-3GlcNAc(4S) |
| G2       | CSC 3-mer        | NGL  | HexUAα-3GlcNAc(6S) |
| G3       | CSC 14-mer       | NGL  | HexUAα-3GlcNAc(6S)-4IdoUAα-3GlcNAc(6S) |
| G4       | HEP 2-mer        | NGL  | HexUAα-2S4GlcNS(6S) |
| H1       | HEP 8-mer        | NGL  | HexUAα-GlcNAcHexUAα-4GlcNα-8Man |
| H2       | K4-mer (C4U)     | NGL  | SAα-3Galβ-4(Fucα-3GlcNAcβ-6Sβ-3Galβ-4GlcNAcβ-4GlcNα) |

* Oligosaccharides A3–B1 and glycosaminoglycan oligosaccharide fractions F2–G3 are heterogeneous. The compositions or sequences of the main components are shown here.

NGL, neoglycolipid; GL, glycolipid; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP, lacto-N-fucopentaose; LNDFH, lacto-N-difucohexose; LNDNFH, lacto-N-neodifucohexose; SA, sialic acid; S, sulfate; CSA, chondroitin sulfate A; CSB, chondroitin sulfate B; CSC, chondroitin sulfate C; HS, heparan sulfate; HEP, heparin; KS, keratan sulfate; P, phosphate; ΔHexUA, 4,5-unsaturated hexuronic acid; anMan, anhydromannose. When the sequence is unknown, the composition is given with a dot inserted between monosaccharide residues.

These O-glycan fractions obtained from bovine submaxillary mucin were described by Chai et al. (49).

![Fig. 5. Oligosaccharide microarray](image_url)

Fig. 5. Oligosaccharide microarray. Thirty sequence-defined oligosaccharides (Table I) as lipid-linked probes (10 pmol) were arrayed on nitrocellulose-coated glass slides as 2-mm bands. Spots H3 and H4 were not used. The slides were overlaid with Dom5His (left panel) and Dom9His (right panel) as described under “Experimental Procedures” to reveal carbohydrate binding.

Results obtained from the oligosaccharide microarray analysis (Fig. 5) on the specific interaction with Man-6-P and, in addition, suggest a weak interaction with mannos.

Acidic pH Dissociation of Dom5His—One key function of the MPRs in the trafficking of lysosomal enzymes to the lysosome is the ability of the MPRs to dissociate from their ligands in the acidic environment of late endosomal compartments. To assess whether Dom5His exhibits pH dependence of carbohydrate binding, Dom5His was subjected to pentamannosyl phosphate-agarose chromatography at pH 3.5 (Fig. 6E) rather than at pH 6.5 (Fig. 6, A–D). The results demonstrate a nearly identical profile of Dom5His at pH 3.5 as in the presence of Man-6-P (Fig. 6, compare B and E), showing little interaction with the resin, indicating that Dom5His exhibits acid-dependent release of carbohydrate. As a control, Dom5His was incubated with acidic pH 3.5 buffer for 4 h, equilibrated in column buffer at pH 6.5, and then subjected to pentamannosyl phosphate-agarose chromatography, which showed an identical profile (data not shown) to that obtained in the absence of pretreatment with an acidic buffer (Fig. 6A). These results demonstrate that no irreversible denaturation of Dom5His occurred upon incubation at pH 3.5. Additional experiments indicated that Dom5His is similar to Dom9His (19) in that it did not undergo acid-dependent dissociation at pH ~4.7 as efficiently as the N-terminal Man-6-P-binding site of the CI-MPR (data not shown).

Frontal Affinity Chromatography of Dom5His to Determine the Kᵢ for Man-6-P—Frontal affinity chromatography is a well established analytical tool used to investigate specific interactions between biomolecules, including various plant and animal lectins, and utilizes relatively weak interactions to quantify protein-ligand interactions and to determine binding constants using equations analogous to those in enzyme kinetics (37). This study has applied this technique for the first time to a P-type lectin. The elution profiles of frontal affinity chromatography of Dom5His on a pentamannosyl phosphate-agarose column in the absence and presence of increasing Man-6-P concentrations (0, 0.1, 1, 2, 5, 10, and 100 mM) are represented in Fig. 7A. The elution volume in the absence of Man-6-P was determined as Vₒ = 27.7 ml. The retardation of the front decreased with increasing Man-6-P concentrations (0.1, 1, 2, 5, 10, and 100 mM) as represented in Fig. 7A. The Vₒ values plotted against (Vᵢ-Vₒ)/[I] give a well fitting straight line (Fig. 7B), with the slope equivalent to the Kᵢ for Man-6-P at 5.3 ± 0.2 mm. The Vᵢ derived from the y intercept of the plot (Fig. 7B) was 7.2 ± 0.6 ml, a value slightly greater than the bed volume (5.3 ml) of the column. The elution volume in the presence of 100 mM Man-6-P (8.4 ml) was greater than Vₒ (7.2
since the concentration of Man-6-P was only ~20-fold higher than the $K_i$ (5.3 mM), thus reaching just over 90% saturation. Taken together, these results demonstrate that domain 5 (Dom5His) harbors a Man-6-P-binding site with an affinity lower by ~300-fold than that observed for the two high affinity binding sites of the CI-MPR localized to domains 1–3 and 9.

### DISCUSSION

Four conserved residues (Gln-66, Arg-111, Glu-133, and Tyr-143) of the CD-MPR have been shown previously to be essential for Man-6-P recognition (31). These residues are also conserved in the two high affinity binding sites (i.e. domains 3 and 9) of all CI-MPRs sequenced to date, and substitution of Gln-66, Arg-111, Glu-133, or Tyr-143 of the CD-MPR (31) or their corresponding residues in domains 3 and 9 of the CI-MPR (21) results in a decrease in the affinity of the receptor for a lysosomal enzyme by ~1000-fold. The crystal structures of the CD-MPR (13, 27) and domains 1–3 of the CI-MPR (28, 29) confirm the importance of these residues by demonstrating that their location is within hydrogen bonding distance of the hydroxyl groups of the mannose ring. To evaluate whether equivalent Gln, Arg, Glu, and Tyr are present in other regions of the CI-MPR, we performed a structure-based sequence align-
a lysosomal enzyme, \(\beta\)-glucuronidase. The inset represents the sensorgram overlays of various concentrations of purified Dom5His (5, 10, 20 \(\mu\)M duplicate), 40, 80, 120, and 200 \(\mu\)M injected over immobilized \(\beta\)-glucuronidase at a flow rate of 40 \(\mu\)l/min in running buffer. An average of the response at equilibrium was determined for each concentration, and the resulting equilibrium response units (RU) were plotted against concentration. The data were fit to a steady-state affinity model using BIAevaluation Version 4.0.1 software to determine the equilibrium dissociation constant (\(K_d\)).

Determination of Identical Polypeptides (45, 46). The CI-MPR appears to contain residues equivalent to Gln-66, Arg-111, Glu-133, and Try-143 of the CD-MPR. These differences in the predicted phosphate-moiety of Man-6-P via the hydroxyl groups of mannose ring. Clearly, additional quantitative studies will be required to corroborate the interaction of domain 5 of the CI-MPR with mannose.

What may contribute to the generation of a low (rather than high) affinity Man-6-P-binding site in domain 5? Despite having the four conserved mannose-binding residues, the sequence alignment (Fig. 1) shows that domain 5 lacks two cysteines (corresponding to Cys-106 and Cys-141 of the CD-MPR) that form a critical disulfide bond that tethers loops C and D within the binding pocket of the CD-MPR (13, 27) and domain 3 of the CI-MPR (28, 29). The absence of this key analogous disulfide bond in domain 5 could result in destabilization of the binding pocket and a reduced affinity for Man-6-P-containing ligands. The structures of the CD-MPR and domains 1–3 of the CI-MPR reveal, in addition, that specific residues in loop C of the CD-MPR (Asp-Asn-His, positions 103–105) and domain 3 (Ser-386) are involved in a network of interactions with the phosphate moiety of Man-6-P. In comparison, domain 5 contains Tyr-Asn-Asn-Glu in the predicted loop C at positions equivalent to Tyr-Asn-Asn-His (residues 102–105) of the CD-MPR (Fig. 1). These differences in the predicted phosphate-binding region of domain 5 may also contribute to a lower affinity for Man-6-P. Crystallographic and mutagenesis studies are needed to elucidate the mechanism of Man-6-P recognition by domain 5 of the CI-MPR.

Multivalency plays an important role in the functional activity of lectins by increasing the avidity and specificity for multivalent carbohydrate ligands. This property can be achieved by clustering multiple carbohydrate recognition domains within a single polypeptide or by existing in an oligomeric state in which carbohydrate recognition domains are clustered via the association of identical polypeptides (45, 46). The CI-MPR appears to utilize both of these mechanisms. This receptor contains three Man-6-P-binding sites (in domains 1–3, 5, and 9) that differ in their affinities for Man-6-P and in their specificity, with domain 9 exhibiting high specificity for phosphomonoesters and
Low Affinity Man-6-P-binding Site of the CI-MPR

The crystal structure of domains 1–3 was used to model the entire 15 domain-containing extracellular region of the CI-MPR (28). The receptor is predicted to bend at several proteolytically accessible sites (between domains 3 and 4, domains 6 and 7, and domains 12 and 13), resulting in a molecule in which the distance between the two high affinity Man-6-P-binding sites (domains 3 and 9) can be modulated between 85 Å in the fully extended form to 45 Å in the fully bent model (28). Domain 9 displays high affinity to domain 5, in conjunction with the two high affinity sites, may facilitate the interaction of the receptor with multivalent ligands. We have recently proposed a model for the 15 domain-containing extracytoplasmic region of the CI-MPR based on the crystal structure of domains 1–3 (28). The model presumes a three-domain unit as the basic structural element for the entire extracytoplasmic region and stacks each of the five three-domain units in a back-to-front fashion with every first and third domain rotated at a relative orientation of ~180°, forming a molecule 210 Å in length (extended conformation) (Fig. 9A). This model also predicts that the two high affinity sites (domains 3 and 9) and the low affinity site (domain 5) are situated on the same face of the molecule. The presence of at least several proteolytically susceptible sites located between domains (18) suggests that the receptor is flexible and that bending at various points would result in a modulation of the spacing between its Man-6-P-binding sites. In contrast to the extended conformation in which domains 3 and 9 are 85 Å apart, this distance is predicted to decrease to 45 Å in the fully bent conformation (Fig. 9A) (28). As the maximum distance between the two phosphate moieties of a diposphorylated high-mannose oligosaccharide is ~30 Å, a single oligosaccharide cannot span the two high affinity sites even in the bent conformation. However, the presence of the low affinity carbohydrate-binding site in domain 5 would allow for a bidentate interaction between the receptor and a diposphorylated oligosaccharide, as the distance between domains 3 and 5 and between domains 3 and 9 is predicted to be <30 Å (Fig. 9A). Given the heterogeneous nature of its ligands, the presence of multiple carbohydrate-binding sites with different specificities, affinities, and spacing within the receptor is likely to enhance binding affinities by increasing the probability of at least two points of interaction between the receptor and its ligand. Furthermore, as recent biochemical and functional data indicate that the CI-MPR can form dimers (14, 41, 42), the dimeric state of the receptor would allow numerous permutations of intermolecular contacts among the six available Man-6-P-binding sites and a multivalent ligand (Fig. 9B). Thus, the proposed flexible nature of the CI-MPR, involving extended, bent, and dimeric conformations, would facilitate the presentation of its carbohydrate-binding sites (domains 3, 5, and 9) in numerous spatial arrays that could serve to “match” the diverse display of phosphomannosyl-containing oligosaccharides found on its numerous ligands.

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REFERENCES

1. Ghosh, P., Dahms, N. M., and Kornfeld, S. (2003) Nat. Rev. Mol. Cell. Biol. 4, 202–213
2. Stein, M., Zijderend-Bleeckemolen, J. E., Geuze, H., Hanlik, A., and von Figura, K. (1987) EMBO J. 6, 2677–2683
3. Sohar, I., Sleat, D., Liu, C.-G., Ludwig, T., and Lobel, P. (1996) Biochem. J. 330, 903–908
4. Griffiths, G. M., and Isaza, S. (1993) J. Cell Biol. 120, 885–896
5. Purdie, A. F., Cooper, J. A., Brunner, A. M., Lioubin, M. N., Gentry, L. E., Kovacina, K. S., Roth, R. A., and Marquardt, H. (1988) J. Biol. Chem. 263, 14211–14215
6. Lee, S. J., and Nathans, D. (1988) J. Biol. Chem. 263, 3521–3527
7. Faust, P. L., Chirgwin, J. M., and Kornfeld, S. (1987) J. Cell Biol. 105, 1947–1955

A, bidentate interaction between the CI-MPR and a biantennary high-mannose oligosaccharide containing two phosphomonoesters (Man-6-P ($M_6P$)). The distance between the two high affinity Man-6-P-binding sites (domains 3 and 9) can be modulated between 85 Å in the fully extended form to 45 Å in the fully bent model (28). B, multivalent interaction between the CI-MPR and a ligand containing six phosphomonoesters, as the distance between domains 3 and 5 is predicted to decrease to 45 Å in the fully bent conformation (Fig. 9A) (28). As the maximum distance between the two phosphate moieties of a diposphorylated high-mannose oligosaccharide is ~30 Å, a single oligosaccharide cannot span the two high affinity sites even in the bent conformation. However, the presence of the low affinity carbohydrate-binding site in domain 5 would allow for a bidentate interaction between the receptor and a diposphorylated oligosaccharide, as the distance between domains 3 and 5 and between domains 3 and 9 is predicted to be <30 Å (Fig. 9A). Given the heterogeneous nature of its ligands, the presence of multiple carbohydrate-binding sites with different specificities, affinities, and spacing within the receptor is likely to enhance binding affinities by increasing the probability of at least two points of interaction between the receptor and its ligand. Furthermore, as recent biochemical and functional data indicate that the CI-MPR can form dimers (14, 41, 42), the dimeric state of the receptor would allow numerous permutations of intermolecular contacts among the six available Man-6-P-binding sites and a multivalent ligand (Fig. 9B). Thus, the proposed flexible nature of the CI-MPR, involving extended, bent, and dimeric conformations, would facilitate the presentation of its carbohydrate-binding sites (domains 3, 5, and 9) in numerous spatial arrays that could serve to “match” the diverse display of phosphomannosyl-containing oligosaccharides found on its numerous ligands.
Identification of a Low Affinity Mannose 6-Phosphate-binding Site in Domain 5 of the Cation-independent Mannose 6-Phosphate Receptor

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