Localization of the Carbohydrate Recognition Sites of the Insulin-like Growth Factor II/Mannose 6-Phosphate Receptor to Domains 3 and 9 of the Extracytoplasmic Region*

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The insulin-like growth factor II/mannose 6-phosphate receptor is a multifunctional receptor that binds to a diverse array of mannose 6-phosphate (Man-6-P) modified proteins as well as nonglycosylated ligands. Previous studies have mapped its two Man-6-P binding sites to a minimum of three domains, 1–3 and 7–9, within its 15-domain extracytoplasmic region. Since the primary amino acid determinants of carbohydrate recognition by the insulin-like growth factor II/mannose 6-phosphate receptor are predicted by sequence alignment to the cation-dependent mannose 6-phosphate receptor to reside within domains 3 and 9, constructs encoding either domain 3 alone or domain 9 alone were expressed in a Pichia pastoris expression system and tested for their ability to bind several carbohydrate ligands, including Man-6-P, pentamannosyl phosphate, the lysosomal enzyme, β-glucuronidase, and the carbohydrate modifications (mannose 6-sulfate and Man-6-P methyl ester) found on Dictyostelium discoideum lysosomal enzymes. Although both constructs were functional in ligand binding and dissociation, these studies demonstrate the ability of domain 9 alone to fold into a high affinity (K_D = 0.3 ± 0.1 nM) carbohydrate-recognition domain whereas the domain 3 alone construct is capable of only low affinity binding (K_D ~ 500 nM) toward β-glucuronidase, suggesting that residues in adjacent domains (domains 1 and/or 2) are important, either directly or indirectly, for optimal binding by domain 3.

Delivery of ~50 different lysosomal enzymes bearing the mannose 6-phosphate (Man-6-P) signal to the lysosomes of higher eukaryotes is mediated by the two members of the P-type lectin family, the cation-dependent Man-6-P receptor (CD-MPR) and the insulin-like growth factor II/MPR (IGF-II/MPR) (1–3). At the cell surface, the multifunctional IGF-II/MPR also recognizes the nonglycosylated polypeptide hormone, IGF-II, targeting this potent mitogenic factor for degradation (46-kDa bovine CD-MPR, CD26 (14), thyroglobulin (15), herpes simplex viral glycoprotein D (16), and varicella-zoster viral glycoprotein I (17). Together, these findings implicate a role for the IGF-II/MPR in a host of cellular pathways due to its lectin activity.

Both of the MPRs are type I transmembrane glycoproteins containing four structural/functional components: an N-terminal signal sequence, an extracytoplasmic region that harbors the ligand-binding site(s), a transmembrane region, and a C-terminal cytosolic tail (Fig. 1). The ~46-kDa bovine CD-MPR, which exists as a stable dimer in membranes, contains a single extracytoplasmic domain per monomer polypeptide that is composed of 154 amino acids and binds to a single molecule of Man-6-P (18–21). Structural and biochemical analyses of a truncated, soluble form of the CD-MPR have demonstrated that its extracytoplasmic domain alone can fold into a high affinity carbohydrate-recognition domain (19, 22–24). The ~300-kDa IGF-II/MPR, on the other hand, appears to exist predominantly as a monomer within the cell, although weak dimeric complexes have been observed in membranes that are stabilized by the binding of multivalent ligands (25–29). The large 2289-residue extracytoplasmic region of the bovine IGF-II/MPR is composed of 15 domains that display amino acid identity (14–38%), similar size (~147 residues) and cysteine distribution to each other and to the CD-MPR, giving rise to the prediction that they exhibit similar disulfide bonding and tertiary structures (30, 31). These observations also led to the early suggestion that perhaps each of the 15 extracytoplasmic domains of the IGF-II/MPR folds into a functional carbohydrate-recognition domain (31). However, equilibrium dialysis experiments demonstrated that the IGF-II/MPR binds only two moles of Man-6-P per mole of receptor (32), and the two distinct carbohydrate-binding sites of the IGF-II/MPR were localized to domains 1–3 and domains 7–9 of its extracytoplasmic region by partial proteolysis experiments and expression of truncated forms of the receptor (33, 34).

Until recently, very little was known about the molecular basis of carbohydrate recognition by the MPRs. The three-dimensional structures of the extracytoplasmic region of the CD-MPR bound to Man-6-P (22) or to pentamannosyl phosphate (23) not only revealed the molecular basis of Man-6-P recognition by the CD-MPR but also provided further insights into carbohydrate recognition by the IGF-II/MPR: a CD-MPR structure-based sequence alignment predicted several amino acids in domains 3 and 9 to be in the N- and C-terminal Man-6-P binding pockets of the IGF-II/MPR (22). Site-directed mutagenesis studies were performed to test these predictions, identifying four residues (Gln-392, Ser-431, Glu-460, and Tyr-
Zeocin were from Invitrogen; GeneMate plasmid DNA minipreps were from Man-6-P was from Roche Molecular Biochemicals; BioMix DNA polymerase was from Bioline; Protein A-Sepharose and lactoperoxidase were from Sigma; and Man-6-S was from V-Labs. MTX 3.2 cells overexpressing human β-glucuronidase were generously provided by Dr. W. Sly (St. Louis University School of Medicine, St. Louis, MO). Phosphomannomann from Hansenula holstii was a kind gift of Dr. M. E. Slodki (Northern Regional Research Center, Peoria, IL). D. discoideum cells were a generous gift of Dr. H. Freeze (The Burnham Institute, La Jolla, CA).

**Synthesis of Man-6-P-OCH₃**—Synthesis of the acid-stable phosphodiester, Man-6-P-OCH₃ was described previously (37).

**Generation of the Dom3His and Dom9His cDNA Constructs**—Previously constructed cDNA constructs encoding extracytoplasmic domains 1–3 (Dom1–3His) or domains 7–9 (Dom7–9His) of the bovine IGF-II/MPR followed by a C-terminal tag of six histidine residues (37) were used as PCR templates to generate constructs encoding either domain 3 alone (Dom3His) or domain 9 (Dom9His) alone followed by a C-terminal His₆-tag (Fig. 1). Briefly, the sequences encoding domain 3 (amino acids 326–476) or domain 9 (residues 1228–1371) followed by six histidine residues (CAC) and a stop codon (TGA) were amplified by PCR and subcloned into the P. pastoris expression vector pGAPZαN (Invitrogen) in-frame with the Saccharomyces cerevisiae α-factor signal sequence using the Xhol (5’ end) and XbaI (3’ end) restriction sites of the pGAPZαN vector. DNA sequencing by the Protein and Nucleic Acid Core Facility (Medical College of Wisconsin) confirmed the predicted sequences.

**Expression and Purification of the Dom3His and Dom9His Constructs**—The Dom3His and Dom9His cDNA constructs (~1 µg) were linearized with BspHI and transformed into P. pastoris by electroporation, and Zeocin-resistant transformants were selected as described previously (38). To obtain purified Dom3His protein, harvested medium (10 ml) from an ~3-day culture was dialyzed against buffer containing 0.5 M NaCl and 20 mM Tris, pH 8.0 (binding buffer). Batch binding of the His₆-tagged receptor was performed by adding 50 µl of nickel-nitri-
olic acid (Ni-NTA, Qiagen) agrose to the dialyzed medium and incubating at 4 °C for ~16 h. The Ni-NTA resin was pelleted and then washed with a 1.5-ml microcentrifuge tube containing 3 x 1 ml volumes of binding buffer prior to eluting Dom3His from the resin with 2 x 0.2 ml of binding buffer containing 250 mM imidazole, pH 7.5. To obtain purified Dom9His protein, harvested medium (10 ml) from an ~3-day culture was modified that yields of ~70 µg and ~55 µg of purified Dom3His and Dom9His, respectively, were obtained from 10 ml of P. pastoris culture medium.

**Endo H Digestion**—Purified Dom3His or Dom9His were incubated with Endo H (1 milliunit) in buffer containing 0.075% SDS, 10 mM β-mercaptoethanol, and 100 mM sodium citrate, pH 6.0 for 3 h at 37 °C. The samples were subjected to SDS-PAGE followed by Western blot analysis using bovine IGF-II/MPR-specific antiserum as described previously (37).

**N-terminal Sequencing**—The purified Dom3His and Dom9His proteins were 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 on tryptic digests of the purified proteins, in which thiohydantoin-derivatized amino acids were separated by reverse-phase HPLC.

**Affinity Chromatography Analysis**—To ascertain whether Dom3His and Dom9His are qualitatively functional in Man-6-P recognition, harvested medium samples (0.2 ml) containing Dom3His or Dom9His were loaded onto a 0.4-ml pentamannosyl phosphate-agarose affinity chromatography. Briefly, the medium samples were loaded on a 0.4-ml pentamannosyl phosphate-agarose column, washed with 3 ml of column buffer, eluted with 2 ml of column buffer containing 5 mM glucose 6-phosphate (non-specific...
ligand), and then eluted with 2 ml of column buffer containing 5 mM Man-6-P. The ability of Dom9His to dissociate at low pH from a 0.4 ml pentamannosyl phosphate column was also tested by eluting the column with low pH buffer (150 mM NaCl, 5 mM β-glycerophosphate, and 75 mM sodium acetate, pH 3.5) before eluting the column with column buffer fractions containing 5 mM glucose 6-phosphate and 10 mM Man-6-P, respectively. As another test of the carbohydrate-recognition ability of Dom3His and Dom9His, purified receptors were passed over 0.4-ml affinity columns composed of lysosomal enzymes from D. discoideum, which contain oligosaccharides modified by Man-6-P residues diesterified to methyl groups (Man-6-P-OCH₃) and Man-6-S (39, 40), washed with column buffer, and eluted with column buffer containing 5 mM glucose 6-phosphate followed by elution with 5 mM Man-6-P. The column fractions were precipitated with 10% trichloroacetic acid followed by SDS-PAGE (10% or 12.5% nonreducing gel) and Western blot analysis as described previously (37).

Binding Affinity Analysis—125I-labeled (1–2 μCi/μg) human β-glucuronidase was prepared and used to perform binding affinity analyses as described previously (24). Briefly, increasing concentrations of iodinated β-glucuronidase were incubated with purified Dom3His or Dom9His, and the receptors and bound ligand were immunoprecipitated with bovine IGF-II/MPR-specific antiserum pre-bound to protein A-Sepharose beads. The beads were washed extensively with column buffer and bound β-glucuronidase was specifically eluted from the receptors with 5 mM Man-6-P. The results were analyzed by nonlinear regression (SigmaPlot version 5.05, SPSS Science) to determine Kᵦ values.

pH Optimum Determination—Purified Dom3His and Dom9His were incubated under equilibrium binding conditions with 125I-β-glucuronidase in the following buffers spanning a broad pH (4–8.5) range: 60 mM sodium acetate, 150 mM NaCl, and 5 mM β-glycerophosphate (pH 4.15, 4.73, 5.23, or 5.75); 60 mM MES, 150 mM NaCl, and 5 mM β-glycerophosphate (pH 5.72, 6.00, 6.28, 6.59, 6.89, or 7.13); or 60 mM 4-2-hydroxyethylpiperazine-1-ethanesulfonic acid (HEPES), 150 mM NaCl, and 5 mM β-glycerophosphate (pH 7.14, 7.64, 8.08, or 8.57). The receptors and bound ligand were immunoprecipitated with bovine IGF-II/MPR-specific antiserum pre-bound to protein A-Sepharose beads and washed extensively with column buffer, and the bound β-glucuronidase was specifically eluted from the receptors with 5 mM Man-6-P. The results were plotted using SigmaPlot version 5.05, version 5.05 (SPSS Science).

Inhibition Studies—Purified Dom9His was incubated with 125I-β-glucuronidase at a concentration ~3× the determined Kᵦ in the presence of increasing concentrations of Man-6-P, Man-6-S, and Man-6-P-OCH₃. The receptors and bound ligand were immunoprecipitated with bovine IGF-II/MPR-specific antiserum pre-bound to protein A-Sepharose beads and washed extensively with column buffer, and the bound β-glucuronidase was specifically eluted from the receptors with 5 mM Man-6-P. The results were analyzed by nonlinear regression (SigmaPlot version 5.05, SPSS Science) to obtain IC₅₀ values, which were then used to calculate Kᵦ values according to the method of Cheng and Prusoff (41).

RESULTS

Expression and Purification of the Dom3His and Dom9His Constructs—Our laboratory has recently demonstrated P. pastoris to be a highly effective heterologous host system for the generation of high yields of a functional, truncated form of the CD-MPR, referred to as Asn¹⁵³/STOP¹⁵⁵ (38). This system was utilized in this report to generate truncated, soluble forms of the bovine IGF-II/MPR composed of extracytoplasmic domain 3 alone (Dom3His) or domain 9 alone (Dom9His) followed by a tag of six histidine residues (Fig. 1). The CDNA constructs were placed into the P. pastoris expression vector pGAPZaA (Invitrogen), which uses the promoter from the glyceraldehyde-3-phosphate dehydrogenase gene for constitutive protein expression (42), in-frame with the 89-residue S. cerevisiae α-factor signal sequence (43). Both of the IGF-II/MPR constructs were expressed well (~5–7 μg/ml) and secreted from P. pastoris into the culture media (data not shown). Only protein secreted into the medium was used in the biochemical assays described below, since proteins destined for secretion that are grossly misfolded are generally retained within cells (44, 45). Consequently, Dom3His and Dom9His were purified from the culture medium by a single-step process, using Ni-NTA-agarose affinity chromatography (Qiagen) or pentamannosyl phosphate-agarose affinity chromatography, respectively (data not shown).

N-linked Glycosylation of Dom3His and Dom9His by Enzymatic Deglycosylation—Because Dom3His and Dom9His each contain two potential N-linked glycosylation sites, the possibility existed that glycosylated and unglycosylated forms of these proteins would be secreted from P. pastoris. Purified Dom3His could be resolved into three distinct banding patterns on 12.5% SDS-polyacrylamide gels: a sharp band of ~17 kDa that is consistent with the predicted molecular mass of unglycosylated Dom3His (17,437 Da), and two diffuse bands of ~22 and ~26 kDa were detected by silver stain analysis (data not shown) and Western blotting using bovine IGF-II/MPR-specific antiserum (Fig. 2). Treatment of Dom3His with endo H, an enzyme that cleaves high-mannose and hybrid-type glycans, converted the majority of the ~22- and ~26-kDa band forms into an ~17-kDa deglycosylated band, confirming the ability of Dom3His to be glycosylated in P. pastoris. In contrast to the presence of glycosylated and unglycosylated forms of Dom3His, purified Dom9His appeared predominantly as a single, diffuse band of ~26 kDa as detected by silver stain (data not shown) and Western blot analyses (Fig. 2). Upon digestion with endo H, virtually all of the Dom9His in the reaction was cleaved to a band of ~17 kDa that is consistent with the predicted molecular mass of unglycosylated Dom9His (17,213 Da), confirming that the ~26-kDa form is glycosylated Dom9His.

N-terminal Sequencing of Dom3His and Dom9His—Purified Dom3His and Dom9His were also subjected to N-terminal sequencing to determine whether the N-terminal signal sequence was processed correctly for each construct. A single N-terminal sequence (Glu-Ala-Glu-Ala-Arg-Asp-Tyr-Leu-Glu-Ser-Arg-Ser-X-Ser-Leu) was obtained for the glycosylated and unglycosylated forms of Dom3His, indicative of efficient Kex2 endopeptidase cleavage of the S. cerevisiae α-factor signal sequence before the two Glu-Ala repeats preceding the start of domain 3 at residue Arg-326 (43, 46). In general, depending on the surrounding amino acid sequence and the tertiary structure of the protein of interest, the Glu-Ala repeats remain after Kex2 cleavage of the signal sequence are removed by the action of Ste13 (43, 46). However, as was also the case for the previously reported CD-MPR construct, Asn¹⁵³/STOP¹⁵⁵ (38), the N-terminal sequencing results did not detect removal of the two Glu-Ala repeats from the N terminus of Dom3His (43). Surprisingly, N-terminal sequencing of the ~26 kDa form of Dom9His detected two slightly different populations of receptors, one in which the two Glu-Ala repeats remained intact at the N terminus (Glu-Ala-Glu-Ala-Val-Val-Arg-Ala-Glu-Gly-Asp-Tyr-X-...
i.e. indicated that the other higher molecular weight species (whereas Dom9His appeared as a band of eluate. Longer exposure of the medium by panels A/H11001 40 kDa, panel B Dom3His was resolved into three bands of tic acid and then analyzed on a 12.5% SDS-polyacrylamide gel followed wash ( ), and eluate samples were precipitated with 10% trichloroacet- ( ), and 5 mM Man-6-P ( ), and finally with Man-6-P. The results show that Dom9His was readily eluted from the pentamannosyl phosphate column with an acidic buffer at pH 4.7 but instead remained on the column until it was eluted with Man-6-P (data not shown). These results support the earlier conclusion that the C-terminal carbohydrate-recognition site (domain 9) of the IGF-II/MPR does not undergo acid-dependent dissociation at low pH as efficiently as the N-terminal Man-6-P binding site (37).

pH profile of Dom3His and Dom9His Binding to β-Glucuronidase—In contrast to qualitative affinity chromatography methods, equilibrium binding assays using the well characterized lysosomal enzyme, β-glucuronidase (47), have provided our laboratory a sensitive and quantitative means to assay the carbohydrate-binding ability of MPR constructs. Therefore, whereas no significant carbohydrate recognition of Dom3His was detected by the affinity chromatography methods described above, specific binding of Dom3His to iodinated β-glucuronidase was demonstrated in equilibrium binding control experiments in which relatively high concentrations of ligand and receptor were used as compared with experiments performed with Dom9His (data not shown). These binding assays facilitated further characterization of the ligand binding and dissociation properties of the two IGF-II/MPR constructs as described below.

Because the complex intracellular pathways traversed by the MPRs subject them to several different pH environments (1–3), Dom3His and Dom9His were incubated with iodinated β-glucuronidase under equilibrium binding conditions in buffers at different pH values spanning pH ~4 to pH ~8.5 (Fig. 4). Similar to the previously reported pH profiles of Dom1–3 and Dom7–9 (37), Dom3His and Dom9His displayed broad pH profiles with optimal binding occurring between pH ~6.5 and pH ~7. These results are consistent with the ability of the IGF-II/MPR to bind lysosomal enzymes in the trans-Golgi network (TGN) (pH ~6.5) and at the cell surface (pH ~7.4) (3). Dom3His and Dom9His also exhibited acid-dependent dissociation of β-glucuronidase, consistent with the ability of the IGF-II/MPR to release its cargo in the acidic (pH < 6) environment of late endosomal compartments. The requirement for a more acidic pH for efficient dissociation of Dom9His from the affinity column (Fig. 3C) versus that observed in the pH profile (Fig. 4) is likely caused by the significant differences in the two assays (i.e. solid-phase assay using a low affinity oligosaccharide ligand performed under non-equilibrium conditions versus a solution-based assay using a high affinity lysosomal enzyme ligand performed under equilibrium conditions).

Recognition of D. discoideum Lysosomal Enzymes—The IGF-II/MPR is distinguished from the CD-MPR by its ability to bind lysosomal enzymes from D. discoideum, which contain oligosaccharides that are modified with Man-6-S residues and Man-6-P groups diesterified to methyl groups (Man-6-P-OCH3) but not the phosphomonoester, Man-6-P (39, 48–51). Our recent studies further demonstrated that the two Man-6-P binding sites of the IGF-II/MPR differ significantly in their ability to bind lysosomal enzymes: the N-terminal Man-6-P binding site facilitates further characterization of the ligand binding and dissociation properties of the two IGF-II/MPR constructs as described below.

Glu-Val) of Dom9His and another population in which one of the two Glu-Ala repeats had been removed (Glu-Ala-Val-Val-Arg-Ala-Glu-Gly-Asp-Tyr-X-Glu-Val).

Pentamannosyl Phosphate-agarose Affinity Chromatography—To determine whether Dom3His and Dom9His are functional in Man-6-P recognition, culture medium containing the secreted receptors was passed over pentamannosyl phosphate-agarose affinity columns. Whereas the Dom3His construct was retained in the run-through, all of the Dom9His appeared to bind to the column and was specifically eluted with Man-6-P (Fig. 3, A and B). These results demonstrate that Dom9His is functional in Man-6-P recognition whereas Dom3His is either incapable of ligand binding or exhibits a much lower affinity than Dom9His for pentamannosyl phosphate.

Based on the observation that N-linked oligosaccharides on proteins expressed in P. pastoris can be significantly elongated as compared with the glycans of higher eukaryotes (43), the possibility existed that the glycans modifying Dom3His were preventing it from binding to the affinity column. Therefore, additional experiments were performed in which Ni-NTA affinity purified Dom3His was enzymatically deglycosylated with endo H under native, non-denaturing conditions (i.e. no SDS or β-mercaptoethanol) prior to pentamannosyl phosphate-agarose affinity chromatography. Despite efficient removal of the N-linked glycans from the ~22- and 26-kDa forms of Dom3His, no detectable binding to pentamannosyl phosphate columns by deglycosylated Dom3His was observed (data not shown).

Since lysosomal enzymes are released from the MPRs in the low pH environment of late endosomal compartments (3), the propensity of purified Dom9His to dissociate from the pentamannosyl phosphate column was also tested by eluting the column sequentially with an acidic buffer (pH 3.5), glucose 6-phosphate, and finally with Man-6-P. The results show that Dom9His is efficiently eluted from the column when exposed to buffer conditions at pH 3.5 (Fig. 3C). Treatment of Dom9His with an acidic buffer does not irreversibly denature the recep-
for their ability to bind affinity columns containing D. discoideum lysosomal enzymes. Similar to the previously characterized Dom7–9 and Dom7–11 constructs, Dom9His bound poorly (~36%) to the column (Fig. 5). Dom3His exhibited no detectable binding by D. discoideum affinity chromatography, consistent with the prediction that this construct has a low carbohydrate-binding affinity.

Inhibition studies were performed with the Dom9His construct to confirm the D. discoideum lysosomal enzyme affinity chromatography results obtained above and to quantitatively determine its affinity for the monosaccharides, Man-6-P, Man-6-S, and Man-6-P-OCH₃. Consistent with its poor ability to bind to the D. discoideum lysosomal enzyme column, Dom9His exhibited ~280- to 335-fold lower affinities toward Man-6-P-OCH₃ (Kᵢ = 6.5 mM) and Man-6-S (Kᵢ = 7.7 mM) than for Man-6-P (Kᵢ = 23 μM) (Fig. 6). Similar results were previously obtained with the Dom7–9His and Dom7–11 constructs: Dom7–9His displayed ~400- and ~800-fold lower affinities for Man-6-P-OCH₃ (Kᵢ = 11 mM) and Man-6-S (Kᵢ = 22 mM) than for Man-6-P (Kᵢ = 28 μM), whereas Dom7–11 exhibited an ~250-fold lower affinity (Kᵢ = 5 mM) for both Man-6-P-OCH₃ and Man-6-S than for Man-6-P (Kᵢ = 22 μM) (37). Thus, not only is domain 9 alone sufficient for high affinity Man-6-P binding, whereas the Dom3His construct is only capable of low affinity carbohydrate recognition.

DISCUSSION

In addition to its role in delivering lysosomal enzymes to lysosomes, the multifunctional nature of the IGF-II/MPR has become increasingly apparent as the list of extracellular ligands recognized by this receptor has grown to include a diverse spectrum of glycosylated and nonglycosylated proteins that are involved in a multitude of cellular processes, including apoptosis, viral entry and transmission between cells, and cellular differentiation and proliferation (3). These findings have prompted detailed structure/function analyses of the extracytoplasmic region of this receptor in order to understand how the IGF-II/MPR recognizes its various target proteins. Previous proteolysis experiments and expression of truncated IGF-II/MPR constructs have determined that the binding site for the nonglycosylated polypeptide hormone, IGF-II, is bipartite: the primary binding determinants reside within the N-terminal portion of extracytoplasmic domain 11 whereas sequence elements within domain 13 contribute an ~5- to 10-fold enhancement to the binding affinity of the receptor for IGF-II (Fig. 1) (53–59). The crystal structure of domain 11 of the human IGF-II/MPR (60) has been reported recently and used to propose a putative IGF-II binding pocket composed of mostly hydrophobic residues, including Ile-1572, a residue that when mutated to threonine abolishes IGF-II binding to the receptor. Furthermore, the Ile-1572 → Thr mutation is associated with loss of heterozygosity of the IGF-II/MPR gene in hepatocellular
cancer (56, 61). In contrast, each of the two Man-6-P binding pockets of the IGF-II/MPR are predicted to consist of a lattice of hydrogen bond and ionic interactions similar to that observed in the structures of the CD-MPR bound to Man-6-P (22) or to pentamannosyl phosphate (23). Not surprisingly, site-directed mutagenesis screens have identified five residues (Gln-392, Ser-431, Arg-435, Glu-460, and Tyr-465) in domain 3 and five residues (Gln-1292, His-1329, Arg-1334, Glu-1354, and Tyr-1360) in domain 9 that are essential for carbohydrate recognition by the IGF-II/MPR (34, 35, 37). However, the two Man-6-P binding sites of the IGF-II/MPR have only been mapped to a minimum of three domains, 1–3 and 7–9 (Fig. 1) (33, 34), and no structural information is yet available for any of these domains. Furthermore, whereas it has been shown that domain 11 alone is sufficient for binding to IGF-II (57, 60), it is not known whether domain 3 or domain 9 alone is sufficient for carbohydrate recognition by the IGF-II/MPR or whether additional domains (i.e. domains 1 and 2 or domains 7 and 8) are necessary. To begin to address these issues, a P. pastoris expression system was used to generate truncated, soluble forms of the IGF-II/MPR composed of either domain 3 (Dom3His) or domain 9 (Dom9His) alone followed by a tag of six histidine residues, and the carbohydrate binding and dissociation properties of these constructs were examined.

Pentamannosyl phosphate-agarose affinity chromatography experiments clearly showed that Dom9His is capable of Man-6-P binding. (Fig. 3B). The ability of the Dom9His construct to bind to β-glucuronidase with an affinity ($K_d$ = 0.3 ± 0.1 nM, Fig. 7B) similar to that previously reported for the Dom7–9His construct ($K_d$ = 0.5 ± 0.1 nM) (35), the Dom7–11 construct ($K_d$ = 0.6 nM) (37), and the full-length IGF-II/MPR ($K_d$ = 2 nM) (52), further demonstrated that extracytoplasmic domain 9 alone can fold into a high affinity carbohydrate recognition. Moreover, Dom9His was functionally equivalent in all aspects tested to the previously characterized IGF-II/MPR C-terminal Man-6-P binding site constructs, Dom7–9 and Dom7–11 (37). Dom9His was readily eluted with low pH buffer from a pentamannosyl phosphate column (Fig. 3C), and the observed pH profile of binding to β-glucuronidase (Fig. 4B) confirmed its ability to undergo acid-dependent dissociation in solution. Consistent with our recent demonstration that the C-terminal Man-6-P binding site of the IGF-II/MPR is highly specific for Man-6-P, Dom9His bound poorly to a column composed of D. discoideum lysosomal enzymes (Fig. 5), which are modified with Man-6-S and Man-6-P-OCH$_3$ groups rather than the phosphomonoester, Man-6-P. Quantitative inhibition studies confirmed these results: Dom9His exhibited 280- to 335-fold lower affinities toward Man-6-P-OCH$_3$ ($K_i$ = 6.5 mM) and Man-6-S ($K_i$ = 7.7 mM) than for Man-6-P ($K_i$ = 23 μM, Fig. 6). Together, these results indicate that domains 7 and 8 are not necessary components of the C-terminal Man-6-P binding site of the IGF-II/MPR; rather, the amino acid determinants of high binding affinity and Man-6-P specificity reside entirely within domain 9.
Dom3His exhibited a pH profile of binding to β-glucuronidase similar to the previously characterized N-terminal carbohydrate binding site construct, Dom1–3His (37), demonstrating that domain 3 alone is sufficient for ligand binding and acid-dependent dissociation (Fig. 4A). However, no binding of Dom3His could be detected by pentamannosyl phosphate-agarose affinity chromatography (Fig. 3A) nor did Dom3His bind to an affinity column containing D. discoideum lysosomal enzymes (Fig. 5), leading to the prediction that its ligand-binding affinity was dramatically lower than that of the Dom1–3His construct. Binding affinity analyses confirmed this prediction, demonstrating that Dom3His has an ~1000-fold lower affinity ($K_d = 500 \text{nM}$, Fig. 7A) for the lysosomal enzyme, β-glucuronidase, than the previously characterized Dom1–3His construct ($K_d = 0.5 \pm 0.1 \text{ nM}$) (35). It is noteworthy that despite its dramatically lower affinity toward β-glucuronidase as compared with Dom1–3His, the Dom3His construct still exhibits a binding affinity that is significantly higher than that of many other lectins, such as C-type lectins, galectins (S-type), and siglec (I-type) that generally exhibit binding affinities in the 100 μM–1 mM range (62).

Several possible explanations could account for the inability of the Dom3His construct to maintain the high affinity interactions that are observed in constructs encompassing a minimum of domains 1–3 of the extracytoplasmic region of the IGF-II/MPR. Because proteins expressed in P. pastoris often contain oligosaccharides that are significantly longer than the glycans generated in higher eukaryotes, we considered the possibility that the glycans modifying Dom3His might be sterically interfering with ligand binding. Enzymatic deglycosylation of Dom3His, however, failed to restore high affinity binding ability (data not shown). A second possibility is that extra residues at the N terminus of the Dom3His could be juxtaposed near the binding pocket, providing an inhibitory influence on its binding ability. Of note, there are eight residues (Cys-His-Arg-Asp-Tyr-Leu-Glu-Ser-Arg-Ser-Cys) in the linker region between the last cysteine residue of domain 2 and the first cysteine of domain 3. A corresponding set of eight linker residues (Cys-Pro-Val-Val-Arg-Ala-Glu-Asp-Tyr-Cys) is found between the last cysteine of domain 8 and the first cysteine of domain 9. In accordance with previous IGF-II/MPR constructs (i.e., Dom5–9 (34) and Dom7–9 (37)) that have been generated in our laboratory that maintain high affinity carbohydrate binding, we elected to begin the Dom3His and Dom9His constructs with the second linker residue such that each polypeptide contained seven of the eight linker residues at its N terminus. Furthermore, N-terminal sequencing revealed that Dom3His contains four additional amino acids (Glu-Ala-Glu-Ala) remaining from the cleavage site of the S. cerevisiae α-factor signal sequence, bringing the total to eleven residues preceding the first cysteine of domain 3. Although the Dom9His construct was clearly unaffected by the nine or eleven residues at its N terminus, we cannot rule out the possibility that the length and/or linker sequence differences present at the N terminus of Dom3His could be preventing it from binding to β-glucuronidase with high affinity.

On the other hand, it is possible that the addition of a tag of six histidine residues to the C terminus of the Dom3His construct is somehow adversely influencing ligand binding, although this seems rather unlikely based on the observation that the addition of a His$_6$-tag to the C terminus of the previously characterized Dom1–3His construct had no detectable effect on its affinity toward β-glucuronidase (35). To test this possibility, Dom1–3 and Dom3 constructs without a C-terminal His$_6$-tag were generated in P. pastoris and tested for Man-6-P binding ability. Whereas the Dom1–3 construct was functional by pentamannosyl phosphate-agarose affinity chromatography, the Dom3 construct exhibited no detectable binding to the column as observed for the Dom3His construct (data not shown). Together these data suggest that domains 1 and/or 2 are necessary for high affinity Man-6-P binding by the N-terminal IGF-II/MPR carbohydrate-recognition site. Analogous to the bipartite nature of the IGF-II binding site, residues in domains 1 and/or 2 may provide additional ligand binding contacts or indirectly facilitate proper folding of the binding pocket to enhance the binding affinity of the N-terminal carbohydrate-recognition site. If proven, this would constitute a dramatic structural/functional difference between the two Man-6-P binding sites of the IGF-II/MPR in addition to the recently described specificity differences (37). Future structural and biochemical studies will be needed to distinguish among these possibilities to determine the minimal elements required for high affinity binding by the N-terminal carbohydrate-recognition site of the IGF-II/MPR.

The ability to generate truncated, soluble forms of the MPRs, as outlined in this report and previously (24, 35, 63), that retain the binding affinity and specificity properties of the native receptors opens the way for many potential MPR-based applications. Several features, including its small size (deglycosylated form is ~17 kDa), high degree of Man-6-P specificity, its μM affinity for Man-6-P, its μM binding affinity for the lysosomal enzyme, β-glucuronidase, and its broad pH range of ligand binding (>50% binding of β-glucuronidase observed between pH ~5.5 and pH ~8.3), render the IGF-II/MPR domain 9-alone construct an especially suitable reagent. For example, it could be useful for the detection and purification of mannose 6-phosphorylated proteins of interest, including lysosomal enzymes for the treatment of lysosomal storage disorders. Alternatively, Dom9His could be used as a tag to facilitate simple, one-step affinity purification of fusion proteins containing this high affinity carbohydrate-recognition domain. This novel IGF-II/MPR derivative could also serve as a high affinity protein inhibitor of the MPRs, with potential applications in the treatment of fibrotic disorders, wound healing, inflammation, cancer, and herpes simplex and varicella-zoster viral infections due to MPR interactions with Man-6-P-containing proteins involved in these processes.

In summary, the results of the current study demonstrate the ability of domain 9 alone of the IGF-II/MPR to fold into a high affinity ($K_d = 0.3 \text{nM}$) carbohydrate-recognition site and suggest that although the primary binding determinants of the N-terminal Man-6-P binding site reside in domain 3, residues in adjacent domains (domains 1 and/or 2) are important, directly or indirectly, for the generation of a high affinity carbohydrate-binding site. Together these findings, in conjunction with the previous assignment of the IGF-II binding site to domains 11 and 13, prompt the question as to what is the function of the other domains of the IGF-II/MPR. Given the size and number of domains, it is likely that at least some of the other domains also function in ligand binding. Along those lines, the binding site for retinoic acid (64–66), a reported IGF-II/MPR ligand, has not been mapped, although evidence has been provided to suggest that it binds to the receptor at a site distinct from the Man-6-P and IGF-II binding pockets. Recently, plasminogen (67) and the urokinase-type plasminogen activator receptor (67, 68) have been shown to interact with the N terminus (i.e., domain 1) of the IGF-II/MPR (69). It is possible that other domains may simply function as scaffolding to maintain the higher order structure of the IGF-II/MPR. Clearly, additional analyses will be required to further unveil the structural/functional properties of each domain of the extracytoplasmic region of the IGF-II/MPR.
Localization of the Carbohydrate Recognition Sites of the Insulin-like Growth Factor II/Mannose 6-Phosphate Receptor to Domains 3 and 9 of the Extracytoplasmic Region

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