Recognition of *Dictyostelium discoideum* Lysosomal Enzymes Is Conferred by the Amino-Terminal Carbohydrate Binding Site of the Insulin-like Growth Factor II/Mannose 6-Phosphate Receptor†

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ABSTRACT: The insulin-like growth factor-II/mannose 6-phosphate receptor (IGF-II/MPR) is a type I glycoprotein that mediates both the intracellular sorting of lysosomal enzymes bearing mannose 6-phosphate (Man-6-P) residues to the lysosome and the bioavailability of IGF-II. The extracytoplasmic region of the IGF-II/MPR contains 15 repeating domains; the two carbohydrate recognition domains (CRDs) have been localized to domains 1−3 and 7−9, and the high-affinity IGF-II binding site maps to domain 11. To characterize the carbohydrate binding properties of the IGF-II/MPR, regions of the receptor encompassing the individual CRDs were produced in a baculovirus expression system. Characterization of the recombinant proteins revealed that the pH optimum for carbohydrate binding is significantly more acidic for the carboxyl-terminal CRD than for the amino-terminal CRD (i.e., pH 6.4−6.5 vs 6.9). Equilibrium binding studies demonstrated that the two CRDs exhibit a similar affinity for Man-6-P. Furthermore, substitution of the conserved arginine residue in domain 3 (R435) or in domain 9 (R1334) with alanine resulted in a similar >1000-fold decrease in the affinity for the lysosomal enzyme, β-glucuronidase. In contrast, the two CRDs differ dramatically in their ability to recognize the distinctive modifications (i.e., mannose 6-sulfate and Man-6-P methyl ester) found on *Dictyostelium discoideum* lysosomal enzymes: the amino-terminal CRD binds mannose 6-sulfate and Man-6-P methyl ester with a 14−55-fold higher affinity than the carboxyl-terminal CRD. Taken together, these results demonstrate that the IGF-II/MPR contains two functionally distinct CRDs.

There are two members of the P-type family of lectins, the 300 kDa insulin-like growth factor-II/mannose 6-phosphate receptor (IGF-II/MPR)[1] and the 46 kDa cation-dependent MPR (CD-MPR), both of which function to target lysosomal enzymes to the lysosome by recognizing mannose 6-phosphate (Man-6-P) residues on newly synthesized lysosomal enzymes. The receptor−lysosomal enzyme complex is transported from the trans Golgi network (TGN) to an acidified late endosomal compartment where the low pH of this compartment causes the complex to dissociate. The lysosomal enzymes move to the lysosome, while the receptors recycle back to the TGN or move to the plasma membrane where they mediate the internalization of exogenous ligands (*1*−*3*).

The MPRs are type I transmembrane glycoproteins which contain four structural domains: an amino-terminal signal sequence, an extracytoplasmic region which contains the ligand binding site(s), a transmembrane region, and a carboxyl-terminal cytoplasmic domain. Biochemical and structural analyses of the 154-residue extracytoplasmic domain of the CD-MPR confirmed that the extracytoplasmic domain alone, which binds a single molecule of Man-6-P (*4*), folds into a functional carbohydrate recognition domain (CRD) capable of high-affinity ligand binding (*5*−*7*). The large extracytoplasmic region of the IGF-II/MPR has a repetitive structure due to the presence of 15 contiguous domains, each of which is approximately 147 residues in length. The existence of significant degrees of sequence homology (14−28% identity) (*8*), including a conserved positioning of cysteine residues, between the extracytoplasmic region of the CD-MPR and each of the 15 repeating domains of the IGF-II/MPR suggests that each of the 15 units in the IGF-II/MPR folds into its own CRD to generate a receptor that contains 15 Man-6-P binding sites. However, equilibrium dialysis experiments revealed that the IGF-II/MPR binds only 2 mol of Man-6-P per polypeptide chain (*9*). Partial proteolysis and expression of truncated forms of

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[1] Abbreviations: MPR, mannose 6-phosphate receptor; CD-MPR, cation-dependent MPR; IGF-II/MPR, insulin-like growth factor II/MPR; Dom 1−3, domains 1−3 of the IGF-II/MPR; Dom 7−9, domains 7−9 of the IGF-II/MPR; Dom 7−11, domains 7−11 of the IGF-II/MPR; Man-6-P, mannose 6-phosphate; Man-6-S, mannose 6-sulfate; Man-P-OCH_3, mannose 6-phosphate methyl ester; Man-P-OGlcNAc, mannose 6-phosphate *N*-acetylglucosamine ester; CRD, carbohydrate recognition domain; DSS, disuccinimidyl suberate; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; Glc-6-P, glucose 6-phosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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the receptor localized the Man-6-P binding sites to domains 1−3 (Dom 1−3) and 7−9 (Dom 7−9), with residues in domain 3 and domain 9 being essential for carbohydrate recognition (*10*, *11*). Our recent studies, which characterized the ability of mutant full-length receptors containing only one functional Man-6-P binding site to target lysosomal enzymes to the lysosome, indicate that the two CRDs of the IGF-II/MPR are not functionally equivalent (*12*). The presence of two different CRDs in the IGF-II/MPR may provide this receptor with a functional advantage over the CD-MPR, which exists as a dimer and, therefore, contains two identical Man-6-P binding sites. In fact, studies in which either MPR-deficient mice (*13*) or MPR-deficient cell lines (*14*) were used indicate that the IGF-II/MPR is more efficient than the CD-MPR in targeting lysosomal enzymes to the lysosome. Furthermore, binding studies performed in vitro demonstrate that the IGF-II/MPR has a higher affinity for and recognizes a broader spectrum of individual lysosomal enzymes than does the CD-MPR (*15*, *16*). Thus, the presence of two CRDs which differ in their carbohydrate binding properties may explain the IGF-II/MPR's enhanced targeting ability, relative to that of the CD-MPR, that is observed in vivo.

The aim of this study is to further characterize the ligand binding and dissociation properties of the two CRDs of the IGF-II/MPR. To analyze the two CRDs of the IGF-II/MPR independently, regions of the receptor encompassing the individual Man-6-P binding sites (Dom 1−3 and Dom 7−9) were produced in a baculovirus expression system. A construct encoding the carboxyl-terminal CRD plus the IGF-II binding site, domains 7−11 (Dom 7−11), was also expressed to determine if the additional domains (10 and 11) influence receptor function. These truncated, soluble forms of the IGF-II/MPR were characterized with respect to oligomeric state, ligand dissociation, and binding to various ligands, including pentamannosyl phosphate, Man-6-P, the lysosomal enzyme β-glucuronidase, and the distinctive carbohydrate modifications [i.e., mannose 6-sulfate (Man-6-S) and Man-6-P methyl ester (Man-P-OCH_3)] found on *Dictyostelium discoideum* lysosomal enzymes.

MATERIALS AND METHODS

Materials. The following reagents were obtained commercially as indicated: Fall Army worm cell line *Spodoptera frugiperda* (Sf9, American Type Culture Collection), Cabbage looper egg cell line, *Trichoplusia ni* 5B1-4 (High Five, Invitrogen Corp.), disuccinimidyl suberate (DSS, Pierce Chemical Corp.), IGF-I and IGF-II (Upstate Biotechnology Inc. or Bachem Inc.), bovine pancreatic insulin (Boehringer Mannheim), pSVL vector (Pharmacia Biotechnology Inc.), Express Five serum-free insect medium (Gibco BRL), Ex-Cell 405 medium (JRH Biosciences), Graces insect medium (Gibco BRL), fetal bovine serum (FBS; Hyclone Laboratories), *Autographa californica* nuclear polyhedris virus (AcNPV) transfer vectors pVL1392 and pVL1393 (PharMingen), modified AcNPV baculovirus DNA (BaculoGold DNA, PharMingen), Ni-NTA−agarose (Qiagen), [1-^{14}C]Man-6-P (American Radiolabeled Chemicals, Inc., 50 mCi/mmol), β-amylase, alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase used for sedimentation, Man-6-P (Sigma), and Man-6-S (V−Labs). Phosphomannan from *Hansenula holstii* was a kind gift of M. E. Slodki of the Northern Regional Research Center (Peoria, IL). L-MPR 13-2-1, MTX 3.2 cells overexpressing human β-glucuronidase were generously provided by W. Sly (St. Louis University School of Medicine, St. Louis, MO). *D. discoideum* cells were a generous gift of H. Freeze (The Burnham Institute, La Jolla, CA).

Synthesis of Man-P-OCH_3. The acid-stable phosphodiester, Man-P-OCH_3, was synthesized by treating Man-6-P with dicyclohexylcarbodiimide in a methanolic solution (*17*). [1-^{14}C]Man-6-P (1 μCi) was added to Man-6-P (0.5 g) to facilitate the monitoring of the completeness of the reaction. The reaction mixture was then applied to a QAE-Sephadex column to isolate the monomethylated species from unreacted Man-6-P and dimethylated species as described previously (*18*). The identity of Man-P-OCH_3 was confirmed using a VG/AutoSpec (Manchester, U.K.) mass spectrometer equipped with a liquid secondary ion mass spectrometry (LSIMS) source.

Generation of Truncated IGF-II/MPRs. The construct encoding the signal sequence and domains 1−3 (Dom 1−3) of the IGF-II/MPR was generated as described previously (*10*). Constructs encoding domains 7−9 (Dom 7−9) or 7−11 (Dom 7−11) were fused to the signal sequence of the IGF-II/MPR (amino acids 1−50, nucleotides 159−302). The constructs were cloned into the pSVL, pVL1392, or pVL1393 expression vector. The resulting plasmids encoding truncated forms of the IGF-II/MPR contain the indicated amino acid residues and nucleotides followed by a stop codon (TGA): Dom 1−3, residues 1−476, nucleotides 1−1580; Dom 7−9, residues 1−50 and 932−1371, nucleotides 159−302 and 2946−4265; and Dom 7−11, residues 1−50 and 932−1657, nucleotides 159−302 and 2946−5120. Cassette mutagenesis was used to generate the Dom 1−3His and Dom 7−9His constructs which contain the indicated amino acid residues and nucleotides as described above for Dom 1−3 and Dom 7−9 except that six histidine residues (CAC) and a stop codon (TGA) were placed at the C-terminus. The oligonucleotide cassettes used were as follows: Dom 1−3HIS, GTGGGATACGAAGTACGCCTGTGTCCACCACCAC-CACCACCACTGAGAATTCT; and Dom 7−9His, CCGAT-TGCTCCTACCTGTTTGAGTGGCGCACGCAGTACGC-CTGCCCGCACCACCACCACCACCACTGAGAATTCT. Single-point mutations in domain 3 (R435A) and domain 9 (R1334A) were generated as described previously (*10*, *12*).

Expression of Recombinant IGF-II/MPRs in Insect Cells. The baculovirus transfer vectors pVL1392 and pVL1393 encoding Dom 1−3, Dom 1−3His (wild-type or R435A), Dom 7−9, Dom 7−9His (wild-type or R1334A), or Dom 7−11 of the IGF-II/MPR (2 μg) and the BaculoGold DNA (0.5 μg) were cotransfected into Sf9 insect cells using the calcium phosphate precipitation method (*19*). Recombinant virus was purified by plaque purification as described previously (*20*). High Five insect cells were cultured in Express Five or Ex-Cell 405 medium under serum-free conditions and maintained at 27 °C. Cells in monolayer cultures were infected with the recombinant virus, and the medium was harvested 4−5 days postinfection.

Affinity Chromatography. Recombinant proteins were purified from the medium of transfected insect cells by pentamannosyl phosphate−agarose affinity chromatography as described previously (*10*). The columns were eluted with glucose 6-phosphate (Glc-6-P) followed by Man-6-P. In some experiments, the columns were eluted with a low-pH buffer

containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 4.6), 150 mM NaCl, 10 mM $MnCl_2$, and 5 mM β-glycerophosphate prior to the elution with Man-6-P. The purified recombinant proteins were also passed over an affinity column substituted with *D. discoideum* lysosomal enzymes, which contain Man-6-P residues diesterified to methyl groups (*21*) and Man-6-S residues (*22*). The columns were eluted with Glc-6-P followed by Man-6-P. The column fractions were precipitated with trichloroacetic acid and analyzed on 10% SDS−polyacrylamide gels followed by Western analysis (see below). Recombinant proteins containing a C-terminal histidine tag were purified by Ni-NTA−agarose affinity chromatography. Following passage of the medium containing the recombinant proteins over the Ni-NTA columns, the columns were washed in buffer containing 20 mM Tris (pH 8.0), 0.5 M NaCl, and 10 mM imidazole and then eluted with buffer containing 250 mM imidazole (pH 8.0).

IGF-II Cross-Linking. Human recombinant IGF-II (2×10^7 cpm/μg) was iodinated by the lactoperoxidase method as described by Tait et al. (*23*). Purified Dom 7−11 (0.8 nM) was incubated with [^{125}I]IGF-II (10 nM) in cross-linking buffer [0.1 M HEPES (pH 8.0) and 0.1 M NaCl] in the presence or absence of a 100-fold molar excess of unlabeled IGF-II, IGF-I, or insulin for 2 h at room temperature. The samples were then incubated with 0.2 mM DSS for 30 min at room temperature, precipitated with 10% trichloroacetic acid, and analyzed by SDS−PAGE and autoradiography (*24*).

IGF-II Binding Affinity. The binding assays were performed like the equilibrium binding assay for β-glucuronidase (see below). Increasing concentrations of iodinated IGF-II (0.05−85 nM) were incubated with purified Dom 7−11, and the MPR and bound ligand were immunoprecipitated with an anti-IGF-II/MPR polyclonal antibody which was prebound to protein A−Sepharose. After a total incubation of ≥2 h, the beads were pelleted and washed. The supernatant and washes were collected, and the radioactivity was counted as a direct measure of the amount of unbound ligand in each reaction mixture. Reaction mixtures without Dom 7−11 were used as a measure of nonspecific binding. In all cases, nonspecific binding was ≤1.5% of the added counts. The results were analyzed by nonlinear regression (SigmaPlot 4.0).

Western Blot Analysis. Western blot and dot blot analyses were performed as described previously (*24*) except that following the incubation with bovine IGF-II/MPR-specific antiserum, the membranes were incubated for 1 h with 20 mM Tris (pH 7.6), 137 mM NaCl, 3.8 mM HCl, and Tween 20 (0.2% v/v) containing bovine serum albumin (0.1% w/v) and the protein A−horseradish peroxidase conjugate (1:2000, Amersham Pharmacia Biotech). The receptor was detected by enhanced chemiluminescence as described by the manufacturer (Pierce Chemical Co.).

N-Terminal Sequencing. Dom 1−3 and Dom 7−11 recombinant proteins were purified from the medium of infected High Five cells by pentamannosyl phosphate−agarose affinity chromatography and subjected to N-terminal amino acid sequence analysis (Medical College of Wisconsin Protein and Nucleic Acids Facility). Fifteen cycles of Edman degradation were performed on each protein, and the PTH-derivatized amino acids were separated by reverse-phase HPLC. The amino-terminal sequence analysis of the recombinant proteins revealed that the signal sequence was processed correctly as the N-terminus of Dom 1−3 (TQGAEFPELXSY) or Dom 7−11 (TQGIRITTDIDQ) was identical to the predicted sequence of the mature protein which starts with residues 48−50 (TQG).

Cross-Linking of the Recombinant Proteins. Dom 1−3, Dom 7−9, and Dom 7−11 proteins purified from baculovirus-infected insect cells were dialyzed against 0.1 M HEPES (pH 8.0) and 0.1 M NaCl and then incubated with the homobifunctional cross-linker, disuccinimidyl suberate (DSS, 0.2 mM), for 1 h. The cross-linking reaction was stopped by the addition of glycine to a final concentration of 10 mM followed by a 10 min incubation (*24*). The samples were precipitated with 10% trichloroacetic acid and analyzed on a SDS−polyacrylamide gel followed by Western blot analysis.

Sucrose Gradients. Purified Dom 1−3, Dom 7−9, and Dom 7−11 (50−100 ng) were run on 5 to 20% continuous sucrose gradients (4 mL) that were equilibrated at 4 °C. The gradients were centrifuged at 250000*g* for 13 h. Fractions (150 μL) were collected from the bottom of the gradient and were assayed for the presence of IGF-II/MPRs by Western blot analysis. β-Amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were run on a separate 5 to 20% sucrose gradient. Fractions were collected and analyzed on a 10% SDS−polyacrylamide gel, and the proteins were visualized by Coomassie staining. The refractive index of each fraction was measured to allow comparison between gradients.

Binding Affinity. The binding assays were performed as described previously (*12*). Briefly, increasing concentrations of iodinated β-glucuronidase were incubated with purified Dom 1−3, Dom 1−3His (wild-type or R435A), Dom 7−9, Dom 7−9His (wild-type or R1334A), or Dom 7−11. The MPR and bound ligand were immunoprecipitated with IGF-II/MPR antisera prebound to protein A−Sepharose. The bound β-glucuronidase was specifically eluted from the antibody-bound MPRs by incubation at 4 °C for 1 h with 10 mM Man-6-P. The protein A−Sepharose beads were counted as nonspecific binding and were ≤1% of the total counts per minute that were added. For inhibition studies, purified Dom 1−3, Dom 1−3His, Dom 7−9, Dom 7−9His, or Dom 7−11 was incubated with [^{125}I]-β-glucuronidase at a concentration equivalent to the determined K_d in the presence of increasing concentrations of Man-6-P, Man-6-S, or Man-P-OCH_3. To determine the pH optimum of ligand binding by each CRD, incubations were carried out at pH intervals of ∼0.25 unit with the following buffers: 50 mM sodium acetate, 150 mM NaCl, 5 mM β-glycerophosphate, and 10 mM $MnCl_2$ (pH 4.5−6.15); 50 mM MES,150 mM NaCl, 5 mM β-glycerophosphate, and 10 mM $MnCl_2$ (pH 5.6−6.6); 50 mM imidazole, 150 mM NaCl, 5 mM β-glycerophosphate, and 10 mM $MnCl_2$ (pH 6.6−7.2); and 50 mM HEPES, 150 mM NaCl, 5 mM β-glycerophosphate, and 10 mM $MnCl_2$ (pH 7.1−7.8). Control experiments were performed which demonstrated that β-glucuronidase did not interfere with the immunoprecipitation of the IGF-II/MPR, and that sufficient antibody and protein A were present to immunoprecipitate all of the IGF-II/MPR at each of the pH values that were tested. The results were analyzed by nonlinear regression (SigmaPlot 4.0).

RESULTS

Expression and Purification of Truncated IGF-II/MPRs. Previous work in our laboratory determined that *S. frugiperda* (Sf9) and *T. ni* 5B1-4 (High Five) insect cells do not contain endogenous MPRs, and that insect cells infected with recombinant baculovirus encoding the CD-MPR produce functional receptors (*7*, *20*). To obtain sufficient quantities of protein to carry out biochemical analyses of the CRDs of the IGF-II/MPR, recombinant baculovirus encoding the various Dom 1−3, Dom 7−9, or Dom 7−11 constructs was used to infect High Five insect cells, and the cells and medium were harvested 4−5 days postinfection. Western blot analysis revealed that a significant fraction (20−60, 75, and 65% for the Dom 1−3, Dom 7−9, and Dom 7−11 constructs, respectively) of the recombinant MPRs were secreted (data not shown). Since secreted proteins which misfold are often retained within the cell (*25*, *26*), all subsequent procedures were carried out on proteins that were purified from the medium of baculovirus-infected High Five insect cells by pentamannosyl phosphate−agarose affinity chromatography. To determine whether the amino-terminal signal sequence was processed correctly by the insect cells, purified recombinant proteins were subjected to N-terminal sequencing which demonstrated that the 44-residue signal sequence was removed at the predicted site. In addition, enzymatic deglycosylation of the Dom 1−3, Dom 7−9, and Dom 7−11 proteins demonstrated that each contains N-linked oligosaccharides based on their sensitivity to endo-*β*-*N*-acetylglucosaminidase H and peptide:*N*-glycosidase F. These results also showed that the three potential N-glycosylation sites in Dom 1−3 are utilized, whereas it was unclear how many of the five potential N-glycosylation sites in Dom 7−9 or Dom 7−11 are utilized due to insufficient resolution on SDS−PAGE (data not shown).

IGF-II Binding of the Dom 7−11 Recombinant IGF-II/MPR. The ability to purify the recombinant proteins with phosphomannosyl-containing columns demonstrates that the proteins have folded into a conformation conducive to the recognition of Man-6-P residues. To further verify that the recombinant proteins are correctly folded, the construct encoding domains 7−11 was evaluated for the presence of a functional IGF-II binding site, which has previously been localized to domain 11 of the IGF-II/MPR (*24*, *27*). Purified Dom 7−11 was incubated with iodinated IGF-II and the cross-linking agent, DSS. The generation of a radiolabeled, cross-linked ~80 kDa species was inhibited by the presence of unlabeled IGF-II, but not IGF-I or insulin (Figure 1A). This ~80 kDa species is consistent with a complex consisting of the 88 kDa Dom 7−11 and the 7.5 kDa IGF-II. To directly determine the affinity of Dom 7−11 for the IGF-II, equilibrium binding assays were performed (Figure 1B) and a K_d value of 8.3 ± 2.6 nM was obtained, which is a lower affinity than that previously reported for the full-length bovine receptor (K_d = 0.2 nM) (*28*). However, a recent study, which has demonstrated that sequences in domain 13 enhance the affinity of the mammalian IGF-II/MPR for IGF-II (*29*), likely explains the 40-fold lower affinity observed for the construct encoding domains 7−11. Taken together, these studies demonstrate that fragments of the IGF-II/MPR can be expressed in a functional form by baculovirus-infected insect cells.

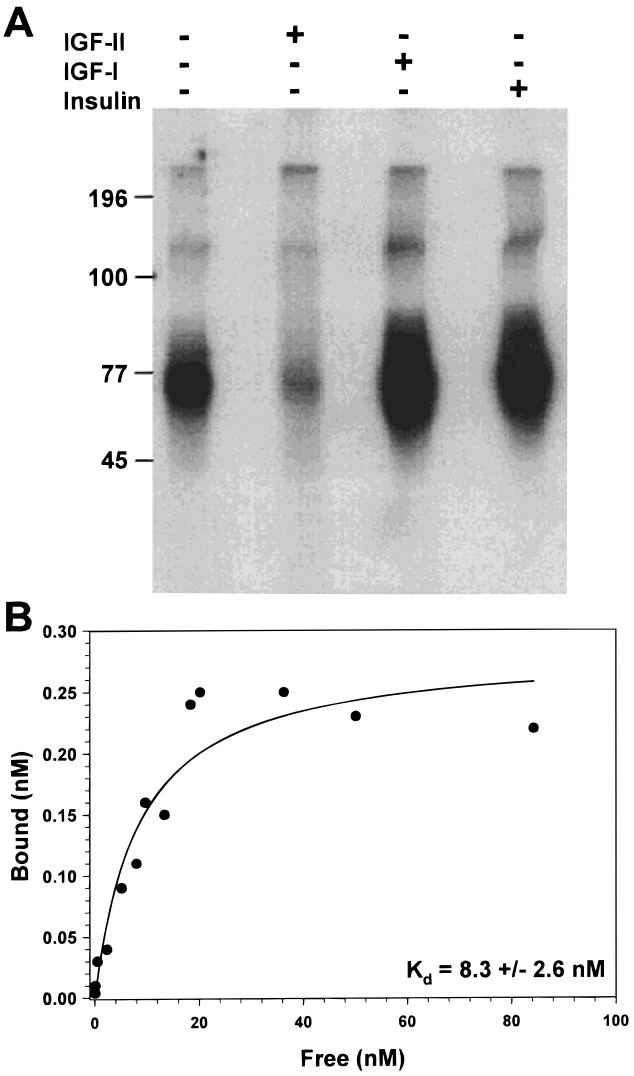

FIGURE 1: Binding of IGF-II to Dom 7−11 recombinant protein. (A) Purified Dom 7−11 was incubated with 10 nM iodinated IGF-II and 0.2 mM DSS in the presence or absence of a 100-fold molar excess of unlabeled IGF-II, IGF-I, or insulin. The samples were precipitated and analyzed on a 7.5% SDS−polyacrylamide gel. (B) Increasing concentrations of iodinated human IGF-II were incubated with purified Dom 7−11. The receptor−enzyme complex was immunoprecipitated with an anti-IGF-II/MPR polyclonal antibody which was prebound to protein A−Sepharose, and the data were analyzed by nonlinear regression (SigmaPlot 4.0). All points are corrected for nonspecific binding, which accounted for ≤1.5% of the added radioactivity.

Oligomeric Structure of Truncated Recombinant IGF-II/MPRs. The oligomeric structure of the full-length IGF-II/MPR is controversial. Studies in which gel filtration and sucrose gradients were used demonstrated that the IGF-II/MPR is monomeric (*30*), whereas cross-linking studies indicated the existence of oligomeric species of the receptor (*31*). In addition, chemical cross-linking and gel filtration analyses of the receptor in the presence of a bound lysosomal enzyme demonstrated a dimeric subunit composition (*32*). Since differences in quaternary structure may provide an explanation for any observed differences in binding affinities, the oligomeric state of the recombinant proteins was analyzed by several methods. Purified Dom 1−3, Dom 7−9, and Dom 7−11 recombinant proteins were run on 5 to 20% continuous sucrose gradients. Figure 2 shows that Dom 1−3, Dom 7−9,

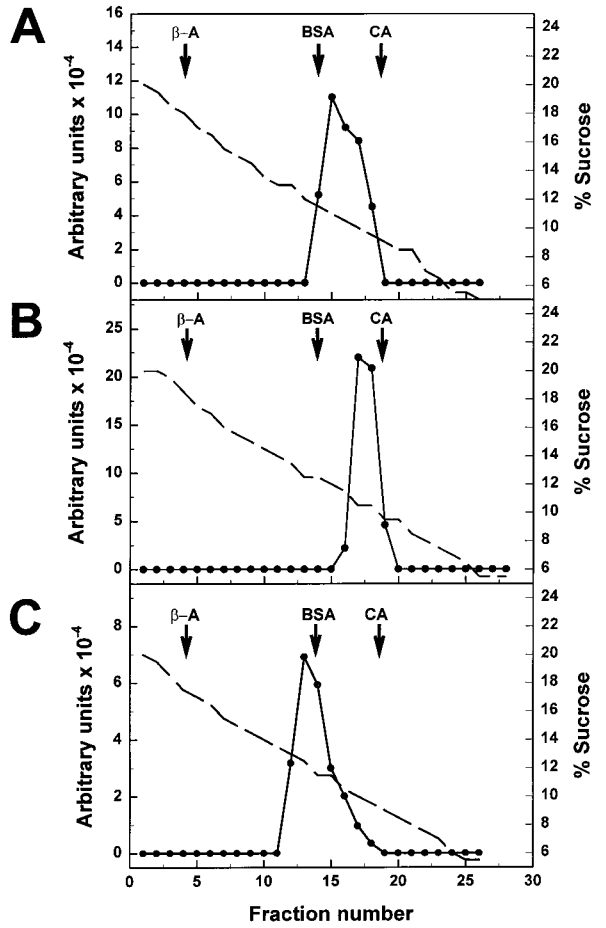

FIGURE 2: Sucrose gradient sedimentation of truncated IGF-II/MPRs. Purified Dom 1−3 (A), Dom 7−9 (B), and Dom 7−11 (C) were run on 5 (fraction 25) to 20% (fraction 1) continuous sucrose gradients shown by the dotted line. Following sedimentation, fractions (150 μL) were precipitated with trichloroacetic acid and assayed for the presence of the receptor by Western blot analysis. The proteins were visualized by enhanced chemiluminescence, and the results were quantified using densitometry. A parallel gradient was run with the following standards: β-amylase (β-A, 200 kDa), bovine serum albumin (BSA, 66 kDa), and carbonic anhydrase (CA, 29 kDa).

and Dom 7−11 sediment mainly in fractions 14−18, 16−19, and 12−17, respectively. In comparison to the predicted molecular masses (including the mass of N-linked oligosaccharides) of 52, 58, and 88 kDa, the Dom 1−3, Dom 7−9, and Dom 7−11 proteins exhibited molecular masses of 49, 38, and 78 kDa, respectively, as determined by sedimentation. Purified truncated proteins were also subjected to gel filtration chromatography using a Sephadex G-150 column. The Dom 1−3 and Dom 7−9 proteins eluted from the column as a single peak with calculated molecular masses of 59 and 88 kDa, respectively. Similar results were obtained for Dom 1−3His and Dom 7−9His (data not shown). Since sedimentation and gel filtration are based on globular protein standards, an independent determination of oligomeric state was also performed. Purified recombinant proteins were incubated in the presence or absence of the homobifunctional cross-linking agent, DSS. Dom 1−3, Dom 7−9, and Dom 7−11 migrated at 45, 50, and 70 kDa, respectively, and in the presence of DSS, no higher-molecular mass species were identified, suggesting all three truncated proteins exist as monomers (Figure 3). Similar results were obtained for Dom

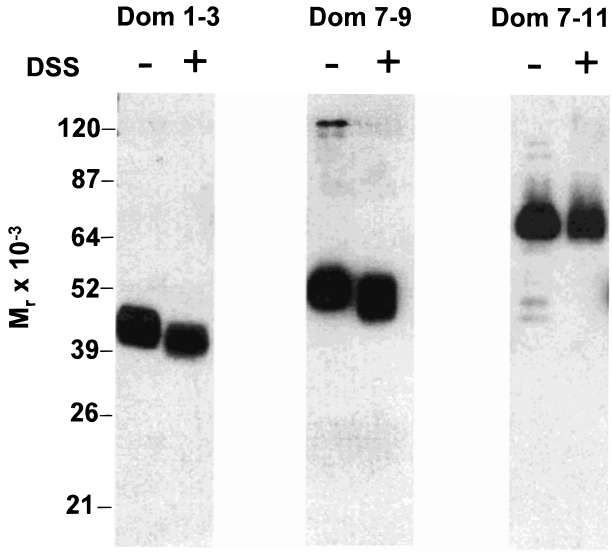

FIGURE 3: Cross-linking of truncated IGF-II/MPRs. Dom 1−3, Dom 7−9, and Dom 7−11 were purified on pentamannosyl phosphate−agarose columns and incubated in the absence (−) or presence (+) of 0.2 mM DSS for 1 h at 25 °C. The samples were precipitated with trichloroacetic acid and analyzed on a 10% SDS−polyacrylamide gel followed by Western blot analysis.

1−3His and Dom 7−9His (data not shown). Addition of DSS to each of the purified truncated receptors resulted in the majority of the receptor migrating with a faster mobility on SDS−polyacrylamide gels than receptors not incubated with the cross-linking agent. These results are consistent with the cross-linking agent forming intramolecular covalent bonds to produce a protein that can no longer be unfolded by SDS, resulting in a more compact conformation upon SDS−PAGE. Taken together, these results demonstrate that Dom 1−3, Dom 1−3His, Dom 7−9, Dom 7−9His, and Dom 7−11 exist as monomeric species.

Pentamannosyl Phosphate−Agarose Affinity Chromatography and Acid-Dependent Dissociation. Prior to the determination of whether Dom 1−3, Dom 7−9, and Dom 7−11 were functional in acid-dependent dissociation, the recombinant proteins purified from the medium of baculovirus-infected insect cells were passed over pentamannosyl phosphate−agarose affinity columns. Figure 4A shows that, as expected, Dom 1−3, Dom 7−9, and Dom 7−11 bound to the column and were specifically eluted with Man-6-P. The targeting of lysosomal enzymes involves both the binding to MPRs in the Golgi and their subsequent dissociation in acidic late endosomal compartments (*1*, *33*). To determine whether the two CRDs of the IGF-II/MPR exhibit ligand dissociation at an acidic pH, purified proteins were passed over an affinity column which was eluted sequentially with an acidic buffer (pH 4.6), Glc-6-P, and Man-6-P. Figure 4B shows that 90% of Dom 1−3 elutes from the column with low pH, whereas only a small percentage (10%) of the carboxyl-terminal binding site (Dom 7−9 or Dom 7−11) dissociates from the column at pH 4.6. To confirm that the difference seen between the two CRDs was not due to a modification or the lack of a modification on the receptor as a result of producing the recombinant proteins in insect cells, Dom 1−3, Dom 7−9, and Dom 7−11 were expressed in mammalian cells; expression of the recombinant MPRs in COS-1 cells yielded similar results in that the carboxyl-

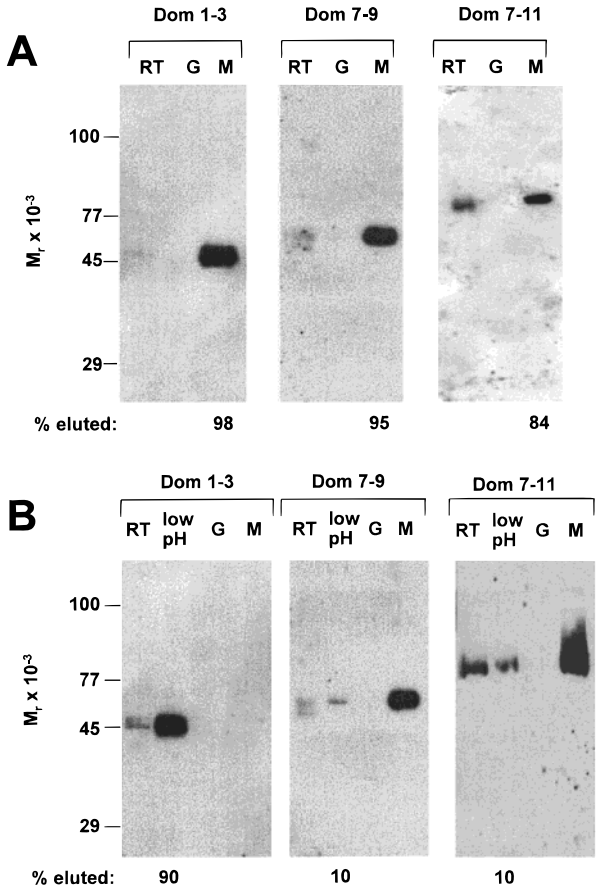

FIGURE 4: Dissociation of truncated IGF-II/MPRs from pentamannosyl phosphate−agarose affinity columns. (A) Dom 1−3, Dom 7−9, and Dom 7−11 purified from the medium of baculovirus-infected insect cells were passed over pentamannosyl phosphate−agarose affinity columns. The columns were washed and eluted with Glc-6-P (G) and then with Man-6-P (M). (B) Purified recombinant proteins were also passed over pentamannosyl phosphate−agarose affinity columns which were washed and eluted with pH 4.6 buffer (low pH), and Glc-6-P (G), followed by Man-6-P (M). The run-through (RT) fractions and eluates were precipitated with 10% trichloroacetic acid. The samples were analyzed on a 10% SDS−polyacrylamide gel followed by Western blot analysis.

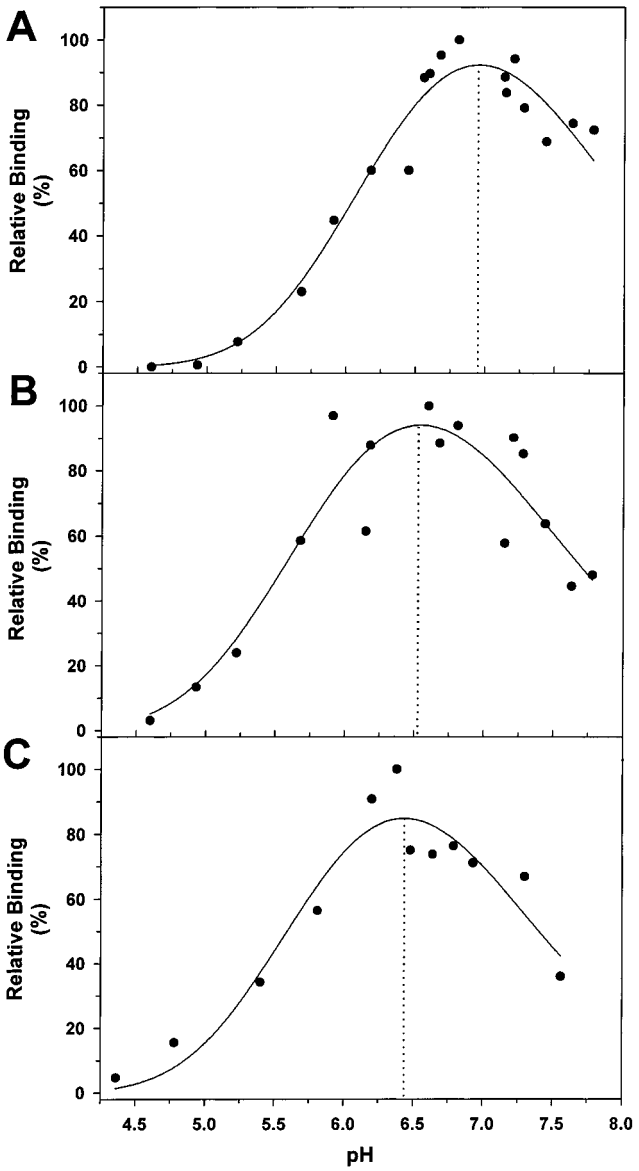

FIGURE 5: pH profile of β-glucuronidase binding. Recombinant proteins (A) Dom 1−3His, (B) Dom 7−9His, and (C) Dom 7−11 were incubated with iodinated β-glucuronidase (2 nM) in buffers at different pH values. The receptor−enzyme complexes were immunoprecipitated with MPR-specific antisera and protein A−Sepharose. [^{125}I]-β-Glucuronidase specifically bound to the recombinant MPRs was eluted by incubation with 10 mM Man-6-P. The vertical dotted lines indicate the pH optimum for each construct.

terminal CRD does not undergo acid-dependent dissociation at pH 4.6 as efficiently as Dom 1−3 (data not shown). To quantitatively compare the two CRDs, a pH profile of binding to the lysosomal enzyme, β-glucuronidase, was performed. The results demonstrate that the carboxyl-terminal CRD (Dom 7−9 or Dom 7−11) exhibits a pH optimum of 6.4−6.5 compared to pH 6.9 for Dom 1−3. Furthermore, in contrast to Dom 1−3, the carboxyl-terminal CRD retains a significant amount of binding activity below pH 5.5 (Figure 5).

Binding Affinity of Man-6-P and β-Glucuronidase. To quantitatively compare the two CRDs, the affinity for Man-6-P was determined by inhibition studies. Purified recombinant proteins were incubated with iodinated β-glucuronidase in the presence of increasing concentrations of Man-6-P. The observed K_i values (range of 6−49 μM) for Dom 1−3 (wild-type or His-tagged), Dom 7−9 (wild-type or His-tagged), and Dom 7−11 (Figures 6 and 9) are similar to previous reports for the full-length IGF-II/MPR which identified a K_i of 5−10 μM (*9*). Although these results demonstrated that the two CRDs of the IGF-II/MPR have a similar affinity for the phosphomonosaccharide Man-6-P, the possibility that the two CRDs differed in their ability to recognize Man-6-P residues in the context of an oligosaccharide chain existed. To address this possibility, the affinity of Dom 1−3, Dom 7−9, and Dom 7−11 for an endogenous ligand was directly determined. The ligand chosen was the lysosomal enzyme β-glucuronidase, which has been well characterized and whose crystal structure is known (*34*). The full-length IGF-II/MPR has been shown to bind β-glucuronidase with a K_d of 2 nM (*35*). Purified Dom 1−3, Dom 7−9, and Dom 7−11 were incubated with increasing concentrations of iodinated human recombinant β-glucuronidase. Analysis of the binding curves by nonlinear regression demonstrated a K_d of 1.0 ± 0.3, 2.5 ± 0.9, and 0.6 nM for Dom 1−3, Dom 7−9, and Dom 7−11, respec-

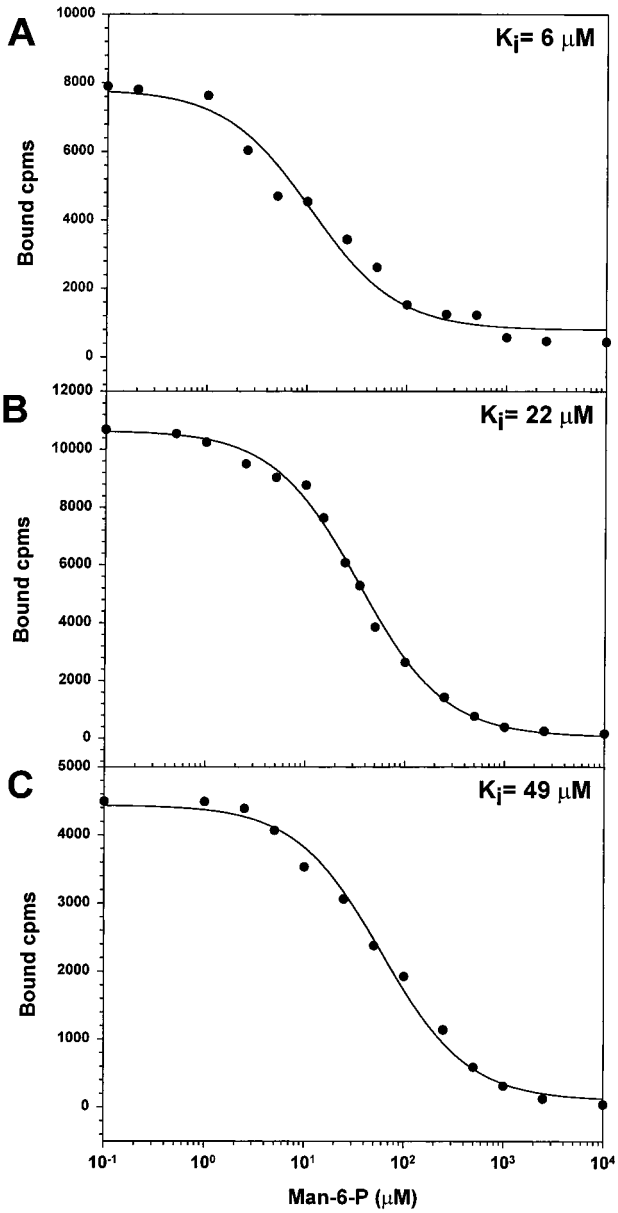

FIGURE 6: Inhibition of β-glucuronidase binding to truncated IGF-II/MPRs by Man-6-P. Purified Dom 1−3 (A), Dom 7−9 (B), or Dom 7−11 (C) was incubated with a concentration of iodinated β-glucuronidase equivalent to the K_d determined in Figure 7 in the presence of increasing concentrations of Man-6-P. Receptor−enzyme complexes were immunoprecipitated with IGF-II/MPR-specific antisera prebound to protein A−Sepharose. [^{125}I]-β-Glucuronidase specifically bound to truncated IGF-II/MPRs was eluted by incubation for 1 h with 10 mM Man-6-P. The results were analyzed with SigmaPlot 4.0.

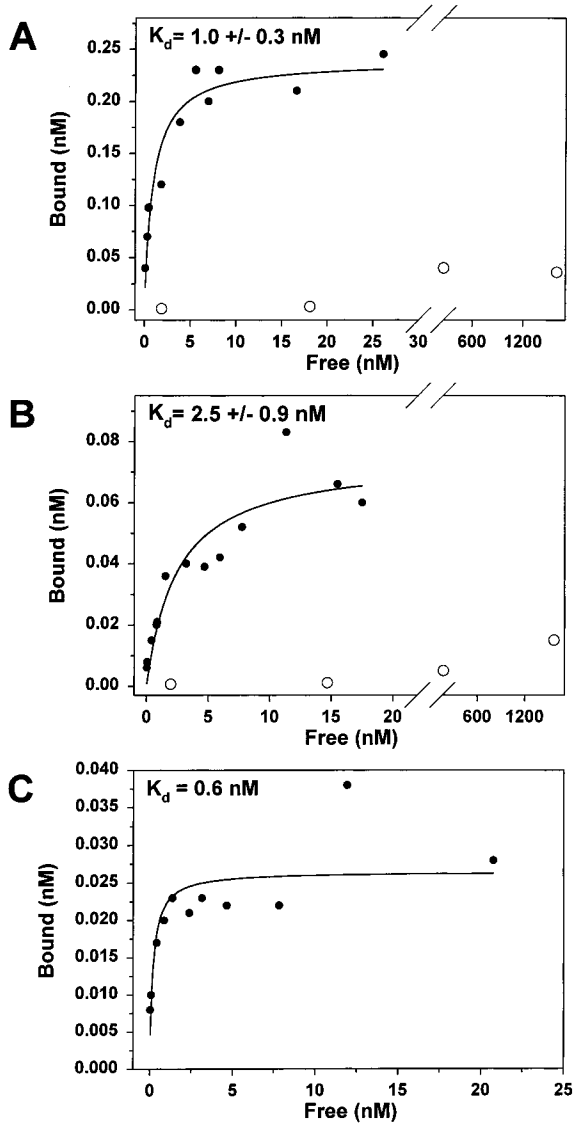

FIGURE 7: Binding of β-glucuronidase to truncated IGF-II/MPRs. Increasing concentrations of iodinated human β-glucuronidase were incubated with Dom 1−3 (A), Dom 7−9 (B), or Dom 7−11 (C) for 2 h at 4 °C. Receptor−enzyme complexes were immunoprecipitated with IGF-II/MPR-specific antisera prebound to protein A−Sepharose. [^{125}I]-β-Glucuronidase specifically bound to the truncated IGF-II/MPRs was eluted by incubation for 1 h with 10 mM Man-6-P. The results were analyzed using nonlinear regression (SigmaPlot 4.0). The mean of two independent binding studies is shown for Dom 7−11: (A) Dom 1−3 (●) and Dom 1−3HisR435A (○), (B) Dom 7−9 (●) and Dom 7−9HisR1334A (○), and (C) Dom 7−11 (●). The amounts of wild-type and mutant receptors used are equivalent.

tively (Figure 7), indicating that the two CRDs of the IGF-II/MPR are similar in their affinity for β-glucuronidase. A structure-based sequence alignment of domain 3 and domain 9, in conjunction with modeling studies based on the crystal structure of the CD-MPR (*6*), predicts that R435 in domain 3 and R1334 in domain 9 are in the carbohydrate binding pockets of the IGF-II/MPR. Substitution of R435 or R1334 with an alanine residue results in a >1000-fold inhibitory effect on carbohydrate recognition (Figure 7), suggesting that R435 and R1334 play similar, critical roles in carbohydrate recognition by both CRDs of the IGF-II/MPR.

Recognition of D. discoideum Lysosomal Enzymes. In contrast to the CD-MPR, the IGF-II/MPR is able to bind lysosomal enzymes from *D. discoideum* (*15*, *36*−*38*). *D. discoideum* lysosomal enzymes have been shown to contain Man-6-P residues diesterified to methyl groups (Man-P-OCH_3) (*21*), rather than the phosphomonoester, Man-6-P, as well as Man-6-S (*22*, *39*) on their N-linked oligosaccharides. To characterize the ability of the two CRDs to bind these carbohydrate modifications, purified Dom 1−3, Dom 7−9, and Dom 7−11 recombinant proteins were passed over affinity columns containing coupled *D. discoideum* lysosomal enzymes. The Dom 1−3 recombinant protein produced in High Five insect cells is similar to the full-length IGF-II/MPR (*9*, *15*) in that 90% bound to the affinity column and was specifically eluted with Man-6-P. Surprisingly, only

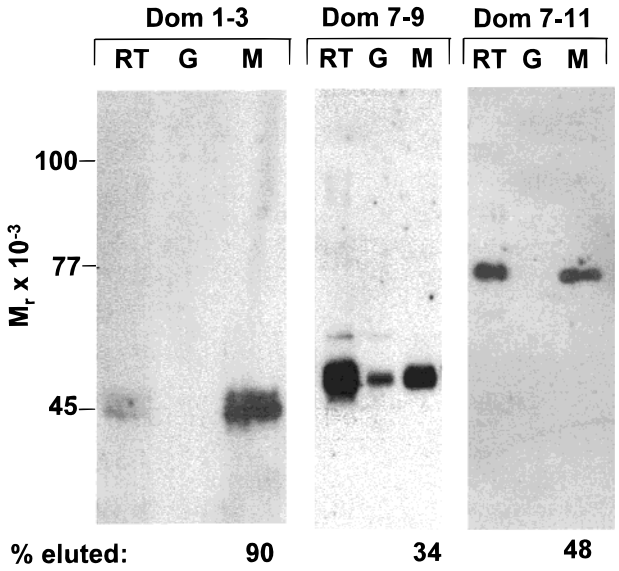

FIGURE 8: *D. discoideum* lysosomal enzyme affinity chromatography. Purified Dom 1−3, Dom 7−9, and Dom 7−11 recombinant proteins were passed over *D. discoideum* lysosomal enzyme affinity columns. Columns were washed and eluted sequentially with 5 mM Glc-6-P (G) and 5 mM Man-6-P (M). The run-through (RT) fractions and eluates were precipitated with trichloroacetic acid and analyzed on a 10% SDS−polyacrylamide gel followed by Western blot analysis.

∼34−48% of the Dom 7−9 or Dom 7−11 construct was retained on the column (Figure 8). Similar results were observed when the recombinant receptors were expressed in the mammalian cell line, COS-1 (data not shown). These results indicate that the two CRDs of the IGF-II/MPR differ significantly in their ability to bind lysosomal enzymes derived from *D. discoideum*.

Binding Affinity of Man-6-S and Man-P-OCH$_3$. To determine whether the difference observed between the ability of the two CRDs to recognize *D. discoideum* lysosomal enzymes is due to differences in the recognition of Man-6-S and/or Man-P-OCH$_3$, the affinity of the amino-terminal and carboxyl-terminal CRD for Man-6-S and Man-P-OCH$_3$ was directly measured. The results of the inhibition studies demonstrate that the amino-terminal CRD exhibits a 14−55-fold higher affinity than the carboxyl-terminal CRD for Man-6-S and Man-P-OCH$_3$ (Figure 9).

DISCUSSION

The IGF-II/MPR functions in the specific recognition of phosphomannosyl residues which have been found on a number of diverse proteins, including soluble lysosomal enzymes (*35*), the growth factors proliferin (*40*) and transforming growth factor-β precursor (*41*), and the cytokine leukemia inhibitory factor (*42*). To understand how this receptor is able to bind these proteins with nanomolar affinity, a knowledge of the molecular interactions between the receptor and carbohydrate is needed. Although several studies using synthetic oligosaccharides and neoglycoproteins have been performed to probe the carbohydrate specificity of the intact IGF-II/MPR (*9*, *38*, *43*), these studies have not examined whether the two CRDs of the receptor are identical in their binding properties. To address this question, regions of the IGF-II/MPR encompassing each of the two CRDs were expressed in baculovirus-infected insect cells and the binding of the recombinant proteins to various ligands was analyzed. In our initial studies, the recombinant proteins were subjected to sucrose gradient centrifugation, gel filtration, and cross-linking which established that the constructs encoding the amino-terminal or carboxyl-terminal CRD exist as monomers (Figures 2 and 3). These results eliminated the possibility that the two CRDs differ in their oligomeric state, a property that could influence ligand binding affinities.

Numerous studies have demonstrated that the MPRs do not bind ligand at acidic pH, which is consistent with their function of releasing ligands in the acidic (pH $\leq$6.0) environment of the late endosomal compartment (*4*, *9*, *15*, *44*). The importance of this process for the correct targeting of lysosomal enzymes is demonstrated by the treatment of cells with the lysosomotropic amines, chloroquine and NH_4-Cl, which raise the intraorganelle pH and impair receptor−enzyme dissociation. The inability to dissociate ligand results in constantly occupied receptors and thus stimulates the secretion of newly synthesized acid hydrolases 2−8-fold (*45*, *46*). To examine acid-dependent dissociation, pentamannosyl phosphate−agarose affinity chromatography was performed which demonstrated that, unlike the carboxyl-terminal CRD, the majority of the amino-terminal CRD eluted from the column under acidic conditions (Figure 4B). These results were confirmed by measuring the pH profile of β-glucuronidase binding; the carboxyl-terminal CRD exhibited significant binding activity below pH 5.5, whereas the amino-terminal CRD did not (Figure 5). Given that only 34% of the sequence of domain 3 and domain 9 of the IGF-II/MPR is identical (*8*), additional studies will be required to identify the structural features of the two CRDs which contribute to their pH dependence of ligand binding and release.

To quantitatively compare the CRDs, inhibition experiments and equilibrium binding assays were performed with Man-6-P and the lysosomal enzyme, β-glucuronidase, respectively. Inhibition curves demonstrated that the amino-terminal and carboxyl-terminal CRD have a similar affinity for Man-6-P (6−49 μM) (Figures 6 and 9), which is very comparable to the affinity exhibited by the full-length IGF-II/MPR (5−10 μM) (*9*). Furthermore, the K_d values of β-glucuronidase binding obtained for Dom 1−3 (1.0 $\pm$ 0.3 nM), Dom 7−9 (2.5 $\pm$ 0.9 nM), and Dom 7−11 (0.6 nM) (Figure 7) compare well with the affinity of the full-length IGF-II/MPR for β-glucuronidase (K_d = 2 nM) (*35*). These results indicate that the two CRDs of the IGF-II/MPR are functionally equivalent in their ability to recognize Man-6-P in the context of an oligosaccharide chain. In addition, the observation that replacement of the conserved arginine residue in domain 3 or domain 9 causes a similar decrease (>1000-fold) in affinity (Figure 7) suggests that residues essential for Man-6-P recognition are shared between the two CRDs.

In mammalian cells, the acquisition of Man-6-P on newly synthesized lysosomal enzymes occurs via the concerted action of two enzymes. The phosphotransferase creates a phosphodiester, Man-P-OGlcNAc, by adding GlcNAc-1-P to the hydroxyl group at the sixth position of selected mannose residues. The phosphodiesterase then acts to remove the GlcNAc residue, leaving a phosphomonoester, Man-6-P. Isolation and characterization of lysosomal enzymes demonstrated the presence of both phosphomonoesters and phosphodiesters (*47*), indicating that in vivo the phosphodi-

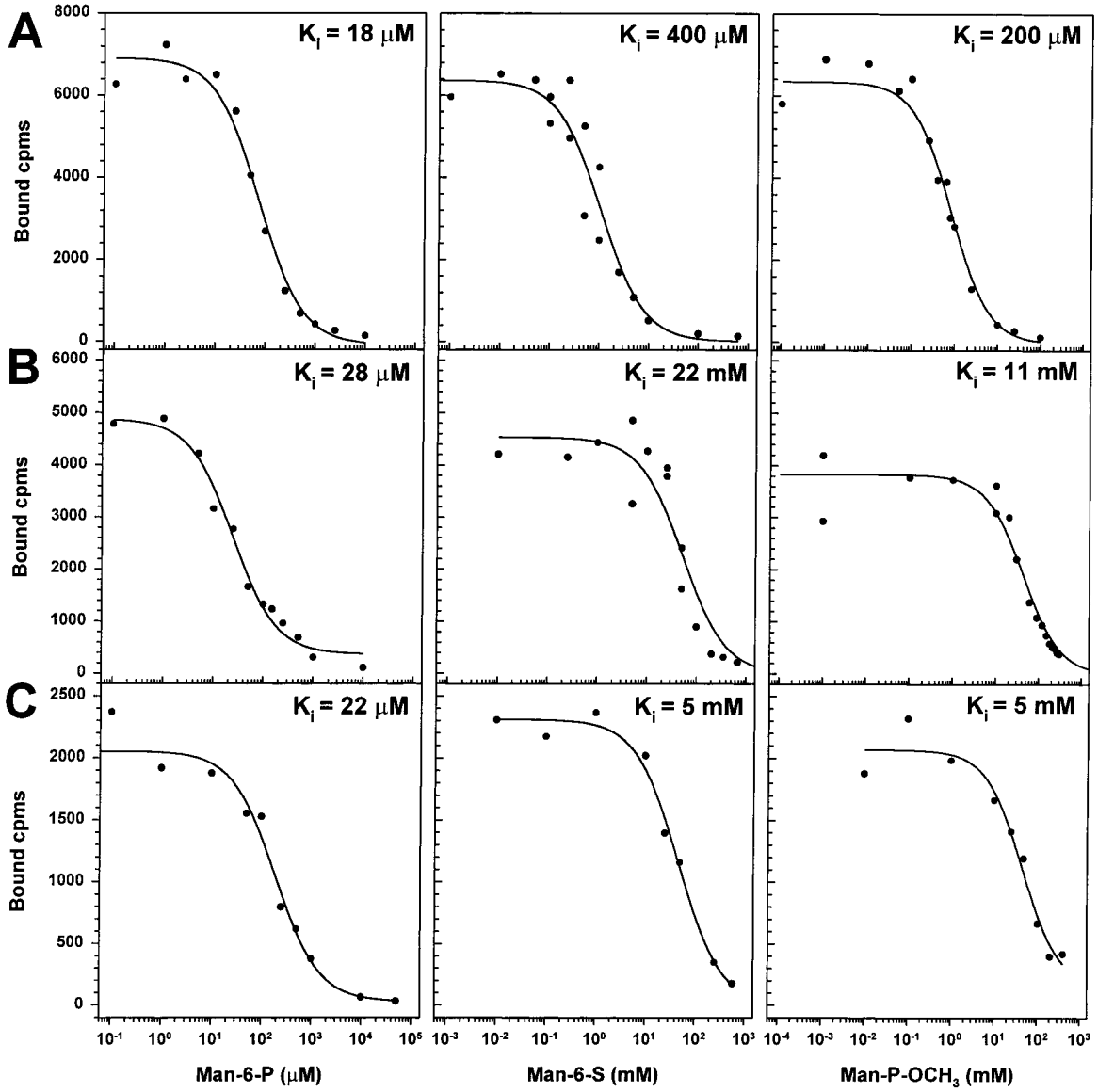

FIGURE 9: Inhibition of β-glucuronidase binding to truncated IGF-II/MPRs by Man-6-S and Man-P-OCH_3. Purified Dom 1−3His (A), Dom 7−9His (B), or Dom 7−11 (C) was incubated with a concentration of iodinated β-glucuronidase comparable to the K_d determined in Figure 7 in the presence of increasing concentrations of Man-6-P, Man-6-S, or Man-P-OCH_3. Receptor−enzyme complexes were immunoprecipitated with IGF-II/MPR-specific antisera prebound to protein A−Sepharose. [^{125}I]-β-Glucuronidase specifically bound to truncated IGF-II/MPRs was eluted by incubation for 1 h with 10 mM Man-6-P. The results were analyzed with SigmaPlot 4.0.

esterase reaction does not go to completion. Thus, the Man-6-P recognition marker on lysosomal enzymes is very heterogeneous due to variability in the presence of phosphodiesters and phosphomonoesters, in addition to differences in the extent of phosphorylation of the oligosaccharide chains and the position of the Man-6-P moiety and its linkage to the penultimate mannose residue. The existence of two CRDs in the IGF-II/MPR that differ in their ligand binding properties may be advantageous for recognizing these heterogeneous ligands. Consistent with this hypothesis are our previous studies which demonstrated that mutant IGF-II/MPRs with only a functional amino-terminal CRD targeted individual acid hydrolases to the lysosome with a different efficiency than receptors containing only a functional carboxyl-terminal CRD (*12*).

The IGF-II/MPR, but not the CD-MPR, is able to bind the phosphodiester Man-P-OGlcNAc (*4*, *9*, *38*). Furthermore, it has been shown that the IGF-II/MPR, unlike the CD-MPR, binds *D. discoideum* lysosomal enzymes, which contain the smaller phosphodiester, Man-P-OCH_3 (*21*), as well as Man-6-S (*22*, *39*), as efficiently as mammalian lysosomal enzymes (*36*, *37*). In this study, we have analyzed the ability of the two CRDs of the IGF-II/MPR to recognize the phosphodiester Man-P-OCH_3 and Man-6-S. Affinity chromatography using a matrix substituted with *D. discoideum* lysosomal enzymes indicated that the amino-terminal CRD bound *D. discoideum* lysosomal enzymes with a higher affinity than the carboxyl-terminal CRD (Figure 8). Inhibition studies demonstrated directly that both Man-P-OCH_3 and Man-6-S are recognized differently by the two CRDs. Dom 7−11 displays an ~250-fold lower affinity for both Man-P-OCH_3 and Man-6-S than Man-6-P. Similarly, Dom 7−9His was shown to exhibit ~400- and ~800-fold lower affinities for Man-P-OCH_3 and Man-6-S, respectively, than Man-6-P (Figure 9). These results, along with the observation that Dom 1−3His binds Man-P-OCH_3 and Man-6-S with only

10- and 20-fold lower affinities than Man-6-P, respectively (Figure 9), demonstrate that the carboxyl-terminal CRD is highly specific for the phosphomonoester, Man-6-P, whereas the amino-terminal CRD can accommodate a phosphodiester as well as a sulfated mannose residue. Although not demonstrated in these experiments, it is likely that the amino-terminal CRD accounts for the ability of the IGF-II/MPR to bind the larger phosphodiester, Man-P-OGlcNAc, found on mammalian lysosomal enzymes (*4*, *9*, *38*). Sulfate residues have been found on the N-linked oligosaccharides of several mammalian lysosomal enzymes, including cathepsin D, arylsulfatase A, and β-hexosamindase (*48*, *49*). However, since these studies did not determine the structure of the sulfated oligosaccharides, it is unclear what role Man-6-S plays in the IGF-II/MPR-mediated targeting of mammalian lysosomal enzymes to the lysosome. Taken together, the results presented in this report demonstrate that it is the presence of its amino-terminal CRD which allows the IGF-II/MPR to bind phosphodiesters and Man-6-S in addition to phosphomonoesters, enabling the IGF-II/MPR to recognize a greater diversity of ligands than the CD-MPR both in vivo (*13*, *14*) and in vitro (*15*, *16*).

The crystal structure of the CD-MPR illustrates the molecular basis for its inability to bind phosphodiesters; the phosphate moiety is almost completely buried within the binding pocket, leaving no room in the structure for accommodation of a phosphodiester (*6*). It is likely that the carboxyl-terminal CRD of the IGF-II/MPR, which binds poorly to Man-P-OCH_3 (Figure 9), has a similar, tight pocket surrounding the phosphate moiety of the ligand. In contrast, the structure of the amino-terminal CRD must be significantly more open to accept a phosphate that is diesterified with a methyl group (Man-P-OCH_3) or, likely, a molecule as large as *N*-acetylglucosamine (Man-P-OGlcNAc). The two CRDs of the IGF-II/MPR also differ significantly in their ability to bind Man-6-S. Although sulfate and phosphate are similar in size and structure, sulfate is fully ionized at physiological pH whereas phosphate exists predominantly as the $H_2PO_4^-$ species below pH 7.2. Thus, it is possible that the lack of a proton(s) for hydrogen bond interactions and/or charge repulsion inhibits the binding of Man-6-S by the carboxyl-terminal CRD. A structure-based sequence alignment between the CD-MPR and IGF-II/MPR predicts residues of domain 3 and domain 9 of the IGF-II/MPR that are involved in phosphomannosyl recognition (*6*). Of the six residues that have been identified, one residue differs between the amino-terminal and carboxyl-terminal CRD. However, since only 34% of the sequence of the amino- and carboxyl-terminal CRDs is identical (*8*), additional studies will be required to determine whether this residue, and/or others, is responsible for the differences observed in the binding specificity of the two CRDs.

To further analyze the binding properties of the carboxyl-terminal CRD, a construct encoding the carboxyl-terminal CRD and the IGF-II binding site (Dom 7−11) was generated. Both cross-linking and equilibrium binding assays (Figure 1) demonstrated the existence of a functional IGF-II binding site in Dom 7−11, which provided additional evidence that this protein has folded into its native state. Our results also showed that Dom 7−11 is very similar to Dom 7−9 in its pH optimum of ligand binding (Figure 5) and in its affinity for binding to the various ligands that were analyzed (Figures 6, 7, and 9). Thus, these results demonstrate that domains 10 and 11 do not influence the carbohydrate binding properties of the carboxyl-terminal CRD of the IGF-II/MPR.

In summary, the expression of functional recombinant proteins in baculovirus-infected insect cells provided a method for characterizing the two Man-6-P binding sites of the IGF-II/MPR independently. We have demonstrated that the two CRDs of the IGF-II/MPR are not functionally equivalent and exhibit differences in their ability to undergo acid-dependent dissociation and to bind phosphodiesters and Man-6-S. Taken together, these results indicate that the IGF-II/MPR has optimized its ability to recognize and target a diverse population of lysosomal enzymes by expressing two different CRDs.

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