Characterization of Truncated and Glycosylation-Deficient Forms of the Cation-Dependent Mannose 6-Phosphate Receptor Expressed in Baculovirus-Infected Insect Cells†

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ABSTRACT: A soluble truncated form of the cation-dependent mannose 6-phosphate receptor (CD-MPR) encoding only the extracytoplasmic region, Stop^{155}, and a truncated glycosylation-deficient form of the CD-MPR, Asn^{81}/Stop^{155}, which has been modified to contain only one N-linked glycosylation site at position 81 instead of five, were purified from baculovirus-infected High Five insect cells. The glycosylated recombinant proteins were functional in ligand binding and acid-dependent dissociation as assessed by pentamannosyl phosphate-agarose affinity chromatography. Gel filtration, sucrose gradients, and cross-linking experiments revealed that both Stop^{155} and Asn^{81}/Stop^{155} are dimeric, demonstrating that the transmembrane and cytoplasmic region of the receptor as well as N-linked oligosaccharides at positions 31, 57, and 87 are not required for dimerization. The K_d of Stop^{155} and Asn^{81}/Stop^{155} for the lysosomal enzyme, β-glucuronidase, was 0.2 and 0.3 nM, respectively. These values are very similar to those reported for the full-length CD-MPR, demonstrating that the extracellular region of the CD-MPR is sufficient for high-affinity binding and that oligosaccharides at positions 31, 57, and 87 do not influence ligand binding.

The cation-dependent mannose 6-phosphate receptor (CD-MPR)[1] functions in the delivery of newly synthesized lysosomal enzymes to lysosomes. Soluble acid hydrolases are specifically modified with phosphomannosyl residues in the Golgi where they are recognized by MPRs and targeted to the lysosome via an acidic endosomal compartment (*1*−*3*). The CD-MPR is an essential component of this mannose 6-phosphate (Man-6-P)-dependent targeting system as demonstrated by immunodepletion and gene disruption studies. Cultured cells depleted of functional CD-MPRs by either the addition of anti-CD-MPR antibodies (*4*) or gene disruption methods (*5*) mis-sort multiple lysosomal enzymes and accumulate undigested substrates in the lysosome while transgenic mice lacking the CD-MPR exhibit elevated levels of Man-6-P-containing glycoproteins in their serum and urine (*5*, *6*).

The CD-MPR, a membrane glycoprotein with a molecular weight of 46 000, exists predominantly as a dimer (*7*, *8*) and, at least for bovine and murine species, exhibits optimal ligand binding in the presence of divalent cations (*8*−*11*). The bovine CD-MPR consists of four structural domains: a 28-residue N-terminal signal sequence, a 159-residue extracytoplasmic region, a single 25-residue transmembrane region, and a 67-residue C-terminal cytoplasmic domain (*12*). Several studies have been conducted to characterize the structural determinants of the CD-MPR that are necessary for binding phosphomannosyl residues. Expression of a construct encoding the signal sequence and extracytoplasmic domain has been shown to produce a protein capable of binding Man-6-P (*7*, *13*). Site-directed mutagenesis studies have indicated Arg^{111} and His^{105} as essential residues for ligand binding (*14*) while the crystal structure of the extracytoplasmic domain has revealed additional residues that are within hydrogen-bonding distance of the bound Man-6-P (*15*). Since the CD-MPR is a heavily glycosylated protein in which carbohydrates constitute ∼20% of the total mass of the receptor, the role of N-linked oligosaccharides in the functioning of the receptor has been studied. The extracytoplasmic domain of the CD-MPR contains five potential N-linked glycosylation sites (Asn-X-Ser/Thr) at positions 31, 57, 68, 81, and 87 (*12*). Analysis of glycosylation-deficient mutants generated by site-directed mutagenesis revealed that these proteins were capable of binding Man-6-P, although with varying capacities (*16*, *17*), suggesting that oligosaccharides are not directly involved in ligand binding. However, studies by Li and Jourdian (*18*) indicate that the CD-MPR bearing sialic acid and polylactosamine residues binds Man-6-P with lower affinity than CD-MPRs which lacked sialic acid and polylactosamine units on their oligosaccharides, suggesting that oligosaccharides influence ligand binding. Assessment of the results concerning the factors required for carbohydrate recognition by the CD-MPR has been difficult due to the lack of quantitative binding studies: all of the above studies have evaluated ligand binding qualita-

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[1] Abbreviations: Man-6-P, mannose 6-phosphate; CD-MPR, cation-dependent mannose 6-phosphate receptor; SDS, sodium dodecyl sulfate; PNGaseF, peptide: *N*-glycosidase F; DSS, disuccinimidyl suberate.

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tively by solid-phase affinity chromatography on columns containing Man-6-P.

In the current study, truncated and glycosylation-deficient CD-MPR constructs were expressed in baculovirus-infected insect cells. The recombinant proteins were purified and characterized with respect to the extent of glycosylation, oligomeric state, binding affinity, and dissociation properties. Our results demonstrate that the extracytoplasmic domain contains all of the information required for formation of dimers and high-affinity binding and that oligosaccharides at positions 31, 57, and 87 are not required for oligomerization and do not contribute to the binding affinity of the CD-MPR for β-glucuronidase.

MATERIALS AND METHODS

Reagents. 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.), peptide: *N*-glycosidase F (PNGaseF, Boehringer Mannheim), Express Five serum-free insect medium (Gibco BRL), Graces insect medium (Gibco BRL), *Autographa californica* nuclear polyhedris virus (AcNPV) transfer vector pVL1393 (PharMingen), modified AcNPV baculovirus DNA (BaculoGold DNA, Pharmingen), and bovine serum albumin, carbonic anhydrase, cytochrome C, and aprotinin used for gel filtration and sedimentation (Sigma). Phosphomannan from *Hansenula holstii* was a kind gift of Dr. 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 Dr. William Sly (St. Louis University School of Medicine, St. Louis, MO).

Expression of Recombinant CD-MPRs. A truncated form of the bovine CD-MPR, Stop^{155}, which encodes the signal sequence and extracytoplasmic region, was constructed as described previously by changing the proline (residue 155) codon in the wild-type bovine CD-MPR sequence (CCA, nucleotide 584−586) to a stop codon (TGA) (*7*). The glycosylation-deficient Stop^{155} mutant, Asn^{81}/Stop^{155}, was generated as described previously by replacing Asn at positions 31, 57, 68, and 87 with Gln, thus eliminating four of the five potential N-linked glycosylation sites (*17*). The baculovirus transfer vector pVL1393 (PharMingen) encoding either the Stop^{155} or the Asn^{81}/Stop^{155} constructs (2 μg) and the BaculoGold DNA (0.5 μg, PharMingen) 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 serum-free medium and maintained at 27 °C. Cells in monolayer cultures were infected with the recombinant virus, and the medium was harvested 4−5 days postinfection.

Pentamannosyl Phosphate-Agarose Affinity Chromatography. The recombinant proteins were purified from the medium by pentamannosyl phosphate−agarose affinity chromatography as described previously (*21*). The columns were eluted with glucose 6-phosphate followed by Man-6-P. In some experiments, the columns were eluted with a low pH buffer containing 50 mM 2-[*N*-Morpholino] ethane-sulfonic 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 column fractions were precipitated with trichloroacetic acid and analyzed on 12% SDS−polyacrylamide gels followed by western analysis (see below).

Western Blot Analysis. Western blot and dot blot analyses were performed as described previously (*22*) except that following the incubation in bovine CD-MPR-specific antiserum, the membranes were rinsed and then 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 protein A/horseradish peroxidase conjugate (Amersham, 1:2000). The receptor was detected by enhanced chemiluminescence as described by the manufacturer (Pierce Chemical Corp.).

N-Terminal Sequencing. Stop^{155} and Asn^{81}/Stop^{155} constructs 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 acid sequence was compared to that of the mature CD-MPR isolated from mammalian cells (*23*).

PNGaseF Digestion. Purified Stop^{155} and Asn^{81}/Stop^{155} were incubated with PNGaseF (200 milliunits) in buffer containing 0.2 M $NaPO_4$ (pH 8.6), 0.1% SDS, 0.6% NP-40, and 10 mM β-mercaptoethanol for the indicated times. The samples were analyzed on a 12% SDS−polyacrylamide gel followed by Western blot analysis.

Gel Filtration. Purified Stop^{155} and Asn^{81}/Stop^{155} were passed over a Sephadex G-75 column (0.7 cm × 50 cm) equilibrated in 50 mM Imidazole, 150 mM NaCl, 5 mM β-glycerophosphate, and 10 mM $MnCl_2$ (pH 6.5) that 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). Column fractions (220 μL) were collected and assayed for the presence of truncated CD-MPRs by a dot blot assay using CD-MPR-specific antisera and protein A/horseradish peroxidase (Amersham). The proteins were visualized by enhanced chemiluminescence, and the results were quantified using an Ambis optical imaging system.

Cross-Linking. Stop^{155} and Asn^{81}/Stop^{155} proteins purified from baculovirus-infected insect cells were dialyzed against 0.1 M Hepes (pH 8.0), 0.1 M NaCl and then incubated with the homobifunctional cross-linker, disuccinimidyl suberate (DSS), for 1 h. The cross-linking reaction was stopped by the addition of glycine to 10 mM followed by a 10 min incubation (*22*). The samples were precipitated with 10% trichloroacetic acid and analyzed on a SDS−polyacrylamide gel followed by Western blot analysis.

Sucrose Gradients. Purified Stop^{155} and Asn^{81}/Stop^{155} were run on 4 mL 5−20% continuous sucrose gradients that were equilibrated at 4 °C. The gradients were centrifuged at 250000*g* for 18 h. Fractions (150 μL) were collected from the bottom of the gradient and were assayed for the presence of truncated CD-MPRs by Western blot analysis. The results were quantified using an Ambis optical imaging system. Bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) were run on a separate

5−20% sucrose gradient. Fractions were collected and analyzed on a 14% 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. Human β-glucuronidase was collected from serum-free conditioned medium from the cell line L-MPR 13−2−1 MTX 3.2, which overexpress and secrete human β-glucuronidase (*24*). β-Glucuronidase was purified by affinity chromatography on a cation-independent (CI)-MPR Affigel-10 column and iodinated using soluble lactoperoxidase (*25*). Following gel filtration on Sephadex G-150, [^{125}I] β-glucuronidase was further purified on a CI-MPR affinity column. The purified β-glucuronidase (1−2 μCi/μg) was dialyzed exhaustively against 50 mM Imidazole, 150 mM NaCl, and 5 mM β-glycerophosphate (pH 6.5) to remove the Man-6-P.

The binding assays were performed as described previously (*26*). Briefly, increasing concentrations of iodinated β-glucuronidase were incubated with purified Stop^{155} and Asn^{81}/Stop^{155}, and the MPR and bound ligand were immunoprecipitated with an anti-CD-MPR polyclonal antibody which was prebound to protein A-Sepharose. After a total incubation of ≥4 h, the beads were pelleted and washed. The supernatant and washes were collected, and the radioactivity was counted as a direct measure of unbound ligand in each reaction. 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 cpms added. Control experiments were performed which demonstrated that β-glucuronidase did not interfere with the immunoprecipitation of the CD-MPR, and that sufficient antibody and protein A were present to immunoprecipitate all of the CD-MPR.

RESULTS

Expression and Purification of Truncated CD-MPRs. The aim of this study was to characterize mutant CD-MPRs in order to determine if the extracytoplasmic domain is sufficient for high-affinity binding and dimerization. In addition, the contribution of N-linked oligosaccharides to ligand-binding affinity and oligomerization was evaluated. Stop^{155} is a construct which contains the N-terminal signal sequence and the extracytoplasmic domain, while Asn^{81}/Stop^{155} encodes the extracytoplasmic region which has been further modified to contain only a single N-glycosylation site (Figure 1). Asn^{81}/Stop^{155} was chosen for subsequent analyses due to its ability to efficiently traverse the secretory pathway and bind to a Man-6-P-containing affinity column when expressed in mammalian cells (*17*). To obtain sufficient quantities of protein to carry out the above studies, we needed to use an expression system that would allow purification of functional recombinant MPRs. Previous work in our laboratory identified that the insect cells Sf9 and High Five do not contain endogenous MPRs, and cells infected with recombinant baculovirus encoding the full-length CD-MPR were shown to produce functional receptor as assessed by affinity chromatography (*20*). Stop^{155} or Asn^{81}/Stop^{155} recombinant virus was used to infect Sf9 and High Five insect

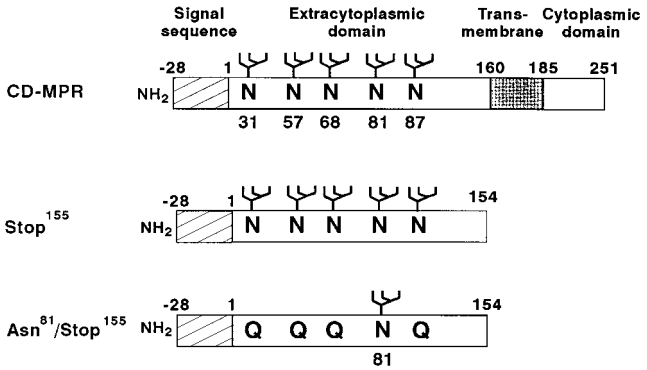

FIGURE 1: Schematic presentation of mutant forms of the CD-MPR. The positions of the five potential N-linked glycosylation sites are indicated by the asparagine residues (N). Stop^{155} was generated by replacing the proline (residue 155) codon in the bovine CD-MPR sequence (CCA, nucleotides 584−586) with a stop codon (TGA), thereby generating a protein lacking the transmembrane and cytoplasmic domains (*7*). Asn^{81}/Stop^{155} also contains this stop codon. However, in addition, four out of the five potential N-linked glycosylation sites were removed by replacing the asparagine residues at positions 31, 57, 68, and 87 with glutamine (Q) (*17*). The predicted molecular weights of the mature truncated polypeptides without oligosaccharides are 17 365 and 17 421 for Stop^{155} and Asn^{81}/Stop^{155}, respectively.

cells, and the cells and medium were harvested 4−5 days post-infection. Western blot analysis with CD-MPR-specific antisera revealed that High Five cells secreted a higher percentage and a greater amount (2−3-fold) of Stop^{155} and Asn^{81}/Stop^{155} than Sf9 cells (data not shown). Pentamannosyl phosphate-agarose affinity chromatography was used to purify the recombinant proteins from the medium of baculovirus-infected High Five insect cells. To confirm that the signal sequence was removed, purified Stop^{155} and Asn^{81}/Stop^{155} were subjected to N-terminal sequencing. The amino acid sequence analysis demonstrated that the 28-residue signal sequence was processed, as the N terminus (TEEKTXDLVGEK) of Stop^{155} and Asn^{81}/Stop^{155} was identical to that of the mature CD-MPR isolated from mammalian cells (*23*).

Pentamannosyl Phosphate-Agarose Affinity Chromatography and Acid-Dependent Dissociation. To determine whether Stop^{155} and Asn^{81}/Stop^{155} were functional in ligand binding and dissociation, we passed recombinant proteins purified from the medium of baculovirus-infected insect cells over a pentamannosyl phosphate-agarose affinity column. Consistent with expression in mammalian cells (*17*), the Stop^{155} and Asn^{81}/Stop^{155} expressed in High Five insect cells bound to the column and were specifically eluted with Man-6-P (Figure 2A). The targeting of lysosomal enzymes involves both the binding of ligand in the Golgi and the dissociation in acidic prelysosomal compartments (*1, 27*). To determine whether recombinant CD-MPRs expressed in insect cells exhibit ligand dissociation at an acidic pH, we passed purified proteins over an affinity column which was eluted sequentially with glucose 6-phosphate, an acidic buffer (pH 4.6), and Man-6-P. Figure 2B shows that >95% of the truncated CD-MPRs elute from the column with low pH. These results demonstrate that the recombinant proteins expressed in insect cells are functional in ligand binding and acid-dependent ligand dissociation.

Enzymatic Deglycosylation of Stop^{155} and Asn^{81}/Stop^{155}. Recombinant Stop^{155} was purified from High Five insect cells

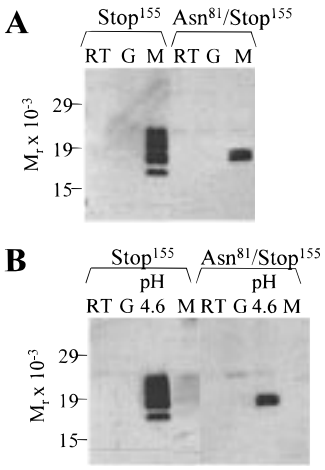

FIGURE 2: Pentamannosyl phosphate-agarose affinity chromatography. (A) Stop^{155} and Asn^{81}/Stop^{155} purified from the media of baculovirus-infected insect cells were passed over pentamannosyl phosphate-agarose affinity columns. The columns were washed and eluted with glucose 6-phosphate (G) and then with Man-6-P (M). (B) Purified recombinant proteins were also passed over pentamannosyl phosphate-agarose affinity columns that were washed and eluted with glucose 6-phosphate (G), acidic buffer (pH 4.6), followed by Man-6-P (M). The runthrough (RT) fractions and eluates were precipitated with 10% trichloroacetic acid. The samples were analyzed on a 12% SDS−polyacrylamide gel followed by Western blot analysis.

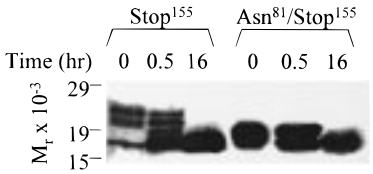

FIGURE 3: Enzymatic deglycosylation of Stop^{155} and Asn^{81}/Stop^{155}. The Stop^{155} and Asn^{81}/Stop^{155} recombinant proteins were purified by affinity chromatography. The purified proteins were incubated with PNGaseF for the indicated times and analyzed on a 12% SDS−polyacrylamide gel followed by Western blot analysis.

as five species with apparent molecular weights of 16.6, 20.9, 23.7, 25.1, and 28.2 kDa (see Figure 2). Previous studies have shown that the bovine CD-MPR contains five potential N-linked glycosylation sites, four of which are utilized in mammalian cells (*12*). To determine whether the multiple forms of Stop^{155} were due to differences in N-glycosylation, we incubated purified recombinant Stop^{155} with PNGaseF for 30 min or 16 h. Enzymatic deglycosylation of Stop^{155} produced in High Five cells showed that after 16 h the number of species decreased to one predominant band with a molecular weight of 16.6 kDa, consistent with that of the predicted size (17.4 kDa) for the nonglycosylated truncated CD-MPR (Figure 3). These results demonstrate that the multiple forms of the Stop^{155} construct are due to varying numbers of N-linked oligosaccharides and that Stop^{155} produced in insect cells contains predominantly 2 or 3 N-linked oligosaccharide chains. The recombinant Asn^{81}/Stop^{155} purified from High Five insect cells contained two species with molecular weights of 17.1 and 21.0 kDa (see Figure 2). This construct has been modified to contain only one N-linked oligosaccharide. To confirm that the two bands represent the nonglycosylated and glycosylated forms, we incubated purified Asn^{81}/Stop^{155} with PNGaseF. Upon incubation with PNGaseF a single band was generated which comigrates with the deglycosylated Stop^{155} (Figure 3). These

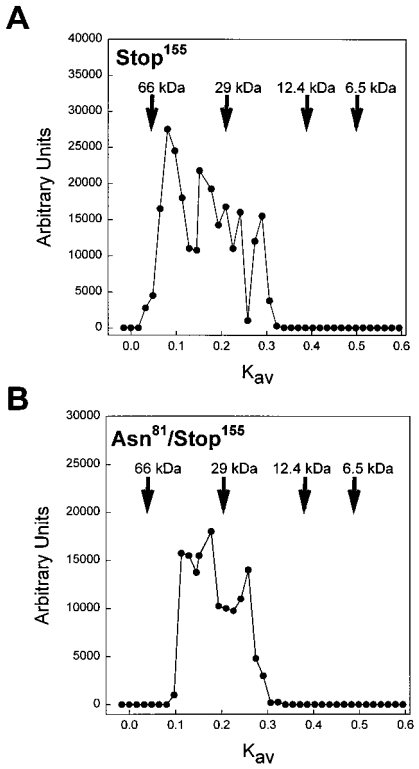

FIGURE 4: Gel filtration of Stop^{155} and Asn^{81}/Stop^{155}. The purified recombinant proteins Stop^{155} (A) and Asn^{81}/Stop^{155} (B) were passed over a Sephadex G-75 column (0.7 cm × 50 cm) which was calibrated with the following proteins: bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa). Column fractions (220 μL) were collected and analyzed for the presence of truncated CD-MPR by a dot blot assay using CD-MPR-specific antisera. The proteins were visualized by enhanced chemiluminescence, and the results were quantified using densitometry.

results confirm that Asn^{81}/Stop^{155} expressed in insect cells contains a single N-linked oligosaccharide.

Oligomeric Structure of Stop^{155} and Asn^{81}/Stop^{155}. Several studies have shown that the native CD-MPR exists predominantly as a dimer in membranes (*7, 8, 23*). Recently, our lab has solved the structure of the extracytoplasmic domain of the CD-MPR (Asn^{81}/Stop^{155}), which revealed a dimer in the crystal structure (*15*). To confirm this result in solution, we passed the purified receptors, Stop^{155} and Asn^{81}/Stop^{155}, over a Sephadex G-75 column. The majority (∼80%) of Stop ^{155} elutes from the column as a broad peak (Kav = 0.065−0.23), with a molecular weight of 32−56 kDa which suggests that this recombinant protein exists as a dimer. The small peaks observed at 19 and 23 kDa likely represent glycosylated and nonglycosylated monomers (Figure 4A). The nonsymmetrical appearance of the major broad peak for Stop^{155} is consistent with this protein containing varying levels of N-glycosylation (see Figure 3). The majority (75%) of the Asn^{81}/Stop^{155} construct elutes from the gel filtration column at a molecular weight of 36−45 kDa, suggesting that this recombinant protein is also dimeric (Figure 4B). As another method to determine the oligomeric state of the recombinant proteins, the purified proteins were incubated in the presence or absence of the homobifunctional cross-linking agent, DSS. Incubation of Stop^{155} or Asn^{81}/Stop^{155} with DSS resulted in >95% of the proteins migrating as a species with an apparent molecular weight of 45 kDa or 32 kDa, respectively (Figure 5). Purified Stop^{155} and Asn^{81}/

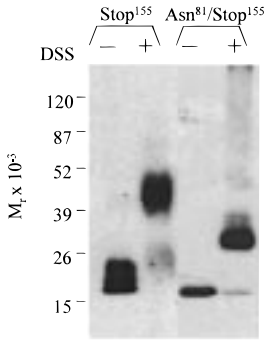

FIGURE 5: Cross-linking of purified Stop^{155} and Asn^{81}/Stop^{155}. Stop ^{155} and Asn ^{81}/Stop ^{155} were purified on pentamannosyl phosphate-agarose columns and incubated in the absence (−) or presence (+) of 0.2 mM DSS for 1 h at 25 °C. The samples were precipated with trichloroacetic acid and analyzed on a 12% SDS-polyacrylamide gel followed by Western blot analysis.

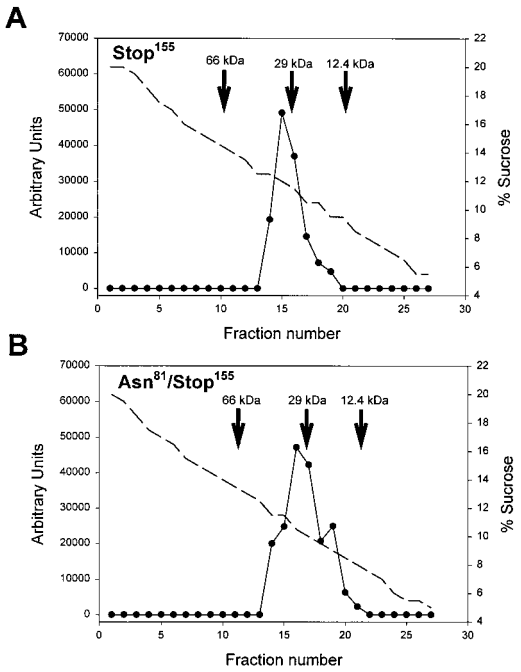

FIGURE 6: Sucrose gradient sedimentation of Stop^{155} and Asn^{81}/Stop^{155}. Purified Stop^{155} (A) and Asn^{81}/Stop^{155} (B) were run on 5% (fraction 27) to 20% (fraction 1) continuous sucrose gradients. Following sedimentation, fractions (150 μL) were precipitated with trichloroacetic acid and 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: bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa).

Stop^{155} were also run on 5−20% continuous sucrose gradients. Figure 6 shows that Stop^{155} and Asn^{81}/Stop^{155} sediment mainly in fractions 14−18, similar to the standard carbonic anhydrase (29 kDa). The molecular weights determined by sedimentation for Stop^{155} and Asn^{81}/Stop^{155} were 40 and 34 kDa, respectively. Taken together, these results demonstrate that the oligomeric state of the truncated CD-MPRs is dimeric.

Binding Affinity. To determine if Stop^{155} and Asn^{81}/Stop^{155} are similar to the wild-type CD-MPR in their ability to bind phosphomannosyl residues, we determined directly the affinity of Stop^{155} and Asn^{81}/Stop^{155} for an endogenous ligand. The ligand chosen was the lysosomal enzyme

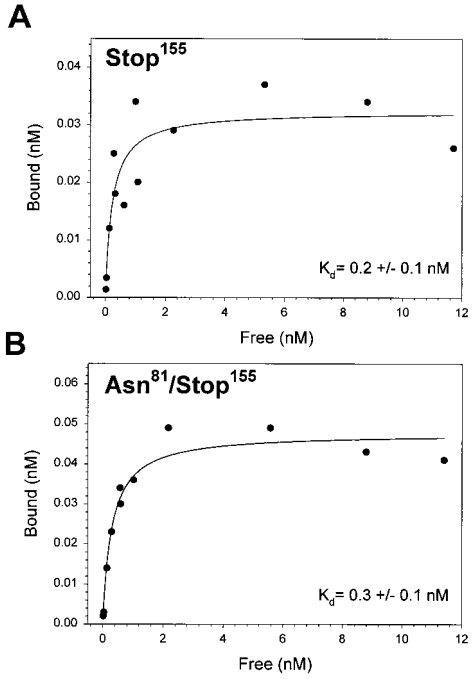

FIGURE 7: Binding of β-glucuronidase to Stop^{155} and Asn^{81}/Stop^{155}. Increasing concentrations of iodinated human β-glucuronidase were incubated with Stop^{155} (A) and Asn^{81}/Stop^{155} (B) for 2 h at 4 °C. Receptor−enzyme complexes were immunoprecipitated with CD-MPR-specific antisera prebound to protein A-Sepharose. [^{125}I] β-Glucuronidase specifically bound to the truncated CD-MPRs was eluted by incubation for 1 h with 10 mM Man-6-P. The results were analyzed using nonlinear regression (SigmaPlot, version 4.0).

β-glucuronidase, which has been well characterized and whose crystal structure is known (*28*). The K_d for the full-length CD-MPR has been reported as 0.28 nM (*29*) and 4−5 nM (*30*). Purified Stop^{155} and Asn^{81}/Stop^{155} were incubated with increasing concentrations of iodinated human recombinant β-glucuronidase. Analysis of the binding curves by nonlinear regression demonstrated K_d values of 0.2 ± 0.1 and 0.3 ± 0.1 nM for Stop^{155} and Asn^{81}/Stop^{155}, respectively (Figure 7). These results indicate that the mutant receptors are similar to the full-length wild-type receptor in their affinity for β-glucuronidase and that the four amino acid substitutions resulting in a decreased level of glycosylation have no effect on ligand binding.

DISCUSSION

The CD-MPR is a member of the P-type family of lectins that mediate the targeting of soluble acid hydrolases to the lysosome. To better understand at the molecular level how this receptor functions in binding lysosomal enzymes, we expressed recombinant forms of the CD-MPR in High Five insect cells. The baculovirus expression system was chosen as a means to provide sufficient amounts of highly purified recombinant CD-MPR for biochemical studies since previous studies have demonstrated that insect cells do not contain any endogenous MPRs, and when transfected with a recombinant baculovirus encoding the full-length CD-MPR, functional protein was produced (*20*). The aim of this study was to determine if the extracytoplasmic domain of the CD-MPR was sufficient for high-affinity binding and oligomerization and if N-linked oligosaccharides influenced ligand binding and/or dimerization.

The Stop^{155} construct encoding the N-terminal signal sequence and extracytoplasmic domain of the CD-MPR was purified from baculovirus-infected insect cells. This recombinant protein was similar to the full-length CD-MPR and Stop^{155} expressed in mammalian cells: the N-terminus of the mature protein was identical to that reported for the CD-MPR purified from human liver (*23*), and the recombinant protein was glycosylated and functional in ligand binding and acid-dependent dissociation (*17*). Stop^{155} expressed in insect cells was found to bind the lysosomal enzyme β-glucuronidase with an affinity ($K_d = 0.2$ nM) very similar to the values reported for the full-length receptor ($K_d = 0.28$ nM, (*29*); $K_d = 4-5$ nM, (*30*)). These results confirm that the extracytoplasmic domain alone of the CD-MPR is sufficient for high-affinity binding.

Studies have indicated that the CD-MPR forms oligomeric species including dimers, trimers, and tetramers (*9, 11, 23, 31, 32*). In membranes, the predominant form of the CD-MPR is a dimer (*7, 8, 33*). In contrast, when the extracytoplasmic region of the bovine CD-MPR was expressed in *Xenopus laevis* oocytes, the recombinant protein was monomeric (*7*), suggesting that the transmembrane region and/or cytoplasmic tail was important for oligomerization. However, studies by Wendland et al. (*13*) demonstrated that a truncated version of the human CD-MPR, containing the N-terminal signal sequence and extracytoplasmic domain, was dimeric when expressed in mammalian cells. Our current studies have also shown that the transmembrane region and cytoplasmic tail are not required for oligomerization. Stop^{155} purified from baculovirus-infected High Five cells was dimeric as shown by gel filtration (Figure 4), cross-linking studies (Figure 5), and sucrose gradients (Figure 6). It is possible that the discrepancy of the observed oligomeric state of the extracytoplasmic region of the CD-MPR expressed in *X. laevis* oocytes versus mammalian or baculovirus-infected insect cells may be explained by the level of receptor expressed in each system. Mammalian fibroblast cell lines typically express 2−3 pmol of CD-MPR/mg of total protein (*34*), whereas the level of CD-MPR detected in microinjected oocytes was ~1000-fold less. In comparison, baculovirus-infected insect cells expressed ~300-fold more CD-MPR than mammalian cells. Furthermore, the full-length CD-MPR or truncated CD-MPRs containing a transmembrane region from the CD-MPR or other receptors formed dimers when expressed in *X. laevis* oocytes (*7*). The inclusion of a transmembrane sequence may have aided in concentrating the proteins such that the close proximity facilitated dimer formation. However, our data clearly show that the transmembrane region is not required for dimerization (Figures 4−6). The dimeric form of Stop^{155} is consistent with the crystallographic structure of Asn^{81}/Stop^{155} (*15*) which revealed two molecules of the CD-MPR per asymmetric unit. The interface observed between the two monomers in the crystal structure covers a large surface area (~20% of one CD-MPR monomer) and is composed of primarily hydrophobic residues. Such a large hydrophobic surface is unlikely to be solvent-exposed, thus suggesting that the dimeric species of the CD-MPR most likely represents the functional form of the CD-MPR. The tight association of the two monomers was demonstrated by performing gel filtration in the presence of high salt (1 M NaCl) or 8 M urea, under which conditions the Asn^{81}/Stop^{155} homodimers did not dissociate (data not shown).

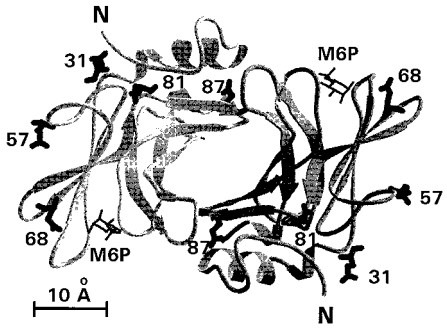

FIGURE 8: Ribbon diagram of the crystal structure of Asn^{81}/Stop^{155}. Asn^{81}/Stop^{155} crystalized as a dimer (*15*) and each monomer (light and dark gray) is shown. The five potential glycosylation sites, residues 31, 57, 68, 81, and 87, are indicated with residues 31, 57, 68, and 87 modified to Gln in this glycosylation-deficient CD-MPR structure. The N-terminus and the ligand, Man-6-P (M6P), are shown for each monomer.

The CD-MPR is a heavily glycosylated protein which contains five potential N-linked glycosylation sites at positions 31, 57, 68, 81, and 87 (*12*). The role of N-linked oligosaccharides in the function of the CD-MPR has been controversial. Early work had suggested that N-linked carbohydrates contribute to ligand binding as enzymatic deglycosylation of the mature receptor impaired binding to an affinity column (*9, 35*). However, further studies involving mutagenesis of the Asn residues involved in N-linked glycosylation showed that the N-linked carbohydrates stabilize the ligand-binding conformation (*14*). Zhang and Dahms (*17*) also generated glycosylation-deficient forms of the CD-MPR and analyzed their ability to bind pentamannosyl phosphate-agarose affinity columns. These qualitative results suggested that N-linked oligosaccharides did not affect ligand binding but facilitated the folding of the CD-MPR into a conformation conducive for ligand binding based on the observation that either the wild-type CD-MPR synthesized in the presence of tunicamycin or a mutant CD-MPR lacking all five N-glycosylation sites bound poorly to the affinity columns. Although these recent studies suggest that N-linked oligosaccharides do not influence Man-6-P binding, studies by Li and Jourdian (*18*) have provided evidence that the type of carbohydrates on the CD-MPR can affect the affinity for phosphomannosyl-containing ligands. Since all of the previous studies were based on qualitative affinity chromatography analyses, we wanted to determine the binding affinity of truncated glycosylation-deficient CD-MPRs. The affinity ($K_d = 0.3$ nM) of Asn^{81}/Stop^{155} for β-glucuronidase was very similar to that of the fully glycosylated truncated CD-MPR, Stop^{155} (Figure 7), as well as the full-length receptor. These results directly demonstrate that the four amino acid substitutions (Asn to Gln) and the loss of four potential N-linked oligosaccharides do not affect the binding of the CD-MPR to β-glucuronidase. These results are consistent with the location of the glycosylation sites with respect to the Man-6-P-binding pocket. The crystal structure shows that Asn 31, 57, 81, and 87 are greater than 20 Å away from the Man-6-P-binding site (Figure 8). A typical N-linked oligosaccharide chain is about 20 Å in length (*36*), and thus, it is unlikely that carbohydrates at Asn 31, 57, and 87 directly affect ligand binding. Although there are five

potential glycosylation sites, it has been shown that only four sites (Asn 31, Asn 57, Asn 81, and Asn 87) are utilized (*12*, *16*, *17*). The receptor may have evolved to utilize Asn68 not for glycosylation but for ligand binding. Analysis of the crystal structure of $Asn^{81}/Stop^{155}$ (Figure 8; ref *15*) reveals that the residue at position 68 is in the Man-6-P-binding pocket, and thus it is possible that a carbohydrate chain at Asn68 would interfere with ligand binding. In addition, our recent structural studies show that residue 68 of the receptor is involved in binding pentamannosyl phosphate (Olson et al., unpublished data).

Studies have shown that the CD-MPR purified from human liver did not efficiently assemble into homodimers when treated with PNGase F, suggesting that oligosaccharides may affect the oligomeric state of the CD-MPR (*35*). However, our experiments demonstrate that $Asn^{81}/Stop^{155}$, the glycosylation-deficient CD-MPR, formed dimers (Figures 4, 5, and 6). Since $Asn^{81}/Stop^{155}$ forms stable homodimers in solution, N-linked oligosaccharides at positions 31, 57, and 87 do not play a role in oligomerization. The crystal structure of $Asn^{81}/Stop^{155}$ reveals that residues involved in N-linked glycosylation (31, 57, 81, and 87) appear on one face of the CD-MPR (Figure 8) and all their side chains face away from the dimer interface. Residue 87 is closest to the dimer interface, but the side chain is positioned so that the presence of an oligosaccharide likely would not interfere with the tight hydrophobic interactions between monomers. The existence of the fully glycosylated $Stop^{155}$ and $Asn^{81}/Stop^{155}$ as dimers suggests that the presence or absence of oligosaccharides does not influence the oligomeric state of the CD-MPR.

In summary, our studies have shown that $Stop^{155}$ and $Asn^{81}/Stop^{155}$ exist as dimers in solution, demonstrating that the transmembrane and cytoplasmic region of the CD-MPR as well as oligosaccharides at positions 31, 57, or 87 do not affect the oligomeric state of the receptor. Our binding studies, which have provided a direct quantitative measurement of ligand binding, showed that the affinity of $Stop^{155}$ and $Asn^{81}/Stop^{155}$ for β-glucuronidase is very similar to the full-length CD-MPR. These data demonstrate that the 154 amino acids of the extracellular region can autonomously fold into a fully functional carbohydrate recognition domain and that oligosaccharides at position 31, 57, or 87 do not influence ligand binding.

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