Mannose 6-phosphate receptors (MPRs) deliver soluble acid hydrolases to the lysosome in higher eukaryotic cells. The two MPRs, the cation-dependent MPR (CD-MPR) and the insulin-like growth factor II/cation-independent MPR, carry out this process by binding with high affinity to mannose 6-phosphate residues found on the N-linked oligosaccharides of their ligands. To elucidate the key amino acids involved in conveying this carbohydrate specificity, site-directed mutagenesis studies were conducted on the extracytoplasmic domain of the bovine CD-MPR. Single amino acid substitutions of the residues that form the binding pocket were generated, and the mutant constructs were expressed in transiently transfected COS-1 cells. Following metabolic labeling, mutant CD-MPRs were tested for their ability to bind pentamannosyl phosphate-containing affinity columns. Of the eight amino acids mutated, four (Gln-66, Arg-111, Glu-133, and Tyr-143) were found to be essential for ligand binding. In addition, mutation of the single histidine residue, His-105, within the binding site diminished the binding of the receptor to ligand, but did not eliminate the ability of the CD-MPR to release ligand under acidic conditions.

The mannose 6-phosphate receptors (MPRs)1 function in the specific recognition of phosphorylated mannose residues, which have been identified on numerous proteins, including lysosomal enzymes (1, 2), several growth factors (3, 4), and a cytokine (5). Although an increasing number of different classes of proteins have been shown to interact with the receptors, the best characterized function of the MPRs in higher eukaryotic cells is their ability to target newly synthesized soluble acid hydrolases to the lysosome (6–8). In the Golgi, the MPRs bind acid hydrolases carrying mannose 6-phosphate (Man-6-P) residues on their N-linked oligosaccharides. The acid hydrolases are then transported from the trans Golgi network to an acidified endosomal compartment where the low pH (pH = 6.0) of this compartment causes the receptor to release its ligand. The acid hydrolases are then packaged into lysosomes, whereas the MPRs travel back to the Golgi to repeat the process or move to the cell surface where they can function in the internalization of exogenous ligands.

The MPRs constitute the P-type family of lectins of which there are only two members: the 46-kDa cation-dependent MPR (CD-MPR) and the 300-kDa insulin-like growth factor II-cation-independent MPR (IGF-II/CI-MPR). Both receptors are type I integral membrane glycoproteins. The bovine CD-MPR, which exhibits optimal Man-6-P binding in the presence of divalent cations (9, 10), is composed of a 159-residue extracytoplasmic domain, a single transmembrane region, and a 67-residue carboxyl-terminal cytoplasmic region (11). In contrast, the IGF-II/CI-MPR contains a large extracytoplasmic region composed of 15 contiguous repeating units, each approximately 147 amino acids in length. Although there are little primary sequence similarities between the transmembrane or cytoplasmic regions of the MPRs, the extracytoplasmic region of the CD-MPR shares 14–28% sequence identity, including a conserved positioning of cysteine residues with each of the 15 repeating extracytoplasmic domains of the IGF-II/CI-MPR (12). The CD-MPR binds 1 mole of Man-6-P/polypeptide chain (10). However, because this receptor exists as a dimer (13–15), the CD-MPR contains two Man-6-P binding sites in its functional state. The IGF-II/CI-MPR contains two Man-6-P binding sites/polypeptide chain, which have been localized to domains 1–3 and 7–9 (16, 17).

Although the carbohydrate binding specificities of the two MPRs have been well characterized, until recently little was known about the molecular interactions that mediate the high affinity binding of Man-6-P by either the CD-MPR or the IGF-II/CI-MPR. Previous chemical modification studies on the human CD-MPR in the presence and absence of Man-6-P suggested that arginine and histidine residues are important in ligand binding (18). Subsequent site-directed mutagenesis studies further supported these observations by indicating that His-105 and Arg-111 of the human CD-MPR are involved in Man-6-P binding (19). Our recent determination of the crystal structure of the extracytoplasmic domain of the bovine CD-MPR complexed with Man-6-P (20) or pentamannosyl phosphate (21) has confirmed the presence of His-105 and Arg-111 in the binding pocket and has identified seven additional residues that are within hydrogen bonding distance of Man-6-P.

To identify the residues that form critical contacts with Man-6-P, we have expressed recombinant proteins that contain single amino acid substitutions of the residues that comprise the carbohydrate binding pocket of the bovine CD-MPR and have analyzed the mutant receptors for their ability to bind Man-6-P by affinity chromatography. Our results indicate that essential ligand interactions are made by residues Gln-66, Arg-111, Glu-133, and Tyr-143, whereas mutations of residues Tyr-45, His-105, or Arg-135 resulted in only a partial loss of the ability of the receptor to bind phosphomannosyl-containing affinity columns. In contrast, mutation of Asp-103 to either alanine or glutamate did not significantly alter the ability of the CD-MPR to bind ligand. Furthermore, our studies demonstrate that
although substitution of His-105 does affect ligand binding, it does not eliminate the ability of the CD-MPR to release ligand at an acidic pH.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were obtained commercially as indicated: EXPRE™ 35S™ [35S]protein labeling mix (NEN Life Science Products, 1200 Ci/mmol), acrylamide (Schwarz/Mann), SDS (BDH Biochemical), prelabeled low molecular weight protein standards, Dulbecco's modified Eagle's medium and trypsin-EDTA (Life Technologies, Inc.), pcdNA3 plasmid (Invitrogen), restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, T7 DNA polymerase, uracil glycosylase, M13K07 helper phage, CJ236 and NM522 Escherichia coli strains (New England Biolabs), Thermo-sequence kit (Amersham Pharmacia Biotech), fetal bovine serum (HyClone Laboratories, Inc.), COS-1 cells (American Type Culture Collection), Man-6-P, protein A-Sepharose at 4 °C for 16–24 h as described previously (22). The immunoprecipitated material was subjected to SDS-polyacrylamide gel electrophoresis. Radioactivity was quantified using a PhosphorImager (Molecular Dynamics Storm 860) and ImageQuant (version 1.1) software.

**RESULTS**

Site-directed Mutagenesis of Residues in the Binding Pocket of the Bovine CD-MPR—We have previously shown that a truncated, glycosylation-deficient form of the bovine CD-MPR (Asn-81/STOP155), which lacks the transmembrane and cytoplasmic regions of the receptor and contains only a single N-glycosylation site, binds the lysosomal enzyme, β-glucuronidase, with an affinity identical to that of the full-length wild-type receptor (24). Our recent success in crystallizing Asn-81/STOP155 in the presence of Man-6-P (20) or pentamannosyl phosphate (21) has resulted in the three-dimensional structure of the extracytoplasmic region of the CD-MPR and has identified nine residues (Tyr-45, Gln-66, Asp-103, His-105, Arg-111, Glu-133, Arg-135, and Tyr-143) that are involved in binding Man-6-P (Figs. 1 and 2). A comparison of the amino acid sequence of the CD-MPR from bovine, human, and mouse species shows that these nine residues are conserved (Fig. 1). To evaluate the relative importance of each of these residues with respect to Man-6-P recognition, eight of the nine residues located within the Man-6-P binding pocket were generated by oligonucleotide-directed mutagenesis according to the method of Kunkel et al. (23). The mutagenic oligonucleotides (each substitution is indicated by the underlined codon) used are as follows: Y45F (5'-AC ATA GCC GAA CAT GTC TGG-3'), Q66E (5'-TTT GAT GAT ATT CAC CAG GC-3'), D103A (5'-ACA GCC AGA CAC AGT-3'), Q133E (5'-GAG TTT GCC TTC CTC CTC AG-3'), H105N (5'-CCT GCC ACA GGA GTT GTC AT-3'), H105S (5'-CCT GCC ACA GGA GTT CTC AT-3'), H111A (5'-TG TCG ATT GCA GGA CAT CAC CAC TCC CGG GCC CTC C-3'), R111K (5'-TG TCG ATT GCA GGA CAT CAC CAC TCC CGG GCC CTC C-3'), E133D (5'-GCC GTC CGT GTC GTC GCA CAG AG-3'), E133L (5'-GCC GTC CGT GTC GCA CAG AG-3'), E133Q (5'-GCC GTC CGT GTC GCA CAG AG-3'), R135E (5'-GAC TTG GCC GTC CGT GTC GCA CAG AG-3'), R135K (5'-GAC TTG GCC GTC CGT GTC GCA CAG AG-3'), and Y143F (5'-CTC AAA GAG GAA ACA ACT-3'). For each construct, the region in the plasmid that was synthesized in vitro by T7 DNA polymerase was subjected to double-stranded DNA sequence analysis to confirm the predicted sequence. All mutants were cloned into the pcDNA3 vector for subsequent isolation and transfection of COS-1 cells.

**Expression of Mutant Forms of the CD-MPR in COS-1 Cells**—COS-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and maintained in a 5% CO2 atmosphere. Transfection of COS-1 cells with plasmid pcDNA3 containing the wild-type or the various mutant forms of Asn-81/STOP155 cDNA was performed by the DEAE-dextran technique as described previously (17). Forty-eight hours after transfection, the cells were labeled in methionine- and cysteine-free Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and 50 μCi/ml EXPRE™ 35S™ [35S]protein labeling mix for 24 h. Following labeling, the medium was harvested and dialyzed exhaustively at 4 °C against 50 mM imidazole (pH 6.5), 150 mM NaCl, 10 mM MnCl2, and 5 mM β-glycerophosphate (column buffer). The cells were solubilized for 1 h at 4 °C in column buffer containing Triton X-100 (1%, v/v), sodium deoxycholate (0.1%, w/v), aprotonin (1%, w/v), antipain (4 mg/ml), benzamidine (20 mg/ml), and 2 mg/ml each of leupeptin, chymostatin, and pepstatin. To determine the percentage of each construct that was secreted into the medium, equal aliquots of the cell lysate and dialyzed medium were subjected to immunoprecipitation and gel electrophoresis as described below.

Pentamannosyl Phosphate-Agarose Affinity Chromatography—Pentamannosyl phosphate-agarose was prepared as described previously (17). To assay the constructs for ligand binding ability, dialyzed media containing the secreted mutant receptors were passed over pentamannosyl phosphate-agarose columns (0.5 × 1.0 cm) that had been equilibrated with column buffer and maintained at 4 °C. Following loading of the sample, the column was washed with column buffer and a total of five fractions (3 ml each) were collected. The column was then eluted with 3 ml of column buffer containing 10 mM glucose 6-phosphate (nonspecific ligand) followed by 3 ml of column buffer containing 10 mM Man-6-P. The flow-through fractions and the material eluted from the column were immunoprecipitated as described below. For acidic pH elution studies, the column was eluted with an acidic pH buffer (MES buffer containing 50 mM MES, 150 mM NaCl, 10 mM MnCl2, and 5 mM β-glycerophosphate prior to elution with Man-6-P.

**Role of Binding Site Residues in Man-6-P Recognition—**
COS-1 cells transfected with the various mutant CD-MPRs were metabolically labeled with [35S]methionine/[35S]cysteine 48–72 h post-transfection. The medium was harvested and subjected to pentamannosyl phosphate-agarose affinity chromatography. The results demonstrate that 73% of the wild-type Asn-81/STOP155 specifically bound to the affinity column, whereas replacement of Gln-66, Arg-111, Glu-133, or Tyr-143 resulted in little or no detectable binding to the column (Table I and Fig. 3). Although the R111A and R111K constructs were poorly secreted, similar results were obtained when these substitutions were generated in the fully glycosylated truncated CD-MPR, STOP155 (22), which resulted in their efficient secretion (80%) by transfected COS-1 cells; no detectable binding of the R111A/STOP155 and R111K/STOP155 constructs to a pentamannosyl phosphate-agarose affinity column was observed.2 In contrast to that observed at positions 66, 111, 133, and 143, replacement of Tyr-45, His-105, or Arg-135 only partially inhibited binding of the CD-MPR to the pentamannosyl phosphate affinity column, whereas substitutions at Asp-103 had no significant effect on Man-6-P binding (Table I and Fig. 3). Studies by Wendland et al. (19) reported similar results for the H105S, R111K, and R135K substitutions performed in the full-length human CD-MPR.

Role of His-105 in Acid-dependent Dissociation from Pentamannosyl Phosphate-Agarose Affinity Columns—To determine whether the replacement of His-105 resulted in a loss of the ability of the CD-MPR to release ligand under acidic conditions, medium from metabolically labeled transfected COS-1 cells expressing the wild-type, H105N, or H105S Asn-81/STOP155 construct was passed over pentamannosyl phosphate-agarose affinity columns. The proteins were then eluted sequentially with glucose 6-phosphate, MES buffer (pH 4.6), and Man-6-P. The results demonstrate that the majority (86%) of the wild-type, H105N, and H105S constructs that bound to the column could be eluted with the acidic MES buffer (Table II and Fig. 4). These results indicate that His-105 is not essential for acid-dependent dissociation of phosphomannosyl residues by the CD-MPR.

DISCUSSION

The MPRs provide a highly specific recognition and delivery system in the cell by carrying Man-6-P-containing proteins from the biosynthetic pathway (i.e. trans Golgi network) or the cell surface to endosomal compartments. The ability of the MPRs to recognize various Man-6-P-containing proteins with nanomolar affinity, such as soluble lysosomal enzymes (27), transforming growth factor-β precursor (28), prolierin (3), and leukemia inhibitory factor (5), suggests that the receptors are involved in a number of diverse cellular events because of their carbohydrate binding activities. How-ever, until recently little was known about the structural features of the MPRs that determine their carbohydrate specific-

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2 N. Dahms, unpublished data.
ity. Our recent determination of the three-dimensional structure of the extracytoplasmic region of the bovine CD-MPR (20, 21) has identified the amino acids that comprise the Man-6-P binding pocket. These residues (Tyr-45, Gln-66, Asp-103, Asn-104, His-105, Arg-111, Glu-133, Arg-135, and Tyr-143), which are conserved among bovine, human, and mouse CD-
TABLE I

Ligand binding and secretion of Asn-81/STOP^{155} constructs

| Constructs expressed | Man-6-P binding^a | Secreted^b | No. of determinations |
|----------------------|-------------------|-------------|----------------------|
| Wild-type            | 73 ± 4            | 80 ± 3      | 16                   |
| Y45F                 | 49 ± 2            | 49 ± 13     | 4                    |
| Q66E                 | <1                | 78 ± 2      | 4                    |
| Q66N                 | <1                | 15 ± 9      | 4                    |
| D103A                | 62 ± 6            | 58 ± 9      | 3                    |
| D103E                | 72 ± 7            | 83 ± 6      | 3                    |
| H105N                | 34 ± 4            | 62 ± 5      | 4                    |
| H105S                | 37 ± 3            | 71 ± 8      | 4                    |
| R111A                | <1                | 8 ± 3       | 4                    |
| R111K                | 2 ± 1             | 7 ± 3       | 4                    |
| E133D                | <1                | 58 ± 4      | 4                    |
| E133L                | <1                | 65 ± 4      | 4                    |
| E133Q                | <1                | 64 ± 4      | 4                    |
| R135E                | 22 ± 3            | 32 ± 11     | 7                    |
| R135K                | 57 ± 7            | 64 ± 5      | 6                    |
| Y143F                | <1                | 54 ± 9      | 4                    |

^a The percent of Man-6-P binding is calculated from the receptor that is secreted into the medium and then subjected to pentamannosyl phosphate-agarose chromatography. The values for the percent of the construct found in the Man-6-P eluate represent the mean ± S.E. for the number of determinations indicated.

^b The values for the percent of the total receptor expressed by transfected COS-1 cells that is found in the medium after a 24-h labeling period (i.e. 48–72 h post-transfection) represent the mean ± S.E. for the number of determinations indicated.

TABLE II

Elution of Asn-81/STOP^{155} constructs under acidic conditions

| Construct expressed | Run through | Wash | G6P pH 4.6 | M6P | Secreted^b |
|----------------------|-------------|------|------------|-----|-------------|
| Wild-type            | 6 ± 2       | 10 ± 4| 2 ± 2      | 79 ± 7 | 3 ± 3 | 86 ± 2 |
| H105S                | 53 ± 5      | 5 ± 3 | 1 ± 1      | 39 ± 3 | 1 ± 1 | 63 ± 7 |
| H105S                | 48 ± 7      | 1 ± 1 | 1 ± 1      | 44 ± 5 | 7 ± 1 | 69 ± 6 |

^b The values for the percent of the total receptor expressed by transfected COS-1 cells that is found in the medium after a 24-h labeling period (i.e. 48–72 h post-transfection) represent the mean ± S.E. for three independent determinations.

Fig. 3. Ligand affinity chromatography of wild-type and mutant Asn-81/STOP^{155} constructs. Media from [35S]methionine-labeled COS-1 cells transfected with various cDNA constructs were passed over pentamannosyl phosphate-agarose columns (0.5 × 1.0 cm). The columns were washed in column buffer and a total of five 3-ml fractions were collected. The columns were eluted first with 10 mM phosphate-agarose chromatography. The values for the percent of the total receptor expressed by transfected COS-1 cells that is found in the medium after a 24-h labeling period (i.e. 48–72 h post-transfection) represent the mean ± S.E. for three independent determinations.

Fig. 4. Effect of substitutions at position 105 on pH-dependent dissociation. SDS-polyacrylamide gels (12.5%) are shown of various cDNA constructs that have been passed over pentamannosyl phosphate-agarose columns. The columns were washed with 15 ml of column buffer (W) before washing sequentially with 3 ml of 10 mM glucose 6-phosphate (G), MES buffer, pH 4.6, and 10 mM Man-6-P (M). The fractions were analyzed as described in the legend of Fig. 3.

MPRs (Fig. 1), were shown to be located within hydrogen bonding distance of Man-6-P (Fig. 2B). The aim of this study was to identify which of these residues provide key contacts for high affinity phosphomannosyl binding by the CD-MPR.

Eight of the nine residues were subjected to site-directed mutagenesis. Asn-104 was not mutated because it interacts predominantly with Man-6-P via a main-chain, rather than a side-chain, atom (Fig. 2B). Of the eight residues altered, only the substitution of Asp-103, with either a conservative (Glu) or nonconservative (Ala) residue, caused no significant effect on ligand binding (Table I). In addition to the amide nitrogen of Asp-103 making hydrogen bond contacts with the phosphate group, its carboxylate side chain is coordinated to the Mn²⁺ present in the binding pocket (Fig. 2). However, the presence of Mn²⁺ has been shown to enhance CD-MPR binding to Man-6-P by only 4-fold (10). Thus, this finding is consistent with changes in the side-chain group at position 103 having no detectable effect on the binding of the CD-MPR to a pentamannosyl phosphate affinity column.

Substitution of Tyr-45, His-105, or Arg-135 resulted in a partial inhibition of phosphomannosyl binding. Our recent crystal structure of Asn-81/STOP^{155} bound to pentamannosyl phosphate reveals that Tyr-45 interacts with the 1-hydroxyl of Man-6-P, which is involved in an O-glycosidic linkage, and the 4-hydroxyl of the penultimate mannose residue (21). Replacement of Tyr-45 with Phe decreases receptor binding to pentamannosyl phosphate columns by 30% (Table I), indicating that Tyr-45 is not critical for ligand recognition.

Substitution of His-105 with Ser or Asn resulted in a 50% decrease in binding to the affinity column (Table I). Although the δ nitrogen of the imidazole ring of His-105 interacts with a phosphate oxygen, the phosphate group of Man-6-P makes several interactions with CD-MPR residues; hydrogen bond contacts are made with the main chain amide nitrogen atoms of Asp-103, Asn-104, and His-105 as well as with the δ nitrogen of His-105 (Fig. 2B). Thus, it is likely that the loss of the imidazole ring does not completely compromise phosphate binding because of the multiple interactions of the polypeptide with the phosphate oxygens of the ligand. Conservative replacement of Arg-135 with Lys had only a moderate effect on receptor binding (~20% decrease), both in the bovine (Table I) and human (19) CD-
Mannose 6-Phosphate Binding by CD-MPR Mutants

Fig. 5. Stereo diagram of potential electrostatic interactions. An orientation similar to that in Fig. 2A is shown. Residues within the Man-6-P binding pocket of Asn-81/STOP155 (21) that are involved in electrostatic interactions are shown. The dotted lines indicate the interatomic distances (angstroms) between charged residues. The aromatic ring of Tyr-102, which stacks against the plane of the guanidinium group of Arg-111, is also shown. ●, nitrogen atom; ○, oxygen atom.

MPR, whereas nonconservative replacement with Glu yields a larger decrease (70%) in receptor binding (Table I). Arg-135 interacts with the 4-hydroxyl of Man-6-P (Fig. 2B). When lysine is modeled at this position, the ϵ-amino group of Lys is within hydrogen bonding distance of the 4-hydroxyl of Man-6-P, which may explain the minimal effect this substitution has on carbohydrate recognition. Modeling of glutamate at position 135 demonstrates that its carboxylate group: 1) is within hydrogen bonding distance of the 4-hydroxyl of Man-6-P and 2) introduces a negative charge adjacent (3.1 Å) to the negative charge of Glu-133, which could destabilize the binding pocket (Fig. 5). Thus, the existence of a potential hydrogen bond could be offset by the close proximity of the two carboxylate groups and may explain why R135E exhibits a greater deleterious effect than R135K.

Four of the amino acid residues present in the carbohydrate binding pocket (Gln-66, Arg-111, Glu-133 and Tyr-143) were shown to play an essential role in phosphomannosyl binding as both conservative and nonconservative substitutions resulted in the complete loss of binding to the affinity column (Table I and Fig. 2A). Modeling of the Gln-66 mutations indicates that the presence of an Asn residue would eliminate the hydrogen bond between position 66 and the 2- and 3-OH of the mannose ring. Because Gln-66 lies on a rigid β-sheet (β-strand 3), substitution with a methylene-carbon shorter residue, Asn, results in the side chain being too distant from the mannose for H-bonding. The introduction of the charged amino acid, Glu, at position 66 is predicted to perturb the electrostatic interaction between Glu-133 and neighboring Arg-135 (Fig. 5), which could alter the binding pocket and result in a decreased affinity for ligand. Previous studies conducted on Arg-111 in the human CD-MPR (19) and the analogous Arg in domains 3 and 9 of the bovine IGF-II/CI-MPR (17) demonstrated that this conserved Arg was essential for ligand binding by both MPRs as assessed by affinity chromatography. Our results are consistent with these findings in that replacement of Arg-111, which interacts with the 2-hydroxyl of Man-6-P (Fig. 2B), with either Lys or Ala in the bovine CD-MPR resulted in the loss of receptor binding to pentamannosyl phosphate-agarose columns. When Lys is modeled into the structure at position 111, the ϵ-amino group is within hydrogen bonding distance of the 2-OH. However, unlike the case observed with R135K, no detectable Man-6-P binding is observed. The presence of a Lys at this position does not appear to introduce any unfavorable electrostatic or steric interactions. However, the observed stacking of the aromatic ring of Tyr-102 with the plane of the guanidinium group of Arg-111, which are located 3.6 Å apart, may aid in the stabilization and/or delocalization of the charge on Arg-111 (Fig. 5). Taken together, these studies suggest that the presence of a guanidinium group at position 111, rather than a positive charge, is essential for high affinity ligand binding. Substitution of Glu-133, which interacts with the 3- and 4-hydroxyl of Man-6-P, with Leu, Glu, or Asp abolished detectable binding to pentamannosyl phosphate-agarose columns (Table I). Replacement of Glu-133 with the aliphatic side chain of Leu eliminates the hydrogen bond acceptor atoms of Glu and results in the loss of three hydrogen bond interactions between the CD-MPR and ligand (Fig. 2B). The introduction of Gln does not alter the hydrogen bonding pattern but does alter the electrostatics of the region, as is also predicted for Leu. In addition to serving as a key contact for the 3- and 4-hydroxyl groups, Glu-133 is within 4.9 Å of Arg-135 and its negative charge, along with Asp-43, may aid in neutralizing the positive charge of this Arg (Fig. 5). Although the presence of Asp at position 133 does not significantly alter the distance between this negatively charged side chain and that of Arg-135, the geometry of the interaction is altered. Furthermore, Asp at position 133 is no longer within hydrogen bonding distance to the ligand. Tyr-143 interacts with the 2- and 3-hydroxyls of Man-6-P (Fig. 2B). The replacement of Tyr-143 with Phe eliminates the ability of the CD-MPR to interact with the affinity column, indicating the important role of these interactions for ligand recognition.

The four residues (Gln-66, Arg-111, Glu-133, and Tyr-143) identified by site-directed mutagenesis to be essential for Man-6-P binding, which combined form all but one of the contacts between the receptor and the 2-, 3-, and 4-hydroxyls of mannose, are located in regions of the receptor with relatively rigid geometry (β-strands 3, 7, 8, or 9 respectively). Residues Gln-66, Arg-111, and Tyr-143 form the region of the binding pocket that interacts with the polar face of the mannose ring, whereas Glu-133 aligns with an edge of the mannose ring (Fig. 2A). The location of amino acids essential for ligand recognition in regions of limited flexibility may contribute to the specificity displayed by the receptor: the fixed geometry of the region may aid in the ability of the receptor to discriminate between mannose and its 2-hydroxyl epimer, glucose.

Residues critical for ligand binding are likely to be conserved between species. This is true for Gln-66, Arg-111, Glu-133, and Tyr-143 of the CD-MPR identified in this study (Fig. 1). Because the CD-MPR and the IGF-II/CI-MPR exhibit a similar carbohydrate specificity for phosphomannosyl residues (10, 29), the possibility exists that the two MPRs share similarities.
in the configuration of their Man-6-P binding pockets. We have previously demonstrated that the two Man-6-P binding sites of the IGF-II/CI-MPR map to domains 1–3 and 7–9 (16), with an essential arginine residue, Arg-435 and Arg-1334, located in domain 3 and domain 9, respectively (17). Arg-435 and Arg-1334 are analogous to Arg-111 in the CD-MPR. Of the four residues found to be essential for Man-6-P binding by the CD-MPR, Glu-66, Arg-111, and Tyr-143 are absolutely conserved between the CD-MPR and domains 3 and 9 of the IGF-II/CI-MPR from various species (Fig. 1). These results suggest that the CD-MPR and the IGF-II/CI-MPR utilize similar amino acids for high affinity binding of phosphomannosyl residues.

The CD-MPR functions to transport acid hydrolases from the trans Golgi network to endosomal compartments, where the acidic pH (≤ 6.0) of the compartment causes the receptor to release its ligand. However, the mechanism that is responsible for triggering this release at low pH is not yet known. A likely amino acid candidate for triggering low pH release is histidine because it has a pK\textsubscript{a} that falls within the range of the normal physiological pH values encountered by the receptor. The CD-MPR contains three His residues in its extracytoplasmic domain: His-58, located in the loop joining β-strand 2 to 3; His-105, located in the ligand binding site; and His-122, located on the loop joining β-strand 7 to 8. The ability of His-105 to regulate low pH dissociation of ligand was tested using the H105N and H105S constructs. Although both constructs demonstrated a diminished overall ability to bind pentamannosyl phosphate-agarose, they were eluted completely from the column by an acidic buffer (pH 4.6) (Table II and Fig. 4). These results indicate that His-105 is not critical for acid-dependent dissociation. However, it is possible that substitution of His-105 resulted in a change in the pH optimum of binding and/or release. Future studies will be required to test this hypothesis and to determine whether His-58 and/or His-122 are involved in the pH dependence of binding and release by the CD-MPR. It is also possible that a residue(s) other than His, whose pK\textsubscript{a} may be altered because of the surrounding microenvironment, is responsible for modulating the interaction of the receptor with ligand under various pH conditions. Structural studies of the CD-MPR under acidic conditions will be required to resolve this issue.

In summary, we have identified four residues (Glu-66, Arg-111, Glu-133, and Tyr-143) of the CD-MPR that are absolutely essential for the specific recognition of lysosomal enzymes. The conservation of Tyr-45, Glu-66, Arg-111, and Tyr-143 between the CD-MPR and the IGF-II/CI-MPR suggests that the two MPRs utilize a similar mechanism for the recognition of phosphomannosyl residues. Future structural studies will be required to confirm whether the architecture of the Man-6-P binding sites of the CD-MPR and the IGF-II/CI-MPR are similar.