Structural Insights into the Mechanism of pH-dependent Ligand Binding and Release by the Cation-dependent Mannose 6-Phosphate Receptor

Received for publication, November 1, 2007, and in revised form, February 5, 2008 Published, JBC Papers in Press, February 13, 2008, DOI 10.1074/jbc.M706994200

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The cation-dependent mannose 6-phosphate receptor (CD-MPR) is a key component of the lysosomal enzyme targeting system that binds newly synthesized mannose 6-phosphate (Man-6-P)-containing acid hydrolases and transports them to endosomal compartments. The interaction between the MPRs and its ligands is pH-dependent; the homodimeric CD-MPR binds lysosomal enzymes optimally in the pH environment of the trans Golgi network (pH ∼ 6.5) and releases its cargo in acidic endosomal compartments (<pH 5.5) and at the cell surface. In addition, CD-MPR binding affinities are modulated by divalent cations. Our previous crystallographic studies have shown that at pH 6.5, the CD-MPR bound to Man-6-P adopts a significantly different quaternary conformation than the CD-MPR in a ligand-unbound state, a feature unique among known lectin structures. To determine whether different pH conditions elicit conformational changes in the receptor that alters ligand binding affinities, we have obtained additional crystal structures representative of the various environments encountered by the receptor including: 1) the CD-MPR bound at pH 6.5 (i.e. trans Golgi network) to a high affinity ligand (the terminally phosphorylated trisaccharide P-Man(1,2)Man(1,2)Man-O-((CH$_2$)$_3$)$_5$COOMe), 2) the CD-MPR at pH 4.8 in an unbound state (i.e. endosome), and 3) the CD-MPR at pH 7.4 (i.e. cell surface). A detailed comparison of the available CD-MPR structures reveals the positional invariability of specific binding pocket residues and implicates intermonomer contact(s), as well as the protonation state of Man-6-P, as regulators of pH-dependent carbohydrate binding.

The two members of the P-type lectin family, the 46-kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) and the 300-kDa cation-independent mannose 6-phosphate receptor (CI-MPR), function to direct the delivery of ∼50 different newly synthesized soluble lysosomal enzymes bearing mannose 6-phosphate (Man-6-P) on their N-linked oligosaccharides to the lysosome. Both MPRs display optimal ligand binding at pH ∼6.5 and no detectable binding below pH ∼5, which is consistent with their function of binding their cargo in the trans Golgi network and subsequently releasing their ligands in the acidic environment of endosomes. The lysosomal enzymes are packaged into lysosomes, whereas the MPRs recycle back to the trans Golgi network to retrieve additional hydrolytic enzymes from the secretory pathway (1–3) or move to the cell surface where the CI-MPR, but not the CD-MPR, binds and internalizes lysosomal enzymes (4, 5). In vitro binding studies support these observations, demonstrating that the CI-MPR retains phosphomannosyl binding capabilities at neutral pH, whereas the CD-MPR displays a bell-shaped pH-sensitive binding profile, with optimum binding occurring at pH ∼6.4 and little or no binding observed at pH values above pH 7.5 or below pH 5.5 (6, 7). Cells treated with reagents that raise the pH of organelles of the endosomal-lysosomal system exhibit decreased sorting of lysosomal enzymes to lysosomes and a concomitant increase in the secretion of lysosomal enzymes into the medium (8). These observations imply a requirement for the MPRs to release their ligands in the acidic environment of endosomes to recycle back to the trans Golgi network in an unbound state, allowing for the acquisition of additional cargo. However, the mechanism by which changes in pH affect the carbohydrate binding pockets of the MPRs to facilitate cargo loading and unloading is unknown.

The CD-MPR is a type I membrane glycoprotein that exists as a dimer, with each subunit containing a single Man-6-P binding site. Unlike C-type lectins that have an absolute requirement for calcium to carry out their sugar binding activities (9), the presence of cations increases the binding affinity of the CD-MPR toward Man-6-P (7) and lysosomal enzymes (10) only 4-fold. We previously reported the crystallization of the extracytoplasmic region (residues 1–154) of the CD-MPR in complex with either Man-6-P (11) or the oligosaccharide pentamannosyl phosphate tetraacetate binding at pH 6.5 and no detectable binding below pH 5,
(P-Man(α1,3)Man(α1,3)Man(α1,3)Man(α1,2)Man) (12). We had also obtained the structure of the CD-MPR in a ligand-free form at pH 6.5, which showed a surprising finding not observed in other lectins; a significant change in the quaternary structure as well as a relocation of a loop in the binding pocket was observed when compared with the structure of the CD-MPR in a ligand-bound state at pH 6.5 (13). However, it was unclear whether the CD-MPR in a ligand-unbound state could adopt other conformations that are dependent upon pH.

In the current report, the crystal structures of the CD-MPR at pH 7.4 and pH 4.8 were determined to evaluate the mechanism by which changes in pH influence the carbohydrate binding ability of the CD-MPR. We also obtained the structure of the CD-MPR in the absence of Mn2+ and another structure in which the receptor is complexed to an oligosaccharide containing α1,2 linkages; these structures allowed for an assessment of the influence of divalent cations and of carbohydrate linkages on the conformation of the CD-MPR. Furthermore, by using the current structures of this report, along with the previously published structures, we are now able to begin to capture the dynamic nature of this cargo-carrying receptor as it encounters various cellular environments.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Crystallization of the Extracytoplasmic Region of the CD-MPR—The glycosylation-deficient extracytoplasmic region (residues 1–154 containing only a single N-glycosylation site at position 81) of the bovine CD-MPR (Asn-81/Stop155) was generated as described previously (14). Recombinant protein was produced in either Trichoplusia ni SBI–4 insect cells (pH 4.8 and pH 6.5 structures) as described previously (11) or Spodoptera frugiperda (Sf9) insect cells (pH 7.4 structures and pH 6.5 structure in the absence of Mn2+). Sf9 cells were grown in serum-free BD BaculoGold Max-XP medium (Pharmingen) as suspension cultures to a density of 3 × 10⁶ cells/ml and were subsequently infected with recombinant baculovirus at a multiplicity of infection of ~3. The medium was harvested 5 days after infection, and recombinant protein was purified to near homogeneity by affinity chromatography as described previously (15) except that phosphomannan-Sepharose was used as the affinity resin instead of pentamannosyl-phosphate agarose. Prior to crystallization, proteins were dialyzed extensively at 4 °C in the indicated buffer (see supplemental Table 1). Some protein preparations underwent additional overnight incubations at 4 °C in the presence of 10 mM Man-6-P or 4 mM α1,2-linked phosphorylated trimannoside (P-Man(α1,2)Man(α1,2)Man-O-(CH₂)₈COOMe). Crystallization was carried out at 19 °C (4 °C was used for crystallization of the pH 4.8 structure) by vapor diffusion using the hanging drop method (16) by mixing equal volumes of the purified protein (12–15 mg/ml ligand bound structures; 20 mg/ml pH 4.8 structure) with the precipitating solution (see supplemental Table 1 for crystallization conditions). The pH of the resulting protein crystallization solutions was measured directly using a microelectrode. The pH of the drop of the pH 4.8 condition was measured to verify that the crystal was grown under acidic conditions. The Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard was used to estimate protein yields. All protein concentrations reflect the application of a 2.2 correction factor as determined previously by amino acid analysis.

Generation of Phosphorylated Trimannoside—Chemical synthesis of the trimannoside containing a terminal Man-6-P (P-Man(α1,2)Man(α1,2)Man-O-(CH₂)₈COOMe) has been described previously (17).

Data Collection—The crystals were flash-frozen in liquid nitrogen after passing through a cryoprotection solution composed of the corresponding precipitating solution with an additional 3% polyethylene glycol 5000 monomethyl ether and 20% glycerol. All x-ray diffraction data were collected at 100 K at the Advanced Photon Source (APS) Structural Biology beam-line 19ID (Argonne, IL) with two exceptions. 1) pH 4.8 diffraction data were collected at the Advanced Photon Source BioCARS beam-line 14-BM-C (Argonne, IL); and 2) diffraction data of the receptor bound to the α1,2-linked trimannoside were collected at 4 °C on an R-AXIS IIC image plate detector system equipped with a Rigaku RU200 rotating anode generator. Data obtained from the pH 4.8 crystals and the trimannoside (P-Man(α1,2)Man(α1,2)Man-O-(CH₂)₈COOMe) crystals were processed using SCALePACK and DENZO (18), whereas all other data sets were processed with HKL2000 (18).

Structure Determination and Refinement—The pH 4.8 structure was solved by molecular replacement using AmoRe (19) using one polypeptide chain of the ligand-free form of the receptor at pH 6.5 (Protein Data Bank code 1KEO) as the search model. All other structures were solved by molecular replacement using AmoRe (19) using a single monomer of the receptor bound to the α1,3-linked oligomannoside, pentamannosyl phosphate (Protein Data Bank code 1C39), as the search model. Models obtained from AmoRe were refined using iterative rounds of CNS (20) followed by manual fitting and rebuilding using the program TURBO-FRODO (21). Each round of CNS refinement consisted of rigid body refinement, conjugate gradient minimization, simulated annealing, and individual B-factor refinement (with bulk-solvent correction). Since the initial models (after the rigid body refinements) of most of the structures already had Rfree/Rwork values less than 30/25%, non-crystallographic symmetry restraints were not used. At later stages of refinement, water molecules were assigned when densities >3σ were observed in the |Fo − Fc| map and were within 3.3 Å of a potential hydrogen-bonding partner. Coordinates for ligand molecules, the trimannoside and the non-phosphorylated branched oligosaccharide, were obtained using INSIGHT II (22), and their topology and parameter files were generated using XPLO2D (23). Data and refinement statistics are provided in Table 1.

Comparative Structural Analyses—Structural comparison of monomers was carried out by overlaying the Ca atoms of residues (47–50, 64–66, 73–76, 83–88, 144–147) located in the β-strands. The root-mean-square (r.m.s.) deviation values were subsequently determined by LSQMAN (24) and used to identify those regions in the receptor that differ in conformation. Differences in quaternary structure were evaluated by overlaying all Ca atoms in the dimer.
Preference for \(1,2\)-linked Phosphomannosyl Residues by the CD-MPR

— Prior to analyzing the effect changes in pH may have on the conformation of the CD-MPR, we probed the interaction between the receptor and its cargo, lysosomal enzymes, by characterizing a complex between the CD-MPR and a high affinity ligand. Previous inhibition studies using synthetic oligosaccharides have shown that phosphorylated dimannosides containing \(1,3\) or \(1,6\) linkages were no better ligands than the monosaccharide Man-6-P, whereas the phosphorylated dimannoside containing an \(1,2\) linkage was a 5–8-fold more potent inhibitor of lysosomal enzyme binding (25). An analysis of oligomannoside structures containing \(1,2\) linkages (i.e. the preferred ligand for the MPRs) is particularly relevant with respect to the interaction of the MPRs with endogenous lysosomal enzymes since of the five possible sites of phosphorylation on \(N\)-linked oligosaccharides, three of the resulting Man-6-P groups are found in \(1,2\) linkages (26).

To address the molecular basis for this linkage specificity and to probe the extended binding pocket of the receptor, we have obtained the crystal structure of the sCD-MPR complexed with the \(1,2\)-linked phosphorylated trimannoside \((P-Man(1,2)Man(1,2)Man-O-(CH_2)_8COOMe)\) (Fig. 1, A and D). A comparison with our previous structure of the sCD-MPR complexed with pentamannosyl phosphate \((P-Man(1,3)Man(1,2)Man-O-(CH_2)_8COOMe)\) (27) (Fig. 1B) revealed that the most striking difference between the two structures is the rotation of the penultimate mannose ring by \(90^\circ\) relative to the corresponding ring in the \(1,3\)-linked oligosaccharide (Fig. 1, A and B, compare ring II). The altered positioning of the penultimate ring allows its non-polar face to orient into the non-polar face of the terminal mannose ring as well as into the protein, minimizing solvent interactions. This observation is consistent with the hypothesis that the favorable gain in energy due to the additional hydrogen bonds with the penultimate and prepenultimate \(1,3\)-linked mannose rings is offset by the unfavorable electrostatic interaction of the penultimate ring with the receptor as the polar face of the penultimate sugar is buried in the

\(\text{FIGURE 1. Stereo view of the binding site of sCD-MPR in the presence of various oligosaccharides.}\)

Residues within the binding site are labeled, and potential hydrogen bonds are shown with dotted lines. Loops A through D are indicated. A, \(1,2\)-linked phosphorylated trimannoside (2RL9) B, pentamannosyl phosphate containing \(1,3\)-linked Man-6-P (1C39). C, non-phosphorylated branched oligosaccharide \((\text{Man}(1,3)\text{Man}(1,6)\text{Man}(1,4)\text{GlcNAc}(1,4)\text{GlcNAc})\) complexed with the receptor at pH 7.4 (2RL6). The terminal (I), penultimate (II), and prepenultimate (III) mannose rings are indicated in A and B. The \(1,3\)-linked (I), bisecting \(1,4\)-linked (II), and \(1,6\)-linked (III) mannose rings of the non-phosphorylated branched oligosaccharide are indicated in C, D and E. Stereo diagram of a model of oligosaccharides fitted into the final electron density map: stereo view of a \(2F_o - F_c\) omit map contoured at 1\(\sigma\) level of the trimannoside P-Man(1,2)Man(1,2)Man(CH_2)_8COOMe (2RL9) (D) or the non-phosphorylated branched oligosaccharide Man(1,3)Man(1,6)Man(1,4)GlcNAc(1,4)GlcNAc (2RL6) (E). Unless otherwise noted, all figures were generated using MOLSCRIPT (36) and rendered using POVRAY.

**RESULTS AND DISCUSSION**

Preference for \(1,2\)-linked Phosphomannosyl Residues by the CD-MPR—Prior to analyzing the effect changes in pH may have on the conformation of the CD-MPR, we probed the interaction between the receptor and its cargo, lysosomal enzymes, by characterizing a complex between the CD-MPR and a high affinity ligand. Previous inhibition studies using synthetic oligosaccharides have shown that phosphorylated dimannosides containing \(1,3\) or \(1,6\) linkages were no better ligands than the monosaccharide Man-6-P, whereas the phosphorylated dimannoside containing an \(1,2\) linkage was a 5–8-fold more potent inhibitor of lysosomal enzyme binding (25). An analysis of oligomannoside structures containing \(1,2\) linkages (i.e. the preferred ligand for the MPRs) is particularly relevant with respect to the interaction of the MPRs with endogenous lysosomal enzymes since of the five possible sites of phosphorylation on \(N\)-linked oligosaccharides, three of the resulting Man-6-P groups are found in \(1,2\) linkages (26).

To address the molecular basis for this linkage specificity and to probe the extended binding pocket of the receptor, we have obtained the crystal structure of the sCD-MPR complexed with the \(1,2\)-linked phosphorylated trimannoside \((P-Man(1,2)Man(1,2)Man-O-(CH_2)_8COOMe)\) (Fig. 1, A and D). A comparison with our previous structure of the sCD-MPR complexed with pentamannosyl phosphate \((P-Man(1,3)Man(1,2)Man-O-(CH_2)_8COOMe)\) (27) (Fig. 1B) revealed that the most striking difference between the two structures is the rotation of the penultimate mannose ring by \(90^\circ\) relative to the corresponding ring in the \(1,3\)-linked oligosaccharide (Fig. 1, A and B, compare ring II). The altered positioning of the penultimate ring allows its non-polar face to orient into the non-polar face of the terminal mannose ring as well as into the protein, minimizing solvent interactions. This observation is consistent with the hypothesis that the favorable gain in energy due to the
protein (Fig. 1B) (27). Although differences are seen in the contacts between the receptor and the oligosaccharide (compare Fig. 1, A and B), analysis of the hydrogen-bonding pattern between the two structures reveals no significant difference in either the overall number of contacts or the length of hydrogen bonds between the receptor and ligand (see supplemental Table 2). Based on the similarity in the number of contacts, the data indicate that the binding preference of the CD-MPR for 1,2-linked oligomannosides is the result of minimization of nonpolar surfaces to solvent that involves the orientation of the penultimate mannose ring.

In addition to providing a characterization of the extended binding pocket surrounding the penultimate and prepenultimate mannose rings, these comparative analyses demonstrated that the interactions between the polypeptide and terminal phosphomannose moiety are essentially the same between the structures containing a bound oligosaccharide and that of the receptor complexed with the monosaccharide Man-6-P (11), with nine residues (Tyr-45, Gln-66, Asp-103, Asn-104, His-105, Arg-111, Glu-133, Arg-135, and Tyr-143) within hydrogen-bonding distance to the terminal phosphorylated mannose (Fig. 1, A and B, and supplemental Table 2). Thus, the presence of α1,2- or α1,3-linked mannose rings does not affect the bonding scheme of the terminal Man-6-P residue to the receptor, the architecture of the binding pocket, or the overall quaternary structure of the sCD-MPR (see below).

Influence of pH (pH 6.5 versus pH 7.4) on the Conformation of the sCD-MPR in the Ligand-bound State—The CD-MPR displays a bell-shaped carbohydrate binding profile that is influenced by pH, with optimum binding occurring at pH 6.4 and little or no binding at pH values above pH 7.5 or below pH 5.5 (5, 7, 28). To identify those regions of the CD-MPR that may undergo structural perturbations dependent upon pH, the receptor was crystallized under different pH conditions, and comparative analyses of all structures were performed. A total of 10 CD-MPR structures have now been obtained under different conditions, which include structures from our previous works and those reported in the current study. A soluble, glycosylation-deficient form of the bovine CD-MPR (i.e. encodes residues 1–154 of the 159-residue extracytoplasmic domain, and four out of the five potential N-glycosylation sites are removed, leaving the site at Asn-81 intact), which is referred to as sCD-MPR, has been used for all previous (11–13) and current crystallographic studies. We have shown that this sCD-MPR binds lysosomal enzymes with the same high affinity as the full-length receptor (14).

To evaluate how pH at the cell surface may negatively impact the ability of the CD-MPR to bind ligand, sCD-MPR crystals were obtained at pH 7.4 in the presence of 10 mM MnCl₂ and in the presence or absence of 10 mM Man-6-P. Although only the structure of the protein crystallized in the presence of Man-6-P was refined due to the limited quality of the other data set, the initial Fo-Fc maps clearly showed that Man-6-P was not present in either structure. Surprisingly, in both cases, the receptor was bound to the α1,3-linked mannose of the non-phosphorylated branched oligosaccharide (Man(α1,3)(Man(α1,6))Man(β1,4)GlcNAc(β1,4)GlcNAc) attached to Asn-81 of a crystallographic neighbor (Fig. 1, C and E). The sCD-MPR protein used here was produced in Sf9 insect cells that synthesize endogenous and recombinant proteins containing N-glycans that are significantly smaller than those observed in mammalian cells, with the paucimannnosidic structure Man₃GlcNAc₂ being the major processed N-glycan produced by insect cells (reviewed in Ref. 29). Despite the presence...
of MnCl₂ during crystallization, Mn²⁺ was not observed in the pH 7.4 structure (Fig. 1C). This is in contrast to all previous structures of the receptor bound to a phosphomannosyl residue at pH 6.5 (Fig. 1, A and B, and see supplemental Table 2) in which Mn²⁺ is present in the binding pocket and is coordinated to one of the carboxylate oxygens of Asp-103, the most solvent-accessible oxygen of the phosphate group of Man-6-P, and four water molecules. To rule out whether any differences observed may be due to the lack of a divalent cation in the binding pocket, we have determined the structure of the sCD-MPR at pH 6.5 complexed with Man-6-P but in the absence of Mn²⁺ (Table 1 and supplemental Table 1). A comparison of the sCD-MPR when complexed to a ligand at pH 6.5 in the presence or absence of Mn²⁺ demonstrates no significant difference between the monomer fold (r.m.s. deviation < 0.2 Å between corresponding monomers), the relative position of residues within the binding pocket, or the dimer conformation (r.m.s. deviation < 0.2 Å) (supplemental Table 2).

A close inspection of the binding site architecture of the pH 7.4 structure (Fig. 1C) shows that it is relatively unaltered when compared with the pH 6.5 structures (Fig. 1, A and B, and see Fig. 3). The α1,3-linked mannose of the branched oligosaccharide makes very similar contacts to the receptor as observed for the terminal mannose ring of Man-6-P, and the bisecting β1,4-linked mannose ring interacts with the sCD-MPR in a similar fashion as observed for the penultimate mannose ring of the α1,2-linked trimannoside and the α1,3-linked oligomannoside. Furthermore, the two GlcNAc residues are in position to make hydrogen bonds with Lys-73 and Asp-103 of the receptor (supplemental Table 2).

Comparative analyses of the structures obtained at pH 6.5 when ligand is present with the structure obtained at pH 7.4 demonstrates that the lack of Mn²⁺ is not the cause of significant structural differences between the two structures. For example, the loop that contains the Asn-81 residue (Fig. 3) is not present in the pH 6.5 structures (Fig. 1, A and B) and is not well defined in the pH 7.4 structures (Fig. 1C). This suggests that the pH dependence of the binding pocket may be due to changes in the conformation of the receptor.

### Table 1: Data collection and refinement statistics

| pH       | Protein Data Bank ID | Data collection | Refinement |
|----------|----------------------|-----------------|------------|
| 4.8      | 2RL7                 | 32769           | 4          |
| 4.8      | 2RL8                 | 57086           | 2          |
| 4.8      | 2RL9                 | 10608           | 2          |
| 4.8      | 2RLB                 | 30104           | 2          |
| 4.8      | 2RL6                 | 35478           | 2          |
| 6.5      | 2RL7                 | 210.0/0.256     | 4          |
| 6.5      | 2RL8                 | 208.0/0.228     | 2          |
| 6.5      | 2RL9                 | 199.0/0.265     | 2          |
| 6.5      | 2RLB                 | 200.0/0.228     | 2          |
| 7.4      | 2RL7                 | 198.0/0.228     | 2          |
| 7.4      | 2RL8                 | 198.0/0.228     | 2          |

### pH Dependence of Man-6-P Binding by the CD-MPR

In the absence of Mn²⁺, the structure of the sCD-MPR at pH 6.5 complexed with Man-6-P demonstrates no significant difference between the monomer fold (r.m.s. deviation < 0.2 Å between corresponding monomers), the relative position of residues within the binding pocket, or the dimer conformation (r.m.s. deviation < 0.2 Å) (supplemental Table 2).
bound, the presence of Mn2
6.5 or pH 7.4 demonstrated that the type of carbohydrate
or the dimer conformation (r.m.s. deviation 0.4–0.9 Å). (Fig.

A

B

FIGURE 2. The crystal structure of sCD-MPR in the presence of ligand.
A, overlay of all monomers solved to date in the bound conformation. Each
polypeptide chain of the homodimer is folded into a flattened β-barrel with
three intramolecular disulfide bonds (gold); an N-terminal α-helix followed by
a four-stranded anti-parallel β-sheet (β1-β4) oriented orthogonally over a
five-stranded, anti-parallel β-sheet (β5-β9), with strand 9 interjecting
between strands 7 and 8. The N and C termini are boxed, and the loops
between strands are labeled as follows: A, β-strands 1 and 2; B, β-strands 3
and 4; C, β-strands 6 and 7; D, β-strands 8 and 9. In the presence of an
oligosaccharide, as opposed to Man-6-P, only a slight collapse of the binding
pocket is observed as loop A (residues 38–43) is drawn toward the binding
pocket by 1–3 Å. B, overlay of the dimer structure of sCD-MPR. For clarity, only
two structures are shown, with each subunit shaded a different color: pH 7.4
(2RL6, brown/tan) and pH 6.5 bound to Man-6-P (2RLB, purple/pink). The loca-
tion of Man-6-P (gold ball-and-stick) in the binding pocket is shown.

The presence of Mn2
6.5 or pH 7.4 demonstrated that the type of carbohydrate
bound, the presence of Mn2
6.5 or pH values of 6.5 or 7.4 do not
significantly influence the monomeric or quaternary structure


4 L. J. Olson, N. M. Dahms, and J.-J. P. P. Kim, unpublished data.

pH Dependence of Man-6-P Binding by the CD-MPR
of the sCD-MPR. Because we were unable to clearly identify any
significant pH-dependent conformational changes, other fea-
tures of the binding pocket of the receptor, particularly the
 electrostatic environment and those involving intermonomer
interactions, which are predicted to be pH-sensitive and may
influence ligand binding, were evaluated.

Proposed Mechanism for Dissociation of Lysosomal Enzymes
from the sCD-MPR at the Cell Surface—Although Man-6-P was
present at high concentrations (10 mM) during crystallization,
only the non-phosphorylated oligosaccharide was located in
the binding pocket of the final model at pH 7.4. Based on this
observation and the known binding properties of the CD-MPR,
we propose a mechanism for how the receptor releases its cargo
under conditions found at the cell surface that involves depro-
tonation effects on both the receptor and Man-6-P (Fig. 3). At
pH 6.4, the optimum condition for binding ligand, the phos-
phate group of Man-6-P (the pKa for the acid dissociation of the
phosphate moiety of Man-6-P has been reported to be 6.4 (31))
would display nearly equivalent populations of HPO4
- and
PO4
- species. Because His-105 is the only residue of the recep-
tor in which a titratable side chain (typical pKa of the side chain
of a histidine residue is ~6.0–6.5) is involved in binding the
phosphate moiety of Man-6-P, it is likely His-105 plays a key
role in modulating ligand interactions on the basic side of the
pH profile. At pH 6.4, His-105 would have a significant popu-
lation in the protonated state, facilitating electrostatic interac-
tion with the negatively charged phosphate group of Man-6-P
Fig. 3A). In contrast, at the more alkaline pH value (pH 7.4), the
equilibrium of Man-6-P has shifted to predominantly the PO4
- species, whereas His-105 is predominantly in the uncharged
state, thereby eliminating its electrostatic interactions with the
sugar phosphate moiety. From the structural information, we
propose that the absence of the “shielding” Mn2
+ in the binding
pocket at pH 7.4 would result in electrostatic repulsion between
the PO4
- moiety of Man-6-P and the negatively charged side
chain of Asp-103 (Fig. 3B). This repulsive force along with the
loss of the electrostatic interaction between the uncharged His-
105 and Man-6-P are likely to work in concert to facilitate dis-
sociation of lysosomal enzymes from the receptor at the cell
surface. Furthermore, the previously reported low concentra-
tion of Mn2
+ in human serum (40–120 nm), along with the
measured binding affinity of the sCD-MPR for Mn2
+ (Kd = 600
µM) (10), is consistent with the receptor lacking bound Mn2
+ at
the cell surface. The observation that Asp-103 and His-105 are
not conserved in the three Man-6-P binding pockets of the
homologous CI-MPR (32, 33), an MPR that does not display
either cation dependence or loss of ligand binding activity at pH
7.4 (6, 7), further supports a role for these residues in modulat-
ing ligand binding by the CD-MPR at the cell surface. Our find-
ing that the receptor is bound to a non-phosphorylated oligo-
saccharide at pH 7.4 suggests a role for the CD-MPR at the cell
surface in binding proteins containing high mannose-type gly-
cans, a result consistent with a previous study that showed that
the non-phosphorylated a1,2-linked mannoside was only a
14-fold weaker inhibitor of lysosomal enzyme binding to the
receptor than Man-6-P (25). Additional studies will be needed
to test this intriguing possibility.

4 L. J. Olson, N. M. Dahms, and J.-J. P. P. Kim, unpublished data.
Influence of pH (pH 6.5 versus pH 4.8) on the Conformation of the sCD-MPR in the Unbound State—We have previously determined the structure of the sCD-MPR at pH 6.5 in the absence of ligand (13). To determine the effect of pH on the conformation of the unbound form of the receptor, we have now determined the structure of the receptor at pH 4.8 in the absence of ligand. Superimposition of the monomers (Fig. 4A) or the dimers (Fig. 4B) at the two pH values shows an average r.m.s. deviation of 0.4 and 0.6 Å, respectively, between all Cα atoms (superimposition of the two dimers in the asymmetric unit of the pH 4.8 structure yields a r.m.s. deviation of 0.8 Å). These results demonstrate that acidic pH conditions do not significantly alter the monomer or dimer conformation of the receptor in an unbound state.

Comparison of the Two Conformations of the sCD-MPR—Surprisingly, all structures of the sCD-MPR obtained to date can be categorized into one of two conformations: an “open” conformation found in all structures containing ligand in the binding pocket and a “closed” conformation found in all structures lacking bound carbohydrate. Unlike other lectins, which do not display any significant conformational change induced by ligand binding, the individual peptide chains of the sCD-MPR showed differences in the positioning of loops A, C, and D, which comprise the majority of the binding pocket of the receptor (Fig. 5, A and B). Loop D (residues Glu-134–Cys-141) exhibits the most dramatic change in position, with Val-138 displaying the largest displacement (Ca-Ca distance of 16 Å). In addition to changes in the location of loops, these two conformations display a significant difference in the orientation of the two monomers relative to each other, with an r.m.s. deviation of 4.1 Å for the 302 Ca atoms of the two dimers (Fig. 5, C and D). This difference in quaternary structure can be described globally as an ~30° scissoring motion between the two subunits of the dimer with respect to the two-fold axis (Z axis) on the XZ plane (Fig. 5C) and an ~12° twist along the Z axis pivoting at the center of the dimer molecule (Fig. 5D). The quaternary structural change resulting from this scissoring and twisting motion preserves the exact molecular two-fold symmetry of the dimeric molecule. In addition, a change in the distance between the individual ligand binding sites is also
observed; the Coα atoms of His-105 located at the tip of loop C
are located ~34 Å apart in the open conformation and ~26 Å
apart in the closed conformation (13).

In addition, structures solved in the absence of ligand over-
whelmingly share the common feature of a highly flexible
region from residues 11–15, a region immediately preceding
the α-helix. This feature is reflected in either significantly
higher than average B factor values (81–97 Å²) for these resi-
dues, as observed in the pH 6.5 unbound structure, or a lack of
sufficient density to place these residues in three out of the four
monomers in the asymmetric unit of the pH 4.8 structure. The
structural data also indicate that loop D serves different func-
tions in these two conformations. In the presence of bound
ligand, residues of loop D form the side of the binding pocket
(Fig. 6A), whereas in the absence of ligand, these residues pivot
into the pocket and participate in the formation of an
intramonomer hydrogen bond network that stabilizes the
binding pocket (Fig. 6B). This region of the receptor also appears to
impact the stability of the quaternary structure of the sCD-MPR.
In the open, ligand-bound conformation, residues of loop D
participate in intermonomer interactions with the N terminus
of the adjacent monomer (Fig. 7A), which results in an order-
ing/stabilization of the N-terminal region preceding the α-he-
lix, whereas in the closed, ligand-unbound conformation, loop
D no longer makes intermonomer contacts (Fig. 7B).

**Proposed Mechanism for Acidic pH-dependent Release of Lyssosomal Enzymes by the sCD-MPR**—Due to
the lack of any significant pH-dependent conformational changes
induced by acidic pH conditions (Fig. 4), the electrostatic environ-
ment of the binding pocket and intermonomer interactions that are
predicted to be pH-sensitive were evaluated. The most obvious poten-
tial participant in modulating the ligand binding activity of the recep-
tor on the acidic side of the pH pro-
file is Glu-133, a residue on loop D that interacts directly with the 3-
and 4-hydroxyl groups of the man-
ose ring of Man-6-P. Under acidic
conditions, protonation of Glu-133
would compromise the ability of the
carboxylate of Glu-133 to engage in
hydrogen bonding with one of the
hydroxyl groups of the sugar and
would eliminate the long range (~5 Å)
electrostatic interaction between
Glu-133 and Arg-135. Glu-133 is
also within ~6 Å of two other resi-
dues essential for Man-6-P binding
(Gln-66 and Arg-111), and thus,
Glu-133 appears to serve as the cen-
tral player coordinating the electro-
static environment of the binding
pocket (Fig. 6A). Therefore, weak-
ening of these interactions upon protonation of Glu-133 would
be predicted to destabilize the binding pocket and start a cas-
cade of events leading to the release of ligand. Once ligand is
released, Glu-133 and Arg-135 are able to shift positions to
those found in the unbound conformation in which Glu-133
and Arg-135 are within hydrogen-bonding distance to each other.
However, if Glu-133 remains unprotonated, Glu-133 and
Arg-135 are in position in the unbound conformation of the
receptor to form an intramonomer salt bridge (Fig. 6A). Cur-
rently, we have no direct evidence to support or refute the abil-
ity of Glu-133 to undergo protonation since the position of
Glu-133 and Arg-135 relative to each other does not vary sig-
nificantly between the unbound structure at pH 4.8 versus that
at pH 6.5. Glu-133 is solvent-accessible as it is within hydrogen-
bonding distance to one conserved water, which in turn is
linked to a series of water molecules leading to the surface of the
protein. Additional studies to directly probe the role of Glu-133
in the acid-dependent release of ligand by the CD-MPR have
been hindered due to the fact that conservative substitution of
Glu-133 with Asp, Leu, or Gln results in no detectable binding
to ligand (27), with a >800-fold reduction in binding affinity
toward a lysosomal enzyme when measured at pH 6.5 (10).

If the pH-sensitive release mechanism is encoded in the
dimer rather than within the binding pocket, one possible
mechanism is the disruption of solvent-accessible intermon-

**FIGURE 5. Comparison of the bound and unbound conformations of sCD-MPR.** A and B, superimposition of
the monomers of the bound (pH 6.5, 2RL8, pink) and unbound (pH 4.8, 2RL7, blue) forms of sCD-MPR. B, the
monomers have been rotated 90° down from the view in panel A. The N and C termini are boxed, and the loops
are labeled in panels A and B. C and D, superimposition of all Co atoms of the dimer of the bound (pH 6.5, 2RL8,
pink) and unbound (pH 4.8, 2RL7, blue) structures. D, the dimers have been rotated 90° down from the view in
panel C. In panels C and D, the axes have been defined such that the center of mass of the dimer is the origin, the
dimer 2-fold axis is the Z axis, and the X axis passes through the longest dimension of the molecule.
mer salt bridges. In the ligand-bound conformation, loop D forms the side of the binding pocket and participates in non-covalent interactions with the N-terminal region of the adjacent monomer that appear to stabilize the binding pocket. In the bound structure, residues located in this region are adjacent to two intermonomer electrostatic interactions (Lys-18–Glu-134, 4.0 Å and Glu-19–Lys-137, 3.9 Å) that participate in linking the N-terminal α-helix (Lys-18, Glu-19) of one monomer to loop D (Glu-134, Lys-137) of the other monomer (Fig. 7A). In the bound form of the receptor, both Glu-19 and Glu-134 are within hydrogen-bonding distance to conserved water molecules and are thus sufficiently solvent-accessible to be affected by changes in pH. A third intermonomer ion pair (Lys-97–Asp-140, 5.1 Å) is present in the bound conformation that links β-strand 6 to loop D (Fig. 7C). All three of these intermonomer electrostatic interactions are absent in the unbound structures (pH 4.8 or pH 6.5) (Fig. 7, B and D). Therefore, a plausible scenario for release of ligand in acidic endosomal compartments involves protonation of Glu-19, Glu-134, and/or Asp-140 resulting in the disruption of intermonomer electrostatic interactions that tie loop D of one monomer to the N-terminal α-helix of the other monomer. Elimination of these ionic interaction(s) “frees” loop D to move into the binding pocket, resulting in the displacement of bound ligand. Protonation of Glu-133 may also occur, which is predicted to facilitate displacement of the sugar from the binding pocket; protonation of Glu-133 would weaken its interaction with the 3- or 4-hydroxyl group of Man-6-P and also would disrupt the electrostatic environment of the entire binding pocket. The repositioning of loop D into the binding pocket eliminates its intermonomer interaction with the N terminus, facilitating the reorientation of the two monomers as the receptor changes its quaternary structure, adopting a more closed conformation in the unbound state.

Maintenance of the sCD-MPR Binding Pocket in the Absence of Ligand and Proposed Mechanism of Ligand Binding—In general, lectins employ shallow grooves rather than pockets for carbohydrate recognition. In the absence of ligand, these same grooves are filled with water molecules, and it is thought that these easily displaceable water molecules act to hold essential side chains in place by forming hydrogen bonds to them (34). The CD-MPR differs dramatically from other lectins in an unbound state. Instead of essential side chain interactions being shifted from the carbohydrate hydroxyls to water, the pocket of the sCD-MPR itself undergoes restructuring, which holds essential side chains in proper orientation for carbohydrate recognition and widens the pocket to facilitate access of ligand.

The phosphate binding region of the pocket is composed of loop C, which contains His-105 (involved in phosphate interactions) and Asp-103 (interacts with Mn$^{2+}$) in the absence of ligand, access to the phosphate portion of the pocket is blocked by a repositioning of the side chains of Asn-104 and His-105 (Fig. 6A). In addition, loop C is elongated relative to the same region in the bound structure such that residues on the N-terminal side of the loop (Asp-101–Asn-104) are in close proximity to residues on the C-terminal side of the loop (Cys-106–Gly-
the receptor to remain in a "ready-state" to accept ligand. This reorganization also creates two intramonomer hydrogen-bonding networks, which appear to stabilize the binding region in the absence of ligand and act to widen the bottom of the binding pocket relative to the bound structures (Fig. 6B).

Taken together, the results indicate that the conformation of the sCD-MPR in the unbound state is poised to interact with an array of structurally diverse oligosaccharides that it encounters as it traffics through the cell. We propose that the sCD-MPR samples potential carbohydrate ligands via interaction with Gln-66, Arg-111, and Tyr-143, the side chains of which are positionally maintained. The fourth residue found to be essential for high affinity binding, Glu-133, is not available in the unbound state for interaction with the ligand as it has been rotated out of the binding pocket through the relocation of loop D. Interaction of Gln-66, Arg-111, and Tyr-143 with the mannose ring may facilitate restructuring of the pocket so that further stabilization of the mannose ring interaction occurs; pivoting of loop D out of the pocket allows Glu-133 to swing into place and form contacts with this sugar ring. Thus, Glu-133 does not participate in the initial recognition phase of mannose binding, but rather, it appears to function as a key stabilizer of the binding pocket in the bound conformation; Glu-133 is within hydrogen-bonding distance to both the 3- and the 4-hydroxyls of the mannose ring and links the entire pocket via electrostatic interactions with Gln-66, Arg-111, and Arg-135 (Fig. 6A).

Conclusions—The CD-MPR functions in the delivery of lysosomal enzymes to the lysosome, an essential component of which involves the binding and release of its cargo in compartments that differ in pH. The structures of the receptor at pH 7.4 and pH 4.8 described in the current study provide insight into the mechanism of cargo release by the receptor at the cell surface and in acidic endosomal compartments, involving alterations in the protonation state of Man-6-P and residues within the binding pocket, as well as disruption of intermonomer ionic interactions. A surprising finding of the comparative analyses of the 10 available structures was that the sCD-MPR was found to exist in only two conformations that were not influenced by
pH or the presence of cations: an open conformation found in all ligand-bound structures and a closed conformation found in all ligand-unbound structures. The implication of this observation is that the CD-MPR must be able to readily transition between these two conformations as it travels to the different cellular compartments, with the unique environment of each compartment impacting the equilibrium between the two states. Future studies will be directed at probing the dynamic nature of this receptor as several questions remain unanswered, including whether the quaternary changes observed in the extracellular region of the CD-MPR transduce across the lipid bilayer to alter the structure of its cytosolic tail, which, in turn, could modulate its intracellular trafficking.

Acknowledgments—We thank Drs. Jianhua Fu, Vaughn Jackson, and Evgenii Kovriguine for careful reading of the manuscript. We also thank the staff at the APS beamlines BioCARS 14BM-C and SBC 19ID for their excellent assistance in data collection. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under Contract No. W-31-109-Eng-38 and Office of Biological and Environmental Research under contract DE-AC02-06CH11357. Use of the BioCARS Sector 14 was supported by National Center for Research Resources Grant RR07707 from the National Institutes of Health.

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