The N-terminal Carbohydrate Recognition Site of the Cation-independent Mannose 6-Phosphate Receptor*

Received for publication, April 26, 2004
Published, JBC Papers in Press, May 28, 2004, DOI 10.1074/jbc.M404588200

Linda J. Olson, Nancy M. Dahms, and Jung-Ja P. Kim‡
From the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

The 300-kDa cation-independent mannose 6-phosphate receptor (CI-MPR) plays a critical role in the trafficking of newly synthesized mannose 6-phosphate-containing acid hydrolases to the lysosome. The receptor contains two high affinity carbohydrate recognition sites within its 15-domain extracytoplasmic region, with essential residues for carbohydrate recognition located in domain 3 and domain 9. Previous studies have shown that these two sites are distinct with respect to carbohydrate specificity. In addition, expression of truncated forms of the CI-MPR demonstrated that domain 9 can be expressed as an isolated domain, retaining high affinity (Kd ≈ 1 nM) carbohydrate binding, whereas expression of domain 3 alone resulted in a protein capable of only low affinity binding (Kd ≈ 1 μM) toward a lysosomal enzyme.

In the current report the crystal structure of the N-terminal 432 residues of the CI-MPR, encompassing domains 1–5, was solved in the presence of bound mannose 6-phosphate. The structure reveals the unique architecture of this carbohydrate binding pocket and provides insight into the ability of this site to recognize a variety of mannose-containing sugars.

The biogenesis of lysosomes, which are key components of the degradative machinery of eukaryotic cells, requires the action of the mannose 6-phosphate receptors (MPRs). Two MPRs, the 300-kDa cation-independent MPR (CI-MPR) and the 46-kDa cation-dependent MPR (CD-MPR), participate in the intracellular delivery of ~50 different lysosomal enzymes to the lysosome by diverting these soluble acid hydrolases from the secretory pathway and delivering them from the trans-Golgi network to endosomal compartments (1). Unlike the CD-MPR, the CI-MPR has been shown to function in the binding and internalization of ligands at the cell surface. The higher capacity of the CI-MPR than the CD-MPR in sorting lysosomal enzymes to the lysosome is due in part to the ability of the CI-MPR at the cell surface to re-capture lysosomal enzymes that may have been secreted, resulting in their internalization and delivery to endosomal compartments (2, 3). In addition to lysosomal enzymes, the repertoire of extracellular mannose 6-phosphate (Man-6-P)-containing ligands has expanded in recent years to include a diverse spectrum of proteins including the precursor form of transforming growth factor-β and renin, proliferin, granzymes A and B, CD26, and herpes simplex viral glycoprotein D (4). Several studies have implicated the interaction of these ligands with the CI-MPR at the cell surface as being essential to their activity and/or function (5–8), thus expanding the role of the CI-MPR from solely an intracellular protein carrier to a cell surface signaling molecule via its lectin activity.

The CI-MPR is a type I integral membrane glycoprotein. Its large 2269-residue extracytoplasmic region is composed of 15 domains that display limited sequence identity (14–38%), similar size (~150 residues), and cysteine distribution to each other and to the CD-MPR (9). The CI-MPR contains two high affinity carbohydrate binding sites (10), with essential residues localized to domain 3 and domain 9 (11, 12). Several lines of evidence indicate that the two carbohydrate binding sites of the CI-MPR are structurally and functionally quite distinct from each other. Expression of truncated forms of the CI-MPR demonstrated that domain 9 could be expressed alone, folding into a high affinity (Kd ≈ 1 nM) carbohydrate recognition domain, whereas the domain 3 alone construct exhibited ~1000-fold lower affinity binding toward a lysosomal enzyme. Furthermore, expression of domains 1–3 resulted in a high affinity binding site, suggesting that residues in adjacent domains (domains 1 and/or 2) are important either directly or indirectly for optimal binding by domain 3 (13). Binding studies have also demonstrated that, like the CD-MPR, domain 9 is specific for phosphomonoesters, whereas domains 1–3 are more promiscuous in that they can recognize mannose 6-sulfate and phosphodiester with only a 10–20-fold lower affinity than Man-6-P (14).

We have recently solved the crystal structure of domains 1–3 of the CI-MPR, which demonstrated that each domain exhibits similar topology, consisting of a flattened 9-stranded β-barrel, to each other and to the CI-MPR (15) and revealed the unique, compact arrangement of the three domains (16). However, the mechanism of phosphomannoseyl recognition by the CI-MPR was not clear since this protein (domains 1–3) contained within its carbohydrate binding pocket a non-phosphorylated oligosaccharide from a neighboring molecule in the crystal lattice (16). We now report the crystal structure of domains 1–3 of the bovine CI-MPR in complex with Man-6-P.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Recombinant protein encoding residues 1–432 (domains 1 residues 1–124), 2 (residues 125–281), and 3 (residues 282–432; domains 1–3) of the mature bovine CI-MPR was expressed in Trichoplusia ni 5B1–4 cells and purified as described previously (14). Briefly, media containing the secreted protein was dialyzed extensively against buffer containing 50 mM imidazole, pH 6.7, 150 mM NaCl, 10 mM MnCl₂, 5 mM β-glycerophosphate before loading...
on a pentamannosyl phosphate-agarose column. The protein was eluted from the column with the imidazole buffer containing 10 mM Man-6-P (Sigma).

Protein Crystalization and Data Collection—Protein was concentrated to ~12 mg/ml before use in crystallization studies. Protein crystals were grown by mixing 2 µl of protein solution with 2 µl of precipitating solution (0.1 M sodium cacodylate, pH 6.35, 25% polyethylene glycol 4000). The drops were allowed to equilibrate over 500 µl of precipitating solution at 19 °C, and thin, rectangular plate-shaped crystals appeared typically within 3–7 days. Crystals were soaked for 15 min in a cryoprotection solution containing 0.1 M sodium cacodylate, pH 6.35, 28% polyethylene glycol 4000, and 20% glycerol before flash-freezing in liquid nitrogen. A native data set and an osmium-soaked data set of domains 1–3 were collected using an R-AXIS IIC image plate equipped with a Rigaku RU200 generator, a Rigaku/MSC X-stream cooling system operating at −175 °C, and Osmic blue confocal mirrors. The osmium derivative crystal was prepared by soaking a crystal in the precipitating solution with an additional 3% polyethylene glycol 4000 and 10 mM K2OsCl6. Although different preparations of the domains 1–3 protein were purified in the same manner and crystallized under the same conditions, the crystals obtained belonged to two different crystal forms, one monoclinic (space group, P21) and the other orthorhombic (space group, P22121) crystal packing to accommodate several sugar residues. Domains 1, 2, and 3 each fold into a flattened 9-stranded β-barrel composed of a four-stranded antiparallel β-sheet (β1–β4) with its strand direction orthogonally oriented over a five-stranded β-sheet (β5–β9) in which β9 interjects between β7 and β8 (Fig. 2B). Each domain contains four disulfide bridges. The domains are joined together through linker regions composed of flexible coils and two short anti-parallel β-strands (−2β and −1β). The overall fold of the two molecules is the same (r.m.s.d. is 0.71 Å for all Cα-atoms) as are the corresponding individual domains (the r.m.s.d. is 0.77, 0.65, and 0.42 Å for domains 1, 2, and 3, respectively). The current structure is also similar to the previously reported structure of domains 1–3 bound to a non-phosphorylated oligosaccharide of a crystallographic neighbor (16), with r.m.s.d. of 0.67 and 0.68 Å for molecules A and B, respectively, for all Ca atoms. The previously reported structure (16) was solved from crystals belonging to an orthorhombic space group (P212121) compared with the monoclinic space group (P21) presented here. These results support the conclusion that the three-dimensional arrangement observed between domains 1, 2, and 3 of the CI-MPR is not the result of crystal packing but is likely the most stable structure in solution.

Carbohydrate Binding Site—Although the crystal structure of the CD-MPR in the presence of phosphomannosyl residues has provided much information concerning the mechanism of carbohydrate recognition by this dimeric receptor (15, 22), the molecular basis for carbohydrate binding by the CI-MPR has

**RESULTS AND DISCUSSION**

**Overall Structure**—The N-terminal 432 residues of the bovine CI-MPR, comprising three of the 15 domains of the receptor extracellular region (Fig. 2A), was expressed and purified from the medium of T. ni 5B1–4 insect cells (14). The purified protein was crystallized in the presence of Man-6-P, and the structure has been refined to 2.1 Å with good geometry (Table I). There are two molecules in the asymmetric unit with the noncrystallographic symmetry axis located between the adjacent carbohydrate ligands. Molecule A has two regions of non-continuous density and is composed of residues 7–82, 88–307, and 312–432. In contrast, molecule B has three regions of discontinuous density, and the model is composed of residues 7–82, 87–229, 234–309, and 311–432. In both molecules A and B, two of the three potential glycosylation sites (Asn-76 and Asn-365) show density for the attached oligosaccharides (Table I). Although no density is detected for an oligosaccharide attached to Asn-400 in either molecule, there is ample room in the crystal packing to accommodate several sugar residues. Domains 1, 2, and 3 each fold into a flattened 9-stranded β-barrel composed of a four-stranded antiparallel β-sheet (β1–β4) with its strand direction orthogonally oriented over a five-stranded β-sheet (β5–β9) in which β9 interjects between β7 and β8 (Fig. 2B). Each domain contains four disulfide bridges. The domains are joined together through linker regions composed of flexible coils and two short anti-parallel β-strands (−2β and −1β). The overall fold of the two molecules is the same (r.m.s.d. is 0.71 Å for all Cα-atoms) as are the corresponding individual domains (the r.m.s.d. is 0.77, 0.65, and 0.42 Å for domains 1, 2, and 3, respectively). The current structure is also similar to the previously reported structure of domains 1–3 bound to a non-phosphorylated oligosaccharide of a crystallographic neighbor (16), with r.m.s.d. of 0.67 and 0.68 Å for molecules A and B, respectively, for all Ca atoms. The previously reported structure (16) was solved from crystals belonging to an orthorhombic space group (P212121) compared with the monoclinic space group (P21) presented here. These results support the conclusion that the three-dimensional arrangement observed between domains 1, 2, and 3 of the CI-MPR is not the result of crystal packing but is likely the most stable structure in solution.

**Carbohydrate Binding Site**—Although the crystal structure of the CD-MPR in the presence of phosphomannosyl residues has provided much information concerning the mechanism of carbohydrate recognition by this dimeric receptor (15, 22), the molecular basis for carbohydrate binding by the CI-MPR has
been unclear. To predict which residues of the CI-MPR are involved in high affinity ligand binding, a structure-based sequence alignment of the CI-MPR with the CD-MPR was performed followed by site-directed mutagenesis of single residues. These studies demonstrated that substitution of Gln-348, Arg-391, Glu-416, Tyr-421, or Ser-387 within domain 3 resulted in a decrease in the receptor affinity for a lysosomal enzyme, β-glucuronidase, by greater than 1000-fold (12). Four of these residues, Gln-348, Arg-391, Glu-416, and Tyr-421, are also conserved in the CD-MPR and in domain 9 of the CI-MPR (Fig. 2A) and are positioned to directly interact with Man-6-P (Fig. 3, A and B). Qualitative and quantitative binding studies demonstrated

![Fig. 2](image-url)
that substitution of Tyr-324 or Ser-386 within domain 3 resulted in a partial (<100-fold) inhibitory effect on carbohydrate recognition by domains 1–3 of the CI-MPR (12). The structure reveals that Tyr-324 interacts with the 1-hydroxyl group of the mannose ring, whereas Ser-386 (both backbone nitrogen and side-chain hydroxyl) interacts with phosphate oxygens and a water molecule (W1), which is one of the three conserved water molecules found in the binding pocket of both molecules A and B (Fig. 3). Comparison of the two molecules of the asymmetric unit reveals that the distances measured between residues of the CI-MPR and Man-6-P are the same within the limit of error (Fig. 3B), indicating a stable binding site.

Additionally, loop D; the hydroxyl group of Ser-387 participates in a hydrogen-bonding network that appears to be an integral part of the binding pocket (Fig. 3). However, the structure reveals that the hydroxyl group of Ser-387 participates in a hydrogen-bonding network that appears to be an integral part of the binding pocket (Fig. 3). In the current structure, the hydroxyl group of Ser-387 is within hydrogen-bonding distance to another conserved water (W3), which bridges the backbone carbonyl group of Asp-418 of loop D. The backbone carbonyl of Asp-418 in turn interacts with the side-chain nitrogen of Lys-98 in domain 1, which in turn makes a hydrogen bond with the backbone carbonyl of Val-417. Thus, substitution of Ser-387 with alanine would disrupt this intricate hydrogen-bonding network, resulting in a perturbation of the binding pocket and interdomain contacts (see below and Fig. 5).

Furthermore, with the exception of a substitution of Arg for Ser at position 386 in the wallaby CI-MPR, Lys-98, Tyr-324, Gln-348, Ser-386, Ser-387, Arg-391, Glu-416, Asp-418, and Tyr-421 are conserved among all species of the CI-MPR sequenced to date (12), supporting the critical role these residues play in the formation of a high affinity carbohydrate binding site.

Comparison to the Carbohydrate Binding Site of the CD-MPR—The sole members of the P-type family of lectins, the CI-MPR and the CD-MPR, are distinguished from all other lectins by their ability to recognize phosphorylated mannose residues. Our previous crystal structures of the CD-MPR in the
FIG. 3. Carbohydrate binding site interactions. A, stereo view of a ribbon diagram showing the carbohydrate binding site of domain 3 (green) with bound Man-6-P (gold). Three conserved water molecules (W1, W2, W3) are shown as red spheres. Potential hydrogen bonds between atoms are indicated with dashed lines. B, a flattened view of the Man-6-P binding site showing potential hydrogen bonds between residues in domain 3 and Man-6-P (gold). Three conserved water molecules (W1, W2, W3) are shown as red spheres. Distances (Å) between atoms are shown. Purple residues, single amino acid substitutions that abolished Man-6-P binding. Silver residues, single amino acid substitutions that resulted in a partial inhibitory effect on Man-6-P recognition. C, stereo view of a ribbon diagram illustrating the potential role of Ser-387 located in domain 3 (green) in maintaining the integrity of the binding pocket. Potential hydrogen bonds are indicated with dashed lines. The disulfide bridge between Cys-385 and Cys-419 is shown in yellow. Lys-98 in domain 1 is in blue, residues within loop D of domain 3 are in silver, and water molecules are shown as red spheres.
Fig. 4. Similarity between the carbohydrate binding site of the CD-MPR and domain 3 of the CI-MPR. A, stereo view of the solvent-accessible surface of domain 3 of the CI-MPR (green) in complex with Man-6-P (gold). Loop D residues and the associated surface have been colored in silver, whereas loop C residues and the associated surface are highlighted in light green. Surfaces in the region for domain 1 (blue) and domain 2 (pink) are also included for clarity. Surfaces were generated using GRASP (32). B, stereo view of the solvent-accessible surface of the CD-MPR (purple) in complex with Man-6-P (gold). Loop D residues and the associated surface are shown in dark purple. The solvent-accessible surface for monomer 2 of the dimer is shown in cyan. C, stereo view of a ribbon diagram showing the binding site of the CD-MPR (purple) superimposed onto domain 3 of the CI-MPR (green). Loop D has been highlighted for clarity (dark purple, CD-MPR; silver, domain 3). The disulfides are shown in yellow: CD-MPR (Cys-106–Cys-141) and domain 3 (Cys-385–Cys-419). Man-6-P (gold ball-and-stick model) is also shown.
presence of Man-6-P (15) or a phosphorylated oligosaccharide (22) or in the absence of ligand (23) have provided much information concerning the mechanism of ligand binding by the homodimeric CD-MPR. Sequence analyses of the two MPRs, which demonstrated significant sequence homology, similar size, and cysteine distribution between the 15 domains of the CI-MPR and the extracellular region of the CD-MPR, led to the proposal that the receptors arose from duplication of a single ancestral sequence (24) and suggests that the mode of carbohydrate recognition is also conserved between the two receptors. The current structure now allows us to determine whether the mechanism of phosphomannosyl recognition has been conserved by comparing the carbohydrate binding site of the CD-MPR with that of the N-terminal binding site of the CI-MPR.

The overall shape of the binding pocket is clearly different between the two MPRs, with domains 1–3 of the CI-MPR forming a much more shallow and open binding site (Fig. 4A) than that observed in the CD-MPR (Fig. 4B). This is particularly apparent when comparing the solvent inaccessibility of the bound ligand; in the case of the CD-MPR, 80% of the phosphate moiety is solvent-inaccessible compared with 45% for domains 1–3 of the CI-MPR. The openness of the binding pocket of domains 1–3 is consistent with the ability of domains 1–3, but not the CD-MPR, to bind phosphodiesters (14, 25).
The carbohydrate binding pocket of the MPRs can be viewed as having two functional regions; one region ("base") interacts with the sugar ring, and the other region ("lid") interacts with the phosphate moiety. The binding pocket of domain 3, like that of the CD-MPR (15, 22), is composed of four finger-like regions. β-Strands 1 and 2 and β-strands 3 and 4 make up two of the finger-like regions. These four strands form a β-sheet, and in conjunction with residues adjacent to loop D (joins β-strands 8 and 9) form the base of the binding pocket. Residues that interact with the mannose ring (Gln-348, Arg-391, Glu-416, Tyr-421) are conserved between the CD-MPR and domain 3 of the CI-MPR and are located in a strikingly similar position, forming the same contacts with the ligand (Fig. 4C). As observed with domains 1–3 of the CI-MPR (12), substitution of these residues has shown that each is critical for high affinity Man-6-P recognition by the CD-MPR (26). Although the base of the binding site is similar between domains 1–3 and the CD-MPR, the major difference between them resides in the lid region, which is involved in binding the phosphate moiety. The third finger forms a lid and is composed of residues in loop C (connects β-strands 6 and 7). The nature of critical residues for ligand binding within loop C differs between the two receptors; Ser-386 and Ser-387 of domain 3, whereas His-105 of the CD-MPR, have been determined to be important for ligand binding (12, 26, 27). However, two differences in the loop C region appear to contribute to the variation in ligands bound by the two receptors. First, loop C in domain 3 is three residues shorter than that of the CD-MPR (Fig. 2A). Second, the positioning of the disulfide differs; in the CD-MPR the disulfide anchors the C-terminal side of loop C (Cys-106) to the C-terminal side of loop D (Cys-141), whereas in domain 3 of the CI-MPR the disulfide (Cys-385-Cys-419) is positioned toward the front of the binding pocket and serves to anchor adjacent residues (Ser-386 and Ser-387) involved in carbohydrate binding. Cys-385 in domain 3 is not in a position analogous to the corresponding conserved cysteine (Cys-106) of the CD-MPR but, rather, is located in a position analogous to Tyr-102 in the CD-MPR (Fig. 4C). This shift in the positioning of the disulfide allows a portion of the binding pocket to remain open (Fig. 4A) and, along with the shortened length of loop C, provides a structural basis for the ability of domains 1–3, but not the CD-MPR, to bind phosphodiesterases (14). Although the three-dimensional position of the disulfide bridge (Cys-385-Cys-419 in domain 3 and Cys-106-Cys-141 in CD-MPR) within loop C differs between the two receptors, their role in maintaining the proper orientation of residues critical for phosphate binding appears to be identical.

The fourth finger-like region of the binding pocket is formed by loop D. In the CD-MPR this loop is composed of seven residues located between β-strands 8 and 9. The corresponding region of domain 3, which is five residues shorter in length than that of the CD-MPR, forms a type-II β-turn with Val-417 and Thr-420 (corresponding to Glu-134 and Phe-142, respectively, in the CD-MPR) acting as anchor points to β-strands 8 and 9 (Fig. 2A and Fig. 4C). Loop D participates in maintaining the position of Glu-416 and Tyr-421 (Glu-133 and Tyr-143, respectively, in the CD-MPR), which are known to be essential for carbohydrate recognition (12, 26). In domain 3 this function is accomplished by residues of loop D being involved in a series of hydrogen bonds from Ser-387 through a conserved water (W3) to Asp-418 (analogous position to Arg-135 in the CD-MPR), which tether loops C and D together (Fig. 3C). In the CD-MPR this function is accomplished through the use of Arg-135, which contacts the 4′-OH of the sugar moiety. The additional five residues in loop D of the CD-MPR, which have no analogous residues in domain 3 of the CI-MPR, are involved in dimer interface interactions and in the maintenance of the integrity of the binding site. The long loop D of the CD-MPR is dynamic and adopts different conformations in the structure depending on the presence or absence of ligand. In the absence of Man-6-P, residues of loop D are positioned within the binding pocket and function to maintain its integrity by forming contacts with residues essential for carbohydrate binding, holding them in the identical position as when carbohydrate is present (23). Clearly, domains 1–3 of the CI-MPR are unable to undergo a similar type of conformational change upon ligand binding and release as the CD-MPR due to the extreme shortness of its loop D. Additional studies are required to determine the nature of the conformational changes that the CI-MPR undergoes triggered by ligand binding and/or release.

**Phosphoester Specificity**—In addition to having significant differences with the CD-MPR, domains 1–3 also differ from the binding site localized to domain 9 of the CI-MPR. Previous studies have shown that the N-terminal binding site of the CI-MPR recognizes the greatest variety of ligands; the CD-MPR and domain 9 of the CI-MPR are highly specific for phosphomonoesters, whereas domains 1–3 of the CI-MPR can also recognize mannose 6-sulfate and phosphodiesterases (14). These inhibition studies demonstrated that domains 1–3 have a greater ability than either domain 9 or the CD-MPR to bind mannose 6-sulfate and mannose 6-phosphate methyl ester with only a 22- and 11-fold lower affinity, respectively, than Man-6-P. Conversely, a construct encoding domains 7–9 displayed almost an 800- and 400-fold lower affinity, respectively, toward these same ligands. We have performed a structure-based sequence alignment of domains 1, 2, 3, and 11 of the CI-MPR and the CD-MPR and interjected domain 9 into this alignment to gain insight into the molecular basis for these specificity differences (Fig. 2A). As described above, a portion of the binding pocket is composed of residues of loop C. The CD-MPR has the longest loop C, with three extra residues compared with both domain 3 and domain 9 (Fig. 2A). Domain 9 and the CD-MPR both have a bulky residue, His-1285 and Asn-104, respectively, in the position analogous to Ser-386 in domain 3 (Fig. 2A). In addition, the CD-MPR has extra residues, Asp-103 and His-105, making the binding cavity even more occluded. Substitution of a histidine in place of the smaller serine into the structure of domains 1–3 at this position is predicted to result in a smaller binding pocket. Thus, the presence of His-1285 in domain 9 may prevent this binding site from being able to accommodate a phosphodiester. Additional structural studies will be required to confirm whether His-1285 of domain 9 functions in a similar fashion to that of His-105 in the CD-MPR to prevent binding of phosphodiesterases.

**Interdomain Contacts**—Expression of truncated forms of the CI-MPR demonstrated that domain 9 can be expressed as an isolated domain, retaining high affinity (Kd ~ 1 nM) carbohydrate binding, whereas expression of domain 3 alone resulted in a protein capable of only low affinity binding (Kd ~ 1 μM) toward a lysosomal enzyme. Furthermore, expression of domains 1–3 resulted in a high affinity binding site (Kd ~ 1 nM), suggesting that residues in adjacent domains (domains 1 and/or 2) are important for the stability and/or formation of this binding site (13). Our current and previous structure (16) of domains 1–3 supports this hypothesis.

The carbohydrate binding pocket region of domain 3 is the only location in the structure where all three domains come into contact with one another. The region around loop C of domain 3 makes several contacts with residues in domain 1 through a series of conserved bridging waters. The γ-amide nitrogen of Glu-390 makes a direct hydrogen bond with the guanidinium group of Arg-118, and both the main chain car-
bonyl and amide nitrogen also utilize three bridging waters to contact the guanidinium group of Arg-118 as well as the carbonyl oxygen of Cys-96 (Fig. 5A). This anchors β-strands 7 and 9 of domain 1 to β-strand 7 of domain 3. These interactions provide stabilization to loop C of the CI-MPR much like the long loop D does in the CD-MPR. Arg-70 in domain 1 interacts with Glu-384 in loop C of domain 3, and Lys-124 and Lys-125, located at the junction of domains 1 and 2, interact with Glu-384 and Ser-386, respectively. As mentioned above (see Fig. 3), Lys-98 in domain 1 is part of a hydrogen-bonding network in which the nitrogen of Lys-98 interacts with both the backbone carbonyls of Val-417 and Asp-418 in loop D of domain 3 (Fig. 3C and Fig. 5A). Thus, these multiple interactions between residues of domain 1 and 2 with residues of loops C and D of domain 3 are likely to aid in the stabilization of this portion of the binding pocket and provide an explanation for the inability of a construct encoding domain 3 alone to generate a high affinity carbohydrate binding site. On the basis of the observation that domain 9 can be expressed as an individual domain, retaining high affinity binding capabilities (13), its mechanism of maintaining and stabilizing its binding pocket must be fundamentally different from that used by the N-terminal binding site of the CI-MPR. Further structural studies will be needed to elucidate how domain 9 folds into a stable conformation that is optimized for the recognition of phosphomonoesters.

Possible Role of the Linker Region between Domains—We have previously reported the structure of domains 1–3, which crystallized with a different ligand (i.e. non-phosphorylated oligosaccharide) and in a different space group (16) than the current crystal. The two crystal forms (orthorhombic versus monoclinic) yielded the same three-dimensional arrangement of domains, strongly indicating that the structure of the N-terminal region of the CI-MPR is representative of the native structure and is not an artifact of the crystal packing. However, when individual domains of the five known structures (the orthorhombic and two molecules from each of the monoclinic crystals presented here) are superimposed and the rest of the molecule is allowed to follow, the observed deviations suggest that the linker regions exhibit some flexibility; superimposition of corresponding domains produces an average r.m.s.d. of ~0.5 Å, whereas the trailing domains have r.m.s.d. ranging from ~1.0 to ~1.4 Å. In addition to the linker regions, the nature of the interface between domains imparts stabilizing and destabilizing features to the structure. Domain 3 shares considerable interface areas with the other two domains: domains 1 and 3, 700 Å²; domains 2 and 3, 1350 Å² (Fig. 5B). The largest interface (~1700 Å²) occurs between domains 1 and 2. All three interfaces have similar numbers of hydrogen bonds (2–3) and salt bridges (2–3) between them. However the interface between domains 1 and 2 is unique from the other two in that it has a large number of hydrophobic interactions (10) compared with the others (0 for domains 1 and 3, 2 for domains 2 and 3). The majority (70%) of the hydrophobic interactions between domain 1 and 2 occurs between β-strand 1 of domain 1 and the linker region connecting domains 1 and 2, which is also part of the proposed site of interaction between the CI-MPR (residues 1–73) and urokinase-type plasminogen activator receptor and plasminogen (28). The enrichment of hydrophobic residues in the interface between domains 1 and 2 is consistent with a mechanism designed to generate a stable cleft between domains 1 and 2 that houses the urokinase plasminogen activator receptor/plasminogen binding site.

An important feature in the intracellular transport of lysosomal enzymes to lysosomes is the ability of the MPRs to release their ligands in the acidic environment of late endosomal compartments. The essential nature of this acidic-dependent release mechanism has been revealed in studies where the neutralization of intracellular compartments results in the excessive secretion of lysosomal enzymes (29, 30). It is intriguing to hypothesize a mechanism for ligand release based on the flexibility of the linker regions connecting the domains and the nature of the interface between domains. The interface between domains 2 and 3 and between domains 1 and 3, both of which participate in stabilizing high affinity Man-6-P binding, are more likely to be influenced by changes in pH or ionic conditions than the highly hydrophobic interface between domains 1 and 2. Thus, the acidic conditions encountered by the CI-MPR as it traverses through endosomal compartments may result in the disruption of key salt bridges, leading to the destabilization of the binding pocket and release of ligand. Assessment of the conformation of the three domains relative to each other under different pH conditions will be needed to test this hypothesis.

In summary, although both the CD-MPR and CI-MPR exhibit a similar affinity for the phosphomonoester Man-6-P (~10 μM), their binding sites are structurally distinct. The strict requirement for a terminal mannose ring by both receptors is reflected in the similarities in that region of the binding pocket responsible for sugar recognition: Glu-416, Tyr-421, Gln-348, and Arg-391 of domain 3 of the CI-MPR are conserved in the CD-MPR and domain 9 of the CI-MPR, and their positioning in the binding pocket is identical between domains 1–3 and the CD-MPR (Fig. 4C). However, diversity in ligand recognition appears to be obtained by alterations in the receptor binding site architecture surrounding the phosphate moiety. The N-terminal binding site of the CI-MPR, unlike the CD-MPR or domain 9 of the CI-MPR, is more promiscuous in that it binds mannose 6-sulfate and phosphodiesterase in addition to phosphomonoesters. This diversity appears to be the result of a shortening of both loops C and D, which effectively makes the binding pocket of domains 1–3 more open than that of the CD-MPR. Although the CD-MPR and domain 9 of the CI-MPR are highly specific for phosphomonoesters, analysis of the primary sequence of domain 9 predicts that the lengths of its loops C and D are short and similar to that of domain 3 (Fig. 2A). Therefore, it will be exciting to determine the structure of domain 9 to decipher the two different mechanisms that have evolved for the recognition of phosphomonoesters.

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J. Biol. Chem. 2004, 279:34000-34009.
doi: 10.1074/jbc.M404588200 originally published online May 28, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404588200

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