Published in final edited form as:

Biochim Biophys Acta. 2011 September ; 1810(9): 815–826. doi:10.1016/j.bbagen.2011.06.016.

Mannose 6-Phosphate Receptor Homology (MRH) Domain-Containing Lectins in the Secretory Pathway

Alicia C. Castonguay, Linda J. Olson, and Nancy M. Dahms*

Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Abstract

BACKGROUND—The Mannose 6-phosphate Receptor Homology (MRH) domain-containing family of proteins, which include recycling receptors (mannose 6-phosphate receptor, MPRs), resident endoplasmic reticulum (ER) proteins (glucosidase II β -subunit, XTP3-B, OS-9), and a Golgi glycosyltransferase (GlcNAc-phosphotransferase γ -subunit), are characterized by the presence of one or more MRH domains. Many MRH domains act as lectins and bind specific phosphorylated (MPRs) or non-phosphorylated (glucosidase II β -subunit, XTP3-B and OS-9) high mannose-type N-glycans. The MPRs are the only proteins known to bind mannose 6-phosphate (Man-6-P) residues via their MRH domains.

SCOPE OF REVIEW—Recent biochemical and structural studies that have provided valuable insight into the glycan specificity and mechanisms of carbohydrate recognition by this diverse group of MRH domain-containing proteins are highlighted.

MAJOR CONCLUSIONS—Currently, three-dimensional structures are known for ten MRH domains, revealing the conservation of a similar fold. OS-9 and the MPRs use the same four residues (Gln, Arg, Glu, and Tyr) to bind mannose.

GENERAL SIGNIFICANCE—The MRH domain-containing proteins play key roles in the secretory pathway: glucosidase II, XTP3-B, and OS-9 are involved in the recognition of nascent glycoproteins, whereas the MPRs play an essential role in lysosome biogenesis by targeting Man-6-P-containing lysosomal enzymes to the lysosome.

1. Introduction

Approximately one third of all proteins are targeted to the endoplasmic reticulum (ER) for their biosynthesis on membrane-bound ribosomes, and most undergo co-translational N-linked glycosylation. A growing list of sugar-binding proteins have been identified in the ER and Golgi compartments that recognize specific N-glycan structures for either the assessment of protein folding in the ER or the trafficking of cargo through the secretory pathway [1]. The P-type lectins, the 46 kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) and 300 kDa cation-independent MPR (CI-MPR), are the most extensively characterized lectins in the secretory pathway [2]. Comprising the only two members of this family, the MPRs are defined by their unique ability to bind phosphomannosyl residues. Studies performed on fibroblasts from patients with the lysosomal storage disorder,

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*Corresponding author: Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226 Tel.: 414-955-4698 Fax: 414-955-6510, ndahms@mcw.edu .

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mucopolipidosis II (ML-II), led to the discovery of the MPRs and the identification of the mannose 6-phosphate (Man-6-P) recognition marker on soluble lysosomal enzymes [3-6] (Fig. 1). The MPRs are dimeric type I membrane proteins. The CD-MPR has a ~160-residue extracytoplasmic region containing six cysteine residues that form three disulfide linkages. In contrast, the CI-MPR contains a large, ~2300-residue extracytoplasmic region with 15 contiguous domains that exhibit a similar size (~150 residues), amino acid homology (15-40% identity), and cysteine profile when compared individually and to the CD-MPR. MPRs are ubiquitously expressed in vertebrate species, including zebrafish (*Danio rerio*) [7], and have been identified in several invertebrates (for review see [8]).

A sequence homology search led to the identification of several proteins that contain a domain similar to the MPRs. Known as the Mannose 6-phosphate Receptor Homology (MRH) domain, MRH domains display a similar size and conservation of residues, including cysteines involved in disulfide bonding [9] (Fig. 2A). These proteins include fungi Mr11 and Yos9p, fly lysosomal enzyme receptor protein (LERP) [10], mammalian glucosidase II β -subunit, OS-9 (ortholog of fungi Yos9p), XTP3-B (also known as Erlectin), and GlcNAc-phosphotransferase γ -subunit, although none to date exhibit the ability to recognize Man-6-P residues (Fig. 2B). Like the MPRs, Mr11 and LERP are type I membrane glycoproteins, with Mr11 containing a single MRH domain and LERP containing five contiguous MRH domains (Fig. 2B). Gene deletion studies indicate that Mr11 functions in concert with Vps10 in the targeting of selected vacuolar hydrolases in *Saccharomyces cerevisiae*. However, the molecular basis for Mr11's interaction with vacuolar hydrolases (*i.e.*, glycan- and/or peptide-dependent) is not known [11]. LERP has been implicated in lysosomal enzyme targeting in *Drosophila melanogaster*, as evidenced by its ability to rescue the missorting of acid hydrolases in MPR-deficient mammalian cells [10]. However, this interaction is not Man-6-P-dependent based on the observations that LERP failed to bind phosphomannan-containing resin and the addition of Man-6-P to the medium did not inhibit LERP-mediated sorting of mammalian acid hydrolases. Clearly, additional studies are needed to elucidate the role of the MRH domain(s) in Mr11 and LERP and the function of these receptors in yeast and *D. melanogaster*, respectively.

Unlike these membrane proteins, the other MRH-containing proteins reside as soluble proteins in the ER (glucosidase II β -subunit, Yos9p, OS-9, XTP3-B) or Golgi (GlcNAc-phosphotransferase γ -subunit) (Fig. 2B). Glucosidase II is a heterodimer that participates in the calnexin/calreticulin cycle by removing the single glucose residue that is added to misfolded glycoproteins by UDP-Glc:glycoprotein glucosyltransferase. Although its interaction with specific glycan structures has been probed, the role of the β -subunit in the deglycosylation of N-glycans carried out by the catalytic α -subunit remains unclear (reviewed in [12]). Yos9p, OS-9, and XTP3-B have been implicated in the recognition of misfolded glycoproteins targeted for degradation through their ability to bind specific high mannose-type N-glycans (reviewed in [1, 13-15]). GlcNAc-phosphotransferase recognizes a conformation-dependent protein determinant on lysosomal enzymes and carries out the transfer of GlcNAc-1-phosphate to mannose residues on their N-glycans [16] (Fig. 1, step 1). The non-catalytic γ -subunit of the $\alpha_2\beta_2\gamma_2$ hexameric complex is not essential for the enzyme's recognition of its substrates [17, 18]. It has been proposed that the γ -subunit binds high mannose N-glycans and favorably presents the glycan to the catalytic α/β subunits, based on the observation that the γ -subunit facilitates the addition of a second GlcNAc-P to a subset of lysosomal enzymes [18]. However, GlcNAc-phosphotransferase γ -subunit binding to glycans has not been directly demonstrated.

To date, the three-dimensional structures of the extracytoplasmic MRH domain of the CD-MPR and 8 (domains 1, 2, 3, 5, 11, 12, 13, 14) out of the 15 MRH domains of the CI-MPR have been determined and are shown to exhibit the same fold [19-24]. The recent crystal

structure of human OS-9 [25] demonstrates the conservation of the MRH fold in proteins that do not bind phosphorylated mannose (Fig. 2C). This review will highlight recent advances in our understanding of the glycan specificity of the MRH-containing proteins and how the MRH fold is ideally suited for mannose recognition.

2. Role of MPRs in the Biogenesis of Lysosomes

2.1. Post-translational modification of lysosomal enzymes

Lysosomes contain a repertoire of ~60 different soluble hydrolytic enzymes (“acid hydrolases”) that function in the degradation of proteins, lipids, and carbohydrates [26]. Lysosomal enzymes, along with thousands of other proteins, are synthesized in the ER and undergo N-glycosylation. Soluble acid hydrolases are marked with a unique tag, Man-6-P, on their N-linked glycans which distinguishes these hydrolytic enzymes from other proteins in the secretory pathway and allows for their specific transport to endosomal/lysosomal compartments by the CI-MPR and CD-MPR [27, 28] (Fig. 1). Optimal ligand binding by the MPRs is observed at pH ~6.4, with little or no ligand binding occurring below pH 5, a finding that is consistent with the acid-dependent dissociation of MPRs from their cargo in the acidic environment of the late endosome [29-34].

Acquisition of Man-6-P residues occurs in a two-step process. In early Golgi compartments, UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase; EC 2.7.8.17) attaches GlcNAc-1-phosphate to the C-6 hydroxyl group of one or more mannose residues to form a phosphodiester, mannose 6-phosphate N-acetylglucosamine ester (Man-P-GlcNAc) [35-38] (Fig. 1, step 1 & Fig. 2B). GlcNAc-phosphotransferase selectively phosphorylates N-linked high mannose-type oligosaccharides in the *cis* Golgi through its ability to recognize surface patches containing two or more lysine residues that are appropriately spaced relative to each other and the oligosaccharide chain on acid hydrolases [39-41]. A second enzyme, N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase, which is referred to as uncovering enzyme (EC 3.1.4.45), removes the GlcNAc moiety in the *trans* Golgi network (TGN) revealing a phosphomonoester, Man-6-P [42-46] (Fig. 1, step 2 & Fig. 2B). The activity of both enzymes is regulated by proteolysis. Proteolytic processing of GlcNAc-phosphotransferase's α/β subunits, by a yet to be identified protease, is required for its optimum activity [47], whereas furin cleaves uncovering enzyme, a type I membrane protein localized to the TGN, to generate the mature, active enzyme [45, 46].

2.2. CI-MPR interacts with multiple ligands at the cell surface

Enzyme replacement therapy for the treatment of patients with lysosomal storage disorders (LSDs) capitalizes on the ability of the CI-MPR, but not the CD-MPR, to function at the cell surface. The missing lysosomal enzyme is intravenously injected and is subsequently internalized by the CI-MPR [48]. Several secreted proteins have also been identified that are modified with Man-6-P, such as leukemia inhibitory factor (LIF) [49] and transforming growth factor- β (TGF- β) precursor [50] (for a list of CI-MPR ligands the reader is referred to [51]). The percentage of proteins identified that acquire the Man-6-P tag is increasing, as recent proteomic approaches using CI-MPR affinity columns have discovered new Man-6-P-containing proteins, including some that are not hydrolytic enzymes (reviewed in [52]). The process by which non-lysosomal proteins acquire Man-6-P residues is not understood, but it is hypothesized that these proteins encounter the same GlcNAc-phosphotransferase as acid hydrolases and are able to avoid the MPRs in the TGN by an unknown mechanism to allow for their secretion.

Unlike the CD-MPR, the CI-MPR interacts with several non-Man-6-P-containing ligands including insulin-like growth factor-II (IGF-II) [53, 54], urokinase-type plasminogen

activator receptor [55], and plasminogen [56] (for a list of CI-MPR ligands the reader is referred to [51]). However, only the CI-MPR's interaction with IGF-II has been extensively characterized. The IGF-II binding site has been mapped to domain 11 (see [57] for review), with the 43-residue fibronectin type II insert in domain 13 enhancing the affinity of this interaction by ~10 fold [23, 58, 59] (Fig. 2B). Furthermore, IGF-II is bound in a homologous position in domain 11 as Man-6-P in domains 3 and 5 [23]. Recent studies have shown Lys residues on the CI-MPR are key for its interaction with plasminogen [60]. Clearly, additional structural information is needed to further understand the CI-MPR's interaction with plasminogen and other non-Man-6-P-containing ligands.

3. Comparison of the carbohydrate binding properties of the MPRs' MRH domains

3.1. The MPRs recognize distinct but overlapping populations of lysosomal enzymes

The existence of ~40 different LSDs, which together, affect an estimated 1 in 5000 live births [61], highlights the importance of the phosphomannosyl recognition system in generating a lysosome that contains a full repertoire of acid hydrolases. Most LSDs are due to a deficiency in a single lysosomal enzyme. However, impaired GlcNAc-phosphotransferase activity results in the mis-sorting and secretion of numerous lysosomal enzymes due to their inability to acquire Man-6-P residues and be trafficked by the MPRs, causing the LSDs, mucopolidosis II and III (also referred to as "I-cell disease" and "pseudo-Hurler polydystrophy", respectively) (for reviews see [62, 63]). As anticipated from these findings, fibroblasts derived from transgenic mice lacking both MPRs demonstrate a similar phenotype to ML-II fibroblasts, as evidenced by the secretion of most acid hydrolases and accumulation of undigested matter in the lysosomes of these cells [64-66].

Studies in mouse fibroblasts lacking either the CD-MPR or CI-MPR have shown that both MPRs are necessary for targeting the entire repertoire of Man-6-P containing acid hydrolases, because individually, neither receptor can completely compensate for the loss of the other [67, 68]. These findings may explain, in part, the observation that both MPRs are expressed in most cell types [69]. The ability of the MPRs to recognize distinct but overlapping populations of lysosomal enzymes was further confirmed by a recent proteomic study which analyzed serum from transgenic mice deficient in either the CD-MPR or CI-MPR. The results indicate that several lysosomal enzymes, including cathepsin D and α -mannosidase 2B1, are selectively transported by the CI-MPR, while heparanase and tripeptidyl peptidase I are recognized preferentially by the CD-MPR [70]. However, the molecular bases underlying the differential binding by the two MPRs to these lysosomal enzymes are not known.

3.2. Phosphomannosyl binding properties of the MPRs

Major differences between the two MPRs include the number of carbohydrate binding sites and the type of phosphomannosyl residues recognized. The CD-MPR contains one Man-6-P binding site per polypeptide chain [29], while the CI-MPR contains three Man-6-P binding sites which are localized to MRH domains 3, 5, and 9 [71, 72] (Fig. 2B). While both receptors bind with similar affinity to the monosaccharide Man-6-P ($K_d = \sim 10 \mu\text{M}$) [29], only the CI-MPR can bind the phosphodiester, Man-P-GlcNAc [73, 74]. In addition, the MPRs bind mannose or glucose 6-phosphate with poor affinity ($K_i = 1-5 \times 10^{-2}\text{M}$), demonstrating that the axial 2-hydroxyl group and the 6-phosphate group are important determinants of binding specificity [29].

To further evaluate the CI-MPR's three carbohydrate binding sites, truncated forms of the CI-MPR were analyzed, revealing the presence of two high affinity Man-6-P binding sites,

which map to domains 1-3 and 9 [75], and a low affinity Man-6-P site located in domain 5 [72]. Unlike domain 9, additional domains influence the binding affinity of domain 3: a construct encoding domains 1-3 exhibits ~1,000-fold higher affinity for lysosomal enzymes than domain 3 alone [75]. Domains 3 and 9 share a similar preference for phosphomonoesters as the CD-MPR. However, unlike CD-MPR, CI-MPR's domain 3 is able to bind carbohydrates found on lysosomal enzymes from *Dictyostelium discoideum*, which contain Man-6-P-OCH₃ phosphodiester and mannose 6-sulfate residues, but with a 10-fold or 20-fold lower affinity than Man-6-P, respectively [33, 76, 77]. Recently, surface plasmon resonance (SPR) analyses using lysosomal enzymes bearing either phosphomonoesters or phosphodiester mapped the location of the CI-MPR's Man-P-GlcNAc binding site, and demonstrated that domain 5 binds Man-P-GlcNAc phosphodiester with a 14-18 fold higher affinity than Man-6-P [78].

The unique ability of the CI-MPR to recognize the product of GlcNAc-phosphotransferase (Fig. 1, step 1) via its MRH domain 5 is further supported by cell-based assays and animal models. Uptake studies using fibroblast cells expressing mutant CI-MPR lacking functional phosphomonoester binding sites in domains 3 and 9 resulted in the internalization of only Man-P-GlcNAc-containing acid hydrolases, with no internalization being detected for the Man-6-P containing acid hydrolases [24]. These results indicate a role for domain 5 in the trafficking of phosphodiester-containing lysosomal enzymes. Transgenic mice that lack the uncovering enzyme (Fig. 1, step 2), which results in their lysosomal enzymes bearing phosphodiester, grew normally and exhibited no histological abnormalities [79]. The normal phenotype observed in these mice suggests that the CI-MPR, due to its ability to bind Man-P-GlcNAc, rescues these mice from the severe ML-II-like phenotype observed in mice lacking GlcNAc-phosphotransferase activity.

3.3. Glycan specificity of the MPRs

N-linked glycans on lysosomal enzymes are heterogeneous in structure, differing in their size, number and location of phosphorylated mannose residues. In addition, the presence of Man-6-P and/or Man-P-GlcNAc further increases the diversity of potential ligands for the MPRs [36, 80]. To understand how the MPRs function as efficient carriers of a diverse population of lysosomal enzymes, a phosphorylated glycan microarray containing purified high-mannose-type glycans with either phosphomonoesters or phosphodiester was probed with the CD-MPR and truncated forms of the CI-MPR [74, 81]. The glycan microarray analyses revealed that the CD-MPR and domains 3, 5, and 9 of the CI-MPR each recognize a distinct repertoire of phosphorylated glycans [74, 81] (Fig. 3). Unlike the CI-MPR, the CD-MPR is unable to recognize phosphodiester-containing glycans on the array (Fig. 3). However, neither receptor is able to bind a Man₇ glycan bearing a phosphomannosyl residue on the B (α 1,3 linked mannose E) and/or C arm (α 1,6 linked mannose F) (Fig. 3). Docking of a Man₇ glycan into the binding pocket of the CD-MPR and domain 3 of the CI-MPR indicates that the α 1,6 linked branch is too short to allow the terminal phosphorylated mannose residue (mannose E or F) to extend into the binding pocket, resulting in steric clashes of multiple regions of the glycan with the receptor (data not shown). However, additional studies are required to confirm this prediction.

Although both domains 3 and 9 of the CI-MPR recognize phosphomonoesters, they exhibit significant differences in their preferred glycan structures. Domain 9 shows a broad range of glycan specificity and recognizes glycan structures Man₆GlcNAc₂ to Man₉GlcNAc₂ bearing one or two phosphomonoesters (Fig. 3). Domain 3, in contrast to domain 9 and the CD-MPR, is unable to interact with glycans that displayed a single phosphate on the terminal α 1,2-linked mannose (I) of the C arm (Fig. 3). To assess the molecular basis of this glycan specificity, we have docked a Man₉ glycan with a phosphate on either the A arm (mannose G) or C arm (mannose I), into the binding pocket of domain 3 and the CD-MPR. The models

suggest that while both glycans can fit into the binding pocket of domain 3, the electrostatics of the interaction differ: the non-binding glycan has the A arm extend over a highly charged region of the receptor, whereas the recognizable glycan has the C arm extend over a neutral/hydrophobic groove of domain 3 (data not shown). Additional studies are needed to test this hypothesis. Structural studies of domain 9, which are currently lacking, are needed to understand how domain 9 is able to accommodate these glycans in its binding pocket. However, based on its glycan specificity, the architecture of domain 9's binding pocket is likely to be more similar to the CD-MPR than to domain 3. In contrast to domains 3 and 9, domain 5 of the CI-MPR recognizes a wide range of phosphodiester-containing glycans, with little to no appreciable binding detected to glycans containing phosphomonoesters (Fig. 3) [81]. The combination of the unique glycan specificities of the three carbohydrate binding domains present in the CI-MPR conveys the ability of this receptor to interact with the structurally diverse repertoire of phosphorylated N-glycans present on lysosomal enzymes, and supports previous *in vitro* [34, 82] and *in vivo* [67, 68, 83, 84] studies which show that the CI-MPR is more efficient in targeting lysosomal enzymes to late endosomal compartments than the CD-MPR.

4. Structural comparison of the phosphomannosyl binding pockets of the MPRs

4.1. Conservation of a signature motif for Man-6-P binding

Studies using chemical modifications were the first to implicate Arg and His as important residues for carbohydrate binding by the CD-MPR [85], and the subsequent crystal structure of the bovine CD-MPR's MRH domain in complex with Man-6-P confirmed the presence of Arg and His residues in the binding pocket [86]. The three-dimensional structures of two (domains 3 and 5) out of the three carbohydrate binding domains of the CI-MPR have now been determined. Analyses of the crystal structures of the CD-MPR [86, 87] (Fig. 4A), domains 1-3 [22] (Fig. 4B), and solution structure of domain 5 [24] (Fig. 4C) in complex with Man-6-P revealed the presence of four residues, Gln, Arg, Glu, and Tyr, that serve as a signature motif for carbohydrate binding. Structure-based sequence alignments of the MRH domains of the CI-MPR revealed that all four signature motif residues (Gln, Arg, Glu, and Tyr) are present in only three (domains 3, 5, and 9) out of the fifteen MRH domains (Fig. 2A), which are the only MRH domains shown to date to exhibit carbohydrate binding activity. These conserved residues (CD-MPR: Q66, R111, E133, Y143; Domain 3: Q348, R391, E416, Y421; Domain 5: Q644E, R687K, E709Q, Y714F) contact the 2-, 3-, and 4-hydroxyl groups of the mannose ring (Fig. 4A-C), and mutagenesis studies have confirmed their critical role by demonstrating that substitution of any of the signature motif residues results in a ~1000 fold decrease in binding affinity [78, 88-90].

4.2. Crystal structure of the phosphomonoester-binding MRH domain of the CD-MPR and CI-MPR

The extracytoplasmic domain of the CD-MPR, in complex with Man-6-P, was the first three-dimensional structure of an MRH-containing protein to be determined [86, 87]. This structure established the overall fold of an MRH domain which comprises a flattened β -barrel structure consisting of nine β -strands organized into two orthogonally oriented anti-parallel β -sheets, β 1- β 4 and β 5- β 9, with β 9 interjecting between β 7 and β 8 (Fig. 2C). The CD-MPR crystallized as a dimer, with extensive contacts observed between the two dimer interface β -sheets (β 5- β 9). The N-terminal region of the CD-MPR contains a single α -helix, which is not present in any of the known structures of the CI-MPR's MRH domains (domains 1, 2, 3, 5, 11-14) [19-22, 24] or OS-9 [25] (Fig. 2C). In contrast to the dimeric CD-MPR, the N-terminal three domains of the CI-MPR function as a compact monomer, with the three domains contacting each other mainly through the residues within their loops and

linker regions [21, 22]. These interactions are critical for stabilization of the carbohydrate binding pocket of domain 3, as expression of a construct encoding domain 3 alone fails to bind lysosomal enzymes with high affinity [75], and indicate that domains 1-3 form a structural unit within the full-length CI-MPR. Extensive structural studies revealed that, unlike other lectins, the CD-MPR is dynamic and undergoes significant changes in quaternary structure upon phosphomannosyl binding [91-93]. To date, no structural information is available assessing whether the arrangement of the CI-MPR's MRH domains is altered upon carbohydrate binding.

Although the position of the four essential residues (Gln, Arg, Glu, and Tyr) is nearly identical, the length and positioning of loops C and D differs between the CD-MPR and domain 3 of the CI-MPR. The position of loop C, which caps the phosphate moiety, together with the longer length of loop D results in a binding pocket that in the CD-MPR (Fig. 4A), but not domain 3 (Fig. 4B), tightly engulfs the phosphate moiety. In contrast to the residues involved in mannose binding, the residues involved in phosphate recognition are not conserved. The CD-MPR utilizes three residues, Asp103, Asn104, and His105 to coordinate the phosphate group of Man-6-P [86, 87], while domain 3 of the CI-MPR uses Ser386 and an ordered water molecule [22] (Fig. 2A). Divalent cations are required for optimal ligand binding by the CD-MPR, but not the CI-MPR [29, 90, 94]. Structural analyses of the CD-MPR revealed the presence of a conserved aspartic acid residue (Asp103) in the binding pocket [86, 87], a residue that is not present in the binding pockets of domains 3 and 5 of the CI-MPR [21, 22, 24]. Mutagenesis studies have confirmed the role of Asp103 in conferring cation-dependence of ligand binding by this receptor [90]. Together, these studies indicate that the presence of a Mn^{2+} cation in the binding pocket of the CD-MPR functions to shield the negative charge of Asp103, thus allowing for high affinity binding of the receptor to the phosphate moiety of Man-6-P [86, 87]. The structure-based sequence alignment (see Fig. 2A, compare residues in loop C) predicts that the mode of phosphate recognition by domain 9 of the CI-MPR will differ from that of both CD-MPR and domain 3 of the CI-MPR. Confirmation of this predication awaits structural studies of domain 9, the CI-MPR's remaining phosphomonoester-specific binding site.

4.3. Structural basis for the recognition of phosphodiester by domain 5 of the CI-MPR

To understand the structural basis of how domain 5 of the CI-MPR functions as the only carbohydrate binding domain with the ability to recognize phosphodiester-containing lysosomal enzymes [78, 81], the solution structure of domain 5 has been determined in ligand-free and Man-P-GlcNAc-bound forms [24]. A comparison of the carbohydrate binding pockets from the MPRs demonstrates the striking positional conservation of the four essential residues (Gln, Arg, Glu, and Tyr) that interact with the 2-, 3-, and 4-hydroxyl groups of mannose, and reveals three major differences with respect to: 1) absence of a disulfide bond tethering loops C and D together, 2) length and positioning of loops C and D, and 3) presence of aromatic residues (Fig. 4A-C). Unlike the CD-MPR and domain 3, domain 5 of the CI-MPR lacks two cysteine residues that form a disulfide bridge in domain 3 and the CD-MPR. However, lack of this disulfide bond does not perturb the overall MRH fold of domain 5 (Fig. 2C).

Domain 5 contains a mixture of structural elements from the ligand binding pockets of both the CD-MPR and domain 3. For example, the short length of loop D and the position of loop C resemble that of domain 3, and the combined orientation of loops C and D results in domain 5 having the most open conformation of the binding pockets (Fig. 2A, compare Fig. 4A-C). However, the presence of an additional aromatic residue in domain 5's binding pocket (Y679) is similar to the CD-MPR (Y102) (Fig. 2A, compare Fig. 4A-C). Y679 of domain 5, which is spatially conserved with the disulfide bond of domain 3 (C385-C419) and Y102 of the CD-MPR (compare Fig. 4A-C), interacts with the methyl group of N-

acetylglucosamine. Furthermore, mutagenesis studies have shown that Y679 of domain 5 is required for high affinity binding to phosphodiester [24]. Although CD-MPR's Y102 residue is evolutionarily conserved, the CD-MPR cannot accommodate the large GlcNAc moiety of a phosphodiester due to the unique architecture of its binding pocket that is formed, in part, by loops C and D (see above).

Unique to the binding pocket of domain 5 is a tryptophan residue (W653), which is absent in the other carbohydrate binding domains of the CI-MPR (Fig. 2A, compare Fig. 4A-C). NMR analyses indicate that W653 in domain 5 orients the 2-hydroxyl group of the mannose ring so that it is positioned toward the side chains of essential residues, R687 and Q644 [24]. The indole ring of W653 is positioned to interact with the guanidinium group of the essential R687, an interaction which is predicted to stabilize domain 5's binding pocket, thus compensating for the missing disulfide bridge. SPR analyses of domain 5 containing single amino acid substitutions were conducted to probe the role of W653 in binding to immobilized lysosomal enzymes bearing either phosphodiester or phosphomonoester glycans. Substitution of W653 with an aromatic residue, phenylalanine, resulted in a ~10-fold decrease in affinity for phosphomonoesters and phosphodiesters, while substitution of W653 with non-aromatic residues, lysine and threonine, resulted in no detectable binding to either ligand (Fig. 4D). Taken together, these results indicate that W653 in domain 5 is important for carbohydrate recognition, and supports the hypothesis that the indole ring of W653 interacts with guanidinium group of R687, as substitution of a Lys or Thr, but not Phe, completely abrogated binding to the GAA monoester and GAA diester surfaces. Although domain 5 displays a similar fold to domain 3 and the CD-MPR, the binding pocket of domain 5 has a unique architecture that allows for the accommodation of a GlcNAc residue, and thus binding of phosphodiester-containing lysosomal enzymes.

5. MRH domain-containing proteins in the ER

5.1. Glycan specificity of ER resident glucosidase II β -subunit, XTP3-B, and OS-9

Glucosidase II is an ER luminal protein comprised of an α - β heterodimer that is involved in the processing of N-glycans by removing α 1,3-linked glucose residues on nascent glycoproteins [95]. The ~55kDa non-catalytic β -subunit contains an MRH domain and a C-terminal ER retention signal (XDEL) (Fig. 2B), and studies in both fungi and mammals implicate its involvement in N-glycan processing (reviewed in [12]). Recent frontal affinity chromatography analyses revealed the requirement of a terminal α 1,2 linked mannose residue on the B or C arms of the N-glycan (with a preference for the C arm) for recognition by the MRH domain of human glucosidase II's β -subunit [96] (Fig. 3). This specificity for mannose residues on the B and C arms is also observed in fission yeast [97].

XTP3-B, also known as Erlectin, is an ER luminal protein that contains two MRH domains (Fig. 2B), of which only the second MRH domain has been shown to mediate the interaction of Kremen2, a coreceptor involved in early *Xenopus* development, in an N-glycan dependent manner [98]. Studies performed on human XTP3-B have implicated MRH domain 2 in mammalian endoplasmic reticulum-associated degradation (ERAD) through the recognition of N-glycans on SEL1L, a resident ER glycoprotein that is part of a ubiquitin ligase complex with HRD1 [99, 100]. Flow cytometric analyses of chimeric proteins containing the N-terminal or C-terminal MRH domain of XTP3-B demonstrated that only the C-terminal MRH domain is able to interact with glycans containing a terminal Man α 1,6Man sequence on the C arm [99] (Fig. 3). Both the N- and C-terminal MRH domains contain the four conserved residues (Gln, Arg, Glu, and Tyr) (Fig. 2A). However, unlike the other MRH domains, the N-terminal MRH domain of XTP3-B contains a stretch of ~45 residues enriched in Lys and Glu residues (Fig. 2A). Additional studies will be required to test the

possibility that this highly charged region negates the lectin activity of XTP3-B's N-terminal MRH domain.

Of the non-MPR MRH-containing proteins, OS-9 is the most extensively characterized biochemically and structurally. OS-9, an ER resident protein that contains a single MRH domain (Fig. 2B) and lacks the ER retention signal found in its yeast ortholog, Yos9p, was first identified as a protein upregulated in human osteosarcomas [101]. The MRH domain of OS-9 has been implicated in glycoprotein ERAD via its recognition of specific N-glycans on misfolded glycoproteins (reviewed in [15]). OS-9 exhibits a similar glycan specificity as XTP3-B, and binds glycans containing a terminal Man α 1,6Man sequence on the C arm [102] (Fig. 3). This binding preference is conserved in yeast Yos9p, with the exception that the yeast ortholog binds with very high affinity to a Man5 glycan containing the terminal Man α 1,6Man sequence [103].

5.2. Crystal structure of OS-9

The recent crystal structure of the MRH domain of human OS-9 in complex with a branched pentasaccharide Man α 1,3(Man α 1,6)Man α 1,6(Man α 1,3)Man shows that this MRH domain adopts a flattened β -barrel structure with the characteristic P-type lectin fold [25] (Fig. 2C). The oligosaccharide binding site of OS-9 contains all four conserved essential residues (Gln, Arg, Glu, and Tyr) (Fig. 2A), which when mutated, abolish or diminish ligand binding [104]. A comparison of the carbohydrate binding pockets of the MPRs and OS-9 reveals the striking positional conservation of these four residues (Fig. 5A). All hydroxyls of the non-reducing α 1,6-linked mannose ring are involved in hydrogen bonding with OS-9 (Fig. 5B). OS-9 utilizes a distinctive double tryptophan motif (WW) to recognize Man α 1,6Man α 1,6Man residues on the processed C-arm of N-linked glycans [25] (Fig. 2A & 5B). The contribution of each of the Trp residues (W117, W118) in the binding pocket of the OS-9 MRH domain was assessed by site-directed mutagenesis. These studies showed that the W117A mutant had a 25-fold reduction in binding to Lec1 cells, which are enriched in Man₅GlcNAc₂ N-glycans on the cell surface, while the W118A mutant completely abrogated binding to the cells [25]. Satoh et al. also suggest that W118 confers linkage specificity of OS-9 and XTP3-B to Man α 1,6Man, whereas the corresponding Tyr found in the MPRs (CD-MPR Y45 Fig. 4A; domain 3 Y324 Fig. 4B; domain 5 Y621 Fig. 4C) and glucosidase II β -subunit (Fig. 2A), may be a critical residue involved in the recognition of α 1,2 linked mannose, the preferred linkage of these MRH domains. Additional studies are needed to test whether a single residue is sufficient to confer linkage specificity.

Given that OS-9 and the MPRs share the same overall fold and the identical four residues that contact the terminal mannose residue, what is responsible for OS-9's specificity for non-phosphorylated high mannose-type glycans? A comparison of the residues in loop C of the MPRs that are involved in phosphate recognition reveal that not only does the position on this loop differ (Fig. 5A), but the residues within this loop are not conserved (Fig. 2A). OS-9 contains Asp182 and Leu183 in its loop C, residues which are not found in the other binding pockets (Fig. 2A, Fig. 5C). Asp182 and Leu183 participate in interactions with the terminal mannose ring. When Man-6-P is modeled into the binding pocket of OS-9 (Fig. 5C), there is no room in the binding pocket for the phosphate moiety. In addition, the presence of the negatively charged Asp182 would electrostatically repel the negatively charged phosphate group of Man-6-P, further negating the ability of OS-9 to interact with phosphorylated glycans. Based on OS-9's subcellular localization in the ER, it is not surprising that its binding pocket has been optimized to recognize high mannose-type glycans. Taken together, these studies suggest that a major contributing factor governing the specificity of an MRH domain for high mannose, phosphomonoester-, or phosphodiester-containing glycans resides in the types of residues within, and the positioning of, loop C.

6. Perspectives

Although much progress has been made in the elucidation of the mechanisms of phosphomannosyl recognition by the MPRs, many important questions are unresolved. It is intriguing that the CI-MPR is currently the only lectin known to recognize the Man-P-GlcNAc phosphodiester, and possesses the ability to bind the products of both enzymes involved in the generation of the phosphomannosyl tag. Does this functional redundancy serve as a protective mechanism for the cell under conditions where either the uncovering enzyme is not activated by furin, or phosphodiester-containing lysosomal enzymes are not efficiently processed by the uncovering enzyme? Unlike the single MRH domain-containing CD-MPR, the CI-MPR can interact with both glycoproteins and non-Man-6-P-containing proteins, such as IGF-II, via its multiple MRH domains. Thus, the MRH fold can function in protein-carbohydrate and protein-protein interactions. It is unclear if all 15 MRH domains of the CI-MPR will eventually be found to serve as ligand binding modules, or will some MRH domains serve solely as a supportive scaffold for a limited number of ligand binding domains? Furthermore, the extent to which the linker region connecting each MRH domain is flexible, and how the various ligand binding sites are oriented with respect to each other in the dimeric CI-MPR, thereby impacting multivalent interactions, is unknown. The crystal structure of OS-9 has revealed that although its MRH domain adopts the characteristic fold observed in the P-type lectins, its mode of mannose recognition differs from both the CD-MPR and CI-MPR. Structural and biochemical analyses of the MRH domains of glucosidase II β -subunit, XTP3-B, GlcNAc-phosphotransferase γ -subunit, LERP, and MrI1 and are needed to clarify their functional role and confirm the prediction that these MRH domains will exhibit a similar fold.

The three carbohydrate binding sites of the CI-MPR evolved to recognize distinct but overlapping populations of phosphorylated N-glycan structures, but it is unclear how these domains acquired the ability to recognize carbohydrates. It is intriguing to speculate that domain 5, which recognizes the product of GlcNAc-phosphotransferase (Fig. 1, step 1), was the first MRH domain of the MPRs to evolve, with domains 3 and 9 of the CI-MPR and the CD-MPR being optimized for phosphomonoester binding following the appearance of the uncovering enzyme (Fig. 1, step 2). *Drosophila* LERP may serve as an intermediate in the evolution of the CI-MPR, as LERP contains five copies of a MRH domain, of which only domains 1 and 4 contain two of the essential residues for Man-6-P binding (for sequence alignment of LERP see [51]). Does fungi MrI1 serve as the primordial MRH domain, as it contains only one of the conserved residues essential for recognition of the mannose ring (for sequence alignment of MrI1 see [51])? Did the ER-resident MRH-containing proteins subsequently evolve, with their binding pockets optimized for high-mannose containing glycans, followed by the Golgi GlcNAc-phosphotransferase, which in turn provided selective pressure in the development of membrane-bound recycling receptors to recognize phosphomannosyl residues? It is interesting to note that the amoeba, *D. discoideum*, expresses a GlcNAc-phosphotransferase lacking a γ -subunit [105]. However, no functional MPRs have been isolated from this organism using classical affinity chromatography approaches. Thus, many questions still remain concerning the evolution and function of the various members of the MRH domain-containing family.

Acknowledgments

We thank Dr. Jung-Ja Kim for helpful discussions and comments on the manuscript. Work described from the authors' laboratory was supported by NIH grant R01 DK042667. The BIAcore 3000 instrument (Protein and Nucleic Acid Core Facility, Medical College of Wisconsin) was purchased through a grant from the Advancing a Healthier Wisconsin program.

Abbreviations

MRH	mannose 6-phosphate receptor homology
MPR	mannose 6-phosphate receptor
CD-MPR	cation-dependent MPR
CI-MPR	cation-independent MPR
Man-6-P	mannose 6-phosphate
ER	endoplasmic reticulum
ML-II	mucopolipidosis II
LERP	lysosomal enzyme receptor protein
GlcNAc-phosphotransferase	UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase
Man-P-GlcNAc uncovering enzyme	mannose 6-phosphate N-acetylglucosamine ester N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase
TGN	<i>trans</i> Golgi network
LSD	lysosomal storage disorder
LIF	leukemia inhibitory factor
TGF-β	transforming growth factor- β precursor
SPR	surface plasmon resonance
ERAD	endoplasmic reticulum associated degradation
IGF-II	insulin-like growth factor-II
GAA	acid α -glucosidase

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Highlights

Recycling receptors, ER proteins, and a glycosyltransferase contain Mannose 6-phosphate Receptor Homology (MRH) domain(s).

Many MRH domains act as lectins and bind specific phosphorylated or non-phosphorylated high mannose-type N-glycans.

Currently, structures are known for ten MRH domains, revealing the conservation of a similar fold.

OS-9 and the mannose 6-phosphate receptors use the same four residues (Gln, Arg, Glu, Tyr) to bind mannose.

Fig. 1.

Subcellular localization of the MRH-containing proteins. Glucosidase II, XTP3-B, and OS-9 are resident, soluble ER proteins that recognize high mannose-type N-glycans, whereas GlcNAc-phosphotransferase is enriched in early Golgi compartments. The MPRs are relatively long-lived (half-life ~20 hrs) proteins that cycle continuously between the TGN, endosomal compartments, and the cell surface [106], an itinerary that requires the recognition of specific motifs in their cytosolic tail by various cytosolic adaptor molecules (reviewed in [27, 28]). In early Golgi compartments, acquisition of the Man-6-P tag on newly synthesized lysosomal enzymes occurs in two steps (see text). The MPRs bind specifically to Man-6-P tagged acid hydrolases in the TGN, and the resulting receptor-enzyme complex is transported to endosomal compartments. Upon acidification of the late endosome, the MPRs dissociate from their cargo and traffic back to the TGN or to the cell surface, where the CI-MPR, but not the CD-MPR, can internalize various Man-6-P and non-Man-6-P containing ligands [27, 107, 108]. For simplicity, the MPRs are depicted as dimeric *green* balls in the cell. On the cell surface the CI-MPR is shown as a dimer consisting of 15 repeating *grey* balls with the three Man-6-P binding domains highlighted in *green*. The GlcNAc moiety of phosphodiesterases on lysosomal enzymes is indicated by a *red square*.

Fig. 2.

Comparison of the MRH-containing proteins. (A) Structure-based sequence alignment of bovine CD-MPR (A27068), domains 3, 5 and 9 of the bovine CI-MPR (A30788) with the MRH domains of human glucosidase II β subunit (CAA04006), human GlcNAc-phosphotransferase γ subunit (Q9UJJ9), the two MRH domains of human XTP3-B (NP_056516), and human OS-9 (BAA24363) (for sequence alignments of LERP and Mrl see [51]). Glucosidase II β subunit, OS-9, XTP3-B, and GlcNAc-phosphotransferase γ subunit contain all four essential residues for Man-6-P recognition (Gln, Arg, Glu, and Tyr), which are shaded in *blue*. The WW motif of OS-9 is boxed in *orange*. W653 and Y679, which are present in the binding pocket of domain 5, are boxed in *blue*. The residues known to interact with the phosphate moiety in the CD-MPR (D103, N104, H105) and Domain 3 (S386) are boxed in *red*. Residues in OS-9 predicted to occlude the binding of the phosphate group of Man-6-P (D182, L183) are boxed in *green*. The cysteine residues are shaded in *yellow*. The secondary structural elements of CD-MPR and OS-9 are shown, with *dark blue* arrows representing the β-strands and the *purple* line representing an α-helix. Location of loops C and D are shown. Sequence alignments were performed using STRAP [109], with minor adjustments performed near residue 709 in domain 5 and residue 1248 in domain 9. (B) Schematic representations of the MRH-containing proteins are shown. The MRH domains are depicted as circles and potential sites of N-glycosylation are shown as *grey* triangles. Mrl1, LERP, CD-MPR, and CI-MPR are type I transmembrane glycoproteins. The 279-residue bovine CD-MPR is a homodimer, and the 2499-residue bovine CI-MPR oligomerizes and most likely exists as a dimer. The CD-MPR contains a single Man-6-P

binding site (outlined in *blue*) per polypeptide. In contrast, the CI-MPR contains three Man-6-P binding sites: two high affinity sites are localized to domains 1-3 and domain 9 (outlined in *blue*), and one low affinity site is present in domain 5. Domain 5 is outlined in *red*, as this domain preferentially binds phosphodiester. Domains 1 and 2 are outlined in *blue* since the presence of these domains have been shown to enhance the affinity of domain 3 for Man-6-P containing lysosomal enzymes by ~1000-fold [75]. Domain 11 (*yellow*) binds IGF-II and domain 13 contains a fibronectin type II insert (*yellow rectangle*). The post-translational modifications, phosphorylation (*P*) and palmitoylation (*jagged line*), are indicated. The chemical structure of Man-6-P phosphomonoester (*blue*) and Man-P-GlcNAc phosphodiester (*red*) are shown. (C) Ribbon diagram of the CD-MPR (PDB ID: 1C39), domain 3 of the CI-MPR (PDB ID: 1SZO), domain 5 of the CI-MPR (PDB ID: 2KVB), and OS-9 (PDB ID: 3AIH). β -strands are numbered from N to C terminus, and loops C and D of the binding pocket are labeled. Only the CD-MPR has a single α -helix at the N terminus. The N- and C-terminus are boxed. The dotted grey line shown in the CD-MPR (between $\beta 1$ and $\beta 2$) and OS-9 (between $\beta 5$ and $\beta 6$) represent regions of unknown structure due to a lack of electron density.

Fig. 3.

Glycan specificity of MRH-containing proteins. Phosphorylated glycan microarray analyses indicate that each of the three carbohydrate binding domains of the CI-MPR and the CD-MPR recognize different populations of phosphorylated glycans. The *inset* shows the structure of the glycan transferred to Asn residues in N-glycosylation, showing glucose (grey triangles), mannose (circles) and GlcNAc (cyan squares). The mannose residues on the three arms of the N-glycan are color coded (arm A green; arm B yellow; arm C orange) and are designated A-I. *Top panel*, Binding of MPRs to phosphorylated glycans. For simplicity, only the Man-6-P (P) containing glycans are shown, with the phosphorylated mannose residue highlighted. Man-P-GlcNAc-containing glycans have the identical structure, except with a phosphodiester at the analogous position as the phosphomonoester. Domains 1-3 and 9 show a preference for Man-6-P-containing glycans (grey bars), whereas domain 5 binds only to Man-P-GlcNAc-containing glycans (red bar). *Bottom panel*, Binding of XTP3-B, OS-9, and glucosidase II β-subunit (GIIβ) to high mannose-type glycans is shown, with the mannose essential for recognition highlighted.

Fig. 4.

Comparison of the carbohydrate binding pockets of the bovine CD-MPR, CI-MPR domains 3 and 5. Close-up view of the binding pockets of the (A) CD-MPR (magenta, PDB ID: 1C39), (B) domain 3 (green, PDB ID: 1SZO), and (C) domain 5 (blue, PDB ID: 2KVB) of the CI-MPR. Disulfide bonds are shown in yellow. Man-6-P is depicted as gold ball-and-stick. Loops C and D are labeled. (D) SPR analyses of the interaction between CI-MPR's domain 5 (wild-type and mutants containing single amino acid substitutions W653K, W653T, and W653F) and immobilized acid α -glucosidase (GAA) containing Man-6-P or Man-P-GlcNAc. W653 in domain 5 was substituted with the conservative residue, Phe, or with a Lys or Thr, as these residues are present in an analogous position in domain 3 and 9, respectively (see Fig. 2A). Expression and purification of mutants and wild-type domain 5 were performed as described previously [24]. To assess binding affinities, purified domain 5 proteins were flowed over the GAA coupled sensor surface as described previously in Olson *et al.* [24]. The response at equilibrium (R_{eq}) to the GAA phosphomonoester (blue) and GAA phosphodiester (red) surfaces is plotted versus the concentration of protein (domain 5 wild-type 2, 5, 20, 40, 80, 120 μ M; domain 5 W635F 2, 5, 10, 20, 40, 80, 120 μ M), and fitted as described previously [24]. W653K and W653T exhibited no response above background to either surface (data not shown).

Fig. 5.

Close-up view of the binding pocket of OS-9. (A) Overlay of the four essential residues (Gln, Arg, Glu, and Tyr) of CD-MPR (*magenta*, PDB ID: 1C39), CI-MPR domain 3 (*green*, PDB ID: 1SZO), CI-MPR domain 5 (*blue*, PDB ID: 2KVB), and human OS-9 (*gold*, PDB ID: 3AIH). Disulfide bridges are shown in *yellow* and loops C and D are labeled. (B) The binding pocket of human OS-9 (PDB ID: 3AIH) is shown with its bound ligand (*gold* ball-and-stick; Mana1,3Mana1,6Man). (C) Molecular surface of OS-9 with Man-6-P modeled into the binding pocket. Asp182 and Leu183 are shown in loop C. The *dotted circle* highlights the position of the phosphate moiety, which is predicted by this model to be excluded from OS-9's binding pocket by the side chains of Asp182 and Leu183. The residue types are color coded as follows: hydrophobic (Ala, Gly, Ile, Leu, Met, Val, *grey*), aromatic (Phe, Trp, Tyr, *light pink*), polar (His, Asn, Gln, Ser, Thr, *wheat*), positive (Lys, Arg, *blue*), negative (Asp, Glu, *red*), cysteine (*pale yellow*).