Identification of residues essential for carbohydrate recognition and cation dependence of the 46-kDa mannose 6-phosphate receptor

Guangjie Sun¹,², Hongtao Zhao³, B. Kalyanaraman³, and Nancy M. Dahms¹,²

¹Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226; and ²Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI 53226

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The 46 kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) plays an essential role in the biogenesis of lysosomes by diverting newly synthesized mannose 6-phosphate (Man-6-P)-containing lysosomal enzymes from the secretory pathway to acidified endosomes. Previous crystallographic studies of the CD-MPR have identified 11 amino acids within its carbohydrate binding pocket. These residues were evaluated quantitatively by assaying the binding affinity of mutant receptors containing a single amino acid substitution toward a lysosomal enzyme. The results show that substitution of Gln-66, Arg-111, Glu-133, or Tyr-143 results in a >800-fold decrease in affinity, demonstrating these four amino acids are essential for carbohydrate recognition by the CD-MPR. Solution binding and surface plasmon resonance analyses demonstrated that the presence of Mn²⁺ enhanced the affinity of the CD-MPR for a lysosomal enzyme by 2- to 4-fold and increased the stoichiometry of the interaction between a heterogeneous population of a lysosomal enzyme and the receptor by ~3-fold. In contrast, substitution of Asp-103 results in a protein that no longer exhibits enhanced binding affinities or altered stoichiometry in the presence of cations, and electron spin resonance demonstrated that the D103S mutant exhibits a 6-fold lower affinity for Mn²⁺ than the wild-type receptor (Kₐ = 3.7 ± 1.4 mM versus 0.6 ± 0.1 mM). Chemical cross-linking revealed that Mn²⁺ influences the stoichiometry of interaction between the CD-MPR and lysosomal enzymes by increasing the oligomeric state of the receptor from dimer to higher order oligomers. Taken together, these studies provide the molecular basis for high affinity carbohydrate recognition by the CD-MPR. Furthermore, Asp-103 has been identified as the key residue which mediates the effects of divalent cations on the binding properties of the CD-MPR.

Key words: lectin/lysosome/mannose 6-phosphate receptor/protein targeting

Introduction

The functional maturation of lysosomes requires the selective delivery of >40 distinct acid hydrolases from their site of synthesis in the endoplasmic reticulum (ER) to the lysosome. The sole members of the P-type family of animal lectins, the 46-kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) and the 300-kDa cation-independent mannose 6-phosphate receptor (CI-MPR), play a key role in mediating this targeting event by binding specifically to phosphomannosyl residues on newly synthesized soluble acid hydrolases (Dahms and Hancock, 2002; Ghosh et al., 2003). The resulting mannose 6-phosphate receptor (MPR)-lysosomal enzyme complex is transported from the trans Golgi network (TGN) to endosomal compartments where the acidic pH of the compartment causes disassembly of the complex. The released lysosomal enzymes are packaged into lysosomes whereas the receptors either return to the Golgi/TGN via a retromer-assisted transport system (Arighi et al., 2004) to repeat the process or move to the plasma membrane where the CI-MPR, but not the CD-MPR, functions to internalize exogenous ligands (Ghosh et al., 2003). The two MPRs display different affinities and capacities for transport of the various acid hydrolases, and studies utilizing receptor-deficient fibroblasts demonstrate that both MPRs are required for the efficient targeting of lysosomal enzymes (Ludwig et al., 1994; Pohlmann et al., 1995; Sleat and Lobel, 1997). In addition to its role in lysosome biogenesis, the CD-MPR has been implicated in other processes, including the regulated secretion of lysosomal enzymes (Chao et al., 1990). Furthermore, recent studies have indicated that elevated expression of the CD-MPR in neuronal cells has a pathogenic role in sporadic Alzheimer’s disease (Mathews et al., 2002).

The CD-MPR, a Type I membrane glycoprotein, exists as a dimer and each subunit is able to bind one mannose 6-phosphate (Man-6-P) molecule (Tong and Kornfeld, 1989; Roberts et al., 1998; Olson et al., 1999b). The crystal structure of the extracytoplasmic region of the bovine CD-MPR complexed with either Man-6-P (Roberts et al., 1998) or a phosphorylated oligosaccharide, pentamannosyl phosphate (Olson et al., 1999b), revealed residues within the binding pocket: nine residues (Tyr-45, Gln-66, Asp-103, Asn-104, His-105, Arg-111, Glu-133, Arg-135, and Tyr-143) interact with the terminal phosphorylated mannose, and two amino acids (Asp-43 and Gln-68) interact with the penultimate and prepenultimate mannose rings of pentamannosyl phosphate. However, only qualitative studies have been performed to assess the role of specific residues in carbohydrate recognition by the receptor (Wendland et al., 1991a,b; Olson et al., 1999a).
The inability to purify the CD-MPR by phosphomannosyl affinity chromatography performed in the absence of cations led to its designation as a “cation-dependent” receptor (Hoflack and Kornfeld, 1985a). The CD-MPR displays a preference for Mn$^{2+}$ as it was shown to exhibit a higher binding capacity for a lysosomal enzyme in buffers containing MnCl$_2$ compared to those containing either CaCl$_2$ or MgCl$_2$ (Hoflack and Kornfeld, 1985a; Ma et al., 1991). However, somewhat surprising were the results from subsequent equilibrium dialysis studies which demonstrated that the bovine CD-MPR displays only a 4-fold enhanced binding affinity to the oligosaccharide, pentamannosyl phosphate, in the presence of MnCl$_2$ ($K_d = 6 \mu M$) versus that observed in the presence of ethylene diamine tetraacetic acid (EDTA; $K_d \approx 25 \mu M$) (Tong and Kornfeld, 1989). This finding differentiates the CD-MPR from C-type lectins which have an absolute requirement for calcium to carry out their sugar binding activities (Drickamer, 1999). The crystal structure of the CD-MPR complexed with ligand revealed the presence of a divalent cation in the binding pocket (Robert et al., 1998; Olson et al., 1999b). This observation led us to propose a role for the metal in enhancing binding affinity: the metal functions as a shield between an electrostatically negative region of the receptor (i.e., Asp-103) and the phosphate moiety of Man-6-P (Olson et al., 1999b).

To test this hypothesis and to further evaluate the role of individual residues in the binding pocket, quantitative solution binding studies, surface plasmon resonance measurements, and electron spin resonance (ESR) analyses were performed on the wild-type CD-MPR and compared to receptors containing single amino acid substitutions. The results demonstrate that four residues of the CD-MPR (Gln-66, Arg-111, Glu-133, and Tyr-143) are key determinants for the metal functions as a shield between an electrostatically negative region of the receptor (i.e., Asp-103) and the phosphate moiety of Man-6-P. The substitution of glutamine for aspartic acid at position 103 necessitates the presence of a divalent cation in the binding pocket to obtain high-affinity ligand binding by functioning to eliminate the inhibitory electrostatic effects of Asp-103 juxtaposed to the phosphate oxygen of Man-6-P. In addition, Mn$^{2+}$ increases the conversion of the CD-MPR from dimer to higher order oligomers which allows the receptor to interact with a larger fraction of a heterogeneous population of a lysosomal enzyme.

**Results**

**Expression and characterization of Asn81His$_6$**

Previous biochemical and X-ray crystallographic studies aimed at defining the mechanism of carbohydrate binding by the CD-MPR utilized a soluble, glycosylation-deficient form of the receptor that encompassed only its extracellular region: Asn81Stop155 encodes residues 1–154 of the mature protein, and four out of the five potential N-glycosylation sites were removed, leaving the site at Asn81 intact (Zhang and Dahms, 1993). Asn81Stop155, expressed in either baculovirus-infected insect cells (Marron-Terada et al., 1998) or Pichia pastoris yeast (Reddy and Dahms, 2002), was shown to bind a lysosomal enzyme with the same affinity as the full-length CD-MPR isolated from mammalian tissues, indicating that the extracellular region contains all of the information required for high affinity ligand binding and that the substitution of asparagine with glutamine at positions 31, 57, 68, and 87, along with the loss of N-linked oligosaccharides at these sites, had no significant effect on ligand binding by the CD-MPR. To facilitate the purification of CD-MPR mutants that lack carbohydrate binding activity, six histidine residues were engineered after residue 154, and the construct (Asn81His$_6$) was placed in the yeast expression vector pGAPZuA in which the native signal sequence of the CD-MPR was replaced by the α-factor signal peptide from Saccharomyces cerevisiae (Figure 1). Western blot analysis of Asn81His$_6$ purified from the medium of P. pastoris revealed multiple species, with a major species migrating at 21 kDa and a minor species migrating at 18 kDa. Enzymatic deglycosylation using endo-β-N-acetylglucosaminidase H (endo H) revealed that the single N-glycosylation site at position 81 is utilized: the 21 kDa species represents the glycosylated form, whereas the 18 kDa form represents the unglycosylated receptor (Figure 2A). N-terminal sequence analysis of the purified Asn81His$_6$ revealed a single sequence (EAEATEEK-TEXDLVGE), demonstrating that the α-factor signal sequence was cleaved between the Arg and Glu residues (Figure 1). Thus, the mature Asn81His$_6$ has an additional four residues (Glu-Ala-Glu-Ala) at the N-terminus compared to the mature CD-MPR isolated from mammalian cells (Stein et al., 1987). Identical results (i.e., glycosylation status and N-terminal processing) were observed for the non-His–tagged version of the receptor, Asn81Stop155, expressed in P. pastoris (Reddy and Dahms, 2002). Asn81His$_6$ bound to a pentamannosyl phosphate-agarose affinity column and could be eluted specifically with Man-6-P (Figure 2B), demonstrating that Asn81His$_6$ is functional with respect to ligand binding. As previously observed for the CD-MPR (Zhang and Dahms, 1993), the glycosylated species bound preferentially to the affinity column (Figure 2B, compare lanes 1.
and 4). Quantitative binding studies were performed using the lysosomal enzyme, β-glucuronidase. Asn81His6 bound with an affinity (K_d = 1.5 ± 0.2 nM) (Figure 2C).

The crystal structure of the CD-MPR complexed with the phosphorylated oligosaccharide, pentamannosyl phosphate, revealed that 11 residues of the CD-MPR are located within hydrogen-bonding distance to the ligand: nine residues (Tyr-45, Gln-66, Asp-103, Asn-104, His-105, Arg-111, Glu-133, Arg-135, and Tyr-143) interact with the terminal phosphorylated mannose, and two amino acids (Asp-43 and Gln-68) interact with the penultimate and prepenultimate mannose rings of pentamannosyl phosphate (Olson et al., 1999b) (Figure 3A). A comparison of the CD-MPRs sequenced to date reveals that all 11 residues are conserved among mammalian species (Figure 3B).

Previous qualitative studies, in which mutant CD-MPRs containing single amino acid substitutions of the residues that comprise the binding pocket were assayed using Man-6-P-containing affinity columns, indicated that four residues (Gln-66, Arg-111, Glu-133, and Tyr-143) are important for carbohydrate binding (Olson et al., 1999a). In the current report, the eight residues assayed by Olson et al. (Tyr-45, Gln-66, Asp-103, His-105, Arg-111, Glu-133, Arg-135, and Tyr-143) (Olson et al., 1999a) plus Asp-43 were evaluated individually and comparable to that of Asn81Stop155 expressed in P. pastoris (K_d = 1.4 nM) (Reddy and Dahms, 2002) and of the full-length receptor isolated from mammalian tissues (K_d = 0.28 nM, Watanabe et al., 1990; K_d = 4–5 nM, Ma et al., 1991).

Taken together, the addition of a C-terminal His6 tag does not significantly alter the function of the CD-MPR.

**Binding affinity analyses of mutant Asn81His6 constructs using the lysosomal enzyme, β-glucuronidase**

Fig. 2. Glycosylation status and binding properties of Asn81His6. (A) Enzymatic deglycosylation of purified Asn81His6 was performed by incubating the protein with (+) or without (−) endo-β-N-acetylglicosaminidase H (Endo H) for 16 h. The samples were analyzed on a 12.5% sodium dodecyl sulphate (SDS)–polyacrylamide gel followed by western blotting. (B) Purified Asn81His6 was passed over a pentamannosyl phosphatase–agarose affinity column. The column was washed (W) and then eluted sequentially with 5 mM glucose 6-phosphate (G) (nonspecific sugar) and 5 mM mannose 6-phosphate (Man-6-P) (M). The run-through (RT), wash (W), and eluate samples were precipitated with 10% trichloroacetic acid and then analyzed on a 12.5% sodium dodecyl sulphate (SDS)–polyacrylamide gel followed by western blotting. (C) Increasing concentrations of iodinated β-glucuronidase were incubated with purified Asn81His6 in the presence of 10 mM MnCl_2, and the receptor and bound ligand were immunoprecipitated with bovine cation-dependent mannose 6-phosphate receptor (CD-MPR)-specific antiserum prebound to protein A-Sepharose beads. The beads were washed extensively and bound β-glucuronidase was specifically eluted from the receptor with 5 mM Man-6-P.

Fig. 3. Residues of the cation-dependent mannose 6-phosphate receptor (CD-MPR) targeted for mutagenesis. (A) Stereo view of a ribbon diagram showing the ligand binding pocket of the CD-MPR in complex with pentamannosyl phosphate (PDB code, 1C39) (Olson et al., 1999b). The sphere indicates the position of Mn^2+ in the binding pocket. Potential hydrogen bonds between side chains and ligand are indicated with dashed lines. (B) Amino acid sequence alignment of the extracytoplasmic region of the CD-MPR of various species. The CD-MPR sequences, with their corresponding National Center for Biotechnology Information (NCBI) protein data base accession numbers, shown are bovine (A27068), human (A32700), mouse (A40399), rat (Kanamori et al., 1998), goat (Suresh et al., 2004), dog (XP534894), and chicken (CAA64755) (Matzner et al., 1996). The sequence of Asn81His6 (top) is shown, with the C-terminal tag (CD-MPR) targeted for mutagenesis. The sequence indicated by italics. N-glycosylation sites that have been mutated are indicated (●) and the single N-glycosylation site remaining intact in Asn81His6 is shown (○). The secondary structure of the CD-MPR is shown above the sequence (wavy line represents the single α-helix, and arrows represent β-strands). The 11 residues that compose the carbohydrate binding pocket are indicated by the arrows. The residues that were subjected to site-directed mutagenesis are in the dark gray shaded boxes.
Cation dependence of the CD-MPR

In the context of the Asn81His6 construct using a quantitative binding assay involving an endogenous ligand, the lysosomal enzyme β-glucuronidase, to assess their role in carbohydrate recognition by the CD-MPR. Asparagine at position 104 was not mutated because a main-chain atom, rather than the side chain, of Asn–104 provides the interaction with Man-6-P (Figure 3A). In addition, glutamine at position 68, which interacts with the penultimate and prepenultimate mannose rings (Olson et al., 1999b) was not mutated because Gln-68 represents a substitution from the wild-type sequence (i.e., Asn-68) in the generation of a glycosylation-deficient form of the receptor (see Figure 3A and Zhang and Dahms, 1993). Consistent with the previous affinity chromatography studies (Olson et al., 1999a), four amino acids (Gln-66, Arg-111, Glu-133, and Tyr-143) are essential for high affinity carbohydrate recognition because constructs containing a single amino acid substitution (i.e., Q66E, R111K, E133D, or Y143F) exhibit at least an 800-fold lower affinity ($K_d$ estimated at >1200 nM) than the wild-type receptor (Figure 4A). In contrast, the affinity of Y45F ($K_d = 6.6 \pm 0.9$ nM, Figure 4B), D103E ($K_d = 9.9 \pm 1.1$ nM, Figure 4C), H105S ($K_d = 20 \pm 4$ nM, Figure 4D), and R135K ($K_d = 4.8 \pm 1.5$ nM, Figure 4E) for β-glucuronidase differs from the wild-type receptor by less than 14-fold. Asp-43, which interacts with the penultimate mannose ring (Figure 3A; Olson et al., 1999b), is predicted to have little contribution to the carbohydrate binding ability of the receptor based on its limited H-bonding interactions with the oligosaccharide. This hypothesis was confirmed by quantitative binding studies that show D43A exhibits a binding affinity ($K_d = 2.2 \pm 0.5$ nM) similar to that of the wild-type receptor (Figure 4F).

Influence of cations on lysosomal enzyme binding by Asn81His6

Previous equilibrium dialysis studies by Tong et al. (Tong and Kornfeld, 1989) demonstrated that the bovine CD-MPR displays a minimal dependence on cations when probed with an oligosaccharide, pentamannosyl 6-phosphate ($K_d = 6 \mu$M in the presence of 10 mM MnCl$_2$ versus $K_d = 25 \mu$M in the presence of a chelator, 10 mM EDTA). To determine whether a similar finding would be observed when a glycoprotein, rather than an oligosaccharide, was used as the ligand, Asn81His6 was incubated with increasing concentrations of iodinated β-glucuronidase, a lysosomal enzyme, in the presence of either MnCl$_2$ or EDTA. The results demonstrate that a similar ~4-fold enhancement in the affinity of the CD-MPR for a lysosomal enzyme occurs in the presence of Mn$^{2+}$ ($K_d = 1.7$ nM in the presence of 10 mM MnCl$_2$ versus $K_d = 7.3$ nM in the presence of 10 mM EDTA) (Figure 5A). Furthermore, the Scatchard analyses reveal that the stoichiometry of binding is also significantly affected by cations, with a 3.4-fold increase in the amount of β-glucuronidase bound per receptor observed in the presence of Mn$^{2+}$ (Figure 5A). These effects were specific to Mn$^{2+}$ because incubations carried out in the presence of 10 mM ZnCl$_2$ did not significantly enhance either the binding affinity or stoichiometry of binding (data not shown). In contrast to the results obtained with a lysosomal enzyme, no significant differences in the stoichiometry of binding were seen in the presence or absence of Mn$^{2+}$ when pentamannosyl phosphate was used as the ligand (Tong and Kornfeld, 1989). Taken together, these results demonstrate that selected divalent cations provide a 4-fold improvement in binding affinity between the CD-MPR and an endogenous ligand.
ligand as well as an ∼3-fold enhancement in the stoichiometry of binding.

Role of Asp-103 in the cation dependence of lysosomal enzyme binding by the CD-MPR

The crystal structure of the CD-MPR indicates that Mn$^{2+}$ 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 to four water molecules (Olson et al., 1999b) (Figure 3A). These data led us to suggest that the presence of a metal cation enhances binding of the phosphate group of Man-6-P by sheltering the solvent-accessible phosphate oxygen from the negatively charged region of the binding pocket (i.e., Asp-103) (Olson et al., 1999b). Removal of the negatively charged side chain of Asp-103 from the vicinity of the phosphate binding site would thus be predicted to eliminate the need of a bound metal to augment binding affinity. To test this hypothesis, Asp-103 was replaced with glutamate, serine, or asparagine, and the binding affinity of these mutant receptors to β-glucuronidase was measured in the presence and absence of MnCl$_2$. Although substitution of Asp-103 resulted in a minimal (<6-fold) perturbation of binding affinity in the presence of MnCl$_2$ (D103E, $K_d = 9.9 \pm 1.1$ nM; D103S, $K_d = 9.9 \pm 1.5$ nM; D103N, $K_d = 4.8 \pm 1.5$ nM) from that of the wild-type Asn81His$_6$ ($K_d = 1.7$ nM, Figure 5A), these mutations substantially eliminated the effect of cations on binding affinity: the affinity for β-glucuronidase in the presence and absence of cations differed by 1.1-fold, 1.1-fold, and 1.2-fold, respectively, for D103E (Figure 5B), D103S (Figure 5C), and D103N (Figure 5D) compared to the 4.3-fold difference in binding affinity observed for the wild-type Asn81His$_6$ (Figure 5A). The Scatchard analyses also revealed that these mutations greatly diminished the effect Mn$^{2+}$ had on the stoichiometry of binding, with...
Asn81His6; (bound method of Scatchard (Scatchard, 1949). The inset shows the amount of 15 nM; D103E, 5 nM; D103S, 60 nM; D103N, 20 nM. (Scatchard analysis of the binding of Fig. 5.

representative of four independent experiments for Asn81His6 and three reactions contained either 10 mM MnCl2 (β-glucuronidase). The amount of receptor used is wild-type Asn81His6,

β-glucuronidase. The amount of receptor used is wild-type Asn81His6, β-glucuronidase as a function of increasing concentrations of mutant Asn81His6 constructs. Binding studies using iodinated

Scatchard analysis of the data demonstrated that neither the affinity nor the stoichiometry of binding by D103S was altered significantly by the presence of Mn2+ (Fig. 7F). In contrast, the presence of Mn2+ resulted in a 2.8-fold increase in the stoichiometry of binding and a 1.8-fold higher affinity of Asn81His6 to the immobilized β-glucuronidase on the sensor chip (Fig. 7E). Similar results were obtained when higher amounts (8-fold) of β-glucuronidase were immobilized on the sensor chip (data not shown). Taken together, the surface plasmon resonance analyses (Fig. 7) are consistent with the solution binding assays (Fig. 5) in that the substitution of Asp-103 with serine eliminates both the enhancement in binding affinity and the increase in the stoichiometry of binding that occurs in the presence of Mn2+.

Surface plasmon resonance analyses

To further characterize the influence of cations on the binding properties of the CD-MPR with a lysosomal enzyme, surface plasmon resonance studies were performed. β-glucuronidase was covalently attached to the dextran matrix of the sensor chip and different concentrations of recombinant CD-MPRs (wild-type Asn81His6 or the D103S mutant) were passed through the flow cell in buffer containing either Mn2+ or EDTA (Fig. 7). Similar to that observed in the binding studies using iodinated β-glucuronidase (Fig. 5), the absence of divalent cations resulted in a significant reduction in the amount of Asn81His6 bound to the sensor chip (compare Figure 7A with Figure 7B) but had little effect on the binding of D103S (compare Figure 7C with Figure 7D). Scatchard analyses of the data demonstrated that neither the affinity nor the stoichiometry of binding by D103S was altered significantly by the presence of Mn2+ (Fig. 7F). In contrast, the presence of Mn2+ resulted in a 2.8-fold increase in the stoichiometry of binding and a 1.8-fold higher affinity of Asn81His6 to the immobilized β-glucuronidase on the sensor chip (Fig. 7E). Similar results were obtained when higher amounts (8-fold) of β-glucuronidase were immobilized on the sensor chip (data not shown). Taken together, the surface plasmon resonance analyses (Fig. 7) are consistent with the solution binding assays (Fig. 5) in that the substitution of Asp-103 with serine eliminates both the enhancement in binding affinity and the increase in the stoichiometry of binding that occurs in the presence of Mn2+.

Influence of cations on the quaternary structure of the CD-MPR

To determine whether the effects of divalent cations on the stoichiometry of ligand binding may be due to changes in the quaternary structure of the CD-MPR, the receptor was incubated with the homobifunctional cross-linking agents disuccinimidyl suberate (DSS; 11.4 Å spacer arm) or ethylene glycol bis(succinimidylsulfamate) (EGS; 16.1 Å spacer arm) in the presence or absence of Mn2+. Incubation with either EGS or DSS resulted in both Asn81His6 and D103S proteins being converted from migrating predominantly as a monomeric species to that of a predominantly dimeric

Fig. 5. Scatchard analysis of the binding of β-glucuronidase to wild-type and mutant Asn81His6 constructs. Binding studies using iodinated β-glucuronidase were carried out as described in Figure 4 except that the reactions contained either 10 mM MnCl2 (●) or 10 mM ethylene diamine tetraacetic acid (EDTA) (○). The data were analyzed according to the method of Scatchard (Scatchard, 1949). The inset shows the amount of bound β-glucuronidase as a function of increasing concentrations of β-glucuronidase. The amount of receptor used is wild-type Asn81His6, 15 nM; D103E, 5 nM; D103S, 60 nM; D103N, 20 nM. (A) wild-type Asn81His6; (B) D103E; (C) D103S; and (D) D103N. The data shown is representative of four independent experiments for Asn81His6 and three independent experiments each for D103E, D103S, and D103N.
incubation with DSS resulted in a predominantly dimeric cross-linked species (Reddy and Dahms, 2002). The results also show that the presence of Mn\(^{2+}\) dramatically increases the percentage of Asn81His\(_6\) that exists as trimers or tetramers or higher order oligomers by 6-fold (Figure 8A, compare lanes 3 and 4) to 8-fold (Figure 8A, compare lanes 5 and 6). In contrast, divalent cations have no significant effect on the oligomeric state of a mutant which binds metal poorly (i.e., D103S, Figure 6), with no significant difference (≤1.4-fold) in the percentage of higher order oligomers in the presence or absence of Mn\(^{2+}\) (Figure 8B, compare lanes 3 and 4, compare lanes 5 and 6). Taken together, these results demonstrate a direct correlation between metal binding ability, higher order quaternary structure, and capacity to recognize a larger fraction of endogenous ligands, namely a lysosomal enzyme.

Discussion

Both the CD-MPR and CI-MPR, which are present in nearly all higher eukaryotic cell types that have been examined to date, are involved in a specific delivery system that functions to divert newly synthesized soluble lysosomal enzymes from the secretory pathway for deposition in endosomal compartments. The observation that both MPRs are required to target the full complement of lysosomal enzymes to the lysosome demonstrates that the CD-MPR and CI-MPR are not functionally redundant. Previous biochemical studies have indicated that the two MPRs exhibit several differences in their binding properties, such as phosphodiester recognition, which are likely to contribute to the differences observed in their lysosomal enzyme targeting capabilities in vivo (Hoflack and Kornfeld, 1985b; Tong and Kornfeld, 1989; Distler et al., 1991). A significant difference between the MPRs, which led to the identification and purification of the CD-MPR (Hoflack and Kornfeld, 1985a,b), is the observation that divalent cations enhance binding affinities of the CD-MPR but not the CI-MPR (Hoflack and Kornfeld, 1985b; Tong and Kornfeld, 1989). The current report investigates the molecular basis for the effect divalent cations have on CD-MPR function.

The crystal structure of the CD-MPR complexed with ligand identified 11 residues within hydrogen bonding distance to the Man-6-P-containing oligosaccharide (Olson et al., 1999b). To provide a quantitative assessment of the relative contribution of each of these residues in carbohydrate recognition, binding assays using a lysosomal enzyme, β-glucuronidase, were performed on CD-MPR constructs containing a single amino acid substitution. Mutation of Asp-43, Tyr-45, Asp-103, His-105, or Arg-135 resulted in a minimal (<14-fold) perturbation in binding affinity (Figure 4B–F). The finding that substitution of Asp-43, which interacts with the penultimate mannose ring (Olson et al., 1999b), results in a less than 2-fold effect on binding affinity (Figure 4F) is consistent with the observation that Man-6-P at a terminal position represents the major determinant of high affinity oligosaccharide binding by the MPRs (Distler et al., 1991; Tomoda et al., 1991). Tyr-45 and Arg–135 have limited interactions with the ligand, with Tyr-45 interacting species (Figure 8, compare lanes 1 and 2 with lanes 3–6).

These results are consistent with our previous studies on a non-His–tagged version of Asn81 which crystallized as a dimer (Roberts et al., 1998; Olson et al., 1999b, 2002) and
with the 1-hydroxyl involved in an O-glycosidic linkage and Arg-135 interacting with the 4-hydroxyl of Man-6-P. Substitution of either residue resulted in only a 3- to 4-fold decrease in binding affinity (Figure 4B and E), demonstrating that Tyr-45 and Arg-135 are not key determinants for carbohydrate recognition by the CD-MPR. Binding studies have shown that the phosphate group is essential for recognition by the MPRs, with mannose exhibiting a $K_d$ greater than three orders of magnitude higher than that for Man-6-P (Tong and Kornfeld, 1989). Although His-105 interacts with the phosphate oxygen of Man-6-P (Roberts et al., 1998; Olson et al., 1999b), the H105S mutant exhibited only a 13-fold decrease in affinity (Figure 4D). Similarly, a minimal 6-fold (D103E, Figures 4C and 5B; D103S, Figure 5C) or 3-fold (D103N, Figure 5D) decrease in binding affinity is observed for the D103 mutants. The small effects observed when a single residue involved in phosphate recognition is altered is likely due to the presence of multiple residues providing side chain (His-105) and main chain (Asp-103, Asn-104, and His-105) interactions with the phosphate oxygens of Man-6-P (Roberts et al., 1998; Olson et al., 1999b). Taken together, these data demonstrate that Asp-43, Tyr-45, Asp-103,
His-105, and Arg-135 provide, individually, only minor contributions to the overall binding affinity of the CD-MPR toward a lysosomal enzyme.

Four residues (Gln-66, Arg-111, Glu-133, and Tyr-143), which are conserved in all CD-MPR species (Figure 3B) as well as in the two carbohydrate recognition domains of the CI-MPR (Hancock et al., 2002), were found to be critical components of the binding pocket because the replacement of any one of these residues (Q66E, R111K, E133D, or Y143F) resulted in a greater than 800-fold decrease in affinity (Figure 4A). The crystal structure of the CD-MPR reveals that these four residues interact with the hydroxyl groups of the mannose ring of Man-6-P, with Arg-111 interacting with the 2-hydroxyl group, Gln-66 and Tyr-143 interacting with the 2- and 3-hydroxyl groups, and Glu-133 interacting with the 3- and 4-hydroxyl groups (Olson et al., 1999b). Thus, substitution of these residues not only will affect hydrogen bonding, but is also predicted to perturb the electrostatic interactions within the binding pocket (Olson et al., 1999a). The observation that mutations which impact the interaction of the receptor with the 2-hydroxyl group of Man-6-P have a significant influence on binding affinity is consistent with previous inhibition studies which measured a $K_d$ value for glucose 6-phosphate that was 3 orders of magnitude greater than the $K_d$ for its 2-hydroxyl epimer, Man-6-P (Tong and Kornfeld, 1989). Comparison of the binding affinities of mutant CD-MPR and mutant CI-MPR constructs, along with the crystal structures of the CD-MPR and CI-MPR carbohydrate binding sites, support the hypothesis that the mechanism of phosphomannosyl recognition is, in part, conserved between the two receptors: (1) substitution of the corresponding conserved residues (Gln, Arg, Glu, or Tyr) in the two carbohydrate binding sites of the CI-MPR results in a similar decrease (>1000-fold) in binding affinity for a lysosomal enzyme (Hancock et al., 2002) and (2) the crystal structure of the N-terminal carbohydrate binding site of the CI-MPR revealed that Gln-348, Arg-391, Glu-416, and Tyr-421 of domain 3 of the CI-MPR are located in a strikingly similar position in the binding pocket as the corresponding residues (Gln-66, Arg-111, Glu-133, and Tyr-143) of the CD-MPR and, importantly, form the same contacts with the mannose ring of Man-6-P (Olson et al., 2004).

Based on the initial observation that a second MPR existed whose carbohydrate binding activity, unlike the 300 kDa MPR, required divalent cations, the 46-kDa MPR was termed a “cation-dependent” receptor (Hoflack and Kornfeld, 1985a). However, subsequent equilibrium dialysis studies demonstrated that the presence of MnCl$_2$ provided only a 4-fold enhancement of binding affinity of the bovine CD-MPR toward the oligosaccharide, pentamannosyl phosphate (Tong and Kornfeld, 1989). Conflicting results have been reported in the literature concerning the cation dependence of the CD-MPR that have been difficult to reconcile because of the use of different species, different types of binding assays, and different ligands among the various studies (Hoflack and Kornfeld, 1985b; Junghans et al., 1988; Watanabe et al., 1990; Ma et al., 1991). Our crystal structures of the bovine CD-MPR indicate that the receptor does, in fact, bind divalent cations. The three-dimensional structure of the bovine CD-MPR revealed a single Mn$^{2+}$ molecule bound per polypeptide of the receptor: the metal is situated within the carbohydrate binding pocket (Figure 3A) and is hexacoordinated (one each to four different water molecules, one to a phosphate oxygen of Man-6-P, and one to a carboxylate oxygen of Asp-103) (Olson et al., 1999b). To confirm the role of Asp-103 in metal binding, Asp-103 was mutated to serine and ESR spectroscopy was employed to measure directly the wild-type and mutant receptor’s binding affinity toward Mn$^{2+}$. The results demonstrate that the wild-type receptor ($K_d = 0.6 \pm 0.1$ mM) displayed a 6-fold higher affinity for Mn$^{2+}$ than D103S ($K_d = 3.7 \pm 1.4$ mM) (Figure 6) and thus support Asp-103 functioning as a key determinant in metal binding.
The D103S mutant was used as a tool to further probe the effect of divalent cations on the binding properties of the CD-MPR toward an endogenous ligand, the lysosomal enzyme β-glucuronidase. Both solution binding studies (Figure 5) and surface plasmon resonance studies (Figure 7) demonstrated that Mn\(^{2+}\) results in a 2- to 4-fold increase in binding affinity of Asn81His\(_6\) to β-glucuronidase, which is consistent with previous studies using pentamannosyl phosphate as the ligand (Tong and Kornfeld, 1989). Furthermore, this effect on affinity is eliminated in the D103 mutants (Figures 5 and 7). As first implicated by the crystal structure of the CD-MPR (Olson et al., 1999b), the observed cation-dependent changes in binding affinity are consistent with Asp-103 providing electrostatic repulsion to the phosphate group of Man-6-P which is offset by the presence of a Mn\(^{2+}\) ideally positioned between a phosphate oxygen of Man-6-P and the carboxylate oxygen of Asp-103 (Figure 3A). Although substitution of D103 with glutamate retains the negative charge, it is predicted from the CD-MPR structure that the additional methylene group of glutamate places its carboxylate group in a location within the binding pocket that would reduce electrostatic repulsion with the phosphate group of Man-6-P. Taken together, the results indicate that Asp-103 is needed for Mn\(^{2+}\) binding by the CD-MPR, whose coordination in the binding pocket allows a favorable interaction with Man-6-P. Asp-103 is conserved in all species except the chicken CD-MPR which has a glycine at position 103 (Figure 3B). Additional studies will be required to test the hypothesis that the binding properties of the chicken CD-MPR would not be influenced by divalent cations because of its lack of an aspartic acid at position 103.

Both solution binding studies (Figure 5) and surface plasmon resonance studies (Figure 7) also demonstrated that Mn\(^{2+}\) results in an ∼3-fold increase in the stoichiometry of binding of Asn81His\(_6\) to β-glucuronidase, and this effect on stoichiometry is dramatically reduced in the D103 mutants. These results differ from previous studies which showed that divalent cations did not appreciably (<10%) affect the CD-MPR’s stoichiometry of binding to an oligosaccharide, pentamannosyl phosphate (Tong and Kornfeld, 1989). The ligand used in the current report, β-glucuronidase, is purified from the medium of a cell line overexpressing the human enzyme. In contrast to the studies using a homogeneous oligosaccharide (i.e., pentamannosyl phosphate), β-glucuronidase, a homotetrameric enzyme with 16 N-linked oligosaccharides, represents a heterogeneous physiological ligand with varying degrees of phosphorylation of its mannosic residues. One possible mechanism that would allow the CD-MPR to capture a larger fraction of a diverse population of β-glucuronidase, whose spatial array of phosphomannosyl-containing oligosaccharides is likely to vary considerably from molecule to molecule, is to alter the oligomeric state of the receptor. Previous chemical cross-linking studies of the full-length CD-MPR support the existence of dimeric and tetrameric forms of the receptor and show that the equilibrium between these forms can be influenced by temperature, ligand, pH, and receptor concentration (Waheed and von Figura, 1990; Waheed et al., 1990). In addition, both dimeric and tetrameric forms have been shown to be functional with respect to binding Man-6-P-containing resins (Li et al., 1990; Waheed et al., 1990). Our chemical cross-linking studies demonstrate that divalent cations influence the oligomeric state of the CD-MPR: Asn81His\(_6\) exists predominantly as a dimer in the absence of divalent cations whereas the presence of Mn\(^{2+}\) resulted in a 6- to 8-fold increase in the fraction of the receptor existing as higher order oligomers (Figure 8A). It is likely that the type of ligand is a key factor in the ability to detect alterations in the binding properties of the CD-MPR as a function of the presence of divalent cations: changes in oligomerization will not affect stoichiometry of binding to a monovalent ligand as shown with pentamannosyl phosphate (Tong and Kornfeld, 1989), whereas interaction of the receptor with multivalent ligands (i.e., β-glucuronidase) can be affected.

It is intriguing to speculate that Mn\(^{2+}\) regulates the activity of the CD-MPR as it traffics between various compartments within the cell. It has been previously shown that the CD-MPR exhibits optimum binding at the pH of the Golgi (~pH 6.4), with minimal binding observed under acidic conditions (<pH 5.5) present in late endosomal compartments or at the pH of the extracellular environment (pH 7.4) (Tong and Kornfeld, 1989). Thus, the CD-MPR, unlike the CI-MPR, binds ligands poorly at the cell surface (Watanabe et al., 1990). It is known that Mn\(^{2+}\) serves as an essential cofactor for glycosyltransferases in the Golgi (Durr et al., 1998), and it is possible that the local concentration of this cation in the Golgi lumen could approach high micromolar concentrations and near the measured K\(_d\) for metal binding by the CD-MPR (K\(_d\) = 600 μM, Figure 6). The concentration of Mn\(^{2+}\) in human serum has been measured and ranges from 40 to 120 nM (Pleban and Pearson, 1979). Thus, this model predicts that high Mn\(^{2+}\) concentrations in the Golgi would facilitate high affinity binding and higher order oligomerization of the CD-MPR to increase binding capacity toward a diverse population of newly synthesized lysosomal enzymes, whereas low concentrations of Mn\(^{2+}\) at the cell surface would trigger release of ligand by lowering the binding affinity and decreasing multivalent interactions by conversion of higher order oligomers of the receptor to dimers. To test this model in vivo will be technically challenging given the low steady state levels of the CD-MPR on the cell surface and the need for use of methodologies that will allow evaluation of both binding properties and oligomeric state in native membranes derived from a particular organelle. Gel filtration analyses of a soluble form (i.e., extracytoplasmic region) of the CD-MPR were unable to consistently detect higher order oligomeric species (tetramer or higher) when the columns were run in the presence of MnCl\(_2\) (N.M. Dahms, unpublished data), suggesting that this interaction is relatively weak. The most compelling evidence to date that Mn\(^{2+}\) concentration plays a role in regulating the function of the CD-MPR in vivo comes from studies on semi-intact cells overexpressing the CD-MPR in which a 1.6-fold increase in the stoichiometry of binding to β-glucuronidase was observed when Mn\(^{2+}\) was included in the incubations (Ma et al., 1991) and a 1.9-fold increase in the percentage of tetrameric CD-MPR, as assessed by chemical cross-linking in the absence of ligand, was reported when the semi-intact cells were incubated in the presence of Mn\(^{2+}\) (Ma et al., 1992). Although higher
order oligomers were not evaluated (Ma et al., 1992), the
data using semi-intact cells (Ma et al., 1991, 1992) are qual-
atively consistent with the results obtained in the current report. Future structural studies will also be required to
determine the architecture of the tetramer and higher order
oligomers to understand the mechanism by which Mn^{2+}
stimulates changes in the quaternary structure of the CD-
MPR.

Materials and methods

Materials

The following reagents were obtained commercially as
indicated: restriction endonucleases (New England Biolabs,
Ipswich, MA); P. pastoris wild-type strain X-33, P. pastoris
expression vector pGAPZαA, T4 DNA ligase, BenchMark
prestained protein ladder, and Zeocin (Invitrogen, Carlsbad,
CA); GeneMate plasmid DNA minipreps (ISC Bioexpress,
Kaysville, UT); glucose 6-phosphate, Man-6-P, ω-aminomethyl
agarose, protein A-Sepharose, and lactoperoxidase
(Sigma, St. Louis, MO); Na_{125}I (carrier free) and protein A-
agarose, protein A-Sepharose, and lactoperoxidase
(Sigma, St. Louis, MO); α-Mannosidase (Zymo Research,
Irvine, CA); GeneMate plasmid DNA minipreps (ISC Bioexpress,
Kaysville, UT); glucose 6-phosphate, Man-6-P, ω-aminomethyl
agarose, protein A-Sepharose, and lactoperoxidase
(Sigma, St. Louis, MO); Na_{125}I (carrier free) and protein A-
agarose, protein A-Sepharose, and lactoperoxidase
(Sigma, St. Louis, MO). The following reagents were obtained commercially as
indicated: restriction endonucleases (New England Biolabs,
Ipswich, MA); D43A (5′-ACA GTG GTT CTC ATA AT-3′), Y45F (5′-AC ATA GCT GAA CAT GTG TGG-3′),
Q66E (5′-TTT GTT GAT CTC CAC GAG-3′), D103E
(5′-ACA GTG GTT CTC ATA AT-3′), D103N
(5′-ACA GTG GTT GAT AAT AAT AT-3′), D103S (5′-ACA
GTG GTT GAT ATA AT-3′), H105S (5′-CCT GCC
ACA GGA GTT GTT ATC-3′), R111K (5′-TG TCG ATT
GCA GGA GAT CAT CAC TGC CCG CTG CTC C-3′), E133D (5′-GCC TCG CTC GTC AGA Cay
AG-3′), R135K (5′-GAC TTT GCC TTC TCT CTC AG-3′), and Y143F (5′-CTC AAA GAG GAA GAA ACA AT-3′).
DNA sequencing analyses (Protein and Nucleic Acid Core
Facility, Medical College of Wisconsin) confirmed the
presence of the predicted sequences.

Expression and purification of wild-type and mutant
Asn81His6 constructs
cDNA constructs (~10 μg) were linearized with BspH1 and
transformed into P. pastoris wild-type strain X-33 by elec-
troporation at 25 μF, 129 Ω, 1500 V using a BTX electro-
porator (Harvard Apparatus, Inc., Holliston, MA). The
transformants were selected on YPDS (1% yeast extract, 2%
peptone, 2% dextrose, and 1 M sorbitol) plates containing
100 μg/mL Zeocin incubated at 30°C. Single colonies were
isolated and inoculated on fresh YPDS plates containing 100
μg/mL Zeocin. The selected clones were inoculated into 3 mL
YPD (1% yeast extract, 2% peptone, and 2% dextrose) and
shaken for 48 h at 30°C. The cells and medium were separated
by centrifugation. Cell lysates were prepared by solubilizing
the cell pellets in buffer containing 65 mM Tris–HCl, pH 6.8,
2.5% sodium dodecyl sulphate (SDS), 10% glycerol, and pro-
tease inhibitor cocktail (1 mg/mL Leupeptin, 1 mg/mL Anti-
pain, 10 mg/mL Benzamidine in aprotonin). As a control,
untransformed P. pastoris strain X-33 cells were used. The
cell lysates and medium were analyzed by western blotting to
identify those colonies expressing high levels of recombinant
protein in the medium. All constructs were efficiently secreted
into the medium, ranging from 54 to 90% secreted, except
D43A (36% secreted) and R111K (21% secreted).

To purify the His-tagged recombinant proteins, media
harvested from P. pastoris cultures were concentrated by
filtration using Amicon stirred cells containing a cellulose
membrane with a 10 kDa nominal molecular mass limit.
Concentrated medium was dialyzed extensively against
metal binding buffer containing 20 mM Tris–HCl, pH 6.8,
0.075% sodium dodecyl sulphate (SDS), 10% glycerol, and pro-
tease inhibitor cocktail (1 mg/mL Leupeptin, 1 mg/mL Anti-
pain, 10 mg/mL Benzamidine in aprotonin). As a control,
untransformed P. pastoris strain X-33 cells were used. The
cell lysates and medium were analyzed by western blotting to
identify those colonies expressing high levels of recombinant
protein in the medium. All constructs were efficiently secreted
into the medium, ranging from 54 to 90% secreted, except
D43A (36% secreted) and R111K (21% secreted).

Enzymatic deglycosylation
Purified Asn81His6 was incubated with endo H (1mU) in
buffer containing 100 mM sodium citrate, pH 6.0, 0.075% SDS,
10 mM β-mercaptoethanol at 16 h at 37°C. The
samples were analyzed on SDS–polyacylamide gels
followed by western blotting.
ESR assay

Proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred electrophoretically to Immobilon P membranes as described previously (Marron-Terada et al., 2000). The membranes were incubated with CD-MPR-specific antiserum, followed by anti-rabbit IgG horseradish peroxidase conjugate (Pierce Chemical). Proteins were detected by enhanced chemiluminescence, as described by the manufacturer (Pierce Chemical).

N-Terminal amino acid sequencing

Purified Asn81His6 was subjected to N-terminal amino acid sequence analysis (Protein and Nucleic Acid Core Facility, Medical College of Wisconsin) to confirm the predicted protein sequence. Fifteen cycles of Edman degradation were performed and the phenylthiohydantoin-derivatized amino acids were separated by reverse-phase high-performance liquid chromatography (HPLC).

Pentamannosyl phosphate–agarose affinity chromatography

Medium samples were subjected to pentamannosyl phosphate–agarose affinity chromatography as described previously (Dahms et al., 1993). Briefly, medium dialyzed against column buffer (50 mM 2-morpholinoethanesulfonic acid (MES); 150 mM NaCl; 10 mM MnCl2; and 5 mM β-glycerophosphate, pH 6.5) was loaded onto a 0.5-mL pentamannosyl phosphate–agarose column, washed with column buffer, then eluted sequentially with column buffer containing 10 mM glucose 6-phosphate (nonspecific ligand) followed by 10 mM Man-6-P (specific ligand).

Binding affinity determinations

Eukaryotic β-glucuronidase was collected from serum-free conditioned medium from cells which overexpress and secrete human β-glucuronidase (MTX 3.2 cells generously provided by Dr. W. Sly). β-Glucuronidase was purified by affinity chromatography on a CL-MPR Affigel-10 column to remove nonphosphorylated enzyme. The same preparations of purified analyte (i.e., wild-type and mutant (D103S) Asn81His6) were injected in a volume of 200 µL over the ligand (β-glucuronidase) and reference flow cells at a flow rate of 40 µL/min. After 5 min, the analyte solution was replaced with running buffer for 2 min to monitor dissociation of receptor and ligand. The surfaces were regenerated with a 10 min injection of running buffer containing 10 mM Man-6-P at a flow rate of 5 µL/min. The association, dissociation, and regeneration phases were followed in real-time by monitoring changes in signals expressed in resonance units, and the data displayed as sensorgrams (response units versus time). The sensorgrams were processed and analyzed using the BIAevaluation software package (version 4.0.1). All response data were double referenced (Myszka, 2000) in which controls for the contribution of the change in the bulk refractive index were performed in parallel with flow cells derivitized in the absence of ligand and subtracted from all binding sensorgrams.

ESR assay

The binding of Mn2+ to wild-type and mutant (D103S) Asn81His6 was measured on a Bruker EMX spectrometer equipped with a Bruker high sensitive cavity. ESR spectra were recorded at room temperature operating at 9.86 GHz. Typical spectrometer parameters were scan range, 1000 G; field set, 3505 G; time constant, 40.96 msec; scan time, 41.94 s; modulation amplitude, 10.0 G; modulation frequency 100 kHz; receiver gain, 0.5–6.32 × 104; microwave power, 50.0 mW; number of scans, 5. Before ESR analysis, the receptors were concentrated to 100 µM in binding buffer (50 mM MES; 150 mM NaCl; and 5 mM β-glycerophosphate, pH 6.5) containing 200 µM Man-6-P. A 25-µL quartz capillary tube was used for all measurements. The receptors were incubated with various concentrations of Mn2+ (10–3000 µM), and the amount of bound Mn2+ was determined by directly comparing the spectral amplitudes of samples containing wild-type or mutant (D103S) Asn81His6 to the corresponding amplitudes observed with a buffered solution containing an equal concentration of Mn2+ in the absence of protein.

Biosensor studies

All surface plasmon resonance measurements were performed at 25°C using a Biacore 3000 instrument. Purified β-glucuronidase was immobilized on CM5 sensor chips by primary amine coupling (Johnsson et al., 1991) as described in the Biacore manual. The reference surface was treated in the same way as the reaction surface except that β-glucuronidase was omitted (i.e., under the same coupling conditions to normalize the chemistries between the two flow cells). Samples of purified wild-type and mutant (D103S) Asn81His6 were prepared in running buffer (50 mM MES, pH 6.5; 150 mM NaCl; 5 mM β-glycerophosphate; and 0.005% (v/v) surfactant P20) containing 10 mM MnCl2 or 10 mM EDTA. Analysis at various flow rates (5, 15, and 75 µL/min) demonstrated that the receptor–ligand interaction was not significantly affected by mass transport. Various concentrations of purified analyte (i.e., wild-type and mutant (D103S) Asn81His6) were injected in a volume of 200 µL over the ligand (β-glucuronidase) and reference flow cells at a flow rate of 40 µL/min. After 5 min, the analyte solution was replaced with running buffer for 2 min to monitor dissociation of receptor and ligand. The surfaces were regenerated with a 10 min injection of running buffer containing 10 mM Man-6-P at a flow rate of 5 µL/min. The association, dissociation, and regeneration phases were followed in real-time by monitoring changes in signals expressed in resonance units, and the data displayed as sensorgrams (response units versus time). The sensorgrams were processed and analyzed using the BIAevaluation software package (version 4.0.1). All response data were double referenced (Myszka, 2000) in which controls for the contribution of the change in the bulk refractive index were performed in parallel with flow cells derivitized in the absence of ligand and subtracted from all binding sensorgrams.
Chemical), or 1 mM EGS (Pierce Chemical) for 1 h at 23°C. The reaction was quenched by the addition of glycine to 100 mM. The samples were resolved by SDS-PAGE followed by western blotting.

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Abbreviations

CD-MPR, cation-dependent mannose 6-phosphate receptor; CI-MPR, cation-independent mannose 6-phosphate receptor; DSS, disuccinimidyl suberate; EDTA, ethylene diamine tetraacetate acid; EGS, ethylene glycol bis[succinimidylsuccinate]; endo H, endo-β-N-acetylglucosaminidase H; ESR, electron spin resonance; Man-6-P, mannose 6-phosphate; MES, 2-morpholinoethanesulfonic acid; MPR, mannose 6-phosphate receptor; SDS, sodium dodecyl sulphate.

References


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