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Glycan Structure Determinants for Cation-Independent Mannose 6-Phosphate Receptor Binding and Cellular Uptake of a Recombinant Protein

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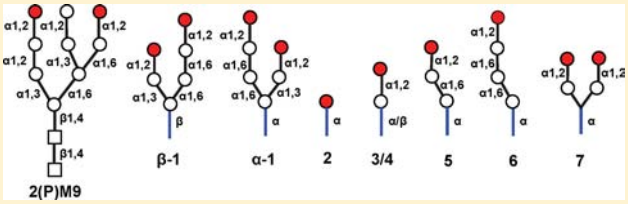
ABSTRACT: The cation-independent mannose 6-phosphate receptor (CI-MPR) plays a critical role in intracellular transport of lysosomal enzymes as well as the uptake of recombinant proteins. To define the minimal glycan structure determinants necessary for receptor binding and cellular uptake, we synthesized a series of glycans containing mono-, di-, tri-, tetra-, and hexamannoses terminated with either one or two phosphates for conjugating to a model protein, recombinant human acid α-glucosidase. A high affinity interaction with the CI-MPR can be achieved for the enzyme conjugated to a dimannose glycan with a single phosphate. However, tightest binding to a CI-MPR affinity column was observed with a hexamannose structure containing two phosphates. Moreover, maximal cellular uptake and a 5-fold improvement in *in vivo* potency were achieved when the bisphosphorylated hexamannose glycan is conjugated to the protein by a β linker. Nevertheless, even a monophosphorylated dimannose glycan conjugate showed stronger binding to the receptor affinity column, higher cellular uptake, and significantly greater *in vivo* efficacy compared to the unconjugated protein which contains a low level of high affinity glycan structure. These results demonstrate that the phosphorylated dimannose moiety appears to be the minimal structure determinant for enhanced CI-MPR binding and that the orientation of the glycan is critical for maximum receptor interaction. In summary, we have improved the understanding of the mechanism of CI-MPR binding and developed a simple alternative for CI-MPR targeting.



INTRODUCTION

Glycans have been demonstrated to play important roles in multiple cellular functions including anti-inflammatory, immune response, and host−microbe interactions.[1] Their roles in protein therapeutics have long been appreciated as having significant impact on pharmacokinetics and pharmacodynamics, as well as glycoprotein stability.[2,3] Several glycoengineered proteins or antibodies have been successfully used in the clinic to treat multiple diseases.[4−6] Recently, the benefit of glycoengineering has also been demonstrated *in vivo* in a murine model of Pompe disease, a lysosomal storage disease with glycogen accumulation in muscle due to mutations in human acid α-glucosidase (GAA).[7−9] Recombinant human acid α-glucosidase (rhGAA) conjugated with synthetic mannose 6-phosphate (Man-6-P) containing glycans (neoGAA) has shown greater efficacy than rhGAA in clearing glycogen in a murine model of Pompe disease,[10] indicating that increasing the amount of phosphorylated glycans in the protein can improve the delivery of the biologic into the disease affected tissue. Thus, neoGAA, with increased cellular uptake through the cation-independent Man-6-P receptor (CI-MPR), may have the potential to be an improved treatment option over the currently approved unconjugated rhGAA.[11−13]

The CI-MPR is essential for uptake and lysosomal delivery of many endogenous as well as recombinant lysosomal enzymes.[14−16] The receptor has been shown to be expressed in a variety of tissues.[17,18] It can transport extracellular proteins from the cell surface through receptor-mediated endocytosis into the lysosomes.[19] The receptor contains 15 homologous extracytoplasmic domains involved in high affinity interactions with various ligands including Man-6-P containing glycans, as well as other noncarbohydrate ligands, such as insulin-like growth factor II (IGF-II).[1,20−22] In contrast to its single domain for noncarbohydrate ligands, the CI-MPR contains three carbohydrate recognition sites including two high affinity sites located within domains 1−3 and domain 9, as well as a low affinity site within domain 5.[21,23,24] There is high sequence homology in these domains among the receptors from bovine, mouse, and human, suggesting their similar substrate specificities.[25−29] Indeed, it has been shown recently that its glycan-binding properties are evolutionarily conserved in vertebrates.[30] Structure−function studies of domains 1−3 revealed the presence of a signature motif in domain 3, in

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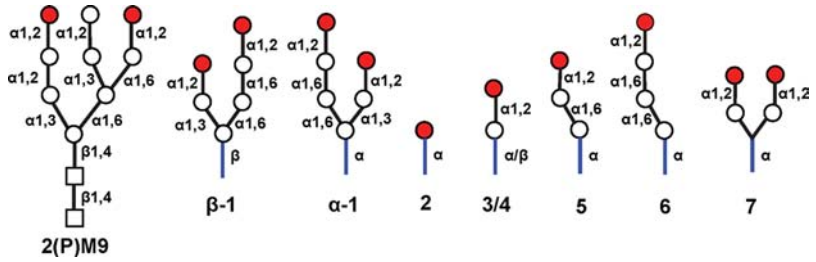

Figure 1. Synthetic Man-6-P containing glycans prepared as mimic of phosphorylated oligomannoses in nature. Synthetic glycans: ***β*-1**, bisphosphorylated *β*-hexamannose; ***α*-1**, bisphosphorylated *α*-hexamannose; **2**, Man-6-P *α*-monomannose; **3**, monophosphorylated *α*-dimannose; **4**, monophosphorylated *β*-dimannose; **5**, monophosphorylated *α*-trimannose; **6**, monophosphorylated *α*-tetramannose; **7**, bisphosphorylated *α*-tetramannose. Phosphorylated oligomannose present in nature: **2(P)M9** or bisphosphorylated oligomannose 9. Symbols: red ball, Man-6-P; black ring, mannose; black open square, *N*-acetylglucosamine; black line, glycosidic bond; blue line, aminooxy linker.

which Gln-318, Arg-391, Glu-416, and Tyr-421 carry out their essential role in Man-6-P recognition by serving as direct hydrogen-bonding partners to the hydroxyl groups of mannose.[31,32] Ser-386 and Ser-387, located within loop C of domain 3, were also identified as critical residues for phosphate recognition.

Although the structure of domains 1−3 of the CI-MPR complexed with Man-6-P has been solved, fully defining the specificity of the 300 kDa transmembrane protein for phosphorylated oligomannose, especially the glycans on complex proteins, can be a challenge. Many approaches have been applied in exploring the receptor specificity for free glycans, including affinity chromatography, immunoprecipitation and equilibrium dialysis, receptor binding inhibition, glycan microarray, and surface plasmon resonance.[23,33−40] Although it is known as an essential epitope for binding, free Man-6-P has low affinity for CI-MPR with a K_D in μM range, suggesting that it is unlikely to be a minimum moiety for strong binding. In contrast, higher affinity interaction (K_D in the nM range) was observed for a natural oligomannose mixture containing two phosphate residues (bisphosphorylated oligomannoses).[33,35] However, there is still uncertainty as to whether there is a minimal structural determinant present in natural glycans associated with strong receptor binding. Although inhibition studies with synthetic glycan analogs demonstrated that oligosaccharides with an α1,2-linked Man-6-P dimannose unit were stronger inhibitors than free Man-6-P as well as the α1,3- and α1,6-linked isomer in CI-MPR binding, none of these synthetic glycans has been proven to be effective in supporting high affinity receptor interaction, especially on a protein molecule.[36,37]

To better understand the glycan structural requirements for CI-MPR binding and to gain insight on the simplest Man-6-P glycan that can still provide high affinity interaction of the conjugate with CI-MPR, we synthesized a series of glycans with mono-, di-, tri-, tetra-, or hexamannoses containing either one or two phosphate residues (Figure 1). Most of these glycans are present as partial structures in natural phosphorylated oligomannoses. Each of these, which were attached to an α or β linker, was then conjugated to oxidized terminal sialic acid residues of a model protein, rhGAA. These conjugates were then evaluated in a screening cascade for conjugation efficiency, CI-MPR binding, myoblast uptake, and *in vivo* glycogen clearance in a mouse model of Pompe Disease (6neo/6neo mouse) to identify the glycan structures that yield meaningful improvement for rhGAA targeting.

EXPERIMENTAL PROCEDURES

Materials. rhGAA was produced by Genzyme Corp. Other reagents, including Man-6-P used in eluting affinity column, were obtained from Sigma (St. Louis, MO) except as otherwise described.

Chemical Synthesis of Various Bis Man-6-P Neoglycans. Preparation of the phosphorylated oligomannose glycans (Figure 2) has been described in a patent application (WO2010075010A2). Briefly, these glycans were prepared by using several differentially protected mannose building blocks to direct the glycosidation sites. A series of glycosidation reactions followed by selective deprotection of the alcohol group in the acceptor mannose allowed the assembly of oligomannose structures. After the desired oligomannose structures were prepared, the 6-OH position was deprotected and the phosphate group installed using benzyl protected phosphate. The intermediate was then globally deprotected to expose all the alcohol, phosphate, and amino groups. The final two steps were the installation of the aminooxy group using a BOC protected aminooxyacetic acid followed by deprotection. The installation of the aminooxy group provides the critical functional group for conjugating these glycans to the protein via an oxime bond.

NeoGAA Conjugation. NeoGAA was prepared using sialic acid-mediated (SAM) conjugation. Various Man-6-P containing glycans were conjugated to sialic acid residues on rhGAA, after initially oxidizing these terminal sialic acid groups with 7.5 mM of sodium periodate according to method described previously,[8,9] except that buffer exchanges were performed by diafiltration on Pellicon XL 50 or Amicon ultra-4 (Millipore, MA) into 25 mM sodium phosphate, 2% mannitol, and 0.005% Tween-80 (pH 6.25). For initial screening, the SAM conjugates were prepared in small scale (~3 mg) by titration with various amounts of individual glycan relative to the oxidized GAA; followed by 200 mg scale preparation of selected conjugates using 17 to 25-fold molar excess of glycan over rhGAA aiming to achieve a similar amount of glycan conjugated for further characterization.

***In Vitro* Characterization.** The molecular weights of rhGAA and neoGAA proteins were measured using MALDI-TOF MS analysis on a Voyager DE-PRO mass spectrometer.[41] The number of glycans conjugated per protein molecule was estimated by subtracting the molecular weight of rhGAA from that of neoGAA before dividing the difference by the molecular weight of individual glycan.

The binding of rhGAA or neoGAA conjugates to CI-MPR was determined by HPLC-based cation-independent Man-6-P

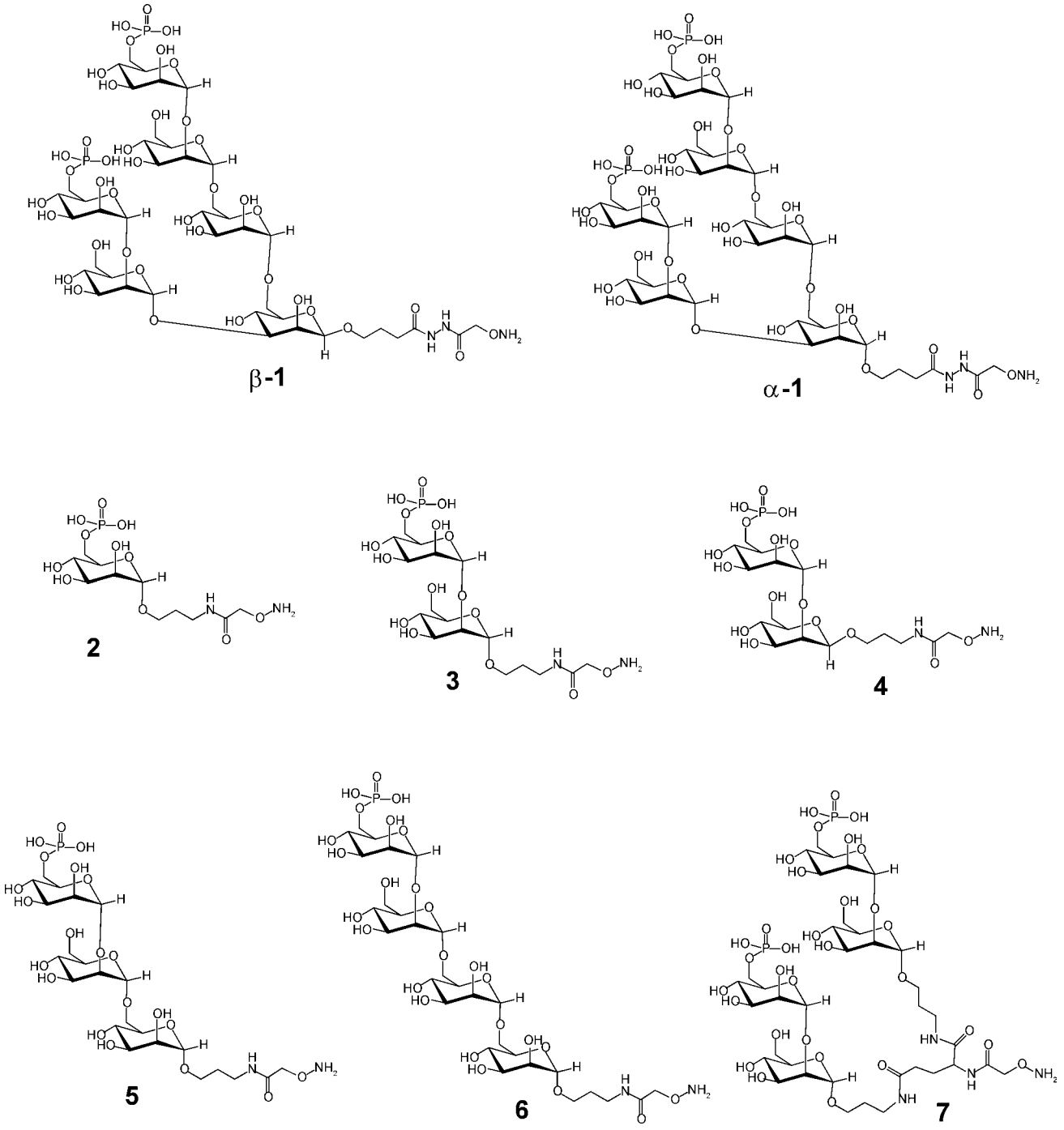

Figure 2. Structure of synthetic Man-6-P containing glycans prepared: ***β*-1**, bisphosphorylated *β*-hexamannose; ***α*-1**, bisphosphorylated *α*-hexamannose; **2**, Man-6-P *α*-monomannose; **3**, monophosphorylated *α*-dimannose; **4**, monophosphorylated *β*-dimannose; **5**, monophosphorylated *α*-trimannose; **6**, monophosphorylated *α*-tetramannose; **7**, bisphosphorylated *α*-tetramannose.

receptor (CI-MPR) affinity chromatography. The soluble bovine CI-MPR, which was purified from fetal bovine serum, was immobilized on Poros EP resin and packed into a HPLC column. The bound proteins were competitively eluted in four fractions using stepwise elution with increasing concentrations of free Man-6-P from Sigma (0.25, 0.85, 5, 20 mM) in the eluting buffer. They were detected by intrinsic protein fluorescence (290 nm excitation/340 nm emission), and the relative amounts of protein eluting at different Man-6-P concentrations were determined by integration of the peak areas at each step. The CI-MPR binding was also determined using surface plasmon resonance on a Biacore 3000 instrument as described.[42]

The enzymatic activity of GAA was determined using *p*-nitrophenyl-*α*-D-glucopyranoside as substrate as described.[42] One unit of activity was defined as the hydrolysis of 1 *μ*mol of substrate per min at 37 °C.

HPLC size-exclusion chromatography was used for protein aggregation measurement.[43] The rate of uptake of rhGAA and neoGAA by rat L6 myoblasts was determined using methods described previously.[8]

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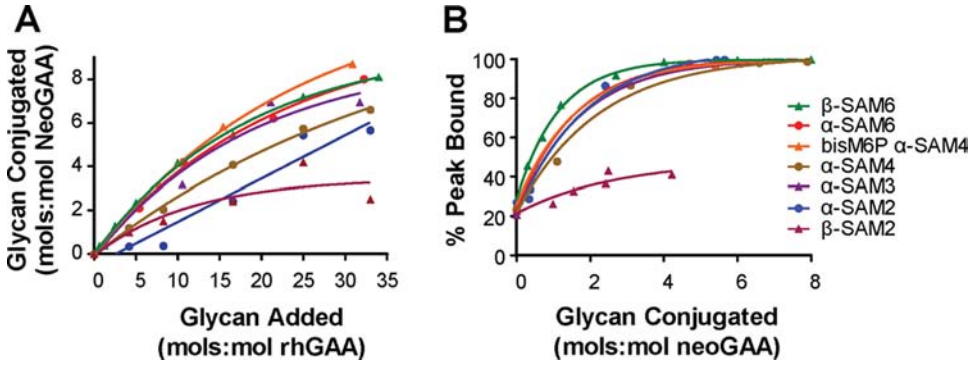

Figure 3. Characterization of neoGAA conjugates prepared with various amounts of synthetic glycans. (A) The mols of glycan coupled to the rhGAA correlated with molar excess of the glycan used. (B) The total receptor bound fractions (peaks 1, 2, 3, and 4) from CI-MPR affinity chromatography of neoGAA conjugates correlated with amount of glycan coupled in the conjugates. One mol of rhGAA contains ~7 mols of sialic acid available for conjugation. α-SAM2 conjugate was prepared with glycan **3**; β-SAM2 with glycan **4**; α-SAM3 with glycan **5**; α-SAM4 with glycan **6**; bisM6P α-SAM4 with glycan **7**; α-SAM6 with glycan ***α*-1**, and β-SAM6 with glycan ***β*-1**.

N-Linked oligosaccharide analysis was performed by releasing glycans with overnight digestion using PNGase F in 50 mM sodium phosphate (pH 7.0) and 10 mM β-mercaptoethanol. The released oligosaccharides were desalted by biodialysis (MWCO 500 Da) with several changes of water. The dialyzed samples were dried in a speed-vac concentrator and reconstituted in 10 mM ammonium formate (pH 4.0) in 50% acetonitrile and 50% water for mass spectrometry analysis. The samples were analyzed using a TSK Gel Amide-80 column (2 × 100 mm, 5 μm particle size) with in-line MS detection in an acetonitrile−water gradient and 10 mM ammonium formate (pH 4.0). An LCT Premier time-of-flight mass spectrometer (Waters, Milford, MA) was used for high mass accuracy mass determination in negative ion mode and scanned from 600 to 2500 *m*/*z*.

***In Vivo* Glycogen Clearance.** The efficacy of the conjugates in clearing glycogen was determined using GAA knockout mice (3−6 months). For comparing neoGAA β-SAM6 and α-SAM2 and rhGAA, the mice were divided into 7 dose groups consisting of 3 males and 3 females per group. Each group received vehicle, 20 or 100 mg/kg rhGAA, and correspondingly 5-fold lower doses of neoGAA at 4 or 20 mg/kg once a week for a total of 4 weeks. For comparing rhGAA and neoGAA β-SAM6, α-SAM6, bis α-SAM4, the mice were divided into 9 dose groups of 3 males and 3 females. Each group received either vehicle, 20, 100 mg/kg rhGAA, or 4, 20 mg/kg neoGAA, once a week for a total of 4 weeks. Animals were euthanized 7 days after the last dose by CO_2 asphyxiation. Heart, quadriceps, triceps, diaphragm, and psoas were harvested and analyzed for glycogen content as described previously.[8,42] One-way analysis of variance (ANOVA) followed by a Newman-Keuls test was used for statistical analysis of the data. A probability value of $p < 0.05$ was considered statistically significant. Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, NIH Publication No. 86−23) and were approved by Genzyme's Animal Care and Use Committee.

RESULTS

Man-6-P Glycan Design. We have previously reported a synthetic glycan, bisphosphorylated Man-6-P β-hexamannose (***β*-1**) (Figure 1 and 2), as an effective mimic of more complex bisphosphorylated oligomannose-type glycans present in nature,[8] including **2(P)M9** (bisphosphorylated oligomannose 9, Figure 1). The mannose on the reducing end of the synthetic glycan ***β*-1** is attached in β linkage to aminooxy functionality for conjugation to mimic β1,4 linkage to one of the core GlcNAc residues in natural *N*-glycans. Although the ***β*-1** is already structurally less complex as compared to **2(P)M9**, the potential to identify even simpler glycan structures to achieve the same receptor binding and *in vivo* results remained. To further understand the minimal glycan determinant required for CI-MPR binding, various simpler glycans were designed and compared to this synthetic glycan (***β*-1**) after conjugation to rhGAA (Figures 1 and 2). They were prepared in either α or β linkage equipped with the aminooxy linker for conjugation. In particular, two dimannoses, α1,2-mannobiose-6-phosphate which mimic the dimannose phosphate group located at the nonreducing end of natural glycans were synthesized, with α (**3**) or β (**4**) orientation of the aminooxy linker. These α or β orientations of the aminooxy linker are also present at the reducing end of bisphosphorylated hexamannose (***α*-1** and ***β*-1**). It had also been demonstrated previously that linear mannose sequences which contain a terminal Man-6-P linked in α1,2 to the penultimate mannose are among the most potent synthetic inhibitors of CI-MPR binding.[36,44] Thus, the tri- and tetramannose versions of glycans with single phosphate, in which α-dimannose was extended with additional one or two mannose residues in α1,6 linkage (**5**, **6**, respectively), were also synthesized to evaluate whether any additional enhancement of the extra mannose groups will contribute to the interaction with the receptor. These tri- and tetramannose are present in the 6-arm of natural **2(P)M9** (Figure 1). Mannose-6-phosphate with an aminooxy linker (Man-6-P, **2**) was synthesized as the smallest glycan in the series. In addition, an initial estimation of the role of bivalency in receptor interaction using the simpler glycan was made by attaching two of dimannose to a glutamate spacer resulting in **7**. The synthetic bivalent bisphosphorylated Man-6-P glycans offers the potential to mimic natural bisphosphorylated oligomannoses after conjugation.

Conjugation Efficiency of Various Glycans. Process efficiency was investigated by measuring the number of glycans coupled to rhGAA when the conjugates were prepared with various molar equivalents of individual glycan relative to enzyme. The number of glycans conjugated was estimated from the difference in molecular weight of rhGAA before and after conjugation by using MALDI-TOF intact protein analysis. As

Table 1. *N*-Linked Biantennary Glycans in neoGAA *β*-SAM6 and *α*-SAM2 Detected by LC/MS Analysis[a]

		β-SAM6		*α*-SAM2	
parent glycan on rhGAA	resulting glycan on neoGAA	theoretical *m/z*	detected *m/z*	theoretical *m/z*	detected *m/z*
NA2	native	1639.58(1-)	1639.55(1-)	1639.58(1-)	1639.50(1-)
NA2F	native	1785.64(1-)	1785.63(1-)	1785.64(1-)	1785.63(1-)
A1	native	1930.68(1-)	N/D	1930.68(1-)	N/D
	oxidized +1 conjugated glycan	1586.48(2-)	1586.49(2-)	1200.89(2-)	1200.86(2-)
A1F	native	1037.87(2-)	N/D	1037.87(2-)	N/D
	oxidized +1 conjugated glycan	1659.51(2-)	1659.51(2-)	1273.92(2-)	1273.90(2-)
A2	native	1110.38(2-)	N/D	1110.38(2-)	N/D
	oxidized +1 conjugated glycan	1701.01(2-)	1700.98(2-)	1315.42(2-)	1315.40(2-)
	oxidized +2 conjugated glycans	1568.78(3-)	1568.77(3-)	1582.49(2-)	1582.46(2-)
A2F	native	1183.41(2-)	N/D	1183.41(2-)	N/D
	oxidized +1 conjugated glycan	1774.04(2-)	1774.00(2-)	1388.45(2-)	1388.41(2-)
	oxidized +2 conjugated glycans	1617.47(3-)	1617.44(3-)	1655.52(2-)	1655.50(2-)

[a]NA2, asialobiantennary; NA2F, asialobiantennary core fucosylated; A1, monosialylated biantennary; A1F, monosialylated biantennary core fucosylated; A2, disialylated biantennary; A2F, disialylated biantennary core fucosylated. A1, A1F, A2, and A2F are the major glycan species in rhGAA, which were used for conjugation.

Table 2. CI-MPR Binding of neoGAA Conjugated with Various Glycans

Conjugates	Glycans in the conjugates	% Unbound	% Peak 1 (0.25mM Man-6-P eluate)	% Peak 2 (0.85mM Man-6-P eluate)	% Peak 3 (5mM Man-6-P eluate)	% Peak 4 (20mM Man-6-P eluate)
rhGAA	-	65.3	22.3	8.5	3.8	0
α-SAM1	**2,**	56.4	35.5	6.5	1.6	0
β-SAM2	**4,**	59	28.1	8.6	3.9	0.4
α-SAM2 *	**3,**	1.4	1.8	13	74.5	9.4
α-SAM3	**5,**	0	1.2	11.8	72.3	14.7
α-SAM4	**6,**	1.3	3.0	9.4	55.5	30.7
BisM6P α-SAM4 *	**7**	2.5	0.9	4.4	62.7	29.4
α-SAM6 *	**α-1,**	1.7	0.2	0.8	34.4	63.0
β-SAM6 *	**β-1,**	1.6	1.2	2.6	32.1	62.5

*The conjugates prepared in 200 mg scale. The smaller scale preparations had similar MPR binding results in general (data not shown). As for conjugates (first column), *α* and *β* represent the configuration of linker attached to glycans that were used for coupling to rhGAA. The number after "SAM" represent the number of mannose in the glycans. Symbols: red ball, Man-6-P; black ring, mannose; black open square, *N*-acetylglucosamine; black line, glycosidic bond; blue line, aminooxy linker.

shown in Figure 3A, most of the glycans were conjugated to rhGAA at similar level, which showed a positive correlation between the number of glycans conjugated and the molar excess of glycan used. However, *β*-SAM2 (a neoGAA conjugate with monophosphorylated *β*-dimannose, **4**) showed relatively low coupling even when 33-fold molar excess of glycan was used in the conjugation as compared to other glycans.

Structures Analysis of the Conjugates. The structures of the *N*-linked conjugated glycans in *α*-SAM2 (a conjugate with monophosphorylated *α*-dimannose, **3**) and *β*-SAM6 (a conjugate with bisphosphorylated *β*-hexamannose, ***β*-1**) were confirmed by time-of-flight mass spectrometry analysis of PNGase F-released oligosaccharides from these samples (Table 1). Biantennary *N*-linked glycans, which are the major sites for conjugation, are present in rhGAA primarily as asialo-, mono-, and disialylated forms.[42] Conjugation of single monophosphorylated *α*-dimannose to sialylated biantennary glycans from rhGAA adds approximately 472 Da mass (552.2 Da glycan − 62 Da lost during oxidation of sialic acid − 18 Da lost during conjugation) and 1243 Da molecular weight is added when the bisphosphorylated *β*-hexamannose (***β*-1**) is conjugated (glycan mass 1323 Da). Conjugation of both monophosphorylated *α*-dimannose and bisphosphorylated *β*-hexamannose was found to occur predominantly through sialic acid residues, with masses corresponding to the addition of 1 glycan coupled to the monosialylated structures (A1 and A1F) and 1 or 2 glycans conjugated to the disialylated structures (A2 and A2F). It is interesting to note that conjugation of two molecules of

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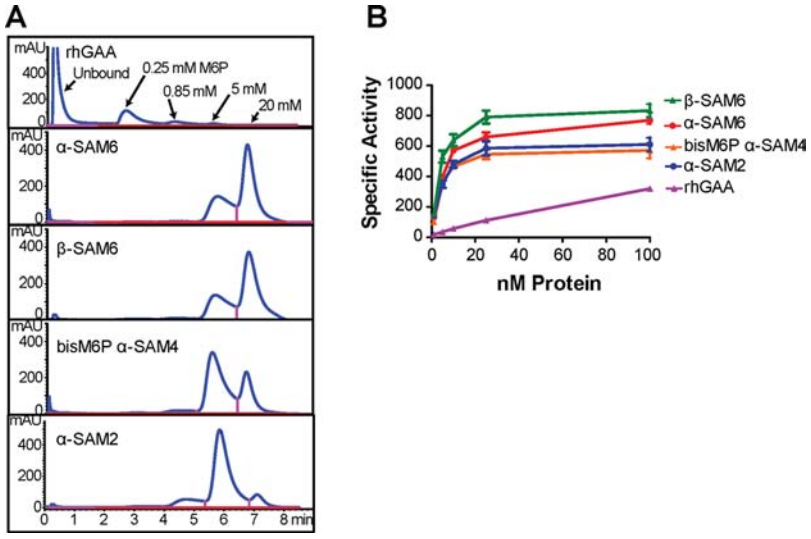

Figure 4. Characterization of neoGAA conjugated with similar level of glycan. (A) CI-MPR affinity chromatography analysis of the conjugates with various glycans. (B) L6 myoblast uptake of the same conjugates. α-SAM2 conjugate was prepared with glycan **3**; bisM6P α-SAM4 with glycan **7**; α-SAM6 with glycan ***α*-1** and β-SAM6 with glycan ***β*-1**.

Table 3. Summary Profile of neoGAA Conjugated with Various Glycans

Conjugates	Glycans in the conjugates	% Aggregation	% Peak 4 of CI-MPR column eluate	Myoblast uptake (fold over rhGAA) *	% Glycogen clearance (Quad) **	% Glycogen clearance (Heart) **
rhGAA	-	0.4 ± 0.06	0	1 ± 0.1	24 ± 5.1	49 ± 8.4
α-SAM2	**3,** 	0.8	9	10 ± 1.0	49 ± 8.2	88 ± 45.1
bisM6P α-SAM4	**7** 	1.4 ± 0.01	29	10 ± 0.2	42 ± 9.8	79 ± 4.5
α-SAM6	**α-1,** 	1.1 ± 0.02	63	11 ± 0.5	55 ± 10.8	90 ± 51.5
β-SAM6	**β-1,** 	2 ± 0.12	63	15± 1.6	81 ± 66.3	100 ± 0

*The myoblast uptake represents the fold of increased activity measured in cell lysates when the cells were incubated with 5nM of each conjugate as compared to rhGAA; **: represents % of glycogen clearance in muscle quadriceps and heart of mice treated with 20 mg/kg of protein. As for conjugates (first column), the α and β represent the configuration of linker attached to glycans that were used for coupling to rhGAA. The number after "SAM" represent the number of mannose in the glycans. Symbols: red ball, Man-6-P; black ring, mannose; black open square, *N*-acetylglucosamine; black line, glycosidic bond; blue line, aminooxy linker.

monophosphorylated α-dimannose to the disialylated oligosaccharides results in structures that present bivalent Man-6-P terminal residues. The same structures were also identified in β-SAM2, but with less amount of 2 glycans conjugated to the disialylated glycans (data not shown).

In addition, low aggregation was also observed for all conjugates, limiting any possibility of interference of protein aggregation on receptor binding as described below (Table 3).

CI-MPR Binding of the Conjugates As Measured Using Surface Plasmon Resonance. A range of affinities were obtained for α-SAM1 (a conjugate with Man-6-P, **2**), α-SAM2, and β-SAM6 with K_D of 8.3, 2.1, and 2.8nM, respectively. There was approximately a 4.5-fold enhancement in affinity of α-SAM1 to the receptor as compared to rhGAA which has a K_D of 37nM. A larger increase in affinity was observed for α-SAM2 and β-SAM6 with 18- and 13-fold enhancement over rhGAA, respectively.

CI-MPR Binding of the Conjugates As Measured Using CI-MPR Affinity Chromatography. Since no significant difference in affinity was observed on Biacore between α-SAM2 and β-SAM6 with different glycans, the binding of these conjugates to CI-MPR was further characterized using a HPLC-based CI-MPR affinity chromatography. The CI-MPR affinity chromatography separates different fractions of the conjugates with varying affinities for the receptor by competitively eluting them using increasing concentrations of free Man-6-P with relative standard deviation below 10%. When the conjugates coupled with various molar (stoichiometric) excesses of individual glycans were analyzed using affinity chromatography, the percentage of total bound fractions (eluted off the affinity column by free Man-6-P) were found to increase correspondingly with increase in the number of glycans conjugated (Figure 3B). The receptor binding of most conjugates reached saturation levels of more than 97% when they contained

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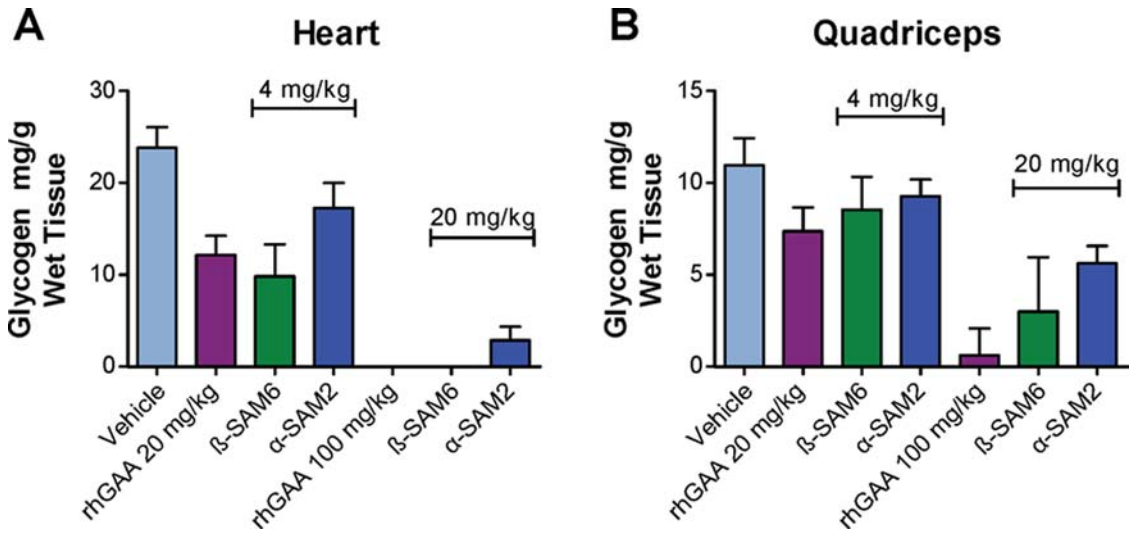

Figure 5. Comparison of rhGAA at 5-fold higher dose with neoGAA β-SAM6 and α-SAM2 in clearance of accumulated glycogen from heart (A) and muscle quadriceps (B) from GAA knockout mice. α-SAM2 conjugate was prepared with glycan **3** and β-SAM6 with ***β*-1**.

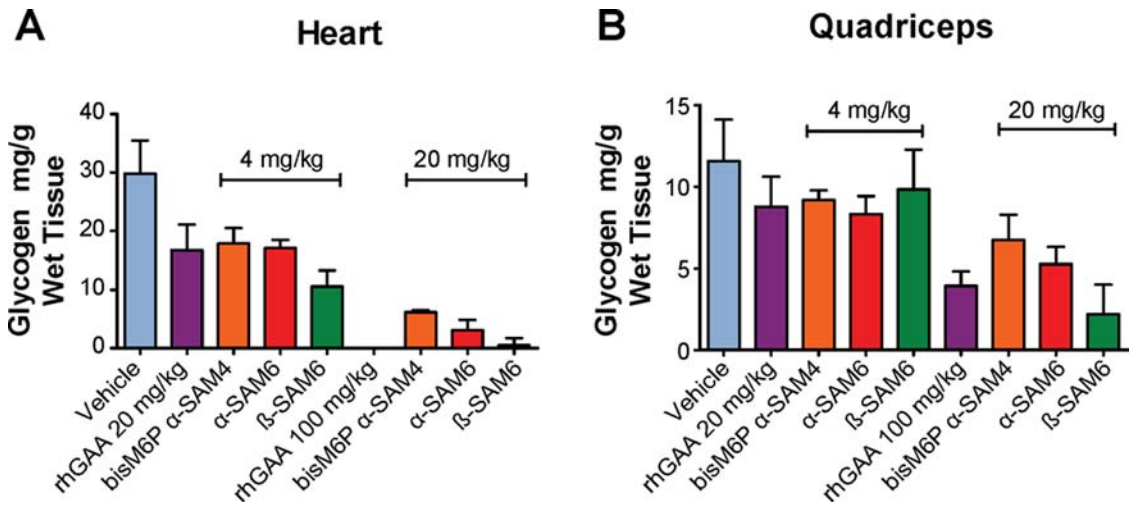

Figure 6. Comparison of rhGAA at 5-fold higher dose with neoGAA β-SAM6, α-SAM6, and bisM6P α-SAM4 in clearance of accumulated glycogen from heart (A) and muscle quadriceps (B) from GAA knockout mice. bisM6P α-SAM4 was prepared with glycan **7**, α-SAM6 with ***α*-1**, and β-SAM6 with ***β*-1**.

more than 6 mols glycan per mol protein. However, β-SAM2 showed only ~40% receptor binding that was close to saturation. The α-SAM1 with ~4 mols glycan per mol protein also showed only 44% binding, while there was more than 80% receptor binding for other conjugates containing similar levels of glycans except for β-SAM2.

Most neoGAA conjugates with approximately 6 glycans per protein prepared using high molar excess of glycans showed different binding profiles on CI-MPR affinity chromatography (Table 2). Compared to rhGAA, there was a slight ~1.5-fold increase in the percentage of total CI-MPR bound fractions observed with α-SAM1 and β-SAM2, which were mainly in the low affinity peaks 1 and 2 fractions (eluted off the affinity column by 0.25 and 0.85 mM of free Man-6-P, respectively). When the high affinity peaks 3 and 4 fractions were collected and analyzed, both conjugates are comparable to rhGAA with 1.6% to 4.3% recovered. In contrast, the other conjugates showed a stronger receptor interaction with the sum of the high affinity peaks 3 and 4 fractions at 84−87% for monophosphorylated and 92−97% for bisphosphorylated glycans (Table 2 and Figure 4A). Among them, β-SAM6 and α-SAM6 (both conjugates contain identical bisphosphorylated hexamannose differing only in the anomeric configuration of the linker) showed a relatively higher percent fraction eluted with 20 mM of free Man-6-P (63% is in Peak 4). On the other hand, α-SAM2, α-SAM3 (a conjugate with monophosphorylated α-trimannose, **5**), α-SAM4 (a conjugate with monophosphorylated α-tetramannose, **6**), and bisM6P α-SAM4 (a conjugate with bisphosphorylated Man-6-P α-tetramannose, **7**) had relatively more peak 3 (56−75%) than peak 4 (9−31%) (Table 2). Interestingly, there was approximately a 3-fold increase in the peak 4 fraction of α-SAM4 and bisM6P α-SAM4 as compared to α-SAM2 and α-SAM3 (~30% vs ~10%). The relative affinities of CI-MPR binding of the conjugates estimated based on the percentage of peak 4 fraction is β-SAM6 ~ α-SAM6 > bisM6P α-SAM4 ~ α-SAM4 > α-SAM3 ~ α-SAM2 > β-SAM2 ~ α-SAM1 ~ rhGAA. Thus, the conjugates containing monophosphorylated α-dimannose (**3**), bisphosphorylated Man-6-P α-tetramannose (**7**), and bisphosphory-

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lated α-hexamannose (**α-1** and **β-1**) were chosen for further characterization.

Cell Uptake of the Conjugates. The rat L6 myoblast cell uptake of these neoGAA conjugates was compared (Figure 4B and Table 3). Although all the conjugates showed a comparable saturable uptake with EC_{50} at ∼5 nM concentration, their maximal uptake as well as uptake at 5 nM were different with a rank order of β-SAM6 > α-SAM6 > α-SAM2 ∼ bisM6P α-SAM4 > rhGAA. As expected, rhGAA did not reach saturation with the amount of protein tested.

***In Vivo* Glycogen Clearance of neoGAA Conjugates.** The ability of the conjugates to clear glycogen from the muscles of GAA knockout mice was investigated in two studies. rhGAA and neoGAA conjugates were administered in four weekly doses via tail vein injection and the amount of glycogen in heart and skeletal muscles of GAA knockout mice was measured one week after the final dose. In one study, α-SAM2 and β-SAM6 were compared in glycogen clearance with rhGAA as control. At the equivalent dose of 20 mg/kg, α-SAM2 cleared glycogen in heart by 88%, while β-SAM6 reduced heart glycogen to below the level of detection. In contrast, rhGAA at the same dose only reduced heart glycogen by 49% (Figure 5A and Table 3). In skeletal muscle, there was also more glycogen cleared by the conjugates than by rhGAA at equivalent doses (20 mg/kg). The muscle glycogen from mice treated with β-SAM6 and α-SAM2 was reduced by 72% and 49%, respectively, compared to vehicle treated animals (Figure 5B and Table 3). However, only a 33% reduction was observed in muscle of mice treated with rhGAA.

In another study, the *in vivo* efficacy of neoGAA conjugates, bisM6P α-SAM4, α-SAM6, and β-SAM6, were compared with rhGAA at 20 mg/kg (Figure 6). In heart, rhGAA reduced glycogen by 44%, while treatment with β-SAM6, α-SAM6, and bisM6P α-SAM4 reduced tissue glycogen by 98%, 90%, and 79%, respectively. In skeletal muscle, the treatment with rhGAA at 20 mg/kg resulted in 24% of glycogen clearance, while 81%, 55%, and 42% of clearance was observed in mice treated with β-SAM6, α-SAM6, and bisM6P α-SAM4, respectively. When the percent glycogen clearance is compared among the conjugates following administration of 20 mg/kg, the skeletal muscle *in vivo* efficacy is illustrated by glycogen clearance in quadriceps with an order of β-SAM6 > α-SAM6 > α-SAM2 > bisM6P α-SAM4 > rhGAA, while in the heart the order was observed as β-SAM6 > α-SAM6 > α-SAM2 > bisM6Pα-SAM4 > rhGAA (Table 3).

■ DISCUSSION

The high affinity CI-MPR binding of natural phosphorylated oligomannoses has been demonstrated with glycans either immobilized on a chip or conjugated to a protein.[40,45] A comparably strong interaction was also obtained with a protein conjugate containing a natural glycan mimic, bisphosphorylated hexamannose **β-1**.[8] However, there are no previous reports on the minimal structural determinants associated with high affinity interactions. To better understand the mechanism of CI-MPR binding and develop simple alternatives, we investigated the interaction of the receptor with a model protein, rhGAA, conjugated by α or β linkage with mono-, di-, tri-, tetra-, and hexamannoses containing one or two phosphate residues. Interestingly, while a monophosphorylated α-dimannose (α1,2-mannobiose-6-phosphate with an α linker, **3**), when conjugated to rhGAA, provided low nM affinity interaction as measured by surface plasmon resonance, it showed less binding in CI-MPR affinity chromatography, less *in vitro* and *in vivo* activities than the conjugate containing bisphosphorylated hexamannose. Nevertheless, the novel conjugates with simpler glycans containing this dimannose moiety demonstrated a significant enhancement in binding to receptor affinity column, myoblast uptake, and *in vivo* efficacy over rhGAA.

We have used multiple methods to more accurately measure CI-MPR binding to a glycoprotein conjugated with Man-6-P glycans. Although surface plasmon resonance is a gold standard for measuring the binding affinity of label-free proteins, it did not differentiate the relative affinity between β-SAM6 conjugated with **β-1** and α-SAM2 conjugated with **3**. By contrast, HPLC-based CI-MPR affinity chromatography provides another dimension for analyzing the interaction with the conjugates. Although both methods use similar immobilization chemistries for CI-MPR through lysine residues, the affinity chromatography method measures the fraction of conjugates binding to the immobilized receptor, while the surface plasmon resonance method captures the property of the total population of conjugates on an average basis. Thus, the former approach provides increased sensitivity so that we were able to differentiate the binding of the conjugates with different phosphorylated glycans.

Results from the CI-MPR mediated cell uptake and CI-MPR affinity chromatography showed similar trends, although the rank orders of the conjugates were not identical. The cell uptake data was more consistent with *in vivo* glycogen clearance. It is likely that the CI-MPR on the cell surface behaves slightly differently from the same receptor artificially immobilized on an affinity column. The receptor seems to be present as a dimer in the cell membrane, although it readily dissociates upon solubilization.[1,46] It has also been shown that the rate of receptor-mediated endocytosis is higher for CI-MPR binding a multivalent ligand, β-glucuronidase containing multiple phosphorylated oligomannoses, than the one binding a monovalent ligand, IGF-II.[47] The increase in ligand internalization may result from the intermolecular cross-linking of the receptor after binding to a multivalent ligand. Thus, the difference in *in vivo* efficacy may reflect not just the binding affinity, but also the efficiency in uptake of the conjugates to lysosome inside the cells. Interestingly, we observed different receptor binding of the conjugates with linear monophosphorylated glycans, including mono-, di-, tri-, and tetramannose. Although the affinity of its conjugate (α-SAM1) determined by surface plasmon resonance was increased 4.5-fold over the unconjugated protein, Man-6-P (**2**) failed to support strong receptor interaction of the conjugate as measured by CI-MPR affinity chromatography, likely due to relatively low affinity of free Man-6-P (K_D at μM range) as reported from an analysis using equilibrium dialysis.[35−37] Indeed, a high number of Man-6-P (n = 46) conjugated to bovine albumin was required for its strong inhibition (IC_{50} at 35nM) of receptor binding.[37] In contrast, a conjugate, α-SAM2, with a monophosphorylated α-dimannose **3** showed maximum affinity as measured by surface plasmon resonance with an 18-fold increase over rhGAA and strong binding in receptor affinity chromatography. Our receptor affinity and cell uptake data are consistent with inhibition studies which showed at least 3- to 9-fold higher inhibitory effect of this dimannose than free Man-6-P.[36,37] According to a recent report from glycan microarray analysis, there is high affinity interaction of CI-MPR with many phosphorylated

oligomannoses containing this dimannose moiety but not a monophosphorylated Man7 isomer that contains only a phosphate attached to mannose in either α1,3- or α1, 6-linkage to penultimate mannose at the 6 arm.[40] Thus, this dimannose moiety seems to be an important structural determinant present in natural phosphorylated oligomannoses having high affinity interaction with CI-MPR. Indeed, other linear phosphorylated glycans containing this dimannose moiety can provide strong receptor binding after coupling to rhGAA. Moreover, among the glycans with one phosphate, α-SAM4 conjugate with longer α-tetramannose (**6**) appeared to bind slightly more strongly than other conjugates with shorter linear glycans (α-dimannose **3** or α-trimannose **5**) in affinity chromatography with a relative ∼3-fold increase in the highest affinity peak 4 fraction. It was reported that a monophosphorylated trimannose appended with a nonglycan component was more potent in inhibiting the receptor binding than the same derivative containing phosphorylated dimannose.[36,37] Although no increase was found in our current study for the conjugate with phosphorylated α-trimannose, a higher affinity interaction was observed with the one containing longer α-tetramannose, probably due to extended presentation of phosphorylated mannose. Alternatively, the penultimate mannose may also contribute to the receptor interaction as proposed by Tomoda et al.[37] Nevertheless, those linear monophosphorylated di-, tri-, and tetramannose conjugates were effective in providing stronger receptor-mediated cell uptake as well as higher glycogen clearance than unconjugated protein.

When the conjugates with glycans containing one or two phosphate residues were compared in binding to the CI-MPR, most of the linear glycans with single phosphate, including di-, tri-, and tetramannose, provided similar but not as strong binding as compared to bivalent bisphosphorylated glycans after conjugation. Our MS results show that bisphosphorylated glycan structures on rhGAA are indeed created in α-SAM2 when two monophosphorylated α-dimannose (**3**) are conjugated to single disialylated complex-type glyans. Additionally, the two molecules of glycan **3** may also be coupled to two monosialylated complex-type oligosaccharides from nearby *N*-glycosylation sites in the protein. However, given the fact that their highest affinity peak 4 fractions never reached the level achieved in β-SAM6, linear glycans presented in a putative bivalent fashion may not be presented in the same optimal orientation as that in the glycan ***β*-1** in β-SAM6. Moreover, even the conjugates with bisphosphorylated glycans were found to have different binding affinity toward CI-MPR. The bisM6P α-SAM4 with bisphosphorylated tetramannose (**7**) also did not bind as strongly as β-SAM6. Similar to the conjugate with linear glycans, bisM6P α-SAM4 was eluted mainly as peak 3. Thus, the high affinity interaction with CI-MPR may require additional structural or conformational features beyond the two phosphorylated dimannose. It is likely that the precise orientation of the two Man-6-Ps plays an important role in the bivalent presentation of the Man-6-P groups.

In addition to the number of phosphate and mannose, the configuration of linker that is attached to glycans at the reducing end also contributed significantly to receptor binding. Although free dimannose, α1,2-mannobiose-6-phosphate, was shown as a strong inhibitor of receptor binding,[36,37] different binding affinity was obtained with the same glycan attached to a linker with either α or β configuration in the current study. Only α-dimannose (α1,2-mannobiose-6-phosphate with α aminooxy linker, **3**) showed strong binding to CI-MPR as described previously, while β-dimannose (α1,2-mannobiose-6-phosphate with β aminooxy linker, **4**) failed to provide similarly strong binding. One possible explanation is that less glycan was coupled to disialylated biantennary species in the latter (β-SAM2) than the former (α-SAM2) conjugate. The fact that the dimannose moiety is always attached via an α linkage to the inner mannose residues of natural phosphorylated oligomannoses indicates that not only is the structure of the dimannose important but also its optimal orientation for high affinity receptor binding. Interestingly, even between the two conjugates containing the same bisphosphorylated hexamannose but with either α or β linker, the β-SAM6 with β-hexamannose (***β*-1**) was superior to α-SAM6 with α-hexamannose (***α*-1**) in receptor-mediated cell uptake and glycogen clearance, perhaps because the natural phosphorylated oligomannoses are attached to the core GlcNAc with a β linkage. Together, these results suggest that the linkage configuration even at the reducing end of the glycan is critical for maximum CI-MPR interaction.

■ CONCLUSION

We have investigated the specificity of CI-MPR recognition by using protein conjugates with various synthetic glycans. A high affinity interaction with the CI-MPR can be observed for rhGAA conjugated to simple glycans containing a dimannose glycan with a single phosphate. Our approach is unique in that the receptor specificity for glycans was illustrated by comparing the binding of a recombinant lysosomal enzyme containing similar amounts of glycans that are structural components of phosphorylated oligomannoses observed in nature. Thus, we can clearly distinguish the impact from the number of mannoses and phosphates as well as the linker orientation on receptor binding, in contrast to the use of modified natural glycan mixtures. We also used multiple characterization methods to demonstrate that not only the phosphorylated dimannose structure determinant but also its optimal orientation are important for maximum receptor recognition. This novel approach for conjugating synthetic glycans to proteins opens the possibility of addressing questions on the structure−function relationships of critical carbohydrate−lectin interactions. Moreover, the simpler glycans we have developed can be used to evaluate targeting of other molecules including proteins and nucleic acids to the CI-MPR.

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Notes

The authors declare the following competing financial interest(s): All authors are employees of Genzyme Corporation, A Sanofi Company.

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■ ABBREVIATIONS

Man-6-P, mannose 6-phosphate; bisM6P, bis mannose 6-phosphate; CI-MPR, cation-independent mannose 6-phosphate receptor; rhGAA, Recombinant human acid α-glucosidase; SAM, sialic acid-mediated; IGF II, insulin-like growth factor II

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