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Biochemistry

A cysteine-rich domain of the "mannose" receptor mediates GalNAc-4-SO_4 binding

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ABSTRACT **A critical element of lutropin bioactivity *in vivo* is its rapid removal from the blood by a receptor, located in hepatic endothelial cells, that recognizes the terminal sulfated carbohydrate structure SO_4-4-GalNAcβ1,4GlcNAcβ1,2Manα (S4GGnM). We have previously shown that the macrophage mannose (Man)-receptor cDNA directs the synthesis of a protein that binds oligosaccharides with either terminal S4GGnM or terminal Man, at independent sites. We now show that the cysteine-rich (Cys-Rich) domain at the N terminus of the Man/S4GGnM receptor accounts for binding of oligosaccharides with terminal GalNAc-4-SO_4, whereas calcium-dependent carbohydrate recognition domains (CRDs) account for binding of ligands containing terminal Man. The Cys-Rich domain is thus a previously unrecognized carbohydrate binding motif. Cys-Rich domains have been described on the three other members of the endocytic C-type lectin family of receptors. The structural relationship of these receptors to the Man/S4GGnM receptor raises the possibility that their Cys-Rich domains also bind carbohydrate moieties and contribute to their function.**

Glycoproteins such as lutropin (LH) and thyrotropin that bear multiple Asn-linked oligosaccharides terminating with the sequence SO_4-4-GalNAcβ1,4GlcNAcβ1,2Manα (S4GGnM) are rapidly removed from the circulation by an S4GGnM-specific receptor found at the surface of hepatic endothelial cells (1, 2). Precise control of the circulatory half-life of LH is thought to be critical for attaining maximal stimulation of the LH receptor located in the ovary during the preovulatory surge in LH levels. We recently reported (3) that the S4GGnM receptor (S4GGnM-R) isolated from rat liver is closely related to the macrophage mannose receptor (Man-R) isolated from rat lung. However, the liver S4GGnM-R and lung Man-R display marked differences in ligand specificity and binding properties. BSA that has been chemically modified to bear multiple S4GGnM trisaccharides (S4GGnM-BSA) is bound by the S4GGnM-R but not by the Man-R. The S4GGnM-R and the Man-R both bind BSA modified with mannose (Man-BSA); however, the kinetics of binding differ for the liver and lung receptors (3). We subsequently determined that a Man-R cDNA prepared from mouse pulmonary macrophages when expressed in Chinese hamster ovary (CHO) cells directs the synthesis of a receptor that binds both S4GGnM-BSA and Man-BSA, at independent sites (4). A fusion protein produced by replacing the transmembrane domain of the Man-R with the constant region of human IgG1 also binds both S4GGnM-BSA and Man-BSA. The ability of recombinant Man-R to bind both types of ligands led us to propose that this receptor be referred to as the Man/S4GGnM receptor to reflect its actual dual specificity (4).

The Man/S4GGnM-R consists of a cysteine-rich (Cys-Rich) domain, a fibronectin type II repeat (FN-II), eight carbohydrate recognition domains (CRDs), a transmembrane domain, and a cytosolic domain (5, 6) (Fig. 1). CRDs 4–8 are known to mediate binding of ligands bearing terminal Man, whereas no binding capacity has been attributed to other regions of the receptor (7, 8). We have generated deletion mutants to locate the region responsible for binding oligosaccharides terminating with GalNAc-4-SO_4 and to determine whether this region is physically distinct from the CRDs that mediate binding of structures terminating with Man.

MATERIALS AND METHODS

Constructs. The Man/S4GGnM-Fc chimera was prepared by replacing the transmembrane and cytosolic domains of the Man/S4GGnM-R with the hinge, C_H2, and C_H3 domains of human IgG1 as described (4). Deletion mutants of the Man/S4GGnM-Fc chimera were prepared by inverse PCR (9), using KLA-*Taq* (10). The primers each contained a 5′–*Sal*I site, which would be in-frame following ligation. The PCR product was digested with *Dpn*I and then with *Sal*I. Following phenol/chloroform extraction and ethanol precipitation the product was ligated overnight at 16°C with DNA ligase. *Escherichia coli*, 1061/P3, were transformed by electroporation and cultured in the presence of ampicilin and tetracycline. Colonies were screened for mutants with the appropriate deletion by PCR. The presence of the appropriate deletion was confirmed by *Sal*I digestion, by cycle sequencing using fluorescently labeled dideoxynucleotides, and *Taq* DNA polymerase under the conditions recommended by the supplier (Applied Biosystems), and by characterization of the protein product following expression (see below). The deletion mutants that were examined are shown in schematic form in Fig. 1.

Transfection of CHO/Tag Cells. Each construct was transfected into CHO/Tag 30A cells by using lipofectamine as described (4). Forty-eight hours after transfection, cells were incubated with Tran^{35}S-label (ICN) (100 μCi/ml) for 3 hr at 37°C in αMEM (GIBCO/BRL). The medium was then changed and the cells incubated for an additional 3 hr. The cells and medium were collected separately for analysis. Cells were lysed with 1.5 ml of 20 mM Tris·HCl buffer, 200 mM NaCl, 2 mM EDTA, 2% Triton X-100, 2 μg/ml aprotinin, 1 mM Pefabloc SC (Boehringer Mannheim), pH 7.4; per 100-mm-diameter culture plate. The cell extract and medium were adjusted to a final concentration of 100 mM borate, pH 8.9, 3.0 M NaCl, 0.05% Triton X-100, 300 mM GlcNAc. The extract and medium were incubated with protein A-Sepharose and washed as described (4). The bound fusion protein was then

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Abbreviations: Man, mannose; Man-R, mannose receptor; S4GGnm, SO_4-4-GalNAcβ1,4GlcNAcβ1,2Manα; S4GGnM-R, SO_4-4-GalNAcβ1,4GlcNAcβ1,2Manα-receptor; CHO, Chinese hamster ovary; Cys-Rich, cysteine-rich; CRDs, carbohydrate recognition domains; FN-II, fibronectin type II; LH, lutropin.
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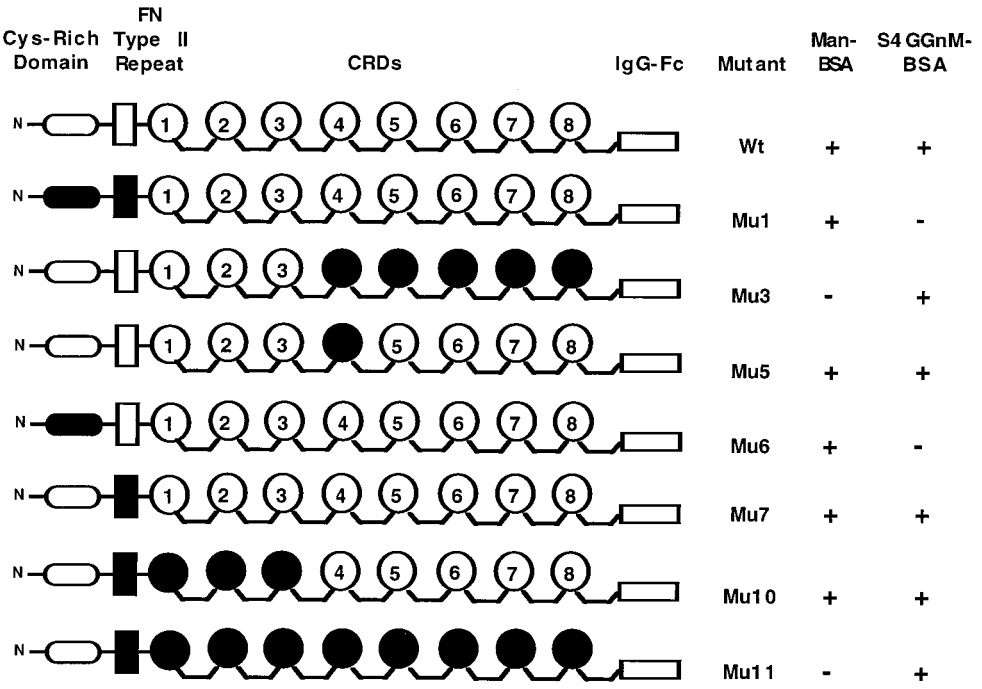

FIG. 1. Schematic representation of the Man/S4GGnM-Fc chimera and the deletion mutants examined for their ability to bind Man-BSA and S4GGnM-BSA. The wild-type chimera (Wt) consists of a Cys-Rich domain (Cys-Rich Domain), a fibronectin (FN) type II repeat, eight CRDs, and the Fc region of human IgG1 (IgG-Fc), which has replaced the transmembrane and cytosolic domains of the native receptor (4). Deletion mutants of the receptor were prepared by inverse PCR (9). Regions shown in black were deleted. The ability to bind (+) or not bind (−) Man-BSA and S4GGnM-BSA is indicated for each mutant based on the results shown in Fig. 3. The EMBL accession number for the murine Man-R is Z11974.

eluted with 100 mM glycine, pH 3.0, 0.1% BSA, 0.05% Triton X-100 and immediately neutralized with 1/10 vol 1.0 M Tris·HCl, pH 8.0. Equal aliquots of the fusion protein prepared from the cell extract and the medium were analyzed by SDS/PAGE (11) and autoradiography. Fusion proteins were prepared for binding studies in the same fashion but were not radiolabeled.

Binding Assays. Man/S4GGnM-Fc chimeras were incubated with ^{125}I-labeled S4GGnM-BSA or Man-BSA ($2–3 \times 10^5$ dpm; 1×10^7 dpm/μg), prepared as described (3), in a final volume of 150 μl containing 20 mM Tris·HCl, 150 mM NaCl, 2 mM $CaCl_2$, 1% (wt/vol) Triton X-100, 0.1% bovine IgG at a pH of 7.5. After 30 min at room temperature, 1.5 ml ice-cold 10% (wt/vol) PEG-8000 (Sigma) in 20 mM Tris·HCl, 150 mM NaCl, and 2 mM $CaCl_2$ was added and precipitated complexes collected after 30 min on ice by filtration of Whatman GF/C filter discs as described (3).

RESULTS

A soluble, secreted chimera of the Man/S4GGnM-R in which the cytosolic and transmembrane domains of the Man/S4GGnM-R have been replaced by the constant region of human IgG1, Man/S4GGnM-Fc, binds Man-BSA and S4GGnM-BSA at independent sites (4). We have used inverse PCR to prepare mutants of the Man/S4GGnM-Fc chimera in which various regions were deleted as illustrated in Fig. 1. Nucleotide sequence analysis confirmed that the appropriate deletion had been introduced in each instance and that the reading frame had been maintained. Each of the mutants when transiently expressed in CHO cells and metabolically labeled with Tran^{35}S-label (ICN) is secreted into the medium and can be isolated by affinity chromatography on protein A-Sepharose as described previously for the wild-type chimera (4). When examined by SDS/PAGE the mutants displayed decreases in apparent mobility, which were consistent with the size of the region deleted (not shown). Each of the mutants can be detected by Western blot analysis using a highly specific rabbit antibody raised to the receptor purified from rat liver (3) (not shown). Finally, as will be shown below, each mutant is able to bind either Man-BSA or S4GGnM-BSA or both.

The wild-type chimera and each of the mutants illustrated in Fig. 1 were tested for their ability to bind Man-BSA and S4GGnM-BSA using an assay in which complexes are precipitated with polyethylene glycol (PEG 8000, Sigma) and collected on glass fiber filters (3, 4). The results of these assays are shown in Fig. 2. Deletion of CRD4 (Mu5), which has been considered the major contributor to binding glycoconjugates with terminal mannose (7), does not result in a loss of Man-BSA binding, whereas deletion of CRDs 4–8 (Mu3) abolishes Man-BSA binding; neither deletion affects binding of S4GGnM-BSA. This implicates the N-terminal half of the receptor as the region containing the binding site for S4GGnM-BSA. Deletion of the Cys-Rich and FN-II domains (Mu1) abolishes S4GGnM-BSA binding, but has no effect on binding of Man-BSA, localizing the S4GGnM-binding site to the Cys-Rich domain and/or the FN-II repeat. Deletion of the Cys-R domain (Mu6), but not the FN-II repeat (Mu7) abolishes S4GGnM-BSA binding. Furthermore, deletion of the FN-II repeat and CRDs 1–8 (Mu11) also does not abolish binding of S4GGnM-BSA, indicating that the Cys-Rich region is both necessary and sufficient to bind oligosaccharides terminating with the sequence S4GGnM. Mu10, obtained by deleting FN-II and CRDs 1–3, retains the ability to bind both S4GGnM-BSA and Man-BSA.

The results summarized in Fig. 1 demonstrate that different domains within the Man/S4GGnM-R account for binding of S4GGnM and Man-terminal ligands. We examined the possibility that the kinetics for binding ligands terminating with either S4GGnM or Man are modulated by regions of the receptor not directly participating in binding. We analyzed saturation curves for Man/S4GGnM-Fc Wt, Mu1, and Mu11 binding Man-BSA and S4GGnM-BSA (Fig. 3). Man/S4GGnM-Fc(Wt) bound Man-BSA and S4GGnM-BSA with

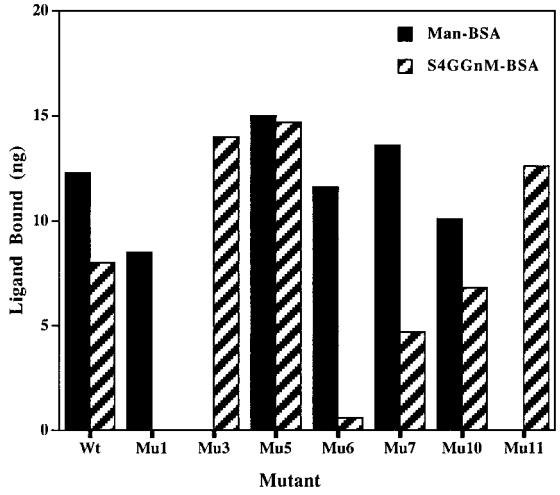

FIG. 2. The Cys-Rich region of the Man/S4GGnM-Fc chimera accounts for binding of oligosaccharides with terminal GalNAc-4-SO_4. Each of the Man/S4GGnM-Fc constructs shown in Fig. 1 was expressed in CHO-Tag 30A cells and isolated from the medium by affinity chromatography on protein A-Sepharose (Pharmacia) as described (4). The purified chimeras were incubated with S4GGnM-[^{125}I]BSA (striped bars) or Man-[^{125}I]BSA (solid bars) for 30 min at room temperature. The complexes formed were precipitated by addition of polyethylene glycol and were collected by filtration on Whatman GF/C filter discs as described (3). The amount of radiolabel precipitated was determined using a γ-counter. Because the efficiency of secretion differed significantly among the Fc chimeras, the amount of protein secreted during metabolic labeling with Tran^{35}S-label (ICN) as compared with the wild-type chimera was used to estimate the relative amount of each construct being produced.

apparent K_ds of 4.4×10^{-9} M and 11.9×10^{-9} M, respectively. When corrected for the differing amounts of Man/S4GGnM-Fc(Wt) used in the two assays, the B_{max} values for Man-BSA and S4GGnM-BSA were identical. Thus, the Man/S4GGnM-Fc(Wt) chimera binds essentially the same amount of Man-BSA and S4GGnM-BSA at saturation.

Man/S4GGnM-Fc(Mu1), lacking the Cys-Rich domain and the FN-II repeat, binds Man-BSA with an apparent K_d of 3.1×10^{-9} M, whereas Man/S4GGnM-Fc(Mu11), containing only the Cys-Rich domain, binds S4GGnM-BSA with an apparent K_d of 13.2×10^{-9} M (Fig. 3 and Table 1). Both values are statistically indistinguishable from those obtained with the Man/S4GGnM-Fc(Wt) chimera, which is able to bind both ligands. Thus, the regions of the Man/S4GGnM-Fc chimera, which mediate binding of Man-BSA and S4GGnM-BSA, function independently. The Cys-Rich domain of the Man/S4GGnM-R is both necessary and sufficient to mediate binding of glycoproteins bearing oligosaccharides terminating with SO_4-4GalNAcβ1,4GlcNAcβ, whereas CRDs 4–8 are necessary and sufficient to mediate binding of glycoproteins bearing oligosaccharides with terminal Man.

DISCUSSION

A feature of the glycoprotein hormones LH and thyrotropin that makes them unique is the presence of Asn-linked oligosaccharides terminating with the sequence SO_4-4-GalNAcβ1,4GlcNAcβ rather than the relatively common sequence Sialic acid α2,3/6Galβ1,4GlcNAcβ (12, 13). The sulfated structures, apparently critical for glycoprotein hormone function, are present on the glycoprotein hormones of all classes of vertebrates from man to fish (14). Thus, like the peptide portion of the glycoprotein hormones, the unique structural features of their oligosaccharides have been conserved throughout vertebrate evolution. We have proposed

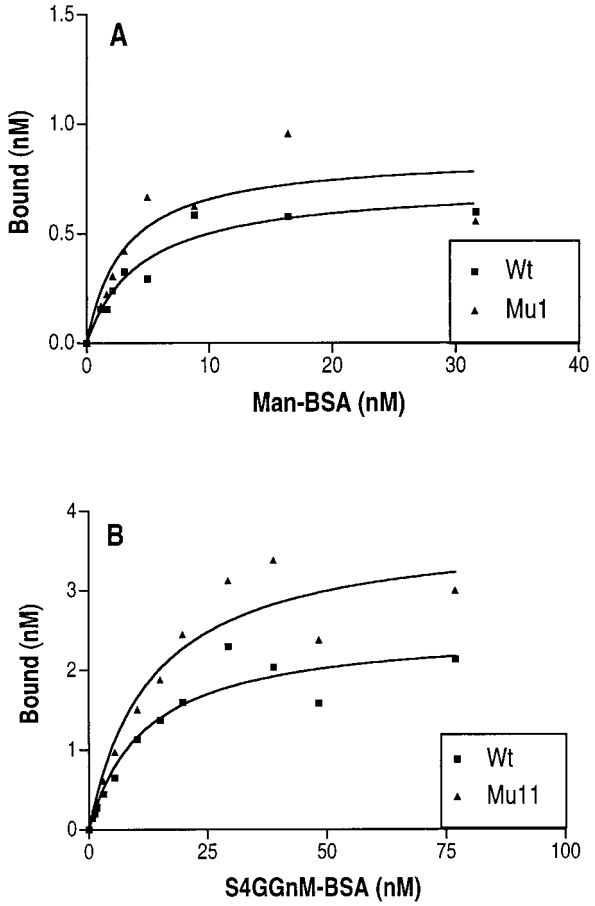

FIG. 3. Comparisons of saturation curves for Man-BSA and S4GGnM-BSA binding curves were obtained for Man-BSA with Man/S4GGnM-Fc(Wt) and (Mu1) (*A*) and for S4GGnM-BSA with Man/S4GGnM-Fc(Wt) and (Mu11) (*B*) using the assay described in Fig. 2. The apparent K_d was determined by using nonlinear regression analysis providing the results summarized in Table 1. Three-fold more Man/S4GGnM-Fc(Wt) was used to analyze S4GGnM-BSA binding in *B* than for analysis of Man-BSA binding in *A*. At saturation, Man/S4GGnM-Fc(Wt) bound the same amount of Man-BSA and S4GGnM-BSA per μg of chimera.

that these unique sulfated structures are important for the expression of full biologic activity by LH (15), because they are recognized by the S4GGnM-R, which is present in large amounts (500,000 receptors per cell) in hepatic endothelial cells (1). The S4GGnM-R mediates the clearance of LH from the circulation (2). In conjunction with secretion of LH from dense core storage granules found in gonadotrophs of the anterior lobe of the pituitary, rapid clearance by the S4GGnM-R produces the episodic rise and fall of LH in the blood. This results in maximal stimulation of the ovarian LH receptor by allowing desensitized receptor to be removed from the cell surface and replaced by unoccupied LH receptors (16–18).

The receptor that recognizes these structures and plays such an important role in the expression of LH bioactivity *in vivo* has a number of remarkable features. We have shown that the recombinant receptor binds oligosaccharides with terminal GalNAc-4-SO_4 and oligosaccharides with terminal Man. We have, therefore, adopted the term Man/S4GGnM-R to reflect this dual specificity. *In vivo*, however, the Man/S4GGnM-R can take on different forms. The Man-R isolated from lung binds Man-BSA but not S4GGnM-BSA, whereas the S4GGnM-R receptor isolated from liver binds both ligands (3). Features of the receptor that seem likely to reflect posttran-

Table 1. Mu1 and Mu11 have the same respective K_d for binding Man-BSA and S4GGnM-BSA as does the wild-type Man/S4GGnM-Fc chimera

	Man/S4GGnM-Fc chimera, mean ± SE		
Oligosaccharide	Wt	Mu1	Mu11
Man-BSA			
K_d	$4.4 \pm 1.1 \times 10^{-9}$ M	$3.1 \pm 1.5 \times 10^{-9}$ M	
S4GGnM-BSA			
K_d	$11.9 \pm 3.4 \times 10^{-9}$ M		$13.2 \pm 3.9 \times 10^{-9}$ M

The results of nonlinear regression analyses of the saturation experiments shown in Fig. 3 are summarized. Additional saturation studies performed with independently prepared batches of each chimera yielded similar results. The standard error (SE) is indicated.

scriptional and/or posttranslational modifications may determine whether Man-specific or S4GGnM-specific binding activity is expressed by the Man/S4GGnM-R in a particular cell type or circumstance.

The present studies demonstrate that the regions that account for Man binding and S4GGnM binding by the Man/S4GGnM-R are physically distinct. The region accounting for Man binding, CRDs 4–8, has previously been extensively characterized (7, 8). We have now shown that the Cys-Rich domain located at the amino terminus of the receptor represents a carbohydrate binding motif that is unrelated to others previously characterized. In contrast with the multiple CRDs on the Man/S4GGnM-R only a single Cys-Rich domain is present. This difference provides a potential explanation for the properties of the receptor isolated from liver and lung. Dimerization or multimerization could present a cluster of Cys-Rich domains and at the same time alter access to CRDs 4–8. Thus, if the S4GGnM-R from liver and the Man-R from lung exist as dimeric/multimeric and monomeric forms, respectively, this could account for their different ligand binding properties. Further studies will be required to address these issues.

A chimeric protein similar to Mu11 in Fig. 1, consisting of the Cys-Rich domain and the Fc region of human IgG1, was recently reported to selectively bind macrophages from the splenic marginal zone and the lymph node subcapsular sinus. During a secondary immune response to ovalbumin, the cells reactive with the Cys-Rich-Fc construct were found to accumulate in developing germinal centers. Notably, multiple proteins were reactive with the Cys-Rich-Fc construct during Western blot analyses of total protein lysates from lymph node and spleen (19). In light of the specificity of the Cys-Rich domain of the Man/S4GGnM-R for the terminal sequence S4GGnM, it is likely that a number of glycoproteins, each bearing terminal GalNAc-4-SO_4, are being synthesized by the cells that have been identified by these immunostains. Expression of the protein-specific β1,4GalNAc transferase and the GalNAc-4 sulfotransferase, and/or their protein acceptors, may be induced in these cells during the secondary immune response or cells expressing these transferases may migrate to these sites. In either case, oligosaccharides bearing the terminal sequence S4GGnM may play different but equally important roles during the hormonal cycle and secondary immune responses.

Four receptors with multiple CRDs have to date been identified: (*i*) the Man/S4GGnM-R found in macrophages and hepatic endothelial cells (5, 6, 20); (*ii*) DEC-205 found in thymic epithelial cells and dendritic cells (21); (*iii*) the phospholipase A_2 receptor, which is widely distributed (22, 23); and (*iv*) a "novel" lectin (MMU56734) found on mouse endothelial cells and chondrocytes (24). They constitute a family of receptors that is collectively referred to as the endocytic C-type lectins (24). Each of these receptors has a Cys-Rich domain followed by a FN-II repeat at its amino terminus. The Cys-Rich domain is the least highly conserved region among these receptors (20, 24), even though this domain is encoded by a single exon and each of the CRDs is encoded by multiple exons (24–26). The Cys-Rich domain of the Man/S4GGnM-R has a defined specificity for a well-characterized carbohydrate. It is possible that the Cys-Rich domains of one or more of the other receptors with multiple CRDs may also bind carbohydrate moieties. In contrast with saccharide binding by CRDs (20, 27), the Cys-Rich domain does not require Ca^{2+} to bind oligosaccharides with terminal GalNAc-4-SO_4. The Cys-Rich domain does have multiple disulfide bonds that may serve to stabilize the receptor when it is exposed to acidic pH during endocytosis.

The Man/S4GGnM-R is an example of a carbohydrate-specific receptor that is capable of binding carbohydrates with different structures independently at different sites. It may also be an example of a carbohydrate-specific receptor whose specificity can be modulated or changed *in vivo*. The presence of the Man/S4GGnM-R on macrophages (28), hepatic endothelial cells (1), and other cells (Y. L. Mi and J.U.B., unpublished observation), along with its dual specificity, suggests that the functions of the receptor differ depending on the setting. In contrast with the oligosaccharide structures recognized by CRDs 4–8, which are found on many glycoproteins and on yeast mannans, oligosaccharides terminating with the S4GGnM sequence have to date been identified in only a small number of glycoproteins. The synthesis of these structures is highly regulated and is generally protein specific (12, 29). Thus, the Man/S4GGnM-R is a critical component of a complex system in which highly specific glycosyltransferases are used to synthesize oligosaccharides terminating with S4GGnM on specific target glycoproteins. This is likely to be a versatile system that fulfills multiple roles *in vivo*.

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