The Oligosaccharide Binding Specificities of CD22β, a Sialic Acid-specific Lectin of B Cells*

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CD22β is a B cell surface glycoprotein involved in cell adhesion and activation. We previously reported that a recombinant soluble form termed CD22βRg is capable of binding α2–6 sialylated complex N-linked oligosaccharides purified from lymphocyte glycoprotein ligands (Powell, L. D., Sgroi, D., Sjoberg, E. R., Stamenkovic, I., and Varki, A. (1993) J. Biol. Chem. 268, 7019–7027). Here, we utilize a number of naturally and enzymatically sialylated oligosaccharides and sialoglycoproteins to further define its lectin specificity and demonstrate that the minimal structure recognized is Neu5Acα2–6Galβ1–4Glc(NAc). Reduction of the glucose residue of Neu5Acα2–6Galβ1–4Glc diminishes the interaction, while truncation of the sialic acid side chain by mild periodate oxidation abolishes it. Branched oligosaccharides with two α2–6-sialyl residues bind better, regardless of whether they were derived from N- or O-linked oligosaccharides or from gangliosides. α2–3-Sialyl residues have no effect on binding, whereas increasing the number of α2–6-sialyl residues on multiantennary oligosaccharides progressively improves binding. No specific feature of the core region affects binding, although the spacing of the α2–6-sialyl residues on tetraantennary chains appears to have a significant effect. Of several model sialoglycoproteins examined, fetuin and transferrin had an apparent affinity no greater than that observed with free sialylated N-linked oligosaccharides. Some subfractions of these proteins displayed unexpectedly weak binding, suggesting that the protein backbone can exert a negative effect. In contrast, a subfraction of α1-acid glycoprotein was identified as having a substantially higher apparent affinity than free oligosaccharides derived from it, indicating that multiple glycosylation sites may increase the apparent binding affinity. Thus, CD22βRg contains a lectin activity specific for the minimal motif Neu5Acα2–6Galβ1–4Glc(NAc), and branched, multisialylated oligosaccharides are better ligands, regardless of the core sequences. Intact sialoglycoproteins can also interact, although with a variable affinity not directly predictable from the precise structure of their sialylated oligosaccharides chains. These data may help to explain why certain T and B cell surface sialoglycoproteins with the Neu5Acα2–6Galβ1–4Glc(NAc) motif are superior ligands, capable of mediating CD22β-mediated adhesion and activation events.

CD22β is a cell surface glycoprotein found on a subset of IgM⁺ B cells and is involved both in cell activation and adhesion to subpopulations of T and B cells (1–7). A soluble chimeric form, termed CD22βRg and created by fusing the three N-terminal domains to the two C-terminal domains of IgG (8), was earlier utilized to identify glycoprotein ligands for CD22β including CD45 and other surface glycoproteins on T and B cells (9). Sialylation of these glycoproteins is essential for binding, as recognition is blocked by pretreatment with sialidase or by mild periodate oxidation under conditions specific for truncation of the exocyclic side arm of sialic acid (3, 5, 9). Moreover, cells that did not express β-galactoside α-2,6-sialyltransferase (α2–6STN)¹ contained no glycoproteins recognized by CD22βRg, whereas transfection of a plasmid coding for this enzyme induced expression of ligands (8, 9).

Studies of the subpopulation of glycoproteins precipitated by CD22βRg from Daudi cells, a B lymphoid cell line, demonstrated a lectin activity specific for complex-type N-linked oligosaccharide structures containing α2–6-linked sialic acid (5). The assay used monitored the elution position of metabolically labeled oligosaccharides applied to a column of CD22βRg bound to protein A-Sepharose. Nonbinding molecules eluted in the column's Vᵢ, whereas binding molecules eluted later, in positions corresponding to increasing degrees of interaction. Binding was eliminated by prior treatment with Arthrobacter ureafaciens sialidase, which cleaves α2–3-, α2–6-, and α2–8-linked sialyl residues, but not by Newcastle disease virus sialidase, which cleaves α2–3- and α2–8-linked residues (5), indicating that CD22βRg bound specifically to chains containing α2–6-linked residues. Moreover, oligosaccharides containing two or three α2–6-linked residues eluted after those containing just one, implying multiple binding sites. However, labeled chains containing two α2–6-linked residues included species that eluted in different positions on the CD22βRg column, indicating that other structural features influence CD22βRg binding.

The complexity of the mixture of oligosaccharides from Daudi cells made further analysis of this question difficult. We, therefore, have examined defined oligosaccharides that were enzymatically resialylated to varying extents with rat liver α2–6STN. This sialyltransferase is specific not only for adding sialic acid to Galβ1–4GlcNAcβ1-R residues found on nonreducing termini of oligosaccharide structures (10), but it also has an established order of addition when sialylating multiantennary N-linked chains (11). Such structurally defined sialylated oligosaccharides were utilized to further define the lectin activity of CD22β. Several model sialoglycoproteins with previously es-

¹ The abbreviations used are: α2–6STN, β-galactoside α-2-6-sialyltransferase (EC 2.4.99.1); α2–3STN, Galβ1,3(4)GlcNAc:CMP-sialic acid α2–3-sialyltransferase; α₁-AG, α₁-acid glycoprotein; Neu5Ac, N-acetylneuraminic acid; Sia, sialic acid, type unspecified; FITC, fluorescein isothiocyanate; Glc-OH, glucitol.

tablished oligosaccharide structures were utilized to further study the contribution of the protein backbone to these interactions.

EXPERIMENTAL PROCEDURES

Sources of Oligosaccharides—Human α_1-acid glycoprotein (α_1-AG) (Sigma) was digested sequentially with *A. ureafaciens* sialidase (Calbiochem) and (following dialysis into the appropriate buffer) peptide:*N*-glycosidase F (5). The released oligosaccharides were desalted and further fractionated on concanavalin A-agarose (Sigma) (5) into nonbinding (tri- and tetraantennary) and binding (biantennary) oligosaccharides. Alternatively, sialylated α_1-AG oligosaccharides released by peptide:*N*-glycosidase F were reduced with NaB[^{3}H]H$_4$ as described (12) with replacement of the paper chromatography step with desalting twice on Bio-Gel P-2 (Bio-Rad). The resulting oligosaccharides were fractionated by negative charge on a Mono Q column (5), and those eluting with four negative charges were desialylated with *A. ureafaciens* sialidase and used as a source of tetraantennary oligosaccharides (with varying degrees of outer $\alpha1$–3 fucosylation). Commercial sources of defined complex oligosaccharides included an asialotriantennary oligosaccharide from fetuin (FT02; Dionex Corp. Sunnyvale, CA), a nonfucosylated tetraantennary oligosaccharide (GP02; Dionex Corp, desialylated by *A. ureafaciens* sialidase), and an asialobiantennary bisected oligosaccharide (C-024311; Oxford GlycoSystems, Rosedale, NY). The branched *neo*-lacto series ganglioside (Ga7, the generous gift of Michiko Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA (13)) was digested concomitantly with 3 milliunits of endo-β-galactosidase and 20 milliunits of *A. ureafaciens* sialidase in 20 mM sodium citrate, pH 5.5, 1.0% taurocholate, and the released oligosaccharides were purified over Amberlite mixed bed resin and Bio-Beads SM-2 (Bio-Rad) to yield the desialylated structure. Bovine submaxillary mucin (14) glycopeptides were obtained by extensive digestion with Proteinase K (Boehringer Mannheim) at 50 °C, followed by chromatography on Sephadex G-50 (pooling the V_i material) and Bio-Gel P-2 (pooling the V_o material), monitoring by acid hydrolysis and the thiobarbituric acid assay (15). A portion of this material was de-*O*-acetylated with 10 mM NaOH for 60 min at 37 °C. A mixture of neutral human bronchial mucin oligosaccharides (fraction Ic as described in Refs. 16 and 17), prepared by alkaline borohydride hydrolysis, ion exchange chromatography, and Bio-Gel P-4 chromatography, was generously provided by Dr. A. Klein.

Sialylation of Neutral Oligosaccharides—Oligosaccharides (0.1–10 nmol) were dissolved in 20–50 μl of 0.1 M sodium cacodylate, pH 6.9, containing 1% Triton CF-54, 1 mg/ml bovine serum albumin, 0.1 μCi of CMP-[9-^{3}H]Neu5Ac (10 Ci/mmol), and 0.6–1.0 milliunits of α2–6STN (Boehringer Mannheim; or the generous gift of Dr. J. Paulson, Cytel Corp., La Jolla, CA). After 4–8 h at 37 °C, the reaction was either terminated (with 50 μl of 0.1 M citrate, pH 3.9, 60 min, 37 °C, to hydrolyze the remaining CMP-[9-^{3}H]Neu5Ac) or further sialylated with 5–30 nmol of CMP-Neu5Ac (Sigma) before termination. Labeled oligosaccharides were subsequently purified away from the free [^{3}H]Neu5Ac on Bio-Gel P-2, eluted in 20 mM pyridineacetate, pH 5.5, and further fractionated according to charge by ion exchange chromatography (5). Tri- and tetrasialylated oligosaccharides required two to three sialylation steps. Oligosaccharides were also sialylated with Galβ1,3(4)GlcNAc: CMP-sia α2–3-sialyltransferase (α2–3STN, also the generous gift of Dr. J. Paulson, Ref. 18). Galβ1–4Glc (0.4 M) or Galβ1–4GlcNAc (0.1 M) (Sigma) were sialylated as above, and the products were purified on Bio-Gel P-4 and Dowex 1 (Bio-Rad, eluted with 1 N formic acid) to remove both the free Neu5Ac and unsialylated disaccharide. [^{3}H]Neu5Acα2–6Galβ1–4Glc-OH was produced by reducing [^{3}H]Neu5-Acα2–6Galβ1–4Glc with 0.1 M NaBH$_4$ as above. Neutral [^{3}H]galactose-labeled α_1-AG oligosaccharides were prepared by de- and regalactosylation utilizing UDP-[^{3}H]Gal (5). A biantennary oligosaccharide containing one α2–3- and one α2–6-linked sialic acid residue was created by partial acid hydrolysis (2 M acetic acid, 1 h at 80 °C) of a α2–6-[9-^{3}H]Neu5Ac bisialylated biantennary structure, isolation of the monosialylated oligosaccharide, resialylation with CMP-Neu5Ac and α2–3STN, and reisolation of the disialylated product.

CD22βRg Chimera—The CD22βRg chimera was purified from transiently transfected COS cells (8) or from a CHO-K1 cell line stably transfected with the CD22βRg-coding plasmid. By SDS-polyacrylamide gel electrophoresis, the CHOK1-derived chimera migrates about 10 kDa larger than the COS-derived chimera, probably due to the presence of polylactosaminoglycans. However, in all binding studies performed, the two chimeras are indistinguishable.

CD22βRg columns were constructed by adsorbing either 200 μg or ~5 mg of protein to 0.6 ml of protein A-Sepharose (Pharmacia LKB

Biotechnology Inc.) in a siliconized 1-ml polystyrene pipette (5). Samples of ^{3}H-labeled oligosaccharides, mixed with the internal nonbinding marker [^{14}C]ManNAc, were applied at 4 °C in Tris-buffered Saline (20 mM Tris-Cl, pH 7.3, 140 mM NaCl, 0.02% sodium azide). Three drop fractions (~80 μl) were collected for 18–28 fractions (as indicated in the text), the column warmed to ambient temperature for 10 min and then eluted for a further 15 fractions with ambient temperature buffer.

FITC-labeled Glycoproteins—The sialylation state of fetuin and transferrin (both from Sigma) were first examined by high pH anion exchange chromotography analysis of the *N*-linked oligosaccharides released by hydrazinolysis (Oxford GlycoSystems GlycoPrep). While the fetuin oligosaccharides were comparable in sialylation to published reports (19), the transferrin oligosaccharides were found to be largely desialylated. Two hundred micrograms of fetuin, transferrin, or α_1-AG were incubated with 4 mg of FITC (isomer I, Sigma) in 300 μl of 50 mM NaHCO$_3$, pH 9.3, 25 mM NaCl, for 2 h at room temperature in the dark. Using ε = 77,000, 1–2 FITC molecules were incorporated per protein molecule. To correct the undersialylation of transferrin, 2.5 μg (~40 pmol) were resialylated sequentially with 1.6 μCi of CMP-[9-^{3}H]Neu5Ac and then 20 nmol of CMP-Neu5Ac. The FITC-tagged samples (1–3 μg of protein) were applied to the CD22βRg column in Tris-buffered Saline with 0.1% Nonidet P-40 to reduce nonspecific binding.

Precipitation of Glycoproteins with CD22βRg—Oligosaccharides or FITC-tagged glycoproteins were precipitated by mixing ~2 μg with 10 μg of CD22βRg chimera in 100 μl of Tris-buffered saline, 0.1% Nonidet P-40, for 4 h at 4 °C, followed by the addition of 50 μl of protein A-Sepharose (1–3 h). Following extensive washing, bound material eluted with 0.1 M acetic acid, 0.1% Nonidet P-40. FITC-labeled samples were titrated to ~pH 9.0 with 0.3 M Tris, pH 9.0, prior to reading fluorescence (excitation, 490; emission, 520).

Determination of Sialic Acid Linkages on α_1-AG—Fifty micrograms of α_1-AG were digested with Newcastle disease virus sialidase or *A. ureafaciens* sialidase (5), and aliquots were removed at 0, 20, 40, and 60 min for quantitation of free sialic acid by the thiobarbituric acid assay. The time course demonstrated complete release by 20 min under the conditions utilized.

RESULTS

Binding of Enzymatically Resialylated Oligosaccharides to CD22βRg—Initially, we examined a mixture of *N*-linked oligosaccharides from asialo-α_1-AG containing bi-, tri-, and tetraantennary structures in approximate molar ratios of 10/40/50, respectively, with about 40% of the structures containing $\alpha1$–3Fuc residues on outer antennae (20). These structures were partially resialylated with CMP-[9-^{3}H]Neu5Ac and either α2–6STN or α2–3STN. By ion exchange chromatography, the products were similar mixtures of mono-, bi-, and trisialylated species (Fig. 1, *A* and *B*, *insets*). When applied to a column containing immobilized CD22βRg, the α2–3-sialylated oligosaccharides failed to bind and coeluted with ManNAc (Fig. 1*A*). In contrast, the α2–6-sialylated oligosaccharides were significantly retarded in their elution (Fig. 1*B*). As seen previously with oligosaccharides from Daudi cells (5), when the column was initially run at 4 °C, two distinct populations of molecules (*I* and *II*) were retarded in elution relative to ManNAc. However, unlike the Daudi cell-derived structures, which gave a single additional peak after warming the column to 22–24 °C (5), two additional distinct populations were seen here (Fig. 1*B*, *III* and *IV*). Thus, CD22βRg has no detectable interaction with oligosaccharides containing α2–3-linked sialic acid residues but a complex pattern is seen with α2–6-sialylated structures.

The Elution Position of α2–6 Sialylated Structures on CD22βRg Is Only Partially Dependent upon the Number of Sialic Acid Residues—The four different pools of oligosaccharides from Fig. 1*B* were analyzed for negative charge content by ion exchange chromatography (Fig. 2). *Pools I* and *IV* contained predominantly mono- and trisialylated structures, respectively. However, *pools II* and *III* both contained bisialylated structures (Fig. 2). Thus, structural features other than the absolute number of α2–6 sialic acid residues are involved in the binding of bisialylated oligosaccharides.

Interaction of Purified Sialylated Oligosaccharides on

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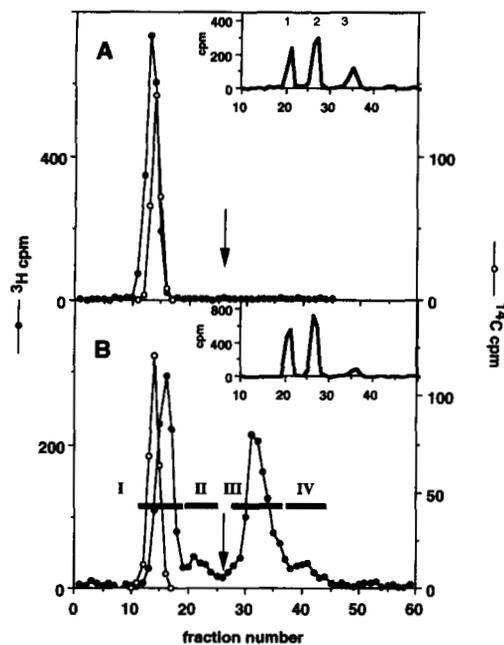
FIG. 1. **Fractionation of differentially resialylated oligosaccharides on a CD22βRg-protein A-Sepharose column.** Asialo-α_1-AG oligosaccharides were enzymatically resialylated with CMP-[9-^{3}H]Neu5Ac and either α2–3STN (*A*) or α2–6STN (*B*). Each sample, mixed with [^{14}C]ManNAc, was applied and eluted at 4 °C for the first 27 fractions (*arrow*); the column was warmed to 22–24 °C for 10 min and then eluted further. The *insets* show the ion exchange chromatograms of the applied samples, indicating the amount of mono-, bi-, and trisialylated structures (*peaks* labeled *1, 2,* and *3,* respectively). *Horizontal bars* in *B* correspond to the positions of pools *I, II, III,* and *IV* analyzed in Fig. 2.

CD22βRg—To determine the structural features other than sialylation that are involved CD22βRg binding, bi- and triantennary oligosaccharides were partially resialylated with α2–6STN and CMP-[9-^{3}H]Neu5Ac, fractionated by preparative ion exchange chromatography, and analyzed on the CD22βRg column. All of the monosialylated structures examined reproducibly eluted just after ManNAc (Fig. 3, *A* and *B*), indicating a weak but measurable interaction with CD22βRg. Bisialylated bi- and triantennary oligosaccharides coeluted in a position approximately 3–5 fractions after the column was warmed from 4 to 22–24 °C (Fig. 3, *C* and *D*). The trisialylated triantennary structure (Table I, structure 11) eluted after its bisialylated counterpart (Fig. 3*F*). The elution positions of the mono-, bi-, and trisialylated structures correspond to those of *pools I, III,* and *IV* from Fig. 1*B* and is in agreement with the ion exchange analysis of those pools shown in Fig. 2. A biantennary oligosaccharide containing one α2–3- and one α2–6-linked sialic acid residue coeluted with a mono-α2–6-sialyl biantennary oligosaccharide showing that an α2–3-linked residue had no effect, either positive or negative, on binding (Fig. 3*E*).

To further demonstrate the unique elution positions of the multisialylated oligosaccharides, they were applied to and eluted from the column entirely at 22–24 °C. At this temperature, the monosialylated oligosaccharide no longer showed detectable interaction, coeluting with ManNAc (data not shown). However, bi-, tri-, and tetrasialylated oligosaccharides (all α2–6 linkages) are clearly resolved, eluting in order of increasing number of sialylated residues (Fig. 4).

CD22βRg-Oligosaccharide Binding Is Influenced by the Location of the Sialic Acid Residues on the Oligosaccharide—A nonfucosylated tetraantennary chain containing 1–4 α2–6 sialic acid residues was examined next. The mono- and trisialylated structures (Fig. 5, *A* and *C*) eluted in positions iden-
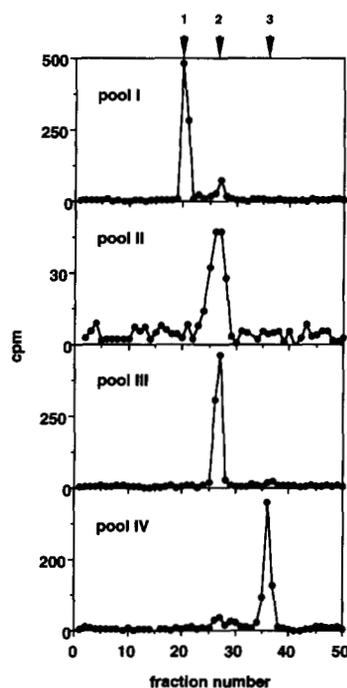
FIG. 2. **Ion exchange chromatograms of CD22βRg-fractionated oligosaccharides.** Oligosaccharides from the profile shown in Fig. 1*B* were analyzed on a Mono Q fast protein liquid chromatography column developed with a gradient of NaCl in 2 mM Tris base. The elution positions of standard oligosaccharides containing one, two, and three negative charges are shown by *arrows*.

tical to those of the similarly sialylated bi- and triantennary structures examined in Fig. 3. The tetrasialylated tetraantennary oligosaccharide eluted approximately three fractions after the trisialylated oligosaccharide, consistent with the results presented in Fig. 4. Of note, naturally occurring oligosaccharides with four α2–6 linked sialyl residues have not been reported.

In contrast, the elution of the bisialylated structures was more complex (Fig. 5*B*), with approximately 60% of the material eluting at 4 °C and the remainder eluting at 22–24 °C in a position identical to bisialylated structures (Fig. 3). The first portion corresponds in elution position to *pool II* of Fig. 1*B* and to the unexplained bisialylated molecules previously described from Daudi cells (see Fig. 3 of Ref. 5). The same pattern was observed with tetraantennary oligosaccharides purified from α_1-AG, which contain some amount of α1–3 fucose residues, and with nonfucosylated tetraantennary oligosaccharides. A plausible explanation for this observation is provided by examining the branch specificity of α2–6STN (11). The first sialic acid residue is generally added to the antenna linked α1–2 to the α1–3-linked mannose. (Fig. 5*E*). However, the location of the second residue varies between tri- and tetraantennary oligosaccharides. For a triantennary structure, the second residue is added to the α1–6-linked antenna 90% of the time, forming a structure similar to a bisialylated biantennary oligosaccharide. In contrast, two different bisialylated tetraantennary structures may be found (11). One of them (Fig. 5*F, left-hand structure*) has the same pattern as in a bisialylated biantennary structure and probably represents the molecules eluting only after the CD22βRg column is warmed (Fig. 5*B*). The other isomer (Fig. 5*F, right-hand structure*) may represent the species eluting earlier at 4 °C. Although the ratios of the two fractions do not precisely correlate with those reported by Joziasse *et al.* (11), this may reflect different conditions of pH, substrate concentrations, and source of sialyltransferase used. Attempts to demonstrate sialylation isomerization in the two different
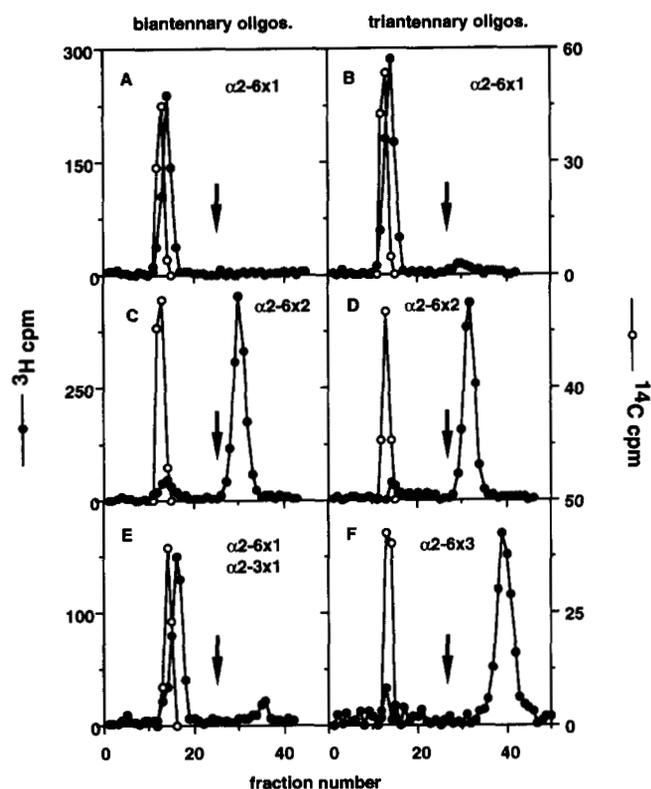
FIG. 3. **Fractionation on the CD22βRg-protein A-Sepharose column of bi- and triantennary N-linked oligosaccharides.** Free asialo-oligosaccharides were partially resialylated with CMP-[9-³H]Neu5Ac and α2–6STN, fractionated by negative charge, and then analyzed separately on the CD22βRg column exactly as in Fig. 1. The oligosaccharides analyzed included mono- (*A*) and bisialylated (*C*) biantennary chains; mono- (*B*), bi- (*D*), and trisialylated (*F*) triantennary chains; and a biantennary oligosaccharide containing one α2–6 and one α2–3 residue (*E*). The *arrow* indicates the point of the warming column from 4 to 22–24 °C.

pools from Fig. 5*B* by glycosidase digestion and lectin chromatography gave ambiguous results and were not pursued further.

The Minimal Structure Recognized by CD22βRg Is Neu5-Acα2–6Galβ1–4Glc(NAc)—Given the identical elution positions of bi-, tri-, and/or tetraantennary oligosaccharides carrying a single α2–6-linked sialic acid residue, smaller structures were examined next. CMP-[9-³H]Neu5Ac and the two different sialyltransferases were used to prepare Neu5Acα2–6Galβ1–4Glc, Neu5Acα2–6Galβ1–4GlcNAc, Neu5Acα2–6Galβ1–4Glcol, and Neu5Acα2–3Galβ1–4Glc. When analyzed on the CD22βRg column employed above, Neu5Acα2–6Galβ1–4Glc eluted one to two fractions after ManNAc, similar to the monosialylated N-linked oligosaccharides (data not shown). To further enhance the sensitivity of this assay, a second CD22βRg column was constructed with 25-fold more chimera. On this high density column at 4 °C, Neu5Acα2–6Galβ1–4Glc elutes six to eight fractions after ManNAc (Fig. 6*A*). All the structures containing one α2–6 sialic acid residue elute in approximately the same position (with the exception of Neu5Acα2–6Galβ1–4Glc-OH, which elutes earlier (Fig. 6*B*)) indicating that the intactness of the Glc ring is important but not critical for recognition. Even on this high density column, Neu5Acα2–3 Galβ1–4Glc continues to coelute with ManNAc (Fig. 6*A*). The α2–6 sialic acid-dependent lectin *Sambucus nigra* is known to recognize Neu5Acα2–6Galβ1–4Glc(NAc) in part through the Galβ1–4Glc(NAc) unit and can be inhibited by high concentrations of lactose (21). In contrast, 0.1 M lactose (Fig. 6*B*) or 0.2 M GlcNAc (data not shown) had no effect on the elution of Neu5Acα2–

6Galβ1–4Glc. Asialo-oligosaccharides from α₁-AG (a mixture of tri- and tetraantennary oligosaccharides) did not bind (Fig. 6*C*). Furthermore, a monosialylated biantennary and a mono-sialylated tetraantennary N-linked oligosaccharide coelute with Neu5Acα2–6Galβ1–4Glc (Fig. 6*C*). Taken together, these results indicate that CD22βRg recognizes specific structural features of the trisaccharide Neu5Acα2–6Galβ1–4Glc(NAc).

Examination of Other Oligosaccharide Structures Containing α2–6 Sialic Acid Residues—Several other oligosaccharide structures containing one or two α2–6 Neu5AcGalβ1–4GlcNAc units, created by partial resialylation with CMP-[9-³H]Neu5Ac and the α2–6STN and preparative ion exchange chromatography, were examined next. The monosialylated species of a *neo*-lacto series glycolipid (Table I, structure 8) coeluted with the other monosialylated structures (data not shown), and the bi-sialylated counterpart eluted immediately after warming the column to room temperature, exactly as seen with the bisialy-lated N-linked structures (Fig. 7*A*). To study O-linked oligosaccharides, which can potentially carry α2–6-linked sialic acid residues, the small neutral oligosaccharides derived by alkaline borohydride treatment of human bronchial mucins (16, 17) were resialylated with α2–6STN. Of the 35 different structures present in this mixture, eight contain a single, and two contain double Galβ1–4GlcNAc units at a nonreducing terminus as potential substrates for the α2–6STN (16, 17). Following sialylation and fractionation by negative charge, 93% of the sialy-lated structures contained a single sialic acid residue, and 7% contained two residues; the predicted structures of the latter are 6 and 7 in Table I. The monosialylated structures coeluted with the other monosialylated structures examined (data not shown). However, the bisialylated mucin-derived oligosaccharides eluted earlier than the other bisialylated structures (Fig. 7*B*). The structures of the two bisialylated oligosaccharides in this mucin population are very similar to those of the bisialy-lated *neo*-lacto-series glycolipid and differ only in the substitution of a N-acetylgalactosaminitol residue for the branching Gal residue, suggesting that the intact ring of the branching hexose residue probably plays a role in properly orienting the two α2–6 sialic acid residues for binding by CD22βRg. The presence of the bisecting GlcNAc residue on the branching mannose residue (Table I, structure 10) had no detrimental effect on binding (Fig. 7*C*). The sialylated glycopeptides from bovine submaxillary mucins, carrying a high density of Siaα2–6GalNAc-O-Ser/Thr, failed to interact with the column (data not shown).

Examination of Model Sialylated Glycoproteins—The intact sialoglycoprotein ligands for CD22βRg derived from lymphoid cells have a better affinity than the best oligosaccharide studied here. While free oligosaccharides from these proteins and those examined in this report cannot be precipitated directly (Ref. 5 and data not shown), the proteins can be precipitated, and the precipitates survive repeated washing and require denaturation for release (9). While the number, location, and arrangement of oligosaccharides on these glycoproteins may explain the improved affinity, there is too little information concerning these polypeptide sequences to permit exploration of these issues. We therefore turned to several well characterized serum glycoproteins that contain complex-type N-linked oligosaccharides of known structures. Serum transferrin contains two biantennary chains that are generally fully sialylated with α2–6-linked residues (22). However, of the lot of commercial transferrin utilized here, only 13% of the chains contained two and 28% contained one sialyl residue (data not shown). Bovine fetuin carries three triantennary N-linked and three O-linked chains. The latter contain a mixture of structures, including Siaα2–3 Galβ1–3(Siaα2–6)GalNAc-Ser/Thr (23, 24); however, the results described above with bovine submaxillary

Binding Specificities of CD22β

TABLE I

Relative binding of sialylated oligosaccharides to CD22βRg

Structure	Relative binding
1. Neu5Acα2-6GalNAc	0
2. Neu5Acα2-3Galβ1-4Glc	0
3. Neu5Acα2-6Galβ1-4Glc-OH	1+
4. Neu4Acα2-6Galβ1-4Glc	2+
5. Neu5Acα2-6Galβ1-4GlcNAc	2+

6. Neu5Acα2-6Galβ1-4GlcNAcβ1-6
                    GalNAc-OH
    Neu5Acα2-6Galβ1-4GlcNAcβ1-3
	3+

7.   Neu5Acα2-6Galβ1-4GlcNAcβ1-6
                    GalNAc-OH
    Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-3
	3+

8. Neu5Acα2-6Galβ1-4GlcNAcβ1-6
                    Galβ1-4GlcNAcβ1-3Gal
    Neu5Acα2-6Galβ1-4GlcNAcβ1-3
	4+

9. Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6
                    Manβ1-4GlcNAcβ1-4GlcNAc
    Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3
	4+

10. Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6
                    GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc
    Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3
	4+

11. Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6
                    Manβ1-4GlcNAcβ1-4GlcNAc
    Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3
        Neu5Acα2-6Galβ1-4GlcNAcβ1-4
	5+

12.   Neu5Acα2-6Galβ1-4GlcNAcβ1-6
    Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6
                    Manβ1-4GlcNAcβ1-4GlcNAc
    Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3
        Neu5Acα2-6Galβ1-4GlcNAcβ1-4
	6+

FIG. 4. **Binding at room temperature of sialylated oligosaccharides to the CD22βRg-protein A-Sepharose column.** In separate runs, a [9-³H]Neu5Ac-labeled (*solid symbols*) bi- or trisialylated triantennary oligosaccharides (from Fig. 3, *panels D* and *F*, respectively) or a tetrasialylated tetraantennary oligosaccharide (from Fig. 5D, indicated by *α2–6Sia × 4*) was mixed with [¹⁴C]ManNAc (*open symbol*), applied, and eluted from the CD22βRg-protein A-Sepharose column entirely at 22–24 °C. The three runs were aligned by the elution position of the ManNAc and plotted together; for clarity, only one of the [¹⁴C]ManNAc profiles is displayed.

mucin glycopeptides indicate that this *O*-linked sequence is not recognized by CD22βRg. The fetuin triantennary oligosaccharides are capped with α2-3- and α2-6-linked residues. Based on high pH anion exchange chromatography analysis of the fetuin utilized here and in comparison with published reports (19), 29% of the *N*-linked chains contain two Siaα2-6Galβ1–4GlcNAc-R structures, 32% contain one Siaα2-3 Galβ1–4(Siaα2–6)GlcNAc-R structure (in addition to other sialic acid

residues), 10% contain both sequences, and the remaining antennae are Siaα2-3 Galβ1–4GlcNAc-R (19). Human α₁-AG contains five *N*-linked chains, consisting of bi-, tri-, and tetraantennary structures in approximate molar ratios of 10/40/50 (20). Digestion with either *A. ureafaciens* sialidase or Newcastle disease virus sialidase indicated that of a total of 18.9 mol of sialic acid/mol of protein, 7.3 mol were α2-3-linked, and thus the remaining 11.6 mol was α2–6-linked. Fully sialylated α₁-AG would be predicted to contain 28 mol of sialic acid/molecule, and this degree of undersialylation is consistent with prior studies (25).

Less than 5% of FITC-labeled fetuin or transferrin could be precipitated by CD22βRg under conditions that precipitate the natural ligands from lymphoid cells (data not shown). However, when applied to the CD22βRg column, complex elution profiles were seen. The FITC-tagged transferrin (not resialylated) showed minimal interaction with the column (Fig. 8A), consistent with its degree of undersialylation. The resialylated transferrin eluted over a broad range, with approximately 24% failing to bind to the column, 43% eluting at 4 °C, and the remaining 32% eluting after warming the column to room temperature (Fig. 8B). The FITC-tagged fetuin also eluted over a broad range, with ~9% failing to bind to the column, ~27% eluting at 4 °C, and the remainder eluting after warming the column to room temperature (Fig. 8C). With all three samples, over 85% of the sample was recovered from the column, and binding was eliminated by prior treatment with *A. ureafaciens* Sialidase (data not shown).

The elution profiles seen with fetuin most likely reflect sialylation linkage microheterogeneity. However, the result with the resialylated transferrin is particularly surprising since all of the molecules should carry two biantennary bisialylated
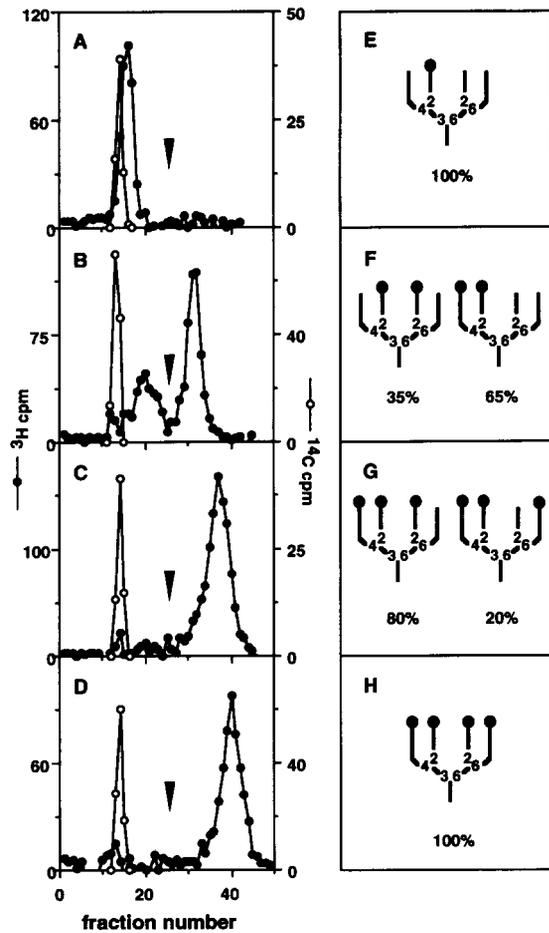
FIG. 5. **Fractionation of sialylated tetraantennary *N*-linked oligosaccharides on the CD22βRg-protein A-Sepharose column.** Tetraantennary oligosaccharides containing 1, 2, 3, or 4 (*panels A–D*, respectively) α2–6-[9-³H]Neu5Ac residues, were analyzed on a CD22βRg column exactly as in Fig. 1. The *arrow* indicates the point of the warming column from 4 to 22–24 °C. In *panels E-H*, the predicted location of the Neu5Ac residues (indicated by ●) is represented, along with the percentage of each form, based on data from Ref. 11. *E*, *F*, *G*, and *H* indicate the predicted structures of the oligosaccharides studied in *A*, *B*, *C*, and *D*, respectively.

chains that are each capable of interacting strongly with the CD22βRg. Thus, the elution of molecules at 4 °C could reflect either a population of protein containing monosialylated oligosaccharides or that the tertiary structure of the protein is capable of interfering with the accessibility of the oligosaccharide to CD22βRg. To examine this question directly, FITC-transferrin, resialylated with CMP-[9-³H]Neu5Ac and α2–6STN, was fractionated on CD22βRg, and the glycoprotein population exhibiting weak interaction (Fig. 8*B*, fractions 15–19) was isolated and treated with peptide:*N*-glycosidase F. The released *N*-linked oligosaccharides were examined by ion exchange chromatography and found to be >90% bisialylated oligosaccharides (data not shown). Thus, a significant fraction of the transferrin molecules carrying two bisialylated biantennary structures eluted *earlier* than the free oligosaccharide itself, indicating that the protein had a negative effect on the binding of the oligosaccharide.

In contrast to these results, a significant portion (26%) of FITC-tagged α₁-AG could be directly precipitated by CD22βRg and required 0.1 M acetic acid for elution (data not shown). Desialylation reduced this binding to ~2%, and a 10-fold excess of non-FITC-tagged α₁-AG blocked over 95% of binding of the native molecule (data not shown), indicating the specificity of
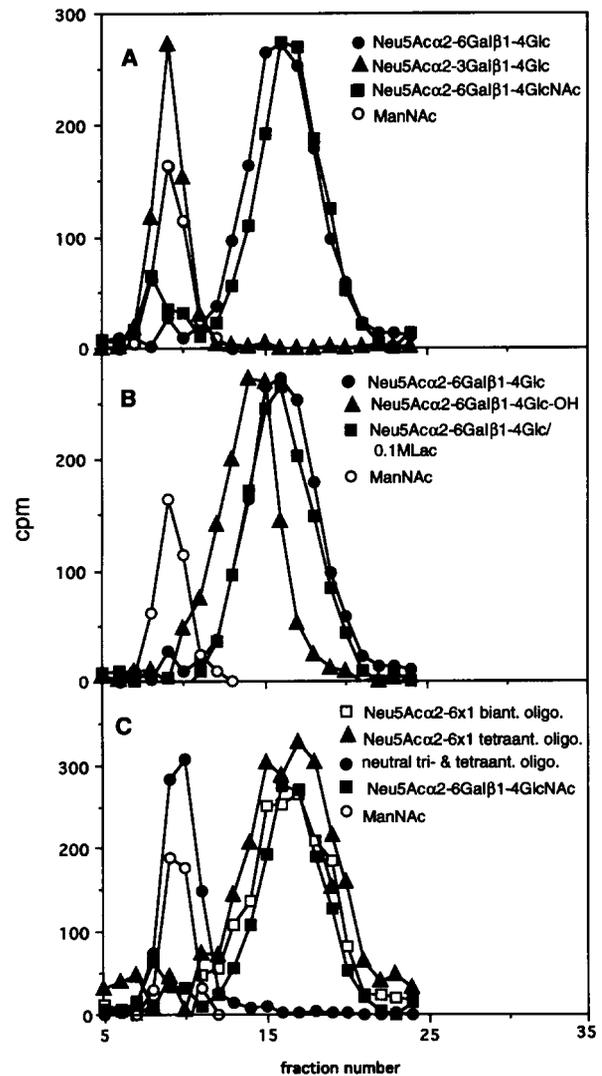
FIG. 6. **Binding of oligosaccharides to a high density CD22βRg-protein A-Sepharose column.** Utilizing a column containing 25-fold more CD22βRg than that used in Fig. 1, the binding of several different oligosaccharides was examined. The ³H-labeled oligosaccharides, mixed with [¹⁴C]ManNAc, were applied to and eluted entirely at 4 °C. In superimposing the different runs in each Panel, only one representative [¹⁴C]ManNAc tracing is presented for clarity. Synthesis of the different compounds with either [9-³H]Neu5Ac or [³H]Gal is described under "Experimental Procedures." In *B*, the elution profile of Neu5Acα2–6Galβ1–4Glc in Tris-buffered Saline in the presence of 0.1 M lactose (*Lac*) is indicated. In *C*, the oligosaccharides analyzed included a mono-α2–6 sialylated biantennary oligosaccharide (from Fig. 3*A*), mono-α2–6 sialylated tetraantennary (from Fig. 5*A*), and a mixture of neutral tri- and tetraantennary structures. The oligosaccharides studied in each panel are indicated in the *inserts*.

the interaction. FITC-tagged fetuin and resialylated transferrin could not be precipitated, indicating a higher apparent binding affinity for α₁-AG.

DISCUSSION

Previously, we demonstrated that CD22β binds to lymphoid glycoprotein ligands if their complex-type *N*-linked oligosaccharides contained α2–6-linked sialic acid residues. Binding was improved by lowered temperatures and by increasing the number of α2–6-linked residues/chain (5). Using the same column binding assay, we demonstrate here that the trisaccharide Neu5Acα2–6Galβ1–4Glc(NAc) is bound with the same apparent affinity as mono-α2–6-sialylated bi-, tri-, or tetraantennary complex *N*-linked oligosaccharides. Oligosaccharides with
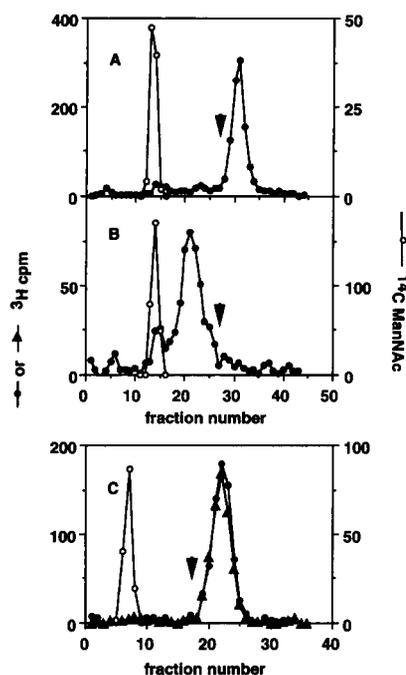
FIG. 7. **Analysis of other sialylated oligosaccharides on CD22βRg.** Utilizing the conditions and column employed in Fig. 1, the following structures were analyzed: bisialylated oligosaccharide from a *neo*-lacto series glycolipid (*A*; Table I, structure 8); a mixture of bisialylated oligosaccharides from reduced bronchial mucin oligosaccharides (*B*; Table I, structures 6 and 7); and a bisialylated bisected *N*-linked oligosaccharide (*C*; Table I, structure 10). The data presented in *C* are from a different column containing ~0.2 mg of protein on 0.4-ml resin, explaining the slightly different elution profile.

FIG. 8. **Analysis of binding of sialylated glycoproteins to CD22βRg.** Samples of transferrin (*A*), resialylated transferrin (*B*), and fetuin (*C*), each tagged with FITC and mixed with [14C]ManNAc, were applied to the CD22βRg-protein A-Sepharose column and analyzed as in Fig. 1. Profiles were monitored for both radioactivity and fluorescence. The point of warming the column to room temperature is indicated by the *arrow*.

higher levels of α2–6 sialylation bound more tightly, probably due to multivalent interactions (discussed below). Siaα2–6GalNAc-*O*-Ser/Thr was not recognized. Fucosylation probably plays no role in binding, as identical results were obtained with fucosylated α_1-AG oligosaccharides as well as with nonfucosylated structures. The structure Galβ1–3(Siaα2–6)GlcNAc-R, found on fetuin, was not examined directly. Table I summarizes the relative binding affinities for the different oligosaccharides examined.

The decreased binding of Neu5Acα2–6Galβ1–4Glcol compared with Neu5Acα2–6Galβ1–4Glc indicates that the inner Glc ring is involved in creating conformational feature(s) recognized by CD22βRg. Additionally, truncation of the exocyclic side chain of the sialic acid residue by periodate oxidation eliminates binding (5, 8). Thus, the minimal structure recognized by CD22βRg is Neu5Acα2–6Galβ1–4Glc(NAc). Moreover, 9-*O*-acetylation of the sialic acid side chain eliminates binding, whereas substitution of a glycolyl for an acetyl group at the 5-position of sialic acid is without effect.[2] One reported solution conformation of Neu5Acα2–6Galβ1–4GlcNAc suggests a "folded" conformation with the sialic acid and GlcNAc residues in proximity to one another (26). CD22β may recognize this folded structure, or it may recognize an extended chain structure utilizing structural features of all three monosaccharide residues.

The apparent binding affinity increases when increasing the number of sialic acid residues, up to 4, the highest number tested. Two different mechanisms may explain these observations. By statistical considerations, ligands with a valency of n will exhibit an apparent affinity of nK_a (where K_a is the association constant between monovalent receptor and ligand), pre-

suming that the different binding sites on the ligand are independent of each other (27). While no affinities were directly measured here, the data presented are consistent with this mechanism with the exception of the early elution of some of the bisialylated tetraantennary oligosaccharides and the bisialylated human bronchial mucins. Alternatively, the binding affinity between a receptor with two combining sites (as is the case for the CD22βRg chimera) and a ligand with two combining sites (as with a bisialylated oligosaccharide) may vary between K_a and K_a^2, depending on factors such as spatial orientation(s) and molecular strain(s) induced by binding (27). Clearly, this second mechanism alone does not explain the higher apparent binding affinities of the tri- and tetrasialylated structures, unless a single oligosaccharide is capable of bridging between multiple CD22βRg molecules (as may potentially occur on the surface of a cell). Most likely, some combination of these two mechanisms is involved, and a direct measurement of binding affinities is currently in progress. The lower binding affinity of some of the bisialylated structures (one of the tetraantennary isomers and the mucin-derived structures) probably is due to differences in strain and/or orientation relative to the other bisialylated structures.

This situation is in many ways analogous to the binding of clustered glycosides containing either Gal or GalNAc to the hepatic Gal/GalNAc receptor, a hexameric protein in which each monomer contains two monosaccharide binding sites (28). Asialo-α_1-AG binds essentially irreversibly, and the inhibitory capacity of small synthetic glycosides increases by 10^3 for each Gal residue added, up to three (29). Thus, the high affinity binding of CD22βRg to intact glycoproteins may reflect multiple interactions. Alternatively, the peptide backbones may present the oligosaccharides in a unique conformation, creating a high affinity epitope, as has been proposed for P-selectin (30).

In this regard, CD45, the one natural ligand identified to

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[2] E. Sjoberg, A. Klein, L. D. Powell, and A. Varki, manuscript in preparation.

date, is known to be heavily glycosylated. Among the different splicing variants, between 11 and 16 N-linked glycosylation sites are predicted and, at least in mouse, most of these are glycosylated (31, 32). Many of these oligosaccharides terminate with the Galβ1–4GlcNAc disaccharide, the acceptor substrate for the α2–6STN. The amount of this disaccharide and the extent to which it is capped by sialic acid (either α2–3 or α2–6 residues) vary between different lymphoid subpopulations (33). This glycosylation heterogeneity may regulate its ability to function as a CD22β ligand.

Initial studies on CD22β demonstrated it was capable of functioning as an adhesion molecule, recognizing several different glycoproteins on different lymphoid cell populations (2, 8). Other examples of proteins with multiple different ligands exist, including the integrins, which recognize different proteins sharing a common polypeptide motif (34), and fibronectin, which contains different binding regions in different protein domains (35). For CD22β, data available to date, including domain subtraction (8) and antibody blocking studies (7), have localized the binding activity to the third domain. Thus, it is unlikely that different regions of CD22β are involved in binding to its many different glycoprotein ligands (9), implying that the adhesive properties of CD22β are due entirely to its lectin specificity. The column assay employed here demonstrates binding interactions that are not demonstrable by direct immunoprecipitation assays. An identical approach was utilized to study the binding of oligosaccharides containing 1 or more phosphomannosyl residues to immobilized mannose-phosphate receptor (36). Subsequent studies determined K_d values of 6 × 10^{-6} and 2 × 10^{-7} M for oligosaccharides containing one and two phosphomonoesters, respectively (37).

The high levels of expression of hepatic α2–6STN are reflected in the abundance of serum glycoproteins expressing α2–6-linked sialic acids. However, even for purified proteins, marked heterogeneity in branching and terminal sialylation has been found. This microheterogeneity is the probable explanation for the complex patterns of interaction between CD22βRg and the model serum glycoproteins examined here. However, the following conclusions are possible. 1) The polypeptide portion of the glycoprotein may interfere with the binding of its oligosaccharide(s) to CD22β, as seen with transferrin. 2) Multiple partially sialylated chains on a single polypeptide may work in concert to increase a protein's affinity for CD22β, as seen with both fetuin and α_1-AG. 3) Highly sialylated glycoproteins, such as α_1-AG, exhibit high affinity binding, as defined by precipitation. Thus, *any* glycoprotein containing a sufficient quantity and/or arrangement of α2–6-linked residues may be a "high affinity" ligand for CD22β. This may explain the finding of Engel *et al.* (7) that ascites fluid and fetal calf serum, both rich sources of sialylated glycoconjugates, contain substances that inhibited CD22β-dependent adhesion assays.

While Neu5Acα2–6Galβ1–4GlcNAc is most commonly found on N-linked oligosaccharides, it can also be found on glycolipids from granulocytes and hepatoma cells (38, 39), cartilage keratan sulfate subfractions (40), and possibly O-linked structures from mucins (16, 17). Also of interest are several epitopes found on lymphoid cell lines that are dependent, in part, on α2–6-linked sialic acid residues. Included here are the CD75, CD76, HB-6, HB-4, and EBU-65 epitopes (41–45). Some of these epitopes show discrete histological localization within lymphoid structures (42). In some cell lines, CD22β itself contains α2–6-linked sialic acid structures (1), indicating that it may potentially participate in homotypic recognition as suggested by Wilson *et al.* (2).

α2–6STN has been extensively studied. It specifically catalyzes the transfer of sialic acid from CMP-Sia to Galβ1–4Glc-

NAc-R but not Galβ1–3GlcNAc-R (10). By Northern blot analyses, striking differences in its level of expression between different tissues have been noted, and several different mRNAs are seen, corresponding to differential usage of different promotors and exons (46–48). Of note, two promotors have been identified in lymphoid cell lines, one of which appears to be unique to lymphoid cells and whose appearance correlates best with the CD75 positive phenotype (48). However, the presence of α2–6STN only partially controls the amount of Neu5Acα2–6Galβ1–4GlcNAc residues on the cell surface. This transferase is both dependent upon and competes with several other transferases in the processing of nascent oligosaccharide chains. The extent to which these other transferases may play a biologically significant role in regulating the expression of CD22β ligands awaits investigation. However, as an example, in the accompanying paper (49), dramatic differences in the level of CD22βRg staining of endothelial cells results from only a 1.4-fold increase in the amount of α2–6-linked sialic acids on N-linked oligosaccharides. Thus, the regulation of biological processes dependent upon CD22βRg-glycoprotein interactions may tightly controlled by glycosyltransferase expression.

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