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Natural Ligands of the B Cell Adhesion Molecule CD22β Carry *N*-Linked Oligosaccharides with α-2,6-Linked Sialic Acids That Are Required for Recognition*

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CD22β is a glycoprotein found on the surface of B cells during restricted stages of development. It is believed to play a role in cell-cell interactions and B cell activation. The accompanying paper (Sgroi, D., Varki, A., Braesch-Andersen, S., and Stamenkovic, I. (1993) *J. Biol. Chem.* 268, 7011–7018) shows that CD22β recognizes multiple glycoproteins on the surfaces of T and B cells and that sialylation of these ligands is essential for binding. To identify the structure(s) of the sialylated oligosaccharide(s) recognized by CD22β, [^{3}H]glucosamine-labeled glycoproteins were purified from Daudi cells by adsorption onto a CD22β recombinant immunoglobulin (CD22βRg) chimera attached to protein A-Sepharose (PAS), and the *N*-linked oligosaccharides were released by peptide *N*-glycosidase F. These released oligosaccharides failed to bind to CD22βRg-PAS under the conditions used initially to adsorb the glycoproteins, but their elution from a column of CD22βRg-PAS was significantly retarded. Populations of oligosaccharides with different affinities could be identified by their order of elution. Specific sialidases were used to determine the content of α-2,3- and α-2,6-linked sialic acid in these different populations and their contribution to binding. Multiantennary oligosaccharides with one α-2,6-linked residue bound marginally, and those with two or more bound more tightly. α-2,3-Linked sialic acid residues were without effect. Binding did not require divalent cations and was abrogated by mild periodate oxidation of the outer side chain of sialic acid. No marked differences in size or fucose content were found between the populations of high and low affinity oligosaccharides. However, the low affinity population could be partially converted into higher affinity by treatment with β-galactoside α-2,6 sialyltransferase and CMP-sialic acid. Thus, CD22β is a mammalian lectin that can recognize specific *N*-linked oligosaccharide structures containing α-2,6-linked sialic acids.

A number of different cell surface proteins specific for unique stages of B cell development have been identified. One of them, CD22, is a glycoprotein whose expression is restricted to resting mature B cells. It is found cytoplasmically in bone marrow-derived pre-B cells, which are sIgM$^-$, and appears on the cell surface just after sIgM expression. Activation of B cells *in vitro* results in the disappearance of CD22, and it does not reappear on plasma cells (1–6). Among B cell neoplasms, CD22 expression is found in about 50% of lymphomas and 100% of hairy cell leukemias and variably in leukemia cells (2, 7, 8). It is not found on other bone marrow-derived cells of hematopoietic origin.

Two isoforms of CD22 (130 and 140 kDa) were originally identified (5, 9) and have recently been cloned (10–12). Two sequences were identified in a Raji cell cDNA library which, when expressed in COS cells, resulted in the expression of 110- and 130-kDa products termed CD22α and CD22β, respectively (10). A clone encoding CD22β has also been isolated from a normal B cell library (12). Sequencing indicated that CD22β contains seven extracellular immunoglobulin-like domains, a short transmembrane sequence, and a 78-amino acid cytoplasmic tail (10, 12). CD22α is identical except that it lacks two of the seven immunoglobulin-like domains. CD22β bears sequence homology to neural cell adhesion molecule and myelin-associated glycoprotein, both of which are involved in intercellular recognition and adhesion (10, 12).

As many members of the immunoglobulin superfamily function as receptors (13), these sequence similarities suggested that CD22 might also play a role in intercellular interactions. Specific ligands for CD22 were searched for using both COS cells transfected with a CD22-encoding plasmid and a recombinant soluble CD22β immunoglobulin chimera (CD22βRg).[1] COS cells expressing CD22α bound to erythrocytes and monocytes but not to other lymphocytes (11). COS cells expressing CD22β bound to tonsillar B cells, CD45RO$^+$ CD4$^+$ T cells, and phytohemagglutinin-induced T cell blasts, but not to circulating B cells (10). Several B cell lines (Daudi, Raji) and T cell lines (CEM, Molt4) bound CD22β-expressing COS cells and a soluble CD22βRg as well (10). Antibody blocking studies suggest that on T cells, one ligand was CD45RO (10) and that the cross-linking of CD45 by CD22 could alter phosphorylation events (14). As shown in the accompanying paper (15), we have extended these observations by identifying distinct glycoprotein ligands for CD22β on several different cell lines.

Several lines of evidence indicate that CD22β ligands are sialylated. The binding of CD22β-expressing COS cells to Daudi or Molt4 cells is prevented by their prior treatment

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[1] The abbreviations used are: CD22βRg, CD22β receptor-globulin chimera; PNGase F, peptide *N*-glycosidase F; PAS, protein A-Sepharose; NDV, Newcastle disease virus; HPLC, high performance liquid chromatography.

with sialidase (10). In several cell lines, the expression of CD75, an epitope formed in part by α-2,6-linked sialic acid, parallels their ability to bind CD22βRg. Moreover, the transfection of a β-galactoside α-2,6-sialyltransferase into two nonlymphoid lines (COS and HeLa), resulted in recognition by CD22βRg (10, 15), suggesting that CD22β recognizes α-2,6-linked sialic acids. Finally, the accompanying paper (15) shows that treatment of cells with mild periodate under conditions that selectively oxidize sialic acids abrogates the binding of CD22β to cells or soluble glycoproteins.

The activity of the α-2,6-sialyltransferase used in these experiments is restricted to adding sialic acid in an α-2,6 linkage to the sequence Galβ1–4GlcNAc (16). This acceptor disaccharide is most commonly found on *N*-linked oligosaccharides. To characterize the carbohydrate-binding specificities of CD22β better we have therefore released the *N*-linked oligosaccharides of CD22β-binding glycoproteins isolated from Daudi cells and studied their binding to CD22β. Comparative analysis of the oligosaccharides that do or do not bind conclusively shows that CD22β specifically recognizes *N*-linked oligosaccharides containing α-2,6-linked sialic acid and that the number of such residues affects the affinity.

EXPERIMENTAL PROCEDURES

Isolation of Radiolabeled CD22β Glycoprotein Ligands—Daudi cells (ATCC CLL 213) were grown in RPMI 1640, 10% fetal bovine serum supplemented with 20–50 μCi/ml [6-^{3}H]glucosamine (Du Pont-New England Nuclear) or [2-^{3}H]mannose (American Radiolabeled Chemicals, Inc.) for 72–96 h. Working at 4 °C, the cells were collected by centrifugation and resuspended in lysis buffer (20 mM Tris-Cl, pH 7.35, 140 mM NaCl, 1 mM $MgCl_2$, 0.2% NaN_3, 0.5% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride and 1% v/v aprotinin (Sigma). The lysates were clarified by centrifugation at 2,000 × *g*, preadsorbed to protein A-Sepharose (PAS), and then adsorbed to the CD22β receptor-immunoglobulin chimera (CD22βRg; for a description of its construction, see Ref. 10) coupled to PAS for 4 h. The resin was transferred to a small column and washed with lysis buffer until no more radioactivity eluted. The bound glycoproteins eluted with 0.5% SDS, 0.1 M ammonium formate heated to 60 °C. The eluted material was concentrated by lyophilization and chromatographed on Sephacryl S-200 (Sigma) equilibrated in 0.1% SDS, 0.1 M ammonium formate to ensure that the glycoproteins were free of any low molecular weight radiolabeled material. The glycoproteins eluting in V_0 were collected, and aliquots of the glycoproteins which bound and failed to bind to the CD22βRg-PAS were analyzed by SDS-polyacrylamide gel electrophoresis on 7.5% gels followed by fluorography using Entensify (Du Pont-New England Nuclear) and Kodak film.

Isolation of N-Linked Oligosaccharides—The glycoproteins isolated after chromatography on Sephacryl S-200 were concentrated by acetone precipitation as described previously (17), resuspended in 50 μl of 20 mM Hepes, pH 8.2, 1% SDS, 20 mM 2-mercaptoethanol, boiled for 5 min, cooled, and diluted with 200 μl of 20 mM Hepes, pH 8.2, 1.25% Nonidet P-40, 10 mM 1,10-phenanthroline. Digestion was performed at 37 °C with 0.5 milliunit of PNGase F, which was prepared according to Plummer and Tarentino (18), with a repeat addition of enzyme at 2–4 h. The reaction was terminated at 4–8 h by boiling and the sample rechromatographed as before on Sephacryl S-200. The radioactive peak eluting after V_0 representing the released *N*-linked oligosaccharides was pooled separately from nondigested glycoproteins eluting in V_0. No radioactivity migrated after V_0 in samples incubated without PNGase F. The released oligosaccharides were purified by precipitating SDS with KCl (2% KCl, 4 °C, overnight), passage over Bio-Rad SM-2 Bio-Beads and finally over Bio-Gel P-2 equilibrated in 25 mM ammonium acetate.

CD22βRg Binding of Released Oligosaccharides—Aliquots of the released oligosaccharides, dissolved in TBS (20 mM Tris-Cl, pH 7.4, 140 mM NaCl, 4 mM $CaCl_2$), were mixed with 20 μl of CD22βRg-PAS, 4 °C, overnight. The resin was washed three times in ice-cold TBS, and the pooled supernatants and the resin (containing the bound material) were mixed with scintillation fluid and counted separately.

Alternatively, the released oligosaccharides were chromatographed on a 0.3 × 15-cm column of CD22βRg-PAS, equilibrated in TBS, at either ambient temperature (22–24 °C) or 4 °C, as indicated. The density of CD22βRg on the PAS utilized in the column was the same as that utilized for the initial adsorption of the glycoproteins. Fractions of 80 μl were collected. The total volume (V_t) fractions of the column were marked by including in each run ~150 cpm of [^{14}C]ManNAc. For each run, the spill of ^{14}C into the ^{3}H channel of the scintillation counter (Beckman LS 1801) was determined and the appropriate correction made when plotting the elution profile of the ^{3}H-labeled oligosaccharides. In addition, [^{14}C]ManNAc and [^{3}H]galactose coeluted from the column exactly, indicating that [^{14}C]ManNAc was not interacting with the CD22βRg-PAS column in any way (data not shown).

Sialidase Digestions—Sialidase digestions were performed with 10–20 milliunits of *Arthrobacter ureafaciens* sialidase (Boehringer Mannheim) in 50 mM ammonium acetate, pH 5.4, 37 °C, for 0.5–12 h (as indicated in the text). Alternatively, samples were digested with 4–6 milliunits of Newcastle disease virus sialidase (NDV sialidase, a preparation of whole virions prepared as per Ref. 19), in 0.1 M sodium cacodylate pH 6.9, 1 mg/ml bovine serum albumin, for 30 min. The *A. ureafaciens* sialidase cleaves α-2,3-, α-2,6-, or α-2,8-linked sialyl residues (including *N*-acetyl- or *N*-glycolylneuraminic acid, even if acetylated in the 7, 8, and/or 9 positions), whereas the NDV sialidase hydrolyzes predominantly α-2,3- and α-2,8-linked sialyl residues. These specificities were validated for the radiochemical amounts of substrate used in this study by digesting preparations of standard *N*-linked oligosaccharides which had been resialylated with [^{3}H]sialic acid in either α-2,3 or α-2,6 linkages by purified sialyltransferases.[2] Under the conditions used, the NDV sialidase released 100% of the α-2,3-linked and less than 5% of the α-2,6-linked sialic acid, whereas the *A. ureafaciens* sialidase released 100% of both.

Following sialidase digestions, the [^{3}H]glucosamine-labeled oligosaccharides were purified away from released sialic acid on Bio-Gel P-6 (minus 200–400 mesh) in 0.1 M ammonium acetate. This step is essential as the [^{3}H]glucosamine is incorporated into sialic acid as well as both *N*-acetylglucosamine and *N*-acetylgalactosamine (20), and the free [^{3}H]sialic acid confounds subsequent analytical steps. For this desalting step, the samples were mixed with *N*-[^{14}C]acetylneuraminic acid so that the desialylated ^{3}H-labeled oligosaccharides could be distinguished clearly from the free [^{3}H]sialic acid.

Ion Exchange Chromatography—Samples were analyzed for negative charges on a Mono Q HR 5/5 column (Pharmacia LKB Biotechnology Inc.) connected to a Pharmacia fast protein liquid chromatography apparatus consisting of dual P-500 pumps driven by an LCC 500 Plus Controller and utilizing a 400-μl sample loop. Elution buffers were water (buffer A) and 0.2 M NaCl, 1 mM Tris base (buffer B), using a gradient of 0% buffer B for 6 min, increasing to 22.4% over 12 min, and then to 90% over 17 min, at 1.2 ml/min and collecting 0.6-ml fractions. Samples were desalted (Bio-Gel P-2, 25 mM ammonium acetate) prior to injection. This system clearly resolves oligosaccharides containing no to five negative charges. Additionally, free sialic acid elutes at a position between oligosaccharides with one and two negative charges. Thus, even if a trace of free labeled sialic acid is present, it is easily identifiable.

Lectin Affinity Chromatography—Chromatography on concanavalin A-Sepharose (2-ml column) was performed at room temperature as described by Merkle and Cummings (21).

Size Fractionation on HPLC—Oligosaccharides were desialylated with *A. ureafaciens* sialidase, desalted over Amberlite (Sigma) mixed bed resin, and chromatographed on a Microsorb-MV amino-bonded silica column (0.46 × 25 cm; Rainin Instruments, Inc.) developed with a gradient of 40–85% water in acetonitrile, 25 mM NaH_2PO_4 at 1 ml/min collecting 0.5-min fractions. The column was standardized with neutral fully galactosylated tri- and tetraantennary oligosaccharides from α_1-acid glycoprotein labeled by digestion with β-galactosidase (from bovine testes; Boehringer Mannheim) and regalactosylation with UDP-[6-^{3}H]galactose and galactosyltransferase (Sigma).

β-Elimination—O-Linked oligosaccharides were released by β-elimination by suspending the protein pellet (from an acetone precipitation) in 200 μl of 0.1 M NaOH (prepared fresh from 10 N stock) containing 1 M $NaBH_4$ and incubating at 37 °C for 18 h (22). Subsequently, the samples were diluted to 5 ml and passed over a 3-ml column (6-fold excess capacity) of Bio-Rad AG 50 (H^+ form), eluted with 15 ml of water, lyophilized, and desalted on P-2 as above.

Mild Periodate Oxidation—Aliquots of isolated oligosaccharides were resuspended in 25 μl of phosphate-buffered saline, pH 7.0, containing 2 mM sodium metaperiodate and incubated at 4 °C for 45 min in the dark. The reaction was quenched with 3 μl of 80 mM

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

sodium sulfite for 15 min at ambient temperature. Calcium chloride (12 nmol) and [^{14}C]ManNAc (150 cpm) were added, and the samples were chromatographed on CD22βRg-PAS at 4 °C as above.

Compositional Analysis—The *N*-linked oligosaccharides from Daudi cells radiolabeled with [2-^{3}H]mannose were hydrolyzed under N_2 with 2 N trifluoroacetic acid for 2 h, at 121 °C. Trifluoroacetic acid was removed by repeated lyophilizations, and the samples were analyzed by descending chromatography on Whatman 1 paper in pyridine:ethyl acetate:acetic acid:water (5:5:1:3, v/v). The elution positions of the radiolabeled monosaccharides were determined by cutting the strips into 1-cm pieces and scintillation counting, and the position of mannose and fucose standards run in parallel was located by silver nitrate staining (23).

Resialylation of Oligosaccharides—[^{3}H]Glucosamine-labeled oligosaccharides purified from Daudi cells by PNGase F digestion were sialylated by resuspension in 20 μl of buffer containing 200 nmol of sodium cacodylate buffer, pH 6.9, 20 μg of bovine serum albumin, Triton CF-54, 4–8 nmol of CMP-neuraminic acid, and 1 milliunit of β-galactoside α-2,6-sialyltransferase (CMP-NeuAc:Galβ1–4GlcNAc-R α-2,6-sialyltransferase; Boehringer Mannheim). Samples were incubated at 37 °C for 12 h, boiled, and desalted over Bio-Rad P-2 as above. The radioactive material eluting in the void volume was collected, lyophilized, and subjected to ion exchange chromatography or fractionation on CD22βRg-PAS.

RESULTS

Isolation of N-Linked Oligosaccharides from Glycoproteins Recognized by CD22βRg—The studies presented in the accompanying paper (15) show that CD22βRg recognizes sialylated glycoprotein ligands on the surface of a variety of T and B cell lines. The precipitation of these glycoproteins is abrogated if they are initially treated with sialidase or with mild periodate under conditions that selectively oxidize sialic acids. We chose to analyze further Daudi cells, a human B cell lymphoma line that contained several ligands precipitable by the CD22βRg after surface labeling with ^{125}I. To establish the specific structural determinants recognized by CD22β, Daudi cells were metabolically labeled with [^{3}H]glucosamine, lysed with a Nonidet P-40-containing buffer, and the lysate adsorbed onto CD22βRg-PAS. After extensive washing with lysis buffer, bound material was eluted with SDS. Approximately 3.5% of the total macromolecular [^{3}H]glucosamine incorporated by Daudi cells was adsorbed by the CD22βRg-PAS. Portions of the bound and nonbound glycoproteins were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1, several labeled glycoproteins are enriched in the CD22βRg-bound material including prominent species at 90 and 220 kDa, a doublet at 155 kDa, and several diffuse bands migrating between 100 and 120 kDa. This is in agreement with data derived from ^{125}I-surface-labeled cells (15), with the exception that several lower molecular weight iodinated proteins were not detected in the glucosamine-labeled material.

To examine directly whether CD22β has an affinity for the oligosaccharides independent of the intact glycoproteins, the CD22βRg-bound [^{3}H]glucosamine-labeled glycoproteins from Daudi cells were treated with PNGase F to release the *N*-linked oligosaccharides from the peptide backbone (24). The initial treatment released approximately 45% of the total radioactivity, as judged by chromatography on a column of Sephacryl S-200, run in the presence of SDS exactly as described previously (17). Control incubations in the absence of PNGase F showed no significant release of radioactivity (data not shown).

Interaction of the Released N-Linked Oligosaccharides with CD22βRg—The *N*-linked oligosaccharides released by PNGase F (hereafter referred to simply as "oligosaccharides") were freed of SDS and salt as described previously (17) and examined for binding to CD22βRg using a batch adsorption protocol similar to that used for the initial isolation of the

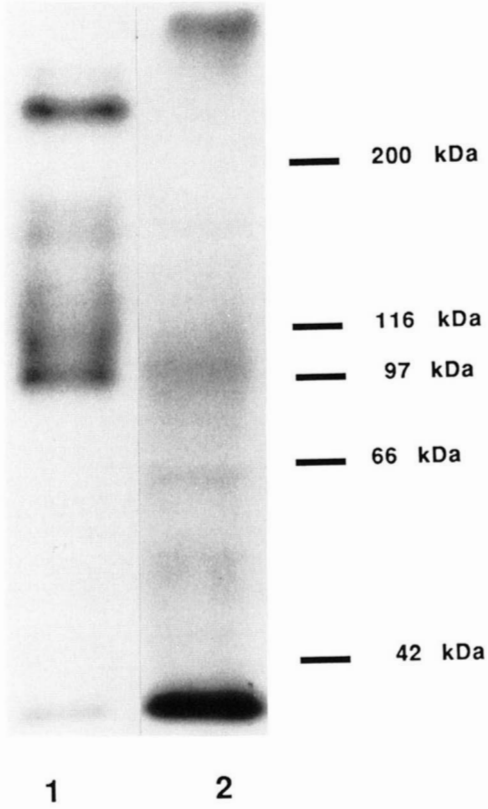

FIG. 1. **CD22βRg selectively recognizes a subset of the total cellular glycoproteins from Daudi cells.** Cells were metabolically labeled for 48 h with [^{3}H]glucosamine, lysed in Nonidet P-40-containing lysis buffer, and the lysate adsorbed onto CD22βRg-PAS as described under "Experimental Procedures." After washing extensively with lysis buffer, bound proteins were eluted with SDS. Aliquots of the adherent (*lane 1*) or nonadherent (*lane 2*) glycoproteins were analyzed on a 7.5% SDS gel and detected by fluorography. The position of molecular mass markers is shown in the *margin*.

glycoproteins. However, only ~2% of the free labeled oligosaccharides remained bound to CD22β under these conditions. Binding of oligosaccharides to lectins can be detected more readily by column chromatography than by batch adsorption (25, 26). A 0.3 × 15-cm column of CD22βRg-PAS was constructed (1-ml total volume, containing approximately 200 μg of chimera). The [^{3}H]glucosamine-labeled oligosaccharides were mixed with [^{14}C]ManNAc (an internal marker to indicate the elution position of nonretarded but completely included material, V_t) and applied to the column. When the chromatography was done at room temperature, only a slight retardation of the oligosaccharides relative to [^{14}C]ManNAc was seen (Fig. 2*A*). At 4 °C, a more pronounced retardation of the oligosaccharides relative to the V_t marker was observed, with a large peak eluting just after [^{14}C]ManNAc, followed by a small shoulder and a long tail that did not return to baseline (Fig. 2*B*). Application of the sample to a similarly constructed column containing pooled IgGs (rather than CD22βRg) adsorbed to PAS showed that both the [^{3}H]glucosamine-labeled oligosaccharides and the [^{14}C]ManNAc coeluted in the unretarded fractions (Fig. 2*C*). This result demonstrates that the interaction of the oligosaccharides with the CD22βRg-PAS column is specific for the CD22 portion of the chimera.

Isolation of the N-Linked Oligosaccharides with Differing Affinities for CD22βRg—The recovery of radioactivity from several different runs done at 4 °C ranged between 60 and 75%, compared with >85% when the runs were done at room temperature. Therefore, the column was loaded at 4 °C and

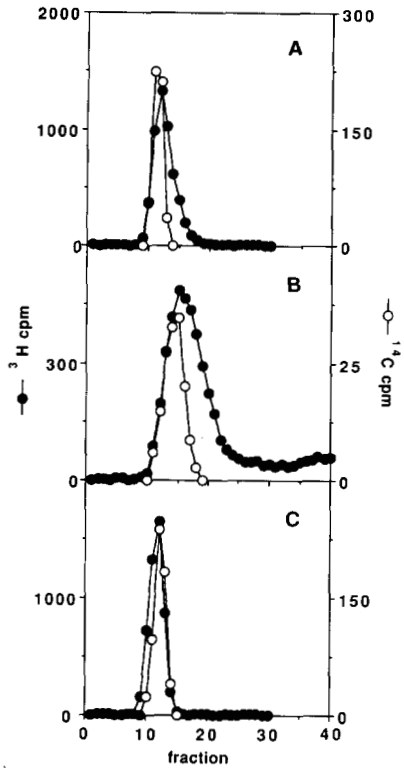

FIG. 2. **CD22βRg-PAS column chromatography of *N*-linked oligosaccharides released from CD22βRg-bound glycoproteins.** *N*-Linked oligosaccharides were released from the [^{3}H] glucosamine-labeled glycoproteins bound to CD22βRg by digestion with PNGase F. Released oligosaccharides were desalted, concentrated, mixed with 150 cpm of [^{14}C]ManNAc, and applied to a 0.3 × 15-cm column of CD22βRg-PAS (*panels A* and *B*) or one of pooled Human IgG-PAS (*panel C*). Fractions (80 μl) were collected and analyzed by liquid scintillation counting. The temperature of the elutions was 22–24 °C (*panel A*) or 4 °C (*panels B* and *C*).

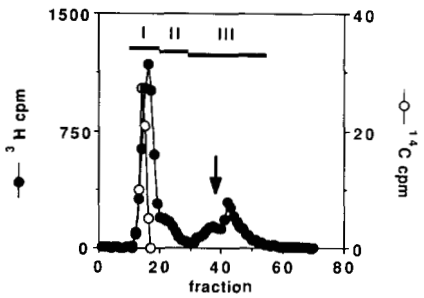

FIG. 3. **Chromatography of *N*-linked oligosaccharides on CD22βRg-PAS.** Oligosaccharides from glycoproteins that bound to CD22βRg were fractionated preparatively on CD22βRg-PAS. The column was loaded and first eluted at 4 °C until fraction 38, warmed to ambient temperature, and then eluted for an additional 10–15 fractions. The point of temperature change is indicated by the *arrow*. Oliogosaccharides from different regions of the run were collected and labeled pools I, II, and III as indicated by the *horizontal bars*.

eluted until fraction 38, when it was brought to room temperature and eluted further (Fig. 3). This strategy clearly demonstrated a more strongly retained (late eluting) oligosaccharide population that required a shift to room temperature for complete recovery. The profile suggested three separate populations of oligosaccharides differing in order of elution and presumably in affinity for CD22βRg. Consequently three pools were made (designated I, II, III) as shown in Fig. 3. To confirm that the different pools represent distinct populations of oligosaccharides, portions were rechromatographed on the CD22βRg-PAS column, with loading at 4 °C (Fig. 4). For these and all subsequent runs, the column was warmed at fraction 28–30, so that pool III would elute in a narrower peak and be easier to detect. Pool III material eluted almost completely in its original position after the temperature elevation. Pool II material also eluted in its original position but contained some label that now eluted in the position expected for pool I. Since the original pool II was small relative to pool I (Fig. 3), this cross-contamination was not surprising. Pool I eluted in its original position, which was consistently found to be slightly *after* V_t, indicated by [^{14}C]ManNAc. This slightly retarded migration of pool I relative to V_t was also seen in the original run shown in Fig. 3 (compare with control column in Fig. 2*C*). This indicates that the pool I oligosaccharides also interact with the CD22βRg, although with weaker affinity than the other pools. The distribution of material among pools I, II, and III was 51 ± 6%, 19 ± 7%, and 30 ± 2%, respectively (n = 4).

Sialic Acid Linkage Is Important in the Binding of N-Linked Oligosaccharides to CD22βRg—Previous work has predicted a role for α-2,6-linked sialic acids in CD22βRg-ligand binding (10, 15). *N*-Linked oligosaccharides may contain α-2,3-, α-2,6-, and (very rarely) α-2,8-linked sialic acids in different combinations on the same *N*-linked oligosaccharide. The type of linkage can be determined by sensitivity to hydrolysis by different sialidases, as the sialidase from *A. ureafaciens* hydrolyzes all three linkages, whereas that from NDV hydrolyzes predominantly α-2,3 and α-2,8 linkages (27). As α-2,8-linked sialic acids are very uncommon on mammalian glycoproteins (28), they will not be considered further in this analysis. As described under "Experimental Procedures," the reported selectivity of these two sialidases toward α-2,3 and α-2,6 linkages was verified for the radiochemical amounts of substrate

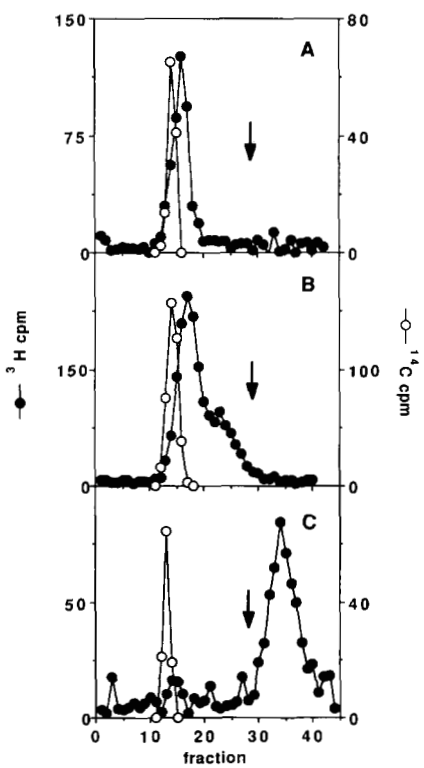

FIG. 4. **Rechromatography of pools I, II, and III on CD22βRg-PAS.** Portions of each of the three pools isolated in Fig. 3 were mixed with 150 cpm of [^{14}C]ManNAc and rechromatographed on the CD22βRg-PAS column as in Fig. 3. The point of changing from 4 °C to ambient temperature is indicated with the *arrow*.

studied here. Digestion of the PNGase F-released oligosaccharides with NDV sialidase was without any effect on the elution profile of the oligosaccharide mixture on CD22βRg-PAS, whereas digestion with *A. ureafaciens* sialidase completely abrogated all interaction (Fig. 5). Subsequent studies (see below) demonstrated that most of these oligosaccharides do contain some α-2,3-linked sialic acid residues that are susceptible to NDV sialidase. Thus, the data presented in Fig. 5 indicate that α-2,3-linked sialic acid residues play no discernible role (either positive or negative) in binding to CD22βRg.

The Number of Sialic Acids in α-2,6 Linkage Affects the Binding of N-Linked Oligosaccharides to CD22βRg—Analytical ion exchange chromatography was utilized in conjunction with the NDV and *A. ureafaciens* sialidases to examine the number and nature of negative charges in each of the three pools isolated from the CD22βRg-PAS column. Portions of each pool were digested with sialidase or treated with buffer alone for 30 min, boiled, and desalted on Bio-Gel P-2 to remove the released ^{3}H-labeled free sialic acid arising from metabolic conversion of [^{3}H]glucosamine (20) (which in Daudi cells was about 20% of the tritium label; data not shown). The desialylated oligosaccharide samples were subsequently analyzed by ion exchange chromatography. As shown in Fig. 6 and Table I, about 90% of the oligosaccharides from each pool contained at least one negative charge, yet >95% eluted as neutral species after treatment with *A. ureafaciens* sialidase. This demonstrates that sialic acids were responsible for all of the negative charge present on these oligosaccharides. Treatment with NDV sialidase reduced almost all of the pool

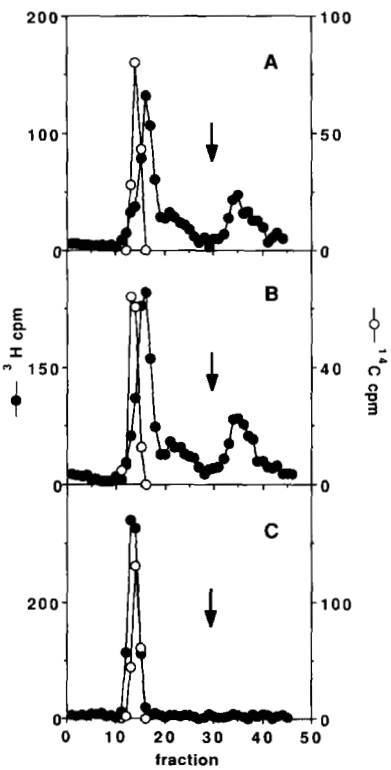

FIG. 5. **Evidence that α-2,6-linked sialic acid is required for binding *N*-linked oligosaccharides to CD22βRg.** PNGase F-released oligosaccharides from CD22βRg-bound glycoproteins were treated either with buffer alone (*panel A*), Newcastle Disease virus sialidase (*panel B*), or *A. ureafaciens* sialidase (*panel C*), boiled, and desalted over Bio-Gel P-2. The oligosaccharides eluting in V_0 were concentrated, mixed with 150 cpm of [^{14}C]ManNAc, and analyzed on the CD22βRg-PAS column as in Fig. 3. The point of changing from 4 °C to ambient temperature is indicated with the *arrow*.

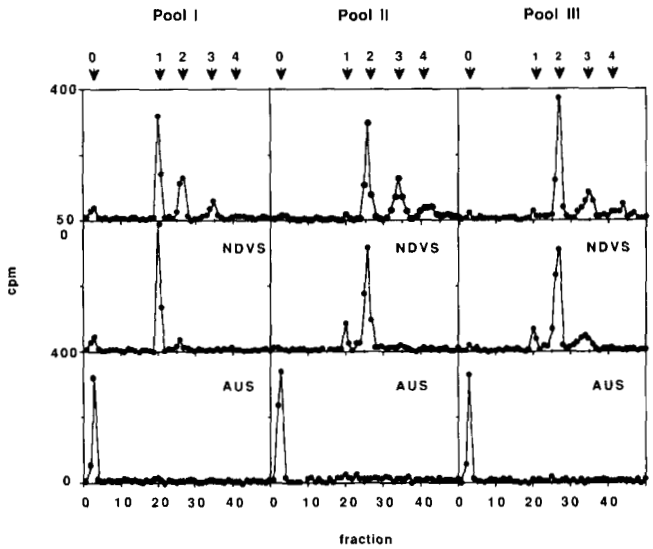

FIG. 6. **Ion exchange chromatography of *N*-linked oligosaccharides before and after sialidase digestion.** Samples of each of the three pools from Fig. 3 were digested either with Newcastle disease virus sialidase (*NDVS*), *A. ureafaciens* sialidase (*AUS*), or buffer alone, desalted over Bio-Gel P-2 (to remove free [^{3}H]sialic acid), and then analyzed on a Mono Q-Sepharose column eluted with a NaCl gradient, as described under "Experimental Procedures." The positions of elution of standard oligosaccharides containing no, one, two, three, or four negative charges are indicated by *0*, *1*, *2*, *3*, and *4*, respectively.

TABLE I

Distribution of charges by Mono Q-Sepharose

For the ion exchange analyses presented in Fig. 6, the amount of radioactivity in each peak, as a percentage of the total radioactivity in all peaks, was calculated. Data for samples analyzed after NDV sialidase or no sialidase treatments are presented.

No. of negative charges	Pool I		Pool II		Pool III	
	None	NDV	None	NDV	None	NDV
0	7	10	2	1	1	2
1	50	80	1	21	3	10
2	29	10	38	72	55	66
3	3		40	9	25	19
4			18		15	3

TABLE II

No. of α-2,6-linked sialic acid residues per pool

From the sialidase digestion data presented in Fig. 6 and Table I, the number of α-2,6-linked sialic acid residues on the oligosaccharides in pools I, II, and III was calculated. For this calculation, the assumption was made that sialic acid residues resistant to NDV but sensitive to *A. ureafaciens* sialidase represented an α-2,6-linked residue.

No. of α-2,6-linked sialic acid residues	Pool I	Pool II	Pool III
None	10	1	0
One	80	21	10
Two	10	72	66
Three	0	9	19

I oligosaccharides to molecules with one negative charge, indicating the presence of one resistant (*i.e.* α-2,6-linked) sialic acid residue. Similar treatment of pools II and III reduced them to species with predominantly two negative charges, indicating the presence of two α-2,6-linked residues. The distribution of the different charged species is tabulated in Table I, and the content of α-2,6-linked sialic acid residues calculated from the sialidase digestions is presented in Table II. These data indicate that pools II and III differ markedly

from pool I in having at least two α-2,6-linked sialic acid residues *versus* only one. By this analysis, pools II and III are relatively similar. Although pool III does have a higher portion of structures with three α-2,6-linked residues (19 *versus* 10%; see Table II), it does contain a significant amount of molecules (66%) with just two α-2,6-linked sialic acid residues. Thus, the basis of the separation between pools II and III on the CD22βRg-PAS column is at present unclear. Because of the low amounts of pool II available and its cross-contamination by pool I, we have not analyzed this issue further.

The Side Chains of the α-2,6-Linked Sialic Acids Are Important in the Binding of N-Linked Oligosaccharides to CD22βRg, but Divalent Cations Are Not—In the accompanying paper (15), we demonstrated that mild periodate oxidation (2 mM at 4 °C) of glycoproteins isolated from Daudi, Raji, and phytohemagglutinin T cell blasts abrogates their binding by CD22βRg. These conditions are well known to oxidize selectively the exocyclic polyhydroxyl side chain (C_7–C_9) on sialic acid without affecting the ring of sialic acid or that of the underlying saccharides (29, 30). To determine whether this requirement for intact sialic acid molecules extends to the isolated oligosaccharides, similar conditions of periodate oxidation were used to oxidize aliquots of the pools I and III oligosaccharides prior to rechromatography on the CD22βRg-PAS column. As shown in Fig. 7, both pools now clearly lost binding and coeluted with the [^{14}C]ManNAc marker. These data, together with the ion exchange data presented above, indicate that the side chain of α-2,6-linked sialic acids is required for binding to CD22βRg.

Previous results have indicated that CD22βRg binding to cells was not dependent upon divalent cations (10). However, the side chain of sialic acid is known to participate in the binding of divalent cations such as calcium (31). Thus, the initial studies presented here were done in buffers including 4 mM $CaCl_2$. However, a requirement for divalent cations was subsequently excluded by replacing the $CaCl_2$ in the column buffer with 5 mM EDTA. EDTA had no effect on the elution profile of the [^{3}H]glucosamine-labeled oligosaccharides on the CD22βRg-PAS column, nor did the inclusion of 0.5 M NaCl (data not shown).

The Underlying Oligosaccharides May Contribute to the Differences in Binding—The neutral oligosaccharide cores of pools I and III were next examined. Portions of each pool were desialylated with *A. ureafaciens* sialidase and desalted as described under "Experimental Procedures." Less than 2% of either sample bound to concanavalin A, indicating that the structures consisted entirely of tri- or tetraantennary or bisected oligosaccharides (data not shown). Portions of each pool were subsequently analyzed by HPLC on amine-modified silica developed with a water gradient (32). As shown in Fig. 8, the elution profiles of the two pools are virtually identical, and the radioactivity eluted at a position consistent with tri- and tetraantennary *N*-linked oligosaccharides.

Since the results presented in the accompanying paper indicate that fucosylated glycoproteins bind CD22βRg (15), the relative fucose content of the different pools was compared with each other and with that of the total population of Daudi *N*-linked oligosaccharides. This analysis was done on material derived from Daudi cells radiolabeled with [2-^{3}H]mannose, since fucose is the only other radiolabeled monosaccharide that can be derived from this isotope (20). Fractions and pools similar to those described above for glucosamine-labeled material were isolated and subjected to monosaccharide compositional analysis as described under "Experimental Procedures." Of the oligosaccharides from the Daudi glycoproteins which failed to bind to CD22βRg, only 6% of the incorporated radioactivity was found in fucose. However, of the radioactivity in pools I, II, and III, 25, 25, and 29%, respectively, were found in fucose. Thus, although *N*-linked oligosaccharides that bound to CD22βRg show 2.5–3 times more fucose incorporation than the total cellular population of glycoproteins, there does not appear to be significant differences in the fucosylation of the different pools.

These observations suggest that the primary difference between pools I and III is in the level of sialylation rather than in the underlying oligosaccharide structure. However, the level of resolution of structural analysis could have missed some subtle differences. A portion of pool I was therefore further sialylated with β-galactoside α-2,6-sialyltransferase and CMP-NeuAc, and the resulting material was analyzed by ion exchange chromatography. Resialylation of pool I resulted in oligosaccharides with one (7%), two (29%), three (45%), and four (18%) sialic acid residues (Fig. 9*A*). Compared with the level of sialylation of native pool I (Table I and Fig. 6), the original oligosaccharides must contain many nonsialy-

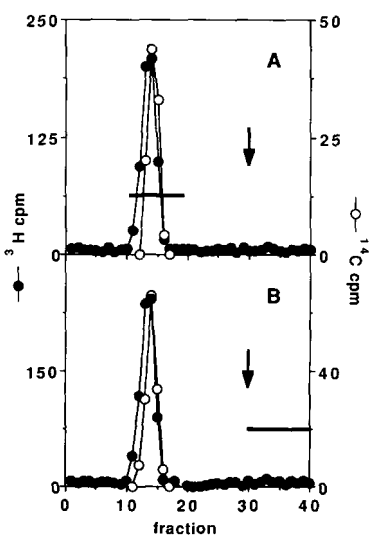

FIG. 7. **Effect of periodate treatment on oligosaccharide binding to CD22βRg.** Samples of pool I (*panel A*) and pool III (*panel B*) oligosaccharides from Fig. 3 were treated with 2 mM periodate on ice as described under "Experimental Procedures" and then chromatographed on the CD22βRg-PAS column. The region of elution of each pool prior to periodate treatment is indicated by the *horizontal bar*. The point of changing from 4 °C to ambient temperature is indicated with the *arrow*.

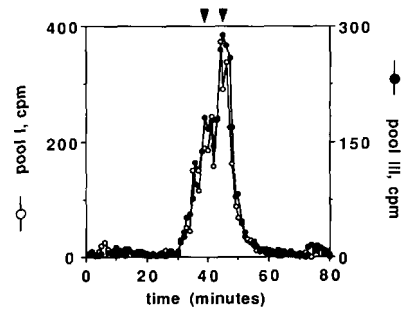

FIG. 8. **Amino-HPLC column analysis of neutral oligosaccharides.** Portions of pools I and III oligosaccharides (from Fig. 3) were treated with *A. ureafaciens* sialidase, analyzed by HPLC chromatography on amine-bonded silica run in acetonitrile:water, and developed with a water gradient, as described under "Experimental Procedures." The elution positions of standard tri- and tetraantennary desialylated *N*-linked oligosaccharide standards are indicated (fractions 39 and 45, respectively).

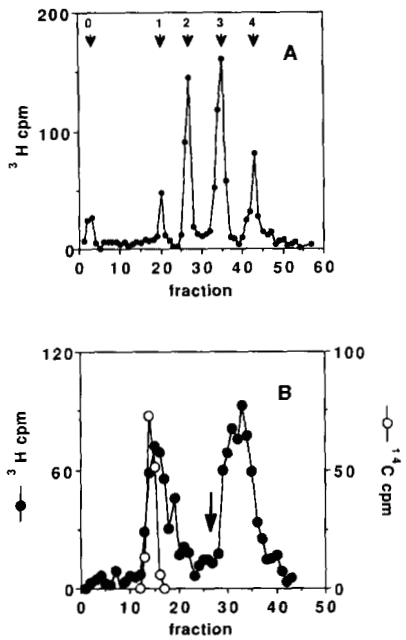

FIG. 9. **Effect of sialylation of oligosaccharides with β-galactoside α-2,6-sialyltransferase.** Portions of the pool I oligosaccharides were resialylated with β-galactoside α-2,6-sialyltransferase and CMP-NeuAc, desalted, and subsequently analyzed by ion exchange chromatography as in Fig. 5 (*panel A*) or on the CD22βRg-PAS column as in Fig. 3 (*panel B*). The point of changing from 4 °C to ambient temperature is indicated with the *arrow*.

lated antennae with terminal β-galactose residues. Following this resialylation, 60% of the pool I material now bound to the CD22βRg-PAS column and eluted only when the column was warmed to room temperature (Fig. 9*B*). Thus, the addition of more α-2,6 sialic acid residues was sufficient to convert pool I oligosaccharides to molecules with interactions similar to those in pool III. This suggests that pool III oligosaccharides may not contain some other unique structural features that are responsible for higher affinity. However, it is also noteworthy that not all of the molecules improved in binding affinity after this treatment.

Glycoproteins That Bind to CD22βRg Contain Some O-Linked Oligosaccharides That Do Not Interact with the Lectin—The PNGase F-resistant material from the CD22βRg-PAS-binding [^{3}H]glucosamine-labeled glycoproteins was examined further. Initially, it was treated a second time with PNGase F, and a further 25% of the labeled material was released. Thus, a total of 65% of the [^{3}H]glucosamine-labeled material which bound to the CD22βRg during the initial adsorption of the Nonidet P-40 lysate could be released by repeated treatment with PNGase F. The oligosaccharides released by the second treatment were analyzed by CD22βRg-PAS chromatography. A profile identical to that seen in Fig. 3 was obtained (data not shown), indicating that this material is a representative of, rather than a subset of, the total *N*-linked oligosaccharide population. The material resistant to repeated PNGase F treatment was subjected to alkaline hydrolysis in the presence of sodium borohydride (β-elimination), conditions that release *O*-linked oligosaccharides (22). Of the PNGase F-resistant material, 76% was released by β-elimination (defined as the inability to bind to the cation exchange resin AG 50). When these free oligosaccharides were freed of salt and SDS and chromatographed on the CD22βRg-PAS column, no interaction was seen (data not shown). These oligosaccharides were not analyzed further.

DISCUSSION

Although soluble cytokines play important roles in the development and regulation of both the cellular and humoral immune system, direct cell-cell contacts between lymphocytes and accessory cells are also important. Many different receptor-ligand pairs involved in intercellular adhesion have been identified to date (for reviews see Refs. 13 and 33). Recent data have indicated that the B cell glycoprotein CD22 may also function as an adhesion molecule, mediating the adhesion of B cells to other B cells, T cells, monocytes and erythrocytes (10, 11). Although some investigators have suggested homotypic recognition between CD22$^+$ B cells involving direct CD22-CD22 binding, recent data (10, 14, 15) have indicated that one of the CD22 isoforms, CD22β, recognizes a number of different sialoglycoproteins on B cells, T cells, and lymphoid cell lines. On T cells, one of these ligands has been identified as CD45RO (10, 14, 15), and work is currently in progress to establish the specific identities of the other ligands.

Data presented previously and in the accompanying paper (15) have implicated sialic acid residues as being involved in the formation of the epitope recognized by CD22β. Most striking was the observation that CD22βRg binding to two nonlymphoid cell types, COS and HeLa, could be induced by transfecting into those cells a plasmid containing the cDNA encoding for the β-galactoside α-2,6-sialyltransferase (10). The sialyltransferase used in these experiments had been previously cloned from a Daudi cell cDNA library (34) and demonstrated to be identical to the β-galactoside α-2,6-sialyltransferase cloned previously from mice (35) and humans (36). The specificity of this sialyltransferase is restricted to adding sialic acid in an α-2,6 linkage to galactose residues linked β1–4 to *N*-acetylglucosamine. This disaccharide is most commonly found on the outer termini of *N*-linked oligosaccharides but can also be found in some extended *O*-linked structures and glycolipids (neolactoseries gangliosides) (37, 38). Although this α-2,6-sialyltransferase is capable of sialylating all of these types of oligosaccharides *in vitro*, it is not yet established whether more than one α-2,6-sialyltransferase exists *in vivo*. To date, however, only one α-2,6-sialyltransferase has been identified by molecular cloning (35).

Prior work has shown that CD22β-expressing COS cells bind to untreated Molt4 or Daudi cells but not to sialidase-treated cells (10). Also, the accompanying paper shows that the binding of the CD22βRg chimera to T cell blasts and several T and B cell lines could be prevented by their prior treatment with 2 mM sodium periodate at neutral pH on ice (15). These conditions result in the selective oxidation of the exocyclic arm (C_7-C_9) of non-*O*-acetylated sialic acid molecules leaving the ring structure of sialic acids and other hexoses intact (29). The data presented here directly demonstrate that CD22β is capable of binding *N*-linked oligosaccharides containing α-2,6-linked sialic acid residues and that the side chains of these residues are important for binding. The oligosaccharide structures studied were obtained by treating Daudi cell glycoproteins with the enzyme PNGase F, which specifically hydrolyzes the glucosaminyl-asparagine bond of *N*-linked oligosaccharides. Both glycoproteins that bound to the CD22βRg chimera and those that did not were examined. Of those isolated from the CD22βRg-adherent glycoproteins, three populations (labeled pools I, II, and III) of oligosaccharides were defined on the basis of their elution position from a column containing CD22βRg coupled to PAS. It is reasonable to assume that the differences in elution position represent different affinities for CD22β. Actual binding constants cannot be extrapolated from these results. However, prior

work using this same approach showed that high mannose-type oligosaccharides bearing mannose 6-phosphate residues eluted in similar positions from a column containing immobilized mannose 6-phosphate receptor, retarded relative to V_t (25). The affinity of such oligosaccharides for the mannose 6-phosphate receptor was later determined to be in the range of $6\text{–}7 \times 10^{-6}$ M (39).

All three populations of oligosaccharides interact with the CD22βRg-PAS column in a manner dependent upon α-2,6 sialic acid, and the interaction is destroyed by mild periodate oxidation. These properties are strikingly similar to the data derived from the binding of the chimera to intact cells and to glycoproteins. Oligosaccharides containing two or more α-2,6-linked sialyl residues bound tighter than those containing just one residue. Moreover, α-2,3-linked sialic acid residues exerted neither a positive nor a negative effect on binding. Taken together with the effects of mild periodate, these results indicate that CD22β-oligosaccharide interaction involves more than just charge recognition and that the receptor recognizes specific conformational features of the oligosaccharide. Studies by ^{1}H NMR have suggested markedly different solution conformations for the two trisaccharides, sialic acid α-2,3-*N*-acetyllactosamine and sialic acid α-2,6-*N*-acetyllactosamine (40, 41). Thus, CD22β can apparently discriminate between these different conformations. Interestingly, the side chain of the sialic acid is also known to play a role in maintaining the conformation of the Sia-α-2,6-*N*-acetyllactosamine (41).

Although these data demonstrate conclusively that CD22β recognizes oligosaccharides containing α-2,6-linked sialic acid residues, additional structural features are clearly required for high affinity CD22β-ligand binding. This conclusion is based on several observations. First, although the intact glycoproteins can be batch adsorbed onto CD22bRg-PAS and survive extensive washing, binding of the free oligosaccharides is difficult to demonstrate under these conditions. Second, no specific chaotropic agents were required for the elution of the free oligosaccharides from the CD22βRg-PAS column, other than warming the column to 22–24 °C. Finally, CD22βRg-*nonbinding* glycoproteins also carried some oligosaccharides that showed retardation on CD22βRg-PAS (data not shown). Thus, it is likely that CD22β recognizes features in addition to α-2,6-sialylated oligosaccharides on high affinity ligands. These features may include clustered oligosaccharides containing α-2,6-linked residues and/or specific polypeptide sequences. Of note, CD22α, an isoform lacking the third and fourth immunoglobulin-like domain found in CD22β, is capable of binding monocytes and erythrocytes, but this binding is not destroyed by treatment with sialidase.[3] Thus, it may be that some domains of CD22 are involved in protein-protein interactions and others in protein-carbohydrate interactions. Other explanations, including the clustering of ligands or receptors on the cell surface, may also be involved. However, it is clear from the data presented here and previously (10, 15) that α-2,6-linked sialic acid residues are an integral component of CD22β-glycoprotein binding. It also remains to be seen if physiologically relevant binding between cells involves α-2,6-linked sialic acids on other types of glycoconjugates, such as glycolipids and *O*-linked oligosaccharide chains.

The data presented here indicate that CD22β prefers oligosaccharides with multiple antennae bearing α-2,6-linked sialic acid. This may indicate that a single CD22β molecule has multiple sites for oligosaccharide binding or that a single *N*-linked oligosaccharide is able to extend between two adjacent molecules. These different possibilities are currently under investigation. Several other structural features of the CD22β-binding oligosaccharides have also yet to be determined. Although the profiles of the neutral core structures from pools I and III were indistinguishable by chromatography on amine-modified silica, this technique is not sensitive enough to detect differences such as bisecting *N*-acetylglucosamine residues, α-galactosylation, short polylactosamine chains, or branching isomers. Moreover, information is not yet available on the location of the fucose residues (core *versus* outer chain) or the relative amounts of Galβ1–3GlcNAc *versus* Galβ1–4GlcNAc on the nonreducing termini. These two structural features are of interest because the β-galactoside α-2,6-sialyltransferase will not sialylate Galβ1–3GlcNAc or Galβ1–3(Fucα1–4)GlcNAc. Despite the similarity of the overall profile of the neutral core structures of pools I and III on amino bonded silica, there are likely to be some structural differences among the pools I, II, and III oligosaccharides, other than sialylation alone. This statement rests on the observation that not all of the pool I material could be converted into pool III material by exogenous sialylation and on the unexplained differences in elution of pools II and III despite their similar sialylation profiles. Experiments are currently under way to elucidate the structural basis for these differences.

The requirement for α-2,6-linked sialyl residues in CD22β-ligand binding indicates that if CD22β functions *in vivo* as a cell adhesion molecule, this function can be regulated at the level of expression of the CD22 protein *and/or* the α-2,6-sialyltransferase. Thus, a given glycoprotein ligand may be constitutively expressed but may only be functionally recognized by CD22β if it is appropriately sialylated. In this regard, the multiple isoforms of CD45 seen in different blood cell types are of interest (42) because they are known to be differentially glycosylated. Alternatively, a cell may use the expression of the α-2,6-sialyltransferase to alter its phenotype between low and high affinity states for binding CD22$^+$ B cells. In this context, it is interesting to note several recent observations on the expression of the α-2,6-sialyltransferase in lymphoid cells. Three different epitopes have been identified which contain, in part, α-2,6-linked sialic acid residues, including CD75 (10), CD76 (43), and the epitope recognized by the monoclonal antibody HB-6 (44). When examining peripheral blood lymphocytes and lymph node sections, different patterns of staining are observed with antibodies directed against these epitopes (10, 43, 44). Given that the different regions of lymph nodes represent lymphoid populations in different stages of maturation, these observations, taken together, demonstrate the regulated expression of α-2,6-sialylated glycoconjugates during lymphocyte development. Within lymph node sections, CD22 is expressed most strongly on marginal zone lymphocytes that surround the follicles (9), the region stained by anti-CD76 and HB-6 (44). Little is known about the regulation of the α-2,6-sialyltransferase in lymphoid or other tissues. Its expression in lymphocytes is cell cycle-dependent (45) and increases with mitogen stimulation (46). Differential processing of α-2,6-sialyltransferase transcripts has been identified in rat kidney (47), but the significance of this in terms of the overall expression of transferase activity is currently not understood.

[3] D. Sgroi and I. Stamenkovic, unpublished observations.

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