A Single N-linked Glycosylation Site Is Implicated in the Regulation of Ligand Recognition by the I-type Lectins CD22 and CD33*

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CD22 is an immunoglobulin superfamily B lymphocyte-specific adhesion receptor and a member of the recently identified I-type class of lectins. Recent work has shown that CD22 specifically recognizes sialic acid linked α2,6 to terminal N-linked oligosaccharides on selected cell surface glycoproteins. CD22-ligand interaction is regulated by the activity of a β-galactosidase α2,6-sialyltransferase that can inactivate CD22-mediated binding by sialylating the CD22 receptor itself. These observations suggest that N-linked glycosylation sites on the CD22 molecule may play a role in the regulation of CD22-mediated adhesion. In this work we have performed site-specific mutagenesis of potential N-linked glycosylation sites on CD22 in an effort to determine whether they might be involved in ligand recognition. We show that mutation of a single potential N-linked glycosylation site in the first immunoglobulin domain of CD2 completely abrogates ligand recognition. Interestingly, this site is characterized by the sequence NCT, where the cysteine is thought to be involved in an intra-chain disulfide bond. Site-directed mutagenesis of similar NC(T/S) motifs in the first or second Ig domains of the I-type lectins myelin-associated glycoprotein, and sialoadhesin did not disrupt their ability to mediate sialic acid binding. In contrast, mutation of a NC(S) motif in the first Ig domain of the I-type lectin CD33 unmasked its sialic acid binding activity. These observations suggest that a single N-linked glycosylation site located at a similar position in the CD22 and CD33 glycoproteins is critical for regulating ligand recognition by both receptors.

Recent work from several laboratories has led to the identification of a novel class of mammalian sialic acid binding lectins composed of immunoglobulin superfamily receptors that recognize sialic acid in linkage-specific fashion (Powell and Varki, 1994). CD22-mediated adhesion requires an intact ligand-associated sialic acid polyhydroxyl side chain (Sgroi et al., 1993), removal or modification of which by mild periodate oxidation or 9-O-acetylation, respectively, abrogates the interaction (Sgroi et al., 1993; Sjoberg et al., 1995). Because endogenous MAG and sialoadhesin bind both NeuAcα2–3Galβ1–3GalNAc, whereas CD33 and sialoadhesin bind both NeuAcα2–3Galβ1–3GlcNAc and NeuAcα2–3GlcNAc oligosaccharides (Kelm et al., 1994; Freeman et al., 1995), COS cells transfected with CD22 adhere to lymphocytes, monocytes, and red blood cells, and the observed adhesion is dependent upon the presence of ligand-associated N-linked oligosaccharides containing α2,6-linked but not α2,3-linked sialic acid (Stamenkovic et al., 1991; Sgroi et al., 1993; Powell et al., 1993; Powell and Varki, 1994). The affinity of receptor-ligand interaction is determined by the number of sialic acid residues associated with the glycoprotein ligand and by appropriate sialic acid presentation by the underlying polypeptide backbone (Powell et al., 1993; Powell and Varki, 1994). CD22-mediated adhesion requires an intact ligand-associated sialic acid polyhydroxyl side chain (Sgroi et al., 1993), removal or modification of which by mild periodate oxidation or 9-O-acetylation, respectively, abrogates the interaction (Sgroi et al., 1993; Sjoberg et al., 1995). Interestingly, co-expression of a β-galactosidase α2,6-sialyltransferase and cell surface or soluble CD22 in COS cells abrogates CD22-mediated adhesion and modulation of T cell activation (Braaesch-Andersen and Stamenkovic, 1994; Sgroi et al., 1995). Because CD22 expressed on B lymphocytes is itself sialylated in α2,6 linkage, it appears likely that the relative α2,6-sialyltransferase activity in B cells and in ligand expressing target cells regulates CD22 interaction with ligands on the same cell surface or on that of adjacent cells.

Unlike CD22, CD33, sialoadhesin, and MAG recognize sialic acid in α2,3 but not in α2,6 linkage. More specifically, MAG recognizes the oligosaccharide NeuAcα2–3Galβ1–3GalNAc, whereas CD33 and sialoadhesin bind both NeuAcα2–3Galβ1–3GlcNAc and NeuAcα2–3GlcNAc oligosaccharides (Kelm et al., 1994; Freeman et al., 1995). COS cells transfected with native MAG and sialoadhesin support adhesion of erythrocytes and neutrophils, whereas CD33 expressing cells require desialylation to uncover their erythrocyte and leukocyte-binding ability (Freeman et al., 1995). Because endogenous glycoconjugates appear to modulate sialoglycoprotein ligand recognition by CD22 and CD33, we performed site-specific mutagenesis of potential N-linked glycosylation motifs within the first two Ig domains of CD22 and the first Ig domain of CD33 in an effort to identify sites necessary for sialic acid binding. Our results demonstrate that mutation of a single potential N-linked glycosylation site is implicated in the regulation of ligand recognition by the I-type lectins CD22 and CD33. Therefore, this site may be hereby marked "advertisement."
linked glycosylation motif in the first immunoglobulin domain of CD22 completely abrogates its adhesion properties, whereas mutation of a homologously positioned N-linked glycosylation site in CD33 umasks its ligand binding function.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cell culture medium (Dulbecco's modified Eagle's medium), cysteine/methionine-free Dulbecco's modified Eagle's medium, and fetal bovine serum were purchased from Irvine Scientific (Santa Ana, CA). L-Glutamine and antibiotics were from Life Technologies, Inc. DEAE-dextran and dimethyl sulfoxide were from Sigma. Monoclonal antibody 9G5 was from Novoceastra Ltd. (Newcastle, U.K.). A monoclonal antibody To15 was from Dako (Glostrup, Denmark). Fluorescein-labeled goat anti-mouse affinity purified antibodies were from Cappel (Malvern, PA). Oligonucleotide synthesis reagents were acquired from Millipore (Bedford, MA). [35S]Methionine was obtained from Du Pont NEN. PNGase F was purchased from Oxford Glycosystems (Oxford, U.K.). FITC-conjugated anti-CD22 mAb sJ 10.1H11 was obtained from Immunotech (Westbrook, ME), and FITC-conjugated anti-CD20 was obtained from Becton Dickinson (Mountain View, CA).

Development of CD22 Mutants—Eleven CD22 mutants were prepared by a modified version of the polymerase chain reaction (PCR)-based method of Ho et al. (1989). All of the mutations targeted potential N-linked glycosylation sites in 1g domains 1 and 2. Oligonucleotide primers were designed to allow creation of a unique XhoI site in the vicinity of sequences encoding N-linked glycosylation sites, such that the forward and reverse primers could contain one or two mutant codons each. PCR amplification of two fragments was performed, one primed by a forward primer starting at the initial ATG of the coding sequence and containing an XhoI site and by a reverse primer starting within the cluster and containing point mutations as well as a novel restriction site and the other primed by a forward primer starting within the cluster and containing point mutations as well as a corresponding novel restriction site and by a reverse primer spanning a unique NheI site at nucleotides 1434–1439 that encode residues within 1g domain 5 of CD22. Amplified fragments were subjected to XhoI and restriction with NheI and endonuclease X and NheI and endonuclease I and inserted into XhoI/NarI cut CD22 in a three-way ligation. A distinct restriction site (X) was created in the vicinity of each of the potential N-linked glycosylation sites and was designed to conserve the wild type amino acids at the site or to provide a conservative substitution. Six potential N-linked glycosylation sites were targeted, four in the first Ig domain, and two in the second. The Asn residue in each potential site was substituted by an Ala or Ile. At potential N-linked glycosylation sites 2 and 3, Thr302 → Ala and Ser344 → Gly substitutions were generated, respectively. CD22 mutants containing Lys100 → Ala and Leu104 → Ala substitutions were also generated. All CD22 mutants were subjected to DNA sequence analysis to ensure that appropriate mutations were introduced during PCR.

Development of CD33, MAG, and Sialoadhesin Mutants—The strategy described above for the generation of CD22 mutants was used in the development of N-linked glycosylation site (CHO) mutants CD33, MAG, and sialoadhesin. Human sialoadhesin cDNA, human CD33 cDNA, and rat MAG cDNA were generously provided by Ajit Varki (UCSD, La Jolla, CA), Brian Seed (Harvard University), and John Roiter (University of Toronto, Toronto, Canada) respectively. A CHO1 mutant in transmembrane CD33 was generated by an Asn100 → Ala substitution. Site-specific mutations surrounding CHO1 were generated by Arg99 → Ala and Leu103 → Ala substitutions. MAG and sialoadhesin CHO1 mutants were generated by Asn99 → Ala and Asn103 → Ala substitutions.

Expression of CD22, CD33, and MAG Mutants—All of the constructs were transiently expressed in COS cells by the DEAE-dextran method (Aruffo and Seed, 1987), and transfectants were tested for reactivity with specific antibodies 48 h following transfection; anti-CD22 mAbs HD39, To15, and HD6 (Mcdenhauer et al., 1986), anti-MAG mAb S13 (generous gifts from T. Tropak and J. Roder), and anti-sialoadhesin HM3-4 (Pharrmeng, San Diego, CA) were used. Transfected COS cells were incubated with 2–5 μg/ml of each antibody for 45 min at room temperature in PBS/5% fetal bovine serum/0.02% sodium azide, washed in PBS, incubated with a secondary, affinity purified, fluorescein-labeled goat anti-mouse antibody for 30 min at room temperature, washed, fixed briefly in 4% formaldehyde, and examined under an epi-fluorescence microscope.

Radioiodination and Immunoprecipitation of CD22 Mutants—COS cells were transfected with the various CD22 mutant cDNAs as described above. 48 h after transfection, the COS cells were washed and incubated for 6 h in cysteine/methionine-free Dulbecco's modified Eagle's medium, 10% dialyzed fetal bovine serum, 100 μCi of [35S]methionine. Cells were washed in PBS three times and lysed in buffer consisting of PBS, 0.5% Nonidet P-40, 20 μg/ml aprotonin, and 0.02% sodium azide. The lysates were preincubated for 6 h at 4°C with 25 μl of protein G-Sepharose beads. Preincubated lysates were incubated with the anti-CD22 mAb, HD6, at 5 μg/ml with 25 μl of fresh packed protein G-Sepharose beads for 8 h at 4°C. Beads were washed four times in PBS/0.05% Nonidet P-40, and precipitates were eluted by boiling in sample buffer in the presence of 2% 2-mercaptoethanol. Samples were exposed to SDS/8% PAGE, and the gel was fixed, incubated in Autorfluor (National Diagnostics; Manville, N.J.) for 1 h, dried, and exposed to x-ray film for 8 h.

Development of Soluble CD22-, CD33-, MAG- and Sialoadhesin-Ig Fusion Proteins—Development of CD22-Ig fusion protein containing the first three Ig-like domains of CD22 has been described previously (Stamenkovic et al., 1991). The strategy used to develop CD22-Ig fusions was applied to the generation of CD33, MAG, and sialoadhesin-Ig chimera.

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Role of Glycosylation in CD22- and CD33-mediated Adhesion

Enzyme-linked Immunosorbent Assay—96-well microtiter plates were coated with 6 μg of CD22Rg, CD44Rg, or PNGase F-treated CD22Rg in 100 μl of PBS/0.02% sodium azide overnight at 4°C. The plate was washed with PBS/0.05% Tween 20 and incubated with 1 mg/ml of anti-CD22 mAb HD6, HD39 or To15, washed with PBS/0.05% Tween 20 and exposed for 1 h at room temperature to alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) diluted 1:4000. After three washes with PBS/0.05% Tween 20, p-nitrophenyl phosphate (Sigma) dissolved in 0.1 M glycine/10 mM MgCl2 solution, pH 9.4, was added. Following 1 h of incubation at room temperature, the colorimetric reaction was read at 405 nm in an EL-310 Microplate Autoreader (Biotek Instruments, Winooski, VT).

Gel Filtration Chromatography—Protein A-Sepharose affinity purified CD22Rg and PNGase F-treated CD22Rg were individually injected onto a Superose-6 HR 10/30 gel filtration column (Pharmacia Biotech Inc.) and subjected to a constant flow rate of 0.5 ml/min utilizing a fast protein liquid chromatography system (Pharmacia). Elution fractions were collected, and protein was detected by spectral absorbance at 226 nm. Elution profiles were generated by plotting protein concentration (spectral absorbance, A226 nm) versus volume (ml).

RESULTS

Mutation of Potential N-linked Glycosylation Sites within the First and Second Ig Domains of CD22—We have shown previously that the first three Ig domains of CD22 are sufficient to provide all of the known adhesive functions and antibody recognition sites of CD22 (Stamenkovic et al., 1991), although the first two domains contain epitopes recognized by several antibodies and provide some adhesive properties. Six potential N-linked glycosylation sites are present in the first two Ig domains (four in domain 1 and two in domain 2), but there are none in the third. To determine the potential role of N-linked glycosylation in the function of CD22 as an adhesion receptor, we substituted the Asn residue in each of the five potential N-linked glycosylation sites with an Ala and that of the sixth with Ile (Fig. 1) and tested each mutant for expression and the ability to support erythrocyte and lymphocyte adhesion in COS cells. COS cells transfected with CD22 mutants containing Asn→Ala, Asn135→Ala, Asn164→Ala, and Asn231→Ile substitutions tested positive with HD39, HD6, and To15 mAb, which recognize distinct epitopes (Table I). However, transfected expressing a mutant containing Asn101→Ala and Asn112→Ala substitutions failed to stain with To15 mAb while maintaining expression of epitopes recognized by HD39 and HD6 mAb (Table I). To determine whether either or both sites are required for expression of the To15 epitope, mutants containing either Asn101→Ala or Asn112→Ala were generated, in addition to mutants containing Thr103→Ala or Ser114→Gly to ensure that any observed change in antibody recognition or functional property is related to the glycosylation defect and not to the Asn substitution per se. Mutants containing Asn101→Ala or Thr103→Ala substitutions displayed weak reactivity with To15 mAb, whereas the mutations Asn112→Ala and Ser114→Gly had no significant effect on the To15 epitope expression (Table I). The surface density of CD22 Thr103→Ala expressed in COS cells was comparable with that of wild type CD22 as determined by FACS analysis (Fig. 2), ruling out the possibility that the weak reactivity with To15 mAb was due to a decrease in surface expression.

Immunoprecipitation of CD22 mutants Asn101→Ala/Asn112→Ala, Asn101→Ala, Thr103→Ala, and Asn112→Ala expressed in COS cells using the HD6 mAb revealed a reduction in molecular weight (Mw) consistent with a loss of N-linked glycans (Fig. 3). Although the shift in Mw of the mutant containing Asn112→Ala was minor, the combined Asn101→Ala/Asn112→Ala substitution resulted in a greater reduction in Mw than substitution of Asn101→Ala alone. These observations are consistent with the notion that Asn112 is glycosylated. Mutation of potential N-linked glycosylation sites Asn67, Asn135, Asn164, and Asn231 resulted in a minimal shift in Mw (Fig. 3).

Mutation of a Single N-linked Glycosylation Site in Ig Domain 1 of CD22 Abrogates Ligand Recognition—Whereas mutation of potential N-linked sites Asn67, Asn135, Asn164, and Asn231 had no significant effect on erythrocyte and lymphocyte adhesion to COS cell transfectants (Table I and Fig. 4), simultaneous mutation of Asn101 and Asn112 completely abrogated adhesion of all cells tested (Table I and Fig. 4). COS cells transfected with CD22 mutants Asn101→Ala and Thr103→Ala (N-linked glycosylation site 2) failed to bind erythrocytes or lymphocytes (Fig. 4). Lymphocyte binding by transfectants expressing mutations of Asn112→Ala and Ser114→Gly (N-linked glycosylation site 3), on the other hand, was similar to that of wild type CD22 expressing counterparts, although the ability to support erythrocyte adhesion was reduced (Fig. 4). COS cells expressing CD22 cDNAs containing the substitutions Lys100→
Ala and Leu\textsuperscript{104} → Ala, which correspond to residues adjacent to N-linked glycosylation site 2, were observed to support adhesion of erythrocytes and lymphocytes in a manner comparable with that of wild type CD22 transfectants (Table I). As expected, immunoprecipitation of CD22 Lys\textsuperscript{100} → Ala and CD22 Leu\textsuperscript{104} → Ala with anti-CD22 mAb did not reveal a shift in \( M_r \), (Fig. 2 and data not shown).

Previous work had demonstrated that α2,6 sialylation of N-linked glycans in the first two Ig domains of CD22 results in the abrogation of its ability to bind sialoglycoprotein ligands (Braesch-Andersen and Stamenkovic, 1994). We therefore used the potential N-linked glycosylation site mutants to determine whether α2,6 sialylation of glycans associated with a specific asparagine residue might be responsible for the observed disruption of CD22 interaction with its ligands. COS cells were co-transfected with human α2,6-sialyltransferase cDNA and cDNAs encoding each of the CD22 N-linked glycosylation site mutants, with the obvious exception of Asn\textsuperscript{103} → Ala, and

### Table I

| Adhesion Assay | Antibody Staining |
|---------------|-------------------|
| RBCs | T cell blasts | Tonsillar lymphocytes | To15 | HD6 | HD39 |
| CD22WT | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn67Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn101Ala/112Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Thr103 | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Ser114Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Thr103 | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Ser114Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn33Ile | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn112Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn164Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn135Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Lys100Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn112Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Ser114Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn135Ala | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Asn231Ile | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Thr103 | ++ | ++ | ++ | ++ | ++ | ++ |
| CD22Ser114Ala | ++ | ++ | ++ | ++ | ++ | ++ |

Fig. 2. Flow cytometry analysis of COS cells transfected with wild type CD22 and CD22Thr103. COS cells expressing wild type CD22 or CD22Thr103 (harvested with EDTA) were stained with FITC-conjugated anti-CD20 (control mAb) or FITC-conjugated anti-CD22 mAb SJ.10.1H11 and analyzed as described under "Experimental Procedures."

Fig. 3. Immunoprecipitation of CD22 and CD22 mutants. SDS/PAGE mobility of CD22 immunoprecipitated from lysates of \( ^{35} \)S-radio-labeled COS cells transfected with CD22 and CD22 mutant cDNAs. Transfectants were lysed in PBS/0.5% Nonidet P-40, and immunoprecipitation performed with the anti-CD22 mAb HD6. Immunoprecipitated CD22 samples were subjected to SDS/7% PAGE. CD22 mutants and molecular mass markers (in kDa) are indicated.

Fig. 4. Adhesion of tonsillar lymphocytes and red blood cells to CD22- and CD22 mutant-transfected COS cells. Tonsillar lymphocyte (A, C, E, and G) and red blood cell rosetting (B, D, F, and H) with COS cells transfected with D22WT (A and B), CD22Asn101Ala/Asn112Ala (C and D), CD22Asn101Ala (E and F), CD22Asn112Ala (G and H).
assayed for their ability to support lymphocyte adhesion. In all cases, co-transfection of α2,6-sialyltransferase cDNA resulted in the abrogation of CD22-mediated adhesion (data not shown).

Deglycosylation of CD22Rg Abrogates Its Recognition of Sialylated Ligands—Because mutation of an N-linked glycosylation site may result in a conformational change due to inappropriate protein folding, we assessed the effect of removing N-linked carbohydrates from mature CD22-Ig fusion protein (CD22Rg) containing the first three Ig domains of CD22 (Fig. 5A). The fusion protein was coupled to protein A-Sepharose and subjected to PNGase F hydrolysis. As a result, PNGase F-treated CD22Rg displayed a reduction in molecular mass marker (Fig. 5A), and a significant decrease in reactivity with lymphocytes (Fig. 5B). The absence of complete abrogation of lymphocyte binding as a result of PNGase F treatment was probably due to the fact that the digestion was performed without SDS to avoid altering the structure of the receptor-globulin. It is therefore likely that removal of N-linked glycans was incomplete. Recognition of PNGase F-treated CD22Rg by anti-CD22 mAb was tested in an enzyme-linked immunosorbent assay, using plates coated with PNGase F-treated and untreated CD22Rg and CD44Rg control. Consistent with N-linked glycosylation site 2 and 3 mutants, PNGase F-treatment of CD22Rg resulted in the loss of the epitope recognized by the To15 mAb but conservation of HD39 and HD6 mAb-specific epitopes (Table II).

Because oligosaccharide side chains can play a role in protein solubility, it is possible that deglycosylation of CD22Rg results in protein aggregation. Such aggregation may mask critical CD22 epitopes thereby providing an alternative explanation for the loss of To15 mAb binding and loss of ligand recognition. Gel filtration chromatography of native and deglycosylated CD22Rg demonstrated a similar retention volume profile (data not shown). Less than 4% of the deglycosylated CD22Rg was present in the void volume, demonstrating that deglycosylation of CD22Rg results in minimal aggregation.

Mutagenesis of the NC(T/S) Motif in the First Extracellular Domain of CD33Rg Unmasks Its Ability to Bind—Comparison of amino acid sequence alignments of CD22 with the other known I-type lectins reveals that each molecule possesses a NC(S/T) motif in either the first or second Ig domain. The NC(S/T) motif is at the same relative location in the first Ig domain of CD22, CD33, and MAG, whereas it is present in the second domain of sialoadhesin. In order to determine whether...
the NC(T/S) motif plays a role in ligand recognition by CD33, MAG, and sialoadhesin, we substituted the Asn residue in the NC(T/S) motif of each receptor with Ala. Soluble receptorglobulins of CD33Asn100Ala, MAGAsn99Ala, and SialoadhesinAsn159Ala (CD33CHO1Rg, MAGCHO1Rg, and SialoadhesinCHO1Rg, respectively, where CHO1 indicates the first N-linked glycosylation site from the NH2-terminus of each receptor) were generated (Fig. 5A), and immunoprecipitation of each revealed a small reduction in M, with respect to the corresponding wild type Rg (Fig. 7), consistent with the loss of N-linked glycans from the mutated site. Binding of each of the mutant receptor-globulins to peripheral blood granulocytes (PBGs) was analyzed by flow cytometry, using binding of CD22Asn101Ala Rg (CD22CHO2Rg) relative to that of CD22wtRg as a control. As expected, soluble CD22Asn101Ala (CD22CHO2Rg) demonstrated a significant loss of binding to PBGs when compared with wild type CD22Rg (Fig. 8A), whereas MAGCHO1Rg and sialoadhesinCHO1Rg stained PBGs with an intensity comparable with that of corresponding wild-type receptor-globulins (Fig. 8, B and C). In contrast, binding of PBG by CD33CHO1Rg was significantly greater than by wild type CD33Rg (Fig. 8D). Treatment of CD33Rg with V. cholerae sialidase resulted in a slight decrease in M, (Fig. 7) and enhanced binding of PBG to a level comparable with that of the corresponding CHO1 mutant.

**DISCUSSION**

The extracellular Ig-like domains of CD22 that are responsible for its adhesion function contain six potential N-linked glycosylation sites. In this work, we have demonstrated that mutation of a single potential N-linked glycosylation site within the first Ig domain of CD22 (Asn101) results in abrogation of CD22-mediated sialic acid binding. Several observations are consistent with the notion that N-linked glycans at Asn101 are required for CD22-mediated adhesion. First, independent mutation of Asn101 → Ala and Thr103 → Ala resulted in the To15 mAb epitope loss as well as the inability of CD22 to bind ligands. Second, mutation of residues immediately proximal and distal to the second N-linked glycosylation motif had no effect on ligand recognition or To15 reactivity. Third, the removal of N-linked glycans from mature CD22Rg, by PNGaseF, resulted in the reduction of its ability to bind cell surface ligands as well as the loss of the To15 epitope. These results suggest that a single N-linked oligosaccharide chain may play a key role in defining the sialic acid binding region of CD22.

The loss of To15 antibody reactivity in COS cells expressing the CD22 Asn101 → Ala mutant suggests that the N-glycosyl chain at Asn101 is necessary for determining the proper three-dimensional conformation of the sialic acid binding site. The mAb To15, which recognizes an epitope in the third extracellular domain and blocks CD22-mediated adhesion (Stamenkovic et al., 1991), reacts weakly with COS cells expressing CD22 Asn101 → Ala and Thr103 → Ala mutants and fails to recognize COS cells expressing the combined Asn101 and Asn112 CD22 mutant. One possible interpretation of this observation is that the Asn101-associated oligosaccharide chain may form part of the To15 epitope and that loss of the oligosaccharide results in diminished mAb reactivity. However, this argument is refuted by the observation that Asn101 and Thr103 reside in the first extracellular domain, which has been shown to be unnecessary for To15 recognition of its epitope (Engel et al., 1995). A more likely possibility may be that loss of Asn101 or simultaneous loss of Asn101 and Asn112-associated glycans result in conformational changes that, respectively, partially or totally mask the To15 epitope.

Recently, the function of at least two structurally unrelated adhesion receptors has been shown to be regulated by N-linked glycans. Recognition of hyaluronan by the cell surface receptor CD44 has been observed to be both positively and negatively regulated by N-linked glycans (Bartolazzi et al., 1996), whereas the single N-linked glycan of the adhesion domain of CD2 has been shown to play a critical role in CD2-CD58 interaction without itself being part of the recognition site (Recny et al., 1992 and Wyss et al., 1995). Cell surface CD2 mutants containing substitutions in the N-glycosylation sequence Asn65,Gly66, Thr67 that preclude attachment of N-glycans at Asn65 display loss of antibody and ligand binding activity, suggesting that the N-linked glycan of CD2 plays a critical role in stabilizing CD2 receptor conformation. These observations suggest that Ig domains of some receptors are not rigid protein scaffolds but appear to maintain the appropriate structure through a dynamic interaction between the polypeptide core and the attached glycan (Wyss et al., 1995). The similar behavior of the...
CD22 Asn101 and the CD2 glycosylation mutant is consistent with the possibility that the second N-linked glycan of CD22, while not necessarily being part of the ligand recognition site, may require for its appropriate conformation.

It has been shown that sialylation of CD22-associated N-linked oligosaccharides abrogates CD22-mediated adhesion (Braesch-Andersen and Stamenkovic, 1994; Hanasaki, Varki and Powell, 1995). Thus, the function of CD22 as an adhesion molecule depends, at least in part, on the reciprocity of receptor-ligand sialylation; desialylated CD22 can bind sialylated glycoprotein ligands whereas sialylated CD22 cannot. Our present data indicate that inhibition of CD22-mediated adhesion by endogenous α2,6 sialylation cannot be ascribed to any single N-linked oligosaccharide and is more likely due to the combined sialylation of several glycans. Because high affinity CD22 ligands have been shown to be those that contain multiple α2,6-linked sialic acid residues, it makes sense that endogenous inhibition of CD22-mediated binding involves more than one of its oligosaccharide chains. However, because our experiments have been performed using cell surface CD22 mutants, we cannot exclude the possibility that the loss of adhesion associated with α2,6-sialyltransferase co-expression may be due to interaction between CD22 and sialoglycoproteins on the same cell surface.

Mutation of the asparagine residue in the NC(T/S) motif in the first or second Ig-like domains of MAGRg and sialoadhesinRg did not disrupt their ability to bind sialic acid ligands, suggesting that the NC(T/S) motif itself does not provide a structure on which the function of all I-type lectins relies. In contrast, CD33-mediated adhesion in COS cells is unmasked by pretreatment with sialidase, suggesting that terminal sialylation of CD33 glycans, like those of CD22, may play a critical role in regulating recognition of ligands. The present study demonstrates that unlike CD22, sialylation of a single N-linked glycan of CD33 is sufficient to inhibit interaction with ligands. However, the site of attachment of the CD33 N-linked glycan that blocks adhesion aligns with that of CD22 required for supporting adhesion. Whether the presence or the absence of N-linked glycans at these sites alters ligand binding properties by interfering with the disulfide bond provided by the juxtaposed cysteine residue remains to be determined. However, these observations highlight the unique properties of these two Ig-like receptors that not only behave as sialic acid binding lectins but whose function is subject to regulation by sialylation of a single, similarly located N-linked glycosylation site.

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