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Human galectin-1 recognition of poly-N-acetyllactosamine and chimeric polysaccharides

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Human galectin-1 is a dimeric carbohydrate binding protein (Gal-1) (subunit 14.6 kDa) widely expressed by many cells but whose carbohydrate binding specificity is not well understood. Because of conflicting evidence regarding the ability of human Gal-1 to recognize N-acetyllactosamine (LN, Galβ4-GlcNAc) and poly-N-acetyllactosamine sequences (PL, [-3Galβ4GlcNAcβ1-]$_n$), we synthesized a number of neoglycoproteins containing galactose, N-acetylgalactosamine, fucose, LN, PL, and chimeric polysaccharides conjugated to bovine serum albumin (BSA). All neoglycoproteins were characterized by MALDI-TOF. Binding was determined in ELISA-type assays with immobilized neoglycoproteins and apparent binding affinities were estimated. For comparison, we also tested the binding of these neoglycoconjugates to *Ricinus communis* agglutinin I, (RCA-I, a galactose-binding lectin) and *Lycopersicon esculentum* agglutinin (LEA, or tomato lectin), a PL-binding lectin. Gal-1 bound to immobilized Galβ4GlcNAcβ3Galβ4Glc-BSA with an apparent K_d of ~23 μM but bound better to BSA conjugates with long PL and chimeric polysaccharide sequences (K_d's ranging from 11.9 ± 2.9 μM to 20.9 ± 5.1 μM). By contrast, Gal-1 did not bind glycans lacking a terminal, nonreducing unmodified LN disaccharide and also bound very poorly to lactosyl-BSA (Galβ4Glc-BSA). By contrast, RCA bound well to all glycans containing terminal, nonreducing Galβ1-R, including lactosyl-BSA, and bound independently of the modification of the terminal, nonreducing LN or the presence of PL. LEA bound with increasing affinity to unmodified PL in proportion to chain length. Thus Gal-1 binds terminal β4Gal residues, and its binding affinity is enhanced significantly by the presence of this determinant on long-chain PL or chimeric polysaccharides.

Key words: galectin; human; neoglycoprotein; poly-N-acetyllactosamine

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Introduction

Galectins are a family of related carbohydrate binding proteins found in all metazoans, including sponges, invertebrates, fungi, and mammals (Cooper, 2002; Leffler, 2001). This wide distribution among species, coupled with a highly evolutionary conserved sequence within the carbohydrate recognition domain, suggests that galectins are involved in conserved biological processes (Barondes *et al.*, 1994a,b; Kasai and Hirabayashi, 1996; Kilpatrick, 2002). In mammalian systems, galectins have been implicated in numerous processes including neutrophil turnover and phagocytosis (Dias-Baruffi *et al.*, forthcoming), cell migration (Levy *et al.*, 2001), immunomodulation (Gabius, 2001; Levi *et al.*, 1983; Rabinovich *et al.*, 1999), growth regulation (Adams *et al.*, 1996; Allione *et al.*, 1998; Blaser *et al.*, 1998; Wells and Mallucci, 1991), apoptosis (Kashio *et al.*, 2003; Perillo *et al.*, 1995), cell adhesion (Rabinovich *et al.*, 1999b), embryogenesis (Cooper and Barondes, 1990; Cooper *et al.*, 1991), tumor spreading (Raz and Lotan, 1987), and pre-mRNA splicing (Vyakarnam *et al.*, 1997). However, the *in vivo* biological functions of galectins in mammals remain enigmatic and are being actively investigated in many laboratories.

There is also uncertainty surrounding the carbohydrate binding requirements of the galectins, particularly in regard to galectin-1, the first galectin family member identified (de Waard *et al.*, 1976; Nowak *et al.*, 1976; Teichberg *et al.*, 1975). Gal-1 homologs occur as homodimers of ~14.6-kDa subunits (29.2-kDa dimer) (Barondes *et al.*, 1994b; Briles *et al.*, 1979). Dimeric bovine galectin-1 was reported to bind weakly to N-acetyllactosamine (Galβ4GlcNAc) and a variety of other related structures (Abbott *et al.*, 1988; Appukuttan, 2002; Solomon *et al.*, 1991), but preferential binding was observed toward poly-N-acetyllactosamine sequences (PL; [-3Galβ4GlcNAcβ1-]$_n$) containing multiple, linear N-acetyllactosamine (LN) units (Merkle and Cummings, 1988; Zhou and Cummings, 1993), suggesting that LN motifs within the PL chains were possibly recognized by bovine galectin-1. These results were later partly confirmed using short chain synthetic PL and to assess the binding of hamster galectin-1 using ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (Di Virgilio *et al.*, 1999). However, crystallographic data suggest that bovine galectin-1 recognizes the terminal nonreducing β4Gal residue and binds weakly to the penultimate GlcNAc residue within the terminal LN sequence (Bourne *et al.*, 1994b; Liao *et al.*, 1994). Other recent results have also shed doubt on the ability of bovine galectin-1 to recognize internal LN motifs within a PL structure (Appukuttan, 2002). Recent studies on the dimeric human galectin-1 (Gal-1) indicate that it binds better to N-glycans containing terminal LN units

than to PL units (Hirabayashi *et al.*, 2002), but there are also suggestions that human Gal-1 may bind chondroitin sulfate B (Moiseeva *et al.*, 2003). These questions about the specificity of Gal-1 for saccharide ligands has prompted us to further investigate the binding characteristics of this protein. It is hoped that a better understanding of these requirements will prove helpful in future studies on the biological functions of Gal-1 and the possible use of Gal-1 as a target for therapeutic intervention, as has been previously suggested (Gabius *et al.*, 2002).

To assess the requirements for saccharide recognition by human Gal-1, we prepared a series of neoglycoproteins in which glycans were covalently attached to bovine serum albumin (BSA). Some of these glycans contained the PL sequence, whereas others contained PL-related sequences with internal modifications, such as the presence of internal -GalNAcβ4GlcNAcβ1- sequences (lacdiNAc or LDN antigen), or Galβ4[Fucα3]GlcNAcβ-R (Lewis x or Lex antigen) in what we have termed chimeric-polysaccharide sequences. These neoglycoproteins were then tested in ELISA-type formats using fluorescently labeled Gal-1 and a number of plant lectins and monoclonal antibodies previously shown to be able to discriminate LN- and PL-containing glycans. Our results demonstrate that human Gal-1 recognizes terminal, nonreducing LN with high affinity and that terminal, nonreducing LN expressed on long-chain PL sequences or chimeric polysaccharide sequences enhance recognition.

GLYCAN STRUCTURE	TRIVIAL NAME
Galβ4Glc	Lactose (L)
Galβ4GlcNAcβ3Galβ4Glc	LNnT
GalNAcβ4GlcNAcβ3Galβ4Glc	LDNT
GlcNAcβ3Galβ4Glc	Triose
Galβ4(Fucα3↓)GlcNAcβ3Galβ4Glc	Lex-L
Galβ4GlcNAcβ3GalNAcβ4GlcNAcβ3Galβ4Glc	LN-LDN-L
Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc	LN-LN-L
Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc	LN-LN-LN-L
GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc	GlcNAc-LN-LN-L
Galβ4GlcNAcβ3Galβ4Glc(Fucα3↓)NAcβ3Galβ4Glc(Fucα3↓)NAcβ3Galβ4Glc	LN-Lex-Lex-L

Fig. 1. Structures of glycans used in the generation of neoglycoproteins.

Results

Characterization of neoglycoproteins

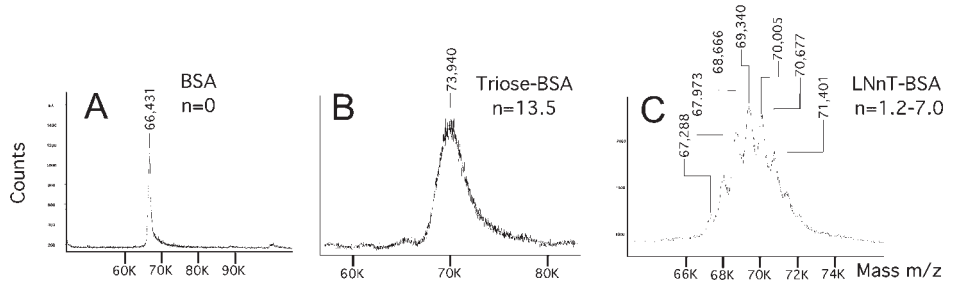
The oligosaccharides LN-LN-LN-L, LN-LN-L, LN-Lex-Lex-L, LN-LDN-L, LNnT, GalNAcβ4GlcNAcβ3Galβ1-4Glc (LDNT), Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (Lex-L), and triose (Figure 1) were prepared by enzymatic synthesis and, along with lactose, were coupled to BSA by reductive amination as described in *Materials and methods*. We attempted to derivatize the glycans to relatively equal densities by matching concentrations during the coupling reactions, but we anticipated that each glycan might demonstrate differential reactivity. To test the density of covalent coupling each conjugate was analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and examples of the MALDI-TOF spectra for unconjugated BSA, triose-BSA, and LNnT-BSA are shown in Figures 2A–C. The compiled data for all neoglycoproteins is shown in Figure 2D. Some neoglycoproteins were found to have a high coupling density, for example, lactosyl-BSA containing ~27 mol glycan/mol BSA (Figure 2D), whereas others had lower coupling densities and demonstrated a range of coupling, for example, LNnT-BSA with a range of 1.2–7.0 (Figure 2C, D).

Although we were concerned that different coupling densities among the neoglycoprotein preparations might confound interpretations of binding data for human Gal-1, our studies demonstrated that in fact, the compounds with the highest coupling densities, for example, LDNT-BSA, triose-BSA, and lactosyl-BSA, binds very poorly to Gal-1, thus indicating that their high coupling densities did not significantly enhance their recognition by the lectin. Interestingly, by using different preparations of the same neoglycoprotein that varied in the density of conjugation, we found that the binding affinity of human Gal-1 and other lectins, as will be discussed in more detail, was not appreciably affected. Thus it is possible that on the large BSA molecule, these glycans are recognized monovalently by the lectin, that is, their densities and orientations are not high enough to promote cross-linking by the lectin and enhanced avidity of binding. This issue may be more formally addressed by crystallographic analyses and other biophysical approaches beyond the scope of the present study.

Binding of Gal-1 to LN-containing ligands LN-LN-LN-L-BSA, LN-LN-L-BSA, and LNnT-BSA

For the study of Gal-1 binding to these neoglycoconjugates, we used the lectin directly fluorescently labeled with the stable fluorescent dye ALEXA-488. The ALEXA-Gal-1 quantitatively rebound to a column of α-lactose-agarose (data not shown), indicating that fluorescence derivatization did not affect the stability of the protein or its ability to bind to the original affinity column used in purification of the recombinant protein.

Microtiter wells were directly coated with neoglycoproteins as described in *Materials and methods*, and the binding of ALEXA-Gal-1 was directly measured toward lactosyl-BSA, LNnT-BSA, LN-LN-L-BSA, and LN-LN-LN-L-BSA (Figure 3). ALEXA-Gal-1 demonstrated the highest apparent binding affinity toward LN-LN-LN-L-BSA (K_d = 11.9 μM) (Figure 3A, B). LN-LN-L-BSA and LNnT-BSA bound with similar affinity to Gal-1 at K_d values equaling 20.9 μM and 21.5 μM, respectively (Table I and Figure 3A, B). (For graphical display, the K_a in μM^{-1} are shown in Figure 3B, and the compiled K_d's of binding are shown in Table I.) Interestingly ALEXA-Gal-1 exhibited no appreciable binding to lactosyl-BSA (Figure 3B, Table I). These results suggest that Gal-1 affinity for PL-containing glycans was not only dependent on the presence of the LN



D

Neoglycoprotein	Observed Mass (Da)	Mol Glycan/Mol BSA
BSA (unconjugated)	66,431	-
Lactosyl-BSA	76,294	27.4
Triose-BSA	73,940	13.5
LNnT-BSA	69,340	1.2-7.0
Lex-L-BSA	72,108	1.1-8.1
LN-LDN-L-BSA	73,940	8.1
LN-LN-L-BSA	73,387	6.6
LN-LN-LN-L-BSA	72,334	2.3-5.1
LN-Lex-Lex-L-BSA	75,224	2.1-7.9

Fig. 2. MALDI-TOF analysis of neoglycoproteins. Glycans from Figure 1 were covalently linked to lysine residues of BSA through reductive amination, and the neoglycoproteins were analyzed by MALDI-TOF, as described in *Materials and methods*. The peak molecular weights are indicated for several examples in (**A**) unconjugated BSA, (**B**) triose-BSA , and (**C**) LNnT-BSA. (**D**) The observed midrange peak masses of all neoglycoproteins and the range of conjugation in mol glycan/mol BSA are listed.

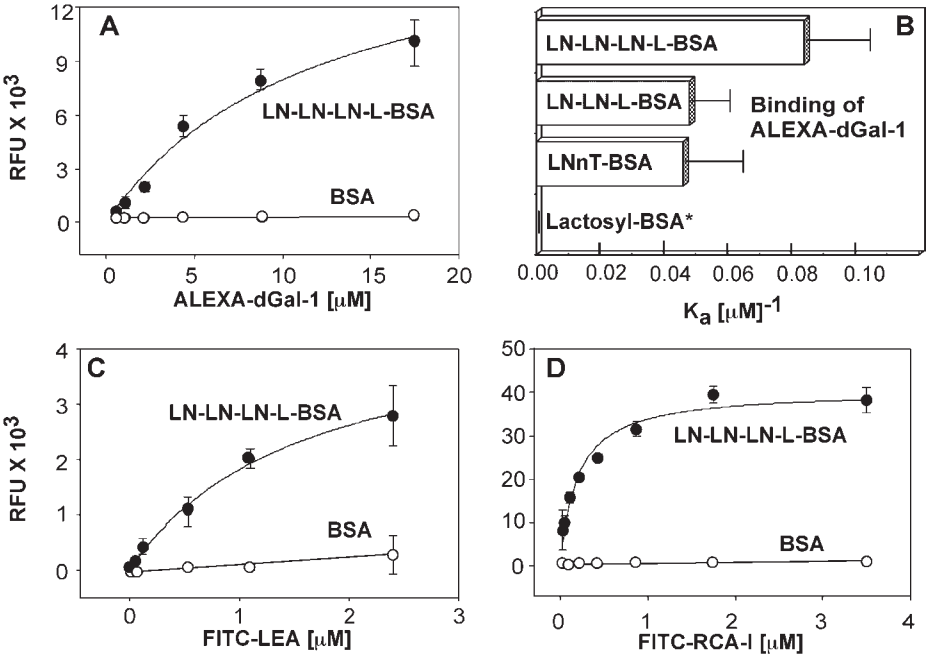


Fig. 3. Binding of ALEXA-Gal-1, FITC-RCA-I, and FITC-LEA to neoglycoproteins. Solid-phase fluorometric binding assays were accomplished as outlined in the *Materials and methods*. (**A**) Binding of ALEXA-Gal-1 to LN-LN-LN-L-BSA and unconjugated BSA. (**B**) Apparent association constants (K_a in μM^{-1}) ($K_a = 1/K_d$) for binding of ALEXA-Gal-1 to indicated neoglycoproteins. (**C**) Binding of FITC-LEA to LN-LN-LN-L-BSA and unconjugated BSA. (**D**) Binding of FITC-RCA-I to LN-LN-LN-L-BSA and unconjugated BSA. All assays were performed in triplicate and the average values are shown. Apparent affinity constants were calculated using a rectangular hyperbola equation to derive the nonlinear curve fitting ($p < 0.001$).

Table I. Apparent binding affinities (K_d [μM]$_{1/2Max}$) of human Gal-1 and plant lectins to neoglycoproteins

	ALEXA-Gal-1	FITC-RCA-I	FITC-LEA
LN-LN-LN-L-BSA	11.9 ± 2.9	0.20 ± 0.03	2.22 ± 0.7
LN-LN-L-BSA	20.9 ± 5.1	0.17 ± 0.08	3.01 ± 1.5
LNnT-BSA	21.5 ± 8.2	0.14 ± 0.02	ND
Lactosyl-BSA	ND	0.30 ± 0.02	ND
LDNT-BSA	ND	0.34 ± 0.02	ND
Lex-L-BSA	ND	ND	ND
LN-Lex-Lex-L-BSA	11.9 ± 2.9	0.28 ± 0.02	ND
LN-LDN-L-BSA	14.5 ± 2.7	0.19 ± 0.01	8.31 ± 6.7
Triose-BSA	ND	ND	ND

The apparent K_d values, taken as the concentration of lectin that gave one-half maximal saturation of binding (1/2Max values) expressed in μM, are shown for each lectin binding to neoglycoproteins in the solid-phase binding assay using fluorescently labeled lectins as indicated. Each value was achieved using nonlinear curve fitting to a rectangular hyperbola. ND indicates those neoglycoproteins to which respective lectins displayed no detectable binding. Neoglycoprotein syntheses and lectin binding analyses were accomplished as outlined in the *Materials and methods*.

unit but was significantly enhanced by extension of LN repeats within the glycans. The results indicate that Gal-1 recognizes long-chain PL sequences with relatively high affinity compared to lactosyl-BSA. It is interesting that Gal-1 failed to bind well to lactosyl-BSA, whereas the lectin does bind to α-lactose-agarose. This could be due to the high density of coupling of lactose on α-lactose-agarose (probably >1 mM) whereas the density of lactose of the lactosyl-BSA is quite low. Thus although the binding affinity to lactose may be low, the relative high coupling density of lactose on α-lactose-agarose is practically useful in purifying the lectin. In addition, the preparation of lactosyl-BSA results in reduction of the glucosyl moiety that may decrease galectin recognition.

To control for the accessibility of the BSA-glycan conjugates, we explored their binding to plant lectins with defined specificities toward either PL sequences of terminal β4Gal residues. To this end we used tomato lectin (*Lycopersicon esculentum* agglutinin; LEA) labeled with fluorescein isothiocynate (FITC) to explore the accessibility of PL sequences. This lectin has been shown to demonstrate high-affinity binding to glycoconjugates containing repeating LN sequences in PL (Merkle and Cummings, 1987). FITC-LEA showed high-affinity binding to LN-LN-LN-L-BSA (Figure 3C) and generally demonstrated increased affinity toward those ligands with increasing number of LN units (Table I). However, LEA binding was different from Gal-1 in the respect that LEA appeared to recognize LN sequences when positioned within the LN-containing glycan, regardless of the presence of a terminal β4Gal residue, as has been previously reported (Merkle and Cummings, 1987). As a result, LEA demonstrated no binding to either LNnT-BSA or lactosyl-BSA (Table I).

To explore binding to neoglycoproteins containing terminal β4Gal residues, we employed *Ricinus communis* agglutinin-I (RCA-I), a plant lectin that recognizes terminal β4Gal residues (Baenziger and Fiete, 1979; Cummings and Kornfeld, 1982; Irimura *et al*., 1981; Kornfeld *et al*., 1981; Narasimhan *et al*., 1985). FITC-RCA bound tightly to LN-LN-LN-L-BSA (Figure 3D). However, FITC-RCA-I displayed relatively uniform affinity for each neoglycoprotein containing terminal β4Gal residues, including lactosyl-BSA, but did not bind to those lacking this residue (Table I). This result also demonstrates that differential coupling densities of glycans to BSA does not appreciably affect the binding affinity of RCA-I, thus arguing for monovalent recognition by the lectin for each glycan, rather than cross-linking of glycans leading to enhanced avidity. The results of using the control lectins FITC-LEA and FITC-RCA further demonstrate that all glycans within the neoglycoproteins are accessible and provide appropriate and predictable expression as neoglycoproteins immobilized in microtiter plates.

Gal-1 binding to modified LN neoglycoproteins

To address the importance of terminal, nonreducing β-linked galactosyl residues and the potential role of the internal LN units in the PL chains for recognition by Gal-1, we generated several novel neoglycoproteins containing chimeric polysaccharide sequences, that is, either LN or lactose (L) disaccharides coupled with other repeating motifs. These included LN-Lex-Lex-L-BSA, containing internal LN residues modified by an α3-linked fucose residue, Lex-L-BSA, containing fucose linked α3 to the penultimate GlcNAc residue, LN-LDN-L-BSA containing an internal LDN sequence, and LDNT-BSA, in which a terminal, nonreducing GalNAc residue replaced the terminal Gal residue linked to lactose (Figure 1). Interestingly, Gal-1 bound equally well to LN-Lex-Lex-L-BSA and LN-LDN-L-BSA (Figure 4A, B) but did not bind to Lex-L-BSA, LDNT-BSA, or triose-BSA (Figure 4A–C and Table I). These data demonstrate that Gal-1 recognizes unmodified terminal Gal residue on a nonreducing LN and that internal modifications of the internal PL sequence do not affect binding.

Interestingly, FITC-RCA-I bound well to LN-Lex-Lex-L-BSA, LN-LDN-L-BSA and LDNT-BSA (Figure 4D–F) but did not bind Lex-L-BSA (Figure 4D). These results indicate that RCA-I requires terminal nonreducing and unmodified LN sequences for high-affinity binding and that the lectin can recognize LDNT-BSA, which contains terminal, nonreducing β4GalNAc residue (Figure 4E). This latter finding is consistent with a previous study documenting the ability of RCA-I to bind such terminal sequences (Saito *et al*., 1978). By contrast, FITC-LEA bound to LN-LDN-L-BSA, although less well than it did to LN-LN-L-BSA (Table I) but did not bind either LDNT-BSA or Lex-L-BSA (Figure 4G and Table I).

Because neither Gal-1, RCA-I, or LEA bound Lex-L-BSA and only FITC-RCA recognized LDNT-BSA, the possibility arose that these glycan determinants on the neoglycoproteins might not be fully accessible to external reagents. To control for this possibility, we used monoclonal antibodies previously prepared toward the Lewis x and LDN antigens (Nyame *et al*., 1997, 1998,

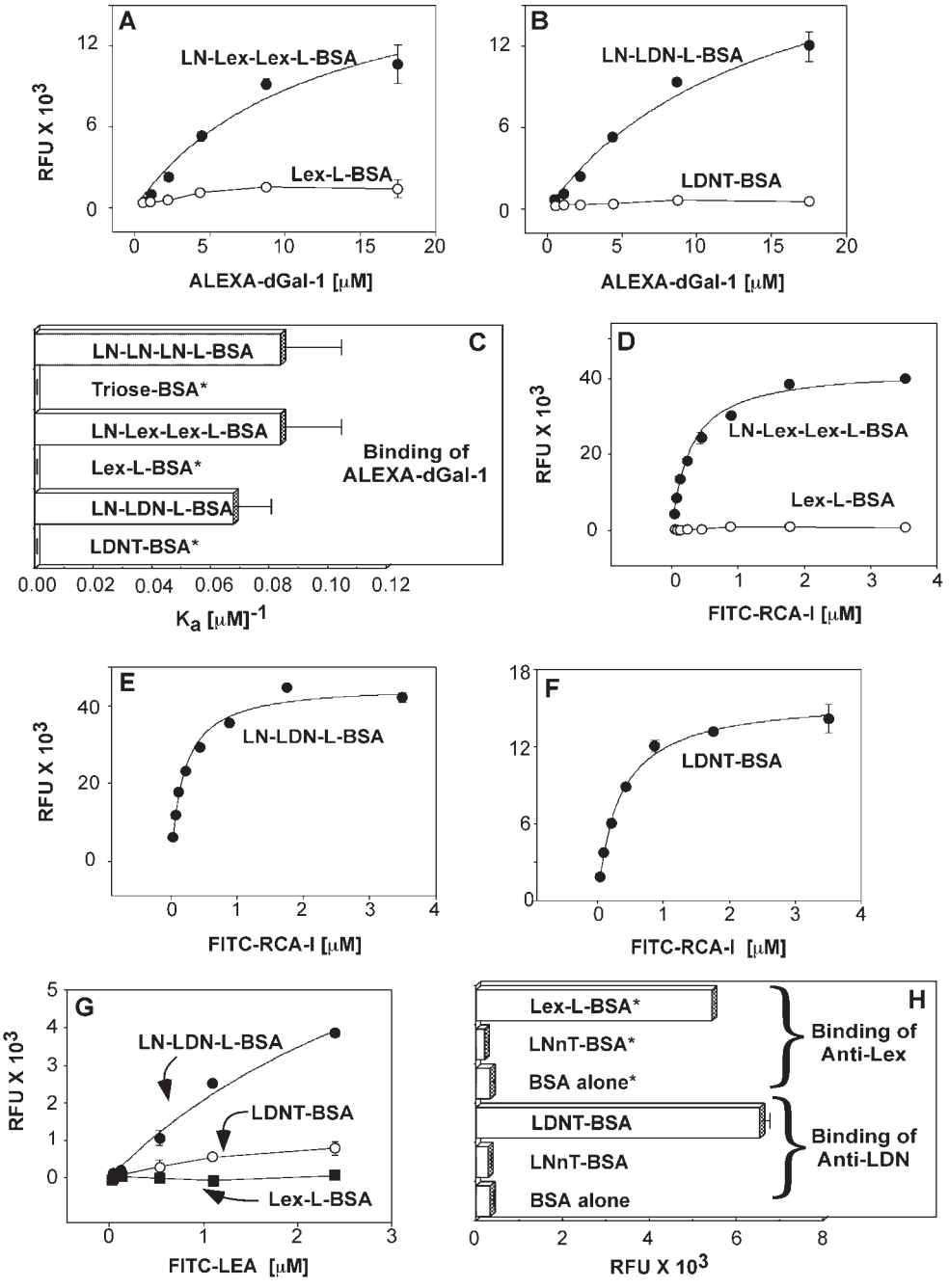


Fig. 4. Binding of ALEXA-Gal-1, FITC-RCA-I, and FITC-LEA to neoglycoproteins. Solid-phase fluorometric binding assays were accomplished as outlined in the *Materials and methods*. (**A**) ALEXA-Gal-1 binding to LN-Lex-Lex-L-BSA and Lex-L-BSA. (**B**) Binding of ALEXA-Gal-1 binding to LN-LDN-L-BSA and LDNT-BSA. (**C**) Apparent association constants (K_a in μM^{-1}) ($K_a = 1/K_d$) for binding of ALEXA-Gal-1 to indicated neoglycoproteins. (**D**) Binding of FITC-RCA-I to LN-Lex-Lex-L-BSA and Lex-L-BSA. (**E**) Binding of FITC-RCA to LN-LDN-L-BSA. (**F**) Binding of FITC-RCA-I to LDNT-BSA. (**G**) Binding of FITC-LEA to indicated neoglycoproteins. (**H**) Binding of monoclonal anti-Lex and anti-LDN to indicated neoglycoproteins. Binding of the primary antibody was detected by fluorescently labeled goat-anti-mouse and binding was quantified as relative fluorescence units (RFUs). Secondary antibody alone in the absence of primary antibody showed no difference in binding from that observed toward unconjugated BSA (data not shown). All assays were performed in triplicate and the average values are shown.

1999). These monoclonal antibodies were able to bind to the appropriate neoglycoprotein conjugate, as shown in Figure 4H, indicating that LDN and Lewis x–containing glyans are available and recognizable by the appropriate reagents. These results demonstrate that neither Lex-L-BSA nor LDNT-BSA is recognized by Gal-1, although both glycan conjugates can be recognized by appropriate monoclonal antibodies to each determinant.

Gal-1 does not independently recognize internal LN units within PL chains

To determine whether Gal-1 can recognize internal, unmodified LN units independently of the terminal LN unit, we treated LN-LN-LN-L-BSA with β-galactosidase from bovine testes to specifically remove terminal, nonreducing β4-Gal residues to generate GlcNAc-LN-LN-L-BSA. FITC-RCA-I was used to determine the degree of removal of terminal β4-Gal residues. Total FITC-RCA binding was reduced over 90% when compared to binding to the untreated LN-LN-LN-L-BSA (Figure 5A). An interesting feature of the solid-phase format we have adopted with fluorescently labeled lectins is that bound lectins can be removed by washing with hapten buffer solutions and the plates can be reused. In this case, plates are washed with phosphate buffered saline (PBS) containing 200 mM lactose to remove all detectable fluorescence from the FITC-RCA-I. (In control experiments, these plates were reused with the FITC-RCA-I and binding during the second experiment was virtually identical to that observed in the first experiment; data not shown). Thus following FITC-RCA-I binding in Figure 5A, the bound FITC-RCA was removed from those plates by incubating wells with buffer containing 200 mM lactose to remove all fluorescently labeled RCA-I. The subsequent binding of these plates to ALEXA-Gal-1 is shown in Figure 5B. Interestingly, Gal-1 was reduced to approximately the same extent demonstrated by FITC-RCA. Residual Gal-1 and RCA binding to GlcNAc-LN-LN-L-BSA was most likely due to the incomplete removal of galactose from some terminal LN units following β-galactosidase treatment. To further control for the integrity of the remaining glycan subsequent to β-galactosidase treatment, FITC-LEA binding affinity was determined subsequent to Gal-1 removal by treatment of plates with 200 mM lactose. FITC-LEA demonstrated similar binding to both LN-LN-LN-L-BSA and GlcNAc-LN-LN-LN-L-BSA (data not shown). These results demonstrate that Gal-1 recognizes terminal non reducing unmodified LN units and will not independently bind LN units located within an LN-containing glycan.

Discussion

Our study shows that human Gal-1 recognizes terminal, nonreducing β4-linked galactosyl residues in PL sequences with high-affinity binding and that the lectin recognizes long chain PL or chimeric polysaccharide sequences even when these sequences have internal substitutions. However, human Gal-1 does not recognize certain terminal sequences, such as those containing terminal LN with α3-linked fucose in the Lex antigen structure or a terminal β4-linked GalNAc residue. In addition, our results indicate that Gal-1 does not independently recognize LN sequences located within the glycan sequence in the absence of an intact, terminal, nonreducing LN unit. These observations extend those of others on the recognition determinants for human Gal-1 and allow predictions to be made concerning the types of biological ligands likely to be recognized by the lectin.

Although there have been many studies on bovine galectin-1, there have only been a few studies to date on the carbohydrate binding specificity of human Gal-1 (Hirabayashi *et al.*, 2002; Kopitz *et al.*, 1998; Lee *et al.*, 1990) and the results have been somewhat confusing. For example, an early study concluded that human Gal-1 (termed galaptin from human spleens) binds branched glycans containing multiple, terminal β4-galactosyl residues in LN sequences and that its binding affinity was not particularly enhanced by clustering the number of terminal β4-galactosyl residues (Lee *et al.*, 1990). However, a recent study found some increased binding affinity to N-glycans with increased branching and numbers of terminal LN units (Hirabayashi *et al.*, 2002). Another study identified the ganglioside GM1 as a human Gal-1 ligand (Kopitz *et al.*, 1998), but a more recent study found no binding of the lectin to a free glycan containing the structure found in GM1 (Hirabayashi *et al.*, 2002). One recent study identified chondroitin sulfate B, which lacks any LN motif, as a potential human Gal-1 ligand (Moiseeva *et al.*, 2003). Importantly, none of these studies used the types of PL or chimeric polysaccharide sequences used herein or addressed the issue of internal modifications of PL sequences in recognition by human Gal-1.

Some of the conflicting information to date about human Gal-1 binding specificity may arise due to different methods

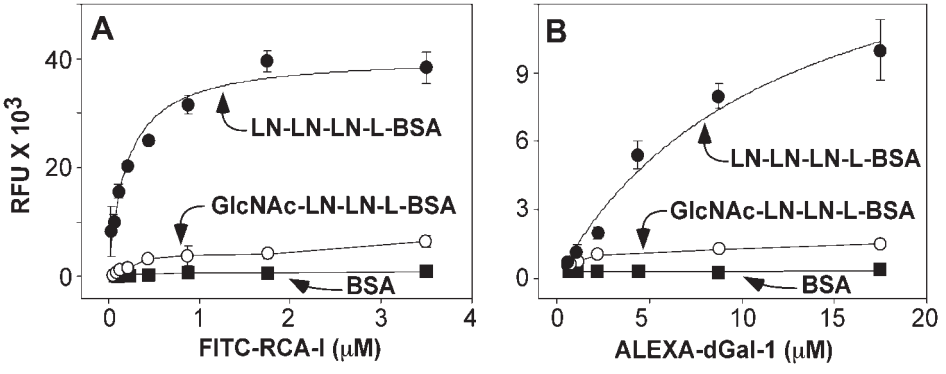


Fig. 5. Lack of binding of ALEXA-Gal-1 and FITC-RCA-I to GlcNAc-LN-LN-L-BSA. (**A**) Binding of FITC-RCA-I to LN-LN-LN-L-BSA and to GlcNAc-LN-LN-L-BSA generated by treatment of the former neoglycoprotein with β-galactosidase and to unconjugated BSA. (**B**) Binding of ALEXA-Gal-1 to indicated neoglycoproteins and unconjugated BSA as in (**A**). All assays were performed in triplicate and the average values are shown.

used for the analysis. For example, frontal affinity chromatography approaches of Hirabayashi *et al.* (2002) requires immobilized human Gal-1 coupled to an affinity support and the use of fluorescently labeled free glycans. This is an equilibrium method, but uses free glycans, rather than glycoconjugates, and also allows for the possibility that cross-linking of the free glycans to immobilized lectin could occur, thereby enhancing the apparent affinity of multi-branched glycans. The approach of Kopitz *et al.* (1998) relied on binding of GM1-coated beads to cells and deduced that Gal-1 was involved in the recognition. Finally, the study of Lee *et al.* (1990) used a variety of synthetic clustered glycosides and glycopeptides in addition to neoglycoproteins containing glycans linked covalently to BSA, with measurements involving inhibition of binding (I_{50} values). We decided to take the approach of using glycoconjugates with glycans covalently linked to a carrier protein to allow direct measurements of their binding to fluorescently labeled Gal-1.

Taken together, our results are generally consistent with those of others (Hirabayashi *et al.*, 2002; Lee *et al.*, 1990) in that terminal LN sequences were found to be recognized by human Gal-1. However, our studies also demonstrate that Gal-1 binds long-chain PL sequences and that internal modifications of these sequences, as seen in the chimeric polysaccharide structures, do not appreciably diminish binding of the lectin. Importantly, Gal-1 did not bind PL sequences terminating in either a β3-linked GlcNAc residue or a β4-linked GalNAc residue, indicating a requirement for a terminal, nonreducing β4-Gal residue. Though it is not clear at present how Gal-1 recognizes long PL sequences, there are several possibilities. The long chains could protrude above the protein matrix making the terminal nonreducing LN unit more accessible to the galectin. Alternatively, the long-chain PL could adopt conformation that enhances recognition by the lectin. Finally, long-chain PL could promote oligomerization of the protein, as suggested by other studies on galectin binding to multivalent ligands (Brewer, 2002).

The inability of human Gal-1 to bind long-chain PL sequences terminating in GlcNAc residues suggests that it is different from bovine Gal-1. Earlier results indicated that the bovine Gal-1 recognizes PL-containing N-glycans (Merkle and Cummings, 1988) even when they contained a terminal, nonreducing GlcNAc residue (Zhou and Cummings, 1993). Other studies on bovine Gal-1 also indicated that it recognizes terminal LN sequences (Abbott *et al.*, 1988; Ahmad *et al.*, 2002; Di Virgilio *et al.*, 1999) as well as weakly binding internal Gal residues within PL chains (Di Virgilio *et al.*, 1999). A recent study on bovine galectin-1 shows that the presence of a terminal, nonreducing α3-linked galactosyl residue in the sequence Galα3Galβ4GlcNAc-R actually enhanced recognition by that lectin (Appukuttan, 2002). Thus the results indicate that both human and bovine galectin-1 bind long-chain PL sequences, although the mode and fine structural determinants required for recognition may differ. Both mature human and bovine galectin-1 contain 134 amino acids but are different at 17 residues for 87% identity, and several differences are nonconservative (Abbott *et al.*, 1989; Hirabayashi *et al.*, 1989b). Whether these differences contribute to differential binding affinity are not known, because crystal structures are only reported for bovine galectin-1 (Bourne *et al.*, 1994a), but not human Gal-1, and cocrystals of bovine galectin-1 have not been done with long-chain PL glycans.

The preference of human Gal-1 for the terminal LN motif is interesting. The terminal sequences of O-glycans and many complex-type N-glycans in most mammalian glycoproteins contain the terminal, nonreducing LN motif, but it is often further modified by sialylation, fucosylation, galactosylation, sulfation, and other types of modifications (Kornfeld and Kornfeld, 1985; Spiro, 2002). Such modifications of the LN unit may be important in regulating the affinity and thereby the degree of biological activity of Gal-1 toward these LN-containing ligands. For example, it has been reported that both human and bovine galectin-1 bind weakly to LN sequences modified by α3-linked sialic acid, and that modification by α6-linked sialic acid blocks binding (Abbott *et al.*, 1988; Ahmad *et al.*, 2002; Hirabayashi *et al.*, 2002; Solomon *et al.*, 1991; Wilson *et al.*, 1989). Similarly, the effects of human Gal-1 on human T cells is blocked by α6- but not α3-sialylation (Amano *et al.*, 2003). We recently found that activation of human neutrophils enhances their binding to human Gal-1, resulting in a signaling event leading to exposure of phosphatidylserine on the cell surface and subsequent phagocytic recognition of the cells by macrophages (Dias-Baruffi *et al.*, forthcoming). We also found that enzymatic desialylation of resting neutrophils did not enhance their sensitivity to human Gal-1 effects, although it did significantly enhance binding of the lectin (Dias-Baruffi *et al.*, forthcoming). In the case of cell lines, such as human HL-60 and MOLT-4 cells, enzymatic desialylation enhanced both their binding and sensitivity to human Gal-1 (Dias-Baruffi *et al.*, forthcoming). Such results may relate to other studies indicating that activation of neutrophils is accompanied by mobilization of an endogenous sialidase to the plasma membrane (Cross *et al.*, 2003; Cross and Wright, 1991).

The ability of Gal-1 to recognize long-chain PL or chimeric polysaccharide sequences may be related to its biological activity. Although there are no direct experimental results indicating that cell surface PL sequences are required for human Gal-1 binding or activity, there is indirect evidence implicating cell surface PL chains in human Gal-1 (Galvan *et al.*, 2000; Rabinovich *et al.*, 2002) and murine galectin-1 activity (Dennis *et al.*, 2002). PL sequences are known to occur on basement membrane proteins, such as laminin, and selected cell surface glycoproteins (Do *et al.*, 1990; Ohannesian *et al.*, 1994; Pace *et al.*, 1999; Perillo *et al.*, 1995; Zhou and Cummings, 1993). The nature and length of long-chain PL extending above the glycocalyx may promote recognition by Gal-1 and increase the ability of Gal-1 to bind and cross-link biological ligands. Recent studies using a mutated form of human Gal-1 to generate a monomeric protein demonstrated that dimerization is required for biological activity toward activated human neutrophils (Dias-Baruffi *et al.*, forthcoming). The presence of other modifications of the PL sequences, such as fucosylation or internal LDN motifs, may not interfere with galectin binding and allow the chains to be multifunctional in that they can also serve as ligands to other carbohydrate-binding

proteins. Future detailed biochemical and structural studies on cell surface macromolecular ligands for human Gal-1 will be required to identify the predicted role of PL chains and/or chimeric polysaccharides in recognition and function of this interesting lectin.

Materials and methods

Enzymatic synthesis of glycans

LNnT was degalactosylated by treatment with β-galactosidase to generate the trisaccharide, GlcNAcβ3Galβ1-4Glc (triose). LDNT was synthesized from triose by addition of a terminal β4 linked GalNAc, as previously described (Nyame *et al*., 1999) with some modifications, using bovine milk β4galactosyltransferase and UDP-GalNAc as the donor. The reaction mixture consisted of 5 μmol triose, 20 μmol UDP-GalNAc, and 5 U β4galactosyltransferase in 100 μl 50 mM sodium cacodylate buffer, pH 7.4, containing 20 mM $MnCl_2$, 0.02% NaN_3, and 10 mg/ml α-lactalbumin. The reaction was carried out at 37°C and aliquots were analyzed daily by high pH anion exchange chromatography-pulsed amperometric detection (HPAE-PAD) chromatography to monitor conversion of the triose to the tetrasaccharide LDNT. The reaction was stopped after 3 days when it had proceeded to ~95% completion. The LDNT product was purified from the reaction mixture by chromatography on a Bio Gel P-2 column (1.5 cm × 160 cm) in H_2O, and 3-ml fractions were collected. The chromatographic profile was monitored by measuring the absorbance of the fractions at 214 nm. Fractions containing the tetrasaccharide product were pooled, and the yield was determined by absorbance at 214 nm using GlcNAc as standard. The reaction yielded 4.8 μmol LDNT. Aliquots of the tetrasaccharide product were hydrolyzed by strong acid and analyzed by HPAE-PAD to confirm its monosaccharide composition.

The pentasaccharide Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (Lex-L) was synthesized from LNnT using GDP-Fuc as donor and recombinant human α3-fucosyltransferase VI (Calbiochem, San Diego, CA). The reaction was carried out in eight aliquots at 37°C in a total reaction volume 200 μl/aliquot. Each reaction mixture contained 740 nmol LNnT, 1 μmol GDP-Fuc, and 5 mU enzyme in 50 mM sodium cacodylate, pH 7.5, containing 20 mM $MnCl_2$, 0.02% NaN_3, and 0.5 U alkaline phosphatase. (Aliquots of the reaction mixture were analyzed daily, as described, to monitor conversion of LNnT to Lex-L.) The reaction was stopped after 72 h, and a 2-nmol sample of the reaction mixture was analyzed by HPAE-PAD chromatography, which showed that all acceptor was converted to product. The product was purified by chromatography on a Bio Gel P-2 column, as described. The total yield of Lex-L, as determined by absorbance at 214 nm, was 5.4 μmol per reaction. The remaining saccharides, LN-LN-LN-L, LN-Lex-Lex-L, LN-LDN-L, and LN-LN-L were prepared as described (Salo *et al*., 2002).

Neoglycoproteins

The oligosaccharides LN-LN-LN-L, LN-LN-L, LN-Lex-Lex-L, LN-LDN-L, LNnT, LDNT, Lex-L, Triose, and lactose were derivatized to BSA by reductive amination (Gray, 1974). Briefly, 1.0 mg of each oligosaccharide was mixed with 670 μg BSA and 520 μg $NaBH_3CN$ in a total volume of 50 μl 0.2 M KH_2PO_4 buffer, pH 7.0. The mixture was incubated at room temperature in the dark for 14 days and the reaction was stopped by addition of water (2 ml). The mixture was dialyzed against water and the protein content was determined by BCA assay (Pierce, Rockford, IL). The mol of sugar derivatized per mol of BSA was determined by MALDI-TOF. The molecular weights and numbers of glycans conjugated in each of the conjugates are described in *Results* and in Figure 1.

Preparation of human Gal-1

Human galectin was polymerase chain reaction–cloned from a human brain cDNA library using known N-terminal and C-terminal sequence (Couraud *et al*., 1989; Hirabayashi *et al*., 1989a) (for 5′ primer; 5′TTAGGATCCGCTTGTG GT-CTGGTC3′ and for 3′ primer, 5′CGCAAGCTTTC AGTCAAAGGCCAC 3′). The recombinant form of human Gal-1 was purified as described for the hamster galectin-1 (Cho and Cummings, 1995) employing affinity chromatography on α-lactose-agarose (Sigma, St. Louis, MO). Following affinity chromatography, the column was washed with five column volumes of PBS (0.01 M Na_2HPO_4, 0.85% NaCl, pH 7.4) containing 2-mercaptoethanol (14 mM), and the bound Gal-1 was eluted with PBS containing 2-mercaptoethanol (14 mM) and lactose (0.1 M).

Preparation of ALEXA-Gal-1

Gal-1 was derivatized with ALEXA-488 as outlined in the protocol accompanying the kit (Molecular Probes protein labeling kit #A-10235, Eugene, OR) with a few minor modifications. Briefly, a PD-10 column (Amersham Pharmacia Biotech, Little Chalfont, UK) equilibrated in PBS was used to desalt the Gal-1 prior to mixing with the ALEXA-488 reactive dye. An aliquot of 400 μl desalted Gal-1 (3–5 mg/ml) was mixed with 50 μl 1 M lactose and 50 μl 1 M NaH_2CO_3 to yield a 500 μl 3–5 mg/ml Gal-1, 100 mM lactose, 100 mM NaH_2CO_3 solution. (Lactose was included in the labeling to block possible ALEXA-488 attachment at or near the carbohydrate recognition domain.) This mixture was then mixed with the ALEXA-488 carboxylic acid, succinimidyl ester, dilithium salt reactive dye (Molecular Probes) at pH 7.4 and allowed to incubate on a microstir plate for 2 h. This mixture was then applied to a Sephadex G25 column equilibrated in PBS containing 0.2 mM NaN_3. Protein quantification was performed as outlined in the protocol accompanying the kit. To confirm that the ALEXA-Gal-1 retained carbohydrate-binding activity, the protein was rechromatographed on α-lactose-agarose. The elution profile of the repurified Gal-1 revealed a single peak of protein that was quantitatively eluted with lactose (data not shown). FITC-LEA and FITC-RCA were purchased from Vector Laboratories (Burlingame, CA).

Monoclonal antibody detection of LDNT-BSA and Lex-L-BSA

Microtiter wells were coated with 50 μl of 0.3 mg/ml neoglycoprotein solution in a PBS buffer followed by a

5% BSA blocking solution. An IgM monoclonal antibody (SMLDN1.1) generated from the spleens of *S. mansoni*–infected mice (Nyame *et al*., 1999) was employed for detection of terminal LDN containing glycans. An IgG monoclonal antibody (F8A1.1.2), previously prepared (Nyame *et al*., 1997, 1998), was used to detect Lex-containing glycans. Bound antibodies were detected by incubation with 50 µl of either 10–20 µg/ml ALEXA-488 rabbit anti-mouse IgG or ALEXA-488 goat anti-mouse IgM for 1 h followed by PBS-Tween 20 (0.5% Tween 20) wash 5× and fluorescent detection using a Perkin Elmer Wallac Victor^2 1420 Multilabel counter. Antibodies were diluted in a PBS, 0.05% Tween 20, 1% BSA solution.

Lectin binding assays

A 50-µl aliquot of 0.3 mg/ml neoglycoprotein solution in a PBS buffer coated each well followed by a 5% BSA in PBS blocking solution. Triplicate analyses of each coating density were performed. Lectin dilutions were carried out in PBS-Tween 20 (0.5% Tween 20 in PBS) containing 1% BSA. A 50-µl volume of each lectin concentration was then allowed to incubate for 1 h, followed by PBS-Tween 20 (0.5% Tween 20) wash 5× and fluorescent detection using the Perkin Elmer counter. Following RCA and Gal-1 fluorescent detection, each lectin was removed by incubating with a 200 mM lactose solution in PBS for 2 h. This allowed for each of the lectin binding determinations to be accomplished repeatedly on the same wells containing immobilized neoglycoproteins. In multiple experiments we tested the reproducibility of the coating density for various neoglycoproteins, as assessed by binding of fluorescently labeled lectins used in this study. Our results indicated that there was less than 10% variation between experiments in coating densities for different neoglycoproteins.

β-*Galactosidase treatment of LN-LN-LN-L-BSA*

Generation of GlcNAc-LN-LN-L-BSA was accomplished by removal of the terminal nonreducing galactose residue from LN-LN-LN-L-BSA by incubating the immobilized conjugate in wells in 60 µl 1 U/ml bovine testes β-galactosidase (Glyko, Novato, CA) for 12 h at 37°C. Enzyme incubation was followed by PBS wash 5× and lectin binding detection.

Data analysis and curve fitting

Dissociation constants for each lectin with neoglycoprotein were calculated using a rectangular hyperbola equation to derive the nonlinear curve fitting (Sigma Plot software). The molecular weights utilized for each lectin in these analyses were the following: Gal-1, 29.2 kDa; LEA, 100 kDa; RCA-I, 120 kDa. Standard deviations were derived using the same software. In all cases the probability value (p-value) was calculated ($p > 0.05$ to $p < 0.001$) and is shown in selected figure legends.

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Abbreviations

BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; Gal-1, dimeric human galectin-1; HPAE-PAD, high pH anion exchange chromatography–pulsed amperometric detection; LEA, *Lycopersicon esculentum* agglutinin; LN, N-acetyllactosamine; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PBS, phosphate buffered saline; PL, poly-N-acetyllactosamine; RCA-I, *Ricinus communis* agglutinin I.

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