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Dimeric Galectin-1 Binds with High Affinity to α2,3-Sialylated and Non-sialylated Terminal *N*-Acetyllactosamine Units on Surface-bound Extended Glycans*

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Galectin-1 is a member of the galectin family of glycan-binding proteins and occurs as an ~29.5-kDa noncovalent homodimer (dGal-1) that is widely expressed in many tissues. Here, we report that human recombinant dGal-1 bound preferentially and with high affinity (apparent K_d ~ 2–4 μM) to immobilized extended glycans containing terminal *N*-acetyllactosamine (LN; Galβ1–4GlcNAc) sequences on poly-*N*-acetyllactosamine (PL; (–3Galβ1–4GlcNAcβ1–)$_n$) sequences, complex-type biantennary *N*-glycans, or novel chitin-derived glycans modified to contain terminal LN. Although terminal Gal residues are important for dGal-1 recognition, dGal-1 bound similarly to α3-sialylated and α2-fucosylated terminal LN, but not to α6-sialylated and α3-fucosylated terminal LN. The binding specificity of human recombinant dGal-1 was similar to that observed with purified bovine heart-derived dGal-1. Unexpectedly, dGal-1 bound free ligands in solution with relatively low affinity and displayed no preference for extended glycans, indicating that dGal-1 preferentially recognizes extended glycans only when they are surface-bound, such as found on cell surfaces. Human dGal-1 also bound to both native and desialylated human promyelocytic HL-60 cells with similar affinity as observed for immobilized long chain PL. Binding to these cells was reduced upon treatment with endo-β-galactosidase, which cleaves PL sequences, indicating that cell-surface PLs are ligands. To test the role of dimerization in dGal-1 binding, we examined the binding of a mutated form of dGal-1 that weakly dimerizes (monomeric Gal-1 (mGal-1)) and a covalently dimerized (chemically cross-linked) form of mGal-1 (cd-mGal-1). dGal-1 and cd-mGal-1 had similar affinities that were both ~3.5-fold higher for immobilized PL than observed for mGal-1, suggesting that dGal-1 acts as a dimer to cross-link terminal LN units on immobilized PL. These results indicate that dGal-1 functions as a dimer to recognize LN units on extended PLs on cell surfaces.

Galectins are a family of soluble β-galactoside-binding lectins, earlier classified as S-type lectins because of their solubility, requirement for reducing thiols for activity, and lack of requirement for Ca^{2+} for ligand binding (1–5). Galectin-1 is a homodimer (dGal-1)[1] consisting of two ~14.5-kDa subunits that are noncovalently associated in a monomer-dimer equilibrium (6). dGal-1 is widely expressed in many vertebrate and invertebrate organisms in various tissues and occurs in the cytosol and is secreted/exported to the extracellular space (7–9). Upon secretion/export, dGal-1 appears to require binding to extracellular ligands to maintain or to acquire activity and stability (8, 10). The biological functions of dGal-1 are probably complex and are not well understood (11–15). It has been proposed that dGal-1 is involved in cell adhesion (10, 16–18), B cell differentiation (19), development (20), inflammation (21), mRNA splicing (22), leukocyte apoptosis (23, 24), neutrophil turnover (25), and cancer (26–28). Recent studies show that the gene encoding human galectin-2, a close relative of galectin-1, is mutated in patients with increased susceptibility to myocardial infarction, implicating galectin functions in vascular inflammation and atherosclerosis (29).

Like many other galectin family members, dGal-1 weakly recognizes lactose (Galβ1–4Glc) and *N*-acetyllactosamine disaccharides such as the type 2 sequence Galβ1–4GlcNAcβ-R (LN) and the type 1 sequence Galβ1–3GlcNAcβ-R (30). However, dGal-1 has been reported to bind with higher affinity to specific glycoproteins containing repeating linear type 2 sequences ((–3Galβ1–4GlcNAcβ1–)$_n$) called poly-*N*-acetyllactosamines (PLs). Such glycoproteins that bind dGal-1 include laminin (31–33), fibronectin (34), and lysosome-associated membrane proteins (35, 36). Other glycoprotein ligands for dGal-1 have also been identified, and they may also contain PL sequences (36–39). dGal-1 also binds to glycopeptides containing PL sequences (40, 41), glycolipids (42), and neoglycoproteins (43). In contrast to these studies, recent data have suggested that dGal-1 may not recognize PL structures with higher affinity compared with its recognition of short non-

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[1] The abbreviations used are: dGal-1, dimeric galectin-1; mGal-1, monomeric galectin-1; cd-mGal-1, cross-linked dimer of monomeric galectin-1; LN, *N*-acetyllactosamine (Galβ1–4GlcNAc); PL, poly-*N*-acetyllactosamine ((–3Galβ1–4GlcNAcβ1–)$_n$); LNnT, lacto-*N*-neotetraose; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; CB, *N*,*N*′-di-*N*-acetylchitobiose; CT, *N*,*N*′,*N*″,*N*‴-tetra-*N*-acetylchitotetraose; GP, glycopeptide; GS-II, *G. simplicifolia* lectin II; RCA-I, *R. communis* agglutinin II; LEA, *L. esculentum* agglutinin; LNnH, lacto-*N*-neohexaose; pLNnH, *para*-lacto-*N*-neohexaose; GCT, galactosylated CT.

extended structures (44, 45). Interestingly, dGal-1 has been suggested to bind primarily to nonreducing terminal LN residues rather than to mid-chain LN residues within a PL chain (42, 43, 46).

Uncertainty also exists about the affinity of dGal-1 binding to complex-type *N*-glycans containing non-extended terminal LN units. Although earlier data suggested that dGal-1 binds weakly to non-extended *N*-glycans (41), more recent studies have suggested that dGal-1 binds to such *N*-glycans with relatively high affinity (42, 45). For example, dGal-1 was reported to bind to *N*-glycans containing non-extended terminal LN units with ~10-fold higher affinity than to long chain linear PL structures in frontal affinity chromatographic analyses (45). The reasons behind these apparently conflicting results are not known, but could result from different methods of analyses and/or different ways to immobilize lectins in chromatographic studies.

Sialylation and fucosylation of glycoconjugate ligands affect their binding to dGal-1. For example, dGal-1 from different sources has been shown to bind α2,3-sialylated (but not α2,6-sialylated) glycans (30, 40, 42, 44, 45, 47). Most of these studies were based on indirect sugar inhibition assays with simple non-extended ligands such as sialylated LN and lactose. More direct binding measurements have been carried out recently with human and bovine dGal-1. Isothermal titration microcalorimetry was used to show that bovine dGal-1 has the same affinity for LN and α2,3-sialyl-LN (44). Bovine dGal-1 has also been shown to bind to immobilized neoglycolipids containing α2,3-sialylated and non-sialylated lacto-*N*-tetraose (Galβ1–3GlcNAcβ1–3Galβ1–4Glc) by TLC overlay and microwell binding assays (42). Human dGal-1 shows significantly lower affinity for α2,6-sialylated complex-type *N*-glycans than for non-sialylated *N*-glycans by frontal affinity chromatography (45). However, the ability of human or bovine dGal-1 to bind to α2,3-sialylated and α2,6-sialylated PL structures has not been compared simultaneously under the same conditions. It has also been reported that α1,2-fucosylated (but not α1,3/4-fucosylated) structures are recognized by dGal-1 (42, 43, 47).

To address many of these questions about dGal-1 specificity and the possible role of its dimerization in binding, we synthesized a panel of biotinylated oligosaccharides and glycopeptides to study the ligand binding specificity of human and bovine dGal-1 and a mutated form of human dGal-1 that behaves as a monomer (mGal-1) rather than a dimer upon gel filtration (25). The glycan structures allowed us to address the role of the terminal sugar residue, PL backbone and chain length, and glycan core structure (*N*-glycan and *O*-glycan) in dGal-1 recognition. We also examined the binding of dGal-1 to human promyelocytic HL-60 cells because dGal-1 (but not mGal-1) binding to these cells has been shown to cause cell-surface expression of phosphatidylserine- and annexin V-binding sites (25). Overall, our results show that dGal-1 recognizes extended structures containing α2,3-sialylated and non-sialylated terminal LN units with high affinity when ligands are immobilized, but not when ligands are free in solution. Our results also demonstrate that lectin dimerization is required for high affinity binding to immobilized glycan ligands.

EXPERIMENTAL PROCEDURES

Synthesis and Biotinylation of Oligosaccharides—The 2-azidoethyl glycoside precursors of compounds LN, lacto-*N*-neotetraose (LNnT), and NGLNnT (see Fig. 1 for explanations of compound abbreviations) were synthesized as described previously (48). The 2-azidoethyl glycoside precursors of compounds LN2, LN3, S3LN2, S6LN2, F2LN2, and (Le^x)3 will be described elsewhere.[2]

Each of the 2-azidoethyl glycoside derivatives LN, LN2, LN3, NGLNnT, LNnT, S3LN2, S6LN2, F2LN2, and (Le^x)3 (50 μmol) was dissolved in methanol/water (1:9 by volume, 0.5–2 ml) and hydrogenolyzed over palladium/carbon (10%, 5–10 mg) in an H_2 atmosphere at room temperature. Sodium hydrogen carbonate (final concentration of 100 mM, pH 8–8.5) and EZ-Link™ Sulfo-NHS-LC-LC-Biotin (sulfosuccinimidyl 6′-(biotinamido)-6-hexanamidohexanoate) or EZ-Link™ Sulfo-NHS-Biotin (*N*-hydroxysulfosuccinimidobiotin) (3 eq; Pierce) were added to the mixture. When the reaction showed completion (analyzed by silica gel TLC, eluent of ethyl acetate/methanol/acetic acid/water, 4:3:3:2 by volume), the crude mixture was filtered and purified by gel filtration chromatography on a Sephadex G-15 column (1 × 70 cm, equilibrated in 5% *n*-butyl alcohol). Appropriate fractions were collected and lyophilized to dryness. The residue was further purified on a reversed-phase chromatography column (silica C_{18}, 0.5 × 20 cm) equilibrated in 5% MeOH. Fractions containing the product was collected and lyophilized to give the biotinylated compound. Typical yields were in the range of 50–70%, and purity was estimated to >95% by ^{1}H NMR spectroscopy (data not shown).

LN3-biotin and LNnT-biotin (7 nmol each) were sialylated at 37 °C for ~66 h using a molar excess of CMP-NeuAc and 10–13 milliunits of α2,3-*N*-sialyltransferase (Calbiochem) in a total reaction volume of 280 μl of 50 mM MOPS (pH 7.5) containing 0.1% Triton CF-54 and 0.1% bovine serum albumin (BSA). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of the reaction mixtures confirmed that sialylation of LN3-biotin and LNnT-biotin was successful. The degree of sialylation was ~60–70% for LN3-biotin and >80% for LNnT-biotin as estimated by reversed-phase HPLC. Sialylated samples (S3LN3 and S3LNnT) were then treated with β-galactosidase as described below to remove non-sialylated terminal galactose residues. The reaction mixtures were diluted with phosphate-buffered saline (PBS) and used directly to immobilize the biotinylated oligosaccharides on streptavidin plates.

N,*N*′-di-*N*-acetylchitobiose (CB) and *N*,*N*′,*N*″,*N*‴-tetra-*N*-acetylchitotetraose (CT) (Dextra Laboratories, Ltd.) were biotinylated using EZ-Link™ Biotin-LC-hydrazide (biotinamido caproyl hydrazide) (Pierce) as will be described elsewhere.[3] CB-LC-biotin and CT-LC-biotin (100 nmol each) were galactosylated using UDP-Gal (200 nmol) and 50 milliunits of bovine milk β1,4-galactosyltransferase (Sigma) in a total volume of 300 μl of 50 mM sodium cacodylate (pH 7.4) containing 20 mM $MnCl_2$. The completeness of the reaction and formation of galactosylated CB (GCB) and galactosylated CT (GCT) were confirmed after 14 h of incubation at 37 °C by reversed-phase HPLC and MALDI-TOF mass spectrometry of an aliquot of the reaction mixture. The reaction mixture was diluted and used directly in a fluorescence-based solid-phase assay.

Synthesis and Biotinylation of Glycopeptides—The glycopeptide precursor GP-1, modeled after amino acids 45–62 of human PSGL-1 (P-selectin glycoprotein ligand-1) with an α-linked GalNAc residue at Thr^{57}, was synthesized on an automated peptide synthesizer as described (49). The core 2-based *O*-glycans of GP-4, GP-4′, GP-4″, and GP-4‴ were synthesized enzymatically as described (50, 51). The purified glycopeptides were biotinylated through their N terminus using EZ-Link™ NHS-LC-LC-Biotin (succinimidyl 6′-(biotinamido)-6-hexanamidohexanoate; Pierce). The biotinylated glycopeptides were purified by reversed-phase HPLC, and the concentration of each peptide solution was determined by UV absorbance at 215 nm upon HPLC.

The NA2 glycopeptide was prepared from bovine fibrinogen (Sigma). Fibrinogen was digested with Pronase, and the glycopeptide fraction was isolated by concanavalin A-Sepharose affinity chromatography. Sialic acids were removed by treating the glycopeptide with mild acid. The desialylated glycopeptide fraction was biotinylated using EZ-Link™ NHS-LC-LC-Biotin, and free biotin was removed on a concanavalin A affinity column. The amount of the glycopeptide was quantified using the phenol/sulfuric acid assay (52). The biotinylated glycopeptide fraction was analyzed by MALDI-TOF mass spectrometry and found to contain a mixture of glycopeptides with a biantennary *N*-glycan (NA2) attached to a single asparagine or to an asparagine residue of a dipeptide (NK) or tripeptides (GEN, ENR).

Biotinylation and Fixation of HL-60 Cells—Cell-surface proteins of HL-60 cells were biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotin (sulfosuccinimidyl 6-(biotinamido)hexanoate; Pierce) according to the manufacturer's instructions. After biotinylation cells, were fixed with 2% paraformaldehyde for 30 min at room temperature and washed with PBS.

[2] O. Blixt, manuscript in preparation.

[3] A. Leppänen and R. D. Cummings, manuscript in preparation.

Preparation and Characterization of dGal-1 and mGal-1 and Chemical Cross-linking—Recombinant forms of human wild-type dGal-1 and a mutated form behaving as a monomer upon gel filtration (mGal-1) were expressed in *Escherichia coli*, and the recombinant proteins were purified as described previously (25). mGal-1 (1.3 mg/ml, total of 2 mg) was chemically cross-linked for 30 min at room temperature using a 50-fold molar excess of disuccinimidyl suberate (Pierce) in PBS containing 0.1 M lactose. The reaction was quenched by addition of 1 M Tris-HCl (pH 7.5) to a final concentration of 50 mM. Disuccinimidyl suberate and Tris were removed using a PD-10 gel filtration column (Amersham Biosciences). A cross-linked dimer of mGal-1 (cd-mGal-1) was fluorescently labeled using Alexa Fluor 488 C_5-maleimide (Molecular Probes, Inc., Eugene, OR) as described below. After removal of lactose, fluorescently labeled cd-mGal-1 was separated from mGal-1 using an α-lactosyl-agarose column (total of 2 ml; Sigma). After sample loading, the column was washed with 10 ml of PBS and eluted with 20 ml of 1 mM lactose in PBS and 10 ml of 0.1 M lactose in PBS. Eluted fractions were analyzed by reducing SDS-PAGE, and protein bands were detected under UV light (data not shown). 1 mM lactose eluted ~90% of the mGal-1 and a small amount of the cd-mGal-1, and 0.1 M lactose eluted most of the cd-mGal-1 (purity > 90%) (see Fig. 8*B*, *inset*).

Purification and Characterization of Bovine Heart dGal-1—Bovine heart dGal-1 was purified essentially as described (53, 54) with some modifications. Briefly, frozen bovine heart was trimmed of fat, cut to small pieces, and minced for 15 s in an Osterizer blender using an S blade (low speed, shred; Sunbeam Products, Inc., Boca Raton, FL). The shredded heart was mixed with 4 liters of cold buffer A (PBS containing 14 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine) and homogenized in several batches (2 min/load) at 4 °C using a blender blade (high speed, liquefy). The homogenate was centrifuged at 5000 × *g* for 45 min. The pellet was washed five times with ~4 volumes of buffer A, suspended in 1.9 liters of 0.1 M lactose in buffer A, and incubated for ~15 h at 4 °C. The extract was centrifuged at 27,000 × *g* for 45 min, and the supernatant (~2 liters) was dialyzed extensively against cold PBS containing 14 mM β-mercaptoethanol to remove lactose. Bovine dGal-1 was affinity-purified by mixing the supernatant with α-lactosyl-agarose beads (5 ml) overnight at 4 °C in several batches. The α-lactosyl-agarose beads containing bound bovine dGal-1 were isolated by centrifugation and washed in a column with PBS until the UV absorbance at 280 nm was <0.1. Bound dGal-1 was eluted using 0.1 M lactose in PBS. Eluted fractions contained bovine dGal-1 as a major component and several large molecular mass proteins as minor contaminants as determined by reducing SDS-PAGE (data not shown). Eluted fractions were pooled and dialyzed against PBS containing 14 mM β-mercaptoethanol. A precipitate was formed during dialysis. It was centrifuged and dissolved in PBS containing 20 mM lactose and 14 mM β-mercaptoethanol (6 ml). Large molecular mass contaminants were separated from bovine dGal-1 in four batches on a Sephadex G-75 column (~36 ml, 1 × 46 cm) in PBS containing 20 mM lactose and 14 mM β-mercaptoethanol. Purification of solubilized bovine dGal-1 on a gel filtration column gave pure homogeneous bovine dGal-1 (total of ~6 mg) with a molecular mass of ~15 kDa as determined by reducing SDS-PAGE of the eluted fractions (data not shown). Non-precipitated supernatant (60 ml) from the final dialysis step was re-chromatographed on an α-lactosyl-agarose column. Reducing SDS-PAGE analysis of the lactose-eluted fractions showed that bound bovine dGal-1 (~10 mg) was free of the large molecular mass contaminants that were present in the precipitated sample after dialysis. This bovine dGal-1 sample showed one very minor contaminating protein at ~33 kDa upon reducing SDS-PAGE (data not shown). This sample was not purified any further. The total yield of membrane solubilized purified bovine dGal-1 was ~16 mg (~12.5 mg/kg of heart tissue).

Fluorescently Labeled Lectins—Fluorescein-labeled *Griffonia simplicifolia* lectin II (GS-II) was from EY Laboratories, Inc. (San Mateo, CA), and *Ricinus communis* agglutinin I (RCA-I) was from Vector Laboratories (Burlingame, CA). Human recombinant mGal-1 and dGal-1 and bovine heart dGal-1 were fluorescently labeled with Alexa Fluor 488 carboxylic acid succinimidyl ester (Molecular Probes, Inc.) or Alexa Fluor 488 C_5-maleimide according to the manufacturer's instructions with minor modifications. Briefly, labeling with Alexa Fluor 488 carboxylic acid succinimidyl ester was performed by incubating 1–2 mg of dGal-1, mGal-1, or bovine dGal-1 with the reactive dye in PBS containing 0.1 M lactose for 1 h at room temperature, and incubation was continued overnight at +4 °C under stirring. Labeling with Alexa Fluor 488 C_5-maleimide was performed by incubating 1–1.5 mg of dGal-1, mGal-1, or cd-mGal-1 with a 10-fold molar excess of the reactive dye in PBS containing 0.1 M lactose overnight at 4 °C under continuous mixing. Free dye and lactose were removed from the labeled Gal-1 samples using a PD-10 gel filtration column in PBS containing 14 mM β-mercaptoethanol. Labeled Gal-1 samples were chromatographed on a small lactosyl-agarose affinity column (1–2 ml volumes) in PBS, and bound Gal-1 was eluted with 0.1 M lactose in PBS. Gal-1 samples labeled with Alexa Fluor 488 carboxylic acid succinimidyl ester were stored in 0.1 M lactose in PBS at 4 °C for extended periods of time without losing activity or, alternatively, in PBS containing 14 mM β-mercaptoethanol at 4 °C for few days. Before each experiment, lactose was removed using a PD-10 column in PBS containing 14 mM β-mercaptoethanol. Gal-1 preparations labeled with Alexa Fluor 488 C_5-maleimide (used in the experiments of Figs. 3*C*, 6*A*, and 8) were stored in PBS containing 14 mM β-mercaptoethanol at 4 °C for at least 3 months without losing activity. All Gal-1 samples used in the assays were at least 80% active as defined by the percentage of protein capable of rebinding to a lactosyl-agarose column.

Lycopersicon esculentum (tomato) agglutinin (LEA) (Vector Laboratories) was dissolved in PBS (4 mg/ml) and incubated with 100 mM sodium *m*-periodate for 30 min at room temperature in the dark. Sodium *m*-periodate was removed using a PD-10 column in PBS. Oxidized LEA was then incubated with Alexa Fluor 488 hydrazide (100 μg/mg LEA; Molecular Probes, Inc.) for 1.5–2 h at room temperature under stirring. Free dye was removed from the labeled LEA sample using a PD-10 column in PBS.

Fluorescence-based Solid-phase Assay—Streptavidin-coated black 96-well microtiter plates (Pierce) were washed three times with 200 μl of PBS and coated for 1.5 h at room temperature with 50 pmol of biotinylated glycopeptides or oligosaccharides in 50 μl of PBS. The wells were washed three times with 200 μl of PBS containing 0.05% Tween 20 and then incubated successively for 1 h at room temperature with 50 μl of Alexa Fluor 488-labeled Gal-1, fluorescein-labeled RCA-I (120 μg/ml), and Alexa Fluor 488-labeled LEA (50 μg/ml) in PBS containing 0.05% Tween 20 and 1% BSA. Bound Gal-1 and RCA-I were removed using 0.2 M lactose in PBS and 0.05% Tween 20 and by washing four times with 300 μl of PBS and 0.05% Tween 20 before incubation with the next lectin. Fluorescence was measured using a VICTOR^2 microtiter plate reader (Wallac, Turku, Finland) with an excitation wavelength at 485 nm and an emission wavelength at 535 nm. The background fluorescence reading after lactose washing was subtracted from each sample.

Biotinylated and fixed HL-60 cells were immobilized on streptavidin-coated 96-well plates (100,000 cells/well) for 1.5 h at room temperature in 50 μl of PBS. The wells were washed three times with 200 μl of PBS containing 1% BSA and then incubated for 1 h at room temperature with 50 μl of Alexa Fluor 488-labeled dGal-1 in PBS and 1% BSA. After washing the wells four times with 300 μl of PBS and 1% BSA, 100 μl of PBS was added to each well, and the fluorescence was measured. In the experiments shown in Fig. 10, incubations with Gal-1 were performed in the presence or absence of 20 mM lactose in PBS and 1% BSA. The control incubations with Alexa Fluor 488-labeled LEA and fluorescein-labeled GS-II shown in Fig. 9 were carried out using lectin dilutions of 100 μg/ml in PBS and 1% BSA.

Glycosidase Digestions—Biotinylated LN (10 nmol), LN2 (10 nmol), LN3 (7 nmol), and NA2 (10 nmol) and the free oligosaccharides lacto-*N*-neohexaose (LNnH; 10 nmol) and *para*-lacto-*N*-hexaose (10 nmol) were treated with 20 milliunits of bovine testis β-galactosidase (Glyko, Inc., Novato, CA) at 37 °C for 17–20 h in a total volume of 100 μl of 0.1 M sodium acetate (pH 4.3) or 50 mM sodium cacodylate (pH 5.0). The reactions were terminated by boiling the samples for 5 min. All β-galactosidase digestions were complete as determined by subjecting a small sample of biotinylated oligosaccharides to reversed-phase HPLC and a small sample of free oligosaccharides to a Dionex HPAEC-PAD (high pH anion exchange chromatography with pulsed amperometric detection) system.

Biotinylated and fixed HL-60 cells were treated with *Arthrobacter ureafaciens* neuraminidase (100 milliunits/ml; Roche Diagnostics, Mannheim, Germany) in PBS at 37 °C for 3 h. After treatment, cells were washed three times with PBS and counted. Neuraminidase-treated and untreated, biotinylated, and fixed HL-60 cells were digested with either *Escherichia freundii* endo-β-galactosidase (250 milliunits/ml; V-Labs, Inc., Covington, LA) or jack bean β-galactosidase (100 milliunits/ml; Glyko) in PBS (pH 5.0) containing 1 mg/ml BSA at 37 °C for 16 h. Control cells were incubated with buffer alone. Digested cells and control cells were directly immobilized on streptavidin-coated microtiter plates (see above).

Radiolabeling of Oligosaccharides and Glycopeptides—LN-biotin, LN2-biotin, LN3-biotin, NA2, LNnH, and *para*-lacto-*N*-hexaose were treated with β-galactosidase (see above) to prepare acceptors for [^{3}H]galactosyltransferase reactions. The degalactosylated oligosaccharides NGLN-biotin, NGLN2-biotin, NGLN3-biotin, NGNA2 (GlcNAcβ1–

2Manα1–3(GlcNAcβ1–2Manα1–6)Manβ1–4GlcNAcβ1–4GlcNAc), NGLNnH (GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc), NGpLNH (GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc), and NGLNnT (100–600 pmol) were incubated overnight at +37° C with a 1.6–5-fold molar excess of UDP-[^{3}H]Gal (specific activity of 4800–5500 cpm/pmol; American Radiolabeled Chemicals) and 50 or 100 milliunits of bovine milk β1,4-galactosyltransferase in a total reaction volume of 50 or 100 μl of 50 mM sodium cacodylate (pH 7.4) containing 20 mM $MnCl_2$. Biotinylated radiolabeled products were separated from UDP-[^{3}H]Gal using Sep-Pak Vac C_{18} cartridges (1 ml; Waters Corp., Millford, MA), and non-biotinylated radiolabeled products were separated from UDP-[^{3}H]Gal using Dowex 1 × 2 (Cl^-) columns (1 ml; Sigma).

Equilibrium Gel Filtration—Equilibrium gel filtration experiments with human dGal-1 and radiolabeled oligosaccharides were conducted in a 3-ml Sephadex G-50 column (0.5 × 15 cm). The column was equilibrated with 5 ml of oligosaccharides, radiolabeled at the terminal galactose residues ([^{3}H]LN-biotin, [^{3}H]LN2-biotin, [^{3}H]LN3-biotin, [^{3}H]LNnH, *para*-[^{3}H]LNnH (p[^{3}H]LNnH), [^{3}H]LNnT, and [^{3}H]NA2), in PBS at a concentration of 1 pmol of terminal galactose/ml (specific activity of 4800–5500 cpm/pmol of galactose). Various amounts of dGal-1 were preincubated with ^{3}H-labeled oligosaccharides in 150 μl of PBS for 5–10 min before applying to the column. Samples were eluted with PBS containing ^{3}H-labeled oligosaccharides, and 150-μl fractions were collected at a flow rate of ~90 μl/min. Radioactivity in the fractions was determined by liquid scintillation counting. The column was washed with PBS before equilibration with ^{3}H-labeled oligosaccharides between experiments with different ligands.

RESULTS

dGal-1 Binds to Extended Poly-N-acetyllactosamines, and Binding Is Highly Dependent on Terminal Galactose Residues—The binding specificity of human recombinant dGal-1 was studied using a fluorescence-based solid-phase assay in which biotinylated oligosaccharides/glycopeptides were captured on a commercial streptavidin-coated microtiter plate (50 pmol/well) (Fig. 1). Fluorescently labeled dGal-1 (40 μg/ml) showed clear preferential binding to extended PL structures on *O*-glycopeptides (GP-4′, GP-4″, and GP-4‴) or on glycopeptide-free oligosaccharides (LN2, LN3, and LNnT) (Fig. 2*A*). Binding to single lactosamine-containing structures was at background levels (GP-4 and LN). The degree of binding increased as the number of lactosamine repeats in the glycans increased (LN3 > LN2 = LNnT > LN or GP-4‴ > GP-4″ = GP-4′ > GP-4). Terminal Gal residues of LN2, LN3, and LNnT were highly important for binding because dGal-1 lacked detectable binding to degalactosylated ligands (NGLN2, NGLN3, and NGLNnT).

After removal of bound dGal-1, the wells were incubated with fluorescently labeled RCA-I, which recognizes terminal Gal residues (55). RCA-I bound well to all immobilized ligands containing a terminal galactose residue, as expected (Fig. 2*B*). These results also demonstrate that all ligands with terminal galactose residues were immobilized at approximately equivalent levels on the streptavidin-coated microplate and were equivalently accessible for lectin binding. After removal of bound RCA-I, the wells were incubated with fluorescently labeled tomato lectin (LEA). LEA binds to long PLs (41). LEA showed increased binding to ligands as the length of the PL chain increased, as expected (Fig. 2*C*). LEA also bound well to degalactosylated PL structures NGLN2 and NGLN3, which dGal-1 was unable to recognize. In summary, the results show that dGal-1 binds to extended PL structures on immobilized glycans and *O*-glycopeptides and that binding is highly dependent on the nonreducing terminal galactose residue.

dGal-1 Binds to Immobilized NA2 and Immobilized LN3 with Similar Affinity—Earlier studies utilizing TLC overlay and microwell binding assays demonstrated that bovine dGal-1 binds to immobilized glycolipids containing linear PL as well as to immobilized neoglycolipids containing bi-, tri-, and tetraantennary *N*-glycans with non-extended terminal LN units (42). However, a more recent study using the frontal affinity chromatography approach has shown that human dGal-1 recognizes biantennary and triantennary *N*-glycans containing non-extended terminal LN units with significantly higher affinity compared with linear PLs (45). Therefore, we compared the binding affinity of human dGal-1 for LN3 *versus* NA2 using the fluorescence-based solid-phase assay. First, we compared the relative binding affinity of human dGal-1 for immobilized LN3 and NA2. Biotinylated LN3 and NA2 were captured on streptavidin-coated microtiter wells, and a fixed concentration of fluorescently labeled human dGal-1 was incubated with immobilized ligands. The results indicate that dGal-1 bound to LN3 and NA2 with similar affinity (Fig. 3*A*, *inset*). To estimate the binding affinity of dGal-1 for immobilized LN3 and NA2, fluorescently labeled dGal-1 was incubated with immobilized glycans. The apparent dissociation constants for dGal-1 derived from the binding curves were 3.5 μM for LN3 and 2.0 μM for NA2 (Fig. 3, *A* and *C*). After removing dGal-1, we then measured the binding of fluorescently labeled RCA-I to the immobilized ligands. RCA-I bound to LN3 and NA2 with equal apparent dissociation constants of 0.14 μM (Fig. 3*B*) and 0.12 μM (Fig. 3*D*), respectively. These results show that both LN3 and NA2 were accessible for lectin binding and were equally immobilized on the plate. Thus, we conclude that human dGal-1 binds to immobilized biantennary *N*-glycan-containing non-extended terminal LN units (NA2) with an affinity similar to that observed for immobilized long chain PL (LN3).

dGal-1 Binds to α2,3-Sialylated or α1,2-Fucosylated (but Not α2,6-Sialylated or α1,3-Fucosylated) PLs—Terminal Gal residues in natural PLs are often substituted with α2,3- or α2,6-sialic acid or α1,2-fucose residues. Moreover, GlcNAc residues of PLs can be substituted with α1,3-fucose residues to form Le^x structures. The effect of sialic acid and fucose residues of PLs on dGal-1 binding was studied using the fluorescence-based solid-phase assay. The α2,3-sialylated structures S3LN2, S3LN3, and S3LNnT; the α2,6-sialylated structure S6LN2; the α1,2-fucosylated structure F2LN2, and the α1,3-fucosylated structure (Le^x)3 and their non-sialylated non-fucosylated derivatives were immobilized on streptavidin-coated plates (~50 pmol/well). Fluorescently labeled dGal-1 (40 μg/ml) bound to all α2,3-sialylated structures (S3LN2, S3LN3, and S3LNnT) to a relatively equivalent extent in comparison with the non-sialylated derivatives LN2, LN3, and LNnT, respectively (Fig. 4*A*). dGal-1 also bound to α1,2-fucosylated F2LN2. However, the α2,6-sialic acid of S6LN2 and the α1,3-fucose residues of (Le^x)3 completely inhibited their recognition by dGal-1. After removing bound dGal-1, the wells were incubated successively with fluorescently labeled RCA-I and LEA. RCA-I bound poorly to α2,3-sialylated structures, and binding to α2,6-sialylated S6LN2 was moderate (Fig. 4*B*). The presence of α1,3-fucose in (Le^x)3 completely inhibited RCA binding, but the presence of α1,2-fucose in F2LN2 only partly inhibited RCA binding. LEA showed slightly higher binding to extended α2,3-sialylated ligands compared with their non-sialylated derivatives (Fig. 4*C*). Binding to S3LNnT was significantly higher that that to LNnT. The presence of α1,3-fucose in (Le^x)3 completely inhibited LEA binding, but α2,6-sialic acid in S6LN2 and α1,2-fucose in F2LN2 only partly inhibited LEA binding. In summary, dGal-1 binds to α2,3-sialylated as well as α1,2-fucosylated PLs, but does not recognize α2,6-sialylated or α1,3-fucosylated PLs.

dGal-1 Binds to Immobilized S3LN3 and Immobilized LN3 with Nearly Equal Affinity—The results of Fig. 4*A* show that dGal-1 bound to immobilized α2,3-sialylated ligands with equivalent affinity compared with their non-sialylated derivatives. The apparent K_d for dGal-1 binding to immobilized LN3 is 3.5 μM (Fig. 3*A*). To compare the binding affinity of dGal-1 for LN3 and α2,3-sialylated S3LN3, the apparent K_d for dGal-1

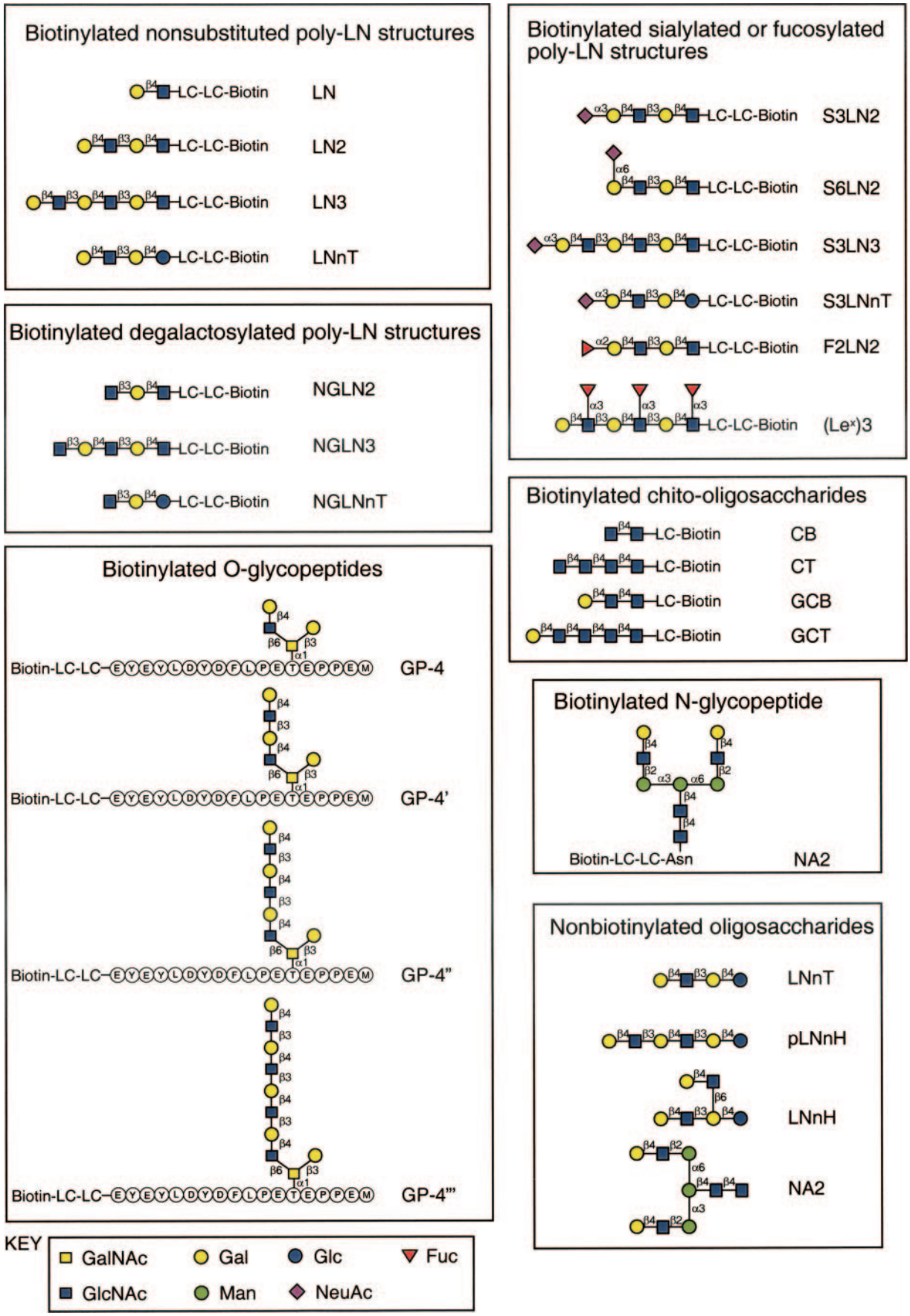

FIG. 1. **Structures of the biotinylated and non-biotinylated oligosaccharides and glycopeptides used in this study.**

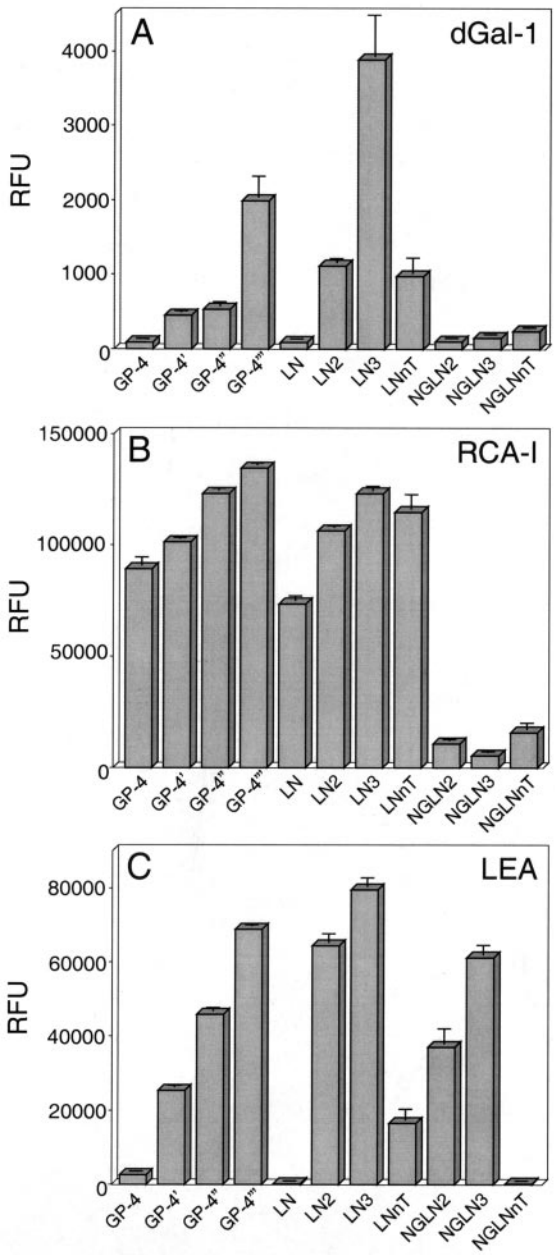

FIG. 2. **Binding of human dGal-1, RCA-I, and LEA to immobilized non-sialylated and non-fucosylated glycopeptides and oligosaccharides in a fluorescence-based solid-phase assay.** Biotinylated glycopeptides and oligosaccharides were immobilized on streptavidin-coated microtiter wells (50 pmol/well). Fluorescently labeled dGal-1 (40 μg/ml; *A*), RCA-I (120 μg/ml; *B*), and LEA (50 μg/ml; *C*) were successively incubated with the immobilized ligands. The data are representative of two independent experiments. All assays were performed in triplicate, and the results are the means ± S.D. of three determinations. *RFU*, relative fluorescence units.

binding to S3LN3 was determined using the fluorescence-based solid-phase assay. dGal-1 bound to S3LN3 with a K_d of ~4.3 μM (Fig. 5), which is only ~20% weaker affinity in comparison with dGal-1 binding to LN3. In summary, dGal-1 binds α2,3-sialylated PLs with an equivalent affinity compared with the non-sialylated derivatives.

dGal-1 Recognizes Terminal LN Units on Non-PL Backbones—The results demonstrate that human dGal-1 bound to immobilized PL structures with apparent high affinity and that binding was strongly dependent on the presence of terminal β-linked galactose residues on these ligands (Fig. 2*A*). These data also suggest that dGal-1 interacts only with the terminal LN residues on long PL chains and that the function of the PL backbone may be to extend the β-linked galactose residues away from the surface. To test this hypothesis, we generated novel non-PL-containing chito-oligosaccharides ((GlcNAcβ1–4)$_n$) carrying the terminal β-linked Gal residue as ligand candidates for dGal-1. Biotinylated CB and CT and their galactosylated derivatives (Fig. 1) were immobilized on streptavidin-coated microtiter wells. The wells were incubated successively with fluorescently labeled dGal-1, RCA-I, and LEA. dGal-1 did not show binding above background levels to non-galactosylated CB or CT, whereas dGal-1 showed significant interaction with galactosylated CT (GCT) (Fig. 6*A*). dGal-1 bound to GCT with an affinity similar to that observed for LNnT and LN2. dGal-1 also showed weak interaction with galactosylated CB, but no interaction with LN. These results support the hypothesis that dGal-1 recognizes only terminal LN units on extended surface-bound glycans and that the PL backbone is not required *per se* for dGal-1 binding. The control binding experiment with RCA-I confirmed that all glycans containing nonreducing terminal β-linked Gal residues were immobilized at equivalent densities since RCA-I binding to those glycans was similar (Fig. 6*B*). LEA showed increased binding to longer chito-oligosaccharides and PL structures; but unlike the results observed with dGal-1, the binding of LEA was independent of the presence of terminal β-linked Gal residues (Fig. 6*C*).

Monomeric Galectin-1 and Dimeric Galectin-1 Have Identical Ligand Binding Specificities—The above studies were performed with dGal-1, but the role of dimerization in the binding of dGal-1 to ligands is unclear. To study the role of dimerization in ligand recognition and affinity, we used the recombinant mutated form mGal-1, which behaves as a monomer upon gel filtration compared with dGal-1, which behaves as a dimer upon gel filtration (25). Fluorescently labeled mGal-1 and dGal-1 were successively incubated with immobilized oligosaccharides. The results show that both mGal-1 and dGal-1 preferred to bind to non-sialylated and α2,3-sialylated extended PLs and that binding was dependent on the length of the PL chain (Fig. 7, *A* and *B*). The binding of either lectin to non-sialylated ligands was highly dependent on the presence of terminal β-linked Gal residues because binding to NGLN3 and NGLN2 (which lack the terminal Gal residue) was undetectable. In summary, mGal-1 and dGal-1 have identical ligand binding specificities, and the mutation that results in monomerization of Gal-1 does not alter the specificity of binding. However, we observed that a much higher concentration of mGal-1 (300 μg/ml) than dGal-1 (40 μg/ml) was required to achieve strong binding signals, suggesting that the affinity of mGal-1 for immobilized ligands is significantly less than that of dGal-1.

Dimeric Galectin-1 Is Required for High Affinity Binding to Immobilized Ligands—It has been shown that hamster dGal-1 has a dimerization K_d of ~7 μM (56). Recent studies on human dGal-1 suggest that it has a lower concentration that promotes dimerization (dimerization K_d ~ 1–2 μM),[4] which is consistent with other studies on human dGal-1 (57). Mutation of the N-terminal residues of both hamster and human mGal-1 cause defects in the ability of the proteins to dimerize, and only monomers are observed upon gel filtration (56). However, dimerization of mGal-1 can occur, albeit at a higher concentration, because chemical cross-linkers can capture dimeric forms of hamster mGal-1 (56). Thus, we reasoned that, at higher concentrations, human mGal-1 is likely to form a noncovalent dimer because mGal-1 at >5 μM is able to agglutinate HL-60 cells.[4]

[4] M. Cho and R. D. Cummings, unpublished data.

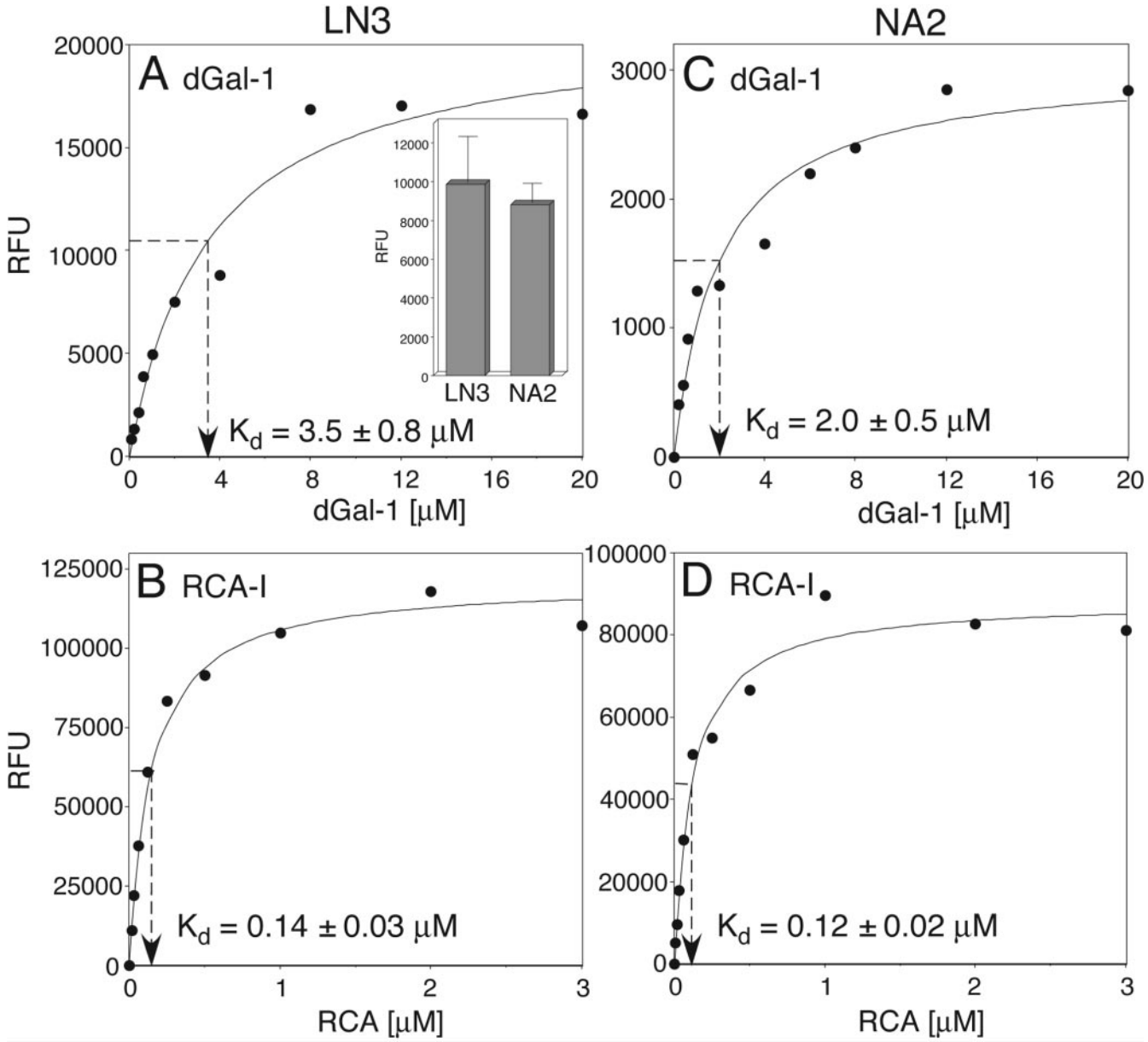

FIG. 3. **Binding affinities of dGal-1 and RCA-I for immobilized LN3 and NA2 in a fluorescence-based solid-phase assay.** Biotinylated LN3 (*A* and *B*) and NA2 (*C* and *D*) were immobilized on streptavidin-coated microtiter wells (50 pmol/well). Various concentrations of fluorescently labeled dGal-1 (*A* and *C*) and RCA-I (*B* and *D*) were successively incubated with the immobilized ligands. All assays were performed in duplicate, and the results are the average of two determinations. The concentrations of dGal-1 and RCA-I were calculated using a monomer molecular mass of Gal-1 and a tetramer molecular mass of RCA-I. The *inset* in *A* shows the relative binding of dGal-1 to immobilized LN3 and NA2 using a single concentration of dGal-1 (40 μg/ml). The assay was performed in triplicate, and the results are the average of three determinations. *RFU*, relative fluorescence units.

The binding affinities of mGal-1 and dGal-1 for immobilized LN3 were compared using the solid-phase binding assay, and the results show that the binding isotherms for the two lectins are very different. Fluorescently labeled mGal-1 was diluted and preincubated for 21 h at 4 °C before the assay to promote equilibration. mGal-1 bound to immobilized LN3 relatively weakly, giving a sigmoidal binding curve (Fig. 8*A*), whereas dGal-1 bound to LN3 with an apparent K_d of ~2.6 μM in a typical rectangular hyperbola binding curve (Fig. 8*C*). The sigmoidal binding curve of mGal-1 suggests that, at low concentrations (<5 μM), mGal-1 may be largely monomeric and that binding affinity is low, whereas at higher concentrations (>5 μM), mGal-1 may dimerize and that these dimers bind to LN3 with higher affinity.

To test this possibility, mGal-1 was chemically cross-linked to form a covalent dimer (cd-mGal-1). cd-mGal-1 was fluorescently labeled and purified on an α-lactosyl-agarose affinity column. The purity of the dimer was confirmed by reducing SDS-PAGE (Fig. 8*B*, *inset*, *lane 2*). Both fluorescently labeled mGal-1 and dGal-1 behaved as monomers upon reducing SDS-PAGE (Fig. 8*B*, *inset*, *lanes 1* and *3*, respectively). The binding affinity of cd-mGal-1 for immobilized LN3 was determined using the solid-phase assay. cd-mGal-1 bound to immobilized LN3 with the same affinity as dGal-1 (K_d ~ 2.3 μM) (Fig. 8*B*), and no sigmoidicity in the binding curve was observed. In contrast to the mGal-1 binding curve, the cd-mGal-1 binding curve was a rectangular hyperbola. These results indicate that dimerization of Gal-1 is required for high affinity binding to immobilized ligands.

dGal-1 Binds to α2,3-Sialylated and Non-sialylated PLs on Immobilized HL-60 Cells—The above data show that human dGal-1 bound to immobilized non-sialylated and α2,3-sialyl-

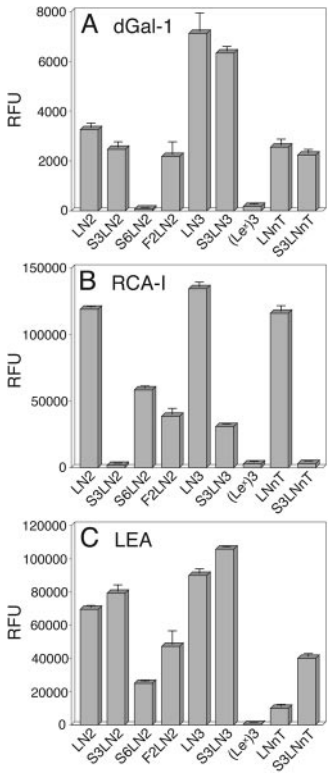

FIG. 4. **Binding of dGal-1, RCA-I, and LEA to immobilized sialylated and fucosylated oligosaccharides in a fluorescence-based solid-phase assay.** Biotinylated glycopeptides and oligosaccharides were immobilized on streptavidin-coated microtiter wells (~50 pmol/well). Fluorescently labeled dGal-1 (40 μg/ml; *A*), RCA-I (120 μg/ml; *B*), and LEA (50 μg/ml; *C*) were successively incubated with the immobilized ligands. The data are representative of two independent experiments. All assays were performed in triplicate, and the results are the means ± S.D. of three determinations. *RFU*, relative fluorescence units.

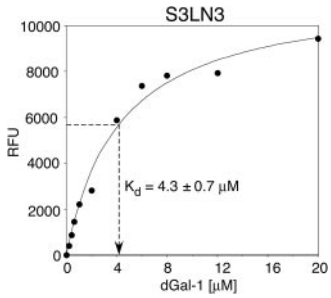

FIG. 5. **Binding affinity of dGal-1 for immobilized S3LN3 in a fluorescence-based solid-phase assay.** Biotinylated S3LN3 was immobilized on streptavidin-coated microtiter wells (50 pmol/well). Various concentrations of Gal-1 were incubated with immobilized S3LN3. All assays were performed in duplicate, and the results are the average of two determinations. *RFU*, relative fluorescence units.

ated PLs with relatively high affinity. Previous studies on human promyelocytic HL-60 cells show that these cells bind dGal-1 and mGal-1, but that only dGal-1 has signaling activity and is able to induce exposure of surface phosphatidylserine (25). HL-60 cells are known to synthesize a variety of PL structures, and many of these have been reported to be terminated by α2,3-sialic acid (58–64). The ability of dGal-1 to bind to cell-surface PL structures on immobilized HL-60 cells was explored using a solid-phase binding assay. HL-60 cells were biotinylated and fixed, and a portion were treated with neuraminidase. Untreated and neuraminidase-treated HL-60 cells were then incubated with either *E. freundii* endo-β-galactosidase or jack bean β-galactosidase to remove PL chains or non-

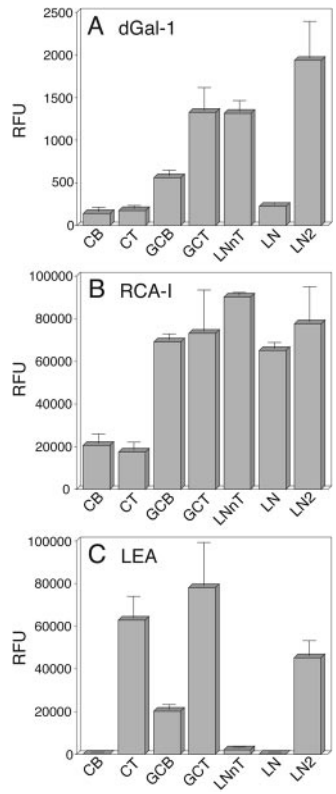

FIG. 6. **Binding of dGal-1, RCA-I, and LEA to immobilized chito-oligosaccharides in a fluorescence-based solid-phase assay.** Biotinylated oligosaccharides were immobilized on streptavidin-coated microtiter wells (~50 pmol/well). Fluorescently labeled dGal-1 (40 μg/ml; *A*), RCA-I (120 μg/ml; *B*), and LEA (50 μg/ml; *C*) were successively incubated with the immobilized ligands. All assays were performed in triplicate, and the results are the means ± S.D. of three determinations. *RFU*, relative fluorescence units.

reducing terminal β-linked Gal residues, respectively. *E. freundii* endo-β-galactosidase cleaves internal β1,4-linked Gal residues within linear PL chains (65–67), whereas jack bean β-galactosidase cleaves only nonreducing terminal β-linked Gal residues (68). Cells were captured on streptavidin-coated plates at equivalent densities (100,000 cells/well). Fluorescently labeled dGal-1 (40 μg/ml) was incubated with the immobilized cells; the wells were washed with buffer; and bound dGal-1 was measured. dGal-1 bound to both untreated and neuraminidase-treated HL-60 cells (Fig. 9*A*). Binding to untreated (sialylated) cells was ~2-fold less than that to desialylated cells, suggesting that α2,6-sialic acid present on glycans of HL-60 cells may inhibit dGal-1 binding. Treatment of desialylated HL-60 cells with either endo-β-galactosidase or β-galactosidase reduced dGal-1 binding by ~50%. These results indicate that dGal-1 bound to nonreducing terminal β-linked Gal residues present on PLs on desialylated HL-60 cells. Endo-β-galactosidase and β-galactosidase treatment of parental sialylated HL-60 cells also reduced dGal-1 binding to immobilized cells by ~63 and ~34%, respectively, in comparison with untreated cells. The smaller effect of β-galactosidase treatment of HL-60 cells on dGal-1 binding suggests that HL-60 cells are probably highly sialylated, as expected, and have few nonreducing terminal β-linked Gal residues. However, a significant effect of endo-β-galactosidase treatment of parental HL-60 cells on dGal-1 binding indicates that dGal-1 can bind sialylated PLs at the cell surface, which is consistent with the results in Fig. 4*A*, which show that dGal-1 bound to α2,3-sialylated ligands with relatively high affinity. dGal-1 binding to desialylated and parental HL-60 cells was sugar-specific since incubation of cells

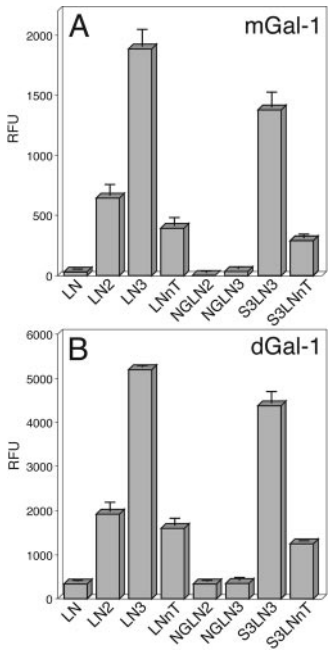

FIG. 7. **Binding specificity of mGal-1 and dGal-1 for immobilized oligosaccharides in a fluorescence-based solid-phase assay.** Biotinylated oligosaccharides were immobilized on streptavidin-coated microtiter wells (~50 pmol/well). Fluorescently labeled mGal-1 (300 μg/ml; *A*) and dGal-1 (40 μg/ml; *B*) were successively incubated with the immobilized ligands. All assays were performed in triplicate, and the results are the means ± S.D. of three determinations. *RFU*, relative fluorescence units.

with 0.2 M lactose after binding with fluorescently labeled dGal-1 removed all bound dGal-1 (data not shown).

To test the effectiveness of endo-β-galactosidase and β-galactosidase in removing PL chains and terminal β-linked galactose residues, respectively, parallel control binding experiments were conducted with plant lectins. Endo-β-galactosidase treatment of cells reduced LEA binding to both desialylated and parental cells by ~50%, indicating that endo-β-galactosidase cleaved ~50% of the cell-surface PLs (Fig. 9*B*). This is consistent with reduced dGal-1 binding to endo-β-galactosidase-treated cells, suggesting that dGal-1 ligands on HL-60 cells are mainly PLs (Fig. 9*A*). Treatment of cells with jack bean β-galactosidase did not have a significant effect on LEA binding to cells (Fig. 9*B*), as expected, showing that nonreducing terminal β-linked Gal residues are not important for LEA binding, consistent with the results shown in Fig. 4*C*. To test exposure of nonreducing terminal GlcNAc residues upon treatment with the glycosidases, we examined the binding of GS-II, which recognizes terminal β-linked GlcNAc residues (69). The binding of fluorescently labeled GS-II increased substantially when desialylated HL-60 cells were treated with endo-β-galactosidase and β-galactosidase. These results show that both glycosidases acted on cell-surface glycoconjugates, exposing terminal β-linked GlcNAc residues (Fig. 9*C*). However, GS-II binding to parental sialylated HL-60 cells was significantly increased only when cells were treated with endo-β-galactosidase, which is consistent with the expectation that endo-β-galactosidase cleaves sialylated cell-surface PLs. β-Galactosidase did not have a significant effect on GS-II binding to parental HL-60 cells, indicating that most of the terminal Gal residues are sialylated in HL-60 cells. We observed high binding of GS-II to desialylated HL-60 cells following treatment with β-galactosidase, but only low binding to desialylated HL-60 cells not treated with β-galactosidase, which indicates that sialidase treatment of HL-60 cells was successful. In summary, these results show that dGal-1 binds to non-sialylated as well as sialylated PLs on

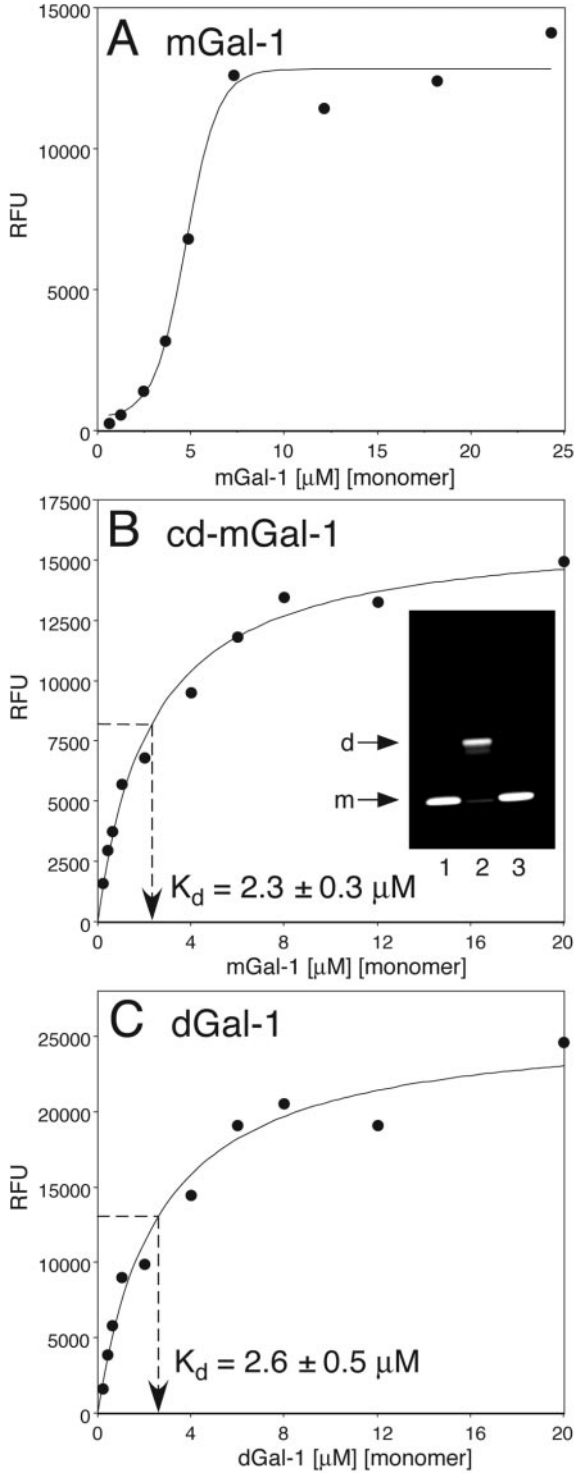

FIG. 8. **Binding affinity of mGal-1, cd-mGal-1, and dGal-1 for immobilized LN3 in a fluorescence-based solid-phase assay.** Biotinylated LN3 was immobilized on streptavidin-coated microtiter wells (~50 pmol/well). Various concentrations of fluorescently labeled mGal-1 (*A*), cd-mGal-1 (*B*), and dGal-1 (*C*) were successively incubated with immobilized LN3. All assays were performed in duplicate, and the results are the average of two determinations. The *inset* in *B* shows the results from reducing SDS-PAGE of the purified fluorescently labeled Gal-1 samples used in this study. *Lane 1*, dGal-1; *lane 2*, cd-mGal-1; *lane 3*, mGal-1. The *arrows* mark the positions of the monomer (*m*) and dimer (*d*). *RFU*, relative fluorescence units.

HL-60 cells. Binding to non-sialylated PLs is dependent on the terminal Gal residues.

dGal-1 Binds to Sialylated and Desialylated Cell-surface PLs on HL-60 Cells with High Affinity—The apparent binding af-

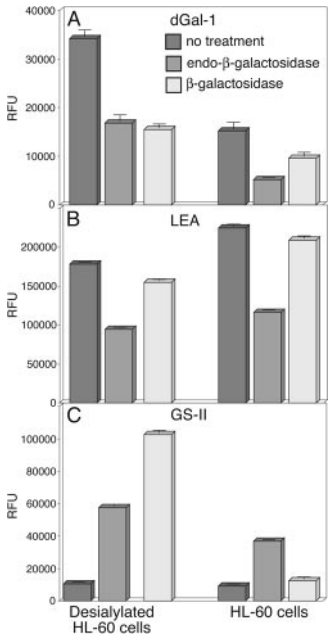

FIG. 9. **Binding of dGal-1, LEA, and GS-II to immobilized desialylated and untreated HL-60 cells in a fluorescence-based solid-phase assay.** Biotinylated and fixed HL-60 cells and desialylated HL-60 cells were first treated with endo-β-galactosidase or β-galactosidase and immobilized on streptavidin-coated microtiter wells (100,000 cells/well). Fluorescently labeled lectins dGal-1 (40 μg/ml; *A*), LEA (100 μg/ml; *B*), and GS-II (100 μg/ml; *C*) were incubated with the immobilized cells. All assays were performed in triplicate, and the results are the means ± S.D. of three determinations. *RFU*, relative fluorescence units.

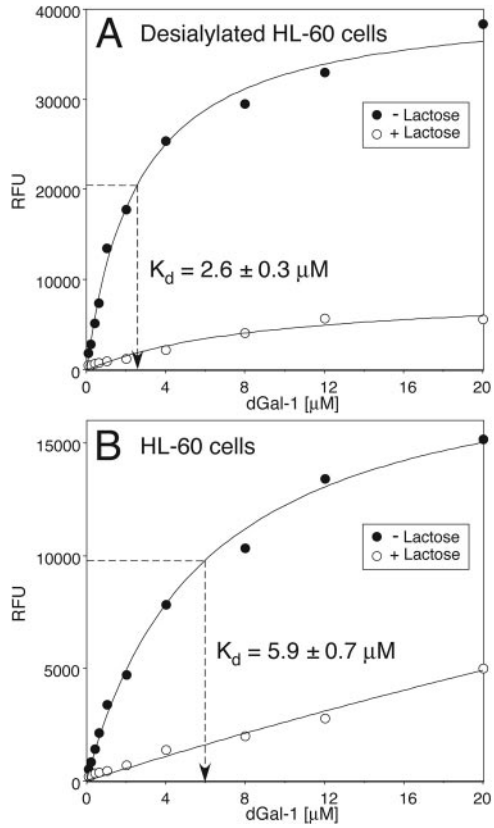

FIG. 10. **Binding affinity of dGal-1 for immobilized desialylated and untreated HL-60 cells in a fluorescence-based solid-phase assay.** Biotinylated, fixed, and desialylated HL-60 cells (*A*) and biotinylated and fixed parental (sialylated) HL-60 cells (*B*) were immobilized on streptavidin-coated microtiter wells (100 000 cells/well). Various concentrations of dGal-1 were incubated with the immobilized cells in buffer with or without 20 mM lactose. All assays were performed in duplicate, and the results are the average of two determinations. *RFU*, relative fluorescence units.

finities of dGal-1 for HL-60 cells and desialylated HL-60 cells were compared using the fluorescence-based solid-phase assay. Biotinylated and fixed HL-60 cells and desialylated HL-60 cells were immobilized on streptavidin-coated microtiter wells. Various concentrations of fluorescently labeled dGal-1 were incubated with the immobilized cells in the presence or absence of 20 mM lactose. dGal-1 bound to immobilized desialylated HL-60 cells with apparent high affinity (apparent $K_d \sim 2.6$ μM), and lactose inhibited >85% of the binding (Fig. 10*A*). The affinity of dGal-1 for parental sialylated HL-60 cells was ~2-fold weaker in comparison with desialylated HL-60 cells (apparent $K_d \sim 5.9$ μM), and lactose inhibited >70% of the binding (Fig. 10*B*). The binding of dGal-1 to desialylated HL-60 cells had an apparent K_d similar to that observed for LN3 (compared Figs. 8*C* and 10*A*). Taken together, our results show that dGal-1 binds to sialylated and desialylated cell-surface PLs on immobilized HL-60 cells with high affinity. The binding affinity of dGal-1 for PLs on HL-60 cells is comparable with the binding affinity of dGal-1 for immobilized PL.

dGal-1 Does Not Show High Affinity Binding to Extended PL Structures upon Equilibrium Gel Filtration—dGal-1 bound to non-sialylated and α2,3-sialylated PLs with apparent high affinity when the ligands were immobilized on plates or at the cell surface. However, a previous study using the frontal affinity chromatography approach indicated that human dGal-1 does not recognize PLs with high affinity (45). In contrast to our solid-phase assay, ligands in solution are free, as in frontal affinity chromatography, and therefore may not be cross-linkable by dGal-1. This prompted us to compare the binding specificity and affinity of dGal-1 for immobilized *versus* free ligands. We determined the equilibrium binding affinity of dGal-1 for some linear and branched oligosaccharides in solution using Hummel-Dreyer equilibrium gel filtration (49, 70, 71). The equilibrium binding affinity of dGal-1 for [^{3}H]LN3-biotin was first measured. Experiments were carried out by loading different concentrations of dGal-1 onto a gel filtration column equilibrated with [^{3}H]LN3-biotin in buffer. The bound [^{3}H]LN3-biotin and free dGal-1 concentrations were calculated from the equilibrium gel filtration data (data not shown), and a K_d of ~21.9 ± 4.5 μM was derived from the binding data (Fig. 11*A*). The equilibrium binding affinities of other ^{3}H-labeled biotinylated and non-biotinylated oligosaccharides ([^{3}H]LN2-biotin, [^{3}H]LN-biotin, [^{3}H]LNnT, *para*-[^{3}H]LNnH, [^{3}H]LNnH, and [^{3}H]NA2) were measured relative to that of [^{3}H]LN3-biotin by loading 9.1 nmol of dGal-1 onto a gel filtration column equilibrated with ^{3}H-labeled oligosaccharides in buffer (Fig. 11*B*). The results show that [^{3}H]LN2-biotin bound to dGal-1 with the same affinity as [^{3}H]LN3-biotin. [^{3}H]LN-biotin and all ^{3}H-labeled non-biotinylated oligosaccharides showed similar but lower binding affinity for dGal-1, ~30–50% relative to [^{3}H]LN3-biotin (estimated K_d values in the range of ~40–70 μM). This weak affinity was independent of the length of the PL chain ([^{3}H]LNnT and *para*-[^{3}H]LNnH) or branching ([^{3}H]LNnH and [^{3}H]NA2) (Fig. 11*B*). These results demonstrate that dGal-1 in solution shows relatively weak affinity for free glycans in solution and shows no preference for those with or without extended PLs.

Bovine Heart dGal-1 Has the Same Binding Specificity as Human Recombinant dGal-1—Because many studies in the past on dGal-1 specificity and activity have been conducted using bovine heart-derived dGal-1, we wanted to directly compare the specificity of bovine heart dGal-1 with the recombinant form of

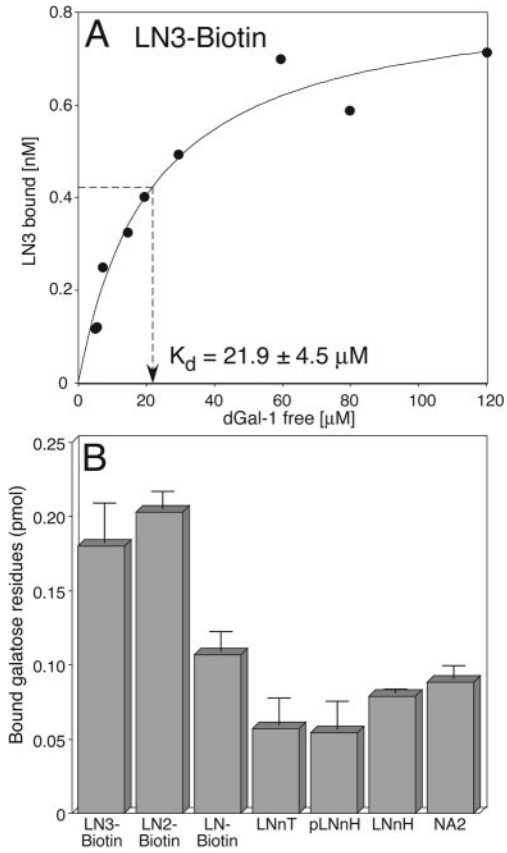

FIG. 11. **Binding affinity of dGal-1 for linear and branched oligosaccharides upon equilibrium gel filtration.** *A*, equilibrium binding affinity of dGal-1 for [^{3}H]LN3-biotin. Equilibrium gel filtration experiments were carried out by loading 2.3, 3.4, 4.6, 6.8, 9.1, 18.2, 27.3, 36.4, and 54.6 nmol of dGal-1 onto a gel filtration column equilibrated with [^{3}H]LN3-biotin in PBS (1 pmol/ml). The bound LN3 and free dGal-1 concentrations were calculated from the equilibrium gel filtration data (data not shown) by dividing the molar amounts of bound [^{3}H]LN3-biotin and free dGal-1 by the peak volume of the dGal-1·LN3-biotin complex. The dissociation constant (K_d ~ 21.9 ± 4.5 μM) was calculated using a rectangular hyperbola equation to derive the nonlinear curve fitting. *B*, relative equilibrium binding affinity of dGal-1 for different biotinylated and non-biotinylated oligosaccharides. Equilibrium gel filtration experiments were carried out by loading 9.1 nmol of dGal-1 onto a gel filtration column equilibrated with the indicated ^{3}H-labeled oligosaccharides in PBS (1 pmol of terminal Gal/ml). All experiments in *B* were performed in triplicate, and the results are the average of three determinations.

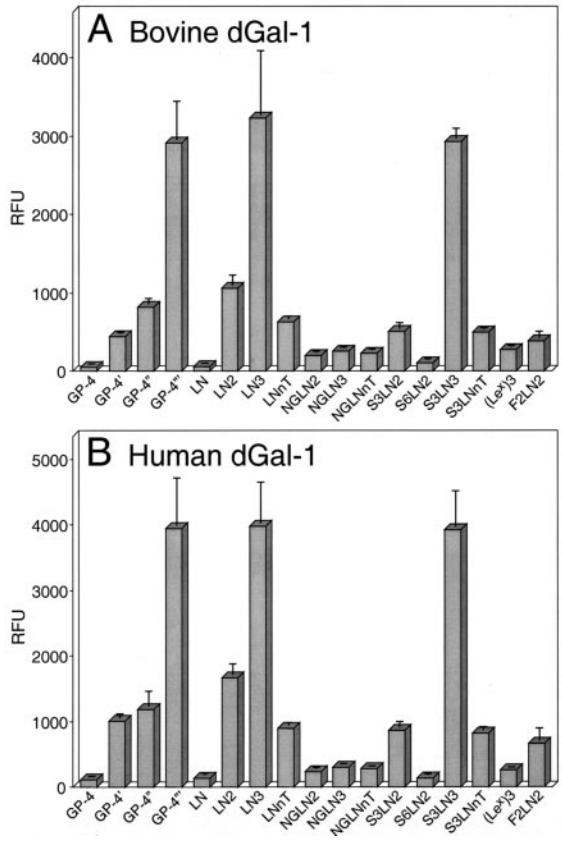

FIG. 12. **Comparison of the binding specificities of bovine heart dGal-1 and human recombinant dGal-1 for immobilized oligosaccharides in a fluorescence-based solid-phase assay.** Biotinylated oligosaccharides were immobilized on streptavidin-coated microtiter wells (~50 pmol/well). Fluorescently labeled bovine dGal-1 (40 μg/ml; *A*) and human dGal-1 (40 μg/ml; *B*) were successively incubated with the immobilized ligands. All assays were performed in triplicate, and the results are the means ± S.D. of three determinations. *RFU*, relative fluorescence units.

human dGal-1. dGal-1 was extracted from bovine heart and purified by affinity chromatography on an α-lactosyl-agarose column as described under "Experimental Procedures." A homogeneous band of bovine dGal-1 (~15 kDa) was detected upon reducing SDS-PAGE (data not shown). Bovine dGal-1 was fluorescently labeled, and the binding specificity for various immobilized ligands was studied using the fluorescence-based solid-phase assay. Bovine dGal-1 bound to non-sialylated and α2,3-sialylated (but not α2,6-sialylated) extended PL structures and the binding affinity increased as the number of lactosamine repeats increased (Fig. 12*A*). α1,3-Fucose residues in (Le^x)3 inhibited the binding of bovine dGal-1, but α1,2-fucose in F2LN2 did not. In non-sialylated ligands, the terminal Gal residue was highly important for binding since no detectable binding to degalactosylated oligosaccharides (NGLN2, NGLN3, and NGLNnT) was observed. Thus, bovine dGal-1 and human dGal-1 showed very similar binding specificity (Fig. 12, *A* and *B*).

DISCUSSION

A variety of biotinylated oligosaccharides and glycopeptides were synthesized to explore the ligand binding specificity of human and bovine dGal-1. To determine the binding affinity of dGal-1 for immobilized and free ligands, we used the fluorescence-based solid-phase assay and equilibrium gel filtration, respectively. Human dGal-1 bound to extended structures with terminal LN units, such as PL-containing glycan structures, with high affinity when ligands were immobilized, but not when ligands were free in solution. However, the PL backbone is not required for dGal-1 binding because dGal-1 also bound to terminal LN units of immobilized biantennary *N*-glycan (NA2) and GCT with high affinity. dGal-1 also recognized immobilized α2,3-sialylated and α1,2-fucosylated extended glycans with nearly the same affinity as the corresponding non-sialylated and non-fucosylated structures, whereas dGal-1 did not bind to α2,6-sialylated and α1,3-fucosylated glycans. Moreover, dGal-1 bound specifically to PL structures on immobilized HL-60 cells, and the apparent binding affinity was equivalent to that of dGal-1 for immobilized synthetic PL-type oligosaccharides. To define the role of Gal-1 dimerization in high affinity binding, mGal-1 was chemically cross-linked to form a covalent Gal-1 dimer. mGal-1 bound to immobilized glycans with lower affinity than wild-type dGal-1 or cd-mGal-1. Both human and bovine heart dGal-1 have similar binding specificities and affinities, further indicating that dimerization of Gal-1 is important for high affinity recognition of immobilized ligands.

There have been conflicting results on the binding specificity of dGal-1, and our studies provide new insights that resolve many of these conflicts. For example, it was found previously

that human and bovine dGal-1 bind with relatively poor affinity to extended PLs and that binding affinity is independent of the length of the glycan (44, 45). These results were based on isothermal titration microcalorimetry and frontal affinity chromatography, in which glycans are free in solution. By contrast, it was reported that bovine dGal-1 binds with relatively high affinity to immobilized extended (neo)glycolipids in TLC overlay and microwell binding assays, whereas binding to non-extended structures was not detected (42). Similarly, human dGal-1 is able to recognize immobilized neoglycoproteins containing PLs with higher affinity compared with immobilized neoglycoproteins with non-extended oligosaccharides in a solid-phase assay (43). The apparent discordance of these results may now be resolved since we have shown that they reflect the differing affinity of Gal-1 for ligands free in solution or immobilized on surfaces.

To address the apparent paradoxical specificity of dGal-1, we were prompted to compare the ability of dGal-1 to recognize extended *versus* non-extended oligosaccharide structures using different approaches, such as equilibrium gel filtration and fluorescence-based solid-phase assays. Our results obtained by equilibrium gel filtration show that dGal-1 bound free linear extended PLs and non-extended glycans with relatively poor affinity and that binding was relatively independent of the oligosaccharide chain length, thus confirming earlier results obtained by isothermal titration microcalorimetry and frontal affinity chromatography (44, 45). However, when the ligands were immobilized on microtiter plates in a solid-phase assay, dGal-1 recognized extended glycans with high affinity and showed only very weak binding to short non-extended glycans. The relevance of the dGal-1 recognition of extended PL structures on surfaces correlates with our finding that dGal-1 also bound with high affinity to PL structures on immobilized HL-60 cells. Taken together, these results demonstrate that, when ligands are surface-bound, dGal-1 binds with high affinity to long chain PL structures and that dimerization is required. Dimers may be required for cross-linking terminal LN units of two extended glycans, leading to high affinity binding. Short chain PL structures immobilized on a surface may not extend far enough to promote efficient cross-linking. Because cross-linking of long chain PL structures cannot occur when the ligands are free in solution, dGal-1 binds to extended and non-extended glycans with the same affinity.

dGal-1 has also been suggested to bind primarily to nonreducing terminal LN units rather than to mid-chain LN units within a PL chain (32, 46). Other results also suggest that dGal-1 is able to bind to extended glycans containing a terminal LN unit attached to a non-PL backbone (42, 43). For example, bovine dGal-1 binds to immobilized neoglycolipids containing complex-type *N*-glycans with non-extended terminal LN units as well as to glycolipids containing PL in microwell binding and TLC overlay assays (42, 43). Our recent results also indicate that human dGal-1 is able to bind to immobilized neoglycoproteins containing a non-substituted terminal LN unit attached to a backbone of eight-sugar $Le^x\beta1–3Le^x\beta1–3Lac$ (where Lac is lactose) or four-sugar $GalNAc\beta1–4GlcNAc\beta1–3Lac$ with an affinity similar to that observed for dGal-1 binding to neoglycoproteins containing a long chain PL structure (43). Nevertheless, none of these studies directly tested the role of the repeating lactosamine sequence $(–3Gal\beta1–4GlcNAc\beta1–)_n$ in galectin recognition.

In this study, we explored the possibility that the PL backbone may not be important for dGal-1 binding by generating extended glycans containing a terminal LN unit linked to a novel non-PL-based backbone in a solid-phase binding assay. We found that dGal-1 bound with similar affinity to the terminal LN unit attached to a non-PL backbone and to the terminal LN unit linked to a PL backbone of equivalent length. For example, dGal-1 bound to immobilized GCT with similar relative affinity compared with LNnT and LN2. dGal-1 also bound to immobilized NA2 with similar affinity compared with LN3. These results clearly demonstrate that dGal-1 binds to terminal LN units and that binding does not require mid-chain LN units on immobilized extended glycans. The data diminish the possibility that dGal-1 has an extended binding site for its ligand or that dGal-1 has a secondary binding site for extended glycans. X-ray crystallographic studies have been carried out with bovine dGal-1 (72, 73) and human galectin-2 (74) and simple non-extended oligosaccharides and show a shallow binding site able to accommodate only a terminal Gal-Glc(NAc) disaccharide unit. However, crystallographic studies utilizing Gal-1 complexed with extended structures should be done to further study the nature of the ligand-binding site of human dGal-1 in more detail.

Recent studies carried out by frontal affinity chromatography show that human dGal-1 binds to bi-, tri-, and tetraantennary *N*-glycans containing non-extended terminal LN units with ~10-fold higher affinity compared with extended linear PL structures (45). By contrast, our results obtained using the fluorescence-based solid-phase assay show that dGal-1 bound to the immobilized biantennary *N*-glycan NA2 with an affinity similar to that for immobilized LN3. Our results also show that dGal-1 was able to recognize only terminal LN units on extended surface-bound glycans. These results suggest that the *N*-glycan core structure of NA2 provides a backbone to extend the terminal LN units and to enhance their recognition and binding affinity. It is conceivable that dGal-1 binding to NA2 might be 2-fold higher because NA2 contains two terminal LN units and LN3 has only one, but we observed a very similar binding affinity of dGal-1 for NA2 compared with LN3. Thus, the two terminal LN residues within an immobilized NA2 molecule may not be able to separately accommodate individual Gal-1 dimers, whereas the free glycan may be more flexible and promote accommodation. Alternatively, only one of the terminal LN units of NA2 might be accessible on the microtiter plate surfaces. For example, it has been shown that the presence of an α1,6-linked fucose in the core of *N*-glycans can dramatically alter the presentation and lectin recognition of the α1,6-linked mannose antenna when the glycans are immobilized (75). We noted that RCA-I binding to NA2 and LN3 was also equivalent (Fig. 3), indicating that RCA-I also does not demonstrate differential binding to branched NA2 compared with linear LN3. In addition, several other studies have also shown that *N*-glycan branching does not improve the binding of dGal-1 (42, 45).

Recent results show that dimerization of Gal-1 is required for its biological activity (25). In this study, we explored the importance of Gal-1 dimerization in binding to its ligand. Our results demonstrate that mGal-1 and dGal-1 have the same binding specificity for immobilized ligands. However, mGal-1 binds to immobilized LN3 with lower affinity compared with dGal-1. In addition, the binding isotherm of dGal-1 for LN3 is a rectangular hyperbola, whereas the binding isotherm of mGal-1 for LN3 is sigmoidal. The sigmoidicity in the binding of mGal-1 probably reflects a level of cooperativity caused by the ability of mGal-1 to form a noncovalent dimer at higher protein concentrations. The higher binding affinity of dGal-1 may result because it readily dimerizes and cross-links surface-bound ligands, whereas mGal-1 is not efficient at dimerization or cross-linking at lower protein concentrations. Our results also demonstrate that the apparent cooperativity for mGal-1 is lost when the protein is covalently dimerized. Furthermore, dGal-1

may efficiently cross-link surface-bound ligands only when the terminal LN unit is extended.

Multiple lines of evidence indicate that α2,6-sialylation (but not α2,3-sialylation) of the oligosaccharides blocks human, rat, and bovine Gal-1 binding to ligand (30, 40, 42, 44, 45, 47). Most of these studies were based on indirect sugar inhibition assays. The binding affinity of human dGal-1 for α2,6-monosialylated and disialylated biantennary *N*-glycans is significantly reduced in comparison with non-sialylated *N*-glycans as determined by frontal affinity chromatography (45). A study utilizing isothermal titration microcalorimetry showed that bovine dGal-1 binds to both α2,3-sialyl-LN and LN with similar affinity (44). Bovine dGal-1 has also been shown to bind to immobilized neoglycolipids containing α2,3-sialyllacto-*N*-tetraose as well as to neoglycolipids containing non-sialylated lacto-*N*-tetraose (Galβ1–3GlcNAcβ1–3Galβ1–4Glc) in microwell binding assays (42). In agreement with these studies, our results show that human and bovine dGal-1 bound to immobilized α2,3-sialylated oligosaccharides (S3LN2, S3LN3, and S3LNnT) with approximately the same affinity as observed for their non-sialylated derivatives. However, the presence of α2,6-sialic acid, as in S6LN2, completely inhibited dGal-1 recognition of the terminal LN unit. The presence of terminal α1,2-fucose, as in F2LN2, only minimally inhibited the binding of dGal-1, whereas the presence of terminal α1,3-fucose, as in $(Le^x)3$, completely inhibited dGal-1 binding, in agreement with earlier results (42, 43, 47).

It has been suggested that expression of core 2 β1,6-GlcNAc-transferase, a key enzyme in core 2-based *O*-glycan synthesis, is important for dGal-1 function (14, 76–78), although other studies have questioned the role of *O*-glycans in galectin signaling (79). In any case, the ability of dGal-1 to bind directly to core 2-based *O*-glycans containing simple and extended PL units has not been studied. Here, we used a series of glycopeptides modeled after the N terminus of human PSGL-1 containing an *O*-glycan at a single Thr residue to study the role of core 2 *O*-glycan and extended core 2 structures in Gal-1 binding (51). Glycopeptides GP-4, GP-4′, GP-4″, and GP-4‴ contain one, two, three, and four linear LN repeats on the core 2 branch, respectively. dGal-1 did not show any detectable affinity for immobilized non-extended GP-4 (Fig. 2*A*). Binding to extended structures increased as the number of LN repeats increased (GP-4‴ > GP-4″ = GP-4′). These results were similar for dGal-1 binding to other immobilized extended oligosaccharides (Fig. 2*A*). In summary, dGal-1 does not appear to bind to the immobilized non-extended core 2 *O*-glycan, but it is able to bind to the immobilized extended core 2 *O*-glycans, consistent with predictions made in earlier studies (14, 77).

Our results with dGal-1 showing increased affinity for some immobilized ligands compared with reduced affinity for the same ligands in solution contrast with our results on the binding of P- and L-selectins to related glycoconjugates. We developed a fluorescence-based solid-phase assay for P- and L-selectin binding to immobilized synthetic glyco(sulfo)peptides modeled after human PSGL-1 (51, 80) and observed that the relative binding affinities between selectins and glyco(sulfo)peptides as determined by the solid-phase assay were in agreement with the binding affinities determined by other methods. Our results predict that these differences in lectin behavior probably reflect the need of dGal-1 to have extended glycans for recognition when the glycans are immobilized, and this need may reflect the biological situation where PL chains are probably extended from cell-surface glycoconjugates. Thus, our results predict that interference in the ability of cells to generate PL-containing ligands will diminish dGal-1 binding.

In our studies, we developed an approach using biotinylated and fixed HL-60 cells in a solid-phase assay to address the binding specificity of dGal-1 and several plant lectins for cell-surface glycoconjugates. These biotinylated and fixed cells can be effectively used for direct binding studies and for testing the stability and sensitivity of cell-surface glycans to exo- and endoglycosidase treatments. In fact, we found no significant differences in the cell binding of lectins whether the biotinylated and fixed cells were treated with enzymes (sialidase, β-galactosidase, and endo-β-galactosidase) before or after capture on streptavidin-coated plates (data not shown). Our study suggests that such solid-phase cell binding assays based on the streptavidin-biotin interaction can be used widely to study interactions of various lectins with cell-surface glycan determinants and might be useful with many types of animal cells and even microorganisms.

In summary, our results obtained by a variety of approaches show that dimerization of Gal-1 is important in its recognition of extended glycans containing terminal LN units when the glycans are surface-immobilized either on cells or on microtiter plates. By contrast, dGal-1 shows no significant difference in recognition of terminal LN units on glycans when both glycans and the lectin are free in solution, as in equilibrium gel filtration. These results are generally consistent with a variety of other studies on dGal-1 and unite a complex set of different interpretations, often based on different methods and approaches, as to how human dGal-1 recognizes ligands. Several studies have now shown that regulation of monomerization/dimerization of Gal-1 and recognition of terminal LN units are critical for signaling functions of Gal-1 (15, 25, 44, 81, 82). The extended LN units may promote cross-linking, dimerization, and cell signaling, as is commonly seen for many other signaling molecules that act independently of sugar recognition, such as the epidermal growth factor/epidermal growth factor receptor, and many other signaling pathways (83–89). Future studies should aim to identify the surface glycoconjugates recognized and cross-linked by dGal-1, which are likely to be glycoconjugates with extended LN units.

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Dimeric Galectin-1 Binds with High Affinity to α2,3-Sialylated and Non-sialylated Terminal *N*-Acetyllactosamine Units on Surface-bound Extended Glycans

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