gal-1 (galectin-1) mediates cell–cell and cell–extracellular matrix adhesion, essentially by interacting with β-galactoside-containing glycans of cell-surface glycoconjugates. Although most structural studies with gal-1 have investigated its binding to simple carbohydrates, in particular lactose and N-acetyl-lactosamine, this view is limited, because gal-1 functions at the cell surface by interacting with more complex glycans that are heterogeneous in size and composition. In the present study we used NMR spectroscopy to investigate the interaction of human gal-1 with a large (120 kDa) complex glycan, GRG (galactorhamnogalacturonate glycan), that contains non-randomly distributed mostly terminal β(1 → 4)-linked galactose side chains. We used 15N–1H-HSQC (heteronuclear single quantum coherence) NMR experiments with 15N-enriched gal-1 to identify the GRG-binding region on gal-1 and found that this region covers a large surface area on gal-1 that includes the quintessential lactose-binding site and runs from that site through a broad valley or cleft towards the dimer interface. HSQC and pulsed-field-gradient NMR diffusion experiments also show that gal-1 binds GRG with a gal-1:GRG stoichiometry of about 5:1 (or 6:1) and with average macroscopic and microscopic equilibrium dissociation constants \( (K_d) \) of \( 8 \times 10^{-6} \) M and \( 40 \times 10^{-6} \) M (or \( 48 \times 10^{-6} \) M) respectively, indicating stronger binding than to lactose \( (K_d = 520 \times 10^{-6} \) M). Although gal-1 may bind GRG in various ways, the glycan can be competed for by lactose, suggesting that there is one major mode of interaction. Furthermore, even though terminal motifs on GRG are Gal-β(1 → 4)-Gal rather than the traditional Gal-β(1 → 4)-Glc/GlcNAc (where GlcNAc is N-acetylgalcosamine), we show that the disaccharide Gal-β(1 → 4)-Gal can bind gal-1 at the lactose-binding domain. In addition, gal-1 binding to GRG disrupts inter-glycan interactions and decreases glycan-mediated solution viscosity, a glycan decongestion effect that may help explain why gal-1 promotes membrane fluidity and lateral diffusion of glycoconjugates within cell membranes. Overall, our results provide an insight into the function of galectin *in situ* and have potential significant biological consequences.

Key words: carbohydrate-binding domain, cell surface, galectin–glycan interactions, heteronuclear single quantum coherence nuclear magnetic resonance (HSQC NMR), NMR diffusion spectroscopy, simple saccharide.

**INTRODUCTION**

Traditionally, galectins have been defined as a subfamily of lectins that selectively bind β-galactosides, and all galectins share significant amino acid sequence conservation within their CRD (carbohydrate-recognition domain) [1]. Although galectins in general can have intracellular functions (e.g. modulating proliferation, apoptosis and pre-mRNA splicing), they are best known for their extracellular activities in mediating cell–cell and cell–matrix adhesion and migration by interacting with various glycan groups of cell-surface glycoproteins and/or glycolipids [2]. gal-1 (galectin-1) interacts with various glycoconjugate ligands of the extracellular matrix [e.g. laminin, fibronectin, the β1 subunit of integrins, ganglioside G_{al} and the Lamps (lysosomal-membrane-associated proteins) Lamp 1 and Lamp 2], as well as those on endothelial cells [e.g. integrins αβ6 and αβ3, ROBO4 [roundabout homologue 4, magic roundabout (Drosophila)], CD36, and CD13] and on T-lymphocytes (e.g. CD7, CD43, and CD45), where it is known to induce apoptosis [2]. Their binding to glycans of cell-surface glycoconjugates can also trigger intracellular activity. For example, gal-1 interacts with the αβ3 fibronectin receptor to restrict carcinoma cell growth via induction of p21 and p27 {Ras–MEK–ERK [Ras GTPase–MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase–ERK1]} pathway [3].

The disaccharide lactose (Gal-β(1 → 4)-Glc) is the simplest carbohydrate to which galectins bind and one that has been used most often to delineate galectin function and structure–activity relationships *in vitro* [1]. High-resolution structures of glycan–galectin interactions have been limited to galectin bound to lactose, N-acetyl-lactosamine, trisaccharide and an N-acetyl-lactosamine octasaccharide [4]. In all these cases, a β-galactoside-containing disaccharide moiety is shown to bind galectin in a similar fashion at its classical CRD, i.e. the quintessential lactose-binding domain, and even the largest one, N-acetyl-lactosamine octasaccharide, has the remaining six saccharide units jutting out from the galectin CRD into solution. Although providing a structural insight into gal-1 saccharide binding, previous studies have provided a biased picture of how galectins interact with, and affect, the properties of complex, heterogeneous cell-surface glycans. Actual galectin-binding glycans of various glycoconjugates in *in situ* have far more varied saccharide compositions and complicated structures than the simple saccharides mentioned above, as they are larger, heterogeneous...
in size and composition, densely packed and therefore self-associating, and it is likely that gal-1 interacts to some extent with saccharide units other than β-galactose in those glycans [5]. The relatively low intrinsic binding affinity (micro- to millimolar) of simple saccharides to galectins is significantly increased by multivalent interactions with larger glycan ligands. Moreover, gal-1, like most galectins, is a dimer with two CRDs that can mediate intermolecular glycan cross-linking [6]. All this and more could determine and/or differentiate galectin structure–function relationships.

In the present paper we report on NMR experiments with human gal-1 and its interactions with GRG (galactorhamnogalacturonate glycan), a relatively large heterogeneous glycan derived from citrus pectins. Citrus pectins are branched heteropolysaccharides of various molecular masses ranging from about 40 kDa to several million Da, and contain multiple branches of oligosaccharides comprised of one to 20 or more moieties of galacturonate, arabinose, galactose, glucose, xylose, mannose etc. [7]. These complex glycans can also include single residues or chains of uronic acid, and their carboxylic groups can be esterified to the extent of 2–30%. Owing to their large size, these glycans tend to form gels in solution [8]. Following processing, the GRG has a weight-average molecular mass of about 120 kDa [9] and can be characterized as having an irregular structure with five principal components: rhamnose, fucose, arabinose, galactose and uronate, which by weight account for about 4.3, 3.7, 19, 37 and 36% respectively [10,11]. The backbone of the GRG polymer is mainly composed of α-(1 → 2)-L-rhamnosyl-α-(1 → 4)-D-galacturonosyl sections. As with most complex glycans, precise structural details for GRG are lacking. However, it is known that the galactose side-chains in GRG are randomly distributed via β(1 → 4) linkages, most of which are at terminal positions [7,10,12], suggesting the potential for interactions with galectins.

In this regard, pectin-derived polysaccharides have been reported to interact with gal-3 [13], as well as to inhibit human cancer cell growth and metastasis in mice [14] and to enhance apoptosis in human prostate-cancer cells [15] and multiple myeloma cells in vitro, probably also by interacting with, and antagonizing, the function of gal-3 and possibly that of other galectins [16]. GRG is currently being investigated and developed as a therapeutic in the oncology arena. Here, we demonstrate that GRG interacts with gal-1 over a large surface area on the polymer, which also decreased hydrophobicity, and hence increased aqueous solubility. Briefly, the crude commercially available citrus pectin (provided as solid crude powder of a kind typically used in food industry) was dissolved in water to pH 10 with 3 M NaOH. After incubation at 50°C for 30 min, 20% (v/v) ethanol was added, and the partially purified polysaccharide was precipitated to remove proteins and pigments. The polysaccharide was then dissolved to 20 g/litre in water, followed by addition of trifluoroacetic acid to a final concentration of 0.5 M for controlled de-polymerization in which the galactorhamnogalacturonan backbone is cleaved to the desired size. After incubation for 24 h at 60°C, the solution pH was adjusted to 4. The solution was cooled to 4°C and centrifuged (15 000 g for 60 min) to remove insoluble matter. The supernatant was then neutralized to a final pH of 8.0 with 1 M NaOH, and 20% ethanol was added to recover soluble polysaccharide. The resulting soluble branched polysaccharide product (GRG) was washed with 70% ethanol or with 100% acetone to provide a final dry powder.

**HSQC (heteronuclear single quantum coherence)**

**NMR spectroscopy**

Uniformly 15N-labelled gal-1 was dissolved at a concentration of 0.3 mM in 20 mM potassium phosphate buffer, pH 7.0, and 0.8 mM dithiothreitol, made up using a \( \text{H}_2\text{O}/\text{D}_2\text{O} (9:1) \) mixture. Using uniformly 15N-enriched gal-1, we performed HSQC NMR experiments to investigate binding of gal-1 to GRG [17]. \( ^{1}H \) and \( ^{15}N \) resonance assignments for the gal-1 have already been reported [17].

All NMR experiments were carried out at 30°C on a Varian Unity Inova 600 MHz spectrometer equipped with an H/C/N triple-resonance probe and \( x/y/z \) triple-axis PFG (pulsed-field gradient) unit. A gradient sensitivity-enhanced version of two-dimensional \( ^{1}H-^{15}N \) HSQC was applied with 256 \( (r1) \times 2048 \ (r2) \) complex data points in nitrogen and proton dimensions respectively. Raw data were converted and processed by using NMRPipe [18] and were analysed by using NMRview [19].

**PFG NMR self-diffusion measurements**

For NMR measurements, gal-1 was dissolved in 0.6 ml of 20 mM potassium phosphate buffer, pH 7.0, and 0.8 mM dithiothreitol, made up using \( \text{H}_2\text{O} \), and the pH was adjusted by adding microlitre quantities of \( \text{NaOH} \) or \( \text{HCl} \). PFG NMR self-diffusion measurements were made on a Varian INOVA-600 spectrometer using a GRASP™ (gradient-accelerated spectroscopy) unit as previously described [20]. NMR spectra for measurement of diffusion coefficients, \( D \), were acquired using a 5 mm triple-resonance probe
Figure 1 1H–15N HSQC spectra for gal-1 with and without GRG

HSQC spectra are shown for 15N-enriched gal-1 (1 mg/ml) alone (A) and at gal-1/GRG molar ratios of 20:1 (B) and 5:1 (C). Spectral expansions are provided below each plot to visualize better the observed resonance broadening as GRG is added to the gal-1 solution. Resonances in the expansion plot to (A) are labelled with assignments reported by Nesmelova et al. [17]. H1, 1H; N15, 15N.

RESULTS
Gal-1 binds to GRG

Figure 1(A) shows a 1H–15N HSQC spectrum of uniformly 15N-enriched gal-1, with cross-peaks labelled as assigned previously [17]. At this concentration (1 mg/ml; 70 × 10⁻⁶ M), gal-1 with a monomer ↔ dimer equilibrium dissociation constant (K_d) of about 2 × 10⁻⁶ M [4], is >99% dimer. As the concentration of GRG is increased, gal-1 resonances are differentially broadened, as exemplified by the 15N-enriched gal-1 HSQC spectra acquired at gal-1/GRG molar ratios of 20:1 and 5:1 (Figures 1B and 1C respectively). Differential broadening is better appreciated in the HSQC spectral expansions shown below each of the full spectra in Figure 1. Because all three HSQC spectra were collected, processed and plotted the same way, it is apparent that some cross-peaks have disappeared, some are greatly decreased in intensity and others appear relatively less affected. These data alone indicate that gal-1 interacts with GRG. Although the increased weight-average molecular mass(es) of the complex(es) formed between gal-1 and GRG likely contribute(s) somewhat to the observed broadening of gal-1 resonances, most of this effect is the result of exchange between or among gal-1 and binding sites on GRG that is occurring on the intermediate chemical-shift time scale [22].
These HSQC data also indicate that the folded structure of gal-1 dimer is not significantly affected by binding to GRG. We reached this conclusion because even though $^{1}H-^{15}N$ resonances are differentially broadened (intensities decreased) during the titration, the chemical shifts of resonances remaining during the titration are mostly unchanged.

Using our HSQC data, we could also estimate the gal-1–GRG binding affinity and stoichiometry by plotting the fractional change in gal-1 HSQC resonance intensities as a function of the concentration (mg/ml) of GRG (Figure 2A). Fractional changes were calculated by subtracting from 1 the intensity of a given HSQC cross-peak divided by that in pure gal-1 at the same protein concentration and with NMR data collected and processed the same way. The average of fractional changes for the 40 most broadened resonances is plotted as a function of the concentration (mg/ml) of GRG. Error bars represent S.D. The continuous line represents a sigmoidal (Boltzmann) fit using the general equation:

$$y = A \cdot e^{\frac{x - b}{c}}$$

where $y$ is fractional intensity, $A$ is the signal amplitude, $x$ is the concentration of gRG and $A$ and $b$ are variable fitting parameters) through the average value at each concentration point. The $\chi^2$ value for the fit was less than 0.0005. An apparent $K_d$ value was estimated at the point on this curve where 50% of the gal-1 molecules are bound to GRG. In this regard, the average fractional intensity change of 0.5 (50% bound) occurs at a GRG concentration of 1 mg/ml, which corresponds to an apparent macroscopic $K_d$ value of about $8 \times 10^{-8}$ M when a weight-average molecular mass of 120 kDa was used for GRG [9]. Furthermore, because gal-1 at 1 mg/ml has about $70 \times 10^{-6}$ M in gal-1 CRD equivalents (14500 Da), each average GRG molecule should saturate about five gal-1 CRDs. This binding stoichiometry of 5:1 (gal-1/GRG) would yield an average microscopic binding constant of about $40 \times 10^{-6}$ M/site.

**Figure 2** Gal-1–GRG binding curve and HSQC resonance broadening mapping

(A) The average of fractional changes for the 40 most broadened resonances is plotted against the concentration (mg/ml) of GRG. Error bars represent S.D. The continuous line represents a sigmoidal (Boltzmann) fit using the general equation $y = A \cdot e^{\frac{x - b}{c}}$ through the average value at each concentration point. The $\chi^2$ value for the fit was less than 0.0005. (B) This plot shows initial fractional changes in gal-1 resonance intensities observed at lower glycan/gal-1 molar ratios (where most gal-1 resonances are still apparent) against the amino acid sequence of gal-1 for GRG. A value of 1 indicates that the resonance associated with that particular residue is no longer apparent, and a value of zero indicates no change in resonance intensity.

Using these HSQC data, we also gained insight into where GRG interacts on the surface of gal-1. We do this in a way that is similar to HSQC chemical-shift mapping [23], which is performed when binding interactions occur in the fast or slow exchange regimes on the chemical-shift time scale. In these instances, resonances are chemically shifted, and little broadened, during the titration with ligand. In our case, gal-1 resonances initially may be shifted somewhat by interaction with GRG, but are primarily broadened due to the exchange process which falls in the intermediate exchange regime on the NMR chemical-shift time scale [22]. There are a number of factors that can contribute to a system falling into a particular NMR exchange regime. However, the general tendency is that as the lifetime of a complex is increased (i.e. binding becomes relatively stronger), the exchange regime on the chemical-shift time scale goes from fast to intermediate to slow. Because interactions occurring on the intermediate exchange time scale may not show discrete resonances, the way in which we present observed broadening effects is different from the way in which we would show $^{1}H$ and $^{15}N$ weighted average chemical-shift changes for a system in the fast or slow exchange regimes.

In the intermediate exchange regime, we show differential broadening at a molar ratio where most resonances are still observed, but are at lower intensities, owing to the broadening. We refer to this as ‘HSQC resonance broadening mapping’ in order to distinguish it from ‘HSQC chemical-shift mapping’. The interpretation is essentially the same as with HSQC chemical-shift mapping, i.e. those resonances that are initially broadened the most are associated with that site(s) on gal-1 that interacts with GRG. We create this map by taking the initial fractional changes in gal-1 HSQC resonance intensities observed at a low GRG/gal-1 molar ratio (where most gal-1 resonances are still apparent) and by plotting them against the amino acid sequence of gal-1. This is illustrated in Figure 2(B), where a value of 1 indicates that the resonance associated with that particular residue is no longer apparent (highly broadened), and a value of 0 indicates no change in resonance intensity (not broadened). From these data it is apparent which gal-1 residues are initially more affected than others.

Figure 3(A) highlights the most affected residues on the folded structure of gal-1 dimer. Resonances whose fractional intensity decrease is more than 0.8, or between 0.6 and 0.8 (Figure 2B), are highlighted in red and orange respectively. The image on the left illustrates the dimer surface with the lactose-binding sites (one per monomer) oriented at the top left and bottom right. For reference, lactose molecules are shown in blue at their previously determined binding sites [24]. Clearly, gal-1 residues at the quintessential lactose-binding site are affected by binding to GRG. However, many other gal-1 residues on this same surface are also significantly affected, indicating that GRG interacts with a larger area on this surface of gal-1. By contrast, GRG binding...
Galectin-1 binding to a galactorhamnogalacturonate glycan

is similar to that of the lactose-binding site and suggests the potential for similar types of protein–glycan interactions, namely hydrogen bonding and hydrophobic interactions, as well as electrostatic interactions between or among positively charged lysine residues (K28, K127 and K129) from gal-1 and negatively charged galactouronate carboxylate groups from the backbone of GRG.

**HSQC mapping with lactose**

Lactose is the quintessential ligand for any galectin. For this reason, we performed the same gal-1 $^{15}$N HSQC titration experiment with lactose. As lactose was titrated (1–10 mM) into solution, we observed that a number of gal-1 HSQC cross-peaks were significantly chemically shifted by the presence of lactose, with saturation being achieved by about 5 mM lactose. Figure 4(A) overlays two HSQC spectral expansions for pure gal-1 (black) and gal-1 plus 5 mM lactose (red). $^{15}$N–$^1$H weighted chemical-shift changes against the amino acid sequence of gal-1 are plotted in Figure 4(B). The simple average over all changes is 0.05 p.p.m. Although a good number of gal-1 residues are affected by lactose binding, those residues that are affected most (mean + 1 S.D.) are highlighted in red on the surface of the gal-1 dimer in Figure 4(C). (Here we used the same surface orientation as illustrated for GRG binding in Figure 3.) Other gal-1 residues that fall above the simple average are highlighted in orange. As expected, the most affected residues are around the lactose-binding site identified previously from X-ray-crystallographic studies [24], and these include many of the same residues affected by GRG binding to gal-1.

Interestingly, lactose binding also elicits an effect on residues at the back side of gal-1 (see Figure 4C), and these are among those also affected by GRG binding (see Figure 3A, right-hand image). Because these residues are relatively well removed from the lactose-binding site, it appears that lactose binding induces conformational changes, however minor, in other regions of the folded protein. This observation with lactose supports the idea that GRG binding to the front face of gal-1 (Figure 3A) does the same.

To determine the $K_d$ for lactose binding to gal-1, we plotted the fractional changes in $^{15}$N–$^1$H weighted chemical shifts for the 15 most shifted resonances as a function of the lactose concentration (Figure 4D). The continuous line represents a sigmoidal (Boltzmann) fit using the general equation $y = A - A_0 / (1 + e^{-c(x-b)})$, through the average value at each concentration point. The $x^2$-value for the fit was <0.0005. A microscopic $K_d$ value of $520 \times 10^{-6}$ M was determined from the lactose concentration at the fractional change of 0.5 (50% bound) and a statistical correction for the number of lactose binding sites, i.e. two in the dimer. Our $K_d$ value is similar to that of $330 \times 10^{-6}$ M reported from calorimetric studies for lactose binding to human gal-1 at 30°C [24] and somewhat higher than that reported for lactose binding to bovine gal-1 ($170 \times 10^{-6}$ M at 30°C) [25]. Nevertheless, these values are all in the same range, and any of these $K_d$ values for lactose binding to gal-1 is considerably greater than the average value we found for gal-1 binding to GRG. The difference in $K_d$ values is consistent with the significant resonance broadening we observed with gal-1 binding to GRG, placing the interaction in the intermediate exchange regime on the chemical-shift time scale [22]. In the case of lactose binding to gal-1, we observed that gal-1 resonances were merely chemically shifted during the titration with lactose, which is what one would expect with fast exchange on the chemical-shift time scale.
Figure 4  HSQC chemical-shift mapping for lactose binding to gal-1

(A) Two $^{15}$N–$^1$H HSQC spectra of gal-1 (1 mg/ml) are overlayed, one without lactose (black cross-peaks) and one with 5 mM lactose (red cross-peaks). Some of the cross-peaks have been labelled, as discussed in the text. Resonances are labelled with assignments reported by Nesmelova et al [17]. $^1$H, $^{15}$N, $^{15}$N–$^1$H. (B) $^{15}$N and $^1$H weighted chemical-shift changes between pure gal-1 and gal-1 upon addition of 5 mM lactose are plotted against the amino acid sequence of gal-1. The broken line indicates the simple average over all values. (C) Residues on the folded structure of gal-1 that have been most shifted by binding to lactose are highlighted in red and orange as discussed in the text. The X-ray structure of lactose-bound human gal-1 has been used in this Figure (Protein Data Base access code: 1gzw) [24]. The orientation at the left shows the face of the dimer where lactose binds. The orientation at the right shows the opposite side of the dimer where lactose binds. (D) The fractional change in gal-1 HSQC weighted chemical shifts is plotted against the concentration of lactose. The continuous line represents a sigmoidal (Boltzmann) fit using the general equation $y = A \cdot e^{x/b}$ through the average value at each concentration point. The $\chi^2$ value for the fit was $<0.0005$.

Gal-\(\beta(1 \rightarrow 4)\)-Gal binds gal-1

Even though we demonstrated that gal-1 binds GRG, the terminal motifs of GRG to which gal-1 most likely interacts are Gal-\(\beta(1 \rightarrow 4)\)-Gal. Galectin/saccharide-binding dogma, however, would say that gal-1 would not bind to a Gal-\(\beta(1 \rightarrow 4)\)-Gal motif, primarily because of the axial $^6$C hydroxy group on the reducing-end Gal unit. In lactose [Gal-\(\beta(1 \rightarrow 4)\)-Glc], the Glc $^4$C hydroxy group is equatorial. For this reason, we performed the gal-1 $^{15}$N HSQC titration with the disaccharide Gal-\(\beta(1 \rightarrow 4)\)-Gal and found that gal-1 HSQC cross-peaks are chemically shifted as they were upon titration with lactose, indicating interaction of Gal-\(\beta(1 \rightarrow 4)\)-Gal with gal-1.

Figure 5(A) indicates which residues in gal-1 are most affected upon binding Gal-\(\beta(1 \rightarrow 4)\)-Gal. If we correlate these chemical-shift changes with those from the binding of lactose (Figure 4B), we get a linear regression coefficient of 0.82 (Figure 5B, inset), which indicates that Gal-\(\beta(1 \rightarrow 4)\)-Gal interacts primarily at the same site on gal-1 as lactose. In Figure 5(B) we plot the fraction bound for the 20 most chemically shifted gal-1 resonances against the concentration of the disaccharide. For this, we assumed that saturation occurs by about 10 mM Gal-\(\beta(1 \rightarrow 4)\)-Gal, and set this value as a bound fraction of 1.0. The continuous line represents a sigmoidal (Boltzmann) fit using the general equation $y = A \cdot e^{x/b}$ through the average value at each concentration point. The $\chi^2$ value for the fit was $<0.0005$. A microscopic $K_d$ value of $1200 \times 10^{-6}$ M was determined at the fractional change of 0.5 (50% bound). This $K_d$ value is significantly greater than that for lactose ($520 \times 10^{-6}$ M), indicating weaker binding of Gal-\(\beta(1 \rightarrow 4)\)-Gal to gal-1.

From the glycan side

In our NMR experiments we actually observe an average effect from GRG glycans that vary in size [9] and from which we cannot get structural detail. Therefore, we performed PFG NMR diffusion experiments to derive $D$ values for insight into what occurs from the perspective of the glycan when it binds gal-1. For the most part, changes in $D$ values reflect changes in molecular size (apparent molecular mass and hydrodynamic radius) and/or solution viscosity due to intermolecular interactions among glycans. A smaller $D$ value indicates an increase in molecular size and/or viscosity and vice versa.

For these diffusion experiments we used a fixed concentration of GRG (4.6 mg/ml) and titrated gal-1 into the glycan solution. During the titration, $D$ values were acquired for the most intense GRG resonances, as identified in the $^1$H NMR spectral trace of the glycan preparation shown in Figure 6(A). If this were a pure single-size glycan, individual resonances would represent
different chemical groups within the GRG molecule and $D$ values derived from any given resonance should be the same or very nearly the same (e.g. differences in internal motions of glycan side chains could affect $D$). Here, this is not the case, because our GRG preparation is composed of heterogeneous galactorhamnogalacturonates, with a distribution of molecular sizes and a weight-average molecular mass of about 120 kDa [9]. Moreover, even though chemical shift is not a function of molecular mass, it is a function of, e.g. chemical composition, folding and supramolecular structure, i.e. anything that affects the chemical environment of a particular saccharide residue. Therefore, depending upon which GRG resonance, or even upon which point within a resonance envelope, $D$ values can and will vary because of both these factors and experimental error. It is for this reason that we measured diffusion-mediated decay curves for several of the GRG resonances.

To show data quality and give a feel for experimental error, Figures 6(B) and 6(C) show diffusion decay curves for two GRG resonances (e and f) as a function of gal-1 concentration. In the absence of gal-1, diffusion decay curves for these resonances appear linear (albeit with different slopes; results not shown), indicating the presence of either a single molecular-size component for each or any number of different glycan components with nearly the same molecular size. Decay curves become increasingly curvilinear as gal-1 is titrated into the GRG solution. If we deconvolute each decay curve into two components simply by approximating slopes for initial and final parts of the decay curves, we can estimate $D$ values for the fast and slow decay components. Broken lines are shown through the final six to eight points of the curves to indicate the slow decay components. Linear fits to these data points are very good, with regression coefficients greater than 0.9. In this regard, differences in $D$...
values are primarily the result of differences in apparent size of the glycan’s internal flexibility and solution viscosity, as opposed to experimental error. The fractional contribution of these slow components to the full decay curves was estimated from the Y-intercept of the linearly extrapolated curves (broken lines).

In Figure 7, D values derived from the slow decay components are plotted as a function of gal-1 concentration. Two types of trends are observed. For some resonances (a, c, e, and i), D values initially decrease upon addition of gal-1, and then they increase somewhat and remain essentially constant (Figure 7A). In the second set (f, g and h), D values merely increase and plateau upon addition of gal-1 (Figure 7B). For the first set, the initial decrease in D indicates an increase in molecular size of GRG upon binding gal-1, as opposed to an increase in solution viscosity. If solution viscosity were responsible for the initial decrease in D, the second set would have behaved similarly, and it did not. If anything, the immediate increase in D values in the second set indicates that solution viscosity is decreased and/or internal flexibility is decreased. Moreover, this trend in the second set suggests that GRG glycans associated with D values derived from resonances f, g and h either do not bind gal-1 or respond differently to interactions with gal-1.

The initial decrease in D occurs at gal-1 concentrations below 1 mg/ml, where gal-1 binding sites on GRG (4.6 mg/ml) are not fully occupied (vis-à-vis Figure 2). As the gal-1 concentration is increased above 1 mg/ml, more and more binding sites on GRG become occupied such that the apparent molecular size of the complex should increase, and yet we observe an increase in D values, indicating the opposite. There are two likely explanations for this apparent conundrum: (1) gal-1 binding disrupts the extensive inter-glycan networks that contribute to the normally high solution viscosity of these polysaccharide solutions; and (2) the hydrodynamic radius of the glycan is decreased by gal-1 binding. In either case, gal-1 binding appears to alter GRG conformation and perturb inter-glycan interactions. Note that, upon gal-1 binding, the fraction of the GRG slow decay component is decreased and tends towards a limiting value (Figure 7C), consistent with saturation of the gal-1-binding sites on GRG (Figures 2 and 7A/7B). Some slow-component fractions are decreased more than others, suggesting different extents of the effect(s) from gal-1 binding.

This gal-1-induced re-organization or conformational change of the GRG glycan network apparently leads to release of lower-molecular-size glycan species, as evidenced by trends in D values derived from the fast components of the decay curves (Figure 8A). If we assume that only two glycan components are represented in each curvilinear decay curve, then the fraction of the fast component can be estimated by subtracting from one the fraction of the slow component (Figure 7B), as plotted in Figure 8(B). During the titration with gal-1, the fraction of the fast component increases rapidly up to the gal-1 saturation point (about 1 mg/ml) and then tends to level off at anywhere from about 20 to 50% of the total glycan species reflected in D values derived from that resonance. On the basis of apparent D values, we estimate that molecular sizes of galectin-mediated GRG-released glycans range from about 20 to 200 saccharide units, compared with 500 to 2000 units prior to addition of gal-1 [9]. The dotted line labelled ‘decasaccharide’ in Figure 8(A) was drawn as a point of reference, because a decasaccharide in dilute solution would have a D value around 2 × 10^{-6} cm^2/s [9]. It is important to note here, however, that because our GRG glycans are not in dilute solution, actual D values will be lower, and changes in D values correlate better with changes in hydrodynamic radii than with molecular mass [9]. It is for this reason that we have provided a very wide range in our estimates of the possible number of saccharide units stated above. Nevertheless, these ranges are reasonable. Furthermore, because gal-1 at these concentrations is a dimer with a molecular mass of 29 kDa, it is unlikely that gal-1 binds to the smallest glycans with D values near to, or larger than, that for the gal-1 dimer (D = 1.04 × 10^{-6} cm^2/s). If gal-1 were to bind to these GRG glycans, the D values would be significantly lower by the end of the titration, and they are not.

On the basis of these data, we estimate a binding stoichiometry of 6:1 (gal-1/GRG) by assuming that saturation of GRG (37 × 10^{-6} M) with gal-1 occurs at a gal-1 concentration of about 240 × 10^{-6} M (Figures 7 and 8). This stoichiometry is essentially the same as that derived from our HSQC binding curve (Figure 2A), which indicated a binding stoichiometry of about 5:1 (gal-1/GRG). Nevertheless, the average microscopic dissociation constants for gal-1 binding to GRG derived either way would be essentially the same, i.e. 48 × 10^{-6} M  at 6:1 compared with 40 × 10^{-6} M at 5:1. This indicates that gal-1 binds to sites on GRG about 10-fold more strongly than it does to lactose.
residues that could potentially form hydrogen bonds with polar groups from the glycan. In addition, negatively charged groups are absent from this domain, whereas positively charged amino acid residues are present for potential electrostatic interactions with negatively charged galacturonate carboxylate groups in the backbone of GRG, as has been reported to occur with the binding of sulfated glycosaminoglycans to platelet factor-4 or sulfated galactosaminoglycans to other galectins [29,30].

Although we do not know which specific groups on GRG are involved in interactions with gal-1, galectins are generally known for their ability to bind to β-galactosides [1]. GRG is composed of about one-third galactose units, which are in either α(1 → 4) or β(1 → 4) anomic linkages. However, most of these are found in the glycan backbone, mainly as sections of α(1 → 2)-L-rhamnosyl-α-(1 → 4)-d-galacturonosyl, and it is unlikely that these galactose units would be accessible to bind to gal-1, as, for example, branched saccharide side chains would likely interfere sterically with the interaction. On the other hand, GRG contains terminal β(1 → 4)-galactose units, which we propose are likely to be involved in interactions with gal-1. This suggestion, however, poses a problem in that these terminal units exist in a Gal-β(1 → 4)-Gal motif to which gal-1 should not bind because, unlike lactose [Gal-β(1 → 4)-Glc], the reducing-end saccharide (galactose) has an axial (not equatorial) C4 hydroxy group. Nevertheless, our HSQC data demonstrate that Gal-β(1 → 4)-Gal does bind at the gal-1 lactose-binding site, albeit not as strongly. Manual docking of Gal-β(1 → 4)-Gal into the lactose-binding site on gal-1 in silico suggests that this galactose C4 axial hydroxy group would be sterically hindered only by the side chain of R48 (Arg48) from gal-1, and that a conformational change in the R48 side chain could 788.0

**DISCUSSION**

We report here several findings that increase our understanding of how gal-1 interacts with larger, more complex, glycans, in the present case with a pectin-derived GRG, galactorhamnogalacturonate. First of all, we found that gal-1 binds relatively strongly to GRG glycans (microscopic $K_d = 40 \times 10^{-6}$ M), more so than it does to lactose ($K_d \approx 520 \times 10^{-6}$ M). The multivalent nature of GRG (five or six gal-1 CRDs per average glycan molecule) contributes to this greater affinity or avidity for gal-1.

The corresponding free energies for binding of GRG glycans and of galactorhamnogalacturonate (GRG) are 6.08 kcal/mol and 4.54 kcal/mol (1 kcal = 4.184 kJ) respectively, which (assuming a single mode of interaction) reflect the greater surface area on gal-1 with which GRG interacts, approx. 1600 Å² (1 Å = 0.1 nm) compared with 700 Å² for the much smaller disaccharide lactose.

The larger binding region on gal-1 GRG not only includes residues of the lactose-binding domain, but extends from there through a broad valley or cleft towards the dimer interface. From the overall dimensions of the protein surface, we estimate that this binding domain could accommodate about eight to ten saccharide units, a number that is consistent with the up to 20 or so saccharide units present in any of the multiple oligosaccharide branches of GRG. Moreover, the amino acid composition of the binding domain is consistent with promotion of protein–glycan interactions, as it contains a significant number of polar

![Figure 8](image-url)  
**Figure 8** D values for GRG fast component  
(A) D values for the fast component of the deconvoluted diffusion decay curves are plotted against gal-1 concentration. The dotted line labelled ‘decasaccharide’ was drawn as a point of reference, because a decasaccharide in dilute solution would have a D value of about 2 × 10⁻⁹ cm²/s [9]. (B) Estimation of the fraction of the fast decay component by linearly extrapolating the slow component line to the Y-intercept is shown. This estimation assumes that the decay curve is composed of only two glycan fractions.
increases in others, but nevertheless eventually increases in all cases as gal-1 is titrated into the GRG solution. Since we know that gal-1 interacts with GRG, the most reasonable explanation for increasing $D$ values is that gal-1 binding decreases the apparent solution viscosity, which most likely occurs via attenuation of inter-glycan interactions. Although it is unclear how this occurs at the molecular level, we suggest that gal-1 binds saccharide groups that would otherwise be involved in intermolecular inter-glycan interactions and that gal-1 binding somehow re-arranges glycan molecules to promote their dissociation. A common analogy would be decongesting a traffic jam or nasal blockage.

Gal-1-mediated glycan decongestion may be biologically relevant. Glycans, in one form or another (glycoproteins or glycolipids), are a major component of any cell-surface micro-environment, and the glycan concentration within micro-environments can be quite high. In fact, we can estimate the glycan concentration in situ. At least for engineered glycan-containing mucins in a plasma membrane, cell-surface packing density is about 50 molecules/μm² [40,41]. Assuming that the glycan moieties of each mucin extend about 100 × 10⁻¹⁰ m from the membrane surface to define the volume of each molecule, we would have $8 \times 10^{-12}$ mol per $1 \times 10^{-17}$ litre, or a concentration of about $8 \times 10^{-8}$ M. Considering that there are numerous glycoproteins on a typical cell surface, this estimate seems reasonable and may even be low, because glycoproteins are not generally homogeneously dispersed on the cell surface (as assumed in our estimation) and are often found in plasma-membrane microdomains [42,43]. Therefore, the concentration of some glycoproteins (and their associated glycans) can be relatively high, possibly in the micro- to milli-molar range, as used here in vitro with GRG.

The functional relevance of galectin-mediated glycan decongestion is further supported by the observation that gal-1 interactions with cell-surface glycans increase membrane fluidity. Gupta et al. used EPR spectroscopy to demonstrate that gal-1 (in a concentration-dependent manner from 1.1 to 4.4 μM) increases erythrocyte membrane fluidity up to a factor of about 3-fold [44]. In this regard, cell-surface viscosity should be decreased, as fluidity is inversely correlated with viscosity. Increased membrane fluidity may be necessary for cell-surface glycoproteins to reorganize within the plasma membrane, as is required, for example, for cell adhesion [45]. Confocal microscopy has demonstrated that, upon exposure to gal-1, CD45 and CD43 receptors cluster on the cell surface of MOLT-4 cells (leucocytes) within 20 min upon exposure to gal-1 [43]. A similar observation has been made with gal-3, which associates and forms clusters when it interacts with cell-surface glycans, thereby explaining its ability to mediate reorganization of cell-surface glycoproteins [46]. In these studies, cell-surface glycoprotein re-organization and clustering are triggered by gal-1–glycan (glycoconjugate) binding and, given the limited time scale over which this process occurs, the phenomenon is likely primarily physically driven. A number of other studies have also proposed that binding of various galectins (i.e. gal-1, -3, -4 and -9) to specific cell-surface glycoproteins contributes to plasma-membrane microdomain assembly and/or maintenance [47–52].

In the light of our present findings, we propose that cell-surface re-organization of glycoconjugates into plasma-membrane microdomains may be promoted by galectin-mediated glycan decongestion. Even though the nature of inter-glycan interactions (specific or non-specific) among various glycoconjugates on the cell surface is unknown, these interactions do occur and would likely attenuate lateral diffusion of glycoproteins and glycolipids within the cell membrane. Galectin interactions with cell-surface glycoproteins may then promote glycan decongestion, increase membrane fluidity and thereby allow glycoconjugate re-organization within the cell membrane to occur.

Conclusions

In the present study we used NMR spectroscopy to investigate interactions between gal-1 and a heterogeneous galactorhamnogalacturonate, GRG. We found that GRG binds to gal-1 more strongly than lactose, and over a broader area on the protein surface, which runs from the quintessential lactose-binding site through a broad valley towards the dimer interface. This phenomenon expands our view of how galectins in general may interact with glycans in situ, and this may play a role in determining and/or differentiating galectin function. Moreover, we found that gal-1 binding to the glycan acts to decongest inter-glycan interactions. Because of this finding, our cell-surface glycan decongestion hypothesis may be biologically relevant in that glycan decongestion could promote re-organization of cell-surface glycoconjugates into plasma-membrane microdomains. Overall, our results provide an insight into galectin function in situ and may help explain how gal-1 mediates cell–cell and cell–matrix adhesion and migration.

AUTHOR CONTRIBUTION

Michelle Miller designed, conducted and analysed the HSQC NMR and diffusion experiments and wrote the paper. Irina Nesmelova performed the HSQC NMR experiment with gal-1 and lactose and helped write the paper. David Platt and Anatole Klyosov helped prepare the glycan GRG and write the paper. Kevin Mayo designed and analysed the NMR experiments, supervised the overall work and wrote the paper.

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