Structural aspects of binding of $\alpha$-linked digalactosides to human galectin-1


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By definition, adhesion/growth-regulatory galectins are known for their ability to bind $\beta$-galactosides such as Gal(1→4)Glc (lactose). Indications for affinity of human galectin-1 to $\alpha$-linked digalactosides pose questions on the interaction profile with such bound ligands and selection of the galactose moiety for CH-$\pi$ stacking. These issues are resolved by a combination of $^{15}$N-$^{1}$H heteronuclear single quantum coherence (HSQC) chemical shift and saturation transfer difference nuclear magnetic resonance (STD NMR) epitope mappings with docking analysis, using the $\alpha$(1→3/4)-linked digalactosides and also Galα(1→6)Glc (melibiose) as test compounds. The experimental part revealed interaction with the canonical lectin site, and this preferentially via the non-reducing-end galactose moiety. Low-energy conformers appear to be selected without notable distortion, as shown by molecular dynamics simulations. With the $\alpha$(1→4) disaccharide, however, the typical CH-$\pi$ interaction is significantly diminished, yet binding appears to be partially compensated for by hydrogen bonding. Overall, these findings reveal that the type of $\alpha$-linkage in digalactosides has an impact on maintaining CH-$\pi$ interactions and the pattern of hydrogen bonding, explaining preference for the $\alpha$(1→3) linkage. Thus, this lectin is able to accommodate both $\alpha$- and $\beta$-linked galactosides at the same site, with major contacts to the non-reducing-end sugar unit.

Keywords: agglutinin / glycolipid / glycoprotein / lectin / sugar code

Introduction

Endogenous lectins translate sugar-encoded information of glycan chains of natural glycoconjugates into cellular responses (for recent reviews, see Gabius 2009). The family of the adhesion/growth-regulatory galectins share a common fold that promotes affinity to substituted $\beta$-galactosides and whose binding at the cell surface triggers a cascade of signaling events that depend on the type of counter-receptor (Barondes et al. 1994; Kasai and Hirabayashi 1996; Cooper 2002; Villalobo et al. 2006; Garner and Baum 2008; Klyosov et al. 2008; Dhirapong et al. 2009). Looking at the proto-type homodimeric galectin-1 (gal-1), extracellular matrix glycoproteins such as fibronectin or laminin, integrins such as the fibronectin receptor, functional markers for cell typing such as CD2, CD3, CD7, CD43 or CD45, and glycolipids such as ganglioside GM1 are specific targets for this effector (Pace and Baum 1997; Gabius 2006). The ensuing interaction, e.g. with CD7 on activated T cells, the $\alpha$5$\beta$1-integrin on susceptible carcinoma cells or ganglioside GM1 on effector T cells, will lead to a negative effect on cell growth (Pace et al. 2000; Fischer et al. 2005; Wang et al. 2009; Sanchez-Rudelsch et al. 2011). At the level of the binding site for glycans, structural investigations therefore have so far been focused on $\beta$-galactosides, mostly the pan-galectin binder lactose (Gal(1→4)Glc), along with other compounds in this class including $N$-acetylactosamine oligomers, lactose mimetics such as its C-linked derivative and the branched pentasaccharide of ganglioside GM1 (Bourne et al. 1994; Asensio et al. 1999; Alonso-Plaza et al. 2001; Siebert et al. 2003; López-Lucendo et al. 2004; Stowell et al. 2004; Nesmelova et al. 2010). However, competitive binding studies have indicated that galectin-1 can, at least to a certain extent, interact with $\alpha$-linked digalactosides as well, especially Galα(1→3)Gal (Sparrow et al. 1987; Ahmed et al. 1990; Lee et al. 1990; Appukuttan et al. 1995; Appukuttan 2002; Hirabayashi et al. 2002; Rapoport et al. 2008). This linear glycan terminus is known as the xenoinherent present in glycolipids and glycoproteins (Macher and Galili 2008). The shift to the $\alpha$(1→4) linkage in galabiose and the establishment of the $\alpha$(1→6) linkage in melibiose (Galα(1→6)Glc) appeared to produce less active compounds (Sparrow et al. 1987; Ahmed et al. 1990; Lee et al. 1990). These results make it attractive to address the issue of the...
binding mode of α-linked digalactosides to human ga-1. Toward this end, we applied a strategy that combines NMR spectroscopic techniques and molecular docking.

In detail, we used 1H–15N HSQC and STD NMR spectroscopy together with molecular dynamics (MD) simulations to investigate structural aspects of ga-1 binding of the two α-linked digalactosides Galα(1→3)Gal and Galα(1→4)Gal as well as of melibiose. Our study demonstrates that these α-Gal-containing disaccharides that differ in their linkage points (i.e. α(1→3); α(1→4); α(1→6)) can all bind to ga-1 at the site of contact for lactose. Of the two sugar moieties, the non-reducing-end Gal unit in the α(1→3/6) anomers is selected for stacking with the Trp residue in the binding site, whereas this interaction appears to be preclude with the α(1→4) anomer. Moreover, processing binding data by a two-site model reveals differences in the binding mode depending on linkage points, i.e. non-cooperatively or with positive cooperativity. Overall, these findings characterize binding of linear α-galactosides to ga-1 structurally and broaden our view on ga-1/glycan interactions.

Results

**HSQC analysis**

Our 1H–15N HSQC data indicate that the two α-linked digalactosides and melibiose all bind to ga-1 at the canonical site with the central Trp (W68) residue. Evidence for this is exemplified in Figure 1, with HSQC spectral expansions of

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**Fig. 1.** Expansions of 1H–15N HSQC spectra for 15N-labeled ga-1 in the absence (black cross-peaks) and presence (red cross-peaks) of α-linked saccharides and lactose. Some cross-peaks have been labeled as discussed in the text. (A) ga-1 ± 10 mM Galα(1→3)Gal; (B) ga-1 ± 10 mM Galα(1→4)Gal and ga-1 ± 10 mM Galα(1→6)Glc and (D) ga-1 ± 10 mM Galβ(1→4)Glc (lactose). 15N-Labeled ga-1 and ligands were dissolved in an aqueous (90% 1H2O/10% 2H2O) solution containing 20 mM potassium phosphate, pH 7, and 2 mM DTT, at 30°C.
$^{15}$N-gal-1 in the presence (red cross-peaks) and the absence (black cross-peaks) of Gal(α1→3)Gal (Figure 1A), Gal(α1→4)Gal (Figure 1B), Gal(α1→6)Glc (Figure 1C) as well as lactose for comparison (Figure 1D). Resonances shown in these HSQC expansions are identified and marked using the assignments published recently (Nesmelova et al. 2008). In all cases, $^{15}$N-gal-1 HSQC resonances are chemically shifted to a similar extent, an observation that is best illustrated with chemical shift maps that plot $^1$H- and $^{15}$N-weighted chemical shift changes, $\Delta \delta$, vs residue number (Rajagopal et al. 1997). These $\Delta \delta$ values are shown in Figure 2 for both backbone (Figure 2A–D) and side-chain (Figure 2E–H) NH groups. For the most part, the largest $\Delta \delta$ values are observed for the same backbone NH residues, i.e. 14–17, 29–31, 44–58, 66–78 and 90–95, which are located either at the lactose-binding site or are affected by lactose binding, as reported previously (Miller, Klyosov, et al. 2009; Miller et al., 2009, Nesmelova et al. 2010). For side-chain NHs, $\Delta \delta$ values are generally rather similar, with the largest shift changes usually arising from N46, R48, N56, N61, W68 and R73. Because the W68 side chain interacts directly with lactose at the ligand binding site, we illustrated ligand-induced chemical shift changes for W68 backbone and side chain NH resonances in Figures 1 and 2, respectively, as labeled.

Linear regression analysis of $\Delta \delta$ correlation plots for any pair of these sugars indicates a relatively high degree of correlation, as exemplified in Figure 3, which plots backbone NH $\Delta \delta$ correlations for the three α-linked disaccharides relative to lactose. Resulting correlation coefficients, $R^2$, are 0.88 for Galα(1→6)Glc (Figure 3A), 0.85 for Galα(1→3)Gal (Figure 3B) and 0.79 for Galα(1→4)Gal (Figure 3C). Moreover, when comparing all disaccharides with each other, $R^2$ values are even higher, falling generally in the range of 0.89–0.93. These relatively high $R^2$ values indicate that the three α-linked disaccharides all bind to gal-1 in a similar fashion, compared with lactose. Of note, there are differences in the chemical shift maps that reflect variations in how each disaccharide interacts with gal-1. This is especially true for binding of Galα(1→4)Gal.

Figure 3D illustrates the structural/spatial relationships among these sequences, where the most significant changes in the $\Delta \delta$ values for any of these ligands are highlighted on the surface of the β-sandwich fold of a gal-1 subunit (López-Lucendo et al. 2004). For lactose-loaded gal-1, a stick structure of this disaccharide is shown in blue. The most affected residues are around the lactose-binding site and within various loops. In this context, the 66–76 loop is proximal to the 110–116 loop, which in turn is proximal to both loops 90–95 and 14–17. In fact, G14 is in the van der Waals contact with D92. Other residues at a distance from saccharide-binding site are also affected, as was previously reported for binding of lactose to gal-1 (Nesmelova et al. 2010).

While binding of Galα(1→3)Gal, Galα(1→4)Gal and Galα(1→6)Glc to gal-1 also involves these residues, it does so with characteristic profiles when compared with lactose (Figure 2). For example, S62 and R111–L112 were considerably more perturbed upon binding of Galα(1→4)Gal, whereas proximal residues R48 and V76 were more affected upon binding of Galα(1→6)Glc. On the other hand, residues R111 and L112 were only minimally perturbed upon binding to lactose. Since

![Fig. 2. HSQC chemical shift mapping for disaccharide binding to gal-1. $^{15}$N- and $^1$H-weighted chemical shift changes, $\Delta \delta$, between gal-1 (no presence of ligands) and gal-1 in the presence of disaccharides are plotted vs the amino acid sequence of gal-1. $\Delta \delta$ values are shown for backbone NH groups (A–D) and for side-chain NH groups (E–H). (A and E) gal-1 ± 10 mM Gal(1→3)Gal; (B and F) gal-1 ± 10 mM Gal(1→4)Gal; (C and G) gal-1 ± 10 mM Galα(1→6)Glc and (D and H) gal-1 ± 10 mM Galα(1→4)Glc (lactose). Inserts in (E)–(H) show expansions from $^{15}$N-$^1$H HSQC spectra for ligand-induced chemical shift changes of the W68 HN group, as labeled on one insert at the top. The x- and y-axes are as labeled in Figure 1, with black and red cross-peaks for $^{15}$N-gal-1 acquired in the presence and absence of α-linked saccharides and lactose.

![Diagram of gal-1 structure](http://gycob.oxfordjournals.org/)

Downloaded from http://gycob.oxfordjournals.org/ at CSIC on February 7, 2012

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the non-reducing galactose unit is the common for all disaccharides investigated here, differences in binding should be accounted for by linkage points, the anomeric linkage and/or the nature of the sugar moiety at the reducing end.

These significant ligand-induced differences at the level of the gal-1 backbone and side-chain groups also reflected changes in apparent strength of ligand binding. We quantified this by plotting $\Delta^\delta$ for the 20 most shifted resonances vs the disaccharide concentration, as shown in Figure 4A–C. Using the procedure described under Materials and methods (Determination of the binding constants by NMR), we performed the Monte Carlo fits to these titration curves (treating $^1$H and $^{15}$N resonances separately, i.e. 40 curves total for each ligand) to obtain association equilibrium binding constants. Because we previously reported NMR-based evidence that processing such gal-1 binding data with a two-site model indicated positive cooperativity for lactose (Nesmelova et al. 2010), we employed a binding model parameterized with two binding constants, $K_1$ and $K_2$, one for each binding site in the gal-1 homodimer (D). Residues in the folded structure of gal-1, whose signals have been shifted significantly by binding of any of these ligand, are colored in red (over 2 SD), orange (1–2 SD) and pink (0.5–1 SD) to illustrate the extent of change by color coding. The X-ray structure of lactose-loaded gal-1 has been used as the platform for this figure (PDB access code: 1gzw; López-Lucendo et al. 2004). The bound lactose molecule is shown in black.

Epitope mapping

For insight into the binding epitope on these disaccharides, we employed the STD NMR technique, using the $\alpha$/

![Fig. 3.](http://glycob.oxfordjournals.org/)

Correlation plots for $^{15}$N- and $^1$H-weighted chemical shift changes, $\Delta^\delta$, are plotted for $\Delta^\delta$ values for $\alpha$-linked disaccharides vs lactose (Galβ(1 → 4)Glc): (A) gal-1 ± 10 mM Galβ(1 → 3)Gal, (B) gal-1 ± 10 mM Galβ(1 → 4)Gal and (C) gal-1 ± 10 mM Galβ(1 → 6)Glc. The largest $^{15}$N-$^1$H-weighted chemical shift changes between gal-1 (no ligands) and gal-1 in the presence of any of these disaccharides are highlighted in the structure of one subunit from the gal-1 homodimer (D). Residues in the folded structure of gal-1, whose signals have been shifted significantly by binding of any of these ligand, are colored in red (over 2 SD), orange (1–2 SD) and pink (0.5–1 SD) to illustrate the extent of change by color coding. The X-ray structure of lactose-loaded gal-1 has been used as the platform for this figure (PDB access code: 1gzw; López-Lucendo et al. 2004). The bound lactose molecule is shown in black.
β-galactoside-binding VAA as a positive control. Binding constants of $1.87 \times 10^3$ M$^{-1}$ ($1.1 \times 10^3$ M$^{-1}$) and $40.7 \times 10^4$ M$^{-1}$ had been found by titration calorimetry for lactose and Galα(1 → 4)Gal, respectively (Bharadwaj et al. 1999; Jiménez et al. 2006), and this high affinity for the α(1 → 4)-linked digalactoside was confirmed by binding assays (Galanina et al. 1997). Under the conditions tested, the Tyr site in the 2γ subdomain is mostly active (Jiménez et al. 2006). VAA basically recognizes the non-reducing terminal galactose moieties, although the nature of the penultimate sugar unit can slightly alter the binding affinity, as revealed by monitoring of different galactosides, disaccharides and β-lactoside derivatives (Lee et al. 1992; Bharadwaj et al. 1999; Alonso-Plaza et al. 2001; Jiménez et al. 2008; André et al. 2010). Overall, our STD NMR results on the disaccharide/VAA systems were used as control models run in parallel with gal-1 experiments using α-linked sugars. In fact, the importance of the non-reducing-end Gal unit provided a standard for the STD-data interpretation (Ribeiro et al. 2010).

STD NMR spectra for VAA and gal-1 are exemplarily shown for the disaccharides Galα(1 → 3)Gal and Galα(1 → 4)Gal in Figure 5A and B, respectively. In each instance, the spectrum at the top is the NMR spectrum for the sugar in the absence of lectin, then the spectrum of the disaccharide in the presence of VAA follows, and the bottom spectrum is for the ligand in the presence of gal-1. For each carbohydrate resonance, the magnitude of the STD effect is given as the ratio of signal intensities from the sugar with or without gal-1.
appears to be the major epitope for recognition by gal-1, as it
key interacting groups are indicated by the size of grey spheres.

In all instances (see Tables I–IV for compilation of signal
intensities), the non-reducing Gal moiety of each disaccharide
appears to be the major epitope for recognition by gal-1, as it
is with VAA. As further control using melibiose, the STD
spectra show that exclusively the non-reducing end (i.e. Gal)
is recognized. Clear STD signals were only observed for this
residue. The STD pattern also is in accord with the differential
tolerance of the two lectins to the two types of sialylation
(α2 → 6 for VAA, α2 → 3 for gal-1) by shifting the major
STD intensities from H4′–H6′ for gal-1 toward H2′–H4′
for VAA. In the case of the human lectin, in addition to the major
STD effects observed for the non-reducing end, interaction
also involves the ligands’ reducing ends. In fact, with
Galα(1 → 3)Gal, subtle differences between VAA and gal-1
Table III. STD intensities of the Galα(1→4)Galα1/4 and Galα(1→4)Galα1/melibiose complexes

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<th>H1α (%)</th>
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<td>H4α</td>
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<td>H5α</td>
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<td>H6α</td>
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Table IV. STD intensities of Galα(1→6)Glcα1/4 and Galα(1→6)Glcα1/melibiose complexes

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<td>H3α</td>
<td>69%</td>
<td>H3β</td>
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<tr>
<td>H4α</td>
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<td>H5α</td>
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<td>H6α</td>
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are evident around the reducing anomeric proton, especially H4 at the reducing end. In contrast, studying Galα(1→4)Gal differences are noted around the reducing H1α proton, whose resonance is significantly more intense in the presence of gal-1 than of VAA. These data furnish experimental input, too, for the computational simulations, which will develop molecular models of the bound state.

**MD simulations**

For additional insight into the molecular recognition process, we performed MD simulations on the gal-1 monomer and these α-disaccharides. In all cases, we started with a docking protocol for each disaccharide by focusing on the carbohydrate recognition site using the X-ray crystal structure of gal-1 (PDB access code 1gzw). For Galα(1→3)Gal and Galα(1→4)Gal, the most optimal binding energies and belonging to the most populated clusters were selected for MD simulations. Other initial poses were also considered, but they were not consistent with the experimental STD data see section on epitope mapping. For Galα(1→6)Gal, two different starting complexes with different relative orientations of the two rings with respect to the protein were chosen. These corresponded to the two most populated clusters found in the docking protocol. For Galα(1→4)Gal, one additional starting geometry was considered with the ligand positioned at a recently reported non-lactose interaction domain, i.e. the α-galactomannan Davanat binding region (Miller, Klyosov, 2009). All simulations were run for 3 ns following an equilibration period of 100 ps. During all simulations, protein structures were stable, and the disaccharides remained at the binding site without diffusing into the solvent. Figure 6 shows the energetically most favorable structures for all three α-disaccharides when bound to gal-1 (Figure 6B and C), as well as for lactose (Galβ(1→4)Glc) (Figure 6D). On this basis, Figure 7A–D illustrates the binding modes with a more detailed view on the ligand-binding pockets. Additional information from these simulations is provided in the Supplementary Section which shows RMSD and torsion angle variations from the trajectories, along with structural illustrations depicting hydrogen-bond interactions and carbohydrate–aromatic residue contacts, as discussed under epitope mapping.

During the simulation with the Galα(1→3)Gal anomer, glycosidic torsional angle variance indicated interconversion between conformers. In this regard, the Φ angle always remained negative, in agreement with the exo-anomeric effect (Batchelor et al. 2001), and different conformers coexisted with distinct Ψ angle values (either negative or positive), each remaining within the minimum energy region for the disaccharide. Apparently, the energetically favored conformation of free Galα(1→3)Gal is not altered by interaction with gal-1, and even though the shape of Galα(1→3)Gal adopted different geometries during the MD trajectory, variations were

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**Fig. 6.** From MD simulations, the most energetically favorable monomer subunit structure of gal-1 bound to each of these disaccharides is illustrated: Galα(1→3)Gal (A), Galα(1→4)Gal (B), Galα(1→6)Glc (C) and Galβ(1→4)Glc (D). From HSQC chemical shift mapping, the largest 13N-1H-weighted chemical shift changes (over 2 SD) between pure gal-1 and gal-1 in the presence of each of these disaccharides are highlighted in blue on each of these gal-1 structures, as discussed in the text.
fairly subtle. In fact, these fluctuations appeared to be primarily related to transient intermolecular interactions with residues of gal-1. Also, during the course of the simulation, it appears that, apart from the typical hydrogen bond between H44 and Gal O4′ (which defines the recognition of galactosides by galectins), only one additional hydrogen bond persisted, i.e. the one between N61 and Gal O6′, several other hydrogen bonds forming transiently between both Gal residues and gal-1 (see Supplementary Section).

Examining the impact of anomericity, the α-glycosidic linkage produced a dramatic variation in the sugar–protein hydrogen-bond pattern at the reducing end, in comparison with that observed for lactose. In the case of this model sugar, there are strong hydrogen bonds between Glc O3 with Glu71 and Arg48 (López-Lucendo et al. 2004). Due to the presence of an α-linkage (rather than the β-linkage) and the 1 → 3 connection, the orientation of the reducing-end residue was rather different, and only transient hydrogen bonds were noted between E71 and hydroxyl groups OH1 and OH2 of this sugar. Various interactions are indicated in the structures shown in Figures 6A and 7A, and others are illustrated in material provided in the Supplementary Section. As opposed to lactose (Figures 6D and 7D), e.g. R48 now remains far from the α-linked galactose residue.

Aside from forming hydrogen bonds, gal-1 also establishes CH–π interactions, shown in the crystal and in solution (Siebert et al. 1997; López-Lucendo et al. 2004). They are formed between the apolar B-face of the core galactose and the indole ring of the suitably positioned Trp residue (W68 for gal-1). In quantitative terms, the frequency of occurrence of this interaction during the MD simulations appeared to be smaller than for lactose. Taken together, our MD results, in accordance with the epitope mapping, define a structural model for binding of α-galactosides, also being consistent with the observed weaker binding of Galα(1 → 3)Gal to gal-1, compared with lactose, determined experimentally, in the range of 10–25-fold in competitive binding assays (Sparrow et al. 1987; Ahmed et al. 1990).

For Galα(1 → 4)Gal, glycosidic torsion angles and the overall conformation also remained fairly stable during the trajectory, with average Φ and Ψ torsion angles of ca. −38° and 0°, respectively, and small fluctuations. In particular, the
Ψ-angle displayed either positive or negative values around the zero value. Overall, both angles sampled an energetically well-defined area of the $\Phi$, $\Psi$-based conformational map (always within the lowest energy region), characteristic for two major conformers. Evidently, energetically privileged conformers are selected for binding, as is common for various types of lectins (Gabius et al. 2011), and for gal-1 binding disaccharides, a branched pentasaccharide or highly flexible $C$-lactose (Asensio et al. 1999; Alonso-Plaza et al. 2001; Siebert et al. 2003). Concerning hydrogen bonding, again, the observed pattern is different from that observed for lactose. Indeed, the orientation of the disaccharide within the binding site is rather different, with the non-reducing end now pointing out of the lactose-binding site. This $\alpha$-anomeric linkage precluded proper positioning of the non-reducing end at the lactose site, and no stacking with W68 appears to take place. Moreover, the non-reducing Gal moiety of Gal$\alpha(1 \rightarrow 4)$Gal does not appear to form stable polar interactions with gal-1, not even the typical one which involves Gal' O4 and H44. In contrast, H44 appears hydrogen bonded to Gal O1, while two hydroxyl groups of the reducing galactose (O2, O3) appear engaged in transient intermolecular hydrogen bonds with N61 and E71. Therefore, for this disaccharide, there seem to be few stabilizing intermolecular interactions (see Figures 6B and 7B, and material provided in the Supplementary Section), consistent with the relatively weak binding affinity detected experimentally.

Melibiose (Gal$\alpha(1 \rightarrow 6)$Glc) is, in principle, more flexible than the two digalactosides with either $\alpha(1 \rightarrow 3)$ or ($\alpha1 \rightarrow 4$) linkage, due to the presence of an additional torsional degree of freedom, i.e. the $\alpha$ angle around the C5–C6 linkage turning the glycosidic linkage into a three-bond system. Our initial docking studies suggested the presence of two different orientations of melibiose in the carbohydrate-binding site, exhibiting similar $\Phi$, $\Psi$ and $\omega$ dihedral angles but with topology of CH–$\pi$ interactions between the galactose ring and W68. Therefore, we performed MD simulations on both complexes. In the first case, the complex remained fairly stable during the MD run, with torsion angles defined by $\Phi \approx 50^\circ$, $\Psi \approx 75^\circ$ and $\omega \approx 56^\circ$ and well-defined fluctuations around these values. In this orientation, the hydrogen-bond pattern and stacking of the apolar face of galactose with W68 persisted throughout the trajectory. Hydrogen bonds were consistently observed between E71 and hydroxyl groups OH3 and OH4 of the galactose ring, and Asn33 with hydroxyl groups OH1' and OH2'. The alternative disaccharide/gal-1 complex also remained stable during the simulation, with disaccharide torsion angles around $\Phi \approx -43^\circ$, $\Psi \approx -45^\circ$ and $\omega \approx -51^\circ$. For this conformation, CH–$\pi$ interactions between the W68 indole ring and galactose protons H1, H2 and H6 were maintained. The Glc unit did not make any significant contacts with groups from the protein, as obvious from Figures 6C and 7C (and material provided in the Supplementary Section). From the experimental side, the STD spectrum for the Galz(1 $\rightarrow$ 6) Glc/gal-1 complex had revealed contacts involving the non-reducing Gal unit. Moreover, the HSQC titration showed a significant chemical shift perturbation for W68, supporting model building based on this MD simulation.

Because a second site with affinity for carbohydrates had recently been reported by testing an $\alpha$-galactomannan (Miller et al. 2009b), we also used MD to explore a reactivity of Galz(1 $\rightarrow$ 4)Gal at this site. We performed docking studies followed by MD simulations for the alternative binding site. As the docking procedure predicted a second cluster (aside from the regular ligand-binding site) for Galz(1 $\rightarrow$ 4)Gal in the region surrounding residues 90–113, the best docked orientation for that region was selected for additional MD simulations with an explicit solvent. Analysis of the trajectory showed the existence of two conformers in equilibrium during the entire simulation, with $\Phi$ angles remaining negative, in agreement with the exo-anomeric effect. Most importantly, there were no significant interactions between the disaccharide and gal-1 that could overcome the energy barrier to alternative geometries. As before, both conformations remained within the lowest-energy regions of this disaccharide. To reconcile experimental observations with MD simulations, various intermolecular distances between Galz(1 $\rightarrow$ 4)Gal and several amino acids were scrutinized. In particular, distances to residues 91, 92, 95, 104, 112 and 113, which showed the largest spectral variations in HSQC experiments, were monitored. In overview, Galz(1 $\rightarrow$ 4)Gal did not show any major conformational changes during the MD simulation. But it significantly changed its position relative to gal-1 during the simulation. Indeed, the distances between the Gal residues and those amino acids remained rather large, always being $>6$ Å.

Discussion

Our results describe the structural characteristics of the interaction of gal-1 with the $\alpha$-linked disaccharides Galz(1 $\rightarrow$ 3) Gal, Galz(1 $\rightarrow$ 4)Gal and Galz(1 $\rightarrow$ 6)Glc. The following structural evidence was provided: (i) gal-1 can accommodate these $\alpha$-linked disaccharides at the lectin site, with stacking to W68 in two cases; (ii) stacking engages the terminal Gal unit, treating the $\alpha(1 \rightarrow 3)$-linked disaccharide akin to lactose, not as an $\alpha(1 \rightarrow 4)$-extended Gal core and (iii) the linkage point can influence the strength (or weakness) of ligand binding including establishment of the typical CH–$\pi$ interaction.

In terms of affinity, we found that these carbohydrates are less potent than lactose, i.e. by $\sim$250-fold relative to Galz(1 $\rightarrow$ 3)Gal and 500-fold relative to both Galz(1 $\rightarrow$ 4)Gal and Galz(1 $\rightarrow$ 6)Glc. These results are clearly less favorable than when obtained by a competitive binding assay which reported about a 25-fold difference for the $\alpha1 \rightarrow 3$-linked digalactoside (Sparrow et al. 1987) or 60-fold for melibiose (Lee et al. 1990). Since clustering in microdomains increases avidity, as shown in vitro for gal-1 and neuroblastoma cells (Kopitz et al. 2010), our data support the possibility that $\alpha$-linked galactosides such as $\alpha1 \rightarrow 3$ (B-type) extensions on $N$-glycans, especially when clustered, may serve as ligands. Of particular note, the level of this reactivity can vary among galectins, and galectin-3 is much more suited to interact with the $\alpha1 \rightarrow 3$-linked digalactoside (Sparrow et al. 1987; Jin et al. 2006; Krzeminiski et al. 2011).
Beyond the NMR-based support of gal-1 reactivity to α-disaccharides, we provide structural information on this interaction at the canonical site by MD simulations. In this regard, the non-reducing end Gal’ residue of Galα(1→3)Gal formed the most stable hydrogen bonds and CH–π interaction. The STD NMR spectra indicated that the Gal’ at the non-reducing end is central for binding, especially at C4′–C6′ positions. In total, both hydrogen bonding and CH–π interaction are less established than those for lactose. Experimentally, W68 resonances showed much smaller chemical shift changes upon binding of this ligand than of lactose. Of the three α-linked disaccharides investigated here, Galα(1→3)Gal nonetheless bound gal-1 the strongest and had the most interactions, in accord with published evidence from competitive binding assays, albeit with quantitative differences. In addition, melibiose also established weak contacts with the reducing-end sugar.

A distinct gal-1 recognition pattern was discerned for galabiose. Especially, the axial orientation of the glycosidic linkage caused the effect that the interaction of this sugar with gal-1 is different. Our MD results, for example, demonstrated that this α-disaccharide has a different orientation with respect to the lectin, with few sugar–galectin hydrogen bonds and impairment of CH–π interaction. Experimentally, W68 shows very minor HSQC chemical shift changes upon binding galabiose. Affinity generation in this case rests on contacts to both sugar units. The combination of the average of two major orientations sampled during the MD run provides direct correlation with STD-derived information in that non-reducing end H3', H4' and H5' protons make contacts with E71 and H1' is close to the βCH2 group of W68, while the reducing-end sugar H1 and H3 protons make contacts with other protein side chains. Experimentally, our HSQC data show only modest ligand-induced chemical shift perturbations of residues at the lactose-binding site. Overall, our results suggest the coexistence of two different orientations of this α-linked disaccharide at the lectin site.

In addition to this site, gal-1 can interact with a polysaccharide at a separate site. We previously reported that gal-1 binds Galα(1→4)Gal non-cooperatively, likely related to its distinct recognition mode for galabiose. By comparison to the α-disaccharides, we reported previously that lactose binds gal-1 with negative cooperativity, \( K_1 = 21 \times 10^3 \text{M}^{-1} \) and \( K_2 = 4 \times 10^2 \text{M}^{-1} \) and a Hill coefficient of \( n = 1.25 \). On the other hand, we found that gal-1 binds Galα(1→4)Gal non-cooperatively, likely related to its distinct recognition mode for galabiose. By comparison to the α-disaccharides, we reported previously that lactose binds gal-1 with negative cooperativity, \( K_1 = 21 \times 10^3 \text{M}^{-1} \) and \( K_2 = 4 \times 10^2 \text{M}^{-1} \) and a Hill coefficient of \( n = 0.8 \) (Nesmelova et al. 2010). When applying a one-site model on calorimetric data, binding data had also been interpretable (López-Lucendo et al. 2004; Dam et al. 2005). It therefore is a challenge for further structural work to identify underlying structural and dynamical changes that mediate a molecular switch for affinity regulation. Also, the structural aspects for the natural substitutions to the core galactose unit, such as α2,3-sialylation or α1,2-fucosylation, deserve to be studied by the strategy applied in this report.

### Materials and methods

#### Chemicals and reagents

All disaccharides evaluated here were commercially available: 3-O-(α-D-galactopyranosyl)-D-galactose (CAS 13168-24-6) and 4-O-(α-D-galactopyranosyl)-D-galactose (CAS 80446-85-1) were purchased from Toronto Research Chemicals; 6-O-(α-D-galactopyranosyl)-D-glucose (CAS 585-09-9) was obtained from Sigma and \( d^{10}\)-dithiothreitol (dDTT) from Cortecnet (Lot EW1711).

#### Gal-1 preparation

Normal and uniformly \( ^{15}\)N-labeled human gal-1 were produced in competent BL21(DE3) cells (Novagen), grown in either TB medium (Roth, Karlsruhe, Germany) or in minimal medium, then purified by a lactose-bearing affinity resin, and any contaminations were removed on a gel filtration column, as described previously (Nesmelova et al. 2008). Typically, 44 mg of purified protein was obtained from 1 L of cell culture. Protein content of the final sample was quantified by using the Biorad protein assay, purity was checked by using one- and two-dimensional polyacrylamide gel electrophoresis. Functional activity of the purified protein was assessed by using a T-cell death assay (Pace et al. 2000), also solid-phase binding and anois assays in pancreatic carcinoma cells expressing the tumor suppressor p16\(^{INK4a}\) (André et al. 2006, 2007).

#### NMR spectroscopy

**HSQC NMR titration experiments.** All gal-1/ligand binding heteronuclear NMR experiments were carried out at 30°C on a Varian Unity Inova 600-MHz spectrometer equipped with a H/C/N triple-resonance probe and x/y/z triple-axis pulse field gradient unit. For these NMR studies, lyophilized uniformly \( ^{15}\)N-labeled gal-1 was dissolved at a concentration of 0.3 mM in 50 mM sodium phosphate buffer at pH 7.0, made up using a 95% H2O/5% D2O mixture. \( ^1\)H and \( ^{15}\)N resonance assignments for recombinant gal-1 have already been reported (Nesmelova et al. 2008). A gradient sensitivity-enhanced version of two-dimensional \( ^1\)H–\( ^{15}\)N HSQC was applied with 256 (\( t_1 \)) × 2048 (\( t_2 \)) complex data points in nitrogen and proton dimensions, respectively. Raw data were converted and processed by using NMRPipe.
Ligand-based STD NMR experiments. Samples for STD NMR experiments were prepared with 50 µM gal-1 and 10 mM ligand (200:1 disaccharide:gal-1) in 99.9% deuterated phosphate-buffered saline buffer ([NaCl] = 138 mM; [KCl] = 2.7 mM; [Na₂HPO₄] = 10.1 mM; [NaH₂PO₄] = 1.8 mM) at pD 7.5. d-DTT (400 µM) was present in the solutions to ensure the protein’s activity and reduced state. Experiments with the Viscum album agglutinin (VAA), purified and checked for activity as described ( Gabius et al. 1992, 2001), were carried out in a similar fashion, with protein and ligand concentrations of 60 µM and 3 mM (50:1 ligand to protein ratio), respectively.

STD NMR data were collected at 20 and 30°C using New Era 5 mm borosilicate tubes (reference NE-SL5-7) on a Bruker Avance DRX 500 MHz NMR spectrometer equipped with a 5 mm inverse probe and using a standard STD pulse sequence with a 15 ms 5 kHz spin lock to minimize background protein resonances, as previously optimized for VAA (Jiménez-Barbero et al. 2009; Ribeiro et al. 2010). Saturation of the protein signals was performed using a train of 40 or 60 selective 70 dB Gaussian pulses of 50 ms duration, totaling a 2 or 3 s saturation time, when VAA or gal-1 systems were analyzed, respectively. The on-resonance frequency was set up at 7.12 (gal-1) and –1 ppm (VAA), and the off-resonance one was applied to 100 ppm. STD spectra were acquired with a total of 8192 (gal-1) or 360/400 (VAA) transients in addition to 16 dummy scans.

Due to the relatively low gal-1 affinity for the disaccharides investigated here (10⁻³ M range), we acquired a relatively large number of transients, ~8192, in order to achieve an acceptable signal-to-noise ratio in STD NMR experiments. Moreover, to avoid over-interpretation of these data, we selected a lower detection limit for any signal that integrated at least three times the root mean square of the noise level (Mocak et al. 1997; Lacey et al. 1999). This STD intensity threshold was used for the analysis of each gal-1/disaccharide system. In addition, all disaccharides were present in solution as a mixture of their α- and β-anomers at the reducing end, with the non-reducing end being identified herein with the prime mark (i.e. Gal'). TOCSY, NOESY and ¹³C-HSQC experiments were initially performed to unequivocally assign ¹H and ¹³C resonances for all carbohydrate ligands studied.

Following a strategically planned protocol, the same ligands were also employed in binding studies using VAA, a galactose-binding protein with documented reactivity to α- and β-anomers (Lee et al. 1992; Galanina et al. 1997). The affinity of lactose for VAA is less than that for gal-1 based on NMR titrations (Ribeiro et al. 2010). Also, the anomeric linkage type does not appear to be a major ligand-discriminatory factor (Lee et al. 1992; Galanina et al. 1997) so that its binding to each of the disaccharides served as an internal positive control.

Determination of the binding constants by NMR. Monte Carlo calculations were used to determine the values of K₁ and K₂ from titration curves acquired using ¹H–¹⁵N HSQC spectra on ¹⁵N-labeled gal-1, as a function of the ligand concentration. Calculations using the binding model described below were performed on 20 individual ¹H and ¹⁵N resonances that were chemically shifted the most during the titration.

For this analysis, we considered sequential binding of a ligand L to the protein with two binding sites, A and B. In this specific case, the gal-1 homodimer harbors one binding site in each monomer. In this model, there are five possible ligand-binding events:

\[ L + A B \overset{K_1}{\underset{C_1}{\rightleftharpoons}} A L B \]

\[ L + A B \overset{K_1}{\underset{C_1}{\rightleftharpoons}} A B L \]

\[ L + A B \overset{K_2}{\underset{C_2}{\rightleftharpoons}} A L B \]

\[ 2L + A B \overset{K_2}{\underset{C_2}{\rightleftharpoons}} A L B \]

where

\[ K_1 = \frac{[A L B]}{[L][A B]} \]

\[ K_2 = \frac{[A L B]}{[L][A B]} \]

\[ K_3 = \frac{[A L B]}{[L]^2[A B]} = K_1 K_2 \]

Ligand binding between the two sites is correlated either when \( K_2 > K_1 \) (positive cooperativity) or when \( K_2 < K_1 \) (negative cooperativity). There is no correlation or cooperativity when \( K_2 = K_1 \). The physical sense of positive/negative cooperativity depends upon the probability of binding to the second site after the first site has been loaded. Note that for equivalent binding sites \([A_1 B_1] = [A_2 B_2]\) and then we can write \( C_L = [L] + 2[A_1 B_1] + 2[A_1 B_2] \), the total ligand, and \( C_P = 2([A B] + 2[A_1 B_1] + [A_1 B_2]) \), the total galectin-monomer concentrations in the sample. The equation for \([L]\) is cubic:

\[ \frac{K_1 K_2 [L]^3}{C_L} + \frac{(K_1 K_2 C_P - K_1 K_2 C_L + 2K_1 )[L]^2}{C_L} + \frac{(K_1 C_P - 2K_1 C_L + 1)[L] - C_L}{C_L} = 0 \]  

\[ 8 K_1 (K_2 - K_1) [A B]^3 + 4 K_1 (2 C_L (K_2 - K_1) + C_P K_2 - 2 K_2) [A B]^2 + 2 (1 + K_1 K_2 (C_L - C_P))^2 + 2 C_L K_1 [A B] - C_P = 0 \]

To determine \( K_1 \) and \( K_2 \), we directly fitted ¹H and ¹⁵N chemical shift changes for each signal in the HSQC spectrum.
during the titration with lactose, using Equations (1)–(3) above and Equation (4). Chemical shift changes, δ(x), of the NMR signals of gal-1 are described by:

\[
\delta(x) = x\delta_f + (1 - x)\delta_l
\]

where \(x\) is the fraction of free (no ligand) gal-1 molecules, and \(\delta_f\) and \(\delta_l\) are the chemical shifts of gal-1 resonances in free and ligand-loaded states, respectively. The value \(x\) in Equation (4) can be written as:

\[
x = \frac{2[A\,B] + 2[AL\,BL]}{C_P}
\]

\(K_1\) and \(K_2\) can also be expressed by the Hill equation using the ratio \(Y/(1 - Y)\), where \(Y\) is the fraction of sites filled by ligand. In a two-site binding model,

\[
Y = \frac{2([A\,B]\) + [AL\,BL]}{C_P}
\]

\(\Phi\) and \(\Psi\) torsion angles of the glycoside linkages were determined during the simulation time. They were defined as \(H1'\)-C1'-O-C3/C1'-O-C3-H3, \(\Phi(\Psi)\), \(H1'-C1'-O-C4/C1'-O-C4-H4, \(\Phi(\Psi)\) and \(H1'-C1'-O-C6/\)

Molecular modeling

The starting coordinates for a monomer form of gal-1 were obtained from the Protein Data Bank (www.rcsb.org), i.e. PDB code 1gzw (1.7 Å resolution). Prior to docking studies, the structure was prepared with the Wizard tool of the Schrödinger package for molecular modeling (Schrödinger 2005). Lactose was removed, polar hydrogens were added and Kollman charges (Weiner et al. 1984) were assigned to all atoms. Protonation of histidine residues was checked manually. For the disaccharides, the low-energy regions in the \(\phi/\psi\) potential energy surfaces were evaluated by employing the MM3 force field in Macromodel (Schrödinger 2005). Resulting minima were in full agreement with those found in the CERMAV database for disaccharides. Gaussian 03 (Frisch et al. 2004) was used to perform the ab initio-based geometry optimization of the disaccharides at the HF/6-31G level of theory. Atomic partial charges were assigned to the molecules using Gaussian with 6-31G(d) basis set functions.

AutoDock 4.0.1 (Garrett et al. 2009) was used for the docking studies, first performed by focusing on the carbohydrate recognition domain of gal-1. The three-dimensional grid (60 × 60 × 80 Å³) was centered in NE1 of W68, central residue of the lectin site, with a spacing of 0.375 Å. Additional docking studies were focused on a putative alternative site of gal-1 for the tested disaccharides based on previous NMR monitoring especially with a galactomannan (Miller, Klyosov, et al. 2009; Miller, Nesmelova, et al. 2010). In this case, the docking grid size was 40 × 40 × 50 Å³ and was centered so as to encompass the region defined by the sequence of residues 90–105. For both docking protocols, the following restraints were applied: dihedral \(\phi/\psi\) torsion angles were adjusted to the lowest energy conformers, whereas the \(\omega\) torsion angle of the non-reducing Gal' residue was fixed in the gt conformation, as is always found in the interaction of galactose-containing oligosaccharides with galectins. The Lamarckian genetic algorithm was selected for ligand conformational searching. For each compound, the docking parameters were as follows: trials of 100 dockings, population size of 100, random starting position and conformation, translation step ranges of 2.0 Å, rotation step ranges of 60°, elitism of 1, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06 and 250,000 energy evaluations. Final docked conformations were clustered by the use of a tolerance of 1.5 Å root-mean-square deviations (RMSDs).

For MD simulations, the AMBER force field with the GLYCAM (Kirschner et al. 2008) and ff99EP parameter sets was employed for the description of the gal-1/digalactoside complexes. All MD simulations were carried out using the Sander module in the AMBER 10 (Case et al. 2008). Three Na⁺ counterions were added to neutralize the system. Each system was then solvated using TIP3P waters (Jorgensen et al. 1983) in a cubic box with at least 10 Å distance around the complex. The Shake algorithm was applied to all hydrogen-containing bonds (Ryckaert et al. 1977), and a 1fs integration step was used. The simulation used periodic boundary conditions, and the electrostatic interactions were represented using the smooth particle mesh Ewald method (Darden et al. 1993), with a grid spacing of 1 Å. Each system was gently annealed from 100 to 300 K over a period of 25 ps. The systems were then maintained at a temperature of 300 K during 50 ps with a solute restraint and progressive energy minimizations, gradually releasing the restraints of the solute followed by a 20 ps heating phase from 100 to 300 K, where after restraints were removed. Finally, the production simulations for each system lasted 3 ns and were also continued in the isothermal–isobaric ensemble. Coordinate trajectories were recorded each 5 ps throughout all equilibration and production runs, which yielded an ensemble of 1500 structures of each complex for further analysis. For Galα(1 → 3)Gal and Galα(1 → 4)Gal, complexes with the most optimal binding energies and belonging to the most populated clusters were selected for MD simulations. For Galα(1 → 6)Gal, two different starting complexes (with two different orientations around the C5–C6 linkage that links both rings) were chosen; these corresponded to the two most populated clusters found in the docking protocol. Additional starting geometries were considered in which the relative orientations of the two rings with respect to the protein were different in order to assess any influence from the starting geometry. In all cases, the only complexes that were consistent with the STD data see section on epitope mapping were those which corresponded to the “lactose-like” interaction mode. Because MD simulations were run for only 3 ns, we have provided RMSD and torsion angle time-series data in the Supplementary Section to demonstrate that all simulations converged.

MD trajectories were analyzed using a combination of the AMBER and VMD packages. Overall, RMSD variations were computed with ptraj (AMBER) after superimposition of the CA, C and N atoms (protein backbone) of gal-1. Hydrogen-bond generation during the 3 ns of the MD run, both those formed between digalactosides and amino acids as well as with water molecules were identified with ptraj (cut off = 4 Å). The dihedral \(\Phi(\Psi)\) torsion angles of the glycoside linkages were determined during the simulation time. They were defined as \(H1'-C1'-O-C3/C1'-O-C3-H3\) \(\Phi(\Psi)\), \(H1'-C1'-O-C4/C1'-O-C4-H4\), \(\Phi(\Psi)\) and \(H1'-C1'-O-C6/\)
C1'-O-C6-C5, for the α(1 → 3), α(1 → 4) and α(1 → 6) glycosidic linkages, respectively. In the case of melibiose involving the exocyclic C6 atom for linkage, the dihedral ω torsion angle, defined as O-C6-C5-H5, was also monitored. Also, the carbohydrate orientation at the binding site was unremoved by measuring the significant distances between the sugar units and the key amino acids.

Conclusions

Gal-1 is commonly known as a receptor for β-galactosides. In contrast, less attention has so far been paid to interactions with α-galactosides. In fact, no structural models have been established to explain such an interaction. The present study fills this gap and provides structural details of such complexes from the perspective of both protein and carbohydrate. Several new findings are reported here: (i) gal-1 can accommodate these α-disaccharides at its lectin site; (ii) the non-reducing-end galactose residue from the two Gal units is engaged in CH–π stacking and (iii) positioning of linkage points (in particular engagement of the axial OH group in interaction). Since this reactivity can differ among galectins, e.g. the chimeric-type gal-3, the tandem-repeat-type gal-4 and the proto-type gal-5 being more reactive than gal-1 to the α1 → 3-linked digalactoside known as a major xenoantigen (Sparrow et al. 1987; Hirabayashi et al. 2002; Wu et al. 2004, 2006; Jin et al. 2006; Macher and Galili 2006; Krzeminski et al. 2011), comparative structural studies are encouraged within the galectin network as an approach to delineate structure–activity relationships and as a means to define galectin-selective blocking compounds.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

DTT, dithiothreitol; gal-1, human galectin-1; HSQC, heteronuclear single quantum coherence; MD, molecular dynamics; NMR, nuclear magnetic resonance; PDB, Protein Data Bank; RMSD, root-mean-square deviation; STD, saturation transfer difference; VAA, Viscum album agglutinin.

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