Sugar-Binding Properties of the Two Lectin Domains of LEC-1 with Respect to the Galβ1-4Fuc Disaccharide Unit Present in Protostomia Glycoconjugates

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Galectins are a group of animal lectins characterized by their specificity for β-galactosides and by an evolutionarily conserved sequence motif in their carbohydrate-binding site. Galectins are involved in a wide variety of biological phenomena, including development, cell differentiation, tumor metastasis, apoptosis, RNA splicing, and regulation of immune function.1–6 A Galβ1-4GlcNAc (N-acetyllactosamine) disaccharide unit is thought to be the endogenous glycoepitope recognized by vertebrate galectins. This recognition has also been observed in galectins from invertebrate species such as Caenorhabditis elegans (C. elegans), including the lectins LEC-1 and LEC-6, which we have isolated previously.7,8 However, the existence of a glycan containing N-acetyllactosamine units has not yet been confirmed in C. elegans.10–15

Recently, we isolated N-type glycoproteins captured by the C. elegans galectin LEC-6 and analyzed their sugar structure by matrix-assisted laser desorption-time-of-flight mass spectrometry (MALDI-TOF MS/MS) in conjunction with glycosidase digestion. We found that LEC-6 interacts strongly with N-glycans containing a Gal-Fuc disaccharide unit that bound to position 6 of the innermost GlcNAc residue.16 In order to identify the linkage between the galactose and fucose residues, and examine the structure of the endogenous glycoepitope recognized by LEC-6, we compared the binding strengths of the chemically synthesized Galβ1-4Fuc and Galβ1-3Fuc,17 and found that LEC-6 interacts more strongly with Galβ1-4Fuc than with Galβ1-3Fuc.18 LEC-1, another major galectin from C. elegans, also showed preferential binding to Galβ1-4Fuc.18 These results suggest that Galβ1-4Fuc is the endogenous unit recognized by the C. elegans galectins.

LEC-1 is a tandem-repeat type galectin, wherein two homologous carbohydrate recognition domains exist within a single polypeptide chain.7,8 We previously reported that although the two domains of LEC-1 showed affinity for sugars containing Galβ1-4GlcNAc units, detailed analysis using fluorescence-labeled oligosaccharides (pyridylaminated (PA) sugars) in frontal affinity chromatography (FAC)19 revealed these domains had different sugar binding properties.20 However, the oligosaccharides used in the previous study20 differed in that they were mainly of vertebrate origin and commercially available in pyridylaminated forms.

For this report, we used recombinant proteins of the whole molecule (LEC-1), the N-terminal lectin domain (LEC-1Nh), and the C-terminal lectin domain (LEC-1Ch), and compared their binding affinities for the Galβ1-4Fuc and Galβ1-3Fuc disaccharide units. Although the two lectin domains showed preferential binding to Galβ1-4Fuc compared to Galβ1-3Fuc and N-acetyllactosamine-containing structure LnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), the binding profiles of the two domains were different. While LEC-1Nh demonstrated a very high affinity to Galβ1-4Fuc with a very low affinity for Galβ1-3Fuc and LnT, LEC-1Ch displayed a moderate affinity for both Galβ1-3Fuc and LnT. Furthermore, both LEC-1Nh and LEC-1Ch had significantly lower binding affinities for the LEC-6-binding glycans. These results suggest that the two domains of LEC-1 bind to different N-glycans containing the Gal-Fuc disaccharide unit, and that the ligands for the two domains of LEC-1 are different from the LEC-6-binding glycans.

MATERIALS AND METHODS

Materials Galβ1-4Fuc labeled with pyridylamine (PA) via a spacer (PA-Galβ1-4Fuc) and Galβ1-3Fuc labeled with pyridylamine via a spacer (PA-Galβ1-3Fuc) (Fig. 1A) were chemically synthesized, as reported previously.7,11
Concentration of 5 nM and applied to an immobilized galectin previously. Briefly, PA-oligosaccharides were dissolved into a stainless steel column (4 × 110 cm; Takara Bio Inc., Shiga, Japan), as described previously. Preparation and purification of pyridylaminated-LEC-6-binding glycans (PA-D3 and PA-E3) were performed, as described previously. PA-Galβ1-4Fuc was also further purified by reversed-phase HPLC on a PALPAK type-R column (0.46 × 25 cm; Takara Bio Inc., Shiga, Japan), as described previously.

**Purification of Recombinant Galectins and Preparation of Afinity Adsorbents** Expression and purification of recombinant proteins (LEC-1, LEC-1Nh, and LEC-1Ch) were performed, as described previously. The recombinant proteins were coupled to HiTrap NHS-activated Sepharose (GE Healthcare, St. Giles, U.K.), and the slurry was packed into a stainless steel column (4 × 10 mm; bed volume, 0.126 ml; GL Sciences, Inc.).

**FAC Analysis** FAC analysis was carried out as described previously. Briefly, PA-oligosaccharides were dissolved in ethylenediaminetetraacetic acid (EDTA)-PBS (1 mM EDTA, 20 mM Na-phosphate, 150 mM NaCl; pH 7.2) to a concentration of 5 nM and applied to an immobilized galectin column (LEC-1, 11.7 mg protein/mL gel; LEC-1Nh, 5.6 mg protein/mL gel; LEC-Ch, 6.0 mg protein/mL gel) through a 2-ml sample loop at a flow rate of 0.25 ml/min at 20°C. Elution of PA-oligosaccharides from the column was monitored by a fluorescence detector at 380 nm (excitation at 310 nm). The elution volume ($V_f$) of the PA-oligosaccharide of interest was determined, as described previously. The elution volume of PA-rhamnose, which has no affinity for galectins, was used as the negative control ($V_n$). In this study, the $K_a$ values for the PA-oligosaccharides tested in each immobilized recombinant galectin column were calculated using the $K_a$ values for the interactions between LEC-1, LEC-1Nh, or LEC-1Ch and PA-LnT by the following basic equation of FAC:

$$K_a = \frac{B}{(V_f - V_n)/[A]}$$

The $K_a$ values were calculated based on the equation: $K_a = 1/K_{A}$.

The structures of the sugars labeled with PA (PA-sugars) used in this study are shown in Fig. 1.

**Inhibition of Galβ1-4Fuc Recognition by Simple Sugars** FAC analysis of PA-Galβ1-4Fuc on immobilized LEC-1Nh or LEC-1Ch columns was conducted in the absence or presence of various concentrations of simple sugars (lactose, galactose, or fucose). The concentrations of competitive sugars used for inhibition were 25, 50, 100, 200, 400, 800, 1600, 3200, and 6400 μM. Retardation ($V_f - V_{0f}$) of PA-Galβ1-4Fuc on the immobilized LEC-1Nh or LEC-1Ch columns in the absence of competitive sugars was set as 100%. The percentage ratios of retardation were calculated.

**RESULTS**

**Analysis of the Binding Properties of the Two Domains of LEC-1 with Respect to Galβ1-4Fuc- or Galβ1-3Fuc-Containing Carbohydrates** In order to analyze the binding abilities of the two carbohydrate-binding domains of LEC-1 (possessing two sugar binding sites on one molecule) with respect to Galβ1-4Fuc and Galβ1-3Fuc, we performed FAC analysis by using immobilized recombinant LEC-1, LEC-1Nh, and LEC-1Ch columns. Chemically synthesized Galβ1-4Fuc and Galβ1-3Fuc, labeled with the fluorophore PA via an appropriate spacer derived from mannitol, were subjected to FAC analysis. PA-labeled Galβ1-4Fuc was further purified after its synthesis by reversed-phase HPLC to remove any remaining traces of contaminants and reduce the occurrence of early non-specific bumps in the FAC elution curve (for example, see Fig. 6 of ref. 18).

Significant retardation was observed for PA-Galβ1-4Fuc, PA-Galβ1-3Fuc, and PA-LnT (PA-Galβ1-4GlcNAcβ1-3Galβ1-4Glc) in the LEC-1 and LEC-1Ch columns (Fig. 2A). The calculated $K_a$ values are shown in Fig. 2B. On the other hand, only PA-Galβ1-4Fuc showed significant retardation in the LEC-1Nh column, with PA-Galβ1-3Fuc and PA-LnT demonstrating very little retardation, as shown in Fig. 2A (the calculated $K_a$ values are also shown in Fig. 2B). These results are consistent with those of our previous report, which showed that the two carbohydrate-binding domains of LEC-1 have different sugar binding profiles, and also suggest that though both of the carbohydrate-recognition domains in LEC-1 (LEC-1Nh and LEC-1Ch) contribute to the recognition of the Galβ1-4Fuc structure, LEC-1Ch is the main site responsible for the recognition of Galβ1-3Fuc and LnT.

**Analysis of the Binding Properties of LEC-1 and the Two LEC-1 Domains with Respect to Carbohydrates Isolated from LEC-6 Binding N-Glycans** Since LEC-1 has a higher affinity for Galβ1-4Fuc than for Galβ1-4GlcNAc, we investigated the binding abilities of LEC-1, LEC-1Nh, and LEC-1Ch with respect to endogenous carbohydrates contain-
In the present study, we have demonstrated that both the two lectin domains of nematode tandem repeat-type galectin LEC-1 (LEC-1Nh and LEC-1Ch) show a greater affinity for Galβ1-4Fuc than for Galβ1-4GlcNAc. Furthermore, the two domains had different binding profiles for Galβ1-4Fuc and Galβ1-3Fuc, i.e., while LEC-1Nh demonstrated a significant affinity for Galβ1-4Fuc but very little affinity for Galβ1-3Fuc, LEC-1Ch showed a moderate affinity for both Galβ1-3Fuc and Galβ1-4Fuc (and for L-NH2). These data are consistent with those of our previous report, which showed that the two lectin domains had different sugar-binding properties when using PA-oligosaccharides obtained mainly from vertebrate glycoconjugates containing Galβ1-4GlcNAc or Galβ1-3GlcNAc.

Since the Galβ1-4Fuc disaccharide unit was identified in the N-glycans from *C. elegans* glycoproteins captured by the lectin domains of LEC-1, we examined using FAC analysis (results are shown in Fig. 3). The concentrations inhibiting 50% of the PA-Galβ1-4Fuc retardation (IC50) were 2000 μM, >6400 μM, and 5300 μM for lactose, galactose, and fucose, respectively, for the LEC-1Nh immobilized column, and 600 μM, >6400 μM, or 3400 μM for lactose, galactose, and fucose, respectively, for the LEC-1Ch column. Since lactose had the smallest IC50 values for both LEC-1Nh and LEC-1Ch by a large margin, the binding sites responsible for the recognition of Galβ1-4Fuc were suggested to be the same sites that recognize lactosamine. Furthermore, the smaller IC50 values for fucose as compared to galactose suggest that fucose has a greater role in the recognition of Galβ1-4Fuc by LEC-1Nh and LEC-1Ch.

**DISCUSSION**

In the present study, we have demonstrated that both the two lectin domains of nematode tandem repeat-type galectin LEC-1 (LEC-1Nh and LEC-1Ch) show a greater affinity for Galβ1-4Fuc than for Galβ1-4GlcNAc. Furthermore, the two domains had different binding profiles for Galβ1-4Fuc and Galβ1-3Fuc, i.e., while LEC-1Nh demonstrated a significant affinity for Galβ1-4Fuc but very little affinity for Galβ1-3Fuc, LEC-1Ch showed a moderate affinity for both Galβ1-3Fuc and Galβ1-4Fuc (and for L-NH2). These data are consistent with those of our previous report, which showed that the two lectin domains had different sugar-binding properties when using PA-oligosaccharides obtained mainly from vertebrate glycoconjugates containing Galβ1-4GlcNAc or Galβ1-3GlcNAc.20

Since the Galβ1-4Fuc disaccharide unit was identified in the N-glycans from *C. elegans* glycoproteins captured by an immobilized LEC-6 affinity adsorbent, we tested whether LEC-1 could also bind to these LEC-6-binding glycans. The IC50 values for LEC-1, LEC-1Nh, and LEC-1Ch with respect to PA-D3 and PA-E3, two of the identified LEC-6-binding glycans labeled with PA, were significantly smaller than those for LEC-6. Furthermore, the affinities of LEC-1 and LEC-1Ch for other LEC-6-binding glycans were similar to those for PA-D3 and PA-E3 (unpublished Results). These data suggest that although both domains of LEC-1 recognize Galβ1-4Fuc units, the natural ligands in *C. elegans* differ from the LEC-6 ligands. Recently, Maduzia et al. reported that the target molecules recognized by LEC-6 and LEC-10 in *C. elegans* are identical and contain the Galβ1-4Fuc struc-
ture. This was demonstrated using labeled recombinant galectins and several C. elegans mutants having a defective Galβ1-4Fuc modification on core N-glycans. Isolation and identification of the LEC-1-binding glycans from C. elegans could reveal the endogenous ligands for the two lectin domains in the LEC-1 molecule and the different ligands that might be cross-linked in the C. elegans system by the entire LEC-1 molecule. Recently, we developed a method using a photoactivatable crosslinker, benzophenone-4-maleimide (BPM), to obtain a covalent complex between galectins and their glycoprotein ligands. In addition to the conventional affinity purification procedure, this new method can be of great help in identifying unknown LEC-1 ligands.

The concentrations required for 50% inhibition (IC$_{50}$) of the interactions between immobilized LEC-1Nh and LEC-1Ch and PA-Galβ1-4Fuc in FAC analysis were determined for three competitive sugars: lactose, galactose, and fucose. Although there is no evidence of Galβ1-4GlcNAc-containing N-glycans in C. elegans, LEC-1 and LEC-6 retain the ability to bind to this disaccharide structure. This property enabled us to isolate two major galectins from C. elegans by using asialofetuin-sepharose and lactose. The IC$_{50}$ values were found to be in the following order: lactose < fucose < galactose, suggesting that the Galβ1-4Fuc binding sites on the two lectin domains of LEC-1 are the same as the sites that recognize Galβ1-4GlcNAc and Galβ1-4Glc (lactose). The X-ray crystal structure of the complex between LEC-1 and the Galβ1-4Fuc disaccharide unit should be helpful in identifying the reason underlying the stronger inhibition ability of fucose compared to that of galactose, and the probable role played by the fucose moiety in the recognition of Galβ1-4Fuc by LEC-1.

The K$_s$ values for LEC-1, LEC-1Nh and LEC-1Ch with respect to Galβ1-4Fuc were 2.9×10$^4$, 2.1×10$^5$, and 1.4×10$^4$ M$^{-1}$, respectively, while the K$_s$ value for LEC-6 with respect to Galβ1-4Fuc was somewhat higher at 6.3×10$^5$ M$^{-1}$. Although LEC-6 and the two lectin domains of LEC-1 show affinities for Galβ1-4Fuc, and their sequences are homologous to other galectin family members of both nematodes and vertebrates, similarities in terms of amino acid identity are not very high, i.e., LEC-6 shares a 28% amino acid identity with LEC-1Nh and 27% identity with LEC-1Ch. Site-directed mutagenesis of the LEC-6 protein followed by FAC analysis revealed that eight amino acid residues in LEC-6, which are conserved among galectins and shown to be important for the recognition of Galβ1-4GlcNAc, function differently when interacting with either Galβ1-4GlcNAc or Galβ1-4Fuc. His$_{60}$ and Glu$_{83}$ in LEC-6, which are conserved among LEC-6, LEC-1Nh, and LEC-1Ch, seemed particularly important for the binding of Galβ1-4Fuc. However, amino acid differences, including substituted amino acid residues, which are otherwise conserved among galectins, such as Ser$_{61}$ (Asn among other galectins) in LEC-1Nh, and Ile$_{204}$ (Val among other galectins) in LEC-1Ch, could be responsible for the differences in Galβ1-4Fuc-binding abilities. Furthermore, there could be some other amino acid residues in the LEC-6 protein responsible for the recognition of carbohydrate moiety other than the Galβ1-4Fuc unit in LEC-6-binding glycans such as D3 or E3. Lacking these amino acids and possibly some structural differences between LEC-1 and LEC-6 may have resulted in lower affinity of the two lectin domains of LEC-1 for D3 or E3 compared to the simple Galβ1-4Fuc unit.

The existence of the Galβ1-4Fuc unit has already been reported in nematodes, squid, octopuses, and keyhole limpets, i.e., in the species belonging to Protostomia. The differences in the binding strengths for Galβ1-4Fuc between the three galectin domains (LEC-6, LEC-1Nh, and LEC-1Ch) seem to indicate that the mechanism responsible for this recognition differs from that responsible for the recognition of N-acetyllactosamine. These observations support our previous suggestion that C. elegans glycans and galectins co-evolved differently compared to the N-acetyllactosamine-containing vertebrate glycans and galectins.

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REFERENCES


