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Caenorhabditis elegans Galectins LEC-6 and LEC-10 Interact with Similar Glycoconjugates in the Intestine

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Galectins are a family of metazoan proteins that show binding to various β-galactoside-containing glycans. Because of a lack of proper tools, the interaction of galectins with their specific glycan ligands in the cells and tissues are largely unknown. We have investigated the localization of galectin ligands in *Caenorhabditis elegans* using a novel technology that relies on the high binding specificity between galectins and their endogenous ligands. Fluorescently labeled recombinant galectin fusions are found to bind to ligands located in diverse tissues including the intestine, pharynx, and the rectal valve. Consistent with their role as galactoside-binding proteins, the interaction with their ligands is inhibited by galactose or lactose. Two of the galectins, LEC-6 and LEC-10, recognize ligands that co-localize along the intestinal lumen. The ligands for LEC-6 and LEC-10 are absent in three glycosylation mutants *bre-1*, *fut-8*, and *galt-1*, which have been shown to be required to synthesize the Gal-β1,4-Fuc modifications of the core *N*-glycans unique to *C. elegans* and several other invertebrates. Both galectins pull down the same set of glycoproteins in a manner dependent on the presence of these carbohydrate modifications. Endogenous LEC-6 and LEC-10 are expressed in the intestinal cells, but they are localized to different subcellular compartments that do not appear to overlap with each other or with the location of their glycan targets. An altered subcellular distribution of these ligands is found in mutants lacking both galectins. These results suggest a model where LEC-6 and LEC-10 interact with glycoproteins through specific glycans to regulate their cellular fate.

First discovered from the electric eel (1), galectins are lectins that bind β-galactoside-containing carbohydrate chains attached to proteins and lipids. Based on their sequence and structure, human galectins have been classified into three major groups (2), which comprise the galectin family: the prototypical galectins, the chimeric galectins, and the tandem repeat galectins. All of the galectins contain a carbohydrate recognition domain (CRD)[2] of ~130 amino acids in length. Prototypical and chimeric galectins each contain a single CRD, whereas the tandem repeat galectins posses two CRDs capable of recognizing and binding two distinct sugar molecules. Although most galectins interact with β-galactose-containing simple carbohydrates such as di- or trisaccharides, the affinities are relatively weak in the range from higher micromolar to lower millimolar concentration. In contrast, they recognize galactose containing natural complex glycoconjugates with affinities in the micromolar or submicromolar range, and each galectin may have highest affinity to different structures. Recent studies began to elucidate the exact sugar structures that are required for this high specificity among galectins from mammals, and it is believed that this interaction is a prerequisite for the cellular function of galectins (2).

The first invertebrate galectin isolated was LEC-1 from the nematode *C. elegans* based on its affinity for lactose containing glycans (3). The *C. elegans* genome contains at least 26 predicted galectin genes (4), 10 of which have been cloned (3, 5, 6). The functions of these *C. elegans* galectins are not known and deletion mutants or gene RNAi knockdown of several of these genes reveal no obvious abnormalities. LEC-1, -2, -3, -4, and -5 are considered tandem repeat galectins because they contain two CRDs and are most similar to each other (6). LEC-8, -10, and -11 contain one CRD with a C-terminal tail of unknown function that classifies them as novel chimeric galectins. LEC-9 is similar to the prototypical LEC-6 galectin in that it contains a single CRD and no C-terminal tail, but it is considered as a novel chimeric galectin based on its diverged sequence (6).

The exact carbohydrate specificity of *C. elegans* galectins remains unclear. Several studies have shown that the presence of galactose in the complex carbohydrate is required for binding, as seen with galectins from other organisms. It has been demonstrated that LEC-1, -2, -3, -4, -6, and -10 are able to bind synthetic oligosaccharides containing β-galactose with high affinity, while LEC-8, -9, and -11 bind poorly to these sugars (6, 7). More recently, it has been shown that recombinant LEC-1 and LEC-6 recognize synthetic galactose-β1,4-fucose (Gal-β1,4-Fuc) with higher affinity than Gal-β1,3-Fuc or galactose-β1,4-*N*-acetylglucosamine (Gal-β1,4-GlcNAc) (8, 9). However, studies have not yet determined whether these glycans are the natural ligands for these galectins.

On the other hand, many studies have focused on the natural glycans, but lack information about the proteins that interact with them. Typical glycan analysis is performed with whole tissues or entire animals. In *C. elegans*, these studies have revealed numerous complex carbohydrates attached to glycoproteins and several of these carbohydrates are unique to *C. elegans* (10). In a study that utilized worms from different developmental stages, it was shown that some of the *N*-glycans are stage-specific (11), implying a role of specific glycans in development. It is not known whether these unique glycans interact with specific proteins.

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[2] The abbreviations used are: CRD, carbohydrate recognition domain; Gal, galactose; GlcNAc, *N*-acetylglucosamine.

Furthermore, such global approaches also cannot reveal where these glycans are localized within the multicellular organism, which is important toward understanding the role of glycans in development or physiology. Carbohydrate-binding lectins derived from animal and plants have been used to examine glycan targets in a variety of experiments, including *in situ* detection of sugar targets (12, 13). However, the specificity of these lectins for complex sugars is not well-defined, although each has been shown to recognize unique structural motifs which can be inhibited by high concentrations of specific monosaccharides or short oligosaccharides (14). Recently, bacterially expressed recombinant lectins have been used successfully to overcome some of the drawbacks associated with using native proteins such as batch to batch variations and internal glycosylation (15).

In this work, we developed a novel method to examine the natural glycan ligands of galectins in *C. elegans*. This approach has allowed us to examine the *in situ* locations of these galectin carbohydrate ligands for the first time within a multi-cellular organism. We have explored the high binding specificity of *C. elegans* galectins to their endogenous glycans and produced recombinant galectins as a tool to examine cell and tissue localization of glycans in a whole organism. Recombinant galectins are also used to identify the glycoproteins that contain specific glycans. In addition, these tools take advantage of SNAP tag technology, which covalently attaches a single label such as a flurophore to a SNAP-tagged fusion protein (16–18). Site-specific labeling eliminates random and sometimes excessive chemical labeling that may interfere with protein activity. We produced SNAP tag fusion proteins with several *C. elegans* galectins (LEC-1, -2, -3, -6, -9, -10, and -11) and found that they recognize overlapping and distinct structures within the worm. We have focused on LEC-6 and LEC-10 in more detail and found they recognize the same glycans located in the intestinal cells. In protein pull-down experiments, the SNAP-tagged LEC-6 and LEC-10 enrich the same set of glycoproteins that contain these specific glycans. In addition, we also examined the expression and subcellular localization of endogenous LEC-6 and LEC-10 using standard immunochemical staining procedures with specific antibodies and GFP fusion protein expression studies. We found that endogenous LEC-6 and LEC-10 are expressed in the intestine, indicating the staining we observed with the recombinant proteins is relevant to their biological function. Taken together, these data suggest that LEC-6 and LEC-10 bind to the same cell surface glycans as their endogenous counterpart. The interaction of LEC-6 and LEC-10 with these glycans might therefore modulate the cellular fate of several glycoproteins.

EXPERIMENTAL PROCEDURES

Worm Strains—Worm strains were cultured and maintained as described previously (19). Wild type N2, *bre-1(ye4)*, *bre-2(ye31)*, *bre-3(ye26)*, *bre-4(ye27)*, *bre-5(ye17)*, *fut-1(ok892)*, *fut-8(ok2558)*, and *F08A8.5(gk453)* were obtained from the *C. elegans* Genetics Center. Deletion mutants *lec-6(tm3706)* and *lec-10(tm1262)* were obtained from the Mitani Laboratory. The double mutant strain, carrying *lec-6(tm3706);lec-10(tm1262),* was generated by performing genetic crosses between single mutant strains. The strain *galt-1(op497)* was a gift from Dr. Markus Künzler.

Recombinant SNAP-galectin Fusions—Coding regions of *C. elegans* galectins cDNA (*lec-1, lec-2, lec-3, lec-6, lec-9, lec-10*, and *lec-11*) were PCR-amplified from a mixed-stage N2 cDNA library and inserted into the C terminus of a modified pSNAP tag® (T7) Vector (NEB) containing a His_6 tag. The *CGL2* gene was PCR-amplified from plasmid PMA 180_pET24-CGL2 obtained from Dr. Markus Künzler. These SNAP fusion proteins from these constructs were expressed in the T7 Express *Escherichia coli* strain (NEB C2566H). 250 mls of LB supplemented with ampicillin (0.1 mg/ml final concentration) was inoculated with 2.5 ml of an overnight culture and grown to an OD of 0.6 at 37 °C. Next, IPTG was added to a final concentration of 1 mM, and the cultures incubated an additional 4 h before cells were pelleted and frozen at −20 °C. Frozen cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, pH 7.2, 300 mM NaCl, 10 mM imidazole) and lysed on ice with 1 mg/ml lysozyme for 30 min. Each cell suspension was then sonicated for 1 min with 10-s pulses at 70% output. The lysate was centrifuged at 10,000 × *g* for 30 min in a Beckman fixed rotor centrifuge. The supernatant was incubated with 1 ml of nickel resin equilibrated with lysis buffer for 1 h rotating at 4 °C. The resin was then packed into a column and washed with 30 ml of wash buffer (50 mM NaH_2PO_4, pH 7.2, 300 mM NaCl, 20 mM imidazole) at 4 °C. Finally, 1-ml elution fractions were collected, and protein concentration determined on the Nanodrop spectrophotometer ND-1000 (Thermo Scientific). Fractions were examined by SDS-PAGE for purity, and those fractions with high protein concentration were pooled and dialyzed overnight in phosphate buffer (50 mM NaH_2PO_4, 50 mM Na_2HPO_4, pH 7.2, 0.1 M NaCl) containing 1 mM DTT.

Labeling of SNAP-galectin—Purified SNAP-galectin protein at a concentration of 5 μM was incubated with 10 μM substrate flurophore in phosphate buffer supplemented with 1 mM DTT for 1 h at 37 °C. Fluorophores used for labeling were SNAP-surface Alexa Fluor 488 (NEB S9129S), SNAP-surface Alexa Fluor 546 (NEB S9132S) and SNAP-surface-IR800. After incubation, each labeled protein was dialyzed overnight in phosphate buffer containing 1 mM DTT to remove nonreacted substrates. Labeled proteins were analyzed by SDS-PAGE and detected with appropriate scanners. Labeled proteins were stored at −20 °C in 50% glycerol.

Worm Fixation—For staining using either SNAP-galectin fusions or antibodies, whole worms were fixed and permeabilized following a method described previously (20) and briefly summarized here. Washed worms were fixed in RFB buffer (160 mM KCl, 40 mM NaCl, 20 mM EGTA, 10 mM spermidine, 30 mM PIPES, 50% methanol) containing 2% formaldehyde. Worms were permeablized by treatments with 1% β-mercaptoethanol, 10 mM DTT, and 0.3% H_2O_2 in various buffers. Finally, samples were incubated with PTC buffer (PBS, pH 7.2, 0.1% BSA, 1 mM EDTA, 0.5% Triton X-100, 0.05% sodium azide) and resuspended in PTB buffer (PBS, pH7.2, 1% BSA, 1 mM EDTA, 0.5% Triton X-100, 0.05% sodium azide).

Generation of Anti-LEC-6 and -10 Antibodies—A commercial service (Genescript USA Inc.) was used for peptide syntheses, immunogen injections, and antibody purification. Peptides for LEC-6 (ADSRFHINLRTPDDC) and LEC-10 (GFHFQRRWDGHVDHC) were conjugated to KLH and then injected into rabbits. Antibodies were affinity purified using the same synthetic peptides. Total *C. elegans* protein lysates from different genetic backgrounds were used in Western blotting analyses with purified antibodies at 1:500 (~2 μg/ml). A secondary anti-rabbit antibody labeled with IR800CW (LiCor, 926–32211) was used to detect the primary antibody followed by imaging with a LiCor Odyssey scanner.

Staining with SNAP-galectin and Antibody—Fixed worms were incubated with labeled SNAP-tagged galectins at a concentration of 50 nM in 500 μl of PTB overnight at room temperature with gentle rotation. When performing sugar inhibition experiments, sugars were added to the worm samples at a final concentration of 0.1 M before the addition of the galectin fusions. The next day, worms were washed four times with 1 ml of PTC for 30 min each at room temperature. Similar procedures were preformed for worms stained with rabbit anti-LEC-6 and anti-LEC-10 antibodies. Each antibody was used at a concentration of 2 μg/ml diluted in PTB and incubated overnight. After several washes in PTC a goat anti-rabbit IgG secondary antibody labeled with Alexa 488 (A11008, Invitrogen) at a 1:2000 dilution in PTB was incubated for 4 h at room temperature. After four washes with PTC, worms were mounted on a 2% agarose pad and examined under a Zeiss Axovert 200 M microscope. Mouse monoclonal antibody MH33 (obtained as supernatant from the Developmental Studies Hybridoma Bank, University of Iowa) was diluted 1:250 and added to the samples in co-staining experiments with either SNAP-galectin, anti-LEC-6, or anti-LEC-10 antibodies. This monoclonal antibody was detected using a goat anti-mouse IgG secondary antibody labeled with Alexa 555 (A21424, Invitrogen) at a 1:2000 dilution.

LEC-6::GFP and LEC-10::GFP Fusion Constructs—A GFP tag was fused in-frame to the C terminus of LEC-6 and LEC-10 using the *C. elegans* GFP expression vector pPD95.75 (A. Fire). PCR was performed on *C. elegans* genomic DNA to amplify ~3 Kb of the promoter regions plus the entire coding regions of each gene using 5′ and 3′ primers containing the restriction sites PstI and KpnI, respectively. The primers used for *lec-6* were the 5′ primer gccgcCTGCAGcctgactcatgtagcactaag and the 3′ primer gccgcGGTACCcgtgagaaacatgggcggaatg (PstI and KpnI restriction enzyme sites are indicated in uppercase). For *lec-10*, the primers used were the 5′ primer gccgcCTGCAGctgagaataatggtgaatatgg and the 3′ primer gccgcGGTACCcctctgtatgggttgaagttttg (PstI and KpnI restriction enzyme sites are indicated in uppercase). These PCR fragments were digested with PstI and KpnI enzymes and inserted into the vector pPD95.75 at these same sites. The constructs were then injected at a concentration of 10 μg/ml along with 50 μg/ml of pRF4 as a co-transformation marker to generate transgenic animals carrying extra chromosomal arrays.

Pull-down Experiments with SNAP-galectin Fusions—100 μl of SNAP-capture magnetic beads (NEB S9145) (with a coupling capacity of 0.5 mg/ml for SNAP protein) were washed twice in immobilization buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM DTT, and 0.1% Tween 20). The equilibrated beads were then added to 200 μl of immobilization buffer containing 200 μg of active SNAP-tagged galectin supplemented with an additional 1 mM of DTT and incubated for an hour at room temperature with rotation. After immobilizing the SNAP-tagged galectin onto the SNAP-capture magnetic beads, the beads were washed three times with immobilization buffer followed by two washes in RIPA buffer (1% Nonidet P-40, 20 mM Tris, pH 7.5, 1 mM EDTA, 1% Triton, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl). *C. elegans* total protein lysates were prepared by homogenizing mixed stage worms in RIPA buffer followed by centrifugation to remove insoluble materials. These protein lysates were precleared with un-reacted SNAP-capture magnetic beads. The SNAP-capture magnetic beads with the immobilized SNAP-tagged lectin were then incubated with *C. elegans* lysates containing ~250 μg of protein for 3 h rotating at room temperature either with or without 50 mM lactose. Finally, the supernatants were removed and the beads washed three to five times for 1 min in RIPA buffer. While washing, the SNAP-Capture Magnetic Beads were separated from solution using a magnetic separation rack (NEB, S1506S). Proteins captured with the magnetic beads were eluted by boiling with 20 μl of SDS-loading buffer and separated by SDS-PAGE. After staining with Coomassie Blue dye, bands that were only present in the wild type lysate were excised for protein identification. The same size regions from controls that did not add any recombinant galectin or from samples that had been co-incubated with lactose were also excised. Protein identities were determined by mass spectrometry analysis at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School.

RESULTS

Production and Fluorescent Labeling of Recombinant SNAP-galectins Fusions—To determine where the glycan ligands are localized within cells, we exploited the affinity that galectins have with their carbohydrate ligands as a tool. We produced recombinant *C. elegans* galectins fused to a SNAP tag and used them to localize their endogenous glycan ligands in whole worms as well as for *in vitro* pull-down experiments to identify proteins that contain these glycans (Fig. 1*A*). This approach allows for the detection of glycoconjugates that naturally interact with these galectins, as opposed to using plant or animal lectins which are derived from other organisms and may potentially interact with different glycan profiles. The SNAP tag is a 20 kDa self-labeling enzyme engineered from O^6-alkylguanine-DNA alkyltransferase (16, 17). Substrate linked to a fluorophore reacts with this enzyme and the fluorescent moiety becomes covalently attached to the protein. By using SNAP tag fusions, recombinant proteins can easily be labeled with a single fluorophore, or covalently attached to beads to pull down interacting molecules. Because there are a variety of commercially available fluorophores attached to the alkyltransferase substrate, this technique also allows for con-

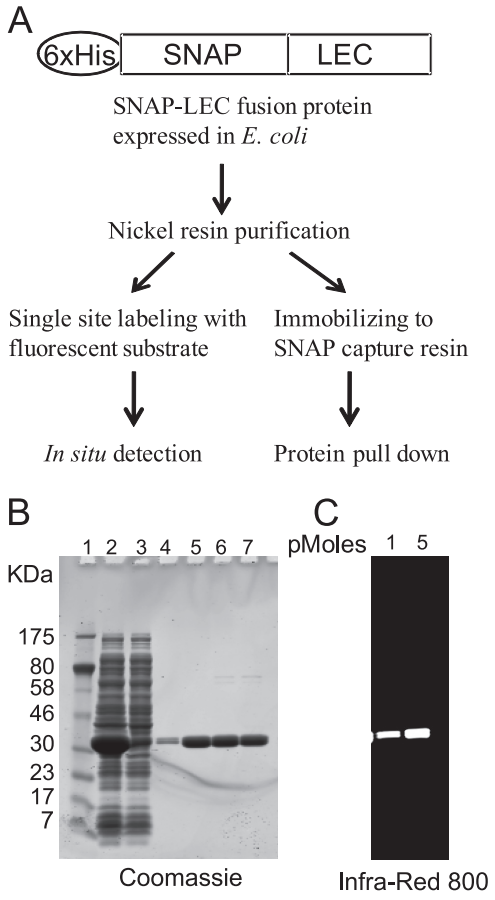


FIGURE 1. **Expression of recombinant SNAP-fusion galectins.** *A*, constructs for producing SNAP-LEC fusions and their uses in *in situ* detection of carbohydrates and in pull down of specific glycoproteins. *B*, expression and purification of SNAP-LEC-6. *Lane 1*, molecular weight marker; *lane 2*, lysate from *E. coli* induced for recombinant protein expression; *lane 3*, lysate from noninduced cells; *lanes 4–7*, consecutive fractions eluted from nickel resin. *C*, purified recombinant SNAP-LEC-6 labeled with infrared dye.

venient double staining between different galectins or with standard immunocytochemical methods.

We fused *C. elegans* galectins LEC-1, -2, -3, -6,-9, -10, and -11 to a SNAP tag by cloning into a vector that allows for efficient recombinant protein expression in *E. coli*. In addition, we also included a fungal galectin CGL2 derived from the mushroom *Coprinopsis cinerea,* which has a weak similarity to LEC-1 when used as a query to perform BLASTP analysis against the *C. elegans* genome (E-value = 1e-05). CGL2 has been shown to bind the *C. elegans* intestine in live worms (21). Each of these galectins was constructed with both a His_6 tag and the SNAP tag at their N-terminal domain (Fig. 1*A*). Most of these fusions resulted in a reasonable amount of protein yield and were considerably pure after purifying with a nickel resin (For example Fig. 1*B*). Purified SNAP fusions were efficiently labeled with a fluorophore (Fig. 1*C*).

Fluorescently Labeled SNAP-tagged Galectins Exhibit Overlapping and Unique Specificities in Different C. elegans Tissues—Labeled SNAP-tagged galectins were used to stain fixed and permeablized *C. elegans* in whole worm *in situ* experiments. These recombinant galectin fusions bind to their specific molecular targets when compared to a control containing the SNAP tag fused with the His_6 tag alone only gave a weak diffuse signal in all tissues. There is some overlap in staining between these galectins and several of them clearly recognize distinct structures within the worm. LEC-1, -2, and -3 bind to targets located in the rectal valve (LEC-1 shown in Fig. 2*A*, LEC-2 and -3 not shown) and spermatheca (data not shown). The most noticeable staining pattern is observed with LEC-6, LEC-9 and LEC-10, all of which are found in the intestine (Fig. 2, *B–D*). LEC-6 and LEC-10, and LEC-9 to a lesser extent, yield a highly concentrated signal along the lumen. LEC-6 and LEC-11 also recognize targets in the buccal cavity and/or the grinder of the pharynx (Fig. 2, *B* and *E*). LEC-9 and LEC-11 show relatively abundant but diffuse signal in the cytoplasm of the intestinal cells (Fig. 2, *C* and *E*) as compared with the other fusions. CGL2 shows intestinal lumen staining as well as a diffuse cytoplasmic staining throughout the intestinal cells (Fig. 2*F*) similar to that of LEC-9. Because these galectins recognize glycan ligands located in distinct cells within the worm, this may indicate that different molecular targets are recognized by each galectin. On the other hand, several galectins recognize targets in similar worm structures, suggesting that some of them might recognize very similar or even identical molecular targets.

Subcellular Localization of Ligands Bound by SNAP-tagged LEC-6 and LEC-10—To study the nature of the staining observed with these recombinant galectins, we decided to focus on LEC-6 and LEC-10 because their targets are abundantly expressed in the intestinal cells. First, we performed co-localization experiments to examine whether their ligands are present on the same subcellular structure. Double staining performed using both SNAP-tagged LEC-6 and SNAP-tagged LEC-10 reveals that the signals obtained with these tagged proteins co-localized to structures under the microscope (Fig. 3*A*), indicting that their ligands are present in the same structure within the intestinal lumen. We further looked at the subcellular location of the LEC-6 and LEC-10 targets relative to the sub-apical marker MH33 that recognizes IFB-2. IFB-2 is an intermediate filament protein which localizes just beneath the apical surface within each intestinal cell (22). Double staining with MH33 indicates that the ligands recognized by SNAP-tagged LEC-6 or LEC-10 most likely lie in a layer outside of the intestinal cells, most probably corresponding to the glycocalyx (Fig. 3, *B–D*). The glycocalyx is a layer lining the intestinal lumen mainly composed of glycoproteins that functions to protect the intestinal surface from pathogenic attack and mechanical injury, to provide a surface support for digestive enzymes and to filter the products of these digestive enzymes so that they may reach the absorptive surface (23, 24).

SNAP-tagged Galectins Recognize Carbohydrate Ligands Containing Galactose and Fucose—To confirm that the staining observed with the labeled SNAP-tagged galectins was due to an interaction with specific glycans, we examined the inhibitory effect of various sugars on the binding abilities of the SNAP-tagged galectins. This approach has been commonly used to probe the sugar structure recognized by carbohydrate-binding proteins such as lectins (14). The monosaccharides galactose, fucose, mannose, or acetylglucosamine (GlcNAc) or the disaccharide lactose was included in staining

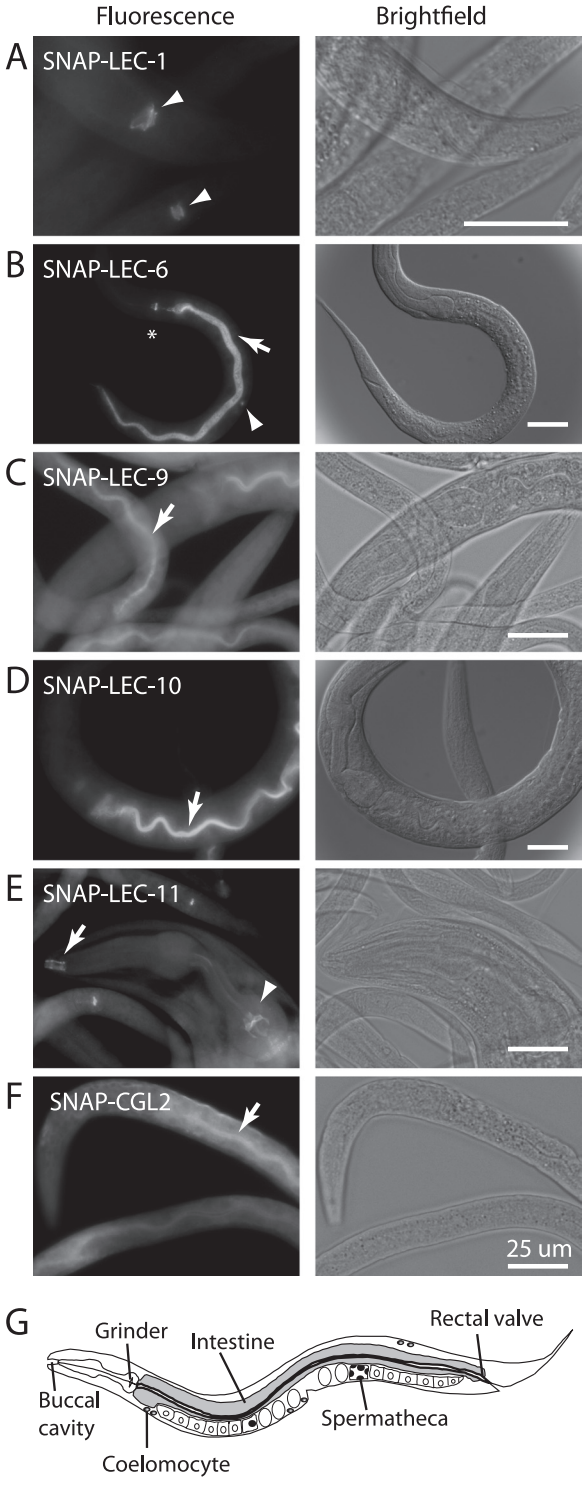


FIGURE 2. **Labeled recombinant SNAP-LECs recognize target molecules located in diverse *C. elegans* tissues.** *A*, LEC-1. Staining is found in the rectal region (*arrowheads*). *B*, LEC-6. Staining is most prominent along the intestinal lumen (*arrow*), the grinder of the pharynx (*star*), and the coelomocytes (*arrowhead*). *C*, LEC-9. Staining is more concentrated along the intestinal lumen (*arrow*) and shows significant but diffuse signal in most tissues. *D*, LEC-10. Staining is primarily along the lumen in the intestine (*arrow*). *E*, LEC-11. Staining is localized in the grinder (*arrowhead*) and the buccal cavity of the pharynx (*arrow*), and a diffuse signal is also present in most cells. *F*, CGL2. Staining is very similar to LEC-9 with concentrated signal localizing along the intestinal lumen with a diffuse signal in most tissues. *G*, diagram of tissues showing staining with recombinant galectins.

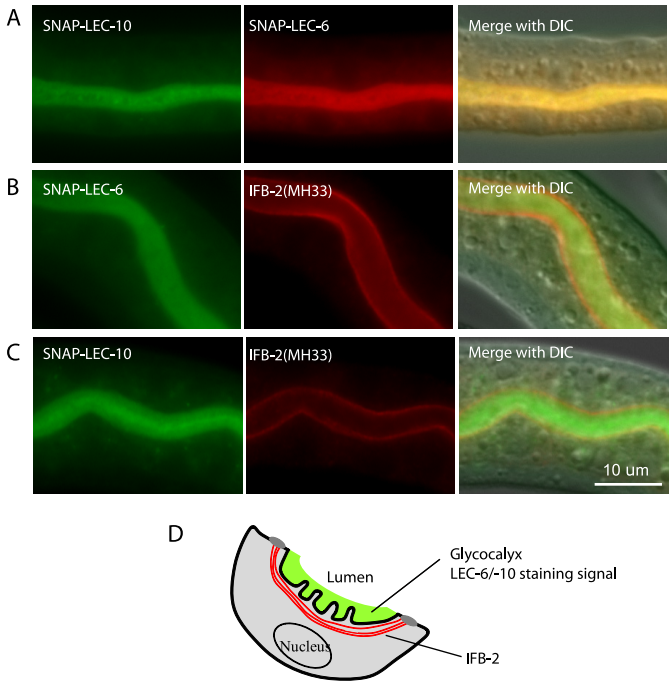


FIGURE 3. **Target molecules recognized by SNAP-LEC-6 and SNAP-LEC-10 are localized along the lumen wall of the intestinal cells.** The subcellular localization of LEC-6 and LEC-10 target molecules are examined relative to each other (*panel A*) or relative to IFB-2 (*panels B* and *C*). IFB-2, which is detected with the monoclonal antibody MH33, is an intermediate filament protein located at the apical edge of the intestinal cells. *A*, co-staining of SNAP-LEC-10 (*green*) with SNAP-LEC-6 (*red*). Complete overlap of green and red signals is observed in the merged image. *B*, SNAP-LEC-6 (*green*) and MH33 (*red*). *C*, SNAP-LEC-10 (*green*) and MH33 (*red*). Staining with recombinant LEC-6 or LEC-10 is localized to the luminal side of the intestine based on its relative position to IFB-2. *D*, diagram of an intestinal cell depicting the location of signals detected with recombinant LEC-6 and LEC-10 relative to the marker IFB-2.

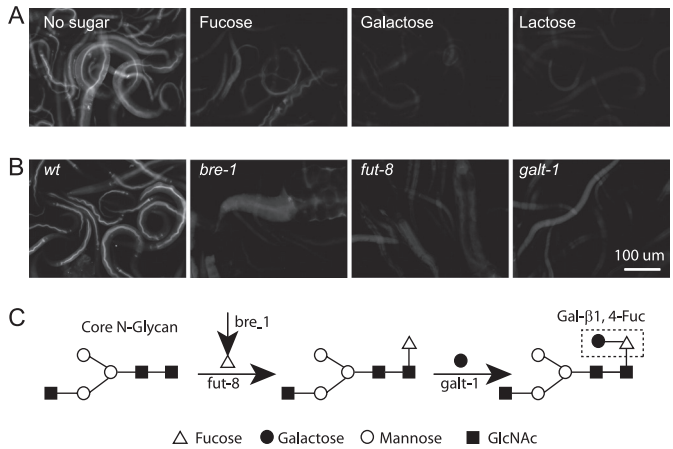


FIGURE 4. **Target molecules recognized by SNAP-LEC-6 contain fucose and galactose.** *A*, recognition of target molecules are disrupted in the presence of fucose, galactose, and lactose (galactose-β1,4-glucose). Images were captured with 0.5 s exposure time. *B*, lack of specific staining signals in mutants lacking fucose (*bre-1*), a fucose transferase (*fut-8*), and a galactose transferase (*galt-1*). Images were captured with 0.2 s exposure time. *C*, biochemical steps in the synthesis of a galactose-β1,4-fucose modification of core *N*-glycans involving *bre-1*, *fut-8*, and *galt-1* genes (21).

experiments with wild-type worms. In the presence of galactose and lactose, the signal level of SNAP-tagged LEC-6 staining (Fig. 4*A*) as well as that of LEC-1–3, LEC-9, and LEC-10 was reduced or eliminated, while LEC-11 staining was unaf-

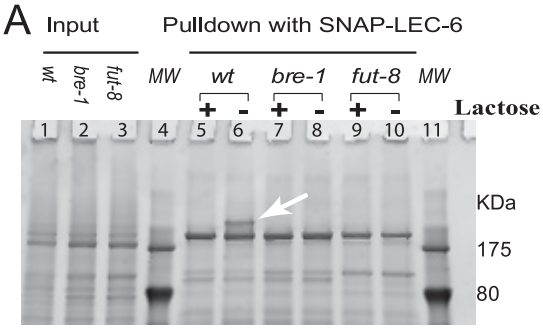
fected (data not shown). Fucose also reduced the staining signals seen with SNAP-tagged LEC-6 (Fig. 4*A*), LEC-1 and LEC-9 (data not shown). However, no dramatic change in the staining signal of SNAP-tagged LEC-10 or LEC-11 was observed in the presence of fucose. In addition, staining patterns produced by any of the SNAP-tagged galectins were unaffected in the presence of either monosaccharide mannose or GlcNAc. These results indicate that the interaction of most SNAP-tagged galectins with their ligands can be inhibited by galactose, fucose or lactose, suggesting that the recognized carbohydrate structures likely contain galactose and fucose.

To further explore the molecular nature of the staining observed with recombinant LEC-6 and LEC-10, we examined a number of glycosylation mutants that lack specific glycan structures. Numerous genes that incorporate galactose and fucose into complex carbohydrates have been identified in *C. elegans*, including *Bacillus thuringiensis* toxin-resistant genes (*bre-1* through *-5*), fucosyltransferases (*fut-1*, *fut-8*, F08A8.5), and a galatosyltransferase *galt-1*. *bre-1* encodes a GDP-mannose 4,6-dehydratase involved in the biosynthesis of GDP-fucose, a precursor used in fucosylation of carbohydrates (25). Four of the *bre* genes (2 thru 5) encode various glycosyltransferases that synthesize glycolipids (26). Several fucosyltransferases are encoded by *fut-1* (α1,3-fucosyltransferase) (27), *fut-8* (α1,6-fucosyltransferase) (28) and F08A8.5 (α1,2-fucosyltransferase) (29). *galt-1* encodes a galactosyltransferase not found in mammals (30). We examined the staining patterns of SNAP-tagged LEC-6 and SNAP-tagged LEC-10 in these various mutant backgrounds. We find that SNAP-tagged LEC-6 (Fig. 4*B*) and SNAP-tagged LEC-10 (data not shown) staining is eliminated from the intestinal lumen in *bre-1*, *fut-8*, and *galt-1* mutant backgrounds. Staining was unaffected in *bre-2*, *bre-3*, *bre-4*, *bre-5*, *fut-1*, and F08A8.5 and was comparable to the staining observed in wild-type animals. Enzymes encoded by *bre-1*, *fut-8,* and *galt-1* are required for the synthesis of Gal-β1,4-Fuc modification on the core *N*-glycan in *C. elegans* (Fig. 4*C*) (21). BRE-1, the GDP-mannose 4,6-dehydrogenase, converts GDP-mannose into GDP-fucose, which is a substrate for FUT-8 α-1,6 fucosyltransferase that incorporates a fucose onto GlcNAc. Additional modification by GALT-1 galactosyltransferase allows for the attachment of a galactose onto the fucose through a β1,4 linkage. Therefore, the lack of staining observed by labeled SNAP-tagged LEC-6 and SNAP-tagged LEC-10 in *bre-1*, *fut-8*, and *galt-1* mutants suggests that these two galectins recognize and bind to Gal-β1,4-Fuc attached to core *N*-glycans.

SNAP-tagged LEC-6 and LEC-10 Interact with Identical Glycoproteins—Since these glycan ligands for LEC-6 and LEC-10 are restricted to a small number of cells, we wondered whether these ligands are associated with specific proteins. We took advantage of SNAP-tag technology to covalently bind the SNAP-tagged LEC-6 and SNAP-tagged LEC-10 to SNAP-Capture magnetic cellulose beads (Fig. 1*A*) and used them to pull down interacting partners. We performed pull-down experiments with *C. elegans* lysates from wild-type animals and performed a control that included lactose to inhibit the specific binding to LEC-6 or LEC-10 ligands (Fig. 5*A*). As a further test for specificity we did the same pull-down experiments with samples prepared from *bre-1* or *fut-8* mutants that lacked targets in our galectin staining experiments. The proteins pulled down were separated by SDS-PAGE and detected using Coomassie stain. Both recombinant LEC-6 and LEC-10 are able to enrich for a prominent band with a size in the 200 kDa range from the wild-type lysates (shown for LEC-6, Fig. 5*A*; data not shown for LEC-10). In addition, this unique band was not observed in wild-type lysates co-incubated with lactose or in lysates from the *bre-1* and *fut-8* mutants regardless of whether or not lactose was present. Because lactose inhibited the binding of the SNAP-tagged galectin in our *in situ* staining experiments, we conclude that the addition of lactose in the pull-down experiments also inhibits specific galectin-carbohydrate interactions. As stated previously, *bre-1* and *fut-8* mutants are unable to synthesize complex carbohydrate structures containing a Gal-β1,4-Fuc attached to a core *N*-glycan (21) and SNAP-tagged LEC-6 and LEC-10 staining was eliminated from the intestinal lumen in these mutant backgrounds (Fig. 4*B*). Therefore, proteins prepared from these mutants might not possess this specific carbohydrate structure and thus could not interact with LEC-6 or LEC-10 in the pull-down experiments.

Mass spectrometric analysis of this 200 kDa band enriched by SNAP-tagged LEC-6 and SNAP-tagged LEC-10 identified the same four novel proteins encoded by F28B4.3, F40F4.6, T25C12.3, and F57F4.3. Each of them is predicted to have ~2100 amino acids (Fig. 5*B*). These proteins were not enriched in samples co-incubated with lactose during the pull down procedure nor from a control sample where no SNAP-tagged galectin was present. Three of these proteins encoded by F28B4.3, F40F4.6, and T25C12.3 show high amino acid sequence homology to each other by BLASTP analysis. F28B4.3 shows 76% amino acid identity to F40F4.6 and 25% identity to T25C12.3 across the entire protein length. When searching the NCBI Conserved Domain database, these proteins are predicted to contain one or two MD domains (31), a C-type lectin domain and one or two von Willebrand factor domains (32) (for example with F28B4.3, Fig. 5*C*). The fourth protein, encoded by F57F4.3, which is 99% identical in the first 2050 amino acids to that encoded by F57F4.4 and thus their identity cannot be resolved by mass spectrophotometric analysis, contains 21 ET modules located throughout the entire polypeptide. The ET module is a domain of unknown function found in several *C. elegans* proteins and it contains 8–10 conserved cysteine residues that are likely to form disulfide bridges. All of these proteins are predicted to have several *N*-glycosylation sites (Fig. 5*B*) and an N-terminal signal sequence, suggesting they are likely cell surface glycoproteins. The fact that both SNAP-tagged LEC-6 and SNAP-tagged LEC-10 identified the same proteins suggests that these two galectins bind to either the same carbohydrate structure attached to each of these proteins or to distinct carbohydrate structures that are located within the same glycoprotein. In either case, they likely share the same Gal-β1,4-Fuc as a key recognition motif.

A



B

Gene model	Number of peptides identified by Mass-spec: Bead -	LEC-6 +	LEC-6 -	LEC-10 +	LEC-10 -	Predicted protein properties: Protein	Domains	Amino acids	N-glycosylation sites
F28B4.3	0	3	40	0	27	CE07153	MD super family, C-type lectin, VWF type A	2229	12
F40F4.6	0	0	53	0	15	CE04536	MD super family, C-type lectin, VWF type A	2214	12
T25C12.3	0	0	26	0	12	CE40820	MD super family, C-type lectin, VWF type A	2103	6
F57F4.3/4	0	0	14	0	9	CE11342 CE11344	21 ET modules	2153	21

C

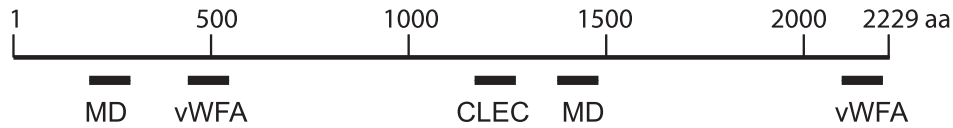


FIGURE 5. **Pull down of specific glycosylated proteins recognized by recombinant LEC-6 and LEC-10.** *A*, Coomassie Blue-stained SDS-PAGE gel of total worm lysates (*lanes 1–3*) or proteins pulled down with SNAP-LEC-6 magnetic beads (*lanes 5–10*). *Lanes 4* and *11*, MW marker. Samples generated from worms with different genotypes (wild type, *wt*, *bre-1*, and *fut-8*; refer to Fig. 5*C* for their gene activity) are used in pull down experiments either with (+) or without (−) the addition of lactose. Only wild type samples enriched for a band with a molecular weight around 200 kDa (*arrow*) and no other bands were detected in samples not inhibited by lactose or absent in the mutants. *B*, identities of proteins enriched by recombinant SNAP-LEC-6 and LEC-10 determined by mass spectrometry. *C*, position of predicted domains in F28B4.3.

Endogenous LEC-6 and LEC-10 Are Localized to the Cytoplasm of the Intestinal Cells—Because the ligands detected with our labeled SNAP-tagged LEC-6 and LEC-10 localize to the intestine, we wondered whether the endogenous LEC-6 and LEC-10 are also expressed in the same cells. To test this, we made C-terminal translational fusions with the green fluorescent protein (GFP) under the control of their own promoters to examine endogenous galectin expression. We find that transgenic worms carrying either LEC-6::GFP or LEC-10::GFP show GFP expression within the intestinal cells, while LEC-6::GFP also expresses in the grinder of the pharynx (Fig. 6*A*). Closer examination reveals that some LEC-10::GFP signal is concentrated in vesicular structures in the apical cytoplasm of the intestinal cells (Fig. 6*C*) while LEC-6::GFP signal is mostly diffuse in the cytoplasm.

We also examined the expression and localization of endogenous LEC-6 and LEC-10 with specific antibodies. To do this, we generated antibodies against LEC-6 and LEC-10. These antibodies recognize specific bands corresponding to the predicted size of either LEC-6 (16 kDa) or LEC-10 (22 kDa) expressed in wild-type animals by Western blot analysis (Fig. 6*B*). These bands are absent in lysates produced from mutant strains carrying *lec-6* or *lec-10* deletion, thus confirming the specificity of these antibodies. When used to stain wild type animals, both antibodies recognize signals in the intestine (Fig. 6, *D* and *E*). These immunochemical signals are absent in mutant strains carrying *lec-6* or *lec-10* deletions, supporting the signals observed in wild type worms are specific. Upon closer examination, the anti-LEC-6 antibody recognizes clustered punctate structures in the cytoplasm of the intestinal cells (Fig. 6*D*). In contrast, anti-LEC-10 antibodies recognize punctate structures found at the sub-apical region of the intestinal cell (Fig. 6*E*). This LEC-10 containing region occupies a similar subcellular region where the LEC-10::GFP-containing vesicular structures are found (compare Fig. 6, *C* and *E*). These results indicate that both endogenous LEC-6 and LEC-10 are expressed in the same intestinal cells where their ligands are detected by the recombinant galectins. How-

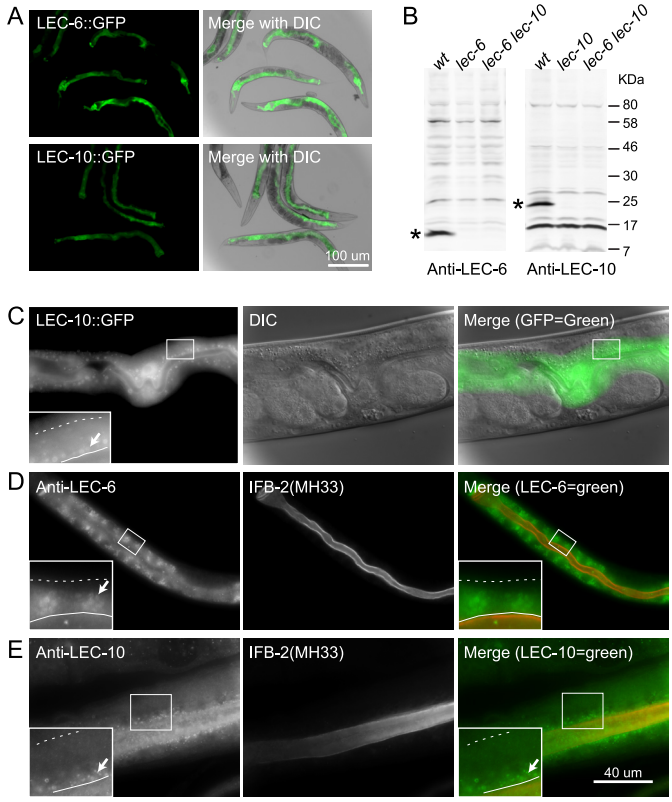


FIGURE 6. **Expression and subcellular localization of endogenous LEC-6 and LEC-10 proteins.** *A*, both LEC-6::GFP and LEC-10::GFP fusions are expressed in the intestine in transgenic worms. *B*, Western blot analyses using specific peptide antibodies to detect LEC-6 and LEC-10 from total protein lysates derived from wild type or deletion mutants. *wt*, wild type; deletion mutants: *lec-6*, *lec-10*, and *lec-6;lec-10*. *, protein bands recognized by respective antibodies and depleted in deletion mutants. *C*, localization of LEC-10::GFP to vesicular structures (*arrow*) that are concentrated in the sub-apical domains. *D*, localization of endogenous LEC-6 detected with a specific antibody. LEC-6 immunostaining signals are localized to punctate structures (*arrow*) that cluster in the cytoplasm. MH33 staining outlines the apical edge of the intestinal cells. *E*, localization of endogenous LEC-10 detected with a specific antibody. LEC-10 immunostaining signals are concentrated to punctate structures (*arrow*) localized at the sub-apical region within intestinal cells. In *C*, *D*, and *E*, an enlarged region containing half of the intestine is shown in the *lower left inset*. A *solid line* marks for the apical side of the intestinal cell and the *dotted line* for the basal lateral side. *Panels C*, *D*, and *E* have the same scaling.

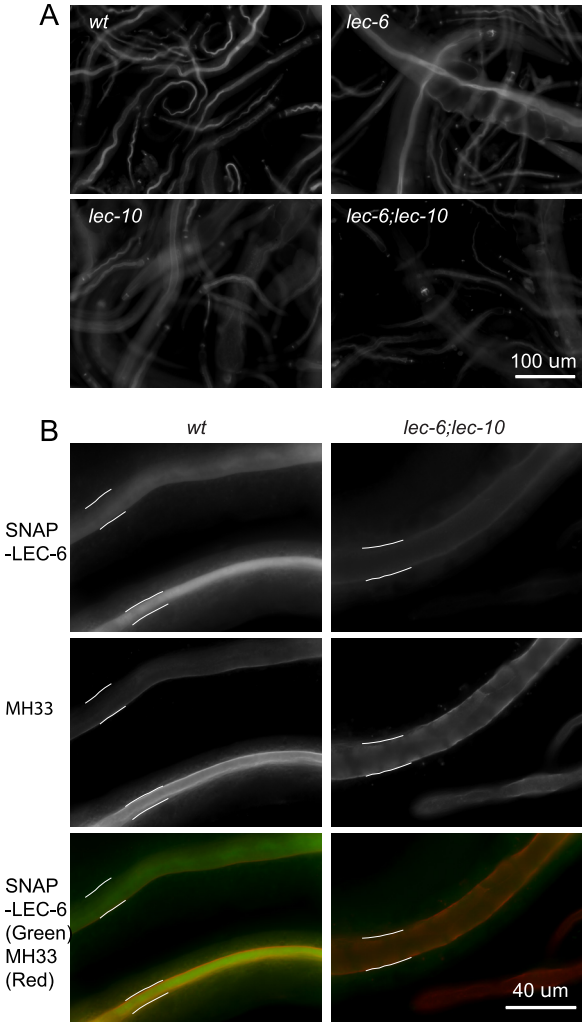


FIGURE 7. **Reduced luminal staining with recombinant SNAP-LEC-6 in *lec-6;lec-10* double deletion mutants.** Images within each panel are captured with equal exposure time. *A*, staining with recombinant SNAP-LEC-6 in wild type (*wt*), *lec-6*, *lec-10*, or *lec-6;lec-10*. *B*, co-staining with SNAP-LEC-6 and MH33 in wild type (*wt*, left column) and *lec-6;lec-10* double mutants. In *wt*, the SNAP-LEC-6 staining (*green* in the merged image) is mostly localized along the lumen wall, which is outlined by the MH33 staining (*red*). In the *lec-6;lec-10* mutant, less SNAP-LEC-6 staining signal is located to the intestinal lumen. The intestinal lumen border is partially outlined in *white*.

ever, they localized to the cytoplasm in non-overlapping subcellular regions, as opposed to the ligands, which were primarily concentrated on the luminal side of the intestinal cells.

The difference in localization of endogenous LEC-6 and LEC-10 *versus* the ligands detected with recombinant galectins prompted us to examine whether these proteins are required for proper localization of the ligands. Toward this goal, labeled SNAP-tagged LEC-6 and SNAP-tagged LEC-10 were used to stain *lec-6* and *lec-10* single deletion mutants as well as *lec-6;lec-10* double deletion mutants. Each of these labeled SNAP-tagged galectins were capable of recognizing their ligands in both single and double mutants; however, the overall staining signal was reduced (Fig. 7*A*) (data not shown for SNAP-LEC-10 staining). This reduction is more noticeable in the double mutant strain. Further examination at higher magnification revealed that less ligand staining is present along the intestinal lumen in the double mutant animals (Fig. 7*B*). Because of this reduced level of staining, these results suggest that endogenous LEC-6 and LEC-10 might play a role in regulating the expression of their ligands or in distributing their ligands subcellularly.

DISCUSSION

The relatively large number of *C. elegans* galectins suggests they have evolved diverse specificities toward carbohydrate ligands. These diverse specificities have been illustrated previously with recombinant galectins based on their affinities to synthetic ligands (6). Here we provide evidence that they interact with different natural ligands found in various tissues. This is achieved by using labeled recombinant galectins to detect endogenous ligands in whole animals, an approach similar to that used on cultured human cells (33) and on mushroom tissues with galectins derived from the same organism (34). We have improved this method by employing a site-specific labeling of recombinant fusion proteins. In prin-

ciple, only ligands with high affinity would allow binding. All the seven galectins (LEC-1, -2, -3, -6, -9, -10, and -11) that we surveyed showed localized staining on different cells, suggesting that they recognize diverse ligands.

The tissue locations of the staining signals are generally correlated with the relatedness of these galectins, indicating that similar galectins interact with ligands with similar structures. For example, LEC-1, -2, and -3 bound to similar structures around the rectal valve. These three proteins, belonging to the tandem-repeat galectins that contain two CRDs, show more sequence similarity to each other than to other *C. elegans* LEC proteins (4, 6). In contrast, LEC-6, -9, and -10, a group of similar galectins with a single CRD (4, 6), gave highly localized staining in the intestine. LEC-11, which branches distinctly from the above two groups on a phylogeny tree (6), gave staining only in the buccal cavity and the grinder. Consistent with this idea, LEC-6 and -10 are found to have similar profiles in experiments assaying for their ability to bind to a panel of synthetic oligosaccharides (6).

We have focused on LEC-6 and LEC-10 as an example to determine the molecular nature of the staining pattern. Multiple lines of evidence support the notion that the endogenous target molecules recognized by labeled recombinant LEC-6 and LEC-10 are identical and contain a Gal-β1,4-Fuc structure. Both fusions stain the luminal wall of the intestine and the cellular location of signals are indistinguishable under the microscope. Monosaccharide galactose and disaccharide lactose inhibit their binding in similar ways, consistent with that they recognize similar galactosides. We analyzed the ligands in glycosylation mutants and observed that the staining targets for both fusions are absent in *bre-1, fut-8*, or *galt-1* mutants, which have defects in the synthesis of the Gal-β1,4-Fuc modification found on *C. elegans* core *N*-glycans (21). In our pull-down experiments, both LEC-6 and LEC-10 interact with the same set of glycoproteins possibly via these carbohydrate ligands. These results are consistent with previous studies showing that recombinant LEC-6 pulled down glycoproteins containing Gal-Fuc disaccharides (8) and that LEC-6 showed a higher affinity for synthetic Gal-β1,4-Fuc than either Gal-β1,3-Fuc or Gal-β1,4-GlcNAc (8, 9). In a global glycome analysis, this Gal-β1,4-Fuc disaccharide attached to a *N*-glycan was found to be a modification unique to *C. elegans* and not present in mammalian cells (35). Our data suggest that both LEC-6 and LEC-10 interact with this unique modification in the intestine.

What proteins contain these unique glycans? The identification of these proteins might elucidate why these unique carbohydrate structures are necessary. In pull down experiments, both LEC-6 and LEC-10 fusions interact with four large glycoproteins by binding to specific glycans. These proteins were also identified among hundreds of glycoproteins enriched with LEC-6 or plant lectins Con A or WGA (36, 37). The functions of these proteins are not known. Three of them show significant similarity to each other and are predicted to have a C-type lectin domain, vWF type A domain and MD domains. The fourth protein contains 21 ET domain repeats. The C-type lectin domain is found in many extracellular proteins and is involved in calcium-dependent carbohydrate interactions (38). The vWF type A domain was initially found in the highly glycosylated von Willebrand factor involved in blood clotting (39). The function of the predicted MD or ET domain is not known. All of these genes are expressed in the intestine as detected by RNA *in situ* hybridization (The Nematode Expression Pattern DataBase), consistent with the intestinal localization of the glycans detected by our recombinant LEC-6 and -10 fusions. Because these proteins contain signal sequences and the detected glycan signals appear to be outside the cell, we predict that they are extracellular surface glycoproteins that contain Gal-β1,4-Fuc modifications to their *N*-glycans.

Toward understanding the role of LEC-6 and -10 and their interactions with Gal-β1,4-Fuc containing glycans, we compared the steady state localizations of endogenous LEC-6 and LEC-10 with that of their glycan ligands. Both LEC-6 and LEC-10 are predominantly localized to punctate structures in the cytoplasm when detected by specific antibodies, although they appear to be localized in different subcellular regions. LEC-6 localized to clustered punctate structures in the central region in the cytoplasm while LEC-10 mostly localized to a sub-apical region beneath the brush border. This sub-apical localized LEC-10 is also seen with the LEC-10::GFP fusion in live worms and the GFP signal appears in vesicular structures. The cytoplasmic localizations of endogenous LEC-6 and LEC-10 are in sharp contrast to that of their ligands, which are not in the cytoplasm but instead highly concentrated at the apical side of the intestinal cells. These differences indicate that at steady state most of the LEC-6 and the LEC-10 proteins are not associated with their targets.

We propose a model to explain the above phenomenon. LEC-6 and LEC-10 may be involved in the apical targeting of newly synthesized glycoproteins containing the Gal-β1,4-Fuc modified *N*-glycan. These glycoproteins are synthesized and glycosylated in the cytoplasm, and then selectively transported to the apical surface. In the cytoplasm, galectins such as LEC-6 and LEC-10 interact transiently with these glycoproteins to promote their apical sorting. This model is supported by our observation that the glycan ligands decrease at the apical surface in mutants lacking these galectins. This apical trafficking of glycoproteins involving galectins has been shown in a few examples in mammalian cells, including galectin-3 (40, 41), galactin-4 (42), and a mannose-binding lectin called VIP38 (43). The *C. elegans* intestine is highly polarized with the apical lumen side specialized in digestion and defense, and containing many components such as the glycoproteins that form the glycocalax layer (24). However, further experiments are necessary to examine whether LEC-6 and LEC-10 are indeed required for the apical trafficking of glycoprotein containing Gal-β1,4-fucose-modified glycans in the intestinal cells.

The fact that LEC-6 and LEC-10 and the specific glycoproteins they interact with are all expressed in the intestine suggests that they have physiological roles associated with this organ. The intestine is the largest organ in *C. elegans* that not only digests food and uptakes nutrients but also has to cope with ingested pathogenic bacteria or toxins. Many microbial pathogens infect the intestine (44), and such infection induces

an increased transcription of many genes (45). Remarkably, the mRNA transcripts of all four genes that we identified to interact with LEC-6 and LEC-10 are up-regulated in worms infected with certain bacterial pathogens (45), suggesting these genes might be involved in a response to bacterial infection. Recently, it has been shown that the toxicity to the mushroom galectin CGL2 is mediated through a Gal-β1,4-Fuc structure found in the intestine (21). Because we showed that LEC-6 and -10 likely recognize the same or overlapping *N*-glycan structures with CGL2, we wondered whether they are involved in CGL2 toxicity. We examined *lec-6* and *lec-10* single or double deletion mutants and found no change in their response to CGL2 toxin compared with wild-type worms. These results may not be surprising, because the glycan ligands only showed a slight decrease in the intestine lumen of the double deletion mutants. Recently, *lec-10* deletion worms are shown to have an increased susceptibility to oxidative stress (46). It would be interesting to find out whether the interaction of LEC-10 with specific glycoproteins is required for the oxidative response, and whether LEC-6 is also involved.

In summary, we have applied a novel approach to study the *in situ* localization of natural galectin ligands and to examine their interactions with galectins, which has been largely impossible before. Together with the study of galectins themselves using standard immunochemistry and GFP fusions, these analyses revealed a complex relationship between galectins and their specific glycan ligands in regards to subcellular localization. This *in situ* information about glycans and associated galectins makes it possible to analyze the role of specific glycosylation at a cellular level.

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