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Caenorhabditis elegans galectins LEC-6 and LEC-1 recognize a chemically synthesized Galβ1-4Fuc disaccharide unit which is present in Protostomia glycoconjugates

Keywords: *Caenorhabditis elegans*/ frontal affinity chromatography/ Galβ1-4Fuc/ Galβ1-4GlcNAc/ galectin

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Running title: *C. elegans* galectins recognize Galβ1-4Fuc

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Abstract

Galβ1-4GlcNAc is thought to be a common disaccharide unit preferentially recognized by vertebrate galectins. Eight amino acid residues conserved in proteins belonging to the galectin family have been suggested to be responsible for recognition. Meanwhile, we isolated and analyzed endogenous *N*-glycans of *Caenorhabditis elegans* that were captured by a *C. elegans* galectin LEC-6, and demonstrated that the unit of recognition for LEC-6 is a Gal-Fuc disaccharide, though the linkage between these residues was not confirmed. In the present study, we chemically synthesized Galβ1-4Fuc and Galβ1-3Fuc labeled with 2-aminopyridine (PA), and demonstrated that LEC-6 interacts with PA-Galβ1-4Fuc more strongly than PA-Galβ1-3Fuc by frontal affinity chromatography (FAC). Galβ1-4Fuc also inhibited hemagglutination caused by LEC-6 more strongly than Galβ1-3Fuc. FAC analysis using LEC-6 point mutants revealed that some of the conserved amino acid residues which have proved to be important for recognition of Galβ1-4GlcNAc are not necessary for the binding to Galβ1-4Fuc. Another major *C. elegans* galectin, LEC-1, also showed preferential binding to Galβ1-4Fuc. These results suggest that Galβ1-4Fuc is the endogenous unit structure recognized by *C. elegans* galectins, which implies that *C. elegans* glycans and galectins may have co-evolved through an alteration in the structures of *C. elegans* glycans and a subsequent conversion in the sugar-binding mechanism of galectins. Furthermore, since glycans containing the Galβ1-4Fuc disaccharide unit have been found in organisms belonging to

Protostomia, this unit might be a common glyco-epitope recognized by galectins in these organisms.

Introduction

Galectins are a family of lectins distributed in animals and fungi (Kasai and Hirabayashi, 1996; Cooper, 2002; Vasta *et al*., 2004). They play roles in various biological phenomena including development, immunity, and tumor metastasis, via recognition of cell-surface glycoconjugates having β-galactosides (Rabinovich *et al*., 2007). A Galβ1-4GlcNAc (*N*-acetyllactosamine) disaccharide unit is thought to be the endogenous glyco-epitope recognized by vertebrate galectins. Such ability has also been found in galectins from invertebrate species including *Caenorhabditis elegans*, and well-conserved 8 amino acid residues of galectins have proved to be important for their *N*-acetyllactosamine-binding ability (Lobsanov *et al*., 1993; Hirabayashi, *et al*., 2002; Pace *et al*., 2002; Vasta *et al*., 2004; Nemoto-Sasaki *et al*., 2008). In the genome of *C. elegans*, over 10 galectin genes have been assigned by database search (designated as *lec*-1 to *lec*-11 and DC2.3a) (Nemoto-Sasaki *et al*., 2008). We have already isolated the products of two of them, LEC-1 and LEC-6, from the worm body, and produced four other products as recombinant proteins (Hirabayashi, Satoh, Ohyama and Kasai, 1992; Hirabayashi, Satoh and Kasai, 1992; Hirabayashi *et al*., 1996; Arata *et al*. 2001; Hirabayashi *et al*., 2002; Nemoto-Sasaki *et al*., 2008). However, existence of the glycan containing *N*-acetyllactosamine units has not been confirmed in *C. elegan*s (Guerardel *et al*., 2001; Schachter, 2004; Cipollo *et al*., 2005; Griffitts *et al*., 2005; Hanneman *et al*., 2006; Paschinger *et al*., 2008). This discrepancy raises a question: What is the endogenous

glyco-epitope that is recognized by *C. elegans* galectins?

Recently, we isolated *N*-type glycoproteins captured by *C. elegan*s galectin LEC-6, and analyzed their sugar structure by mass spectrometry (MALDI-TOF/TOF analysis in conjunction with glycosidase digestion); we found that LEC-6 interacts strongly with *N*-glycans containing a Gal-Fuc disaccharide unit attached to position 6 of the innermost GlcNAc residue (Takeuchi, Hayama *et al*., 2008). The linkage between galactose and fucose residues remained undetermined, because it was difficult to reveal by MS analysis. A β1-4 linkage is more likely, because the presence of Galβ1-4Fuc has been reported in *C. elegans N*-glycans (Hanneman *et al*., 2006; Gutternigg *et al*., 2007), but the possibility of Galβ1-3Fuc cannot be excluded because of its similarity in conformation to Galβ1-4Glc (lactose).

In the present study, we compared the binding strengths of chemically synthesized Galβ1-4Fuc and Galβ1-3Fuc to LEC-6 by frontal affinity chromatography (FAC) analysis (Kasai *et al*., 1986). We used an immobilized LEC-6 column and fluorescence-labeled Galβ1-4Fuc and Galβ1-3Fuc; these disaccharides were conjugated with 2-aminopyridine (PA) via a spacer derived from mannitol (Fig. 1). Retardation of PA-Galβ1-4Fuc was significantly larger than that of PA-Galβ1-3Fuc. It was also found that hemagglutination caused by LEC-6 was inhibited more strongly by non-labeled Galβ1-4Fuc than by Galβ1-3Fuc. These observations support the assumption that Galβ1-4Fuc is the endogenous glyco-epitope present in *C. elegans N*-glycans. Furthermore, from experiments using LEC-6 point mutants, we

found that some amino acid residues of LEC-6 responsible for the binding of Galβ1-4Fuc are not the same as those for binding of Galβ1-4GlcNAc. LEC-1, another major galectin of *C. elegans*, was also shown to preferentially bind Galβ1-4Fuc.

Results

C. elegans galectin LEC-6 interacts with Galβ1-4Fuc more strongly than with Galβ1-3Fuc

In order to examine the structure of the endogenous glyco-epitope recognized by the *C. elegans* galectin LEC-6, we chemically synthesized Galβ1-4Fuc and Galβ1-3Fuc, and labeled them with the fluorophore 2-aminopyridine (PA), which is indispensable to monitor the elution profile when FAC analysis is performed, via an appropriate spacer derived from mannitol. The PA-sugars thus obtained, namely, PA-Galβ1-4Fuc and PA-Galβ1-3Fuc (Fig. 1) were subjected to FAC analysis by using an immobilized LEC-6 column. For comparison, E3, an endogenous ligand oligosaccharide labeled with PA, which was derived from *C. elegans N*-glycan trapped by a high capacity LEC-6 Sepharose column and whose structure was determined by MS analysis previously (Takeuchi, Hayama *et al*., 2008), was also subjected to FAC analysis. Fig. 2A shows the elution profiles of each PA-sugar. The extent of retardation of the elution front compared with that of PA-rhamnose, which has no affinity for LEC-6, is proportional to the binding constant of each PA-sugar. The elution fronts of Galβ1-4Fuc, Galβ1-3Fuc, and E3 showed evident retardation. K_a and K_d values calculated for the LEC-6-PA-sugar complex are shown in Fig. 2B. E3 was shown to have the strongest affinity. The K_a value for Galβ1-4Fuc was lower than that for E3, but it was 4-fold higher than that for Galβ1-3Fuc.

These results suggest that Galβ1-4Fuc, but not Galβ1-3Fuc, is the endogenous disaccharide unit of recognition that preferentially interacts with LEC-6. It was also suggested that in the case of the oligosaccharide E3, portions other than the Galβ1-4Fuc moiety contribute to the binding. We also examined the ability of the corresponding synthesized sugars (not fluorescence-labeled) to inhibit hemagglutination caused by LEC-6 (Fig. 2C). The observed minimum concentrations required for inhibition of hemagglutination were as follows: Galβ1-4Fuc, 2.5 mM; Galβ1-3Fuc, 10.0 mM; and Galβ1-4Glc, 5.0 mM. Galβ1-4Fuc was 4 times stronger than Galβ1-3Fuc.This also supports that Galβ1-4Fuc, not Galβ1-3Fuc, should be the endogenous glyco-epitope recognized by LEC-6.

Amino acid residues of LEC-6 responsible for the recognition of Galβ1-4Fuc are not the same as those of Galβ1-4GlcNAc (or Galβ1-4Glc)

The 8 amino acid residues well conserved among the galectin family were shown to be important for the recognition of *N*-acetyllactosamine (Hirabayashi and Kasai, 1991; Lobsanov *et al*., 1993; Hirabayashi *et al*., 2002). They are also conserved in LEC-6 (Fig. 3A), which is able to bind glycans containing Galβ1-4GlcNAc (Hirabayashi *et al*., 1996; Hirabayashi *et al*., 2002). However, the above results showed that LEC-6 recognizes Galβ1-4Fuc more strongly. Since Galβ1-4Fuc and Galβ1-4GlcNAc (or Galβ1-4Glc) are different from chemical and conformational viewpoints (Fig. 3B and C), it is possible that the mechanism of recognition of

Galβ1-4Fuc by LEC-6 is different from that of Galβ1-4GlcNAc by vertebrate galectins. To test this possibility, we substituted each one of the 8 conserved amino acid residues of LEC-6 and expressed each point mutant as FLAG-tagged recombinant protein in *Escherichia coli*. Three of them (N62, E83, and R85) correspond to the residues essential for Galβ1-4GlcNAc-binding ability in the case of human galectin-1 (Hirabayashi and Kasai, 1991) (Fig. 3A). The extract of *E. coli* was applied to an asialofetuin Sepharose column. Following extensive washing of the column, the bound protein was specifically eluted with lactose (Fig. 4).

In the case of wild-type LEC-6 (WT), most of the recombinant protein was detected in lactose-eluted fractions (Fig. 4A, fraction 14 and 15), though some portion was detected both in follow-through fractions (fraction 1–4) and in retarded fractions (fraction 5–13). On the other hand, most LEC-6 point mutants were detected in retarded fractions, except the N62D and R64H mutants. N62D was mainly detected in lactose-eluted fractions, though its recovery in the eluted fractions seemed slightly smaller than that of WT (data not shown). In contrast, R64H was detected almost exclusively in follow-through fractions. These results suggest that substitution of each one of the 8 amino acid residues resulted more or less in the reduction of binding ability to the glycan parts of asialofetuin, that is, these residues of LEC-6 are also important for the recognition of Galβ1-4GlcNAc, as in the case of vertebrate galectins.

Next, in order to obtain quantitative data, we performed frontal affinity chromatography (FAC) using columns packed with immobilized point mutants on Sepharose. We recovered fractions from asialofetuin affinity chromatography containing each point mutant (except R64H mutant since it showed no retardation) (Fig. 4) and immobilized each mutant protein on NHS-activated Sepharose. FAC analysis was performed by applying fluorescence-labeled sugars to these immobilized mutant LEC-6 columns. The K_a and K_d values obtained are shown in Fig. 5 and Table I. The K_a values of LEC-6 point mutants for sugars containing Galβ1-4GlcNAc units (NA2, NA3, and NA4) were found to be significantly lower in comparison with WT. Therefore, these conserved residues seem to be important for the recognition of Galβ1-4GlcNAc. However, though H60F and E83Q have little or no affinity for sugars containing Galβ1-4Fuc (E3 and PA-Galβ1-4Fuc), N62D and V71A were found to retain affinity for these sugars. N73D, W80F, and R85H have reduced affinity for E3, but they retain almost similar affinity for Galβ1-4Fuc.

These results suggest that these 8 conserved residues function differently when the counterpart is Galβ1-4GlcNAc or Galβ1-4Fuc. His60 and Glu83 seem to be important for the binding of Galβ1-4Fuc. The others residues seem to be important for the binding of Galβ1-4GlcNAc but not of Galβ1-4Fuc. Since the K_a value of the R85H mutant for E3 was lower than that for Galβ1-4Fuc, such a mutation seems to cause hindrance to the interaction with a portion of E3 other than the Galβ1-4Fuc moiety. In the cases of N73D and W80F, the

affinity for E3 was reduced to half from WT, though that for Galβ1-4Fuc did not change significantly. This implies that these residues are involved in the interaction with a portion of E3 other than the Galβ1-4Fuc moiety. Collectively, these results indicate that a different binding mode exists when LEC-6 interacts with Galβ1-4Fuc instead of Galβ1-4GlcNAc.

C. elegans galectin LEC-1 also binds Galβ1-4Fuc preferentially

The above observation that Galβ1-4Fuc is the endogenous disaccharide unit recognized by LEC-6 raised a possibility that other *C. elegans* galectins also recognize the same unit. Therefore, we examined LEC-1, which is another major galectin, and moreover, the first galectin found from *C. elegans*. It was first isolated by affinity chromatography on immobilized asialofetuin and has been extensively studied (Hirabayashi, Satoh, Ohyama and Kasai, 1992; Hirabayashi, Satoh and Kasai, 1992; Arata *et al*., 2001). Here, we performed FAC analysis using an immobilized recombinant LEC-1 column, and the results are shown in Fig. 6A. Significant retardation was observed for PA-Galβ1-4Fuc, PA-Galβ1-3Fuc, and PA041 (PA-Galβ1-4GlcNAcβ1-3Galβ1-4Glc). The K_a and K_d values calculated are shown in Fig. 6B. It is evident that LEC-1 also preferentially recognizes β1-4-linked Gal-Fuc. Affinities for Galβ1-3Fuc or Galβ1-4GlcNAc (present in PA041) were weaker. Inhibition of hemagglutination caused by LEC-1 was also examined (Fig. 6C). The observed minimum concentrations required for inhibition were as follows: Galβ1-4Fuc, 1.25 mM; Galβ1-3Fuc,

5.0 mM; Galβ1-4Glc, 2.5 mM. Galβ1-4Fuc was found to be more effective than Galβ1-3Fuc and Galβ1-4Glc as an inhibitor. Therefore, LEC-1 also exhibited preferential recognition of Galβ1-4Fuc.

Discussion

In the present study, we showed that the major *C. elegans* galectins LEC-6 and LEC-1 prefer Galβ1-4Fuc to Galβ1-4GlcNAc. Since the existence of glycan having multiple *N*-acetyllactosamine units, which is the endogenous unit recognized by vertebrate galectins, has not been confirmed in *C. elegans* glycoconjugates (Guerardel *et al*., 2001; Cipollo *et al*., 2005; Griffitts *et al*., 2005; Hanneman *et al*., 2006; Paschinger *et al*., 2008), these results suggest that Galβ1-4Fuc is the endogenous glyco-epitope for *C. elegans* galectins instead of Galβ1-4GlcNAc. The existence of the Galβ1-4Fuc unit has already been reported in nematode, squid, octopus, and keyhole limpet (Zhang *et al*., 1997; Takahashi *et al*., 2003; Wuhrer *et al*., 2004; Hanneman *et al*., 2006; Gutternigg *et al*., 2007), i.e., species belonging to Protostomia. This implies that the Galβ1-4Fuc disaccharide unit may be used as a common glyco-epitope for galectins or galectin-like lectins in the organisms belonging to Protostomia, although no report has appeared so far that showed the existence of galectins in these species except for nematode.

Since Galβ1-4Fuc is structurally different from Galβ1-4GlcNAc, the binding site of LEC-6 may also be different from those of vertebrate galectins in order to accommodate the former. However, the 8 conserved amino acid residues of galectins thought to be important for the recognition of Galβ1-4GlcNAc are also present in LEC-6 (Nemoto-Sasaki *et al*., 2008). In the present research using LEC-6 point mutants, these residues were also found important for

binding of Galβ1-4GlcNAc by both asialofetuin-binding assay and FAC analysis. On the other hand, some of them proved to be unnecessary for the binding of Galβ1-4Fuc. Since His60, Asn62, Arg64, Asn73, Trp80, and Glu83 of LEC-6 were thought to be necessary for the interaction with the Gal moiety (See, Fig. 3B), substitution of these 6 residues was expected to result in reduction in binding not only of Galβ1-4GlcNAc but also of Galβ1-4Fuc. However, N62D, N73D, and W80F mutant proteins retained the ability to recognize Galβ1-4Fuc, though some extent of reduction in binding affinity for E3 was observed in the cases of N73D and W80F mutant proteins. Among residues supposed to be responsible for the recognition of the GlcNAc moiety of *N*-acetyllactosamine, Val71 was found to be nonessential for the recognition of Galβ1-4Fuc. Significant reduction in Galβ1-4GlcNAc recognition was observed for all mutant proteins.

On the basis of these results, Galβ1-4Fuc seems to be accommodated in the binding site of LEC-6 in a mode somewhat different from Galβ1-4GlcNAc, though the influence of substitution of residues on the conformation of the binding site should not be neglected, and we have no information on the conformation of Galβ1-4Fuc docked in the binding site. We are undertaking X-ray crystallography of the complex between LEC-6 and Galβ1-4Fuc. It should also be noted that Asp62 and Val71 of LEC-6, which seem to be necessary for the recognition of Galβ1-4GlcNAc, but are not required for the binding of Galβ1-4Fuc and E3, are relatively less conserved in *C. elegans* galectins in comparison with the other 6 highly

conserved residues (Nemoto-Sasaki *et al*., 2008).

LEC-1 was also found to bind Galβ1-4Fuc more strongly than Galβ1-4GlcNAc. However, the binding strength of LEC-1 for Galβ1-4Fuc was 4 times lower than that of LEC-6, and E3, an oligosaccharide isolated as an endogenous ligand *N*-glycan of LEC-6 having a Galβ1-4Fuc disaccharide unit linked to position 6 of innermost GlcNAc, was poorly recognized by LEC-1 (unpublished result). Therefore, it seems that the structures of endogenous ligand oligosaccharides of LEC-1 are not the same as those of LEC-6. Isolation and identification of the endogenous ligand glycans of LEC-1 are in progress.

There exists a variety of differences between structures of glycans of *C. elegans* and those of vertebrates. Although no evidence of the presence of glycans containing the Galβ1-4GlcNAc units in *C. elegans* has been presented so far (Schachter, 2004; Cipollo *et al*., 2005; Paschinger *et al*., 2008,), *C. elegans* galectins actually retain Galβ1-4GlcNAc-binding ability (Hirabayashi *et al*., 2002; Nemoto-Sasaki *et al*., 2008). This property enabled us to discover galectins from *C. elegans*, because we first isolated LEC-1 and LEC-6 from the worm body using asialofetuin Sepharose (Hirabayashi, Satoh, Ohyama and Kasai, 1992; Hirabayashi, Satoh and Kasai, 1992; Hirabayashi *et al*., 1996). In the present study, we showed that the *C. elegans* galectins LEC-6 and LEC-1 recognize Galβ1-4Fuc more strongly than Galβ1-4GlcNAc, and the mechanism of this recognition seems to be different from that of the recognition of Galβ1-4GlcNAc. These observations suggest the occurrence of

co-evolution between *C. elegans* glycans and galectins. Structures of *C. elegans* glycans have been altered during their evolutional process in a way different from those of vertebrates. Animals belonging to Protostomia acquired Galβ1-4Fuc, but those belonging to Deuterostomia acquired Galβ1-4GlcNAc as the unit of recognition for galectins. Because fungi and sponges have galectins (Cooper, 2002), ancestors of the galectin family should have existed before the divergence of Protostomia and Deuterostomia. After the divergence, Protostomia galectins and Deuterostomia galectins seem to have evolved in order to more efficiently accommodate Galβ1-4Fuc and Galβ1-4GlcNAc, respectively. If this is true, the importance of the galectin-glycan interaction has never declined in Protostomia and Deutrostomia in spite of alterations in the glycan structure. Therefore, research on the galectins of *Drosophila melanogaster* (another species in which galectins were found) and their endogenous glyco-epitopes seems to be important (Pace *et al*., 2002; Pace and Baum, 2004). Synthesized Galβ1-4Fuc and PA-Galβ1-4Fuc should be very useful tools for further research on the sugar recognition phenomena occuring in Protostomia.

Materials and methods

Materials

Galactoseβ1-4Fucose (Galβ1-4Fuc), Galactoseβ1-3Fucose (Galβ1-3Fuc), Galβ1-4Fuc labeled with pyridylamine via a spacer (PA-Galβ1-4Fuc), and Galβ1-3Fuc labeled with pyridylamine via a spacer (PA-Galβ1-3Fuc) were chemically synthesized (details regarding the synthesis will appear elsewhere). Their structures were confirmed by ^{1}H-NMR analyses.

Construction of a recombinant LEC-6 expression plasmid

The open reading frame of LEC-6 containing the *Bam*HI and *Xho*I sites was amplified by a polymerase chain reaction (PCR) using 5′-GGATCCATGATCGGAGGAGGAAT-3′ as the forward primer and 5′-CTCGAGTTAGTGAGAAACATGGG-3′ as the reverse primer. The PCR fragment thus obtained was cloned into the *Xcm*I-digested pGEM-TX plasmid generated by inserting the double-strand DNA fragment that was composed of 5′-TACCATATGTTAACTGGGGAGTCGACGCCCCAGCTGACTAGTGG-3′ and 5′-CATGCCACTAGTCAGCTGGGGCGTCGACTCCCCAGTTAACATATGG-3′ into the *Nco*I and *Nde*I sites of the pGEM-T plasmid (Promega, Madison, WI, USA). The pGEM-TX-LEC-6 plasmid was then digested with *Bam*HI and *Xho*I, and the DNA fragment thus obtained was inserted into the *Bam*HI and *Xho*I sites of the pET-FLAG vector (Takeuchi, Sennari *et al*., 2008). Point mutants of LEC-6 were generated by PCR using the

pGEM-LEC-6 plasmid and the following primers (substitution sites are underlined): H60F, 5′-TTCTTCAACGCTAGATTCGATG-3′ and 5′-AAGAACAATGTCGTCTGGAGTG-3′; N62D, 5′-GACGCTAGATTCGATGAGGG-3′ and 5′-GAAGTGAAGAACAATGTCGTC-3′; R64H, 5′-CACTTCGATGAGGGAGCTGTTG-3′ and 5′-AGCGTTGAAGTGAAGAACAATG-3′; V71A, 5′-GCGAACAACTCGACCAGCGG-3′ and 5′-AACAGCTCCCTCATCGAATC-3′; N73D, 5′-GACTCGACCAGCGGAGGAGG-3′ and 5′-GTTCACAACAGCTCCCTCATC-3′; W80F, 5′-TTCCAATCCGAGGATCGTCAC-3′ and 5′-ACCTCCTCCGCTGGTCGAGT-3′; E83Q, 5′-CAGGATCGTCACGCCAAT-3′ and 5′-GGATTGCCAACCTCCTCC-3′; R85H, 5′-ATCACGCCAATCCATTCCAG-3′ and 5′-GATCCTCGGATTGCCAAC-3′. Insert DNA fragment of the pGEM-LEC-6 point mutant plasmid was then subcloned into the pET-FLAG vector.

Expression and affinity purification of recombinant galectins on asialofetuin-Sepharose

Escherichia coli strain BL21 (DE3) transformed with a FLAG-tagged LEC-6 expression plasmid was cultured overnight at 37°C in LBA medium (Luria-Bertani medium supplemented with 50 μg/ml of ampicillin), transferred to a 25-fold volume of 2×YT medium containing 50 μg/ml of ampicillin, and then incubated at 37°C for 3 h. Following chilling in ice-cold water, isopropyl-1-thio-β-D-galactopyranoside was added to the culture at a final concentration of 0.2 mM and the culture was further incubated overnight at 20°C. The cells

were then harvested and suspended in phosphate-buffered saline-EDTA (PBS-EDTA; 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl, pH 7.4, supplemented with 2 mM EDTA) and then lysed by sonication. Following centrifugation of the lysate, the recombinant LEC-6 contained in the supernatant was adsorbed on asialofetuin-Sepharose packed in a column (bed volume 7ml for LEC-6 WT, N62D, E83Q, and R85H; bed volume 10 ml for the other mutants) prepared as described previously (Arata *et al*., 1997). The adsorbed protein was eluted with PBS-Lac (PBS-EDTA containing 0.1 M lactose). These purification procedures were performed at 4°C. The fraction volume was set to 6 mL throughout the experiment. Each fraction was subjected to SDS-PAGE and stained with Bio-Safe™ Coomassie (BIO-RAD, Hercules, CA, USA). The protein concentration of each fraction was determined using a BIO-RAD Protein Assay (BIO-RAD) with bovine serum albumin as the standard. For the preparation of immobilized mutant LEC-6 columns, the following fractions of asialofetuin affinity chromatography (Fig. 4) were recovered: WT, Fr. 14 and 15; H60F, Fr. 5–7; N62D, Fr. 14 and 15; V71A, Fr. 5–8; N73D, Fr. 4 and 5; W80F, Fr. 6–10; E83Q, Fr. 5 and 6; R85H, Fr. 6 and 7. Expression and purification of recombinant LEC-1 protein was performed basically as described above, with the exception that the pET-LEC-1 plasmid (Arata *et al*., 1997) was used for transformation of *E. coli*, and LBA medium was used instead of 2×YT medium.

Frontal affinity chromatography analysis

Immobilization of recombinant galectins on HiTrap NHS-activated Sepharose (GE Healthcare, St. Giles, UK) and FAC analysis were performed basically as described previously (Takeuchi, Hayama *et al*., 2008). In brief, each PA-sugar at a concentration of 5 nM was applied to an immobilized galectin column (1.6 mg LEC-6, 5.4 mg LEC-6 H60F, 1.6 mg LEC-6 N62D, 7.2 mg LEC-6 V71A, 8.2 mg LEC-6 N73D, 6.4 mg LEC-6 W80F, 3.6 mg LEC-6 E83Q, 6.6 mg LEC-6 R85H, or 11.7 mg LEC-1 protein/mL gel) at a flow rate of 0.25 mL/min at 20 °C, and the elution profile was monitored by a fluorescence detector. The K_d value for the interaction between galectin and PA-sugar was determined according to the following basic equation of FAC: $K_d = B_t/(V_f - V_0) - [A]_0$. In this equation, B_t is the effective ligand content, V_f is the volume of the elution front, V_0 is the V_f of PA-rhamnose that is not bound by LEC-6 and LEC-1, and $[A]_0$ is the initial concentration of the PA-sugar. If the $[A]_0$ is negligibly smaller than K_d, the equation can be simplified as $K_d = B_t/(V_f - V_0)$. In this study, the B_t value of each immobilized galectin column was calculated from the data obtained by concentration-dependence analysis with various concentrations of Galβ1-4Fuc, and that of LEC-1 column was calculated from the reported K_d values for the interaction between LEC-1 and PA041 (See below) (Arata *et al*., 2001). The K_a values were calculated based on the equation, $K_a = 1/K_d$. PA-oligosaccharides used are as follows. PA001 (NA2, Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcAc-P

A), PA002 (NA3, Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-4)Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-PA), PA004 (NA4, Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-4)Manα1-3(Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-6)Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-PA), PA041 (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA). These PA-oligosaccharides and PA-rhamnose were purchased from Takara Bio (Shiga, Japan). E3, a pyridylaminated endogenous ligand *N*-glycan of LEC-6, was isolated from *C. elegans* as reported previously (Takeuchi, Hayama *et al*., 2008).

Hemagglutination assay

Rabbit erythrocytes (Nippon Biotest Labo, Tokyo, Japan) were treated with trypsin and then with glutaraldehyde. The erythrocytes were then washed with phosphate-buffered saline (PBS) containing 0.1 M glycine, after which they were extensively washed with Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl). To examine the ability of the competitive sugars to inhibit the hemagglutination activity of galectins, an appropriate concentration of galectin (25 μg/mL LEC-6 or 12.5 μg/mL LEC-1), 50 μg/mL of bovine serum albumin, 1% (v/v) rabbit erythrocytes, and two times serially diluted competitive-sugars (at a final concentration of 0.005–10 mM) were mixed in 100 μL of TBS in a 96-well V-shaped titer plate, and then incubated at room temperature for 1 h.

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Legends to Figures

Figure 1. Structures of chemically synthesized PA-sugars

The structures of Galβ1-4Fuc and Galβ1-3Fuc labeled with 2-aminopyridyne (PA) via a spacer derived from mannitol are depicted. These conformations are based on ^{1}H-NMR spectra. (A) PA-Galβ1-4Fuc. (B) PA-Galβ1-3Fuc.

Figure 2. Frontal affinity chromatography analysis of PA-sugars containing Gal-Fuc on an immobilized LEC-6 column and hemagglutination inhibition assay of disaccharides

(A) Elution profiles of the PA-sugars from an immobilized LEC-6 column. The structure of each PA-sugar is depicted in each panel of elution profile. Open circle with diagonal line, hexose; open circle, mannose; filled circle, galactose; filled square, *N*-acetylglucosamine; open triangle, fucose. The elution profile of each PA-sugar (solid line) was superimposed on that of PA-rhamnose (broken line) which has no affinity for LEC-6. (B) The K_a and K_d values for the interaction between LEC-6 and PA-sugars were calculated as described in Materials and Methods. (C) Inhibition of the hemagglutination activity of LEC-6 by competitive sugars. The final concentration of LEC-6 was fixed at 25 μg/mL. Competitive sugars were added to each well at varying concentration. The final concentrations of competitive sugars are indicated.

Figure 3. Alignment of human galectin-1 and LEC-6 and comparison of the structures of Galβ1-4Glc with Galβ1-4Fuc

(A) Alignment of amino acid sequences of human galectin-1 (hGal-1) and LEC-6. The three amino acids (N46, E71, and R73 corresponding to N62, E83, R85 of LEC-6, respectively) that were demonstrated to be essential for sugar binding function of hGal-1 (Hirabayashi and Kasai, 1991) are denoted by closed arrowheads. The 8 amino acids (H, N, R, V, N, W, E, and R) conserved among proteins belonging to the galectin family are denoted by closed circles. Conserved or homologous amino acids are denoted by asterisks. The initiating methionine residue is not contained in amino acid sequence of hGal-1, because the residue of hGal-1 has been proved to be processed in its mature form (Hirabayashi and Kasai, 1988), and for consistency with the previous report (Hirabayashi and Kasai, 1991). (B) A simulated model of interaction between Galβ1-4Glc and the binding site of LEC-6 modified from the report on the crystallography of human galectin-2 and the Galβ1-4Glc complex (Lobsanov *et al*., 1993). Hydrogen bonds are denoted by broken lines. Van der Waals interactions are denoted by grey circles. (C) Galβ1-4Fuc depicted on the assumption that it takes the same conformation as that of Galβ1-4Glc docked in the binding site.

Figure 4. Results of affinity chromatography of point mutant recombinant LEC-6 proteins on an asialofetuin-Sepharose column

Recombinant LEC-6 protein was expressed in *E. coli* BL21 (DE3), and the cells were then disrupted. After centrifugation, the supernatant fraction was applied to an asialofetuin-Sepharose column (bed volume 7 ml for WT, N62D, E83Q, and R85H; bed volume 10 ml for the other mutants). After the column was washed with PBS-EDTA, the adsorbed protein was eluted with 0.1 M lactose. Successive 6 mL fractions were collected and subjected to SDS-PAGE. (A) Wild type. (B) H60F. (C) N62D. (D) R64H. (E) V71A. (F) N73D. (G) W80F. (H) E83Q. (I) R85H. Recombinant LEC-6 proteins are indicated by arrowheads.

Figure 5. FAC analysis of PA-sugars on columns immobilized with WT and point mutants LEC-6

(A) Structures of the PA-sugars examined by FAC analysis. Symbols are as in Figure 2. (B) The K_a values for the interaction between LEC-6 and PA-sugars. ND indicates not determined, because no retardation was observed and it was unable to calculate.

Figure 6. FAC analysis of PA-sugars on an immobilized LEC-1 column and the ability of sugars to inhibit hemagglutination caused by LEC-1

(A) Elution profiles of the PA-sugars. The structure of each PA-sugar is depicted in each elution profile. Symbols are as in Figure 2, and grey circles indicate glucose residue. The elution profile of each PA-sugar (solid line) was superimposed on that of PA-rhamnose (broken line). (B) The K_a and K_d values for the interaction between LEC-1 and PA-sugars. (C) Inhibition of hemagglutination activity of LEC-1 by competitive sugars. Final concentration of LEC-1 was fixed at 12.5 μg/mL; final concentrations of competitive sugars are indicated.

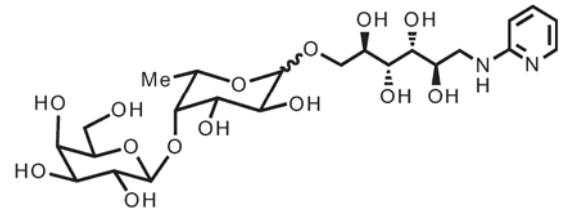
Table I. Summary of the K_d values (μM) between PA-sugars and LEC-6 wild type (WT) or its point mutants, determined by frontal affinity chromatography

	NA2	NA3	NA4	E3	Galβ1-4Fuc
WT	37.8	19.1	16.1	6.9	17.9
H60F	ND	ND	ND	ND	ND
N62D	155.7	83.1	80.1	7.6	22.7
V71A	752.7	74.8	68.8	6.7	22.3
N73D	ND	ND	ND	15.0	21.4
W80F	172.0	66.2	57.3	12.7	32.1
E83Q	ND	ND	ND	ND	ND
R85H	892.0	341.8	382.7	42.8	24.4

ND indicates not determined because of no retardation.

Figure 1

A. PA-Galβ1-4Fuc



B. PA-Galβ1-3Fuc

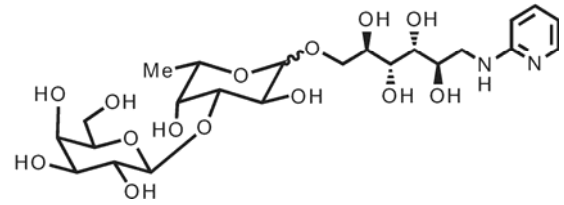


Figure 2

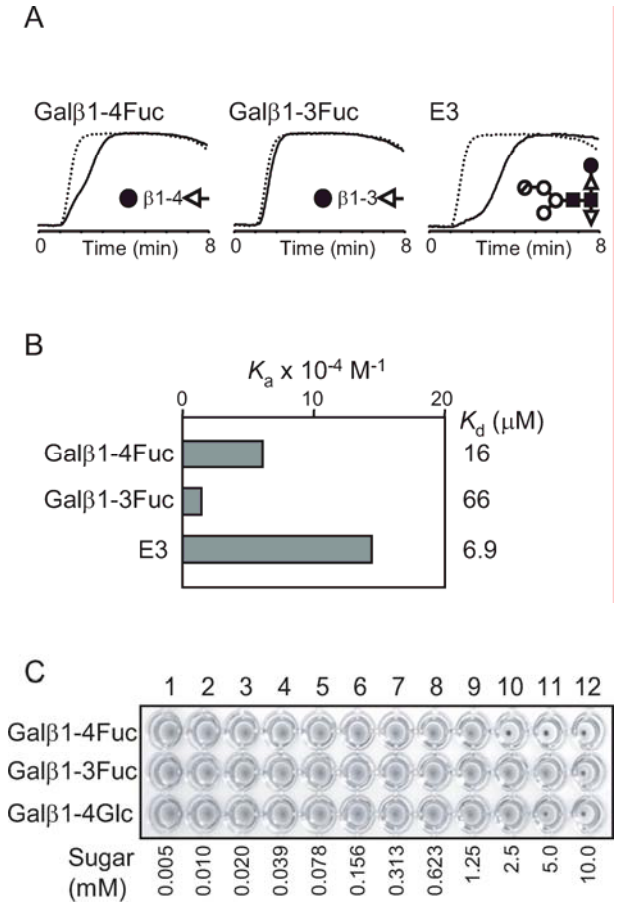

A
Galβ1-4Fuc
Galβ1-3Fuc
E3
β1-4
β1-3
0
Time (min)
8
B
K_a x 10^{-4} M^{-1}
0
10
20
K_d (μM)
Galβ1-4Fuc
Galβ1-3Fuc
E3
16
66
6.9
C
1 2 3 4 5 6 7 8 9 10 11 12
Galβ1-4Fuc
Galβ1-3Fuc
Galβ1-4Glc
Sugar (mM)
0.005
0.010
0.020
0.039
0.078
0.156
0.313
0.623
1.25
2.5
5.0
10.0

Figure 3

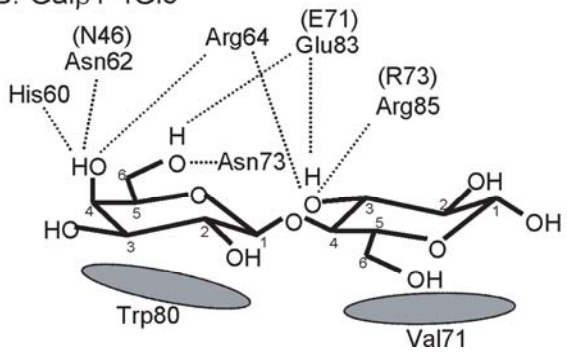
A

```
hGal-1   1:--------------------ACGLVASNLN-LKPGECLRVRGEVAPDAKSFVLNLGKDSNN 40
LEC-6    1:MIGGGIGISFCNEFFNPQTPVNI---PVQGFSNGARLRLVLLPTSADSRFHINLRTPD-D 56
                                          *  **           *  **

hGal-1  41:LCLHFNPRFNAHGDANTIVCNSKDGGAWGTEQREAVFPFQPGSVAEVCITFDQANLTVKL 100
LEC-6   57:IVLHFNARFDEG---AV-VNNSTSGGGWQSEDRHAN-PFQQNKIYTLEFVSNGGIISIFV 111
            **** **         * **  ** *  * * *  ***

hGal-1 101:PDGYEFK-FPNRLNLEAINYMAADGDFKIKCVAFD-                          134
LEC-6  112:NGA-HFADFVERTPSHGVHLIEIEGGVHVHSAHVSH                          146
                  *  *  *           *
```

B. Galβ1-4Glc



C. Galβ1-4Fuc

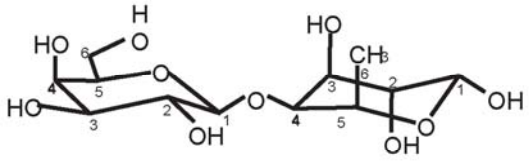


Figure 4

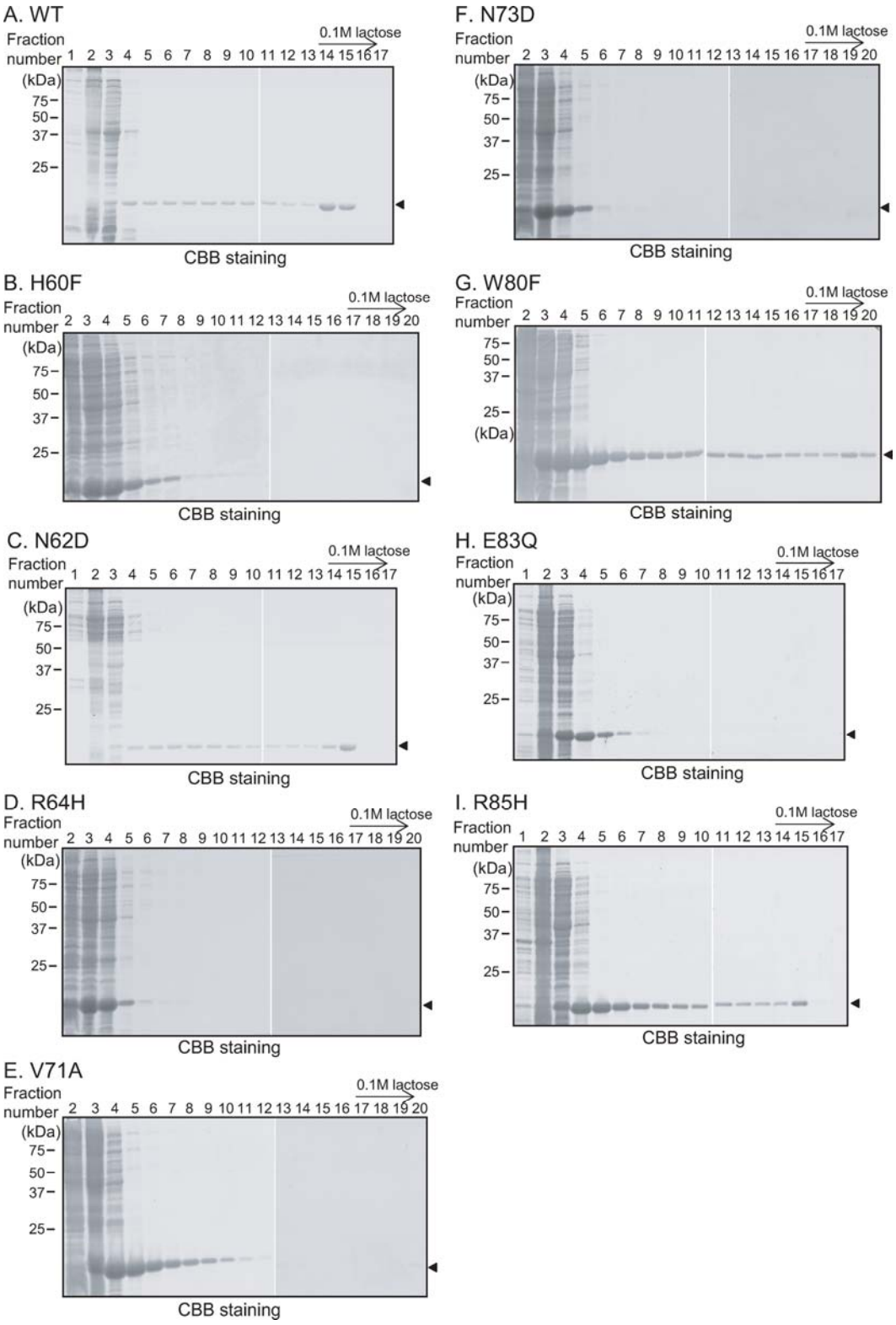

A. WT
Fraction number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0.1M lactose
(kDa)
75
50
37
25
CBB staining
F. N73D
Fraction number 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
0.1M lactose
(kDa)
75
50
37
25
CBB staining
B. H60F
Fraction number 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
0.1M lactose
(kDa)
75
50
37
25
CBB staining
G. W80F
Fraction number 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
0.1M lactose
75
50
37
25
(kDa)
CBB staining
C. N62D
Fraction number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0.1M lactose
(kDa)
75
50
37
25
CBB staining
H. E83Q
Fraction number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0.1M lactose
(kDa)
75
50
37
25
CBB staining
D. R64H
Fraction number 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
0.1M lactose
(kDa)
75
50
37
25
CBB staining
I. R85H
Fraction number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0.1M lactose
(kDa)
75
50
37
25
CBB staining
E. V71A
Fraction number 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
0.1M lactose
(kDa)
75
50
37
25
CBB staining

Figure 5

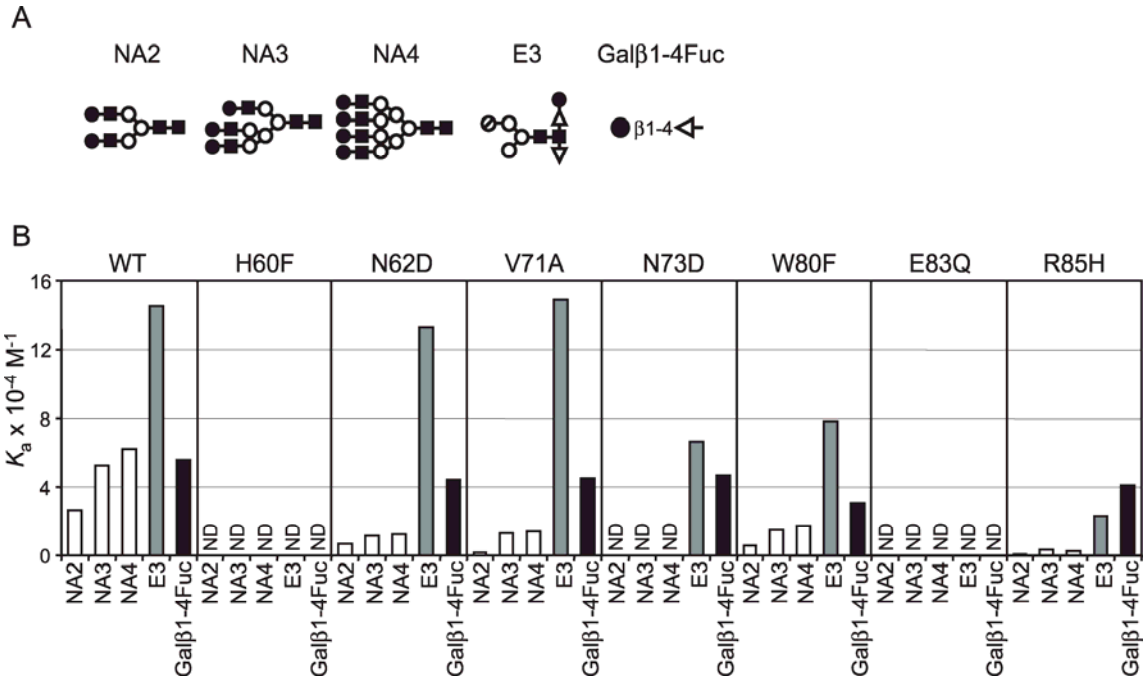
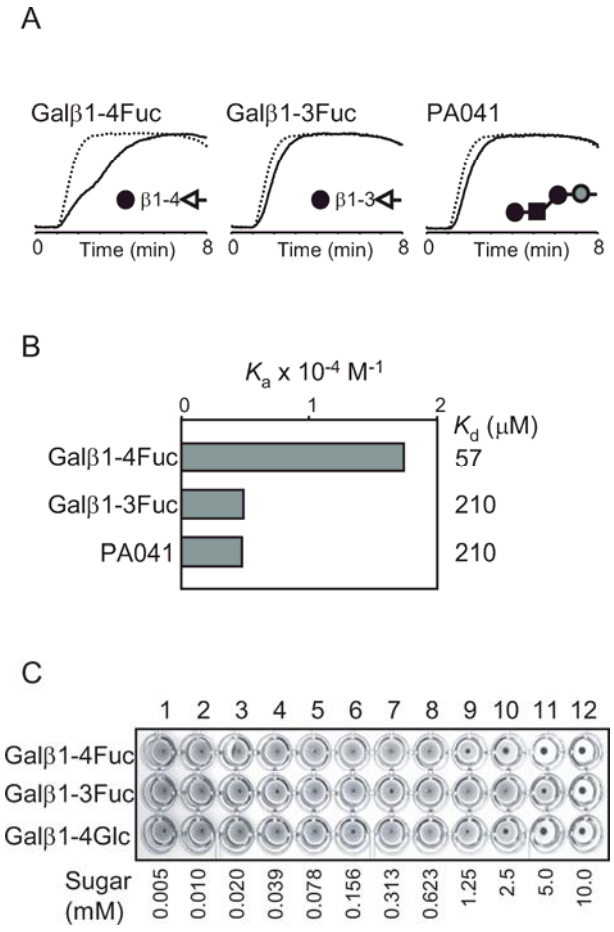

A
NA2
NA3
NA4
E3
Galβ1-4Fuc
β1-4
B
WT
H60F
N62D
V71A
N73D
W80F
E83Q
R85H
K_a x 10^{-4} M^{-1}
16
12
8
4
0
ND
NA2
NA3
NA4
E3
Galβ1-4Fuc

Figure 6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Galβ1-3Fuc
PA041
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β1-3
0
Time (min)
8
B
K_a x 10^{-4} M^{-1}
0
1
2
K_d (μM)
Galβ1-4Fuc
57
Galβ1-3Fuc
210
PA041
210
C
1 2 3 4 5 6 7 8 9 10 11 12
Galβ1-4Fuc
Galβ1-3Fuc
Galβ1-4Glc
Sugar (mM)
0.005
0.010
0.020
0.039
0.078
0.156
0.313
0.623
1.25
2.5
5.0
10.0