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Caenorhabditis elegans proteins captured by immobilized Galβ1-4Fuc disaccharide units: Assignment of 3 annexins

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Abbreviations: ACN, acetonitrile; EDTA, ethylenediaminetetraacetic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; TFA, trifluoroacetic acid

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Abstract

Galβ1-4Fuc is a key structural motif in *Caenorhabditis elegans* glycans and is responsible for interaction with *C. elegans* galectins. In animals of the clade Protostomia, this unit seems to have important roles in glycan–protein interactions and corresponds to the Galβ1-4GlcNAc unit in vertebrates. Therefore, we prepared an affinity adsorbent having immobilized Galβ1-4Fuc in order to capture carbohydrate-binding proteins of *C. elegans*, which interact with this disaccharide unit. Adsorbed *C. elegans* proteins were eluted with ethylenediaminetetraacetic acid (EDTA) and followed by lactose (Galβ1-4Glc), digested with trypsin, and were then subjected to proteomic analysis using LC-MS/MS. Three annexins, namely NEX-1, -2, and -3, were assigned in the EDTA-eluted fraction. Whereas, galectins, namely LEC-1, -2, -4, -6, -9, -10, and DC2.3a, were assigned in the lactose-eluted fraction. The affinity of annexins for Galβ1-4Fuc was further confirmed by adsorption of recombinant NEX-1, -2, and -3 proteins to the Galβ1-4Fuc column in the presence of Ca^{2+}. Furthermore, frontal affinity chromatography analysis using an immobilized NEX-1 column showed that NEX-1 has an affinity for Galβ1-4Fuc, but no affinity towards Galβ1-3Fuc and Galβ1-4GlcNAc. We would hypothesize that the recognition of the Galβ1-4Fuc disaccharide unit is involved in some biological processes in *C. elegans* and other species of the

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Protostomia clade.

Keywords: Galβ1-4Fuc; Annexin; Galectin *Caenorhabditis elegans*; NEX

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1. Introduction

Galectins are a family of lectins distributed in animals and fungi and characterized by their conserved carbohydrate-recognition domain having affinity for β-galactosides.[1–3] In vertebrates, a Galβ1-4GlcNAc disaccharide unit in glycoconjugates was known to be the endogenous motif recognized by galectins. On the other hand, although galectins are also present in *Caenorhabditis elegans*, the presence of the Galβ1-4GlcNAc structure had not been confirmed in this species.[4–6] This discrepancy was resolved by our recent finding that the motif recognized by LEC-6, a *C. elegans* galectin, is Galβ1-4Fuc, rather than Galβ1-4GlcNAc.[7,8] Other *C. elegans* galectins, namely LEC-1 and LEC-10, were also found to have an affinity for Galβ1-4Fuc.[8,9] These findings indicate that Galβ1-4Fuc is a principal disaccharide motif recognized by *C. elegans* galectins, taking the place of Galβ1-4GlcNAc in the vertebrate galectins. Furthermore, this raises the possibility that Galβ1-4Fuc serves as a recognition motif for other sugar-binding proteins in *C. elegans*.

In the genome of *C. elegans*, genes encoding various families of carbohydrate-binding proteins, such as galectins, C-type lectins, and annexins, have been assigned, and sugar-binding abilities of some of their products have been demonstrated[10–15] : as for *C. elegans* annexin proteins, their binding abilities toward

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heparan sulfate and chondroitin were analyzed, and NEX-1 and -3 were found to bound only to heparan sulfate, whereas NEX-2 bound to the both.[14,15] However, Galβ1-4Fuc-binding ability of most of these proteins has not been reported. Therefore, we prepared an agarose derivative containing immobilized Galβ1-4Fuc, and tried to identify proteins bound by this adsorbent. We found that various galectins and annexins have an affinity for Galβ1-4Fuc.

2. Results and discussion

2.1. Preparation of an immobilized Galβ1-4Fuc column

In order to capture proteins of *C. elegans* that interact with the Galβ1-4Fuc disaccharide unit, we prepared an adsorbent by immobilizing Galβ1-4Fuc on agarose gel via hydrophilic spacer modified with a free amino group (Fig. 1A). The quality of the adsorbent was assessed by applying purified wild-type (WT) LEC-6 protein and an extract of *E. coli* expressing the LEC-6 N73D mutant, which is known to have only weak affinity for the Galβ1-4GlcNAc disaccharide unit,[8] to the column. After extensive washing, 0.1 M lactose solution was added to the column (Fig. 1B, C). Both the LEC-6 WT and N73D proteins were recovered in the lactose eluate. This indicates their strong binding to the immobilized Galβ1-4Fuc. The immobilized Galβ1-4Fuc column proved

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to be useful to capture carbohydrate-binding proteins that interact with this unique sugar structure.

2.2. Capture and identification of *C. elegans* proteins interacting with the Galβ1-4Fuc motif

Several families of carbohydrate-binding proteins are present in *C. elegans*, including galectins, C-type lectins, and annexins. Binding of galectins to β-galactosides is not dependent on the presence of Ca^{2+}, while C-type lectins and annexins require Ca^{2+} for binding.[16,17] Therefore, we applied an extract of *C. elegans* containing Ca^{2+} to the immobilized Galβ1-4Fuc column. After extensive washing with a buffer containing Ca^{2+}, the column was treated with EDTA solution, and then with lactose solution (Fig. 2A). SDS-PAGE showed the presence of a number of proteins in each eluate. Protein bands were excised and digested with trypsin, and the resultant peptides were subjected to LC-MS/MS analyses (Fig. 2B, C) for assignment of proteins.

In the EDTA-eluted fraction (Fig. 2B), 3 proteins belonging to the annexin family, namely NEX-1, -2, and -3, were assigned.[17] Annexins are a family of proteins found in multicellular organisms, and had been considered as Ca^{2+}-dependent phospholipid-binding proteins. However, their interaction with sugars and proteins

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including galectin has also been reported.[17] In *C. elegans*, 4 annexin proteins, NEX-1, -2, -3, and -4, have been found, and their binding of phospholipids and glycosaminoglycans of vertebrate origin has been studied.[14,15] Because their ability to bind endogenous glycans of *C. elegans* has not been studied so far, the present finding is remarkable because the Galβ1-4Fuc disaccharide unit is found on the *N*-glycans of *C. elegans*.

In the lactose-eluted fraction (Fig. 2C), we found several galectins, namely, LEC-1, -2, -4, -6, -9, -10, and and a galectin-like protein DC2.3a. Among them, LEC-1, -6, and -10 have already been reported to have affinity for Galβ1-4Fuc.[7–9] On the other hand, binding of LEC-2, -4, -9, and DC2.3a to Galβ1-4Fuc was observed for the first time. Although LEC-9 was also found in the EDTA-eluted fraction, this was probably due to its relatively weak binding to the column. Of 12 putative galectin genes in the genome of *C. elegans*,[12] products of at least 7 proved to have binding ability towards Galβ1-4Fuc. This suggests that the Galβ1-4Fuc disaccharide unit is an important motif in the endogenous counterpart glycans in *C. elegans*. We also identified NRA-1 in the EDTA-eluted fraction and UCR-2.1 and C41G7.9 in the lactose-eluted fraction. Though their sugar-binding abilities have not been reported so far, we focused on annexin proteins in the following experiment.

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2.3. *C. elegans* annexins NEX-1, -2, and -3 interact with Galβ1-4Fuc in a Ca^{2+} ion-dependent manner

For further studies on the binding of the 3 annexins to Galβ1-4Fuc, we prepared recombinant GST-tagged NEX-1, -2, and -3 proteins. Extracts of *E. coli* expressing these recombinant proteins were applied to an immobilized Galβ1-4Fuc column in the presence of Ca^{2+}. After washing with a buffer containing Ca^{2+}, the column was successively washed with solutions of lactose and EDTA (Fig. 3A).

The recombinant annexin proteins, exhibiting an increased molecular weight due to the GST tag,[15] were recovered in the EDTA-eluted fraction. This suggests their Ca^{2+} dependent interaction. Lactose failed to elute the proteins, probably because it was too weak as competitor.

Interaction between annexin and Galβ1-4Fuc was also examined by frontal affinity chromatography (FAC) analysis. Retardation of fluorescent-labeled sugars through an immobilized recombinant GST-NEX-1 column was compared to the elution profile of PA-rhamnose, which we assume should not interact with NEX-1 (Fig. 3B, C). Distinct retardation of Galβ1-4Fuc-Man-ol-PA was observed, though Galβ1-3Fuc-Man-ol-PA and L*N*nT-PA, which contain the Galβ1-4GlcNAc disaccharide

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unit, were not retarded at all. Therefore, the interaction of NEX-1 with Galβ1-4Fuc is specific. This may be due to the conformation of the hydroxyl groups of the reducing end monosaccharide, which participate in the formation of a glycoside bond. The hydroxyl group at position C^4 of fucose is axial, while that at C^3 is equatorial. In the cases of lactose and Galβ1-4GlcNAc, the hydroxyl group at C^4 of the Glc or GlcNAc residues is also equatorial. Glycoside bonds, including the equatorial hydroxyl group of the reducing end sugar, seem to be unfavorable for the interaction with NEX-1. The binding ability of NEX-1 to an oligosaccharide of *C. elegans* origin that contains a Galβ1-4Fuc unit (E3, obtained as one of the endogenous ligand *N*-glycans of *C. elegans* galectin LEC-6) was also analyzed (Fig. 3B, C). No retardation of E3-PA through the column was observed. It is possible that moieties other than the Galβ1-4Fuc unit interfere with the interaction with NEX-1.

Besides galectins and annexins, other *C. elegans* sugar-binding proteins such as C-type lectins might interact with the Galβ1-4Fuc disaccharide unit *in vivo*. In the present investigation, neither C-type lectin nor any other sugar-binding protein was captured. This might be due to a very low expression level or to some technical reason, such as a failure in solubilization. The Galβ1-4Fuc disaccharide unit has been found only in Protostomia species, such as octopus, squid, keyhole limpet, and *C. elegans*.[18–21]

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However, the galactosyl transferase genes responsible for the synthesis of the Galβ1-4Fuc unit are widely distributed in invertebrate species.[22] This structural unit might have an important role as a fundamental motif recognized by a variety of sugar-binding proteins in invertebrate species. The sugar derivative used in the present study, such as Galβ1-4Fuc-NH_2, and also Galβ1-4Fuc-Man-ol-PA,[23] should serve as very useful tools for invertebrate glycobiology.

3. Experimental

3.1. Synthesis of a Galβ1-4Fuc derivative with a hydrophilic spacer modified with a free amino group (Galβ1-4Fuc-Man-ol-NH_2)

Galβ1-4Fuc-Man-ol-NH_2 (Fig. 1A) was chemically synthesized by combining a Galβ1-4Fuc derivative with a trichloroacetimidate group at the C^1 of the Fuc residue[23] with an azidated linker derived from mannitol under appropriate protection of the hydroxyl groups. The product was then deprotected, and the azide group of the linker was reduced. The structure of Galβ1-4Fuc-Man-ol-NH_2 (Fig. 1A) obtained was confirmed by ^{1}H-NMR analysis, and the fucose residue was confirmed to have closed ring structure. Details regarding the synthesis will be published elsewhere.

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3.2. Preparation of an immobilized Galβ1-4Fuc column

Chemically synthesized Galβ1-4Fuc-Man-ol-NH_2 was dissolved in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) at a concentration of 4.0 mg (about 10 μmol)/mL. Then, 10 mL of the solution were mixed with 10 mL of NHS-activated Sepharose (GE Healthcare, Chalfont St. Giles, UK). The procedure was followed essentially according to the manufacturer's instructions. The immobilized Galβ1-4Fuc adsorbent was packed in a disposable column (Bio-Rad, Hercules, CA), prior to use.

3.3. Expression of recombinant proteins in *Escherichia coli*

E. coli expression plasmids, pET-FLAG-LEC-6N73D,[8] pGEX-3X-NEX-1, pGEX-6P-NEX-2, and pGEX-6P-NEX-3[14,15] were used in this study. Expression of recombinant proteins and preparation of *E. coli* extracts were performed as previously described,[8] with the exception that TBS-$CaCl_2$ (20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5) was used for preparation of extracts of *E. coli* expressing GST-tagged NEX-1, NEX-2, and NEX-3. In brief, *Escherichia coli* strain BL21 (DE3) transformed with expression plasmid was cultured overnight at 37°C in LBA medium (Luria-Bertani medium supplemented with ampicillin), transferred to a 25-fold volume of 2×YT medium containing ampicillin, and then incubated at 37°C for 3 h. After chilling,

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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 10 mL TBS-$CaCl_2$, and then lysed by sonication. After centrifugation, the resulting *E. coli* extract (about 10 mL) was subjected to affinity chromatography.

3.4. Affinity chromatography of recombinant proteins on the immobilized Galβ1-4Fuc column

Affinity purified FLAG-LEC-6 protein and extracts of *E. coli* expressing either FLAG-LEC-6N73D, GST-NEX-1, GST-NEX-2, or GST-NEX-3 were applied to the immobilized Galβ1-4Fuc column (bed vol., 10 mL). In the cases of FLAG-LEC-6 and FLAG-LEC-6N73D, after extensive washing with 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 ethylenediaminetetraacetic acid [EDTA]), the bound materials were eluted with PBS-EDTA containing 0.1 M lactose. In the cases of GST-NEX-1, -2, and -3, after extensive washing with TBS-$CaCl_2$, the bound materials were eluted with TBS-$CaCl_2$ containing 0.1 M lactose, then with TBS-EDTA (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 7.5). These procedures were performed at 4°C. Of each fraction, 6 mL

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were collected. Each fraction was then subjected to SDS-PAGE using 15% polyacrylamide gels and stained with Bio-Safe™ Coomassie (Bio-Rad).

3.5. Isolation and assignment of *C. elegans* proteins adsorbed to the immobilized Galβ1-4Fuc column

Mixed stages of *C. elegans* N2 strain (8 g, wet weight), prepared as described previously,[24] were suspended in ice-cold TBS-EDTA and then disrupted by sonication. After centrifugation, $CaCl_2$ was added to the supernatant at a final concentration of 7 mM, and the solution was applied to the immobilized Galβ1-4Fuc column (bed vol., 10 mL). After extensive washing with TBS-$CaCl_2$, adsorbed materials were eluted with TBS-EDTA and then with TBS-EDTA containing 0.1 M lactose. All procedures were performed at 4°C. Six milliliters of each fraction were collected. Portions of the fractions were concentrated by TCA precipitation, and the precipitates were subjected to SDS-PAGE, followed by staining of proteins with Bio-Safe™ Coomassie. For TCA precipitation, 100 (w/v) %TCA was added to the fractions at a final concentrations of 10%. After chilling on ice, the mixtures were subjected to centrifugation. The precipitates thus prepared were washed twice with EtOH, and then were dried. They were re-suspended in SDS-PAGE sample buffer (1.0% SDS, 50 mM Tris-HCl, 10%

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glycerol, 0.01% bromophenol blue, 2.0% 2-mercaptoethanol, pH6.8) and used for SDS-PAGE. The samples were concentrated about 100-fold by the TCA precipitation.

Selected protein band were excised, washed first with 50% acetonitrile (ACN) in 25 mM NH_4HCO_3 and then 100% ACN, and vacuum-dried. The proteins were subjected to reduction with 10 mM dithiothreitol in 25 mM NH_4HCO_3 at 56°C for 1 h. The gel pieces were then washed with 25 mM NH_4HCO_3 and incubated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 at room temperature for 45 min in the dark. The gel pieces containing *S*-carboxymethylated proteins were washed with 50% ACN in 25 mM NH_4HCO_3, and vacuum-dried. Then, 10 μg/mL of trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 was added, and the solution was incubated overnight at 37°C. The trypsinized peptides thus produced were extracted with 50% ACN containing 5% trifluoroacetic acid (TFA), and the peptide solutions were vacuum-dried. Then, the peptides were resuspended in 2% ACN with 0.1% TFA, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis basically as described.[25]

3.6. Frontal affinity chromatography (FAC) analysis

Immobilization of recombinant GST-NEX-1 protein, which was purified by the

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immobilized Galβ1-4Fuc column, on NHS-activated Sepharose (GE Healthcare) was performed according to the manufacturer's instructions. We used 1.4 mg of GST-NEX1 protein for immobilization and the amount of immobilized protein was calculated from concentration of the protein solution before and after the immobilization process. Protein concentration was determined by using BIO-RAD Protein Assay (Bio-Rad). FAC analysis was performed essentially as described previously,[7,8] with the exception that TBS-$CaCl_2$ was used. To an immobilized GST-NEX-1 column (1.9 mg GST-NEX-1 protein/mL gel), solutions of fluorescent sugars were applied. Galβ1-4Fuc-Man-ol-PA and Galβ1-3Fuc-Man-ol-PA, sugars labeled with pyridylamine via a spacer derived from mannitol, were chemically synthesized.[23] Lacto-*N*-neotetraose-PA (L*N*nT-PA; Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA), and PA-rhamnose were purchased from Takara Bio (Shiga, Japan). E3-PA, a PA derivative of natural *N*-glycan which contains the Galβ1-4Fuc unit isolated from *C. elegans* (structure shown in Fig. 3B), was prepared as reported previously.[7]

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Figure Legends

Fig. 1. Assessment of the capacity of the immobilized Galβ1-4Fuc column

(A) Structure of the chemically synthesized ligand containing the Galβ1-4Fuc unit. Its conformation is based on a ^{1}H-NMR spectrum. About 10 mg of purified FLAG-LEC-6 protein (B) and extract of *E. coli* expressing over 30 mg of FLAG-LEC-6N73D protein (C) were applied to an immobilized Galβ1-4Fuc column. After extensive washing, the bound materials were eluted with 0.1 M lactose. Each fraction was subjected to SDS-PAGE, and proteins were stained with Coomassie brilliant blue. The numbers on the left of the panel are the molecular masses of standard proteins. Arrowheads indicate the positions of recombinant LEC-6 proteins. Arrows indicate lactose-eluted fractions.

Fig. 2. Assignment of *C. elegans* proteins bound to the immobilized Galβ1-4Fuc column

(A) Extract from a mixed stage preparation of *C. elegans* N2 strain was applied to the immobilized Galβ1-4Fuc column. After extensive washing, the bound materials were eluted with 2 mM EDTA, and then with 0.1 M lactose. Each fraction was subjected to TCA precipitation, followed by SDS-PAGE, and proteins were stained with Coomassie brilliant blue. (B) Fraction 21, and (C) Fraction 24, of the eluate from the Galβ1-4Fuc

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column. Proteins assigned by LC-MS/MS analyses are indicated. The names of the proteins used are from the WormBase (http://www.wormbase.org/). Details on the assignment are shown in a supplementary table..

Fig. 3. *C. elegans* annexins NEX-1, -2, and -3 have affinity for Galβ1-4Fuc

(A) An extract of *E. coli,* expressing either GST-tagged NEX-1, NEX-2, or NEX-3, was applied to the immobilized Galβ1-4Fuc column. After extensive washing, the bound materials were eluted with 0.1 M lactose, and then with 2 mM EDTA. Each fraction was subjected to SDS-PAGE, and the proteins were stained with Coomassie brilliant blue. The numbers on the left of the panel are the molecular masses of standard proteins. (B) Elution profiles of PA-sugars from an immobilized GST-NEX-1 column. The structure of each PA-sugar is depicted in each panel of the elution profile. Open circle with diagonal line, hexose; open circle, mannose; gray circle, glucose; 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 GST-NEX-1. (C) The K_a and K_d values for the interaction between GST-NEX-1 and PA-sugars were calculated. N.D. means not determined because of no retardation.

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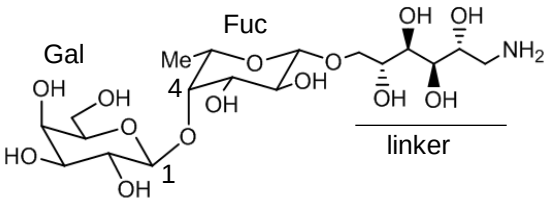
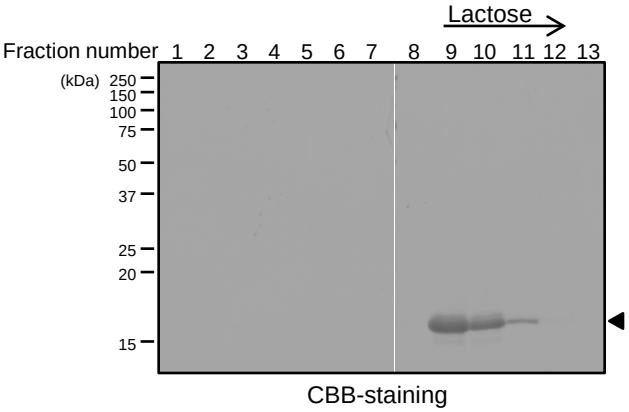
Figure 1. Takeuchi et al.

A. Galβ1-4Fuc-Man-ol-NH_2



B. LEC-6WT



C. LEC-6N73D

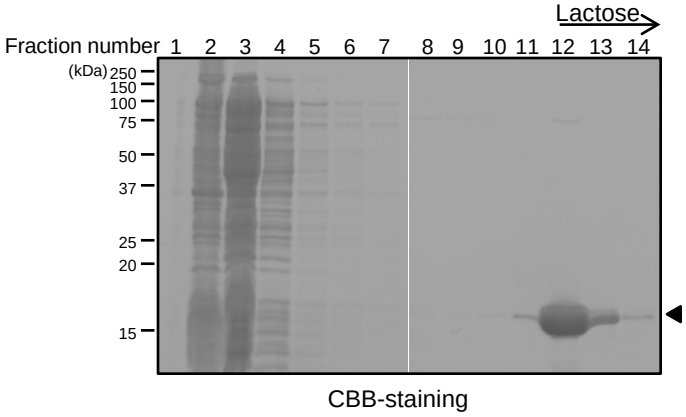


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Figure 2. Takeuchi et al.

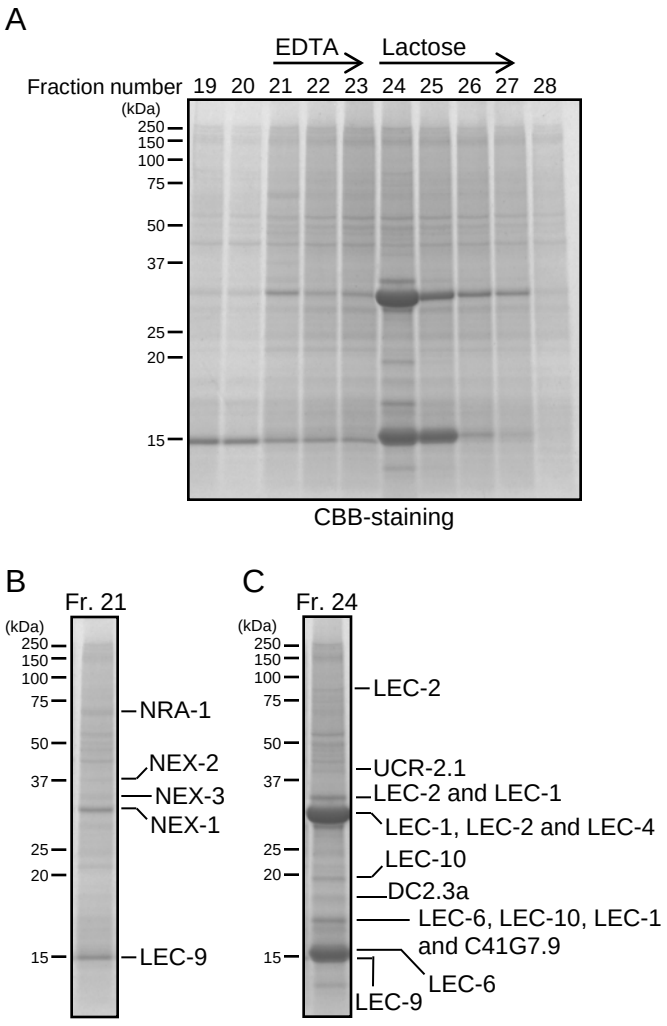

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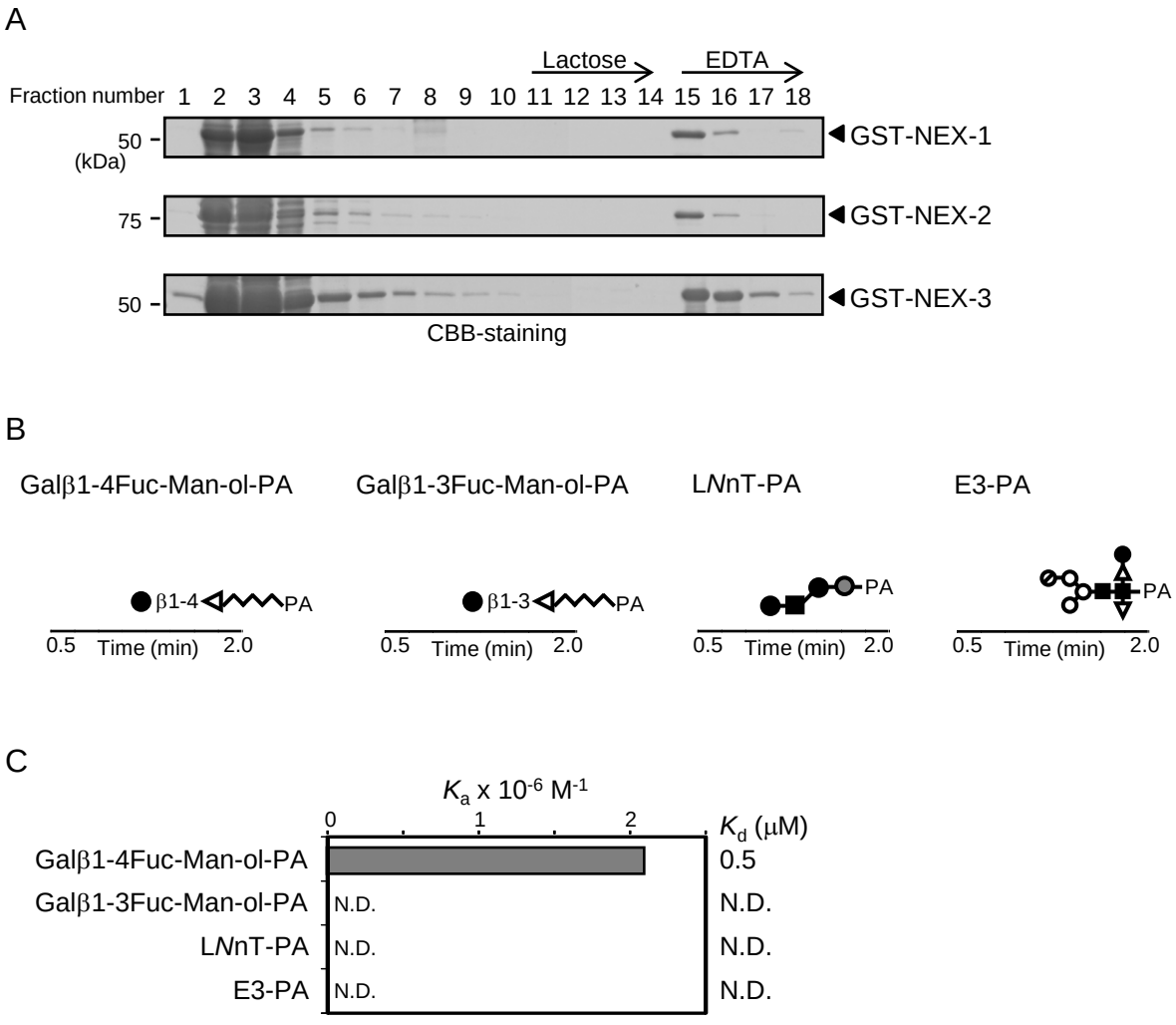
Figure 3. Takeuchi et al.



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Highlights

1. Preparation of an immobilized Galβ1-4Fuc column

2. *C. elegans* proteins bound to an immobilized Galβ1-4Fuc column were identified

3. Multiple *C. elegans* galectins have affinities for Galβ1-4Fuc

4. *C. elegans* annexins, NEX-1, -2, and -3, interact with Galβ1-4Fuc

5. NEX-1 specifically recognizes Galβ1-4Fuc but not Galβ1-3Fuc or Galβ1-4GlcNAc

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Supplementary table. *C. elegans* Galβ1-4Fuc binding proteins identified by nanoLC-MS/MS with a significant score.

MainName	Unique Peptides	Theoretical MW (kDa)	Coverage (%)	Score
C41G7.9	5	18.6	62.0	45
DC2.3a	1	19.9	10.1	10
LEC-1	14	31.8	65.2	340
LEC-2	12	31.2	61.9	184
LEC-4	4	32.4	22.6	17
LEC-6	10	16.0	92.5	423
LEC-9	12	15.5	83.6	153
LEC-10	5	22.0	33.3	68
NEX-1	13	35.7	46.0	167
NEX-2	4	49.4	14.1	47
NEX-3	6	36.1	22.7	47
NRA-1	14	69.7	41.3	107
UCR-2.1	7	42.7	39.0	47