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Journal Article

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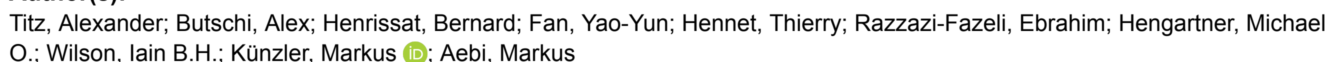
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Molecular Basis for Galactosylation of Core Fucose Residues in Invertebrates

IDENTIFICATION OF CAENORHABDITIS ELEGANS N-GLYCAN CORE α1,6-FUCOSIDE β1,4-GALACTOSYLTRANSFERASE GALT-1 AS A MEMBER OF A NOVEL GLYCOSYLTRANSFERASE FAMILY[*] [S]

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Galectin CGL2 from the ink cap mushroom *Coprinopsis cinerea* displays toxicity toward the model nematode *Caenorhabditis elegans*. A mutation in a putative glycosyltransferase-encoding gene resulted in a CGL2-resistant *C. elegans* strain characterized by *N*-glycans lacking the β1,4-galactoside linked to the α1,6-linked core fucose. Expression of the corresponding GALT-1 protein in insect cells was used to demonstrate a manganese-dependent galactosyltransferase activity. *In vitro*, the GALT-1 enzyme showed strong selectivity for acceptors with α1,6-linked *N*-glycan core fucosides and required Golgi-dependent modifications on the oligosaccharide antennae for optimal synthesis of the Gal-β1,4-fucose structure. Phylogenetic analysis of the GALT-1 protein sequence identified a novel glycosyltransferase family (GT92) with members widespread among eukarya but absent in mammals.

Carbohydrate-binding proteins or lectins are found in all domains of life. They are the key mediators between carbohydrate signals and biological processes (1). Galectins constitute a distinct family of lectins with a characteristic fold (sandwich of two antiparallel β-sheets) and a conserved signature of β-galactoside-coordinating residues (2). Galectins have been implicated in various cellular and extracellular processes (3), such as apoptosis, cancer, cell adhesion, infection, and innate immunity (4).

We have identified three closely related galectin-like proteins (CGL1, CGL2, and CGL3) from the ink cap mushroom *C. cinerea*, primarily expressed in the fruiting body developmental stage (5–9). Simultaneous silencing of *cgl1* and *cgl2* genes by small hairpin RNA did not alter the fruiting body formation, making an essential function in fruiting body development unlikely (10). The carbohydrate binding specificity of recombinant CGL2 is specific for β-galactosides (8, 9), and the structure of CGL2 in complex with various β-galactosides was determined by x-ray crystallography (7).

The 16-kDa galectin CGL2 is toxic for the model nematode *Caenorhabditis elegans* in a carbohydrate-dependent manner,[3] suggesting that galectins might have a direct role as effectors in defense against predators, parasites, or pathogens and that such a lectin-mediated defense, known primarily from bacteria (12, 13) and plants (14), may be present in fungi as well. In a forward genetic screen, we identified several CGL2-resistant *C. elegans* mutant strains. The mutations conferring resistance to the nematode are related to GDP-Fuc[4] biosynthesis and *N*-glycan biosynthesis. Mutation in either the *fut-8* gene or in open reading frame M03F8.4 shows a resistant phenotype, whereas deficiency in FUT-1 activity (*i.e.* core α1,3-fucosylation (15)) does not result in CGL2-resistant worms.[3] Interestingly, Hanneman *et al.* (16) identified β-1,4-galactose linked to core α-1,6 fucose at the reducing end GlcNAc in *N*-glycans. Because CGL2 binds β-galactosides and FUT-8 (17) is responsible for the core α1,6-fucosylation of *N*-glycans, we hypothesized that open reading frame M03F8.4 might encode the galactosyltransferase required to generate the core β-galactoside absent in the corresponding deletion strains.[3]

Here we show that the *C. elegans* open reading frame M03F8.4 indeed encodes a manganese dependent UDP-galactose galactosyltransferase that adds β-galactose to position 4 of α-1,6-linked fucose at the reducing end GlcNAc in *N*-glycan cores. Therefore, the protein encoded by M03F8.4 was termed GALT-1.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S3.

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[4] The abbreviations used are: Fuc, fucose; FLAG, FLAG peptide (DYKDDDDK); HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MES, 4-morpholineethanesulfonic acid; MS, mass spectrometry; MS/MS, tandem MS; TOF, time of flight; DIC, differential interference contrast.

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EXPERIMENTAL PROCEDURES

Chemicals

UDP-Gal was obtained from VWR International and Sigma; UDP-Glc, UDP-GlcNAc, and UDP-GalNAc were all from Sigma; UDP-[^{14}C]Gal was from GE Healthcare; and GnGnF^6 was from Dextra Laboratories.[5] Dabsyl-GEN[GnGnF^6]R (18), dabsyl-GEN[MMF^6]R (19), dabsyl-GEN[MMF^3]R (19), and dansyl-N[GnGnF^6]ST (20) were obtained according to previously published methods.

Isolation of C. elegans cDNA and Expression of GALT-1

Isolation of C. elegans M03F8.4 cDNA—Methods for culturing *C. elegans* have been described (21). The wild type Bristol N2 strain was grown at 20 °C on standard nematode growth medium agar plates seeded with *Escherichia coli* OP50. A *C. elegans* mixed culture was harvested from one standard nematode growth medium agar plate and washed twice in sterile M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$). Total RNA was extracted using the NucleoSpin® RNA II RNA isolation kit (MACHEREY-NAGEL AG). cDNA synthesis was performed with 0.5 μg of total RNA using the first strand cDNA synthesis step of the SuperScript™ III Platinum two-step quantitative reverse transcription-PCR kit (Invitrogen AG).

Construction of the pFastBac1 Donor Plasmid for Recombinant Gene Expression in Sf9 Insect Cells—galt-1 cDNA was amplified from cDNA by PCR using Phusion high fidelity DNA polymerase (Finnzymes). For construction of an untagged version, forward and reverse primers flanked with SalI and XbaI restriction sites, respectively, were used. The resulting fragment was digested with the appropriate restriction enzymes and cloned into the pFastBac1 donor plasmid (Invitrogen). For construction of an N-terminally FLAG-tagged version, a forward primer lacking the start codon was used. The resulting fragment was cloned into a pFastBac1 donor plasmid containing an N-terminal FLAG sequence (22).

Recombinant baculoviruses containing the *C. elegans galt-1* cDNA (with and without N-terminal FLAG tag) and a vector control were generated according to the manufacturer's instructions (Invitrogen). After infection of 2×10^6 *Spodoptera frugiperda* (*Sf9*) adherent insect cells with recombinant baculoviruses and incubation for 72 h at 28 °C, cells were lysed with shaking (4 °C, 15 min) in 150 μl of Tris-buffered saline (pH 7.4) containing 2% (w/v) Triton X-100 and protease inhibitor mixture (complete EDTA-free, Roche Applied Science). The lysis mixtures were centrifuged (2000 × *g*, 5 min), and the postnuclear supernatant was recovered and used for all further enzymatic studies.

Denaturing Gel Electrophoretic Analysis and Immunoblotting

Infected *Sf9* cells (2×10^6 cells, see above) were lysed in 200 μl of reducing sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS (v/w), 5% β-mercaptoethanol (v/v), 10% glycerol (v/v), 0.01% bromophenol blue (w/v)), and proteins were denatured by heating (95 °C, 5 min) prior to SDS-PAGE (12% acrylamide, 120 V) and subsequent analysis by either silver staining or immunoblotting on nitrocellulose. After blocking the membrane (5% bovine serum albumin in PBST (136 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 0.1% Tween-20 (v/v))), immunodetection was performed by incubation with anti-FLAG antibody M2 (Sigma; dilution 1:2000 in PBST, supplemented with 1% bovine serum albumin) followed by anti-mouse IgG coupled to horseradish peroxidase (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); dilution 1:10,000 in PBST, supplemented with 1% bovine serum albumin). After extensive washing (PBST), horseradish peroxidase activity was detected using the ECL reagent kit (Pierce) and exposure to photographic film.

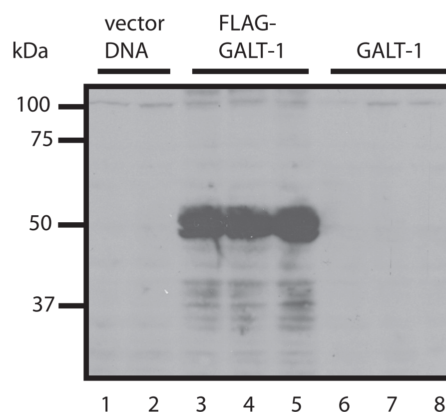


FIGURE 1. **Expression analysis of GALT-1 in baculovirus infected insect cells.** Sf9 cells were transfected with bacmids containing vector DNA (*lanes 1* and *2*), bacmids containing *N*-terminally FLAG-tagged *galt-1* DNA (*lanes 3–5*), and bacmids containing untagged *galt-1* DNA (*lanes 6–8*). Extracts were prepared, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was probed with FLAG tag-specific antibodies. The position of the molecular mass markers in kDa is given at the *left*.

Glycosyltransferase Assays

Enzymatic activity using different carbohydrates or glycoconjugate acceptors was assessed using 0.5 μl (0.1 μl only in case of kinetics analysis) of the raw extract of Sf9 cells (transfected with either the vector bacmid, GALT-1-expressing bacmid, or a FLAG-tagged GALT-1-expressing bacmid) in a 2.5-μl final volume of MES buffer (pH 6.5, 40 μM) containing Mn(II) chloride (10 μM), UDP-galactose (1 mM), and the acceptor fucoside (glycan or glycopeptide, 40 μM). Glycosylation reactions were run for 2 h at room temperature, unless noted otherwise. For donor specificity analysis, UDP-galactose was replaced by equal amounts of UDP-Glc, UDP-GlcNAc, or UDP-GalNAc, respectively. For co-factor specificity analysis, $MnCl_2$ was replaced by equal concentrations of the various metal chlorides or Na_2EDTA. To quantify the incorporation of galactose into the acceptor glycans, UDP-Gal was mixed with UDP-[^{14}C]Gal (GE Healthcare) to a final specific activity of 28.5 mCi/mmol. Excess substrate was removed by loading the reaction mixture (quenched with 100 μl of H_2O) onto a column of anion exchange resin (AG1-X8, Cl^- form (Bio-Rad); 200 mg) and elution of the uncharged products with H_2O (900 μl).

[5] Complex glycans are abbreviated according to the Schachter (34) nomenclature as follows: GnGnF^6, GlcNAc-β1,2Manα1,6(GlcNAcβ1,2Manα1,3)-Manβ1,4-GlcNAcβ1,4(Fucα1,6)GlcNAc; MMF^6, Manα1,6-(Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc; MMF^3, Manα1,6(Manα1,3)Manβ1,4GlcNAc-β1,4(Fucα1,3)GlcNAc.

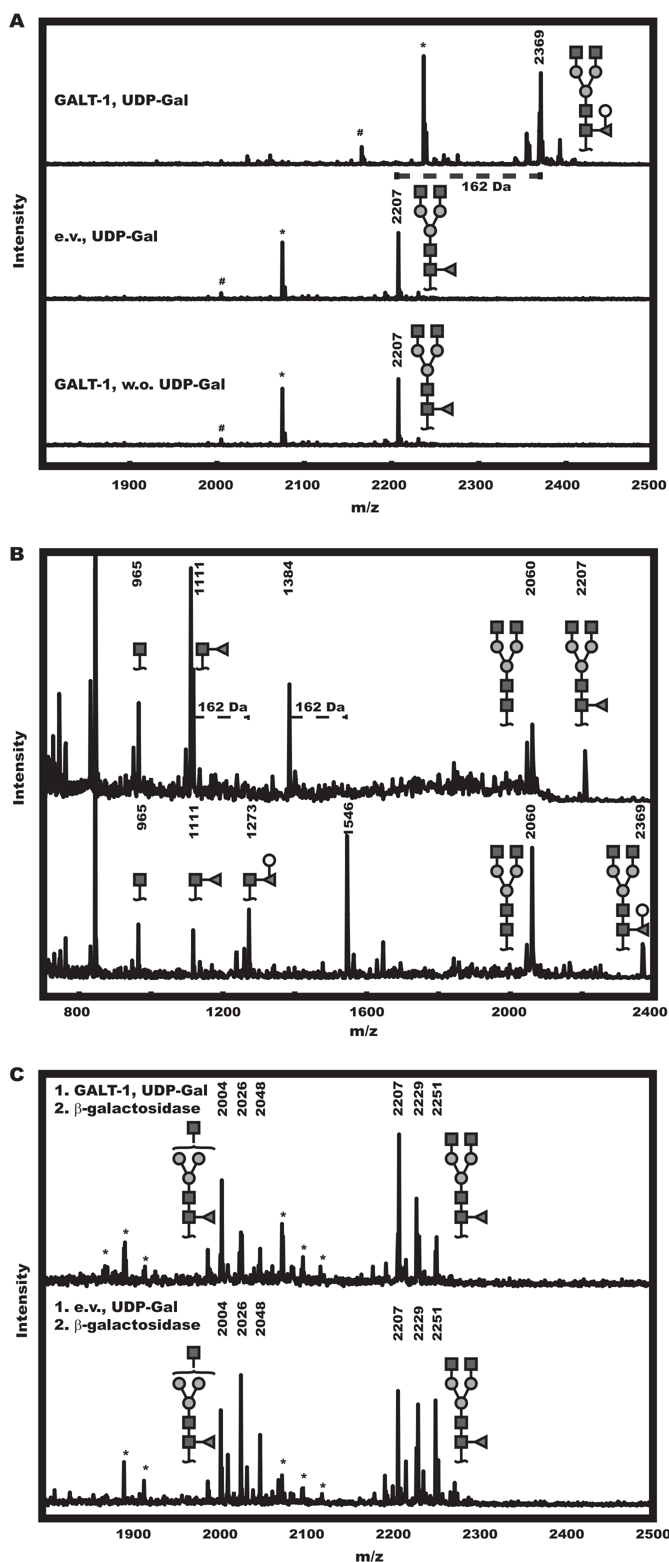


FIGURE 2. **MALDI-MS-based galactosyltransferase assays and determination of position and linkage of the galactose residue resulting from GALT-1 catalysis.** *A*, dabsylated GnGnF^6 glycopeptide was incubated with the following: UDP-Gal and insect cell extract derived from infection with *galt-1*-containing baculovirus (*upper trace*, complete reaction), UDP-Gal and insect cell extract derived from infection with vector DNA-containing baculovirus (*e.v.*) (*middle trace*, control reaction), or insect cell extract derived from infection with *galt-1*-containing baculovirus in the absence of (*w.o.*) UDP-Gal (*lower trace*). In *B* and *C*, the reaction products of the complete reaction (*upper trace*) and the control reaction (*lower trace*) were analyzed. *B*, product analysis by MS/MS for determination of regioisomers. *C*, product analysis for linkage anomericity by enzymatic reaction with *Aspergillus* β-galactosidase followed by MS. Laser breakdown peaks are indicated with an *asterisk*, and peaks from residual hexosaminidase activity (23) originating from the insect cell extracts are marked (#).

Analysis of the reaction products was performed either by direct MALDI-TOF mass spectrometry, HPLC analysis of fluorescently labeled glycopeptides for donor specificity, or scintillation counting of radiolabeled product. Thin layer chromatography was performed on Cellulose F TLC plates (Merck) with MeCN/H_2O (65/35). Radioactivity on TLC plates was detected with a TLC plate reader, the System 200 imaging scanner (Bioscan, Washington, D. C.).

Structural Analysis of Oligosaccharide Products

After exposing dabsyl-GEN[GnGnF^6]R to galactosylation conditions, the resulting crude mixture was adjusted to 50 mM sodium citrate and pH 4.5, digested with *Aspergillus oryzae* β-galactosidase (27 milliunits) (23) for 2 days at 37 °C. The samples were analyzed by MALDI-TOF mass spectrometry (see below).

HPLC Analysis

Dansyl-N[GnGnF^6]ST acceptor substrate was separated from the reaction product using an isocratic solvent system (flow rate of 1.5 ml/min, 8.5% MeCN (v/v) in 0.05% aqueous trifluoroacetic acid (v/v)) on a reversed phase Hypersil ODS C18 column (4 $\times$ 250 mm, 5 μm) and fluorescence detection (excitation at 315 nm, emission detected at 550 nm) at room temperature. The Shimadzu HPLC system consisted of a SCL-10A controller, two LC10AP pumps, and a RF-10AXL fluorescence detector controlled by a personal computer using Class-VP software (V6.13SP2). Dansyl-N[GnGnF^6]ST eluted at a retention time of 9.09 min, and the galactosylated reaction product eluted at 8.06 min.

Mass Spectrometry

Glycans were analyzed by MALDI-TOF mass spectrometry on a BRUKER Ultraflex TOF/TOF machine using α-cyano-4-hydroxycinnamic acid as matrix. A peptide standard mixture (Bruker) was used for external calibration. Mass spectra were analyzed using Bruker software and the mMass version 2.4 software package (24).

Scintillation Counting

The eluates of the anion exchange resin column were thoroughly mixed with scintillation fluid (Irga-Safe Plus (Packard), 4 ml) and measured with a Life Sciences Tri-Carb 2800TR scintillation counter (Perkin Elmer).

Use of M03F8.4::mCherry Fusion for Localization of GALT-1 Expression

Transgenic strains (*opEx* and *opIs* alleles) were generated by microparticle bombardment of constructed strain *unc-119(ed3);galt-1(op497)* using a biolistic particle delivery system (PDS-1000, Bio-Rad). The protocol was carried out as previously described (25) using *unc-119(ed3)* as a transformation marker. Resulting strains containing integrated arrays were

unc-119(ed3);galt-1(op497);opIs444 and *unc-119(ed3);galt-1(op497);opIs445*. Transgenic animals were analyzed for expression of *mCherry* by fluorescence microscopy and for rescue of sensitivity toward CGL2-mediated developmental arrest.

For construction of the extrachromosomal and integrated arrays for localization of *galt-1*, a region comprising 1.8 kb upstream of the predicted start codon and the complete coding region without the stop codon of *galt-1* were PCR-amplified from genomic DNA obtained from worm lysates using Phusion® high fidelity DNA polymerase (Finnzymes). The resulting 4.3-kb *galt-1* fragment was verified by DNA sequencing and cloned in frame upstream of the mCherry coding sequence and the *let-858* transcriptional terminator in the bombardment vector pLN022 (kindly provided by Lukas Neukomm) containing the *unc-119(ed3)* gene as transformation marker. The final *galt-1::mCherry::let-858* fusion construct (pAB15) was verified by DNA sequencing. Primers used are shown in supplemental Table S1).

A plate assay was devised to examine the toxicity of wild type and mutant CGL2 toward *C. elegans*. Nematode growth medium plates containing 1 mM isopropyl 1-thio-β-D-galactopyranoside and 50 μg/ml kanamycin were seeded with *E. coli* BL21(DE3) expressing either wild type CGL2 or mutant CGL2(W72G) as described above. As a control, plates were seeded with *E. coli* BL21(DE3) containing vector DNA. The plates were incubated overnight at 37 °C seeded with synchronized populations of *C. elegans* (26) for the developmental assay. Quantitative data on the effect of CGL2 on *C. elegans* development was acquired by placing 50–100 newly hatched L1 larvae of the indicated genotypes on the plates. After 72 h, the fraction of animals that reached L4 stage was determined.

Differential Interference Contrast (DIC) and Fluorescence Microscopy of Nematodes

For general worm handling, a Leica MZ 12.5 stereomicroscope was used. To analyze transgenic lines for expression of the *galt-1*-mCherry construct, we used a Leica MZ 16 FA stereomicroscope equipped with the appropriate filter sets (DsRed). Pictures were taken with a Nikon Coolpix 990 digital camera. For fluorescence microscopy, worms were placed on 2% agarose pads in M9 (27), anesthetized with levamisole (3–5 mM; Sigma), and mounted under a coverslip for observation using a Leica DM-RA or Zeiss Axiovert 200 microscope equipped with DIC (Nomarski) optics and standard epifluorescence with a DsRed filter set for detection of mCherry. Pictures were taken with a Hamamatsu ORCA-ER camera. Images were false-colored using OpenLab software.

Bioinformatics

Amino acid sequences were retrieved from the National Center for Biotechnology Information by iterative BLAST searches starting from the *C. elegans* sequence with accession number NP_504545. Sequences were aligned using MUSCLE (28). The aligned sequences were clustered using the SECATOR algorithm (29), which relies on BIONJ (30) to build a tree from the multiple-sequence alignment and subsequently collapses the branches from subtrees after identification of the nodes joining different subtrees (29). The neighbor-joining tree was made from the

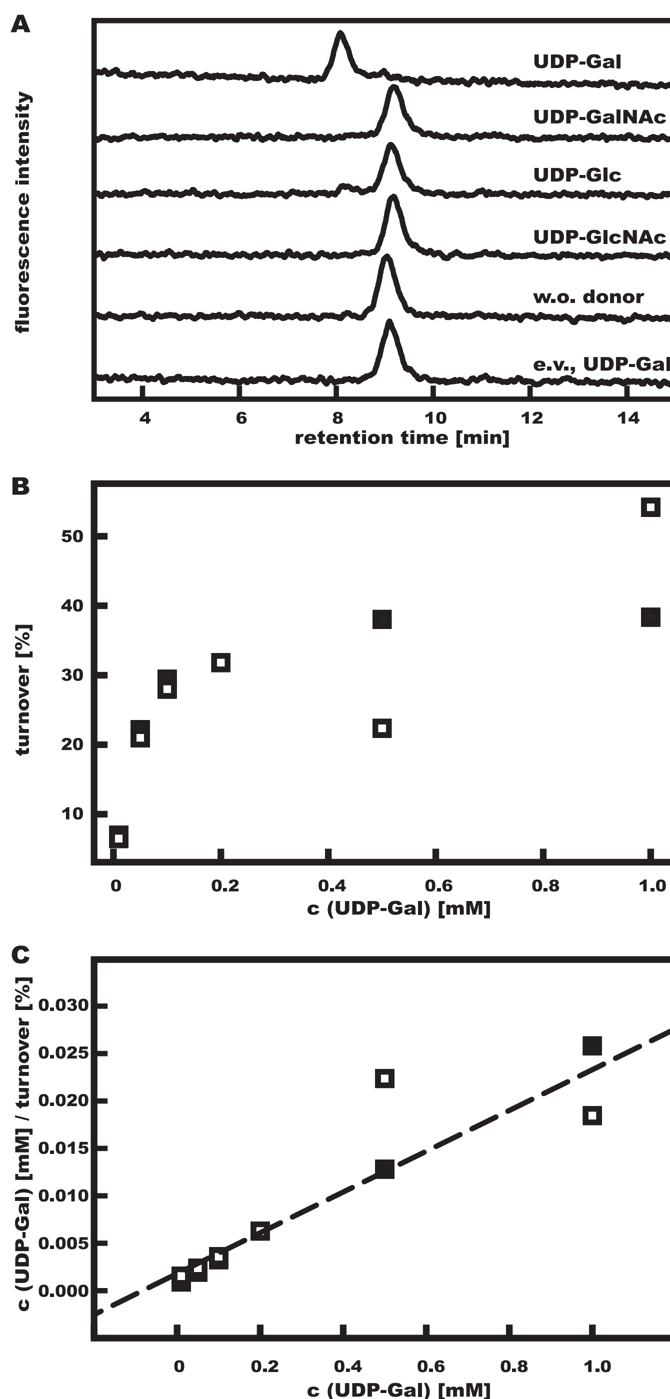


FIGURE 3. *A*, the donor specificity of GALT-1 was analyzed by HPLC separation of reactions of dansyl-N[GnGnF[6]]ST glycopeptide with insect cell extract derived from infection with *galt-1* containing baculovirus and different donor substrates (UDP-Gal, UDP-GalNAc, UPD-Glc, and UDP-GlcNAc) in the absence of a donor substrate (*w.o. donor*) and in the presence of UDP-Gal and insect cell extract derived from infection with vector DNA-containing baculovirus (*e.v.*; UDP-Gal). *B*, kinetics of GALT-1 (*B* and *C*) for UDP-Gal was analyzed by HPLC (turnover describes the ratio of the peak areas of product to the sum of product and reactant multiplied by 100%). *C*, Hanes plot of the data obtained in *B* for graphical determination of the K_m value for GALT-1 and UDP-Gal. Two independent data sets are shown (*black and white squares*), the linear regression is derived from the average values.

resulting distance matrix using Blosum62 substitution parameters (31). Visualization of the tree was done with Dendroscope (32). The multiple sequence alignment was displayed using ESPript (33).

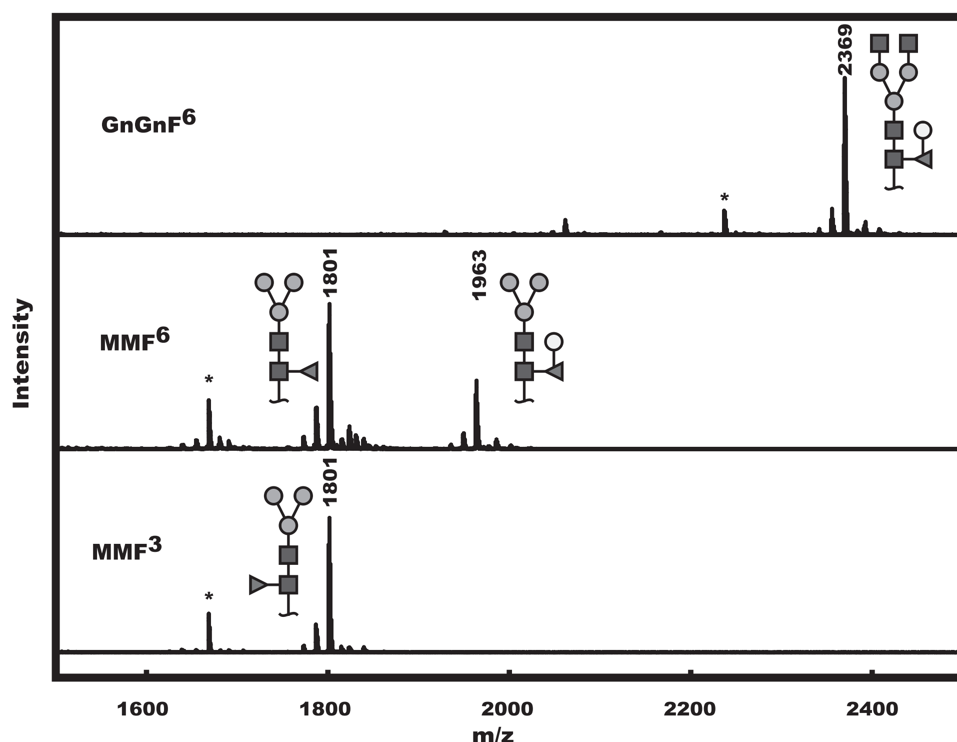


FIGURE 4. **Analysis of the acceptor specificity of GALT-1 by MALDI-MS.** Dabsylated tetrapeptides with varying glycan structure (GnGnF[6], MMF[6], and MMF[3]) were incubated with UDP-Gal and insect cell extract derived from infection with *galt-1*-containing baculovirus, and the reaction products were analyzed by MS. Laser breakdown peaks are indicated with an *asterisk*.

RESULTS

Expression of GALT-1 in Baculovirus-infected Insect Cells—The cDNA encoding the putative nematode galactosyltransferase GALT-1 was isolated, and the protein coding region was cloned into the pFastBac1 vector for expression of both the authentic protein and the *N*-terminally FLAG-tagged variant in baculovirus-infected *Sf9* insect cells. The latter protein was produced for detection of its expression by immunostaining. After incorporation of the corresponding vectors into the bacmid, *Sf9* cells were transfected, and viruses were amplified. After the third passage, protein expression was analyzed by SDS-PAGE of whole cell extracts and detection of the FLAG tag by immunoblotting. Two strong signals close to the 50 kDa marker band were detected in extracts derived from cells infected with FLAG-GALT-1 virus (Fig. 1). Because the protein sequence of GALT-1 contains two putative *N*-glycosylation sites (Asn^{109} and Asn^{152}), whole cell extracts derived from FLAG-GALT-1-expressing virus infection were treated with peptide:*N*-glycosidase F. This resulted in a mobility shift and the detection of a single protein species upon SDS-PAGE and immunoblotting (data not shown). We concluded that these cells expressed the *C. elegans* protein and that the partially glycosylated glycosyltransferase domain was oriented toward the lumen of the secretory apparatus.

Enzymatic Characterization of GALT-1—Extracts of insect cells expressing GALT-1 were tested for galactosyltransferase activity. Considering that the *galt-1* mutant strain lacks the galactose modification on core α1,6-fucosylated *N*-glycans,[3] an *N*-terminally dabsylated glycopeptide bearing a core α1,6-fucosylated biantennary asialo agalacto *N*-glycan (referred to as GnGnF[6]) was used as a substrate in the presence of manganese ions, UDP-galactose, and the GALT-1-expressing insect cell extracts. Incorporation of an additional hexose (mass increase of 162 Da) into this acceptor glycopeptide was observed by MALDI mass spectrometry of the reaction products (Fig. 2*A*). The same activity was observed in extracts containing recombinant forms of either the N-terminally FLAG-tagged (data not shown) or non-tagged GALT-1. When extracts of the baculovirus-infected insect cells carrying only vector DNA were tested, no activity was detected. Experiments with functional GALT-1 but in the absence of UDP-galactose did not change the mass of the acceptor glycopeptide, indicating a specific transfer of a galactose residue from UDP-galactose by GALT-1. Product analysis by tandem mass spectrometry (Fig. 2*B*) revealed the addition of the galactose moiety to the fucose at the reducing terminal GlcNAc of the *N*-glycan core. The indicatory secondary ion with *m/z* 1273 corresponded to the glycopeptide bearing a Hex-dHex-HexNAc sugar structure and was only observed after fragmentation of the galactosylated glycopeptide (parent ion *m/z* 2369) and not with the acceptor GnGnF[6]-glycopeptide (parent ion *m/z* 2207). Treatment of substrate and product with *Aspergillus* β-galactosidase led to identical mass spectra, indicating that the nature of the additional hexose is a β-linked galactoside bound to the core fucose of the *N*-glycan (Fig. 2*C*). In addition, permethylation of the reaction product, released by peptide:*N*-glycosidase F and subsequent MALDI-MS/MS analysis, revealed the same characteristic y fragmentation ion with *m/z* 678 as reported for the native Galβ1,4Fuc structure (supplemental Fig. S3) (16).

The donor specificity of GALT-1 was addressed in an HPLC-based assay with detection of dansylated GnGnF[6] glycopeptides by fluorescence (Fig. 3*A*). The retention time of the acceptor glycopeptide (t_R = 9.09 min) shifted when the glycosylation reaction was performed in the presence of UDP-galactose (t_R = 8.06 min). We concluded that the galactosylated product eluted with a retention time of 8.06 min. In the case of UDP-Gal*N*Ac, UDP-GlcNAc, or the controls, no change was observed, whereas with UDP-glucose, a minor peak (<10%) with the same retention time of the galactosylated product (t_R = 8.06 min) became visible. This quantitative detection of the reaction product was used to determine the kinetic parameters for the UDP-Gal substrate in the galactosyltransferase reaction. Visualization of the obtained data in a Hanes plot (Fig. 3*C*) gave a graphically determined apparent K_m value of 84 μM for UDP-Gal, consistent with values for nucleotide sugar donors of other

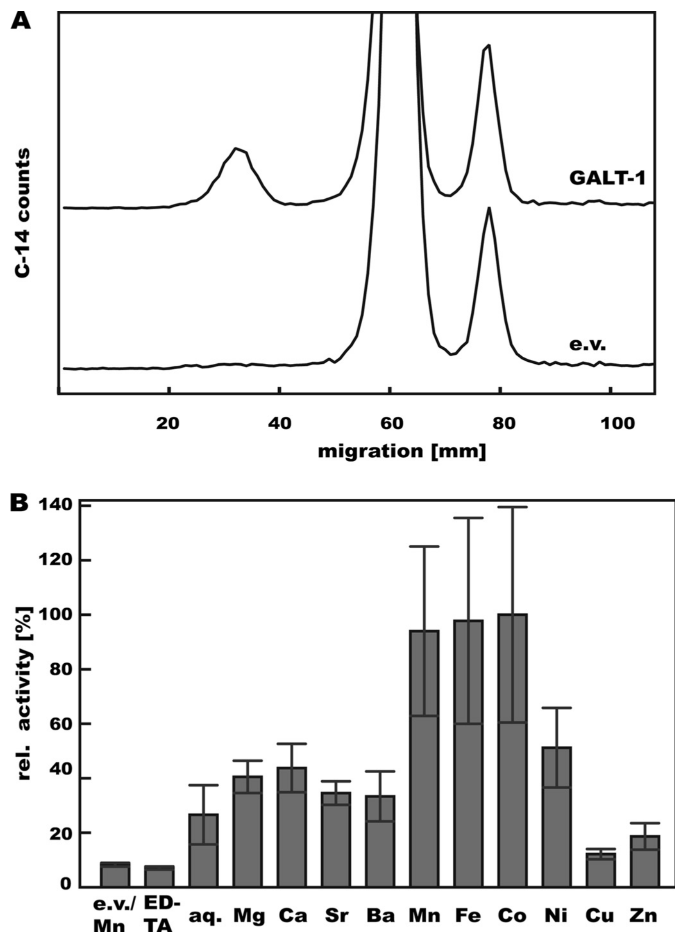
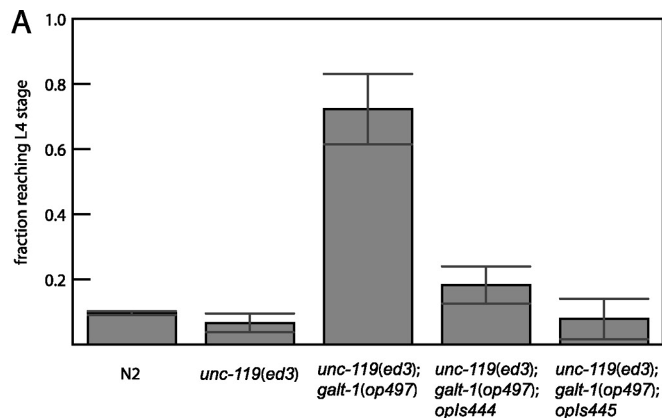


FIGURE 5. **Determination of the co-factor dependence of GALT-1.** *A*, GnGnF[6] was incubated with UDP-[^{14}C]Gal and insect cell extract derived from infection with *galt-1*-containing baculovirus (*GALT-1*) or insect cell extract derived from infection with vector DNA-containing baculovirus (*e.v.*) for 12 h, and the reaction products were separated by TLC on cellulose-F plates and analyzed by counting with a TLC plate reader. *B*, the reaction was performed for 2 h as described for *A* in the presence of various bivalent metals, EDTA, residual co-factors from the insect cell extract (*aq.*), or insect cell extract derived from infection with vector DNA containing baculovirus (*e.v.*), and the reaction products were analyzed by scintillation counting after the removal of excess substrate using anion exchange resin. Activities are shown relative to the highest average activity (corresponding to cpm), which was arbitrarily set to 100%; *error bars*, S.D. of 3–7 independent experiments.

ER- and Golgi-localized glycosyltransferases (35). Fucose and 4-nitrophenyl-α-L-fucoside were not accepted by GALT-1 as substrates (data not shown). Due to limited amounts of the oligosaccharide substrate, we were unable to determine the kinetic parameters for this acceptor.

We determined the specificity of GALT-1 for different acceptor glycans by product analysis using mass spectrometry (Fig. 4). Dabsylated glycopeptides bearing the GnGnF[6] carbohydrate structure were completely galactosylated in a 2-h reaction time as observed from the complete disappearance of the peak corresponding to the acceptor mass (*m/z* 2207) and the appearance of the peak of the galactosylated product (*m/z* 2369). Interestingly, when the non-reducing terminal GlcNAc on the antenna were removed (MMF[6]), only a partial turnover from the acceptor (*m/z* 1801) into the galactosylated product (*m/z* 1963) was observed. The positional isomer of the latter structure, bearing an α1,3-fucose instead of the α1,6-fucose at



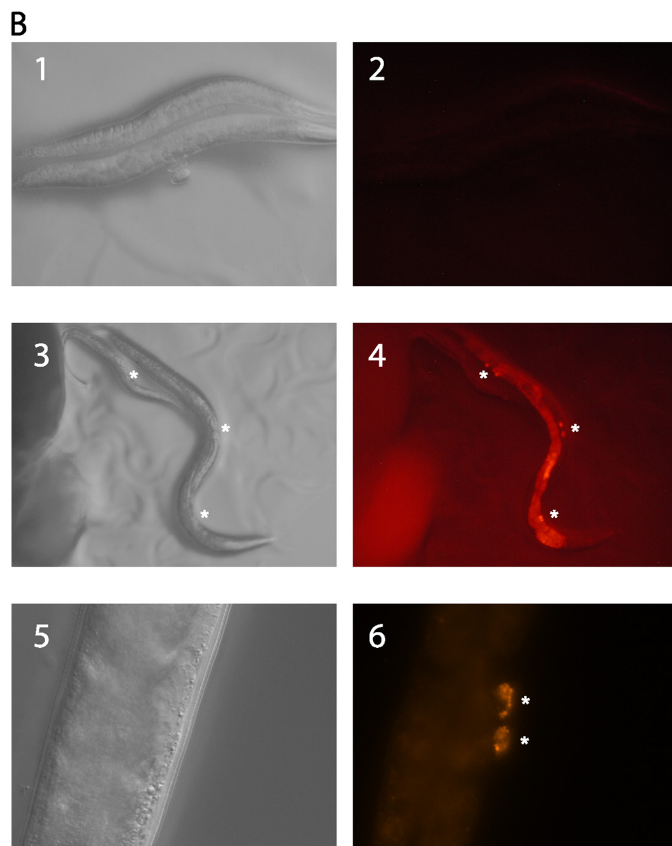


FIGURE 6. **Localization of a functional galt-1(M03F8.4)-mCherry fusion protein in *C. elegans*.** Transgenic worms carrying the integrated *galt-1(M03F8.4)-mCherry* fusion construct were generated by biolistic transformation of *unc-119(ed3);galt-1(op497)*. The resulting strains *unc-119(ed3); galt-1(op497);opIs444/opIs445* were analyzed for rescue of CGL2 sensitivity of the CGL2-resistant *galt-1* (M03F8.4) mutant strain (*A*) and red fluorescence (*B*). For the rescue (*A*), *C. elegans* of the indicated genotypes were analyzed for development from L1 to L4. *Columns* represent the average of three replicates. *Error bars* indicate S.D. For the localization (*B*), the expression of the *galt-1* reporter construct was examined by DIC (*1*, *3*, and *5*) and fluorescence microscopy (*2*, *4*, and *6*) of *unc-119(ed3);galt-1(op497)* (*1* and *2*) *versus unc-119(ed3);galt-1(op497);opIs444/opIs445* worms (*3–6*). The *asterisks* indicate the pronounced expression of the *galt-1* reporter construct in the three coelomocyte pairs along the stained intestine. *5* and *6* show a magnification of the frontal coelomocyte pair. A Leica MZ 16 FA stereomicroscope equipped with a DsRed filter set was used in *1–4*, and a Leica DM-RA microscope equipped with DIC (Nomarski) optics and standard epifluorescence with an appropriate filter set for higher magnification was used in *5* and *6*.

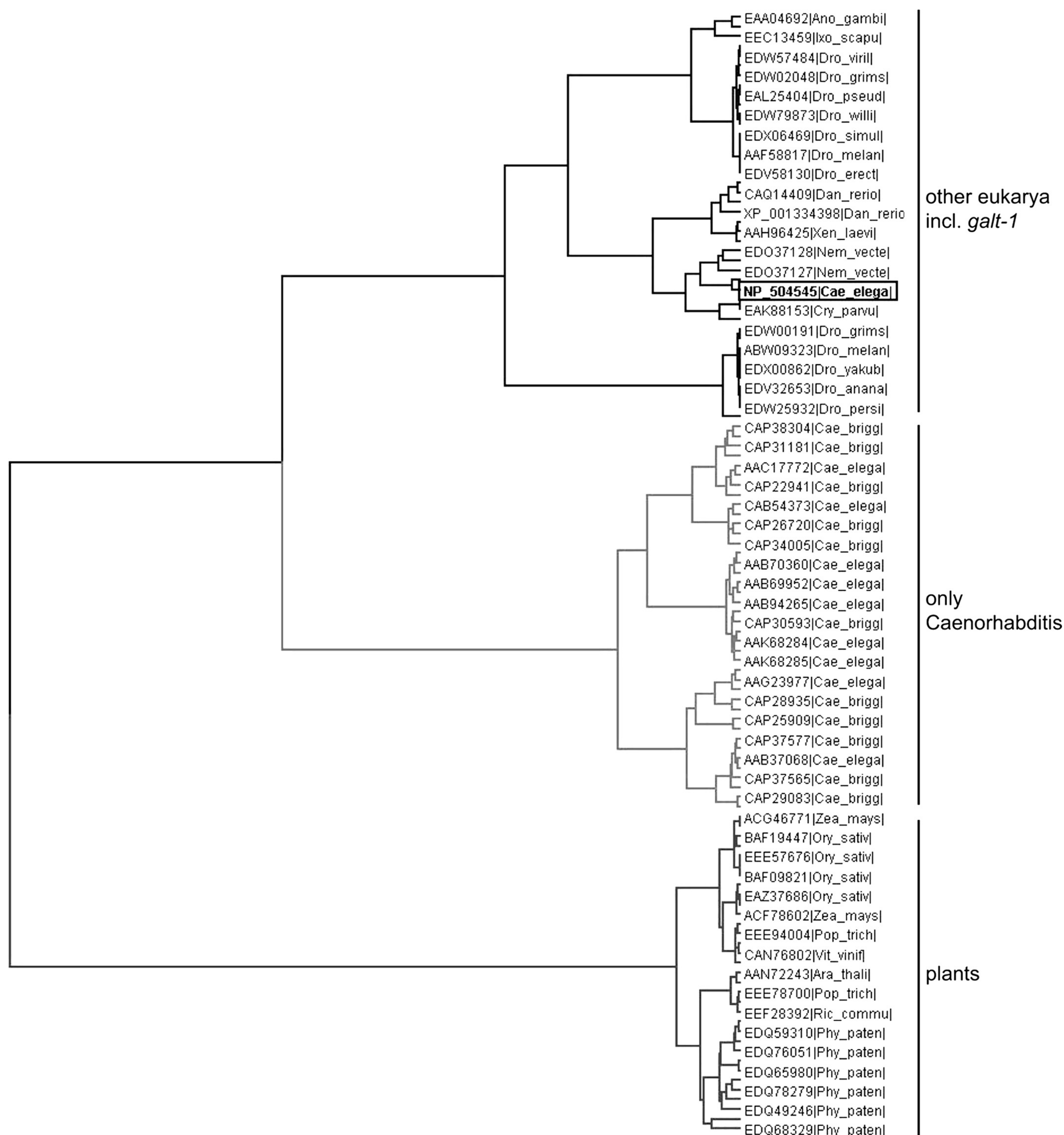


FIGURE 7. **Secator-BIONJ tree of the new glycosyltransferase family (GT92).** The GenBank™ accession numbers are indicated along with abbreviated species names. The *boxed number* shows the enzyme studied here (GALT-1, *C. elegans* NP_504545).

the innermost GlcNAc (MMF[3]) remained unchanged when exposed to GALT-1 under standard galactosylation conditions.

For quantification of a possible metal co-factor dependence of GALT-1, the acceptor substrate GnGnF[6] was used as an untagged free glycan. Galactosylation under standard conditions was performed using radiolabeled UDP-[^{14}C]Gal. The specific incorporation of [^{14}C]galactose into GnGnF[6] was visualized after separation of the reaction mixture on cellulose TLC plates and quantification of the reaction product.[6] The specificity of incorporation into the acceptor glycan could be visualized after separation by thin layer chromatography, and the resulting radioactive nonasaccharide was retained on the cellu-

[6] The TLC plate reader is semiquantitative because soft radiation resulting from C-14 is absorbed by the thin layer, and therefore only material at the surface of the layer is detected.

```
          90        100          110                        120
A  SAYTDDRNG.....NMGYKYVRVLMFITSQD.....NFSCEI..NGRK...............STDVSLY
B  SAYYDWRID.....RIRIN...SLIPSNFYD...RIEMECAIILDKNI............YTGTIKKVIH
C  GAYYDIRRTSLLGPTVRIL....GMIDRIEP...KVKTYCQFWFDGQK.......EP...FIVKTFEYKY
D  SAF.VYPDQI....SIVTT......AFHTYG...K.RAVCIYYDCNRR.......EI...PSSRFASR..
E  AAF.VYKDQI....TVTLT......SENQYN...K.TVYCRYFDCQRR...............EIPDQFY
F  YAY.EFEHEI....TVTTT......SWKRMG...H.RVYCRYLDDNNI.......EI....GIPFESL..
G  GAYRGGPRTFA...VVGLA....SKPLHVYG..RP.WYKCEWISSNGS..............ASLRAKAY
H  SAYRQSLRTFF...VNGIS...SY.VARSFDKDKV.AHTCEWHPGNGSLPLSMHTDPGAFVETTAEHIYM
I  SAYRGGVNSFA...VIGLS....SKPLHVYG..HP.SYRCEWVSLDPT.............QDPISTTGF

     130         140         150        160             170           180
A  EFSENHKM....KWQMFILNCKLPDG..IDFNNVSSVKVIRST.....TKQFVDVPIR....YRIQDEKI
B  KEHHNKE.....YVSSTLLCEIAKNEIKFEDISRKVLITILENGNSTNKSEIWITLKKIPKNSS.....
C  IWYNKWGNYKQGIYQPYLIACQIPK...PFHGVVPS.SVSMVE..KECDTATNNLRVI....YNRP.....
D  ............VIPLTVVSCP......RRHGAE...YMSVSF..NKDEEIPEPIKLT.FRAYEQ.....
E  SV.........IFPQSTVFCA......RRPG..AK.YISIAR..NFTDTPEFPVPII....PRL.....
F  ...........TYPEYIVSCK......KRDGTKKI.GLSVEK.....NGDFFPLPII.DRMLKK.....
G  KMLPDWG..YGRVYTVVVVNCTFAEN..PNEDNAGG.KLVLRA..YYGESPRKFEKFTVLEEQPGSYNES
H  KSDENRGT.....YSPTIINCTFKVP..VGANRLGG.LLVLRI.STGYDRWEKNLPILALEEQVNEVNIV
I  KILTDWG..YGRIYTTVVVNCTFSSISAVNPQNSGG.TLILHA.TTGDPTLNLTDSISVLTEPPKSVDFD

             190      200                    210          220      230
A  ITPDE...YDYKMSICVPAL.FGN............GYDAKRIVEFIELNTLQ..GIEKIYIYTNQKELD
B  ........NNHELTVCVRPW.WGEPIKNGNLGNKQKFNNSGLMLEFINSYLFL..GANKFYLYQNYLDID
C  ..PDD...QKKGFAVCVKGLDFLY............DDLSVRLIEWIEMLNIL..GADKIYFY..NLQVH
D  ........PVHELSVCVGPL.YGS............ESKWLEVVEYVEHYRLL.................
E  ..EKE...PPHYFTVCMATL.YGD............EPKFLQIVDFIEYYKLQ..GATFFHIY..LRNVT
F  ........PKYELSMCVASI.YGD............EPKWLMFIEMIEHFKLQ..GVQHFYLH..IHHAS
G  NYRPP..YQYEYLYCGSSL.YGS.............LSAARIREWMAYHAWFFGPKSHFVFH.DAGGVS
H  STPPEK..IPFKYAFCGAPM.HGT.............VRADWILHWMKYHHYLTEGSAHFFFY.NLGGLA
I  LYNSTKKTKKYDYLYCGSSL.YGN.............LSPQRVREWIAYHVRFFGERSHFVLH.DAGGIH

        240         250        260                      270        280
A  GSMKKTLKYYSD.NHKITLIDYTLPFRED............GVWY..HGQLATVTDCLLRNTGITKYTFF
B  EDVRNIINYYSNIKNVLEIIPYSLPIIPFK...........QVWD..FAQTTMIQDCLLRNIGKTKYLLF
C  PNITKVLNHYEQ.EGKVQVIPLTLPGGQPNVPGFQHLYLTKKTNHKRQNEVIPYNDCLYKNLYLYDYIAL
D  .........................................................ECHHRSRFHSKWVIN
E  DYDRVILDDYVR.TGDIEIIKMHDHHW.............RDDF..MWHNSQINDCHHRNKYYSKWTAL
F  EYDMRVINDYVR.TGEVEVHYLIERDM.............RADN..HWHMVNIADCLIWSRGETKWTIF
G  PAVRAALEPWVR.AGWATIQDIRGQAEF............DGYY..YNQFLVVNDCLHRYRHAANWTFY
H  NSDRPVFNEFIN.AGLLSITDILDPNLSWDY.........PTWY..FHQVLLINDCLHRSRFMAERVFF
I  EEVFEVLKPWIE.LGRVTLHDIRDQERF............DGYY..HNQFMIVNDCLHRYRFMTKWMFF

   290         300         310                320        330
A  NDFDEFFVPVI.KSRTLFETISGLFEDP..TI.....GSQRTALKYINAKI..................
B  VDTDEFVFPNLKNY..NLMDFLNLLEANNPYY......KNKVGAMWIPMYFHFLEWESDKNNLKKYS...
C  LDIDEVIMP..KGGAVLWSELMDKVRPESRKIKPDGFHSYNFRNVYFLDDQQH......EHGWHK.....
D  MDIDERVIYTGPNN..FIHFFRSIPSNF........SEISLSSNRVLKTHEL......PERFKNEN...
E  VDIDERIEIKNEAH.KTILSYLNSIHNS..SI.....VNLHFQVQWVIKQNNT......PARYKSDEQVR
F  ADLDERIYMTNYTG..TILDYVQVVKNE..SI.....ASIQFRQQWIMKTELM......PPKYEGDR...
G  FDVDEYIYL..PDG.RTLESVMEEFSDY.........TQFTIEQNPMSSVLCL......NDSSRN.....
H  FDYDEFLQVPAPDS...LKMFMRRHAKE.........PWISFGSIYANTKDCRLPKPGESETLF......
I  FDVDEFLHV..PVK.ETISSVMESLEEY.........SQFTIEQMPMSSRICY......SGDGPAR....

                               340            350             360
A  ...........KSAPY.......SLKNIVS........EKRIETRF.......TKCVVRPEMVFEQGIHH
B  ...........TIEKK.IKKKMANIEFVLY.....RKTCRMLSSGTKKSDKTRRKVIIRPERVLYMGIHE
C  ...........DIPKY........MHMLQH....VHRAKNYTKPNQ......YVKCFHDPERVLTLHNHF
D  ...........ELRAE........MMFLKY......NQTTEISWYN.......LKGIIRPEMVALLFYHW
E  HTSSVNPAVQFQLTRE........MIFLKY.....QNVSQVGDIWD......QPKCIIRPEKVVAMTIHV
F  ...........QLDKW........MPTHRW.....HSSSGIGPPGH......TAKCIVDTSKVFIMFIHY
G  ...........YSREW.......GFEKLLF......RDSRTRIRRD.......RKYAIQAKNAYSTGVHM
H  ...........ERMVWRRPRPECRIQQAKYEGPPVDALHCIGPLGR.......RKYVANPRKTFAAGVHW
I  ...........TYRKW.......GIEKLAY......RDVKKVPRRD.......RKYAVQPENVFATGVHM

   370        380         390
A  TSRVIQD.....NYKTVSH..........GGSLLRVYHYK
B  TEEML.......SKKFHFIRAPVINVGGGNELSIYLHHYR
C  PLSCL.....GGVCKSYPV..........DTKDAQLQHYR
D  SCRQFD......ETHVMSV..........PKRFAYVRHYR
E  PTAVY.....SGERFTFI..........PPSVGVVRHYR
F  VTQFF.PATNGSNYVQIRV..........DPEEGLVRHYR
G  SENVI.......GKTLHKT..........ETKIRYYHYH
H  VTI........PKNGLDL..........SGNGAHILHYH
I  SQNLQ.......GKTYHKA..........ESKIRYFHYH
```

lose stationary phase through carbohydrate-carbohydrate interactions to a higher extent than both starting material and the nonspecific hydrolysis product. The thin layer chromatogram (Fig. 5*A*) showed the incorporation of radioactive galactose into GnGnF^6 by GALT-1 as an additional slowly migrating peak (32 mm) that was absent when vector control extracts were used. Excess UDP-[^{14}C]Gal migrated at 60 mm, and the fast migrating peak (78 mm) most likely resulted from substrate hydrolysis due to extended reaction time. For the quantification of the metal co-factor specificity of GALT-1, excess donor was removed from the reaction mixture by anion exchange chromatography and a reduced incubation time was chosen to obtain a minimum of substrate hydrolysis (Fig. 5*B*). The addition of EDTA prevented the transfer of galactose. Bivalent alkaline earth metals and transition metals were tested, and the highest turnover was observed with manganese, iron, and cobalt, consistent with results obtained for other glycosyltransferases (37). In comparison, when earth alkaline metals (magnesium, calcium, strontium, and barium) or nickel were added, the activity was slightly increased as compared with the control with the addition of water (Fig. 5*B*, *column aq.*). The presence of copper or zinc ions inhibited incorporation of [^{14}C]galactose into GnGnF^6.

Expression of GALT-1 in Nematodes—For *in vivo* localization of the expression of GALT-1, a CGL2-resistant *C. elegans unc-119(ed3);M03F8.4(op497)* mutant was transformed with a galt-1::mCherry translational fusion. The fusion construct comprised 1843 bp of the genomic DNA upstream of the translational start codon. Rescue of the inability to form Dauer stages associated with the *unc-119(ed3)* mutation was used to select for low copy integrants (25), which were examined both for CGL2-sensitivity and for fluorescence in comparison with the untransformed *unc-119(ed3)* single and *unc-119(ed3);M03F8.4(op497)* double mutant strains. The observed rescue of CGL2 sensitivity by the transformed fusion construct suggested that the encoded fusion protein was functional (Fig. 6*A*). Examination of these integrants by fluorescence microscopy revealed specific expression of *galt-1* in the intestine and in coelomocytes (Fig. 6*B*). Upon magnifying the fluorescence image at altered contrast, expression of GALT-1 appears to be localized in vesicular structures or organelles within the coelomocytes.

GALT-1 as a Member of a New Glycosyltransferase Family—Data base searching with the sequence of *C. elegans* GALT-1 resulted in the identification of 115 proteins of previously unknown function. On the basis of the presented data, these proteins can now be classified as a new family of glycosyltransferases in the Carbohydrate-Active Enzymes data base (38) (Fig. 7), namely as family GT92. This family can be phylogenetically clustered into three main subgroups. The subgroup containing GALT-1 features homologues from various species, among which are vertebrates (*Xenopus* and *Danio rerio*), invertebrates (*Caenorhabditis* and *Drosophila*), and protozoa (*Cryptosporidium*). Whereas the second subgroup consists of homologues exclusively from *C. elegans* and *Caenorhabditis briggsae*, the third one comprises proteins of plant origin (*e.g.* from *Arabidopsis*, *Oryzae*, and *Physcomitrella*). The sequence alignment of the homologous domain of the entire family is exemplified with three selected proteins of each branch (Fig. 8).

DISCUSSION

Our previous study[3] revealed the necessity of a functional gene in *C. elegans* open reading frame M03F8.4 for the presence of the galactosyl-β1,4-fucosyl-α1,6 moiety (16) at the reducing end GlcNAc in *N*-glycans *in vivo*. To determine the biochemical activity of the GALT-1 protein encoded by this gene and displaying sequence homology to other glycosyltransferases, the corresponding cDNA was isolated and expressed in baculovirus-infected insect cells. GALT-1 is likely to be a Golgi glycosyltransferase due to its *N*-terminal signal sequence targeting it to the secretory pathway. With a $D^{289}X^{290}D^{291}$ sequence, it shares with many other Golgi glycosyltransferases a D*X*D motif (39) often associated with a requirement for manganese (or occasionally magnesium) for coordination of the phosphates of the activated sugar during catalysis; the subsequent enzymatic characterization of GALT-1 was compatible with such a requirement for bivalent metal ions.

Three types of enzymatic assay methods (based on MALDI-TOF MS, HPLC, and radioactivity) indicated transfer of galactose by GALT-1 to core α1,6-fucosylated *N*-glycans. This preference was absolute, and an isomeric core α1,3-fucosylated glycopeptide was not galactosylated by this enzyme. Despite the presence of other hexose modifications on the core α1,3-fucoses on the distal and proximal GlcNAc residues of the core region of *C. elegans N*-glycans (16, 40), these epitopes are probably not synthesized by GALT-1 *in vivo*. Therefore, the action of GALT-1 as a bifunctional enzyme also acting on *N*-glycans with different remote (*i.e.* on the non-reducing ends) sugar structures or on core α1,3-fucose cannot be fully excluded but seems rather unlikely. Considering the preference of GALT-1 for UDP-Gal, the sensitivity of its enzymatic product to a β1,4-specific galactosidase, our detailed product analysis, and the lack of galactosylated core α1,6-fucosylated glycans in the *galt-1* mutant, we postulated that this enzyme is a functional β1,4-galactosyltransferase necessary for the transfer of galactose to core α1,6-fucose in *C. elegans*.

Interestingly, non-reducing terminal GlcNAcs on the antenna had an important role on the rate of galactosylation by GALT-1. The GlcNAc-bearing glycopeptide (GnGnF^6) was completely transformed into the corresponding galactoside. Under identical reaction conditions, but in the absence of these terminal GlcNAcs, the MMF^6 glycopeptide was only partially galactosylated. Thereby, our *in vitro* results are compatible with the observed accumulation of glycans carrying

FIGURE 8. **Representative alignment of the homologous domain of nine selected sequences (three of each branch, *A–C*, *D–F*, and *G–I*) of the phylogenetic tree according to the entire new enzyme family (for full alignment, see supplemental material).** Amino acid residues highly conserved throughout the entire family are *boxed*. *A*, GALT-1 (*C. elegans* NP_504545); *B*, *Cryptosporidium parvum* EAK88153; *C*, *Drosophila melanogaster* ABW09323; *D*, *C. elegans* AAC17772; *E*, *C. briggsae* CAP30593; *F*, *C. elegans* AAB37068; *G*, *Vitis vinifera* CAO66312; *H*, *Physcomitrella patens* EDQ50855; *I*, *Arabidopsis thaliana* CAB79017. Sequence numbering refers to GALT-1 (*A*).

both Galβ1,4Fuc and non-reducing terminal GlcNAc in the *hex-2* hexosaminidase-deficient worm strain (23).

A similar influence of non-reducing GlcNAc is described for FUT-8, responsible for the addition of the core α1,6-fucose (41). FUT-8 requires one remote GlcNAc on the antenna of the *N*-glycan for the addition of the α1,6-fucose, but in turn its activity is inhibited by the presence of a bisecting GlcNAc on the β-mannose or an α1,3-fucose on the reducing end GlcNAc. We concluded that the prior action of GlcNAc-TI was an important prerequisite for formation of the Galβ1,4Fuc epitope during biosynthesis of *C. elegans N*-glycans (42), despite the absence of the non-reducing terminal GlcNAc in many of the wild-type *N*-glycans. Thus, our data also partly account for the less complex *N*-glycome of nematodes lacking functional forms of all three GlcNAc-TI genes in *C. elegans* (43) and are compatible with the premise that the Golgi hexosaminidases in the worm act after GALT-1.

Using the sequence of GALT-1 for a search of homologous proteins present in the data bases, an entire new family of glycosyltransferases was revealed, which was classified as GT92 in the CAZy data base. Three subfamilies were identified in the tree, one of which included the *C. elegans* enzyme studied here, and two others that are more distantly related. Interestingly, although all GT92 members are of eukaryotic origin, no mammalian sequence was identified. Furthermore, a large recent expansion of one of the subfamilies in *C. elegans* and *C. briggsae* was observed. With only two exceptions among over 100 family members, all sequences bear the conserved D*X*D motif commonly used in glycosyltransferases for manganese coordination. As observed for eukaryotic Golgi glycosyltransferases, family GT92 glycosyltransferases appear to have a single pass transmembrane located *N*-terminally of the catalytic domain (in some instances, the *N*-terminal transmembrane domain is lacking probably due to incomplete protein models).

Although the β-1,4-galactose epitope linked to core α-1,6 fucose at the reducing end GlcNAc in *N*-glycans is not only observed in *C. elegans* (16) but was first discovered as a feature of octopus rhodopsin (44) and later on *N*-glycans of squid rhodopsin (45) and keyhole limpet hemocyanin (46), at present, no GALT-1 related sequences are found among the published expressed sequence tag data base of these species. Due to relatively high sequence divergence with GALT-1, it is presently impossible to predict the substrate specificity of the distant relatives (the members of the other two subfamilies). It is interesting to note that none of the newly identified plant sequences were predicted by Hansen and co-workers (36) in a bioinformatics approach to identify novel glycosyltransferase sequences, showing that experimental investigation is still essential for enzyme discovery.

The observed intestinal localization of GALT-1 was in accordance with the reported localization of the CGL2-ligand to the intestinal epithelium and the observed damage of the *C. elegans* intestine upon CGL2 intoxication.[3] The significance of the initial binding event between CGL2 and its Galβ1,4Fuc ligand at the intestinal epithelium and the expression of *galt-1* in coelomocytes, cells implicated in *C. elegans* innate immunity (11), for the mechanism of CGL2-mediated nematotoxicity remains to be clarified. Furthermore, the molecular basis for the transfer of previously identified hexose modifications to core α1,3-fucose of *C. elegans N*-glycans as well as the subsequent modification of the Galβ1,4Fuc epitope by additional hexoses (16, 40) remains to be resolved. It is possible that the distantly related subfamily consisting exclusively of *C. elegans* and *C. briggsae* genes is responsible for the biogenesis of these further epitopes. Considering the enzymological and structural complexity of such glycosylation events, these and other aspects of the unusual modifications of the core region of nematode *N*-glycans, as well as the function of GALT-1 homologues in other species, represent challenges for future studies.

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