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Characterization of Wheat Germ Agglutinin Ligand on Soluble Glycoproteins in *Caenorhabditis elegans*

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Some mutants of *Caenorhabditis elegans* show altered patterns of ectopic binding with wheat germ agglutinin (WGA). Some of these mutants also have defects of morphogenesis and movement during development. To clarify the structures of WGA-ligands in *C. elegans* that may be involved in developmental events, we have analyzed glycan structures capable of binding WGA. We isolated glycoproteins from wild-type *C. elegans* by WGA-affinity chromatography, and analyzed their glycan structures by a combination of hydrazine degradation and fluorescent labeling. The glycoproteins had oligomannose-type and complex-type *N*-glycans that included agalacto-biantenna and agalacto-tetraantenna glycans. Although the complex-type glycans carried β-GlcNAc residues at their non-reducing ends, they did not bind to the WGA-agarose-resin. Thus, it was suggested that these *N*-glycans were not responsible for WGA-binding of the isolated glycoproteins. Hydrazinolysis of the glycoproteins also released a considerable amount of GalNAc monosaccharide. It was surmised that *N*-acetylgalactosamine was derived from mucin-type *O*-glycans with the Tn-antigen structure (GalNAcα1-*O*-Ser/Thr). WGA-blotting assay of neoglycoproteins revealed that a cluster of Tn-antigens was a good ligand for WGA. These results suggested that the WGA-ligand in *C. elegans* is a cluster of α-GalNAc monosaccharides linked to mucin-like glycoprotein(s). The observations reported in this paper emphasize the possible significance of mucin-type *O*-glycans in the development of a multicellular organism.

Key words: *Caenorhabditis elegans*, pyridylamination, *srf* mutants, Tn antigen, WGA.

Abbreviations: PA, pyridylamino; HPLC, high performance liquid chromatography; GN2, *N,N′*-diacetylchitobiose-PA; HSA, human serum albumin; HRP, horse radish peroxidase.

The body of the nematode *Caenorhabditis elegans* is surrounded by a collagenous cuticle. This cuticle is secreted by the underlying hypodermis, which consists of a single layer of cells. The hypodermis not only secretes the cuticle but is also involved in guiding the attachment and migration of underlying muscle, neuronal, and gonadal cells. Link *et al.* have shown that the surface of the cuticle is specifically modified at the hermaphrodite vulva and the male copulatory bursa by demonstrating that these regions specifically bind wheat germ agglutinin (WGA) (*1*). They also established six mutants that showed an altered ectopic WGA-binding phenotype (*2*). Three of the six mutants, *srf-2*, *-3*, *-5*, are grossly wild-type except for their lectin-binding phenotype. The other mutants, *srf-4*, *-8*, *-9*, have a suite of defects including abnormal egg laying, uncoordinated movement, and defective copulatory bursae morphogenesis. Such pleiotropic mutations interact with mutations in the *lin-12* gene, which is an orthologue of the *Notch* gene. These observations suggest that WGA-ligands in *C. elegans* include molecule(s) closely involved in developmental events.

Although several studies have been carried out on glycosylation in *C. elegans* (*3–5*), the component(s) bound by WGA have not been identified. WGA specifically binds to some GlcNAc- or sialic acid–containing oligosaccharides. In particular, polysialic acids and *N*-glycans with bisecting GlcNAc are known as high affinity ligands to WGA. However, genomic analysis has revealed that *C. elegans* has no homologous gene to *mgat3*, which encodes the bisecting GlcNAc-synthesizing enzyme, and Basic *et al.* have shown that sialic acid is absent in *C. elegans* (*6*). These facts suggest that *C. elegans* has a new category of WGA-ligand(s). Therefore, we initiated the present study to characterize these ligands, and as described below, we present the possibility that Tn-antigens on the mucin-like glycoprotein(s) are responsible for WGA binding in *C. elegans*.

EXPERIMENTAL PROCEDURES

Standard PA-Oligosaccharides—The structures and abbreviations of the authentic PA-glycans used in this study are listed in Fig. 1. AG12, AG1234, M3GN-1 and M3GN-2 were purchased from Takara Biomedicals (Kyoto, Japan). AG123F was from Seikagaku Kogyo (Tokyo, Japan). All the others were prepared as described previously (*7*).

Preparation of WGA-Binding Glycoproteins from C. elegans—Mixed cultures of *C. elegans* (Bristol N2) grown on NGM agar were collected from 40 plates (ϕ90 mm), then washed with M9 buffer to separate the baits (*8*). The

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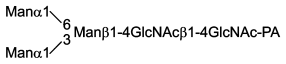
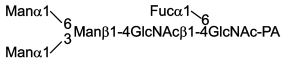
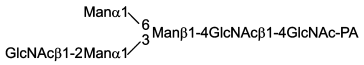
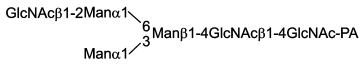
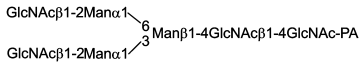
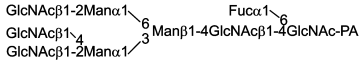
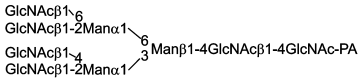
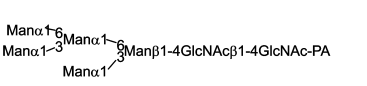
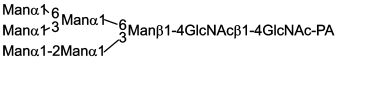
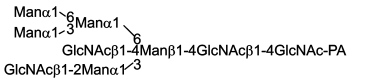
Abbreviation	Structure	Elution Position in HPLC	
		RP (Gu)	Size (Gu)
M3B		6.64	4.50
M3F6		8.14	4.91
M3GN-1		6.55	5.37
M3GN-2		8.33	5.37
AG12		7.93	6.25
AG123F		11.52	7.14
AG1234		8.12	7.46
M5A		6.93	6.45
M6B		6.36	7.40
Hyb		8.06	7.62
GN2	GlcNAcβ1-4GlcNAc-PA	–	1.75

Fig. 1. **Structures and elution positions on HPLC of standard PA-oligosaccharides.**

worms were homogenized using Polytrone in 30 ml of ice-cold PBS. Soluble materials were collected by centrifugation at 17,000 × *g*, and the precipitates were washed with 25 ml of ice-chilled PBS and again centrifuged. The first and second supernatants were combined and applied to a WGA-agarose column (0.8 × 4 cm). After washing out unbound materials with PBS, bound materials were eluted with 0.1 M GlcNAc in PBS. Chromatography was carried out at 4°C. Eluted materials containing WGA-binding glycoproteins were exhaustively dialyzed against PBS to remove free saccharides, and then concentrated by lyophilization.

Preparation of PA-Glycans—Glycans were liberated from glycoproteins by hydrazinolysis at 100°C for 10 h as described previously (*9*). Reducing ends of the liberated glycans were tagged with a fluorophore, 2-aminopyridine, in the following manner. Lyophilized samples were heated at 90°C for 60 min with 20 μl of the pyridylamination reagent. After addition of 70 μl of the reducing reagent, the reaction mixture was heated at 80°C for 35 min (*9*). Excess reagents were removed by a TSK-gel HW-40F column (0.8 × 4 cm) (Tosoh Ltd., Tokyo, Japan) equilibrated with 0.1% acetic acid. The fraction containing PA-glycans was further purified with a SepPak Plus C18 cartridge (Waters, Milford, MA) as described previously (*10*).

High Performance Liquid Chromatography for PA-Glycan Separation—Size-fractionation HPLC was performed on a Shodex NH2P-50 column (0.46 × 15 cm, Showa Denko, Tokyo, Japan) at a flow rate of 0.8 ml/min. The column was equilibrated with 50 mM ammonium acetate, pH 7.0, containing 86.5% acetonitrile. After a sample had been injected, the acetonitrile concentration was decreased linearly from 86.5 to 37.5% over a period of 50 min. The PA-glycans were detected with a fluorescence spectrophotometer at an excitation wavelength of 310 nm and an emission wavelength of 380 nm. The molecular size of each PA-glycan is given in glucose units based on the elution times of the PA-isomaltooligosaccharides. Reversed phase HPLC was performed on a Cosmosil 5C18-P column (0.46 × 15 cm, Nacalai Tesque, Kyoto, Japan) at a flow rate of 1.5 ml/min. The column was equilibrated with 100 mM ammonium acetate, pH 4.0, containing 0.025% 1-butanol. After the injection of a sample, the 1-butanol concentration was increased linearly from 0.025 to 0.5% over a period of 55 min. The PA-glycans were detected at an excitation wavelength of 315 nm and an emission wavelength of 400 nm. The retention time of each PA-glycan is given in glucose units based on the elution times of the PA-isomaltooligosaccharides. PA-monosaccharides were separated on a TSKgel Sugar AX-I column (0.46 × 15 cm, Tosoh Ltd., Tokyo, Japan) equilibrated with 10% acetonitrile in 0.8 M potassium borate, pH 9.0, at a flow rate of 0.3 ml/min at 74°C, and detected with a fluorescence spectrophotometer at an excitation wavelength of 310 nm and an emission wavelength of 380 nm.

Glycosidase Digestion—PA-oligosaccharides were digested with 0.1 unit of jack bean β-*N*-acetylhexosaminidase (Seikagaku Kogyo, Tokyo, Japan) in 20 μl of 50 mM sodium citrate buffer, pH 5.0, for 5 h at 37°C. The neoglycoprotein with Tn-antigen (Dextra Laboratories, Ltd.) was digested with 0.1 unit of *Acremonium* sp. α-*N*-acetylgalactosaminidase (Seikagaku Kogyo, Tokyo, Japan) in 50 mM sodium citrate buffer, pH 4.5, for 20 h at 37°C.

Blotting Analysis by WGA—Neoglycoproteins with T-antigens (Galβ1-3GalNAc-HSA) or Tn-antigens (GalNAcα1-Ser-HSA) were purchased from Dextra Laboratories Ltd. (Reading, UK). Proteins were separated on a 10% SDS-PAGE gel and transferred to an Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA). In the case of WGA-lectin blotting, HRP-conjugated WGA (Honen Corporation, Tokyo, Japan) and diaminobenzidine were used for detection of the ligand glycoproteins.

RESULTS

Isolation and Structural Analysis of WGA-Ligand in C. elegans—To analyze the structures of WGA-ligands in *C. elegans*, we isolated glycoproteins that could bind to WGA. An extract of *C. elegans* was subjected to WGA-agarose affinity chromatography (Fig. 2A). Bound materials eluted with hapten sugar GlcNAc were collected and dialyzed to remove free saccharides. The affinity chroma-

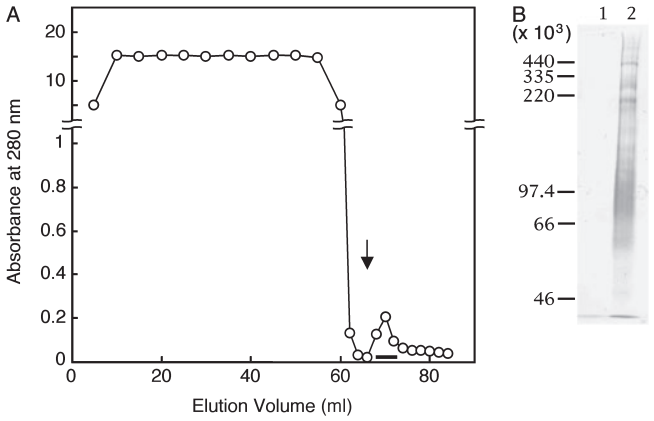


Fig. 2. **Preparation of WGA-binding glycoproteins.** (A) Extract from *C. elegans* was applied to a WGA-agarose column followed by elution of the bound materials with 0.1 M GlcNAc. Eluted proteins were monitored by absorbance at 280 nm. The arrow indicates the change of the elution buffer. The fraction assigned by the thick bar was collected and analyzed further. (B) The pass-through and bound fractions were subjected to WGA-lectin blotting assay. The method is described in "EXPERIMENTAL PROCEDURES." The numbers 1 and 2 indicate the pass-through and bound fractions, respectively. The numbers on the left of the panel are the molecular weights of the standard proteins.

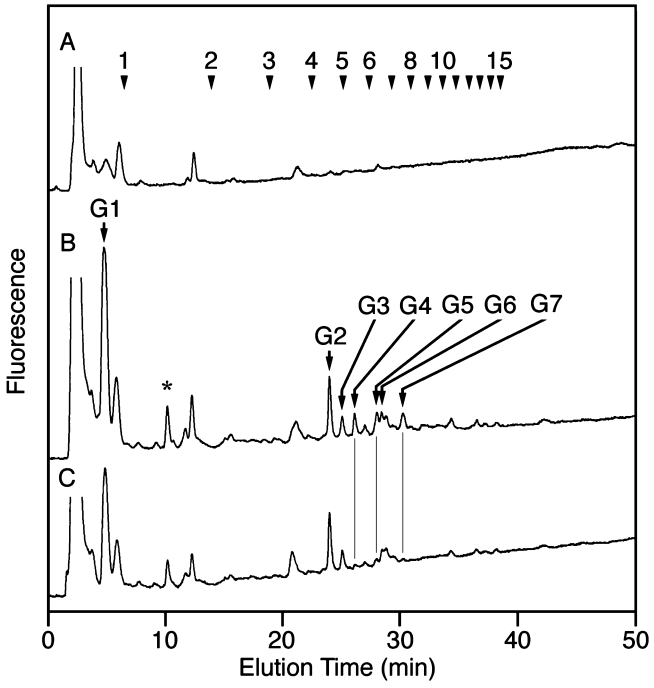


Fig. 3. **Size-fractionation HPLC of PA-glycans derived from the WGA-bound fraction.** (A) PA-Saccharides derived from the bound fraction without hydrazinolysis. (B) PA-Glycans liberated from glycoproteins in the bound fraction by hydrazinolysis. Peaks G1–G7 were collected as the glycans liberated from glycoproteins. (C) PA-Glycans after digestion of the glycans in (B) with jack bean β-*N*-acetylhexosaminidase. Arrowheads indicate the elution positions of PA-isomaltooligosaccharides with the corresponding degrees of polymerization.

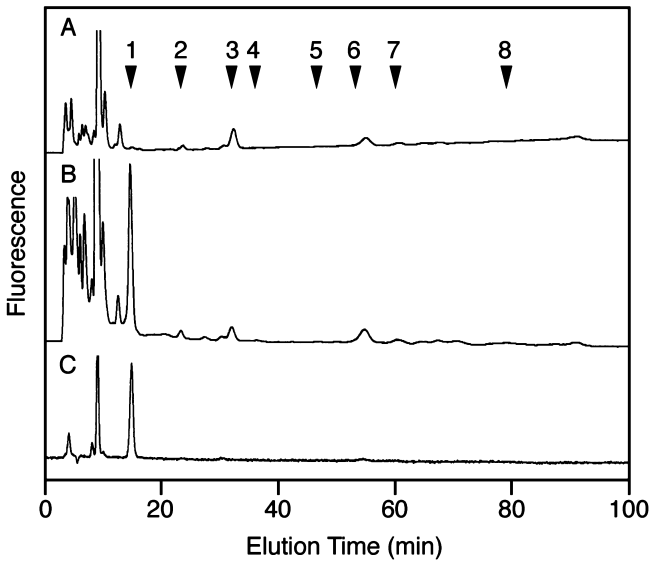


Fig. 4. **PA-monosaccharide analysis of WGA-bound fraction.** (A) PA-Saccharides derived from the bound fraction without hydrazinolysis. (B) PA-Glycans liberated from glycoproteins in the bound fraction by hydrazinolysis. (C) The fractionated peak of G1 in (B) was subjected to HPLC for PA-monosaccharide analysis. Numbered arrowheads 1–8 indicate the elution positions of PA-GalNAc, PA-ManNAc, PA-GlcNAc, PA-Rib, PA-Glc, PA-Man, PA-Fuc and PA-Gal, respectively.

tography successfully concentrated the WGA-ligands (Fig. 2B). Though *C. elegans* has chitin as a component of its cuticle, chitinase digestion did not alter the blotting pattern of the materials bound by WGA (data not shown). This shows that the material isolated by the WGA-affinity column was not chitin. The glycans were liberated from the glycoproteins by hydrazinolysis and made fluorescent with a 2-aminopyridine tag. The PA-glycans were separated by size-fractionation HPLC (Fig. 3B). To ensure that the peaks were derived from glycans liberated from glycoproteins, the HPLC profile was compared with that of a sample without hydrazine degradation (Fig. 3A). As a result, peaks G1–G7 were confirmed to be glycans released from glycoproteins. The peak indicated by an asterisk gave no reducing end saccharide by acid hydrolysis analysis. To determine why some high affinity ligands of WGA have β-GlcNAc at the non-reducing end terminal, we carried out β-*N*-acetylhexosaminidase digestion of the glycans. Three glycans, G4, G5 and G7, were susceptible to the digestion (Fig. 3C). Since the G1 peak showed a smaller size than PA-glucose by size-fractionation HPLC, we subjected the glycans to monosaccharide analysis (Fig. 4). The hydrazine-treated sample specifically contained PA-GalNAc (Fig. 4B), while the sample without hydrazine degradation had no PA-GalNAc (Fig. 4A). This result showed that the monosaccharide GalNAc was released from glycoprotein(s), and was not residual free saccharide after the dialysis process. The peak G1 isolated by size-fractionation HPLC was analyzed by HPLC for PA-monosaccharides and confirmed to be PA-GalNAc (Fig. 4C). Peaks G2–G7 were separated further by reversed phase HPLC. Peak G4 was split into two peaks, G4-1 and G4-2, by reversed phase HPLC. The elution positions of the isolated PA-glycans

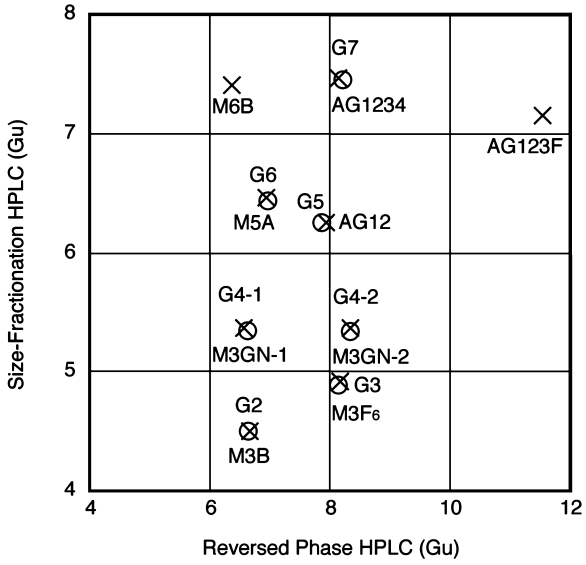


Fig. 5. **Two-dimensional map of PA-glycans derived from the WGA bound fraction.** The elution position of each PA-glycan by size-fractionation and reversed phase HPLC is given in glucose units (Gu) based on the elution times of the PA-isomaltooligosaccharides, and then plotted on the map. The positions of the PA-glycans are indicated by circles, those of the standard PA-glycans by crosses.

Fraction	Estimated Structure	Abbreviation
G1	GalNAc-PA	GalNAc-PA
G2	Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA	M3B
G3	Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA, Fucα1-6	M3BF6
G4-1	Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA	M3GN-1
G4-2	GlcNAcβ1-2Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA	M3GN-2
G5	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA	AG12
G6	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA	M5A
G7	GlcNAcβ1-6(GlcNAcβ1-2Manα1-6)(GlcNAcβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA	AG1234

Fig. 6. **Estimated structures of PA-glycans from WGA-binding glycoproteins of *C. elegans*.**

on the two kinds of HPLCs were plotted on a two-dimensional map and compared with those of standard PA-glycans (Fig. 5). The positions of G2, G3, G4-1, G4-2, G5, G6 and G7 showed good coincidence with those of M3B, M3F6, M3GN-1, M3GN-2, AG12, M5A and AG1234,

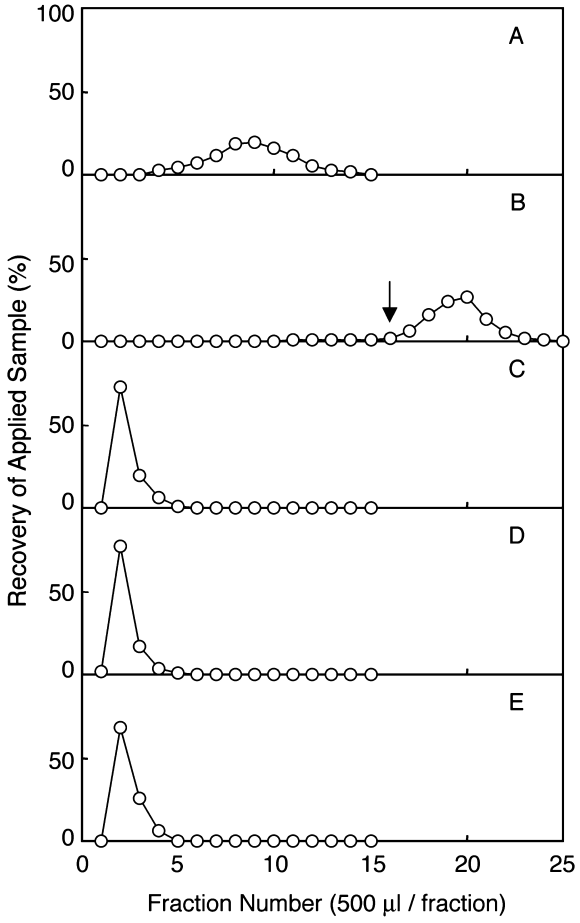


Fig. 7. **Binding assay of PA-glycans to WGA.** PA-glycans (A, GN2; B, Hyb; C, M5A; D, AG12; E, AG1234) were subjected to WGA-lectin-affinity-chromatography. The protocol is described in "EXPERIMENTAL PROCEDURES." The arrow indicates the point where the buffer was changed to PBS containing 0.1 M GlcNAc.

respectively. The structures of the standard PA-glycans are listed in Fig. 1. The result obtained with the β-*N*-acetylhexosaminidase digestion shown in Fig. 3C supported the structures estimated from two-dimensional mapping of PA-glycans. The estimated structures of PA-glycans derived from WGA-binding glycoproteins are listed in Fig. 6.

Binding Assay of PA-N-Glycans to WGA—The AG12 and AG1234 found in the glycans, had two and four *N*-acetylglucosamine residues at their non-reducing ends, respectively. Thus, we analyzed their ability to bind WGA, since this lectin is known to recognize GlcNAc (Fig. 7). GN2, which had moderate affinity to WGA, was retarded on the WGA-agarose column (panel A). The positive control Hyb, which has high affinity for WGA, was bound by the column and was eluted with 0.1 M GlcNAc (panel B). The oligomannose-type glycan M5A**,** fraction G6, passed though the column as expected (panel C). The glycans AG12 and AG1234, corresponding to fractions G5 and G7, respectively, also showed no binding ability to WGA (panels D and E). These results suggested that the *N*-glycans on the isolated glycoproteins were not responsible for the binding of WGA.

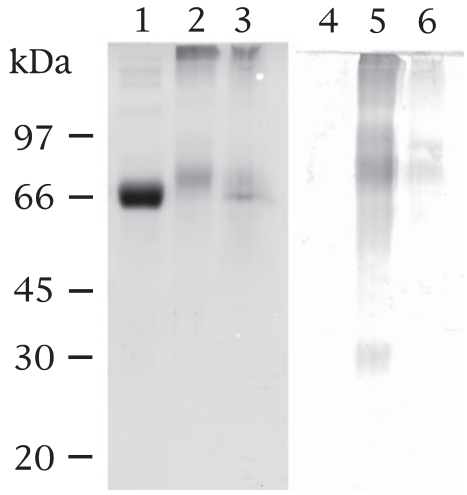


Fig. 8. **Lectin-blotting of neoglycoproteins with multiple sugars.** Neoglycoproteins with T-antigen-HSA (Galβ1-3GalNAc) (lanes 1 and 4), Tn-antigen-HSA (GalNAcα1-) (lanes 2 and 5), and α-*N*-acetylgalactosaminidase treated Tn-antigen-HSA (lanes 3 and 6) were subjected to SDS-PAGE, and stained with CBB (lanes 1–3) or blotted with HRP-conjugated WGA (lanes 4–6).

Binding Assay of Multiple GalNAc Residues to WGA—Since WGA has high affinity to chitooligosaccharides and polysialic acids, WGA is considered to recognize *N*-acetyl groups of saccharides. A monosaccharide, GalNAc, which was identified as a major glycan of WGA-binding glycoproteins from *C. elegans* as described above, also contained an *N*-acetyl group. It has been reported that methyl α-GalNAc has a very weak affinity for WGA (*11*). We therefore investigated the possibility that the α-GalNAc residues of glycoproteins, which were known as the Tn-antigen structure, will produce sufficient affinity to bind WGA due to a cluster effect. We tested this possibility using human serum albumin with α-GalNAc residues, a neoglycoprotein. As shown in Fig. 8, the T-antigens of the neoglycoprotein did not bind to WGA**.** However, the neoglycoprotein with the Tn-antigens clearly bound to the lectin, and the binding ability was abolished by α-*N*-acetylgalactosaminidase digestion. These results indicated that clustered α-*N*-acetylgalactosaminides on a protein were capable of acting as a good ligand of WGA.

DISCUSSION

The aim of this study was to identify WGA ligands in *C. elegans*. We therefore prepared WGA-binding glycoproteins from a *C. elegans* extract. These glycoproteins proved to be highly heterogeneous, similar to mucins that exhibited a smeared image on SDS-PAGE. Analysis of the structures of the glycans showed that oligomannose and agalacto-complex-type *N*-glycans were present on the glycoproteins. However, we found that *N*-glycans from the glycoproteins did not have sufficient affinity to be retained on the WGA-agarose column. We also showed that there were many GalNAc monosaccharide residues on the glycoproteins. Hydrazinolysis of the glycoproteins released considerable amounts of GalNAc monosaccharides, indicating that these residues are covalently linked to the proteins. Our results suggest that there are Tn-antigen structures (GalNAcα1-Ser/Thr) on the WGA binding glycoproteins.

Neoglycoprotein with multiple Tn-antigens could bind to WGA, and α-*N*-acetylgalactosaminidase digestion abolished the binding ability. A single GalNAc residue cannot be a suitable ligand for WGA because of its low binding affinity to WGA (*11*). Thus, a cluster of the Tn-antigens may be required to act as a ligand for WGA in *C. elegans*. Although we attempted to determine whether α-*N*-acetylgalactosaminidase digestion removed ligand activity from *C. elegans* glycoproteins, we were unsuccessful because of interference by endogenous proteinase actions on the ligand glycoproteins during glycosidase digestion. For further analysis of this question, we require purified ligand glycoproteins from *C. elegans*.

In this study, the WGA-ligands in *C. elegans* were suggested to have clustered α-GalNAc residues. The observations reported in this paper emphasize the possible significance of mucin-type *O*-glycans in development of a multicellular organism.

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