Neural complex-specific expression of xylosyl N-glycan in Ciona intestinalis

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Glycobiology vol. 18 no. 2 pp. 145–151, 2008
doi:10.1093/glycob/cwm128
Advance Access publication on December 3, 2007

We herein report N-glycosylation profiles of the individual tissues derived from the ascidian Ciona intestinalis. Multidimensional HPLC mapping revealed that the C. intestinalis expresses high-mannose-type oligosaccharides as major N-glycans, along with paucimannose-type and complex-type oligosaccharides, in a tissue-specific manner. Notably, the trimannosyl core carrying β1,2-xylose and α1,3-fucose residues was identified as a principal N-glycan in the neural complex. As far as we know, this is the first description of xylosyl N-glycan expressed in deuterostome. Furthermore, we found that this xylosyl N-glycan is exclusively displayed on a membrane-associated protein so far described as a putative protein whose gene expression is specific for the neural complex. These data suggested that the xylosyl N-glycan is associated with some neural functions of C. intestinalis.

Keywords: ascidian/Ciona intestinalis/neural complex/N-glycan/xylose

Introduction

N-glycans play important roles in mediation of cell-cell communication and virus infection, determination of the fates of their carrier proteins in and out of cells, and modulation of protein functions as hormones and antibodies (Helenius and Aebi 2004; Kannagi et al. 2004; Suzuki 2005; Kato and Kamiya 2007).

Cellular expression patterns of N-glycans depend on developmental stages and environmental factors (Cipollo et al. 2005; Takemoto et al. 2005; Aoki et al. 2007; Ishii et al. 2007). The recently emerging glycomics projects aim at comprehensive identification and characterization of N- and O-glycans expressed by whole cells, tissues, organs, and bodies. They encompass some model organs such as Caenorhabditis elegans (Cipollo et al. 2002, 2005; Natsuka et al. 2002), Drosophila melanogaster (Aoki et al. 2007), and Danio rerio (Takemoto et al. 2005; Guèrardel et al. 2006), as well as mammals (http://glycomics.scripps.edu/CFGad.html) (Comelli et al. 2006). However, N-glycosylation profiles of deuterostomes other than vertebrates have scarcely been characterized.

Ascidians, or sea squirts, are valuable model animals for studying the development of the nervous system from evolutionary aspects, because urochordates (including ascidians) are the closest living relatives of vertebrates (Bourlat et al. 2006; Delsuc et al. 2006). Recently, the genomic information of the ascidian Ciona intestinalis has become available at a web-accessible database (ghost database: http://ghost.zool.kyoto-u.ac.jp/indexr1.html) (Dehal et al. 2002; Satou et al. 2005), which shows that the Ciona genome contains approximately 16 000 protein-coding genes including those encoding putative enzymes catalyzing biosynthesis and processing of N-glycans. In view of the situation, we undertook this study to examine N-glycosylation profiles of C. intestinalis, which provide a missing link in comparative glycomics. Herein, we report N-glycosylation profiling of the individual tissues derived from C. intestinalis. We found that the neural complex specifically expresses a xylosyl N-glycan. Furthermore, we identified a 180-kDa protein bearing this carbohydrate moity.

Results

N-glycosylation profiling of C. intestinalis tissues

We performed N-glycosylation profiling of the egg, larva, tunic, body wall muscle, gut, gonad, heart, and neural complex derived from C. intestinalis by the multidimensional high performance liquid chromatography (HPLC) mapping method. Since virtually no anionic N-glycan was detected by a preliminary profiling on a diethylaminoethyl (DEAE) anion-exchange column (data not shown), we subjected the PA-glycans from the individual tissues directly to the profiling on an amide-silica column (Figure 1). The individual fractions were further applied onto an ODS column, giving rise to subfractions such as d-1, d-2, and d-3. The PA-oligosaccharide was identified on the basis of coincidence of elution time normalized in GU with those on the HPLC map (Tomiya et al. 1991; Takahashi et al. 1995; Kamiya et al. 2005). For example, the glycan corresponding to peak a was eluted at 5.5 GU on the amide column and at 6.0 GU on the ODS column. The elution data set is in good agreement with the elution profiles on an ODS column, giving rise to subfractions such as d-1, d-2, and d-3. The PA-oligosaccharide was identified on the basis of coincidence of elution time normalized in GU with those on the HPLC map (Tomiya et al. 1991; Takahashi et al. 1995; Kamiya et al. 2005). For example, the glycan corresponding to peak a was eluted at 5.5 GU on the amide column and at 6.0 GU on the ODS column. The elution data set is in good agreement with the elution profiles on an ODS column, giving rise to subfractions such as d-1, d-2, and d-3. The PA-oligosaccharide was identified on the basis of coincidence of elution time normalized in GU with those on the HPLC map (Tomiya et al. 1991; Takahashi et al. 1995; Kamiya et al. 2005).
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Fig. 1. N-Glycosylation profiles on an amide column derived from C. intestinalis.

co-chromatography and the MALDI-TOF-MS analyses, we confirmed the structure of this PA-oligosaccharide. In a similar way, we identified 10 kinds of N-glycans derived from C. intestinalis. In addition, structures of PA-oligosaccharides corresponding to peaks d-2 and d-3 were estimated based on MALDI-TOF-MS data and the calculated HPLC map for high-mannose-type PA-oligosaccharides (Tomiya et al. 1991). Table I summarizes the structures of the identified N-glycans with their incidence. In this table, only carbohydrate compositions based on the MALDI-TOF-MS data were presented for the PA-oligosaccharides corresponding to peaks c, e, and g, which were observed in tunic, body wall muscle, and heart, because no HPLC data have so far been available for these low-abundance complex-type glycans. The MS/MS data suggested that the branching structures of the oligosaccharides corresponding to peaks c and e are as follows:

The PA-glycans corresponding to peaks c, e, and g were all resistant to α-mannosidase, α-galactosidase, β-galactosidase, and β-N-acetylhexosaminidase treatments for further structural characterization (data not shown), suggesting that the nonreducing terminal hexose linkages are different from those commonly observed for mammals.

Thus, our glycosylation profiling data demonstrate that the N-glycans derived from C. intestinalis consist mainly of high-mannose-type oligosaccharides, along with paucimannose-type and complex-type oligosaccharides, while anionic glycan such as sialylated oligosaccharides could not be detected. Each tissue exhibited a characteristic pattern of N-glycosylation. It is particularly noteworthy that the neural complex exhibited a unique N-glycan having α1,3-fucose and β1,2-xylene residues as a major (70%) component.

Detection and identification of glycoprotein exhibiting the xylosyl N-glycan in the neural complex
To detect glycoproteins bearing the xylosyl N-glycan, we performed an immunoblot analysis with polyclonal anti-HRP antibodies, which are directed against N-glycans exhibiting β1,2-xylene and core α1,3-fucose residues (Kurosaka et al. 1991; Strasser et al. 2000; Benc ´urov´a et al. 2004). One major band (corresponding to apparent molecular mass of 180 kDa; indicated by arrow in Figure 2B) was detected in the membrane-associated fraction with the polyclonal anti-HRP antibody. No reactivity was detected by the cytosolic fraction (Figure 2B), indicating that the xylosyl N-glycan is expressed exclusively on the membrane-associated 180-kDa glycoprotein in the neural complex. The total proteins in the neural complex were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), and then stained with CBB (Figure 2A). A piece of polyacrylamide gel containing the 180-kDa glycoprotein was excised, digested with trypsin, and then analyzed by LC-MS/MS. The 180-kDa glycoprotein was identified as a translation product of the C. intestinalis cDNA, clone: cieg020o17 (GeneBank accession no. AK114875). The observed sequence covered 19% of that of the 180-kDa glycoprotein (Figure 3).

Discussion
The present HPLC mapping revealed that the N-glycans of egg and larva of C. intestinalis are composed exclusively of high-mannose-type oligosaccharides, while mature adult expressed high-mannose-type, paucimannose-type, and...
Neural complex-specific xylosyl N-glycan in *C. intestinalis*

### Table I. *N*-Glycans derived from *C. intestinalis*

<table>
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<th>GU(OIDS)</th>
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<th>T</th>
<th>M</th>
<th>Gt</th>
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<th>H</th>
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* Calculated from the peak areas in Figure 1.

* Below the detection limit.

* The carbohydrate composition was estimated based on MALDI-TOF-MS data.

* These structures were estimated based on MALDI-TOF-MS data and the calculated HPLC map for high-mannose-type PA-oligosaccharides (Tomiya et al. 1991).
complex-type oligosaccharides in a tissue-specific manner. Hence, N-glycosylation of *C. intestinalis* exhibits metamorphic changes. The *N*-glycosylation of matured *C. intestinalis* shares common features with those of protostomes rather than vertebrates. For examples, *N*-glycans possessing α1,3-fucose residue linked to the proximal GlcNAc residue are expressed by insect (Staudacher et al. 1992; Kubelka et al. 1993, 1994; Hsu et al. 1997; Aoki et al. 2007), nematodes (Natsuka et al. 2002), helminths (Haslam et al. 1996; Khoo et al. 1997, 1998; Gutternigg et al. 2004) and parasites (Satou et al. 1997; Natsuka et al. 2002), and mollusk (Takahashi et al. 2003). Yet this is the first description of α1,3-fucosylated *N*-glycan expressed in deuterostomes. Also, the terminal HexNAc-(DeoxyHex-)HexNAc unit, exhibited by the minor complex-type oligosaccharides in body-wall muscle and heart, has been reported for snails and helminths (Geyer et al. 2005; Lehr et al. 2007).

Most interestingly, the major (70%) *N*-glycans expressed on the neural complex possess β1,2-xylene residues. It is well known that plant glycoproteins express the β1,2-xyllosylated *N*-glycans, which has so far been found only in gastropods (van Kuik et al. 1985, 1987; Gutternigg et al. 2004) and parasites (Khoo et al. 1997, 2001) among the animal kingdom. Furthermore, we identified the 180-kDa glycoprotein bearing the xylosyl *N*-glycan, which is a putative membrane-associated protein with 17 possible *N*-glycosylation sites (Figure 3). The genome database of *C. intestinalis* shows that the gene of this protein is specifically expressed in the neural complex (Satou et al. 2005).

The abundance of the β1,2-xyllosyl *N*-glycan (Figure 1) strongly suggests that *C. intestinalis* possesses β1,2-xyllosyltransferase. Indeed, the *C. intestinalis* genome database contains two β1,2-xyllosyltransferase-homologous genes, one of which intriguingly exhibits a neural complex-specific expression (Satou et al. 2005). On inspection of all these data, we suggest a possible role of this enzyme in the expression of the xylosyl *N*-glycan. More interestingly, these genes are highly conserved among animals including mammals. In the vertebrate nervous system, cell-cell and cell-extracellular matrix interactions during development and regeneration are regulated by *N*-glycans with polysialic acid chains expressed on a neural cell adhesion molecule (NCAM), a membrane-associated protein possessing six *N*-glycosylation sites (Kleene and Schacher 2004). Although it is obviously necessary to perform further studies including *O*-glycosylation profiling for the elucidation of functional roles of the xylosyl glycoprotein, our findings raised the possibility that xylosyl *N*-glycans play roles in cell-cell communication in the neural complex in ascidians and, more generally, in neural systems in animals.

Materials and methods

**Materials**

Mature adults of *C. intestinalis* was collected in Aioi Bay and Murotsu Port, Hyogo, Japan during April to June or November to January and kept in artificial seawater (ASW) at 18°C under constant illumination. The larvae were obtained by artificial insemination. Frequently, the eggs were not fertilized and the embryos became deformed. To prevent the contamination of unfertilized eggs and deformed eggs, the eggs taken from each adult oviduct were individually collected in a dish and inseminated with the mixture of different sperms. Among them we took the dishes in which about 100% of larvae were hatched normally, and the larvae at 6 h posthatch were collected by centrifugation to removed ASW and then frozen with liquid nitrogen. The larvae of more than five batches were mixed and used as the starting materials for *N*-glycan analyses. Adult tissues (egg, tunic, body wall muscle, gut, gonad, and heart) were dissected from a few adults. Neural complexes, which are much smaller than the other tissues, were collected from 50 adults for *N*-glycosylation profiling.

Glycoamidase A from sweet almond, α-mannosidase, β-galactosidase, and β-N-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). α-Galactosidase from coffee bean was purchased from Oxford.
Characterization of N-glycan derived from C. intestinalis

The residue after chloroform–methanol extraction of each tissue of C. intestinalis was used as a starting material. All experimental procedures used, including the chromatographic conditions and glycosidase treatments, have been described previously (Nakagawa et al. 1995; Takahashi et al. 1995, 2001). The extract was proteolyzed with chymotrypsin and trypsin mixture and further digested with glycoamidase A to release N-glycans. After the removal of the peptide materials, the reducing ends of the N-glycans were derivatized with 2-aminopyridine (Wako, Osaka, Japan). This mixture was applied onto a DEAE column (Tsoh, Tokyo, Japan) or a TSK-gel Amide-80 column (Tsoh), and then each fraction separated on the amide column was applied to a Shim-pack HRC-ODS column (Shimadzu, Kyoto, Japan). The final yields of the PA-glycans was 10 to 900 pmol per milligram of the chloroform–methanol extract of the individual tissues, i.e., egg (70 pmol), larva (20 pmol), tunic (10 pmol), body wall muscle (730 pmol), gut (140 pmol), gonad (100 pmol), heart (830 pmol), and neural complex (900 pmol). The elution times of the individual peaks onto the amide-silica and ODS columns were normalized with respect to the PA-derivatized isomalto-oligosaccharide of polymerization degree, and represented in units of glucose (GU). Thus, a given compound from these two columns provided a unique set of GU values, which corresponded to coordinates of the 2D HPLC map. The PA-oligosaccharides were identified by comparison with the coordinates of ~500 reference PA-oligosaccharides in a homemade web application, GALAXY (http://www.glycoanalysis.info/) (Takahashi and Kato 2003). The calculated HPLC map based on the unit contribution values was used for the estimation of some high-mannose-type PA-oligosaccharides (Tomiya et al. 1991). The PA-oligosaccharides were co-chromatographed with reference to PA-oligosaccharides on the columns to confirm their identities.

Mass spectrometric analyses of PA-glycans

PA-oligosaccharides were subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF-MS) analysis. The matrix solution was prepared as follows: 10 mg of 2,5-Dihydroxybenzoic acid (Sigma) was dissolved in 1:1 (v/v) of acetonitrile/water (1 mL). Stock solutions of PA-glycans were prepared by dissolving them into pure water. One microliter of sample solution was mixed on the target spot of a plate with 1 µL of matrix solution and then allowed to air-dry. MALDI-TOF-MS data were acquired in the positive modes using AXIMA-CFR (Shimadzu) operated in the linear mode. MS/MS analyses were performed using a 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA) in the positive mode with the collision energy set to 1 kV with air as the collision gas.

Detection of glycoprotein bearing neural specific xylosyl N-glycan

A neural complex was homogenized using a needle in 500 µL of phosphate-buffered saline (PBS) (0.02 M phosphate, 0.15 M NaCl, pH 7.4), containing 1 mM EDTA and protease inhibitors (Nakalai Tesque, Kyoto, Japan). The homogenATE was centrifuged at 20 000 × g for 30 min at 4°C. The resultant supernatant was used for the following experiments as cytosolic fraction. The precipitate was re-suspended overnight in PBS containing 1% Nonidet P-40, which was then centrifuged at 20 000 × g for 30 min at 4°C. The resultant supernatant was used as membrane fraction. Each fraction was subjected to 3–10% gradient SDS–PAGE and subsequent transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, CA). After blocking with 2% bovine serum albumin in PBS containing 0.05% Tween 20, the membrane was incubated with polyclonal rabbit anti-horseradish peroxidase (HRP) antibodies (Sigma), followed by incubation with HRP-conjugated goat antirabbit Ig antibody (Amersham Pharmacia Biotech., Piscataway, NJ). Anti-HRP antibody directed against N-glycans exhibiting β1,2-xylose and core α1,3-fucose residues (Kurosaka et al. 1991; Strasser et al. 2000; Bencúrová et al. 2004). The protein bands were visualized by immunochromatological staining with Immobilon Western (Millipore).

Identification of glycoprotein by LC/MS/MS analyses

Protein spot from the Coomassie Brilliant Blue (CBB)-stained gel was manually excised with a round bottom dermal slicer of 3-mm diameter. The gel piece was destained in 30% acetonitrile in 25 mM ammonium bicarbonate buffer, pH 8.0, dehydrated with 100% acetonitrile, and then dried in a centrifugal evaporator. The dried gel piece was incubated in the presence of dithiothreitol (10 mg/mL) for 1 h and then in the presence of iodoaceticamide (10 mg/mL) for 45 min in the dark. The gel piece was washed in 25 mM ammonium bicarbonate buffer and subsequently in 30% acetonitrile in 25 mM ammonium bicarbonate buffer, dehydrated with 100% acetonitrile, dried in a centrifugal evaporator and then rehydrated in 20 µL of trypsin solution (10 µg/mL) in 25 mM ammonium bicarbonate buffer. After removing the supernatant, the gel pieces were extracted with 2.5% formic acid/50% acetonitrile, and the extracts were then dried under vacuum. The digested peptides were reconstituted in 0.1% formic acid and analyzed by a NanoFrontier L (Hitachi High-Technologies, Tokyo, Japan), which consists of a nano HPLC system and an electrospray ionization linear ion trap time-of-flight mass spectrometry (ESI-LIT-TOF). The peptides were directly infused into the ESI source through a packed PicoFlit column (0.075 × 100 mm, New Objectives) equilibrated in 0.1% formic acid at a flow rate of 200 nL/min, and were eluted with an acetonitrile gradient from 2 to 45% over 90 min. The detection of peptides was performed in which the mass spectrometer was set as a full scan MS followed by data-dependent MS/MS. All the MS/MS spectra were searched against full insert sequences of eDNA clones from ghost database (Satou et al. 2005) using the MASCOT search engine (Matrix Science).

Funding

Grants-in-Aid for Scientific Research (18390016 and 17046017) from the Ministry of Education, Culture, Sports,
Science and Technology of Japan; Japan Society for the Promotion of Science Research Fellowships for Young Scientists.

Conflict of interest statement
None declared.

Abbreviations
ASW, artificial seawater; CBB, Coomassie Brilliant Blue; DEAE, diethylaminoethyl; DeoxyHex, deoxyhexose; Fuc, fucose; GlcNAc, N-acetylglucosamine; GU, Glucose Unit; Hex, hexose; HexNAc, N-acetylhexosamine; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; Man, mannose; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NCAM, neural cell adhesion molecule; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Xyl, xylose.

References
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