Sulfated and sialylated N-glycans in the echinoderm *Holothuria atra* reflect its marine habitat and phylogeny

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Among the earliest deuterostomes, the echinoderms are an evolutionary important group of ancient marine animals. Within this phylum, the holothuroids (sea cucumbers) are known to produce a wide range of glycoconjugate biopolymers with apparent benefits to health; therefore, they are of economic and culinary interest throughout the world. Other than their highly modified glycosaminoglycans (e.g. fucosylated chondroitin sulfate and fucoidan), nothing is known about their protein-linked glycosylation. Here we used multistep N-glycan fractionation to efficiently separate anionic and neutral N-glycans before analyzing the N-glycans of the black sea cucumber (*Holothuria atra*) by MS in combination with enzymatic and chemical treatments. These analyses showed the presence of various fucosylated, phosphorylated, sialylated, and multiply sulfated moieties as modifications of oligomannosidic, hybrid, and complex-type N-glycans. The high degree of sulfation and fucosylation parallels the modifications observed previously on holothuroid glycosaminoglycans. Compatible with its phylogenetic position, *H. atra* not only expresses vertebrate motifs such as sulfo-- and sialyl--Lewis A epitopes but displays a high degree of anionic substitution of its glycans, as observed in other marine invertebrates. Thus, as for other echinoderms, the phylum- and order-specific aspects of this species’ N-glycosylation reveal both invertebrate- and vertebrate-like features.

Sea cucumbers (holothuroids) are a group of organisms living in the benthic zones of seas across the world. As one of the five clades of the Echinodermata (Fig. 1), sea cucumbers are primitive deuterostomes and are thus related to the ancestors of vertebrates. Around 100 of the 1500 extant sea cucumber species are consumed by humans, in part because of their intrinsic nutritional value and the proposed beneficial effects of various unusual phosphorylated, fucosylated, sialylated, and multiply sulfated N-glycans that were released by serial digestion with PNGase-F and then PNGase-A and subsequently analyzed by HPLC, MS, and enzymatic and chemical treatments. In addition to a number of common oligomannosidic types, unusual phosphorylated, fucosylated, sialylated, and multiply sulfated N-glycans were identified that potentially represent phylum- and order-specific aspects of echinoderm glycosylation.

Results

**Workflow and analytical strategy**

N-glycans of *H. atra* were released by serial digestion with PNGase-F and then PNGase-A, resulting in free N-glycan pools that were subfractionated on graphitized carbon into neutral and anionic subpools prior to pyridylamination (PA). The initial survey of the complete N-glycome showed some mass shifts of 78 Da between positive and negative MS modes, compatible with the presence of either phosphate or sulfate modifications (Fig. 1). Subsequently, the neutral and anionic PNGase F–released N-glycans were subject to reverse-phase (RP) and normal-phase (NP) HPLC; all fractions were analyzed by MALDI-TOF MS in positive and negative modes (Fig. 2 and Fig. S1). This offline LC-MS approach revealed that the neutral N-glycan pool was relatively simple, containing primarily well-
known oligomannosidic structures, whereas the anionic pool contained numerous unusual charged hybrid and complex N-glycans.

### Oligomannosidic-type N-glycans

The neutral pool contained a series of Hex$_{4-12}$HexNAc$_2$ glycans (m/z 1151–2447). These could be assigned as isomers of Glc$_0$–Man$_{4-6}$GlcNAc$_2$ because of their retention time on RP/NP HPLC as well as positive MS/MS fragmentation patterns before and after mannosidase digestion. As these common isomers (see Table S1 for a comparison of elution times) were identified previously in other organisms (11, 12), they are not discussed further.

We also observed unusual N-glycans in both positive and negative modes; they were predicted to be phosphorylated forms of Hex$_{10-12}$HexNAc$_2$ (m/z 2203, 2365, and 2527; Fig. 3, A and E, and Fig. S2, A–C). The most abundant of these (m/z 2527) was dephosphorylated with either alkaline phosphatase or HF, yielding an MS/MS spectrum and retention time characteristic of the basic endoplasmic reticulum Glc$_3$Man$_x$GlcNAc$_2$ N-glycan precursor (m/z 2447). The thereby predicted P$_1$Glc$_3$Man$_x$GlcNAc$_2$ glycan was also treated with α-mannosidase, resulting in loss of up to five mannose residues regardless of whether the phosphate had been removed, and with endo-α2-mannosidase, which removed a P$_1$Glc$_3$Man$_x$ unit (Fig. 3 and Fig. S2); these data indicated that the phosphate residue is on the triglucosylated A arm. Considering also the MS/MS B$_1$ ions at m/z 241 (P$_1$Hex$_1$) in negative mode, we concluded that the terminal glucose residue is the location of the phosphate modification.

### Neutral hybrid-type N-glycans

Within the H. atra glycome, there were five neutral glycans with predicted compositions of Hex$_{4-6}$HexNAc$_2$Fuc$_0-2$ (m/z 1500–1970). These potentially hybrid structures were analyzed using HPLC, MS, and exoglycosidase treatments to define the nature of their antennae. For instance, a 2D HPLC-purified form of Hex$_4$HexNAc$_3$ (m/z 1678, Fig. 4, A and I) was sensitive to β3-galactosidase (Fig. 4, G and J; loss of one Gal) and α-mannosidase (Fig. 4, H and K; loss of two or three Man) but not to β4-galactosidase (Fig. 4F). This indicated the presence of a type 1 antenna (neo-LacNAc, Galβ3GlcNAcβ-R) on a hybrid backbone, a conclusion confirmed by comparison with a later-eluting isomer with a type 2 antenna (LacNAc, Galβ4GlcNAcβ-R; prepared by in vitro β4-galactosylation; Fig. 4B). The β3-galactosylated hybrid structure appeared to be the basis for a number of sialylated and sulfated glycans, as desialylation of S$_1$Hex$_2$HexNAc$_3$ (m/z 1756) and desialylation of NeuGc$_1$Hex$_3$HexNAc$_3$ (m/z 1856) resulted in a coeluting Gal$_1$Man$_3$GlcNAc$_2$ structure (Fig. 4, C and D).

### Sulfated hybrid-type N-glycans

Monosulfated fucosylated structures of S$_1$Fuc$_1-2$Gal$_1$Man$_3$-GlcNAc$_2$ were analyzed via negative MS and MS/MS (Fig. 5, A, F, H, K, and M), whereby key B ions and neutral losses aided definition of the sulfate and fucose positions. For instance, the
Two fucosylated isomers of \( S_1 Fuc_1 Gal_1 Man_5 GlcNAc_3 \) (m/z 1902, eluting at 4.7 g.u. and 7.8 g.u.) exhibited different negative MS/MS fragmentation patterns (Fig. 5, H and K); the first one with \( m/z \) 590 (\( S_1 Fuc_1 Gal_1 GlcNAc_1 \)) and 1603 (loss of GlcNAc-PA) is concluded to possess an antennal fucose, whereas the second one with \( m/z \) 444 (\( S_1 Gal_1 GlcNAc_1 \)) and 1457 (loss of Fuc1GlcNAc-PA) is core/fucosylated, as it was released by PNGase-F. The related difucosylated \( m/z \) 2048 structure not only presented an \( m/z \) 590 B fragment but also an \( m/z \) 1603 ion, indicative of loss of Fuc1GlcNAc-PA (Fig. 5M). Treatment of the antennally fucosylated isomer with either \( \alpha3/4\)-galactosidase (also no digestion even after \( \alpha\)-fucosidase), \( \alpha\)-mannosidase (loss of two or three Man), \( \alpha3/4\)-fucosidase and HF (both resulting in loss of one Fuc) (Fig. 5, A–E) aided definition of the A arm as Lewis motifs with sulfated galactose residues. An alternative position for sulfation (rather than on galactose) is concluded for a hybrid-type \( S_1 Fuc_1 Gal_1 Man_3–GlcNAc_3 \) glycan, which was \( \beta3\)-galactosidase–sensitive; the
m/z 241 and 1213 fragments in particular indicated that the sulfate is, in this case, on mannose (Fig. S3).

For sulfated structures in general (13), positive mode MS/MS of [M-SO₄]⁺ pseudomolecular ions is useful to define the underlying backbones. Here, positive mode Y-ions at 446 (Fuc₆GlcNAc₋PA), 1297/1459 (Fuc₆Man₆₋GlcNAc₋PA), and m/z 503–1313 (Man₅₋GlcNAc₋PA) as well as the B ions at m/z 366/512 (Gal₁GlcNAc₁Fuc₀₋₁) provided full-sequence coverage of the core and the antennae of the hybrid structures (Fig. 5, I, J, L, and N). Therefore, considering the presence of β₃-galactose on nonfucosylated glycans (Fig. 4) and the available LC-ESI-MS data (Fig. S4), the antennal motif for these Lewis-modified glycans is concluded to be (HSO₄)⁴Galβ₃(Fuco⁺)GlcNAc-R, i.e., sulfo-Lewis A.

For disulfated S₂Fuc₂₋₃Gal₂Man₅₋GlcNAc₃ structures (m/z 1842–2150; Fig. 6, A–D), diagnostic negative B ions are consistent with two sulfates substituting either the Gal, GlcNAc, or α₃-Man residues on the A arm. Negative-mode MS/MS could indeed distinguish disulfated m/z 2150 isomers (eluting at 42.5 and 48.4 min on NP HPLC); the pattern for the first isomer with m/z 241 (S₁Gal₁), 854 (S₁Fuc₁Gal₁GlcNAc₁Man₁), and 1537 (S₁Fuc₁Man₁₋GlcNAc₋PA) suggests the presence of sulfate on the terminal Gal and lower α₃-Man residues (Fig. 6F). For the second one, the occurrence of fragments of m/z 241 (S₁Gal₁) and 692 (S₂Fuc₁Gal₁GlcNAc₁), together with loss of the latter upon HF treatment while retaining both sulfate residues, is compatible with the presence of a disulfo-Lewis motif (Fig. 6, D, E, I, and J), thereby the possibility of sulfation of the fucose is excluded, but sulfation of the Gal and GlcNAc residues is confirmed.

For the disulfated S₂Fuc₁₋₂Gal₆Man₅₋GlcNAc₃ structures (m/z 2515 and 2661 as [M-2H+Na]⁻; eluting at 50 min on NP HPLC), two mannooses were removed by α-mannosidase treatment (Fig. S5, A and B), proving their hybrid-type backbones. MS/MS analyses of the parental ion, as well as the [M-SO₄]⁻ and [M+H-2SO₄]⁺ ions resulting from in-source loss of sulfate, indicated the presence of two sulfo-Lewis–type antennae, presumably β₁,2- and β₁,4-linked to the α₃-linked mannose (Fig. S5, C–E).

Trisulfated S₃Fuc₀₋₂Gal₁Man₁₋GlcNAc₃ structures eluted rather late on the NP HPLC column (65–68 min) and were best detected when supplementing the matrix with sodium acetate (Fig. S6, A, B, G, and H; m/z 1960 and 2252). Negative- and positive mode MS/MS of the “real” [M-Hₙ⁺Naₙ₋₁]⁻ and “in-source” parent ions yielded fragments (Fig. S6, C–F and I–L) consistent with the three sulfation positions on the A arm (terminal Gal, antennal GlcNAc, and α₃-Man), as established for the aforementioned disulfated species.

Sialylated hybrid-type N-glycans

The results of offline LC-MS/MS led us to predict a number of sialylated glycans in the H. atra N-glycome (Fig. 2B and Fig. S1B). To resolve some of these, a 2D HPLC approach was applied. NP HPLC–fractionated monosialylated structures (NeuGc₁Fuc₀₋₁Gal₁Man₁₋GlcNAc₃; m/z 1985/2131) were reinjected onto RP HPLC before or after α₃-sialidase S treatment, and isomers with different sialylation and sialidase sensitivity were identified (Fig. 7, A, B, E, and F). Only rather subtle differences in positive- and negative-mode MS/MS between the
monosialylated isomers could be observed, with the main diagnostic sialylated negative/positive B ions at 306/308 (NeuGc1) and 671/673 (NeuGc1Hex1HexNAc1) for NeuGc-modified antennae being shared (Fig. 7, K, M, and R). In case of a sialidase S–resistant isomer, 3-galactosidase treatment resulted in loss of one galactose residue and best revealed a diagnostic m/z 511 NeuGc1GlcNAc1 fragment (Fig. 7, G–J). Thus, the conclusion was that there were two positions for sialylation (“externally” on Gal or “internally” on GlcNAc), and sialidase S only removed the former but not the latter.

There were also related disialylated NeuGc,Fuc0/1Gal1Man5-GlcNAc3 glycans (m/z 2292 and 2438); the former was also rechromatographed before and after 3-sialidase S treatment. Removal of only the terminal NeuGc occurred and resulted in altered retention time (Fig. 7, C and D) and minimal changes in MS/MS fragmentation (Fig. 7, P and R). Negative-mode MS/MS of the m/z 2292 glycan and positive MS/MS of the core-fucosylated m/z 2438 structure resulted in detection of either an m/z 996 C fragment or an m/z 980 B fragment compatible with disialylation of Hex1HexNAc1 (Fig. 7, Q and S). Some sialylated glycans were also sulfated, and MS/MS (Fig. 7, N, O, and T) could show the presence of either a sulfo-sialyl–Lewis–containing motif (B ion at m/z 897, S1NeuGc1Fuc1Gal1GlcNAc1) or a sulfated NeuGc (B ion at m/z 386, S1NeuGc1).

**Complex-type N-glycans**

MS predicted a large number of complex-type N-glycans in *H. atra*, but the relatively low abundance of these structures “overloaded” with fucose and sulfate residues meant that their analysis was challenging. On RP HPLC, glycans such as...
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$S_{3-4}Fuc_{2-4}Hex_{5-6}HexNAc_{4-5}$ (m/z 2439–3052) were particularly concentrated in the fraction eluting at 14.5 min (Fig. 2B), whereas on NP HPLC, many eluted after 60 min (Fig. S1B). The RP HPLC fraction was analyzed by negative MS before and after digestion with $\beta$3/4-galactosidase and $\alpha$3/4-fucosidase, which resulted in no loss of the sulfated galactose residues but removal of up to four fucoses (Fig. 8, A–D). MS/MS spectra of such bi- and triantennary complex-type N-glycans (see Fig. 8, E–J, and Fig. S7 for examples) showed the presence of similar B ions (e.g. sulfo–Lewis A at m/z 590) as described above; however, possibly because of their low abundance, no multisulfated fragments were detected, as was the case for di- or trisulfated hybrid glycans, but positive-mode MS/MS facilitated definition of the core and antennal fucose residues. Unlike the hybrid structures, desulfation of multisulfated glycans was inefficient and led to unspecific hydrolysis; thus, an unambiguous definition of all galactose linkages ($\beta$3 or $\beta$4) is expected for a Lewis-type antenna regardless of sulfation. $H,N$: negative and positive MS/MS of these glycans, also compared with a core fucosylated isomer eluting at 7.8 g.u., showed a number of key diagnostic variations. Although the presence of a negative-mode B-fragment ion at m/z 241 (S, Hex, H, K, and M) is compatible with sulfation of galactose, the position of fucose on either the antenna or core can be deduced on the basis of the sulfated B ions at m/z 444/590 (S, Fuc, Gal, GlcNAc). The neutral losses of either $\beta$299 (GlcNAc-PA, $Fuc_{1}GlcNAc_{1}$, Fuc, Gal) or $\beta$445 (Fuc, GlcNAc-PA, $Fuc_{1}GlcNAc_{1}$) are indicative of the absence ($H$) or presence ($K$ and $M$) of core fucosylation. On the other hand, positive-mode MS/MS of these glycans and an HF digestion product (I, J, L, and N; m/z 1824, 1678, or 1970 resulting from in-source loss of sulfate) reveals the underlying backbones. Specific diagnostic $Y$ ions at m/z 300/446 (Fuc,$\beta$4GlcNAc,$\beta$-PA) and 1151/1297 (Fuc,$\beta$4Man,$\beta$-GlcNAc,$\beta$-PA) are characteristic of core and hybrid-type structures, whereas m/z 512 B ions are indicative of fucosylated antennae. Highlighted in green and purple are, respectively, fragments aiding definition of the antennae and the core. Asterisks indicate ions $[M-179]$ deriving from $\alpha$2A cross-ring cleavage of the core PA-labeled GlcNAc, as also observed previously in negative-mode MS/MS of sulfated N-glycans from lepidopteran species (13). Losses of Fuc ($F$), Hex ($H$), HexNAc ($N$), or HexNAc-PA (NPA) are indicated.

Figure 5. MALDI-TOF MS analysis of monosulfated hybrid-type N-glycans. A–G, $S_{n},Fuc_{1/2}Gal_{1}Man_{5}GlcNAc_{3}$ glycans (m/z 1902/2048 as [M−H]−, eluting at 4.7 and 6.8 g.u.; see Fig. 2B) were analyzed by negative MS before (A and F) and after enzymatic or chemical treatments (B–E and G). The hybrid nature and lack of sulfation of fucose were shown by sensitivity to jack bean $\alpha$-mannosidase (C, removing up to three mannoses) and removal of fucose by either $\alpha$3/4-fucosidase (D) or HF (E and G) treatments without loss of the negative charge, whereas resistance to $\beta$3/4-galactosidase (B) is expected for a Lewis-type antenna regardless of sulfation. $H,N$: negative and positive MS/MS of these glycans, also compared with a core fucosylated isomer eluting at 7.8 g.u., showed a number of key diagnostic variations. Although the presence of a negative-mode B-fragment ion at m/z 241 (S, Hex, H, K, and M) is compatible with sulfation of galactose, the position of fucose on either the antenna or core can be deduced on the basis of the sulfated B ions at m/z 444/590 (S, Fuc, Gal, GlcNAc). The neutral losses of either $\beta$299 (GlcNAc-PA, $Fuc_{1}GlcNAc_{1}$, Fuc, Gal) or $\beta$445 (Fuc, GlcNAc-PA, $Fuc_{1}GlcNAc_{1}$) are indicative of the absence ($H$) or presence ($K$ and $M$) of core fucosylation. On the other hand, positive-mode MS/MS of these glycans and an HF digestion product (I, J, L, and N; m/z 1824, 1678, or 1970 resulting from in-source loss of sulfate) reveals the underlying backbones. Specific diagnostic $Y$ ions at m/z 300/446 (Fuc,$\beta$4GlcNAc,$\beta$-PA) and 1151/1297 (Fuc,$\beta$4Man,$\beta$-GlcNAc,$\beta$-PA) are characteristic of core and hybrid-type structures, whereas m/z 512 B ions are indicative of fucosylated antennae. Highlighted in green and purple are, respectively, fragments aiding definition of the antennae and the core. Asterisks indicate ions $[M-179]$ deriving from $\alpha$2A cross-ring cleavage of the core PA-labeled GlcNAc, as also observed previously in negative-mode MS/MS of sulfated N-glycans from lepidopteran species (13). Losses of Fuc ($F$), Hex ($H$), HexNAc ($N$), or HexNAc-PA (NPA) are indicated.

Core $\alpha$3-linked N-glycans

The glycopeptides remaining after PNGase-F digestion were treated with PNGase-A to identify possible core $\alpha$3-fucosylated N-glycans. This residual pool was also separated in neutral and
anionic subpools prior to labeling and injection onto RP HPLC. Although some of the masses in the fractions were the same as those identified previously in the PNGase F digest, two HPLC fractions contained hybrid or complex N-glycans displaying the presence of an additional fucose (i.e. S Fuc, Gal, Man, GlcNAc, at m/z 2194 and S Fuc, Gal, Man, at m/z 2893; Fig. 9, A and C). While HF treatment resulted in loss of all fucoses except the core α6-linked one (Fig. 9, B and D), negative-mode MS/MS of the hybrid structure (m/z 2194) showed neutral losses of the difucosylated core as well as the B ions, showing occurrence of a sulfogalactosyl Lewis motif (Fig. 9E). On the other hand, positive MS/MS of the corresponding [M - SO₃]⁺ pseudomolecular ion (m/z 2116) yielded a core Y ion at m/z 592 (Fuc, GlcNAc, PA), which is a further proof of difucosylation of the innermost core GlcNAc (Fig. 9F).

Discussion

The N-glycome of H. atra, the first to be described of any sea cucumber, is characterized by 74% of neutral structures (mainly oligomannosidic-type N-glycans) and 26% of anionic structures (1% phosphorylated, 24% sulfated, and 1% sialylated), as judged by RP HPLC fluorescence and MS intensities (Fig. 10 and Table S2). The relatively high amount of sulfated hybrid and complex-type N-glycans were enriched in the anionic pool, whereas isoforms with different positions of the fucose (core or antennal), sulfate (four different positions; i.e. either on Gal, GlcNAc, Man, or NeuGc) or sialic acid residues (on Gal or on GlcNAc) could be resolved by NP or RP HPLC (Fig. 5A and 5B). The enrichment and separation as well as addition of Na⁺ to enhance sulfate detection by MS proved to be crucial for the in-depth sulfo- and sialoglycomic investigation, as isolation, enrichment, and detection of anionic glycans remains a challenging task for which specific protocols involving either fluorescent labeling (14–17) or permethylation (18) have been employed previously.

Overall, our data suggest that at least four sulfates can modify the N-glycans of H. atra, and indeed, most LacNAc-like antennae are not just sulfated but are most commonly fucosylated; sulfated forms of β3-galactose, β-GlcNAc, α3-mannose, and α3-sialic acid residues could be proven by MS/MS. Sulfation of galactose is similar to that in the oyster (19), but the relative dominance of sulfation of α3-mannose is in contrast to insects, in which sulfation of α6-mannose or core fucose is more common (13). Unlike the highly sulfated keratan-like N-glycans of unfertilized eggs of a fish, Tribolodon hakonensis (20), with repetitive sulfated neo-LacNAc motifs, no obvious repeating

![Figure 6. MALDI-TOF MS analysis of disulfated hybrid N-glycans.](Figure 6. MALDI-TOF MS analysis of disulfated hybrid N-glycans. A–D and F–I, disulfated hybrid-type S Fuc, Gal, Man, GlcNAc, glycans present in different normal phase fractions (Fig. S1B) were analyzed by negative MS (A–D), exhibiting some in-source loss of sulfate (indicated by Δ102 and asterisks), and MS/MS (F–I). Monosulfated (M−H⁻) and disulfated (M−2H⁺+Na⁺ or K⁺) B-fragment ions were observed at m/z 241, 282, or 444 (S, Gal, GlcNAc, ), 546/562 (S, Gal, GlcNAc, ), 708/724 (S, Gal, GlcNAc, Man, ) and 854/870 (S, Fuc, Gal, GlcNAc, Man, ) in addition to Y ions at m/z 1375/1537 (S, Fuc, Man, GlcNAc, PA) aid definition of sulfation of Gal, GlcNAc, or Man. The fragmentation patterns of the two m/z 2150 isomers (H and J) indicated either sulfation on the terminal Gal and lower α3-Man or two sulfate residues on a Lewis motif (blue arrows). E and J, HF treatment of the latter only removed the antennal α4-fucose (whereby the m/z 546 fragment corresponds to a disulfated hexaHexNAc), as the 80-Da modification was not lost, it was neither linked to the Lewis-type fucose nor was it an isobaric phosphate. The distinct elution of the disulfated isomers is the result of the spatial arrangement of sulfate residues causing weaker or stronger binding to the HIAX column. Losses of Fuc (F), hexose (H), or reducing terminal Fuc, GlcNAc, PA (–NPA or –FNPA) are indicated.)
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units were detected in this study. Although the function of glycans in echinoderms is unclear, sulfation is implicated as a critical determinant mediating a diverse range of biological recognition functions on N- and O-glycans (21).

Other hybrid and complex N-glycans in *H. atra* are sialylated, and some structures are even carrying antennal sialic acid in combination with sulfate and/or fucose modifications. Interestingly, like mammalian fetuin (22), sialylation occurs on either antennal galactose or antennal GlcNAc residues; there may, of course, be biosynthetic competition with sulfation for these positions. In the proven β3-galactosylated/α4-fucosylated structures in *H. atra*, the sialyl–Lewis A element corresponds to the human CA19-9 epitope with roles in cancer (23). Such motifs have a potential role in cell–cell interactions; in the case of echinoderms, it is conceivable that a sialylated glycan could be important for regeneration. Compared with the brittle star described in the accompanying study (24), sialylation is less common in *H. atra* (Fig. 10). Nevertheless, the ability of this species to sialylate N-glycans on two different residues (α2,3 on Gal or α2,6 on GlcNAc) correlates with expansion of the sialyltransferase gene family in echinoderms (25). Compared with the evolutionarily more primitive protostome phyla (Fig. 10), nematodes have no sialylation capacity at all, whereas most insect species have single homologs of α2,6-sialyltransferase and CMP-NeuAc synthase (26, 27); only for *Drosophila* are there M5 data indicative of sialylation of N-glycans in a nonevolved insect system (28). However, to date, glucuronic acid and sulfate have been proven to be the most recurrent anionic modifications of invertebrate N-glycans (29).

All sialylated N-glycans proposed for *H. atra* contain NeuGc rather than NeuAc, even though both have been reported previously on glycolipids from other sea cucumbers (4, 5, 30). Certainly, the CMP-NeuAc hydroxylase required for NeuGc transfer is known in echinoderms (31), and NeuGc also occurs in many higher deuterostomes, including cephalochordates, fish, and mice (32–34), but not humans (35). However, unlike the brittle star, there is an apparent lack of methylated NeuGc on *H. atra* N-glycans.

A rather unusual anionic feature detected in *H. atra* is phosphorylation of three oligomannosidic-type N-glycans with a tri-glycosylated A arm (P<sub>2</sub>GlcNAc<sub>3</sub>Gal<sub>3</sub>GalNAc<sub>3</sub>) carrying the phosphate on the terminal glucose; such an N-glycan modification has not been reported previously, in contrast to the “famous” mannos-6-phosphate involved in intracellular trafficking via the cognate receptor for lysosomal enzymes (36). The terminal localization of glucose-6-phosphate could have an important role in glycoprotein folding regulation during calnexin/calreticulin cycles in the endoplasmic reticulum (37). This phosphorylation position contrasts with the presence of phosphate on antennal GlcNAc residues of the brittle star, as described in the accompanying study (24).

The fucosylation level in *H. atra* is very high in the hybrid and complex sub-N-glycans, with many of the sulfated and/or sialylated glycans displaying antennal fucosylation; as the galactose residue on the hybrid glycans is clearly β3-linked, this means that the fucose residue on such antennae is α4-linked, a feature found on complex plant N-glycans as well as some human glycans (38, 39). Furthermore, a small minority of glycans are core α3-fucosylated, a feature known to be common in nematodes, insects, and plants (40), whereas core α6 fucosylation in *H. atra* is frequent. Thus, there must be at least three fucosyltransferases capable of modifying N-glycans in this echinoderm species.

Another obvious difference to the brittle star is the relative dominance of hybrid structures compared with complex forms in the sea cucumber. Also, the maximal number of branches appears to be three in *H. atra* rather than four. This would suggest low processing by Golgi α-mannosidase II but also the presence of GlcNAc transferases I, II, and IV in the sea cucumber; some of the hybrid glycans actually display processing by both GlcNAc transferase I and IV, which results in disubstitution of the α3-mannose (Fig. S5), as observed also in insects or birds, for example (41, 42). The high abundance of the same hybrid β3-galactosylated “backbone” in *H. atra*, regardless of whether the N-glycans are sialylated or sulfated, suggests that these classes of structures are biosynthetically related and not random contaminants from the diet.

In conclusion, the N-glycome of *H. atra* contrasts with that of the brittle star, but galactosylation, sialylation, and sulfation of the antennae are common features. The presence of fucose, sulfate, and sialic acid has also been reported in other glycoconjugates of various sea cucumbers, such as glycolipids with a fucose-modified trisialylated glucosyl ceramide, chondroitin sulfate with sulfate-modified difucose branches, and triterpene glycosides, which can also be sulfated (4–7). The glycome of *H. atra* may reflect a high expression level of sulfo- and fucosyltransferases as well as their associated metabolites; thus, if genetic manipulation becomes possible, then it could prove to be a good model to study the regulation, mechanisms, and functions of fucosylation and sulfation. From an evolutionary per-
The occurrence of β3-galactosylation and core α3-fucosylation on one hand but of sialylation or antennal α4-fucosylation on the other shows that this echinoderm species does present both invertebrate- and vertebrate-like features in its glycome.

Experimental procedures

Enzymatic release of N-glycans

3 g (wet weight) black sea cucumber (*H. atra* adult form) shredded into 2- to 4-mm cubes were suspended in boiling water and denatured for 5 min prior to addition of 0.1 M ammonium bicarbonate (pH 8.0), 20 mM CaCl₂, and 3 mg of thermolysin in a final volume of 15 ml. Proteolysis was allowed to proceed for 2 h at 70 °C, and then the sample was centrifuged to remove residual insoluble material. The resulting glycopeptides were enriched by cation-exchange chromatography (Dowex AG50, Bio-Rad) and gel filtration (Sephadex G₂₅, GE Healthcare), yielding 30 mg of purified glycopeptides. N-glycans were released using peptide:N-glycosidase F (PNGase-F, Roche) in 100 mM ammonium carbonate (pH 8), overnight at 37 °C; the remaining glycopeptides were then digested using peptide:N-glycosidase A (PNGase-A, Roche) in 50 mM ammonium acetate (pH 5) overnight at 37 °C. PNGase-F- and PNGase-A–released N-glycan fractions were further purified by a second round of
cation-exchange chromatography (Dowex, Bio-Rad) and separated by a nonporous graphitized carbon column (Supelco) using 40% acetonitrile to elute the neutral glycans, followed by 40% acetonitrile with 0.1% TFA to elute the anionic glycans (43). All N-glycan fractions were then pyridylaminated as described previously (44). Compared with the RP HPLC fluorescent signal of 10 pmol of a purified PA-labeled N-glycan from a commercial source (30 mV at the detector gain used), the yield of total labeled N-glycans was 7 nmol for the neutral pool and 3 nmol for the acidic pool. Sea cucumbers feed on planktonic algae, amoebae, and small animals; as no pentose-containing glycans were detected, we conclude that no algae were coanalyzed.

**MALDI-TOF MS analysis**

The pyridylaminated N-glycans were fractionated by RP or NP HPLC columns and profiled by MALDI-TOF MS (Autoflex Speed, Bruker Daltonics) in positive- and negative-ion modes using FlexControl 3.4 software. All HPLC peaks were collected, freeze dried, redissolved in 10 \( \mu L \) and examined by MALDI-TOF MS, using 6-aza-2-thiothymine as matrix (45). Sample and matrix solutions (1 \( \mu L \) each) were sequentially spotted and dried under a vacuum. To enhance formation of \([M+H]^+\) or \([M-H_n+Na_m]^+\) ions, either 1 \( \mu L \) of 20 mm ammonium sulfate or 1 \( \mu L \) of 10 mm sodium acetate was spotted on top of the matrix. MS/MS to confirm the composition of all proposed structures was performed by laser-induced dissociation (the precursor ion selector was generally set to ±0.6%). The detector voltage was generally set at 1977 V for MS and 2133 V for MS/MS; 500–1000 shots from different regions of the sample spots were summed. Spectra were processed with the manufacturer’s software (Bruker FlexAnalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS/MS (four times smoothed). All MS and MS/MS spectra were manually interpreted on the basis of the mass fragmentation pattern and results of chemical and enzymatic treatments; isomeric structures present in different RP HPLC or NP HPLC fractions were defined on the basis of comparisons of the aforementioned parameters. At least four MS/MS fragment ions were used to aid definition of each of the structures, which are depicted according to the Symbol Nomenclature for Glycans (46). For further details, refer to the supporting information.

**HPLC purification of N-glycans**

Separation of PA-labeled glycans was carried out on a Shimadzu HPLC system equipped with a fluorescence detector (RF-20AXS) using a Kinetic 5-\( \mu m \) RP column (XB-C18 100A, 250 \( \times \) 4.6 mm, Phenomenex, Torrance, CA) with a gradient of methanol in 0.1 m ammonium acetate (pH 4) up to 16.5% over 44 min applied at a flow rate of 0.8 ml/min as follows: 0–30 min, 0%–9% methanol; 30–35 min, 9%–12% methanol; 35–40 min, 12%–16.5% methanol; 40–44 min, 16.5% methanol; and 44–50 min, return to 0% methanol. For separation based on size and...
charge, a HIAx IonPac AS11 NP column (Dionex) was used with 800 mM ammonium acetate (pH 3.85) (buffer A) and 80% (v/v) acetonitrile (buffer B). The following gradient was applied at a flow rate of 1 ml/min: 0–5 min 99% B, 5–50 min 90% B, 50–65 min 80% B, and 65–85 min 75% B. PA-labeled glycans were detected by fluorescence with excitation/emission wavelengths of 320/400 nm. The RP HPLC column was calibrated daily in terms of glucose units using a pyridylaminated dextran hydrolysate, whereas the NP HPLC column was calibrated daily using a mixture of pyridylaminated N-glycans (Man3–9GlcNAc2) derived from white beans; the order of elution of the standards was confirmed by MALDI-TOF MS of collected calibrant fractions (43).

Structural elucidation using exoglycosidases and chemical treatment

The following glycosidases were employed: recombinant Aspergillus niger β3/4-galactosidase (prepared in-house (47)); Xanthomonas manihotis β3-galactosidase (New England Biolabs); Bacillus fragilis β4-galactosidase (New England Biolabs); bovine kidney α-fucosidase (Sigma-Aldrich); almond α3/4-fucosidase (New England Biolabs); jack bean α-mannosidase (Sigma-Aldrich); purified recombinant Bacteroides xylanisolvens BxGH99 α2-endo-mannosidase, which catalyzes removal of a disaccharide from Glc3Man3GlcNAc2 but not from unglycosylated Man3GlcNAc2 (48); recombinant Aspergillus saitoi α2-mannosidase (Prozyme); and Streptococcus pneumoniae α3-sialidase S (New England Biolabs). In general, 10% of an HPLC fraction (1 l) was incubated overnight at 37 °C with 0.8 l of 100 mM ammonium acetate (pH 5.0) and 0.2 μl of a glycosidase (see above). For removal of phosphate- or α3/4-linked fucose, 30% of an HPLC fraction (3 l) was dried under a vacuum and incubated overnight on ice with 20 μl of 0.05 M methanol-HCl (methanolysis) prior to drying again. For removal of sulfate, 30% of an HPLC fraction (3 μl) was dried under a vacuum and incubated for 4 h at 37 °C with 20 μl of 0.05 M methanol-HCl (methanolysis) prior to drying again. Enzymatically or chemically treated N-glycans were generally reanalyzed by MALDI-TOF MS and MS/MS without further purification unless rechromatographed by RP HPLC (see the relevant figure legends). The β4-galactosylated Hex3HexNAc3 standard was generated by treatment of a Man5GlcNAc2 structure with bovine milk galactosyltransferase (Fluka) in the presence of UDP-Gal and Mn(II) ions (49).

Figure 10. Semiquantitative analysis of the H. atra N-glycome and comparisons within the Deuterostoma. The signal intensities of HPLC and MALDI-TOF peaks containing characterized N-glycans were used to estimate the ratio of each individual class and subclass of N-glycans to provide an overview of their relative abundance. Particular proven epitopes include variable antennal α4 fucosylation, β3 galactosylation, 4-linked sulfation of galactose, α3 sialylation of galactose, α6 sialylation of GlcNAc, sulfation of GlcNAc (putatively 6-linked if otherwise not sialylated), and sulfation of mannose; phosphorylation of triglycosylated glycans and core difucosylation of hybrid/complex glycans were also detected (the latter accounting for some 0.3% of the total N-glycome of H. atra). For a full list of predicted compositions, refer to Table S2. The simplified evolutionary tree (left panel, based on Vaughn et al. (53)) exhibits the division between protostomes and deuterostomes in the Animalia (500 million years ago) as well as example resulting species. The depiction of the Deuterostoma (center panel) shows the phyla of the Echinodermata and Chordata. An overall comparison of N-glycomic features (right panel) of H. atra (sea cucumber, this study), Ophiactis savignyi (brittle star, accompanying study (24)), and Vertebrata (e.g. human and bovine) shows selected similarities (e.g. disialylated motif) and differences (e.g. variation in fucosylation, sulfation, and sialylation levels).
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