**Hemocytes and Plasma of the Eastern Oyster (Crassostrea virginica) Display a Diverse Repertoire of Sulfated and Blood Group A-modified N-Glycans**

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**Background:** The eastern oyster, an important estuarine species, is parasitized by a protozoan in a galectin-dependent manner.

**Results:** A variety of paucimannosidic, hybrid, and complex neutral and acidic N-linked oligosaccharides was found.

**Conclusion:** The oyster possesses a complex repertoire of glycans with some features reminiscent of vertebrates.

**Significance:** The N-glycome of the eastern oyster correlates well with the specificity of its own galectin CvGal1.

The eastern oyster (Crassostrea virginica) has become a useful model system for glycan-dependent host-parasite interactions due to the hijacking of the oyster galectin CvGal1 for host entry by the protozoan parasite Perkinsus marinus, the causative agent of Dermo disease. In this study, we examined the N-glycans of both the hemocytes, which via CvGal1 are the target of the parasite, and the plasma of the oyster. In combination with HPLC fractionation, exoglycosidase digestion, and fragmentation of the glycans, mass spectrometry revealed that the major N-glycans of plasma are simple hybrid structures, sometimes methylated and core α1,6-fucosylated, with terminal β1,3-linked galactose; a remarkable high degree of sulfation of such glycans was observed. Hemocytes express a larger range of glycans, including core-fucosylated paucimannosidic forms, whereas bi- and triantennary glycans were found in both sources, including structures carrying sulfated and methylated variants of the histo-blood group A epitope. The primary features of the oyster whole hemocyte N-glycome were also found in dominin, the major plasma glycoprotein, which had also been identified as a CvGal1 glycoprotein ligand associated with hemocytes. The occurrence of terminal blood group moieties on oyster dominin and on hemocyte surfaces can account in part for their affinity for the endogenous CvGal1.

Host-pathogen interactions in higher organisms are highly complex due to the diverse receptors and ligands involved as products of co-evolutionary processes, the still unfa...
or F (Roche Applied Science). The released N-glycans, which did not bind a second Dowex 50 column, were pyridylaminated overnight prior to gel filtration (Sephadex G-15) and MALDI-TOF MS; residual glycopeptides bound to Dowex 50 were subject to gel filtration prior to reductive β-elimination and LC-MS. Released O-glycans were cleaned up as described previously (14).

MALDI-TOF MS of Glycans—Monoisotopic MALDI-TOF MS was performed using either Bruker Ultraflex TOF-TOF (equipped with a 50 Hz nitrogen laser) or Autoflex Speed (equipped with a 1000 Hz SmartbeamTM-II laser) instruments in either positive or negative reflectron mode with 6-aza-2-thiophenamine as matrix. MS/MS was performed by laser-induced dissociation. 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). Glycan spectra were manually interpreted on the basis of the masses of the predicted component monosaccharides, differences of mass in glycan series, fragmentation pattern, and exoglycosidase digestions. Calculated theoretical masses were verified using GlycoWorkbench 2.0.

LC-MS of Glycans—PA-labeled N-glycans were also analyzed by LC-MS and LC-MS” using a 10 cm × 250 μm inner diameter column, prepared in-house, containing 5-μm porous graphitized carbon particles (Thermo Scientific, Waltham, MA). N-Glycans were eluted using a linear gradient from 0 to 40% acetonitrile in 10 mM ammonium bicarbonate over 40 min at a flow rate of 10 μl/min. The eluted N-glycans were detected using an LTQ XL ion trap mass spectrometer (Thermo Scientific, San Jose, CA) in negative ion mode with an electrospray voltage of 3.5 kV, capillary voltage of −33.0 V, and capillary temperature of 300 °C. Air was use as a sheath gas, and mass ranges were defined dependent on the specific structure to be analyzed. Specified ions were isolated for MS” fragmentation by collision-induced dissociation with the collision energy set to 30%. The data were processed using Xcalibur software (version 2.0.7, Thermo Scientific).

Glycan Fractionation and Exoglycosidase Digestion—Complete pyridylaminated N-glycomes were fractionated, as described previously (15, 16), by either reversed-phase HPLC (Hypersil ODS from Thermo Scientific or Ascentis Express RP-amide from Sigma; gradient of 0.3% methanol per min in 100 mM ammonium acetate, pH 4), normal-phase HPLC ( Tosoh TSKgel Amide-80; inverse gradient of acetonitrile in 10 mM ammonium formate, pH 7), or combined hydrophobic interaction anion-exchange HPLC (HIAx, Dionex IonPac AS11; inverse gradient of acetonitrile in 800 mM ammonium acetate, pH 3). Glycans were detected by fluorescence with excitation/ emission wavelengths of 320/400 or 310/380 nm. NP- and RP-HPLC columns were calibrated daily in terms of glucose units using a pyridylaminated dextran hydrolysate, whereas the HIAx column was calibrated with a set of oligomannosidic glycans isolated from beans. All fractions were analyzed by MALDI-TOF MS and MS/MS in positive and negative modes.

Further analysis by MALDI-TOF MS was performed after treatment overnight with either β-galactosidases (Aspergillus or bovine testes from Sigma, recombinant β1,3-specific from...
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New England Biolabs or recombinant β1,4-specific from Prozyme), α-fucosidases (bovine kidney from Sigma, *Xanthomonas* α1,2-specific from New England Biolabs or almond α1,3-specific from Prozyme), α-mannosidases (jack bean from Sigma, α1,2/3-specific from New England Biolabs or α1,6-specific from New England Biolabs), chicken liver α-N-acetylgalactosaminidase (Sigma or Prozyme), or jack bean β-N-acetylhexasaminidase (Sigma) in 50 mM ammonium acetate, pH 5, at 37 °C.

**Purification of Galectin-binding Proteins**—Hemocyte lysates were purified on a CvGal1 column (CvGal1 cross-linked to Affigel 15, Bio-Rad) as described previously (12). Bound proteins were eluted in phosphate-buffered saline containing 50 mM lactose with or without 1% Triton X-100 (for Triton and aqueous extracts respectively) prior to lyophilization.

**Intact Protein Mass Spectrometry and Top-down Sequencing**—The molecular mass of the major protein in plasma and in the galectin-binding fractions was estimated by MALDI-TOF MS on an Autoflex Speed mass spectrometer using 2,5-dihydroxyacetophenone as matrix with a Savitzky-Golay algorithm. Top-down sequencing was performed using the Protein Calibration Standard 1 (Bruker Daltonics), and the data were processed using FlexAnalysis 3.3 (peak detection with a Centroid algorithm, peak width 1000 m/z; one cycle of smoothing (20 m/z) with a Savitzky-Golay algorithm). The molecular mass of the major protein in plasma and in the galectin-binding fractions was estimated by MALDI-TOF MS on an Autoflex Speed mass spectrometer using 2,5-dihydroxyacetophenone as matrix (17) and 500 ng of sample; calibration was performed using the Protein Calibration Standard 1, m/z; one cycle of smoothing (20 m/z) with a Savitzky-Golay algorithm). The results were subject to BLASTp searching using the NCBI server.

**Glycoproteomic Analysis of Dominin**—After SDS-PAGE on 12% gels, Coomassie-stained bands of the major galectin-binding protein of hemocytes and of the major plasma protein were excised, washed, reduced, and carboxymethylated prior to overnight incubation with pepsin. After extraction of the peptides from the gel pieces using acetonitrile/water/TFA, aliquots of each digest were dried, dissolved in water, and heat-inactivated; ammonium bicarbonate buffer and PNGase F were added and incubated overnight. The released glycans were purified first on Dowex 50, eluted with 2% acetic acid, and lyophilized prior to passage through a mini-column consisting of LiChroprep C18 and Supelclean ENV1-Carb (19); the glycans were eluted using 40% acetonitrile followed by 40% acetonitrile containing 0.1% trifluoroacetic acid prior to small scale derivatization with 2-aminopyridine also as described previously (19). For tryptic mapping by LC-MS, a Novex 4-12 BisTris gel (NuPAGE, Invitrogen) was used. Coomassie Blue-stained bands were also excised, reduced, and carboxymethylated before trypsin digestion. The resultant peptides were subjected to nano-LC-electrospray ionization MS/MS analysis with an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Peptide MS/MS spectra were searched against NCBI nonredundant oyster database using Mascot software (version 2.2.04, Matrix Science Inc.). Only peptides with a mass deviation lower than 10 ppm were accepted, and the MS/MS of two peptides were manually inspected for protein identification.

**RESULTS**

**Glycomic Screening of Oyster Hemocytes and Plasma**—As an initial test of the glycomic potential of the eastern oyster, *C. virginica*, blotting with selected lectins and anti-carbohydrate antibodies was performed using hemocyte and plasma proteins of the oyster. Positive reactions were observed (Fig. 1) for both samples with the fucose-specific lectin from *Lens culinaris* agglutinin (LCA), known to react with core α1,3-fucose and β1,2-xyllose residues present in plants and various invertebrates, was only observed with hemocytes. Finally, an anti-blood group A (anti-BGA) monoclonal antibody reacted in a manner sensitive to chicken α-N-acetylgalactosaminidase (Fig. 1). In blots with plasma, the binding pattern was dominated by a major band of around 30 kDa, compatible with the presence of a single major protein species in this sample as judged by Coomassie Blue staining. Indeed, a protein with approximately this apparent molecular mass has been previously reported and named “dominin” (20).

**Glycomic Screening of Hemocytes and Plasma**—N-Glycans from oyster plasma were released using either PNGase A or PNGase F, whereas N-glycans from oyster hemocytes were released using PNGase A alone as only limited material was available. The resulting N-glycomic pools were examined by MALDI-TOF MS before and after fluorescent labeling using 2-aminopyridine. Both pools of glycans from plasma are qualitatively similar, although some differences in relative peak heights are apparent. However, at first sight, the hemocyte glycan spectrum is more complicated than those of plasma N-glycans, especially in the lower mass range (see also Tables 1 and 2); this is not unexpected as hemocytes represent cellular material, whereas there is only one major protein in oyster plasma.

The positive mode spectra of free plasma glycans were dominated by ions of m/z 1312, 1414, 1458, and 1675 (Fig. 2A); upon
pyridylation, the major structures are shifted by 56 Da as compared with the free glycans (Fig. 2B). MALDI-TOF MS of the pyridylaminated N-glycans from hemocytes yielded a positive mode spectrum dominated by species of m/z 989, 1149, 1295, 1368, and 1514 and some putatively methylated variants (Fig. 2C). Example Δm/z values within the spectra include 14, 46, and 102, which would respectively indicate the presence of methyl, fucose, and sodiated sulfate or phosphate residues. In the negative mode, quasimolecular ions of 1446, 1592, and 2160 and putatively methylated variants thereof were the most common species in spectra of both hemocyte and plasma N-glycans (Fig. 2D); these values are 78 mass units higher than the major

### TABLE 1

| Structure | Predicted Structures for Neutral N-Glycans Common to Both Oyster Plasma and Hemocytes |
|-----------|-------------------------------------------------------------------------------------|
| 987.41    | ![Structure](image1)                                                                   |
| 1003.41   | ![Structure](image2)                                                                 |
| 1017.43   | ![Structure](image3)                                                                 |
| 1135.45   | ![Structure](image4)                                                                 |
| 1149.47   | ![Structure](image5)                                                                 |
| 1163.48   | ![Structure](image6)                                                                 |
| 1206.49   | ![Structure](image7)                                                                 |
| 1352.55   | ![Structure](image8)                                                                 |
| 1354.53   | ![Structure](image9)                                                                 |
| 1355.55   | ![Structure](image10)                                                                |
| 1368.54   | ![Structure](image11)                                                                |
| 1382.56   | ![Structure](image12)                                                                |
| 1500.58   | ![Structure](image13)                                                                |
| 1514.60   | ![Structure](image14)                                                                |
| 1528.62   | ![Structure](image15)                                                                |
| 1530.59   | ![Structure](image16)                                                                |
| 1557.61   | ![Structure](image17)                                                                |
| 1662.64   | ![Structure](image18)                                                                |
| 1676.67   | ![Structure](image19)                                                                |
| 1690.67   | ![Structure](image20)                                                                |
| 1706.66   | ![Structure](image21)                                                                |
| 1717.68   | ![Structure](image22)                                                                |
| 1719.66   | ![Structure](image23)                                                                |
| 1731.69   | ![Structure](image24)                                                                |
| 1733.67   | ![Structure](image25)                                                                |
| 1745.71   | ![Structure](image26)                                                                |
| 1824.70   | ![Structure](image27)                                                                |
| 1838.71   | ![Structure](image28)                                                                |
| 1849.72   | ![Structure](image29)                                                                |
| 1852.72   | ![Structure](image30)                                                                |
| 1863.74   | ![Structure](image31)                                                                |
| 1865.72   | ![Structure](image32)                                                                |
| 1877.75   | ![Structure](image33)                                                                |
| 1879.73   | ![Structure](image34)                                                                |
| 1891.77   | ![Structure](image35)                                                                |
| 1893.75   | ![Structure](image36)                                                                |
| 2011.77   | ![Structure](image37)                                                                |
| 2068.80   | ![Structure](image38)                                                                |
| 2082.81   | ![Structure](image39)                                                                |
| 2096.83   | ![Structure](image40)                                                                |
| 2110.84   | ![Structure](image41)                                                                |
| 2124.85   | ![Structure](image42)                                                                |
| 2228.87   | ![Structure](image43)                                                                |
| 2242.88   | ![Structure](image44)                                                                |
| 2431.95   | ![Structure](image45)                                                                |
| 2445.96   | ![Structure](image46)                                                                |
| 2447.94   | ![Structure](image47)                                                                |
| 2453.98   | ![Structure](image48)                                                                |
| 2473.99   | ![Structure](image49)                                                                |
| 2578.01   | ![Structure](image50)                                                                |
| 2592.02   | ![Structure](image51)                                                                |
| 2606.04   | ![Structure](image52)                                                                |
| 2811.10   | ![Structure](image53)                                                                |
| 2957.15   | ![Structure](image54)                                                                |
predicted presence of either phosphate or sulfate. The species detected in positive mode and so also indicate the theoretical presence of either phosphate or sulfate.

HPLC Fractionation of Oyster Glycans—Prior to subsequent analysis, the PA-labeled glycans were fractionated by a variety of methods. Hemocyte glycans were fractionated by NP-HPLC and plasma glycans by either NP-HPLC and RP-HPLC on classical reversed-phase or RP-amide columns as well as by HIAX chromatography. In all cases, fractions were collected manually and subject to MALDI-TOF MS and MS/MS. In the case of NP-HPLC (Tosoh Amide 80) of hemocyte N-glycans, some 30 fractions eluting between 2 and 10 g.u. were collected; the chromatograms of plasma glycans were, in keeping with the glycomic MS data, simpler and consisted of two groups of peaks respectively centered around 2–4.5 and 5–8 g.u. (Fig. 3). By use of positive and negative mode MALDI-TOF MS, the first group of peaks was concluded to tend to display a modification of 80 Da as compared with the second group. Therefore, it appears that NP-HPLC separates by both charge and mass with an earlier elution time for the anionic glycans as compared with that expected for their size; methylation was associated also with a shift to lower elution time as compared with the “parent” glycan.

Complementary information and different types of elution resulted when using both types of RP-HPLC columns as well as HIAX chromatography of the plasma glycans. HIAX, like NP-HPLC, separates basically by both mass and charge (21); however, MALDI-TOF MS of the some 20 fractions revealed that mono- and di-anionic species elute later than their neutral parent structures (supplemental Fig. 1A). The amide-capped RP-column (Ascentis Express RP-amide) had, however, the highest resolution of all columns used, and some 30 fractions were collected. In particular, the larger fucosylated and methylated glycans eluted late on this column, whereas anionic glycans were shifted to earlier elution times as compared with the parent glycans (supplemental Fig. 1B). Using a classical RP-HPLC column (Hypersil ODS), approximately 20 fractions were collected; glycans that ionized well in negative mode eluted earlier than their parents, whereas many fucosylated species eluted late (supplemental Fig. 1C), which is a potential indication of core α,1,6-fucosylation as compared with the literature (22).

These different chromatographic approaches enabled us to often separate the same glycans in different mixtures of varying relative intensities or to isolate different isomers as well as structures of low abundance, and by combining the original “whole glycome” spectra and the spectra of individual fractions, we could detect glycans as large as m/z 2900 (sodiated free) and 2956 (protonated pyridylaminated). Further analyses, including MS/MS and exoglycosidase treatments, were then performed on individual fractions.

Oligomannosidic N-Glycans—Particularly the spectra of hemocyte glycans indicated the presence of putative oligomannosidic species (Hex⁴HexNAc₂); due to their simple composition, they were not studied further. However, reminiscent of other molluscs as well as planaria, some of these glycans as isolated from hemocytes putatively carried methyl groups (Hex₅HexNAc₂Me₃, Hex₆HexNAc₂Me₁–2, and Hex₇HexNAc₂Me₁). It is assumed that the methyl groups are terminal as indicated by neutral loss of 176 Da and resistance of these structures to jack bean α-mannosidase. An MS/MS spectrum of an example structure is shown in supplemental Fig. 2A.

Paucimannosidic N-Glycans—A feature of many plant and invertebrate glycomes is the presence of paucimannosidic glycans (23), sometimes core-fucosylated, based on Man₁–5GlcNAc₂ and lacking N-acetyllactosaminyl antennae. These glycans were most obvious in the hemocyte glycome and display m/z values of between 811 and 1325 for the pyridylaminated forms with compositions, as judged by MS/MS varying from m/z 446 (a single core fucoside; Fig. 4, A and B) and 592 (core difucoside; Fig. 4C). Difucosylation of the core was solely detected on hemocyte glycans and was assumed to indicate α,1,3- and α,1,6-fucosylation of the same reducing terminal GlcNAc residue as found in many invertebrates; this would account for the anti-HRP reactivity of only the hemocyte sample. Enzymatic treatments verified the structural assumptions. For instance, a difucosylated glycan of m/z 1309 (Hex₃HexNAc₂Fuc₂Me₂) was sensitive to

### Table 2

**Predicted structures for neutral N-glycans unique to oyster hemocytes**

| m/z | Structure |
|-----|-----------|
| 957.40 | ![Structure](image1.png) |
| 989.39 | ![Structure](image2.png) |
| 1003.41 | ![Structure](image3.png) |
| 1119.46 | ![Structure](image4.png) |
| 1133.47 | ![Structure](image5.png) |
| 1149.47 | ![Structure](image6.png) |
| 1151.45 | ![Structure](image7.png) |
| 1165.46 | ![Structure](image8.png) |
| 1179.48 | ![Structure](image9.png) |
| 1281.51 | ![Structure](image10.png) |

Structures of N-glycans found in hemocytes, but not in plasma, are depicted according to the nomenclature of the Consortium for Functional Glycomics; the given m/z values are theoretical values for pyridylaminated glycans in their protonated form.

![Supplemental Figure 1](image11.png)
bovine α-fucosidase, a treatment accompanied by the loss of the \( m/z \) 592 fragment (Fig. 4, C and D).

A particularly intriguing fragment was one of \( m/z \) 462 that would suggest hexosylation of the core GlcNAc (Fig. 4E). Glycans of this type had compositions of Hex\(_3–4\)HexNAc\(_2\)Me\(_0–2\); sometimes this fragment was also observed in combination with core fucosylation giving rise to a fragment of \( m/z \) 608 in glycans of Hex\(_3–4\)HexNAc\(_2\)Fuc\(_1\)Me\(_0–2\). These fucosylated versions (e.g. Hex\(_4\)HexNAc\(_3\)Fuc\(_1\)Me\(_2\); \( m/z \) 1325, see supplemental Fig. 3A) were also sensitive to bovine kidney α-fucosidase, resulting in loss of the \( m/z \) 462 and 608 fragments in the digested product, whereas the \( m/z \) 462 was still present (Fig. 4G). Therefore, the \( m/z \) 608 fragment in oyster glycans contrasts with the fragment with the same \( m/z \) value in glycans from nematodes and trematodes as it is associated in hemocytes with an uncapped core \( \alpha_1,6 \)-fucose residue and not with a “GalFuc” epitope. Attempts to digest these core-hexosylated glycans with other exoglycosidases, other than fucosidase, were unsuccessful; thus, we can only conclude that we have discovered an unknown and novel hexose modification of oyster N-glycan cores. Further analysis was impeded by the rather low abundance of these structures.

As with the oligomannosidic glycans, methylation was also a feature of some of the paucimannosidic structures; fragments of \( m/z \) 542, 717, and 841 are compatible with the presence of a methyl group on the \( \alpha_1,3 \)- and/or \( \alpha_1,6 \)-linked mannose (Fig. 4, A, C, E, and F).

**Simple Hybrid and Biantennary N-Glycans**—The vast majority of N-glycans from hemocytes and plasma were predicted to contain three or more N-acetylhexosamine and four or more hexose residues; this was suggestive of the presence of simple hybrid and complex-type oligosaccharides. Often these glycans contained methyl and fucose moieties; the simplest examples are Hex\(_4\)HexNAc\(_3\)Fuc\(_0–1\)Me\(_0–1\) (\( m/z \) 1354, 1368, 1500, and 1514 in positive mode; see supplemental Fig. 3A for a mass spectrum of Hex\(_4\)HexNAc\(_3\)Fuc\(_1\)). These glycans, eluting between 5 and 8 g.u. from the NP-HPLC column, were treated with exoglycosidases to determine their nature more exactly. The fucosylated structures were sensitive to bovine kidney α-fucosidase (supplemental Fig. 3B), and MS/MS revealed that the typical \( m/z \) 446 fragment (supplemental Fig. 3E) was lost; therefore, a core \( \alpha_1,6 \)-linkage was demonstrated in monofucosylated glycans from both the PNGase A and F digests. Another question was whether the galactose is \( \beta_1,3 \)-linked (type 1) or \( \beta_1,4 \)-linked (type 2). Digestions overnight with native bovine testes \( \beta \)-galactosidase and recombinant Xanthomonas \( \beta_1,3 \)-specific galactosidase (supplemental Fig. 3C) of the neutral \( m/z \) 1500 glycan were complete, whereas use of native and recombinant Aspergillus.

**FIGURE 2.** Mass spectrometric analysis of N-glycans from the Eastern Oyster. Positive mode MALDI-TOF MS spectra of free (A) or pyridylaminated (B and C) glycans derived from either PNGase F digestion (A) or PNGase A digestion (B) of oyster plasma glycopeptides and of PNGase A digestion of hemocyte glycopeptides (C) indicate the higher complexity of the hemocyte N-glycome as opposed to the plasma N-glycome; selected quasimolecular [M + H]\(^+\) ions are annotated for the pyridylaminated glycans and [M + Na]\(^+\) for the free glycans. Negative mode spectra of pyridylaminated plasma and hemocyte glycans were similar to each other; the negative mode spectrum of hemocyte glycans is shown (D), and selected [M – H]\(^–\) ions are annotated. Structures of neutral and anionic glycans are shown in Tables 1–3.
enzymes with a tendency to more efficiently cleave N-acetylhexosamine residues with or without methyl groups; the simplest is a glycan of m/z 1849 (Hex$_3$HexNAc$_4$Fuc$_1$), whereas the most complex include the glycans of m/z 2810 and 2956 predicted to be Hex$_5$HexNAc$_5$Fuc$_2$Me$_1$. In addition, glycans with one or two fucose residues, but lacking the hallmark of core fucosylation (i.e., a fragment of m/z 446, GlcNAc$_1$Fuc$_1$-PA), were also detected (Hex$_{4,5}$HexNAc$_{4,6}$Fuc$_{1,2}$Me$_{1,2}$). MS/MS spectra of methylated hybrid and biantennary glycans (m/z 1676, 1838, 1733, and 1879) are compatible with the proposed structures (supplemental Fig. 2).

Histo-blood Group A Modification of N-Glycans—A number of glycans from both hemocytes and plasma had predicted compositions suggestive of two or more fucose and four or more N-acetylgalactosamine residues with or without methyl groups; the simplest is a glycan of m/z 1849 (Hex$_3$HexNAc$_4$Fuc$_1$), whereas the most complex include the glycans of m/z 2810 and 2956 predicted to be Hex$_5$HexNAc$_5$Fuc$_2$Me$_1$. In addition, glycans with one or two fucose residues, but lacking the hallmark of core fucosylation (i.e., a fragment of m/z 446, GlcNAc$_1$Fuc$_1$-PA), were also detected (Hex$_{4,5}$HexNAc$_{4,6}$Fuc$_{1,2}$Me$_{1,2}$). MS/MS spectra of methylated hybrid and biantennary glycans (m/z 1676, 1838, 1733, and 1879) are compatible with the proposed structures (supplemental Fig. 2).

Among glycans of the simple complex class, those of m/z 1719, 1865, and 1879 (Hex$_3$HexNAc$_4$ and Hex$_3$HexNAc$_4$Fuc$_1$Me$_{0,1}$) were found to lose two hexose residues upon β,1,3-specific galactosidase treatment, a result also indicative of their biantennary nature (supplemental Fig. 5). However, Hex$_{5,6}$HexNAc$_3$Fuc$_1$ glycans (m/z 1662 and 1824) were both galactosidase-sensitive (with the loss of one galactose residue) and mannosidase-sensitive (with the loss of one or two mannose residues), which is an indication of their hybrid nature (data not shown), whereas their methylated variants (Hex$_{5,6}$HexNAc$_3$Fuc$_1$Me$_1$; m/z 1676 and 1838) lost either none or only one hexose upon jack bean α-mannosidase digestion as methylated mannose is resistant to this enzyme. MS/MS spectra of methylated hybrid and biantennary glycans (m/z 1676, 1838, 1733, and 1879) are compatible with the proposed structures (supplemental Fig. 2).

One abundant example of these complex glycans, present in the NP-HPLC fraction of 7.5 g.u., was subject to exoglycosidase digestion and subsequent MALDI-TOF MS and MS/MS analysis; the glycan of m/z 2082 (Hex$_3$HexNAc$_4$Fuc$_1$Me$_1$; Fig. 7A) lost one hexose upon incubation with either β,1,3-specific galactosidase or bovine β-galactosidase (product of m/z 1920; Fig. 7B) and an N-acetylgalactosamine after jack bean β-hexosaminidase digestion of the degalactosylated glycan (product of m/z 1717; Fig. 7C). Chicken α-N-acetylgalactosaminidase digestion resulted in loss of one methylated N-acetylgalactosamine, and subsequent Xanthomonas α,1,2-fucosidase treatment liberated one fucose (products of m/z 1500 and 1354: Fig. 7D and E), accompanied by loss of the m/z 217 and 511 fragments, respectively (Fig. 7, I and J). Finally, subsequent β,1,3-galactosidase treatment could remove one galactose residue to yield a product with m/z 1192 (Fig. 7F). In contrast to the sensitivity of antennal fucose toward α,1,2-fucosidase, such residues were resis-
tant to hydrofluoric acid and almond α1,3-fucosidase treatments (data not shown). Therefore, we concluded that the novel modification is MeGalNAc (i.e. methylated blood group A) on one antenna, whereas a standard Gal1,3GlcNAc was present on the other; the other two minor components in this fraction contained either two Gal1,3GlcNAc-modified antennae (m/z 1865) or two blood group A antennae (m/z 2445). Also, nonmethylated forms of terminal N-acetylhexosamine were detected in other fractions, as judged by the results of exoglycosidase digestion, on other glycans such as Hex4HexNAc4Fuc2Me0–1, Hex5HexNAc5Fuc1–2, and Hex5HexNAc6Fuc3Me1 (m/z 1849, 1863, 2068, 2215, and 2578).

A later eluting NP-HPLC fraction (8 g.u.) contained some of the largest glycans detected during this study (m/z 2237, 2470, 2600, and 2833 as [M + Na]+; supplemental Fig. 6A); after digestion with chicken β-N-acetylgalactosaminidase and Xanthomonas α1,2-fucosidase (supplemental Fig. 6B), subsequent treatment with β1,3-specific galactosidase resulted in the loss of up to three galactose residues (supplemental Fig. 6C). These degalactosylated glycans also lost up to three N-acetylhexosamine residues when then treated with jack bean N-acetylglucosaminidase (supplemental Fig. 6D); thus, we conclude that the glycans with m/z 2448 and 2810, as [M+H]+, are triantennary complex structures with one or two blood group A epitopes. The mass spectrometric data are thereby in accordance with the blotting data showing reactivity toward anti-blood group A.

**Sulfation of Hybrid and Biantennary N-Glycans**—Earlier eluting NP-HPLC fractions (2–4.5 g.u.) often contained glycans with the same apparent m/z value in positive mode MS as later eluting ones (5–8 g.u.), but they were resistant to β-galactosidase treatment (data not shown). However, closer inspection revealed that these positive mode ions were accompanied by an ion of 102 mass units higher, which in turn was associated with further sodium and potassium adducts (Fig. 8A); MS/MS of such a sodiated adduct resulted in sequential loss of 80, 162, and 203 mass units (Fig. 8F). These early eluting glycans were also easily detected in negative mode as an ion 78 mass units higher than the predominant positive mode ion (see example in Fig. 8B). Addition of sodium acetate to the matrix when analyzing an early/late eluting fraction “pair” (NP-HPLC fractions 3.8 and 5.4 g.u.) resulted in shifts in the spectra in the positive ion mode (Fig. 8, C and D). Under consideration of our previous experience with charged glycans from *Dictyostelium* and also observations described in the literature (26), we concluded that these early eluting glycans are sulfated, rather than phosphorylated, and the ions observed were concluded to be [M + SO3 + H]+ and [M + Na]+ in positive mode and [M − H]− in negative mode (see also Table 3). In the sodium-supplemented samples, only [M + Na]+ forms were observed, and the in-source loss...
of sulfate was suppressed in the positive ion mode for the early eluting fraction; thus the composition of the 3.8 g.u. glycan is Hex$_4$HexNAc$_3$Me$_1$[SO$_3$]$_1$.

MS/MS in negative mode of these sulfated $N$-glycans typically yielded fragments of $m/z$ 241 and 444 (Hex$_3$HexNAc$_{2-1}$[SO$_3$]$_1$; Fig. 8E). This suggested that terminal hexose residues were modified probably in the context of Gal$_1$3GlcNAc motifs, thereby explaining the galactosidase resistance of these anionic glycans. The aforementioned MS/MS of this glycan in the positive mode can thereby be explained by the loss of sulfate, galactose, and $N$-acetylglucosamine from one antenna. LC-MS$^3$ analysis of Hex$_4$HexNAc$_3$Me$_1$[SO$_3$]$_1$ resulted in fragments of 139 and 199, which were interpreted as being the result of 0,2- and 0,4-cross-ring cleavages and so indicative of 6-sulfation of terminal galactose (supplemental Fig. 4, C and D).

Among the late-eluting HIAX glycans as well as in NP-HPLC fractions, there are also disulfated species such as Hex$_3$HexNAc$_2$[SO$_3$]$_2$ and Hex$_3$HexNAc$_2$Fuc$_1$Me$_1$[SO$_3$]$_2$ ($m/z$ 1899 and 2262). The former (Fig. 8H) is a typical biantennary glycan with two sulfated Gal$_1$3GlcNAc motifs; a fragment of 444 (Hex$_3$HexNAc$_2$[SO$_3$]$_2$) as well as a loss of 466 (Hex$_3$HexNAc$_2$[SO$_3$]$_2$Na$_1$) were observed. The glycan with $m/z$ 2262 (Fig. 8I), however, has one sulfated Gal$_1$3GlcNAc motif and one sulfated blood group A motif as shown by the presence of fragments of 444 and 807 as well as the loss of 829 (Hex$_3$HexNAc$_2$Fuc$_1$Me$_1$[SO$_3$]$_2$Na$_1$); the latter fragment is indicative of methylation and fucosylation close to the sulfated hexose.

In addition, monosulfated glycans with the blood group A motif were also detected (e.g. $m/z$ 1795 and 2523); in
one case the putatively terminal GalNAc residue is not methylated as indicated by the fragment of m/z 793 (Hex$_3$HexNAc$_2$Fuc$_1$[SO$_3$)$_1$; Fig. 8G). In the second example, the fragment of m/z 807 (Hex$_2$HexNAc$_3$Fuc$_2$Me$_1$[SO$_3$)$_1$) was again observed in the negative mode as was a fragment of m/z 387 suggestive of fucosylation and sulfation of the same hexose residue (Fig. 8F). The GalNAc residue of such sulfated blood group A glycans can be removed by chicken N-acetyl-galactosaminidase (data not shown).

**O-Glycans of Oyster Hemocytes and Plasma**—In contrast to the complicated N-glycomes, O-glycans found in oyster hemocytes and plasma were simple; only core 1 (Galβ1,3GalNAc) and extended core 1 (GlcNAcβ1,3Galβ1,3GalNAc) were detected. An O-glycan with the composition of Hex$_4$HexNAc$_2$ ([M – H]$^-$ ion of m/z 587) was identified as a single isomer. The presence of ions at m/z 202, 220, 384, and 479 in the MS/MS spectrum (supplemental Fig. 7) is indicative of a modified core 1 O-glycan, HexNAc-Galβ1,3GalNAc. In addition, this struc-
structure was resistant to chicken α-N-acetylgalactosaminidase and showed same MS² spectra and retention time as an authentic β1,3GlcNAc extended core 1. Thus, this structure was annotated as an extended core 1 O-glycan, which was also present after performing β-elimination on tryptic peptides of gel-separated proteins (data not shown).

Identification of CvGal1 Glycoprotein Ligands on Hemocytes and Plasma—Considering the expectation that glycoproteins in oyster hemocytes are the endogenous ligands for the galectin CvGal1, affinity purification of hemocyte glycans on immobilized CvGal1 was performed, and the bound glycoproteins were specifically eluted with lactose (50 mM). Mass spectrometry indicated that the major protein eluted has a molecular mass of 22,000, similar to that of dominin from oyster plasma (supplemental Fig. 8A); subsequent “top-down” sequencing (ISD) of the galectin-binding protein (Fig. 9A) yielded a sequence (Ile-/Leu)-His-(Ile/Leu)-Ser-Asp-Asp-Glu-Asp-Pro-Asn-Tyr-Ala-(Hex4HexNAc3Fuc0–1Me1) with a Gal and plasma (Fig. 9, PNGase treatment of the peptic peptides followed by pyridyloric acid treatment and almond exoglycosidase digests, our analyses reveal a number of unexpectedly complex and varied set of core and antennal modifications of the N-glycans of the main immune cell type and plasma of the eastern oyster C. virginica (summarized in Tables 1–3).

Core α1,3-fucosylation, a characteristic of many invertebrate and plant species (27), was found to be a feature of hemocyte, but not of plasma, glycans; this is also compatible with the anti-HRP staining observed only with the hemocyte extract. Core α1,6-fucosylation, a universal animal modification present on many N-glycans of both sources, probably accounts for the lentil lectin (LCA) reactivity (28, 29). A novel modification of the core of hemocyte glycans was also observed and is predicted to be the direct modification of the reducing terminal GlcNAc with a hexose as shown by the fragment of m/z 462. Although a fragment of m/z 608 was also observed, it is not indicative of galactosylation of the core fucose as found in planaria and nematodes (15), but it is due to concomitant uncleaved α1,6-fucosylation; thus, this core hexose is concluded to be attached to the C3-hydroxyl as the other positions (C2, C4, and C6) are already substituted by an N-acetyl group, the second core GlcNAc and the α1,6-fucosyl, respectively.

The question as to the type of antennal fucosylation was initially more difficult to resolve; preliminary data from blotting with the lectins from Ulex europaeus (UEA; normally considered H-type α1,2-fucose-specific (30)) and Lotus (LTA; normally considered Lewis-type α1,3-fucose-specific (31)) suggested the presence of α1,2-fucose (data not shown). However, both α1,2- and α1,3-fucosyltransferase homologues are found in the genome of the related oyster Crassostrea gigas, but one or more of the α1,3-fucosyltransferase homologues may be responsible for core α1,3-fucosylation. Indeed, the terminal fucose modification was also seemingly insensitive to hydrofluoric acid treatment and almond α1,3-fucosidase, which ruled out a Lewis-type α1,3- or α1,4-fucosyl linkage to GlcNAc (both types of linkage being sensitive to hydrofluoric acid in our hands), whereas strong, yet inhibitable, reactivity toward antiblood group A was observed, and digestion of the glycans with chicken α-N-acetylgalactosaminidase and Xanthomonas α1,2-fucosidase was successful. Digestion with both the latter two enzymes was a prerequisite for removal of the underlying β1,3-galactose residue to which antennal fucose is attached. The presence of terminal α-linked N-acetylgalactosamine on N-glycan was resistant to treatment with jack bean β-hexosaminidase (C), chicken α-hexosaminidase (D), and Xanthomonas α1,2-fucosidase (E), and finally β1,3-specific galactosidase (F). The progress of digestion of the major structure in this fraction is shown using red dashed lines and is compatible with the different anomeric specificity (α or β) of the two hexosaminidases employed; glycans are annotated in their protonated forms. MS/MS analysis also shows the progress of digestion; shown are the fragmentation patterns for the major species in A, C, D, and E with the respective m/z values (G) 2082, (H) 1717, (I) 1500, and (J) 1354.

**DISCUSSION**

**N-Glycosylation of Oyster Hemocytes and Plasma**—Using various glycomic tools, including mass spectrometry of whole N-glycomes, of HPLC-purified glycans, and of one specific glycoprotein in combination with Western blotting, MS/MS, and exoglycosidase digestions, our analyses reveal a number of unexpectedly complex and varied set of core and antennal modifications of the N-glycans of the main immune cell type and plasma of the eastern oyster C. virginica (summarized in Tables 1–3).

Core α1,3-fucosylation, a characteristic of many invertebrate and plant species (27), was found to be a feature of hemocyte, but not of plasma, glycans; this is also compatible with the anti-HRP staining observed only with the hemocyte extract. Core α1,6-fucosylation, a universal animal modification present on many N-glycans of both sources, probably accounts for the lentil lectin (LCA) reactivity (28, 29). A novel modification of the core of hemocyte glycans was also observed and is predicted to be the direct modification of the reducing terminal GlcNAc with a hexose as shown by the fragment of m/z 462. Although a fragment of m/z 608 was also observed, it is not indicative of galactosylation of the core fucose as found in planaria and nematodes (15), but it is due to concomitant uncleaved α1,6-fucosylation; thus, this core hexose is concluded to be attached to the C3-hydroxyl as the other positions (C2, C4, and C6) are already substituted by an N-acetyl group, the second core GlcNAc and the α1,6-fucosyl, respectively.

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cans could also account for the reactivity toward *Vicia* lectin (VVA), which has been previously shown to bind N-acetylglucosamine-capped N-glycans, O-glycans, and blood group A structures (29, 32). The data overall show that oyster glycans are capped in part with a frequently methylated form of the A-type histo-blood group. Indeed, histo-blood group A has been detected in oysters before, which was of interest due to a possible interaction with noroviruses (33). Some of the glycans modified with blood group A were triantennary as judged by A-type histo-blood group. Indeed, histo-blood group A has been capped in part with a frequently methylated form of the Hex5HexNAc5Fuc1Sia1SO3 (42).

Squid *Todarodes pacificus* carries methylated oligomannose and simple hybrid structures (41); however, the Japanese oyster protein *C. gigas* genome is interesting (34). In nematodes, probably the only form of charged modification of N-glycans is the addition of phosphorylcholine, a zwiterion, that is also found in the trematode *Echinococcus* (19). Among unicellular organisms, sulfation and methyl phosphorylations of N-glycans are known in *Dictyostelium* (16), and some N-glycans of *Trichomonas vaginalis* carry phosphate or phosphorylethanolamine (13).

**Comparison with Glycans of Other Molluscs** —A number of other studies have been published over the years regarding the N-glycans of molluscs, including bivalves, cephalopods, and gastropods. Seemingly, rather few studies have specifically dealt with the glycosylation of bivalves such as oyster and clam. In the case of cephalopods, such as squid and octopus, probably the most notable feature is galactosylation of core fucose (36, 37), a modification also found in nematodes (15, 38) and in a flatworm (39). In another study, β1,3-galactosylation of the type found in this study on oyster was found on the N-glycans of the squid *Todarodes pacificus* (40). The clam lectin tridacnin carries methylated oligomannose and simple hybrid structures consisting otherwise only of mannose and N-acetylgalcosamine residues (41); however, the Japanese oyster protein known as nacrein was predicted to carry a glycan with the composition Hex3HexNAc5Fuc5Sia5SO3 (42).

Probably, the large majority of such studies has been performed on the N-glycans of various gastropods, including various snails. Here, a focus has been on glycans of hemocyanins (43–48) from various species, including *Helix pomatia*, *Lymnaea stagnalis*, *Rapana thomasiana*, *Rapana venosa*, *Halioitis tuberculata*, and *Megathura crenulata* (keyhole limpet). Furthermore, tissues or hemolymphs derived from various snails, including *Biomphalaria glabrata*, have been glycomically analyzed (49). A range of compositions have been proposed such as Hex3HexNAcPntFucMe1 in *L. stagnalis* and Hex4HexNAc3FucMe1 in *R. thomasiana*; terminal motifs such as [HexMe]HexNAc, MeHex(FucX3L3)-GlcNAc, or fucosylated LacdiNAc have been concluded to be terminal elements of snail glycans.

Interestingly, a fragment of m/z 729 (Hex, HexNAc, Fuc, Me) was previously found during MS/MS analyses of a snail glycan; a fragment of the same predicted composition was found during our study. However, in the case of *R. thomasiana*, this fragment was suggested to represent a disubstituted fucose linked to GlcNAc (50). In the case of oyster, we show that a methylated blood group A is present. The elements of the novel oyster modification (GlcNAc, GalNAc, fucose, and methyl residues) are found as antennal elements of other snail glycans, but the proposed structures are quite different. Considering that there has been no systematic study of mollusc glycans and that many different methods have been used in different laboratories, there is the possibility that individualized interpretations of analytical data may hide common structural themes; however, we can state that we have verified MS/MS data with exoglycosidase digestions and blotting data. Therefore, we have not relied on a single method.

**Potential Ligands for the Oyster Galectin CvGal1** —Previous reports have suggested that the oyster galectin, which is expressed and secreted by many oyster cell types, including hemocytes, has an affinity for terminal galactose and N-acetylgalcosamine; N-glycans with such terminal residues were found by us both in hemocyte and plasma samples. Galectin affinity purification of proteins from hemocyte lysates was employed to identify potential glycoprotein ligands for CvGal1 (see also accompanying paper by Feng et al. (12)). The resulting bound fractions were analyzed, and the major protein in them was found by mass spectrometry to be dominin. This protein, with a molecular weight of ~22,000 as judged by MALDI-TOF MS and which has been previously found to be the major protein in the plasma of *C. virginica* (20), contains a single potential N-glycosylation site.

Dominin has homology to two other hemolymph proteins, cavortin from the related oyster species *C. gigas* and pernin from the green-lipped mussel (*Perna canaliculus*); both of these proteins also have superoxide dismutase motifs, but rather seem to bind iron (51). Therefore, it is possible that dominin may function as an iron transporter or iron storage protein. Glycomic analysis indicated that dominin present in plasma and in galectin-binding fractions contains predominantly sim-
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ple type 1 hybrid N-glycans with terminal galactose with and without sulfate; a minor proportion is decorated with the blood group A epitope. The glycans of dominin thereby are a subset of the total N-glycans of hemocytes and represent also the major N-glycans found in plasma.

As dominin can be isolated on a CvGal1 affinity column, we assume that dominin is decorated with potential ligands for the galectin and thereby associates with the hemocyte surface, but only low quantities of glycans substituted with blood group A were observed in the galectin-binding fractions even though type 2 glycans with this modification were distinctly the best ligands for CvGal1 on a microarray (12) (data are available via the Consortium for Functional Glycomics website). However, testing lectins with the currently available arrays is handicapped by the lack of nonmammalian glycans on these platforms; in this instance, 6-sulfated β1,3-linked galactose (never mind in the context of methylated and nonmethylated hybrid glycans) and the newly discovered core hexosylated glycans are absent, and so binding of oyster-specific N-glycan motifs could not be tested. Another factor is that Nature does not necessarily adopt the “optimal” glycan structure for biological activity; a well known example is the “brake function” on antibody-dependent cellular cytotoxicity mediated by the presence of core α1,6-fucose on most IgG molecules (52). Thus, it may well be that the 45% lower binding of the galectin to blood group A type 1 glycans, as opposed to type 2, may not be biologically significant in vivo or that evolution has by chance arrived at a lower affinity lectin-ligand combination. In this context, low abundance glycoproteins (e.g. β-integrin; see Feng et al. (12)) or lower affinity glycan ligands, including those not present on the glycan array, may well also contribute to the overall formation of a galectin-glycoprotein lattice known to exist on the oyster hemocyte surface.

**Potential Function of CvGal1**—Like many invertebrate lectins, CvGal1 binds to multiple bacterial species and microalgae. As a major hemocyte subset is phagocytic and is involved in uptake of microbial pathogens and phytoplankton, a dual role in defense and feeding was proposed for CvGal1; this was supported by the finding that blocking the hemocyte surface CvGal1 by pre-treatment with a specific antibody reduces phagocytic activity (8). These functions, however, have been subverted by the protozoan parasite *P. marinus*, which may outcompete the above ligands to gain entry into the hemocytes where it survives oxidative killing and proliferates (5). Although it is not known which glycans are recognized by CvGal1 in the parasite surface, experimental evidence indicates that these do not display blood group A glycotopes. Certainly, some EST sequences have been identified from *P. marinus*, which indicate that it should have the capacity to produce Glc3Man9GlcNAc2 N-glycans (53), which is not universal for protozoan parasites; the preliminary genomic data also indicate the potential for fucosylation and galactosylation. Furthermore, glycotyping of the trophozoite surface in several *P. marinus* strains with plant lectins (54) has revealed the potential presence of N-acetyl-

**TABLE 3**

Predicted structures for anionic N-glycans common to both oyster plasma and hemocytes

| m/z 1432.47 | m/z 1446.48 | m/z 1460.50 | m/z 1578.53 | m/z 1592.54 | m/z 1606.56 | m/z 1608.54 | m/z 1754.59 | m/z 1795.62 | m/z 1797.60 | m/z 1809.63 | m/z 1811.62 | m/z 1823.65 |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| S | Me | S | Me | S | Me | S | Me | S | Me | S | Me | S |
| 1899.95 (+Na) | 1943.66 | 1955.59 | 1957.67 | 2160.75 | 2174.77 | 2188.78 | 2262.71 (+Na) | 2306.81 | 2320.83 | 2523.91 |

Structures of anionic N-glycans found in hemocytes and in plasma are depicted according to the nomenclature of the Consortium for Functional Glycomics; the given m/z values are theoretical values for pyridylaminated glycans in their [M − H]⁻ form, other than two disulfated glycans detected as [M + Na − 2H]⁻ species.

**FIGURE 9.** Proteomic and glycomic analysis of oyster dominin. Top-down sequencing of the major proteins in the galectin-binding fraction of hemocyte lysates (A) and of plasma (B) indicated a common sequence of HISSDDEPNYANTMHYAK as found in oyster dominin. MALDI-TOF MS in positive (C and E) and negative (D and F) modes of the pyridylaminated N-glycans isolated from dominin present in the galectin-binding fraction of hemocyte lysates (C and D) and of plasma (E and F) indicating a very similar range of glycans; species marked with an asterisk represent a contaminating polyhexose series; example structures are shown for the m/z 1446, 1512/1536, 1592, 1753, 2104 and 2160 species. The insets in C and D constitute a region of the MS/MS of the major species found in positive and negative modes. In the positive mode, the protonated form of the HexHexNAcFucMe glycans (m/z 1514), rather than the sodiated (m/z 1536), was fragmented and shows fragments corresponding to GlcNAc-PA and GlcNAcFuc-PA (fragments of m/z 300 and 446); in the negative mode, the sulfated form of HexHexNAcFucMe was subject to MS/MS, and the resulting spectrum shows the presence of sulfated HexHexNAcMe (fragments of m/z 241 and 444).
galactosamine and galactose (see also Feng et al. (12)) which may, despite the absence of blood group A on the parasite, act as biologically effective ligands.

As the parasite *P. marinus* requires iron for anti-oxidative activity mediated by the two superoxide dismutases PmSOD1 and PmSOD2 (55, 56) and expresses three iron uptake transporters (PmNramp1–3) (57) as well as active secreted proteases (58), our finding that dominin (which is, as discussed above, a potential iron-binding protein) carries CvGal1 ligands suggests the intriguing possibility that the parasite not only “hijacks” CvGal1 to enter the host hemocyte but also to access either soluble or hemocyte-associated iron pools in the oyster. Ongoing studies are aimed at addressing this interesting question.

A gallecin of CRD organization similar to CvGal1 has been identified in the freshwater snail *B. glabrata*, an intermediate host of the parasitic helmint *Schistosoma* sp. (59). Although similarities in the glycosylation of both the schistosome and snail are known (60, 61), the actual ligands of the snail gallecin remain unknown. As a parallel with the oyster-*Perkinsus* interaction (54), it was proposed that the snail gallecin may participate in host-*Schistosoma* interactions, yet to be determined (59). However, N-glycans are not the only parasite ligands for host gallecins, as shown by the binding of *T. vaginalis* lipoglycan to human gallecin-1 on epithelia (62). Because gallecins are a highly conserved lectin family along the metazoan lineages, it has been hypothesized that some protozoan parasites have evolved their glycomes to take advantage of the host gallecin-mediated recognition to facilitate adhesion and establish infection (5).

**Conclusion**—The N-glycans of the oyster analyzed in this study are another indication that the N-glycosylation of invertebrates can be rather complex (23) and surprisingly similar to mammalian structures. Indeed, these glycans are unusual for a number of reasons as follows: first, the large degree of sulfation; second, the relatively low amount of paucimannosidic species for an invertebrate; third, the modification of core GlcNAc directly with a hexose residue; fourth, the modification with Galβ1,3GlcNAc; and fifth, the occurrence of modified forms of the human histo-blood group A antigen. The latter two types of structures probably represent a set of endogenous ligands for the oyster gallecin CvGal1.

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