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Characterization of a Novel Sulfated Carbohydrate Unit Implicated in the Carbohydrate-Carbohydrate-mediated Cell Aggregation of the Marine Sponge *Microciona prolifera**

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Species-specific cell reaggregation in the marine sponge *Microciona prolifera* is mediated by an adhesion proteoglycan. Two interactions are involved in the process: a Ca^{2+}-dependent homophilic binding between proteoglycan molecules and a Ca^{2+}-independent binding between the molecule and cells. Both interactions are mediated by the glycan moieties of the proteoglycan. The interaction of the proteoglycan with itself has been characterized as a carbohydrate-carbohydrate interaction of multiple low affinity sites. The monoclonal antibodies Block 1 and Block 2 raised against the purified aggregation proteoglycan and selected for inhibition of aggregation bind to these glycans. In a previous report the structure,

$$\mathrm{Pyr}\langle^{6}_{4}\rangle\mathrm{Gal}\beta1\text{-}4\mathrm{GlcNAc}\beta1\text{-}3\mathrm{Fuc}$$

STRUCTURE 1

was assigned to the oligosaccharide reacting with Block 1 antibody (Spillmann, D., Hård, K., Thomas-Oates, J., Vliegenthart, J. F. G., Misevic, G., Burger, M. M., and Finne, J. (1993) *J. Biol. Chem.* 268, 13378–13387). By the technique of attaching the water-soluble acid-degraded fragments to a lipid carrier for immunochemical detection and by chemical, enzymatic and spectroscopic methods the structure,

$$\begin{array}{l}\mathrm{GlcNAc}\beta1\text{-}3\mathrm{Fuc}\\ \quad\;\;|\\ \quad 3\mathrm{SO_3}\end{array}$$

STRUCTURE 2

was assigned to the oligosaccharide reacting with the aggregation-blocking monoclonal antibody Block 2. The structure,

$$\begin{array}{l}\mathrm{Gal}\alpha1\text{-}2\mathrm{Gal}\beta1\text{-}4\mathrm{GlcNAc}\beta1\text{-}3\mathrm{Fuc}\\ \qquad\qquad\quad\;\;|\\ \qquad\qquad\;\; 3\mathrm{SO_3}\end{array}$$

STRUCTURE 3

was assigned to a major nonreactive oligosaccharide, which outlined the molecular requirements of antibody binding of the two aggregation-associated epitopes. These data demonstrate that two different functional sites with distinct structural characteristics and antibody reactivities are involved in the reaggregation of sponge cells, a model of carbohydrate-carbohydrate-mediated cell interactions.

The species-specific reaggregation of dissociated cells from the marine sponge *Microciona prolifera* has been studied as a model of cellular interactions of higher animals (1, 2). The reaggregation depends on the presence of a large extracellular adhesion proteoglycan and a high concentration of Ca^{2+} ions (3–5). Two functional domains have been identified: one a homophilic, Ca^{2+}-dependent proteoglycan-proteoglycan interaction site and the second a heterophilic, Ca^{2+}-independent proteoglycan-cell interaction site (5).

The carbohydrates of the proteoglycan form a major part of the molecule and, in polymeric form, have the capacity to mediate the species-specific reaggregation process in the absence of a protein backbone. This has led to the conclusion that multiple low affinity carbohydrate-carbohydrate interactions form the basis of cell reaggregation in the sponge system (6, 7). Low affinity carbohydrate-carbohydrate interactions have also been suggested to mediate cell interactions in higher animals during embryonic development and in tumor cell interactions (for reviews see Refs. 8–10).

The proteoglycan is composed of uronic acid, fucose, and other neutral monosaccharides in the same glycan chains (4, 11) without having close similarity to classical glycosaminoglycan structures. The monoclonal antibodies Block 1 and Block 2, prepared against the purified proteoglycan and selected for aggregation-inhibiting activity are directed against the carbohydrate portions of the proteoglycan (6, 12). The oligosaccharide structure reacting with the Block 1 antibody was previously characterized and was shown to be a unique structure containing pyruvate acetal and an internal fucose residue, Pyr-4,6Galβ1–4GlcNAcβ1–3Fuc (13). In this report we describe the isolation and structural determination of the saccharide epitope of Block 2 antibody and of a major nonreactive control oligosaccaride. Both saccharides represent novel sulfated oligosaccharides with an internal fucose moiety, and together with the previously characterized saccharide outline the structural

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requirements of antibody reactivity with the two reaggregation-associated oligosaccharide epitopes.

EXPERIMENTAL PROCEDURES

Materials—Live specimens of *Microciona prolifera* were collected by the Supply Department of the Marine Biological Laboratory (Woods Hole, MA).

N-Acetylneuraminyl α2–3 lactose (3′-sialyl lactose) was purified from bovine colostrum as described previously (14). Galβ1–3GlcNAcβ1–3Galβ1–4Glc (lacto-*N*-tetraose) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (dipalmitoyl phosphatidylethanolamine) were from Sigma. NaB^3H_4 (500 mCi/mmol) was from Amersham International (United Kingdom). Poly(isobutylmethacrylate) (M_r 300,000) was supplied by Aldrich, 4-chloro-1-naphtol by Merck, and Dextran T 70 by Pharmacia Biotech Inc. Dihydroxybenzoic acid matrix originated from Finnigan MAT (U.K.). ^2H_2O (99.8 and 99.96 atom % ^2H) was purchased from MSD Isotopes (Montreal, Canada).

The monoclonal antibody clones Block 1 and Block 2 were purified as described (6, 12). Horseradish peroxidase-coupled rabbit anti-mouse antibody was received from Dakopatts (Glostrup, Denmark). Enzymes used were as follows: β-galactosidase from *Charonia lampas* (Seikagaku Kogyo, Tokyo, Japan) and from *Escherichia coli*, α-galactosidase from jack beans (both from Boehringer Mannheim), and β-*N*-acetylhexosaminidase from jack beans (Sigma).

TLC aluminium-backed Silica Gel 60 sheets and Fractogel TSK DEAE-650 were from Merck. Bio-Gel P-2 (200–400 mesh) and Dowex AG 50W-X8, H^+ form (100–200 mesh), were obtained from Bio-Rad. The CarboPac PA1 HPLC[1] columns and the anionic micromembrane suppressor cartridge were from Dionex (Sunnyvale, CA), and the Dextro-Pac cartridge was from Waters (Milford, MA). Kodak X-OMAT AR x-ray films were purchased from Eastman Kodak (Rochester, NY).

Purification of the Adhesion Proteoglycan Glycans—Isolation of the adhesion proteoglycan and its glycans was carried out essentially as described (6). Partial acid hydrolyses were performed in conical Teflon-sealed glass vials as indicated in the figure legends. The samples were neutralized and evaporated under vacuum and either linked to lipid for analysis or purified further by anion exchange chromatography.

Synthesis and Immunochemical Detection of Neoglycolipids—Aliquots of dried oligosaccharides were lipid-linked and identified by immunochemical detection of the separated neoglycolipids on TLC plates essentially as described before (13, 15, 16).

Column Chromatography—Anion exchange chromatography on Fractogel TSK DEAE-650 was performed as described before (13). For small scale separation of radiolabeled oligosaccharides into neutral and charged pools mini-anion exchange columns (1-ml bed volume) of Fractogel TSK DEAE-650 were equilibrated with water and eluted with water (5 column volumes) and 100 mM pyridine-acetic acid buffer, pH 5.0 (5 column volumes). Recovery of applied material was confirmed to be quantitative by scintillation counting of the eluents. Gel filtration was achieved on a Bio-Gel P-2 column (1.6 × 130 cm) as desribed before (13). High performance anion exchange chromatography was carried out using a Dionex System (Series 4500i) (17) as described before (13). Postcolumn addition and detection was omitted during preparative runs and instead eluents were desalted on-line (13).

Thin-layer Chromatography of Oligosaccharides—Equal amounts of radiolabeled oligosaccharides (~1000 cpm) were applied to TLC sheets, and the sheets were developed in butanol/acetic acid/water (20:10:15, v/v). For fluorography the dry sheets were sprayed with diethylether/methylanthranilate (1:2, v/v). The sheets were exposed for 1–7 days to Kodak x-ray films.

Chemical Treatments—Fragmentation of purified oligosaccharides was achieved by comparison of mild hydrolysis procedures as described in the figure legends (10 mM HCl for 2 h at 100 °C; 40 mM HCl for 90 min at 100 °C; 60 mM HCl/MeOH for 24 h at 4 °C). Reduction was achieved in 0.2 M $NaBH_4$, 1 mM NaOH for 2 h at room temperature. Methanolysis was carried out as described (18) except that a higher temperature (80 °C) was used and the methyl glycosides were analyzed on a Waters Dextro-Pac column (8 × 100 mm) (19).

Enzymatic Digestions—Enzymatic digestions of ^3H-labeled oligosaccharides were carried out as described before (13).

Chemical Derivatizations—Desulfation was carried out in 200 μl of 50 mM methanolic HCl at room temperature for 3.5 h, and the volatile reagents were removed under vacuum. Permethylation was achieved using a modification of the method of Ciucanu and Kerek, as described (20), and the methylated sulfated tetrasaccharide was recovered using a C18 minicartridge equilibrated in water. The salts were removed by washing with 10 ml of water, and the methylated sulfated product was eluted using the following stepwise gradient: 1.5 ml of 15% aqueous acetonitrile, 1.5 ml of 30%, 1.5 ml of 50%, and finally 1.5 ml of 75% acetonitrile solution. The fractions were collected together and dried under vacuum.

Linkage Analysis—Partially methylated alditol acetates were prepared as described (20) and analyzed by gas chromatography-mass spectrometry (GC-MS) on a JEOL JMS-AX505W mass spectrometer fitted with a Hewlett Packard 5890 gas chromatograph (Department of Mass Spectrometry, Utrecht University) as described before (13). Mass spectra were obtained under conditions of electron impact and were recorded using linear scanning from m/z 50–500 (for monosaccharide derivatives) or m/z 50–600 (for the disaccharide derivative) at an accelerating voltage of 3 kV.

Matrix-assisted Laser-desorption Ionization Mass Spectrometry (MALDI-MS)—The mass spectra were recorded in the negative mode using a Lasermat Mass Analyzer (Finnigan MAT) employing a time-of-flight analyzer. One μl of the samples was loaded onto dihydroxybenzoic acid matrix. Mass calibration was performed using substance P (M_r 1348.6).

Fast Atom Bombardment Mass Spectrometry (FAB-MS)—FAB mass spectra were obtained in either the positive or negative ion modes using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer (Department of Mass Spectrometry, Utrecht University) at either +10 kV or −10 kV accelerating voltage as described before (13).

^1H NMR Spectroscopy—Compounds were exchanged twice in ^2H_2O (99.9% ^2H) with intermediate lyophilization and then dissolved in 450 μl of ^2H_2O (99.96% ^2H). 500- and 600-MHz ^1H NMR measurements were carried out on a Bruker AMX-500 and AMX-600 spectrometer, respectively (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University). The probe temperature was 293 K unless stated otherwise. Suppression of the $^1HO^2H$ was performed by a modified water-eliminated Fourier transform sequence (21) for one-dimensional experiments or by presaturation for 1 s for two-dimensional experiments. ^1H-chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225) at all probe temperatures. The two-dimensional HOHAHA experiments were recorded using MLEV-17 mixing sequence cycles (22), with the offset placed on the $^1HO^2H$ frequency position. The spectrum of compound Block 2 was recorded at 600 MHz at a probe temperature of 283 K using a 76-ms MLEV-17 mixing sequence at a field strength of 10.0 kHz (90° pulse width of 25.0 μs). The spectral width was 5555 Hz in each dimension. The spectrum of compound C-1 was recorded at 500 MHz using a 57-ms MLEV-17 mixing sequence at a field strength of 9.1 kHz (90° pulse width of 27.4 μs). The spectral width was 4000 Hz in each dimension. The experiments consisted of a 400 × 2 K data matrix, which was zero-filled and multiplied by a π/2 shifted sine bell prior to Fourier transformation, resulting in a 1 × 4 K spectral data matrix. A double quamtum-filtered ^1H-^1H COSY spectrum (23) of the Block 2-reactive oligosaccharide was recorded at 500 MHz at a probe temperature of 300 K using a spectral width of 4000 Hz. The 450 × 2 K data matrix was zero-filled and multiplied by a π/2 shifted sine bell prior to Fourier transformation, resulting in a 1 × 4 K spectral data matrix.

RESULTS

Purification of the Block 2-reactive Oligosaccharide—For the identification and isolation of the epitope reactive with the Block 2 antibody, the purified glycans of the aggregation proteoglycan were fragmented by partial acid hydrolysis, and the resulting oligosaccharides were linked to dipalmitoyl phosphatidylethanolamine for immunochemical analysis (15). As compared with the previously characterized Block 1-reactive oligosaccharide (13), determination of the conditions for partial acid hydrolysis revealed a narrower window of conditions for the optimal release of the oligosaccharide, which indicated a higher sensitivity to acidic conditions (results not shown). Fig.

[1] The abbreviations used are: HPLC, high performance liquid chromatography; Fuc, L-fucose; Hex, hexose; Pyr, pyruvate; MALDI, matrix-assisted laser desorption ionization; FAB, fast atom bombardment; MS, mass spectrometry; GC, gas chromatography; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; MLEV, M. Levitt (Composite pulse sequence); COSY, correlated spectroscopy; G_{M3}, sialosyl-lactosylceramide (Siaα2–3Galβ1–Glcβ1–1′-Cer); Cer, ceramide (*N*-fatty acyl sphingosine); Gg3Cer, gangliotriaosylceramide (GalNAcβ1–4Galβ1–4Glcβ1–1′-Cer); LacCer, lactosylceramide; Gb3Cer, globotriaosylceramide (Galα1–4Galβ1–4β1–1′-Cer).

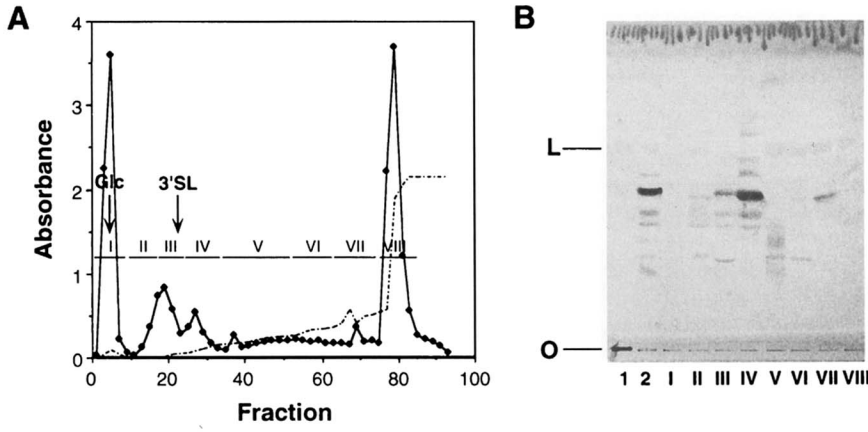

FIG. 1. **Fractionation of adhesion proteoglycan-derived oligosaccharides by ion exchange chromatography.** *Panel A*, adhesion proteoglycan-derived oligosaccharides (20 mg, hydrolyzed in 10 M acetic acid at 80 °C for 8 h) were fractionated on a column of Fractogel TSK DEAE. Elution was achieved by washing with water followed by a linear gradient of pyridine-acetic acid buffer, pH 5.0, from 0 to 0.1 M, and final elution with 0.5 M pyridine-acetic acid buffer. The gradient was measured at 260 nm (-·-·-). The hexose content was determined by the anthrone reaction at 620 nm (♦----♦). The elution positions of the reference compounds glucose (*Glc*) and 3′-sialyl lactose (*3'SL*) are indicated. Fractions *I-VIII* were pooled as indicated. *Panel B*, analysis of the oligosaccharides linked to dipalmitoyl phosphatidylethanolamine by TLC and immunodetection with monoclonal antibody Block 2. *Lane 1*, nonhydrolyzed glycans; *lane 2*, unfractionated partial acid hydrolysate. *Lanes I-VIII* correspond to pools I-VIII as indicated in *panel A*. The positions of the origin (*O*) and free lipid (*L*) are indicated on the *left*.

1 shows the immunochemical analysis of the neoglycolipids derived from the oligosaccharides fractionated after partial acid hydrolysis by anion exchange chromatography. Whereas the material not bound on anion exchange column contained antibody-unreactive, neutral monosaccharides, all antibody-reactive oligosaccharides were retained on the column, which indicates that the epitope structure is charged. The main antibody-reactive component was eluted in fractions III and IV. In addition to the main component, some slower moving components were also present. These were extremely sensitive to acid, since they were rapidly lost if more acidic conditions were used for the fragmentation of the glycans.

The antibody-reactive oligosaccharide pools III-IV were subjected to chromatography on a Dionex semipreparative PA1 anion exchange column using an anionic micromembrane suppressor for on-line desalting during the purification of the oligosaccharides (Fig. 2*A*). Individual peaks were analyzed for their immunoreactivity, and their purity was monitored by rerunning aliquots on an analytical column under the conditions used for the isolation and at high pH, under which conditions ionized hydroxyl groups participate in the separation (17) (Fig. 2, *B* and *C*). The exhibition of single peaks under both conditions suggests that the peaks represent single oligosaccharide components.

Analysis of the immunoreactivity of the individual oligosaccharides identified one (*peak c*) as the component recognized by antibody Block 2 (Fig. 2D). As a control, antibody Block 1 did not react with this oligosaccharide, whereas it reacted with the previously characterized Block 1 oligosaccharide (*peak a*). The most prominent oligosaccharide in the pool (*peak b*), which did not react with either antibody, was designated C-1 and was also subjected to structural characterization.

Structural Characterization of the Block 2-reactive Oligosaccharide—The apparent size of the purified, reduced Block 2-reactive oligosaccharide was, in gel filtration, 3.6 glucose units (Table I). Matrix-assisted laser desorption ionization mass spectrometry yielded a pseudomolecular ion at m/z 446 ($[M-H]^-$)consistent with a $HexNAc_1Deoxyhex_1$ disaccharide, bearing either a sulfate or a phosphate group. Compositional analysis after methanolysis indicated the presence of equimolar amounts of *N*-acetylglucosamine and fucose. The presence of fucose as the reducing terminal residue was indicated by the loss of fucose on reduction (fucitol is not detected in the HPLC monosaccharide analysis system). These results suggested that the Block 2-reactive saccharide consists of the disaccharide GlcNAc-Fuc with a charged group attached to one of the two sugars.

The sugar composition analysis did not reveal the nature of the negative charge in the oligosaccharide. However, mild hydrolysis or methanolysis of the Block 2-reactive saccharide under conditions used to release sulfate esters (24), resulted in the release of the neutral disaccharide (Fig. 3). This, together with the reported presence of sulfate in the glycans (4) and the MALDI-MS data suggests that the charged residue in the disaccharide is a sulfate.

^{1}H NMR Spectroscopy of the Block 2-reactive Oligosaccharide—The chemical shifts of the ^{1}H resonance of the Block 2-reactive saccharide are compiled in Table II. The one-dimensional 600 MHz ^{1}H NMR spectrum demonstrates one α-anomeric signal and two β-anomeric signals. The α-anomeric signal at 5.229 ppm ($^3J_{1,2}$ = 3.8 Hz) and the β-anomeric signal at 4.568 ppm ($^3J_{1,2}$ = 8.1 Hz) were found at the same positions as the Fuc α- and β-anomeric signals in Pyr4,6Galβ1–4GlcNAcβ1–3Fuc (13), respectively. The corresponding Fuc CH_3 signals were established at 1.202 ppm for the α-anomer and at 1.243 ppm for the β-anomer. The third anomeric signal at 4.816 ppm ($^3J_{1,2}$ = 8.9 Hz), together with a NAc methyl signal at 2.017 ppm, revealed the presence of a β-GlcNAc or β-GalNAc residue. The latter residue can be excluded, because the two-dimensional HOHAHA spectrum did not show the typical pattern of a Gal-like spin system (marked by Gal $^3J_{3,4}$ = 3 Hz and Gal $^3J_{4,5}$ = 1.5 Hz). A COSY experiment (data not shown) was used to assign the GlcNAc H-2, H-3, H-4, and H-5 signals. Notably, GlcNAc H-3 did not resonate in the region of the bulk of skeleton protons (4.0–3.5 ppm) but was found at the downfield position of 4.407 ppm instead. This is indicative of the presence of an acidic group. The absence of a signal for an acetal methyl group excludes a pyruvate moiety, and the existence of phosphate can be eliminated because no phosphate coupling is detected on GlcNAc H-3. In addition, the effect of the acidic group on the resonance position of H-3 is too large to be attributed to posphate (compare GlcNAc H-3 at 4.407 ppm in the Block 2-reactive oligosaccharide with Glc H-3 at 3.99 ppm in P-(O-3)-Glcβ1–4Galβ1- (25)). This leaves sulfate, which indeed induces the appropriate downfield chemical shift (compare GlcNAc H-3 at 4.407 ppm in the spectrum of the Block

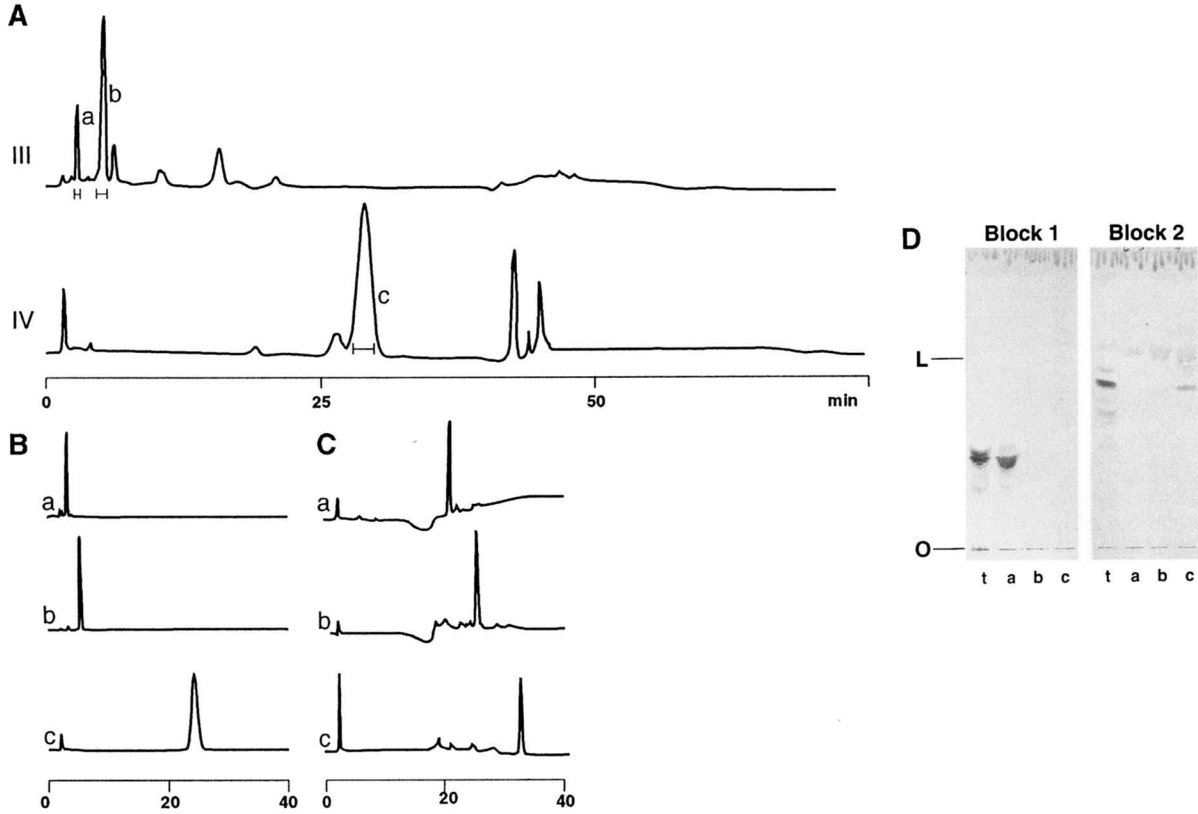

FIG. 2. **Purification of adhesion proteoglycan-derived oligosaccharides by HPLC.** Pool III and IV oligosaccharides (Fig. 1) were separated on a semipreparative PA1 column, and fractions were collected after on-line desalting. *Panel A*, aliquots of the oligosaccharide pools III and IV run on a PA1 analytical column eluted isocratically with 80 mM sodium acetate, 10 mM NaOH. Peak fractions pooled are indicated by a *bar* and the letters *a* (oligosaccharide Block 1), *b* (oligosaccharide C-1), and *c* (oligosaccharide Block 2). *Panel B*, aliquots of three individual oligosaccharides rechromatographed on a PA1 analytical column eluted isocratically with 80 mM sodium acetate, 10 mM NaOH, detector sensitivity 300 nA. *Panel C*, the same oligosaccharides as in *panel B* analyzed with isocratic 100 mM NaOH and a gradient of 0–250 mM sodium acetate started at 10 min and completed at 30 min. *Panel D*, analysis of total unfractionated oligosaccharides (*t*) and the purified oligosaccharides as neoglycolipids by TLC and immunodetection with monoclonal antibodies Block 1 and Block 2. The positions of the origin (*O*) and free lipid (*L*) are indicated on the *left*.

TABLE I
Molecular size and monosaccharide composition of the native and reduced oligosaccharides Block 1 and C-1

The apparent molecular sizes of the oligosaccharides were determined by gel filtration on a Bio-Bel P-2 column standardized with a dextran hydrolysate and are expressed as glucose units. The molecular mass values were obtained by matrix-assisted laser desorption ionization mass spectrometry in the negative mode. The monosaccharide compositions of the native oligosaccharides (N) and the oligosaccharides reduced with $NaBH_4$ (R) were determined after methanolysis by HPLC as their methyl glycosides. The compositions (3 independent determinations) are expressed per mol of *N*-acetylglucosamine. The reduced forms of the monosaccharides are not detected in the HPLC system employed.

	Block 2		C-1	
	N	R	N	R
Apparent size (Glc units)		3.6		6.5
Molecular mass (Da)	447.0		771.2	
		mol/mol		
Constituent				
Gal	0.0	0.0	2.2	2.9
GlcNAc	1.0	1.0	1.0	1.0
Fuc	1.2	0.0	1.3	0.0

2-reactive oligosaccharide with Gal H-3 in Gal3SO_3β1–4GlcNAcβ1-, which resonates at 4.34 ppm (21, 26, 27)). In conclusion, the results derived from the ^{1}H NMR data of the Block 2-reactive oligosaccharide indicate a GlcNAc3SO_3^-β-Fuc disaccharide (Fig. 4).

GC-MS of the Block 2-reactive Oligosaccharide—In order to determine the site of substitution of the GlcNAc on the fucose

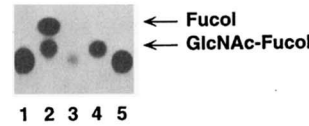

FIG. 3. **Desulfation of Block 2-reactive oligosaccharide by mild acid treatment and mild methanolysis.** The purified Block 2-reactive oligosaccharide labeled by reduction with NaB^3H_4 (*1*) was treated with 40 mM HCl at 100 °C for 90 min (*2* and *3*) or 60 mM HCl in methanol at 4 °C for 24 h (*4* and *5*). By a DEAE ion exchange column the hydrolyzates were separated into neutral (*2* and *4*) and acidic (*3* and *5*) products. Equal aliquots of each fraction were analyzed by TLC and fluorography. The positions of the reference compounds fucitol (*Fucol*) and *N*-acetylglucosaminyl-(β1–3)-fucitol (*GlcNAc-Fucol*) are indicated on the *right*.

residue, the oligosaccharide was desulfated and permethylated. The success of the derivatization was assessed using FAB-MS in the positive ion mode, which yielded a very intense $[M+H]^+$ pseudomolecular ion at m/z 466, corresponding to a fully methylated $GlcNAc_1Fuc_1$ disaccharide. Partially methylated alditol acetates were produced and analyzed using GC-MS, and monosaccharide derivatives corresponding to terminal HexNAc and 3-substituted Deoxyhex were identified, consistent with a GlcNAc1–3Fuc linkage. This assignment was confirmed by analysis of the disaccharide derivative produced on desulfation, sodium borodeuteride reduction, and permethylation. The success of this derivatization was confirmed using positive ion FAB-MS, which yielded an $[M+H]^+$ pseudomolecular ion at m/z 483, consistent with the expected product. The disaccharide derivative was then analyzed using GC-MS under

TABLE II
^{1}H chemical shifts of oligosaccharides Block 2 and C-1

Chemical shifts are relative to acetone at 2.225 ppm in ^2H_2O at 293 K.

Residue	Reporter group	Chemical shift	
		Block 2[a]	C-1[b]
		ppm	
Fuc	H-1α	5.229	5.228
	H-1β	4.568	4.568
	H-2α	4.051	ND[c]
	H-2β	3.850[d]	3.830[e]
	H-5α	4.164	4.159
	H-5β	3.755[d]	3.743[e]
	$CH_3\alpha$	1.202	1.202
	$CH_3\beta$	1.243	1.242
β-GlcNAc3SO_3^-	H-1	4.816	
	H-2	3.834[d]	
	H-3	4.407	
	H-4	3.629[d]	
	H-5	3.514[d]	
	NAc	2.017	
β-GlcNAc	H-1 (α)		4.685
	H-1 (β)		4.674
	NAc		2.044
β-Gal3SO_3^-	H-1		4.718
	H-2		3.912[e]
	H-3		4.417
	H-4		4.286
α-Gal	H-1		5.411
	H-2		3.765[e]
	H-3		3.953
	H-4		4.006
	H-5		4.338

[a] Recorded at 600 MHz.
[b] Recorded at 500 MHz.
[c] Not determined.
[d] From the HOHAHA experiment, recorded at 283 K.
[e] From the HOHAHA experiment.

conditions of electron ionization in order to induce fragmentation of the deoxyhexitol residue. Intense fragment ions arising by A^+-type cleavage of the nonreducing terminal GlcNAc residue were observed at m/z 260 and 228 (corresponding to elimination of methanol), whereas ions at m/z 423, 379, 392, and 436, arising from cleavage within the deoxyhexitol residue, unambiguously define the attachment of the GlcNAc residue to *O*-3 of the deoxyhexitol (see fragmentation, Scheme 1).

Taken together the analytical data allow the structure,

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GlcNAcβ1−3Fuc
|
3SO3
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STRUCTURE 2

to be assigned to the Block 2-reactive oligosaccharide.

Structural Characterization of Oligosaccharide C-1—The main nonreactive oligosaccharide C-1 eluted slightly after the Block 1-reactive oligosaccharide from both the DEAE anion exchange and the HPLC columns. Its apparent size on gel filtration was 6.5 glucose units. MALDI-MS yielded a pseudomolecular ion at m/z 770 ($[M-H]^-$). Analysis of the sugar composition indicated that C-1 is a tetrasaccharide containing two galactose residues, one *N*-acetylglucosamine, and one fucose residue (Table I). As in the Block 1- and Block 2-reactive saccharides, fucose is the reducing terminal residue.

Enzymatic Degradation of Oligosaccharide C-1—Attempts to digest the native oligosaccharide with α- or β-galactosidases or hexosaminidase failed, apparently because of the presence of the charged moiety (sulfate, see below) in the oligosaccharide. In order to attempt the removal of the charged group from the oligosaccharide, a ^{3}H-reduced sample was hydrolyzed in 10 mM HCl for 2 h at 100 °C. The products were separated on an anion exchange column into neutral and acidic fractions. Analysis of the two fractions by gel filtration revealed two acidic and four neutral components (Fig. 5, *A* and *D*). The first acidic component (aI) eluted at the same position as the native oligosaccharide. The second acidic component (aII) was smaller and was shown to contain 1 mol of galactose less on monosaccharide analysis (data not shown). Both acidic components were resistant to α- and β-galactosidase digestion (Fig. 5, *B* and *C*). Under exhaustive conditions (100-fold higher enzyme concentration) a minor degradation of component aI by α-galactosidase could be observed.

In contrast to the charged degradation products, the neutral products were susceptible to galactosidase digestion. Component nI (the native oligosaccharide without the charged group) was degraded by α-galactosidase but not by β-galactosidase and gave a product that eluted at the position of component nII (Fig. 5*E*). The incomplete degradation of component nI by α-galactosidase could be improved by prolonged incubation with higher amounts of enzyme, which indicates that it is an unfavorable substrate for the enzyme. Component nII could be degraded by β-galactosidase but not by α-galactosidase (Fig. 5*F*) and was converted into a product that eluted at the position of component nIII. Component nIII was in turn digested by prolonged incubation with hexosaminidase into component nIV, which coeluted with fucitol (not shown).

The results of the enzymatic degradation of the components obtained by mild acid treatment of the C-1 oligosaccharide indicate that the tetrasaccharide consists of the core disaccharide GlcNAc-Fuc extended with one α-Gal and one β-Gal residue. Because the β-Gal residue could only be removed after the removal of the α-Gal residue, the α-Gal residue was suggested to be distal to the β-Gal residue. However, because of possible steric hindrance effects, a branched structure could not be excluded by these data.

FAB-MS of Oligosaccharide C-1—Positive ion FAB mass spectrometric analysis of C-1 yielded an intense $[M+H]^+$ pseudomolecular ion at m/z 772 accompanied by sets of less intense ions at m/z 789 ($[M+NH_4]^+$), 794 ($[M+Na]^+$), 806 ($[M-H+2NH_4]^+$), and 811 ($[M-H+2Na]^+$), consistent with a phosphorylated or sulfated $Hex_2HexNAc_1Deoxyhex_1$ tetrasaccharide. An intense fragment ion was also observed at m/z 692, which indicates cleavage of a sulfate moiety. In order to determine the sequence, branching pattern, and site of sulfate substitution of the tetrasaccharide, it was permethylated. Negative ion FAB-MS analysis of the product yielded an $[M-H]^-$ pseudomolecular ion at m/z 938, corresponding to full methylation of the oligosaccharide portion, without incorporation of any methyl groups into the acidic moiety, again consistent with the assignment of a sulfate (formation of monomethyl esters of phosphate groups under the conditions used is expected (28)). Collision-induced dissociation MS-MS analysis of the m/z 938 precursor ion resulted in extensive fragmentation (Fig. 6). The structures of the fragment ions produced in this collision-induced dissociation experiment and the mechanisms of their formation appear to be directly analogous to those described by Dell *et al.* (29) and Pfeiffer *et al.* (30) for fragment ions generated in the ionization source from permethylated monosulfated oligosaccharides. The fragment ions allow the structure of the tetrasaccharide sulfate to be defined as the linear sequence Hex-Hex-HexNAc-Deoxyhex, in which the sulfate group is attached to the internal Hex, which is linked either to *O*-4 or *O*-6 of the adjacent HexNAc (Fig. 6).

^{1}H NMR Spectroscopy of Oligosaccharide C-1—The chemical shifts of the ^{1}H resonance of C-1 are listed in Table II. The one-dimensional 500-MHz ^{1}H NMR spectrum of C-1 (not shown) reveals two α- and four β-anomeric signals. A comparison of the spectra of oligosaccharide C-1 and the Block 2-reac-

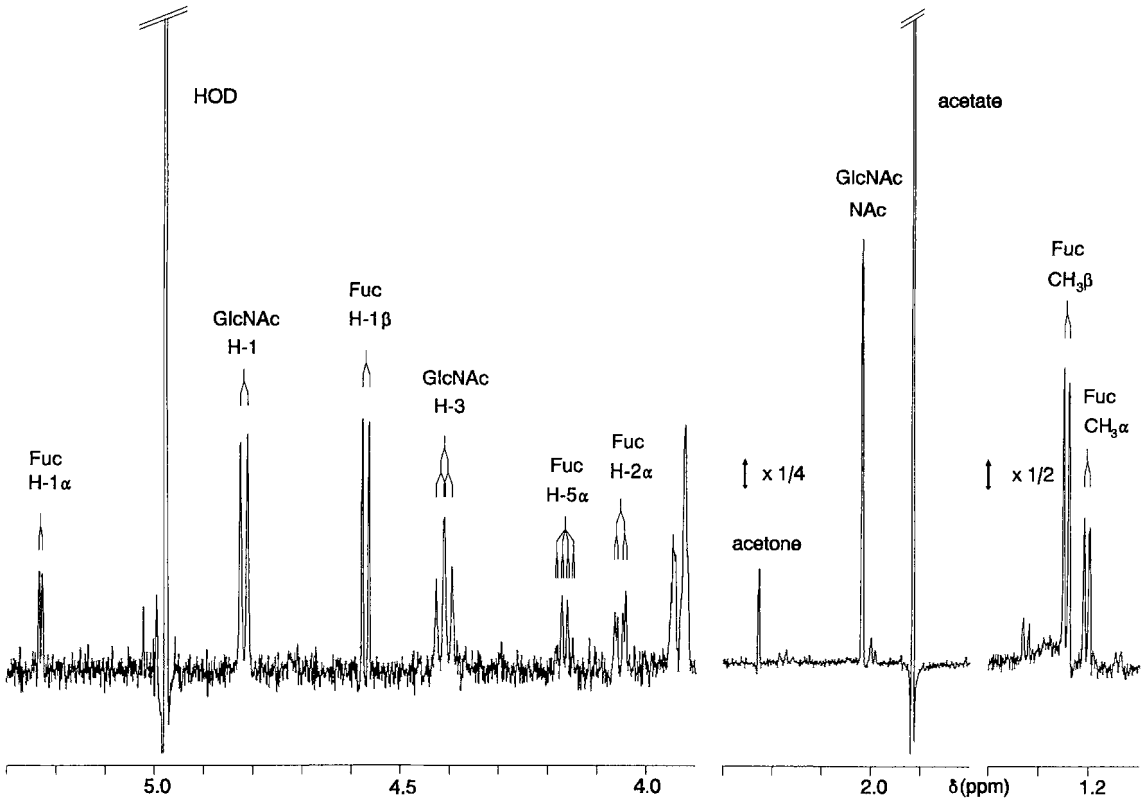
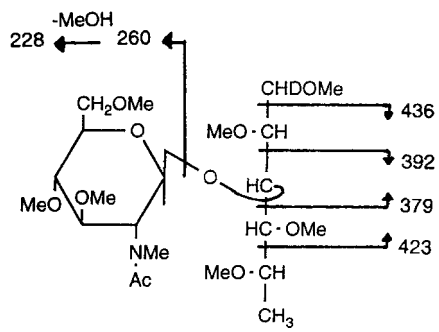


FIG. 4. **^{1}H NMR of the Block 2-reactive oligosaccharide.** Selected regions of the resolution-enhanced 600 MHz ^{1}H NMR spectrum, recorded in ^2H_2O at 293 K, are shown. The relative intensity scale of the *N*-acetyl and Fuc-CH_3 proton regions differ from that of the other parts of the spectrum as indicated.

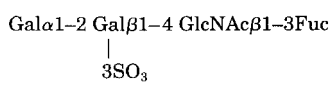


SCHEME 1

tive oligosaccharide disclosed the presence of Fuc in the reducing position for oligosaccharide C-1. The Fuc chemical shifts are essentially the same for both structures. This accounts for the anomeric signals at 5.228 and 4.568 ppm, which were attributed to α-Fuc H-1 and β-Fuc H-1, respectively. Together, these signals have the intensity of one proton. The overlapping doublets at 4.685 and 4.674 ppm are both assigned to the same anomeric proton that experiences the presence of neighboring reducing Fuc. This is in accordance with the intensities of these signals. Thus, the number of residues is limited to four, previously identified as Fuc, GlcNAc, Gal, and Gal, by compositional analysis. The identification of these residues is corroborated by the patterns of the monosaccharide spin systems in the HOHAHA spectrum. The α-anomeric signal at 5.411 ppm ($^3J_{1,2}$ = 4.0 Hz) and the β-anomeric signal at 4.714 ppm ($^3J_{1,2}$ = 7.7 Hz) both belong to a Gal type pattern, and the β-anomeric signals at 4.68 ($^3J_{1,2}$ = 8.0 Hz) agree with a Glc type spin system. The β-Gal H-3 and H-4 signal were shifted downfield from the bulk of skeleton protons (4.0–3.5 ppm) to 4.417 and 4.286 ppm, respectively. The assignments of β-Gal H-3 and H-4 are based on the coupling constants $^3J_{2,3}$ = 10.1 Hz and $^3J_{3,4}$ = 3.4 Hz. The position of β-Gal H-3 indicates the presence of sulfate, following the same reasoning as for Block 2. Sulfate is linked to C-3 and not to C-4, although both β-Gal H-3 and H-4 are shifted downfield from the bulk of skeleton protons (compare β-Gal H-3 and H-4 from oligosaccharide C-1 with Gal H-3 at 4.34 ppm and Gal H-4 at 4.29 ppm in Gal3SO_3^-β1–4GlcNAcβ1- (21, 26, 27)). If sulfate were bound to Gal C-4 instead of C-3, only Gal H-4 (and not H-3) would be shifted downfield from the bulk of skeleton protons (*e.g.* Gal H-4 resonates at 4.692 ppm in GalNAc4SO_3^-β1-GlcNAcβ1- (31)). Thus the oligosaccharide C-1 is a tetrasaccharide with Fuc at the reducing terminus and β-GlcNAc linked to Fuc and additionally containing β-Gal3SO_3^- and α-Gal (Fig. 7).

GC-MS of Oligosaccharide C-1—GC-MS analysis of the partially methylated alditol acetates obtained from desulfated C-1 allowed identification of derivatives corresponding to terminal hexose, 2-substituted hexose, 4-substituted HexNAc, and 3-substituted deoxyhex residues, allowing the following linear sequence to be defined: Hex1–2Hex1–4HexNAc1–3Deoxyhex.

Taken together, the analytical data allow the following structure to be assigned to oligosaccharide C-1.



STRUCTURE 3

DISCUSSION

The involvement of carbohydrates in the aggregation process of dissociated cells from the marine sponge *M. prolifera* has been suggested on the basis of studies using carbohydrate-degrading treatments (5, 32). In subsequent studies, the iso-

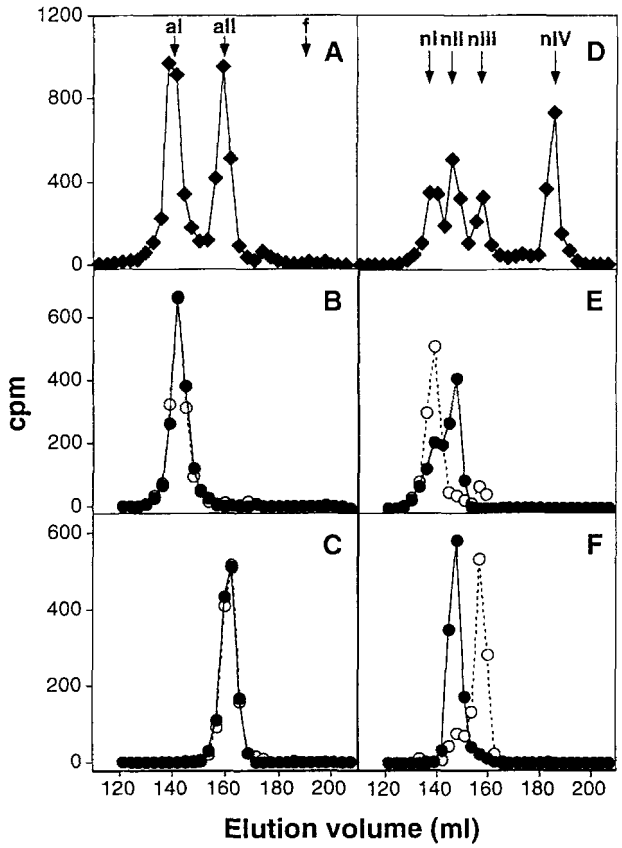


FIG. 5. **Sequential degradation of oligosaccharide C-1 with glycosidases.** The purified C-1 oligosaccharide labeled by reduction with NaB^3H_4 and subjected to mild acid treatment (10 mM HCl for 2 h at 100 °C) was separated into acidic (*panel A*) and neutral (*panel D*) products on a DEAE ion exchange column and analyzed by gel filtration on a Bio-Gel P-2 column. The elution volumes of the acidic (*aI* and *aII*) and neutral (*nI–nIV*) saccharides and of fucitol (*F*) are indicated by *arrows*. *Panels B* and *C*, *peaks aI* and *aII*, respectively, rechromatographed after α-galactosidase (●——●) and β-galactosidase digestions (○——○). *Panels E* and *F*, *peaks nI* and *nII*, respectively, rechromatographed after α- and β-galactosidase digestions.

lated and polymerized glycans of the adhesion proteoglycan were shown to promote species-specific aggregation in the absence of the protein backbone (6). The self-interaction of these glycan polymers suggested a carbohydrate-carbohydrate interaction mediated by multiple low affinity interactions. Low affinity interactions are more difficult to study than interactions based on high-affinity binding. The development of functional antibodies that block sponge cell aggregation and recognize epitopes in the carbohydrate portion of the molecule (6, 12) was therefore used as an approach to identify the carbohydrate deteminants involved in the interaction.

By the technique of attaching the highly water-soluble acid-degraded fragments to a lipid carrier for immunochemical detection (13, 33) and by the combination of chemical, enzymatic, and spectroscopic methods of structural analysis Structure 2 was assigned to the oligosaccharide reacting with the aggregation-blocking monoclonal antibody Block 2. Glucosamine sulfated at *O*-3 is a rare component of heparin, but because of the biosynthetic pathway, it occurs only in combination with *N*-sulfation and is part of the highly specific sequence required for binding of antithrombin (34). However, neither *N*-acetylglucosamine 3-sulfate nor *N*-acetylglucosamine 3-sulfate linked (β1,3) to fucose have been described before. The Block 2-reactive oligosaccharide thus represents a unique novel structure.

Although the oligosaccharide isolated was the most prominent fragment reacting with the Block 2 antibody, the possibility cannot be excluded that the complete epitope may include portions proximal to the reducing fucose residue. The sulfate ester on the Block 2-reactive oligosaccharide is considerably more acid-sensitive than the pyruvate residue on the Block 1-reactive structure. Because of this acid sensitivity the selection of the conditions of partial acid hydrolysis had to be made carefully. Whereas the window of stability was quite broad for the Block 1 epitope, the corresponding characteristics for the Block 2-reactive species were narrower, and the acid lability resulted in a reduced recovery of this epitope. Some slower moving bands with reactivity to Block 2 antibody rapidly lost this reactivity and were not obtained in amounts amenable to structural characterization. It was also not possible to recover Block 2 oligosaccharides in a yield corresponding to the theoretical amount of antibody binding sites determined for the intact adhesion proteoglycan (12).

The distinct reactivity of the isolated saccharide with the Block 2 antibody suggests that it represents an integral part of the epitope. Among the isomeric *N*-acetylglucosamine 3-, 4-, and 6-*O*-sulfates examined as inhibitors of the antibody binding, only the 3-*O*-sulfate exhibited some weak inhibitory activity,[2] which on one hand suggests that the position of the sulfate ester is critical and on the other hand indicates that the fucose residue is an important part of the epitope. The approximately 10-fold lower apparent affinity of the Block 2 antibody for its saccharide as compared with that of the Block 1 antibody with its epitope oligosaccharide[2] may be explained by changed reactivity caused by the opened fucose ring of the lipid-linked derivative in the TLC immunoassay.

In order to obtain information on the structure of the adhesion proteoglycans and to isolate a control oligosaccharide for antibody specificity testing, the most prominent oligosaccharide in the mixture of acid-degraded oligosaccharides that showed similar properties during purification was isolated. By employing chemical and enzymatic analyses in combination with NMR and FAB-MS Structure 3 was assigned to the C-1 oligosaccharide. The Galα1–2 Gal sequence is a rare constituent of glycoconjugates, being previously characterized in earthworm cuticle collagen (35) and in the capsular polysaccharide (type S-15B and S-15C) from *Streptococcus pneumoniae* (36). The latter structure is remarkably similar to that of the C-1 oligosaccharide in that it contains the same tetrasaccharide sequence with a phosphate instead of the sulfate ester and a galactose instead of the fucose residue. However, a structure identical to the C-1 oligosaccharide seems not to have been described before. For the determination of the molecular requirements of sponge cell aggregation, the C-1 oligosaccharide serves as an important charged control molecule with structural features similar to those present in the Block 1 and Block 2 oligosaccharides.

No information is as yet available on the overall structure of the glycans of the sponge adhesion proteoglycan. Although the glycans contain *N*-acetylglucosamine, galactose, uronic acid, and sulfate, they seem not to represent any of the main classes of glycosaminoglycans of higher animals, as indicated by the large amount of fucose and their resistance to common glycosaminoglycan-degrading enzymes (12). Preliminary NMR analyses of the intact glycan chains indicate such structural diversity that the possible presence of a common backbone with a repeating saccharide unit cannot be determined.

The oligosaccharides so far characterized all contain the same GlcNAcβ1–3Fuc disaccharide unit at their reducing termini. The presence of reducing terminal fucose seems to be

[2] D. Spillmann and J. Finne, unpublished results.

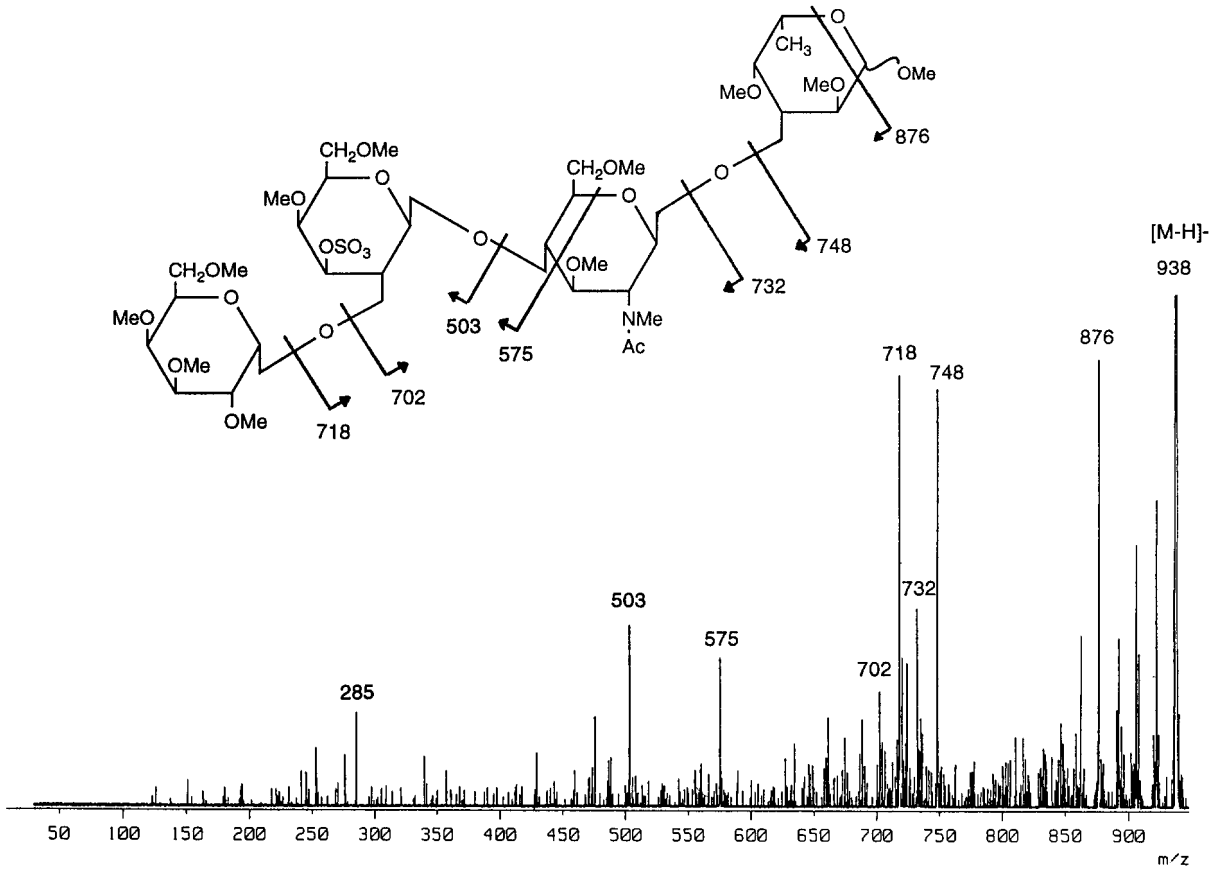


FIG. 6. **Negative ion collision-induced dissociation MS-MS of permethylated C-1 oligosaccharide.** The ion at *m/z* 285 is a double cleavage ion arising by combination of the cleavage that produces the ion at *m/z* 718 with that producing the ion at *m/z* 503.

caused by the acid lability of the fucosyl-linkage (37). On the other hand the occurrence of the same disaccharide unit in all the fragments characterized may suggest that it is part of a linear repeating unit or that it represents a common branch unit. The use of alternative cleavage methods would be needed to solve this question, but these have so far not been used because of a loss of the antibody reactivity of the fragments obtained. Endoglycolytic enzymes should be useful for the analysis of large structures consisting of repeating units. However, enzymes such as chondroitinase or heparinase/heparitinase that cleave sulfated glycosaminoglycans do not cleave the sponge adhesion proteoglycan. The difficulty with most exolytic enzymes is that the presence of a charged group such as a pyruvate acetal or sulfate ester prevents the action of the enzymes and that unusual sequences like the rare Galα1–2Gal and GlcNAcβ1–3Fuc units are cleaved only slowly.

The two epitope saccharides characterized for the Block 1 and Block 2 antibodies as well as the C-1 oligosaccharide represent distinct molecular species both in their structure and reactivity (Table III). The existence of distinct epitopes involved in the carbohydrate-carbohydrate-mediated interaction suggests that the species specificity of sponge cell aggregation is not determined by the arrangement of single monomeric residues distributed in proper spacing but rather by the specific sequences that mediate the interaction. Although the same "core" disaccharide is present in the structures, it is evident that the Block 2 antibody recognizes the 3-*O*-sulfate on the *N*-acetylglucosamine moiety but does not interact with the corresponding nonsulfated sequence within the other two structures or with the 3-*O*-sulfate on the galactose of C-1. On the other hand, the terminal charged pyruvate group of the Block 1-reactive oligosaccharide is similarly an essential part

TABLE III
Oligosaccharide fragments of the sponge adhesion proteoglycan and their reactivities with the aggregation-blocking monoclonal antibodies Block 1 and Block 2

Oligosaccharide	Block 1	Block 2
Pyr<6,4>Galβ1–4GlcNAcβ1–3Fuc	+	–
Galβ1–4GlcNAcβ1–3Fuc	–	–
GlcNAcβ1–3Fuc | 3-SO_3	–	+
Galα1–2Galβ1–4GlcNAcβ1–3Fuc | 3-SO_3	–	–

of the Block 1 epitope (13). In view of the finding that sulfate can subsitute for sialic acid in sialyl Le^a and sialyl Le^x interaction of E-selectin (38), it is of interest that the charged pyryvate group on the galactose moiety in Block 2 cannot be substituted with the sulfated galactose structure present in C-1, although the additional α-galactose moiety may also be inhibitory. Both charged groups may potentially represent binding sites for the Ca^{2+} ions needed in the aggregation process.

Carbohydrate-carbohydrate interactions between mammalian cells are suggested to be mediated by both heterotypic interactions between different structures as in the interaction of the G_{M3} ganglioside with the Gg3Cer, LacCer, or Gb3Cer glycolipids (39, 40) or homotypic interactions involving similar structures as in the case of the Le^x or H glycolipids (41). Although all the evidence available indicates that the aggregation of sponge cells involves a carbohydrate-carbohydrate interaction of the glycans in the adhesion proteoglycan and both

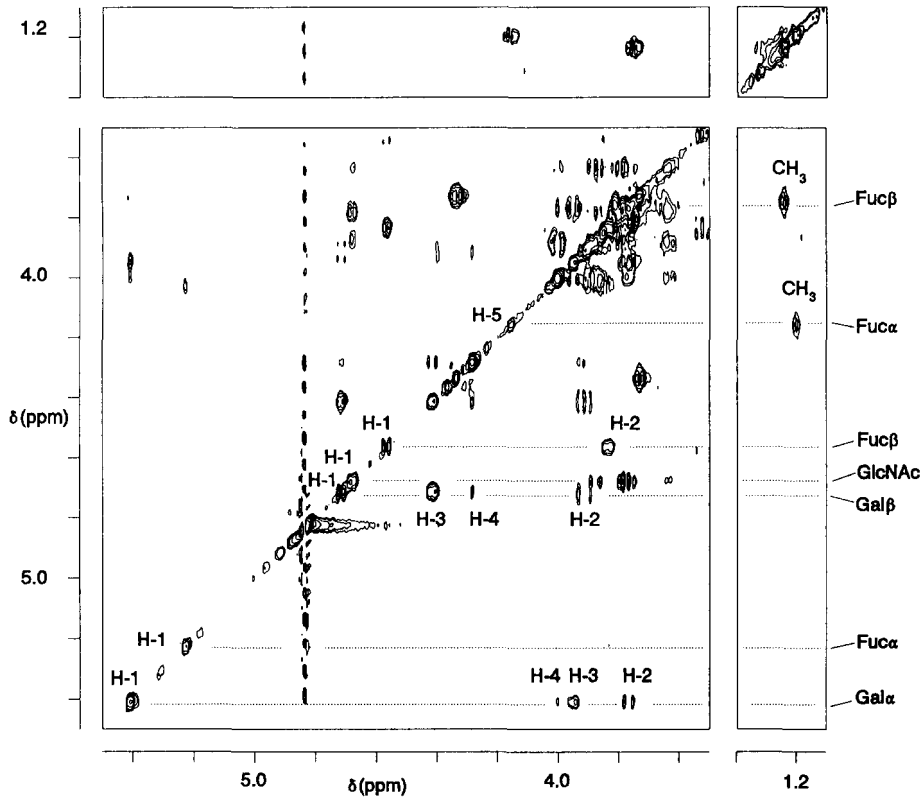


FIG. 7. **NMR spectrum of oligosaccharide C-1.** 500-MHz ^1H NMR two-dimensional HOHAHA spectrum with a mixing time of 57 ms of oligosaccharide C-1, recorded in ^2H_2O at 293 K.

Block 1 and Block 2 antibodies inhibit the aggregation (6, 12), the specific roles of the two structures characterized remain open. The presence of two distinct sites, Bock 1 and Block 2, that participate in the interaction whereas a similar charged structure like the C-1 is not recognized suggests that the binding is not just caused by nonspecific electrostatic interactions of the acidic carbohydrate chains. The inhibition of the aggregation by either of the two antibodies could be explained by a heterologous interaction of several carbohydrate sites (Block 1) on one chain with several sites (Block 2) on the other chain. However, it is also possible that homologous sites could interact with one another and that an interruption by only one antibody is sufficient to disrupt the entire interaction. The phenomenon of multiple low affinity interaction sites is a feature difficult to follow experimentally but undoubtly of large biological potential not restricted to carbohydrate-carbohydrate interactions (10, 42). The identification of the structures recognized by functional antibodies will now make possible studies designed to test the different alternatives in order to reveal the molecular basis of sponge cell adhesion.

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