Carbohydrate-Carbohydrate Interactions of a Novel Acidic Glycan Can Mediate Sponge Cell Adhesion*

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Cell recognition and adhesion were first demonstrated in marine sponges. These phenomena were later shown in *Microciona prolifera* sponge to be mediated by a Ca$^{2+}$-dependent self-association of adhesion proteoglycans (APs) attached in a species-specific manner to cell-surface receptors. Using the same experimental system we now provide three lines of evidence that highly polyvalent Ca$^{2+}$-dependent carbohydrate-carbohydrate interactions of a novel AP glycan represent the basis of AP-AP self-binding and thus of cell adhesion. 1) A specific monoclonal antibody which blocks cell aggregation and AP bead adhesion identified a highly repetitive novel carbohydrate epitope (2500 sites) in an acidic glycan of $M_r = 200 \times 10^3$ (g200) from AP. 2) Reconstitution of the Ca$^{2+}$-dependent self-interaction activity of AP was achieved by cross-linking the purified protein-free g200 glycan into polymers of similar valency as the native AP. 3) Beads coated with the protein-free g200 glycan showed a Ca$^{2+}$-dependent aggregation equivalent to that of AP beads. Carbohydrate and amino acid analyses of the g200 glycan purified by gel electrophoresis, high performance liquid chromatography gel filtration, and ion exchange chromatography yielded six components in the following proportions; 68 fucose, 32 glucuronic acid, 2 mannose, 18 galactose, 19 N-acetylglucosamine, and 1 asparagine residue. These unique chemical features together with immunological and enzymological analyses suggest that the g200 glycan is a high and fucosylated, acidic, N-linked polysaccharide with a novel structure distinct from that of other known glycosaminoglycans.

Species-specific reaggregation of dissociated marine sponge cells was the first experimental system to provide direct evidence for the existence of cell recognition and adhesion (Wilson, 1907; Gaitsoff, 1923). Later studies (Humphreys, 1963; Henkert et al., 1973; Cauldwell et al., 1973) with *Microciona prolifera* sponge revealed that both of these cell-interaction processes are mediated by an adhesion proteoglycan molecule (AP), originally known as *Microciona* aggregation factor. Further analyses of the molecular mechanism of AP-promoted cell aggregation have shown that two different functions reside within AP, namely a Ca$^{2+}$-independent cell-binding and a Ca$^{2+}$-dependent AP-AP self-association activity (Jumblatt et al., 1980; Misevic et al., 1982; Misevic and Burger, 1986). The cell-binding site of AP was localized in one of the major AP glycans of $M_r = 6.3 \times 10^5$ termed g6 (Misevic and Burger, 1990a, 1990b). Although g6 monomers have very low and experimentally unmeasurable affinity for the receptor, the species-specificity and high affinity of AP-cell interactions are brought about by the cooperative binding of 1000 copies of the g6 glycan in a single AP molecule. The mechanism of such highly polyvalent, but very low single site affinity binding of glycan adhesion molecules to their receptors is conceptually different to that of the well known, higher affinity monovalent or tetravalent interactions of: 1) the immunoglobulin cell adhesion molecules (Moran and Bock, 1988); 2) the integrin receptor family (Aumaillely et al., 1987; Hautanen et al., 1989); 3) lectins and specific glycans (Baenziger and Fiete, 1979; Nybroe et al., 1988; Roberts et al., 1986; Roberts et al., 1987); and 4) the cadherins (Takeichi, 1991). Jumblatt et al. reported in 1980 that the cell-binding function of AP alone was not sufficient to promote cell adhesion, but adhesion did occur when AP-AP self-interaction was activated by the addition of physiological seawater containing 10 mM Ca$^{2+}$. It was also shown in a cell-free system that the AP-AP binding can mediate aggregation of AP-coated beads. The authors concluded that the major force promoting cellular adhesion was provided by AP-AP self-binding.

The Ca$^{2+}$-dependent AP-AP self-interaction domain was localized within the carbohydrate portion of AP using specific monoclonal antibodies (Misevic et al., 1987). Characterization of the functional self-interacting AP glycan(s) and the molecular mechanism by which they mediate cell adhesion required further study. In this article we report that the novel acidic glycan of $M_r = 200 \times 10^3$ (g200) isolated from the sponge AP promotes cell adhesion via Ca$^{2+}$-dependent homophilic carbohydrate-carbohydrate interactions. Chemical analyses combined with enzymatic and immunological studies of purified g200 revealed that this N-linked highly fucosylated and negatively charged polysaccharide of unique carbohydrate composition (fucose, glucuronic acid, galactose, N-acetylgalactosamine, and mannose) suggests a novel structure distinct from that of previously characterized glycosaminoglycans.

**MATERIALS AND METHODS**

*Sponges—*M. prolifera* sponges were collected by the Marine Biological Laboratory Marine Resources Department or by the authors.

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1 The abbreviations used are: AP, adhesion proteoglycans BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay, HPLC, high performance liquid chromatography.
during summer periods in the area of Woods Hole, Massachusetts. Only freshly collected spongoids were used for preparation of cells and AP.

**Isolation and Radioiodination of AP**—AP was isolated according to the previously described procedure of Henkart et al. (1973). Final purification was performed as described by Misevic et al. (1987). AP was isolated using cross-counter chromatography (Misevic, 1989).

**Isolation and Radiolabeling of AP Glycans**—The protein part of purified AP was extensively digested with Pronase as previously described. Final purification was performed as described by Misevic et al. (1987; Finne and Krusius, 1982). Typically, 9 ml of AP (1 mg/ml) in CaCl₂ and Mg²⁺-free artificial seawater buffered with 20 mM Tris, pH 7.4 (CSW), and supplemented with 2 mM CaCl₂, was digested with Pronase type B (Boehringer Mannheim) which have been preincubated for 30 min at 60 °C and was added three times at 24-h intervals, during which digestion was carried out at 60 °C. Amino acids and small peptides were removed from total AP glycans by gel filtration and ion exchange chromatography (Misevic et al., 1987). One part of the total glycans were N-acylated by 1H-labeled acetic anhydride (25 μCi/ml, 500 μCi/mmol) as described by Finne and Kruisius (1988).

**Cross-linking of Glycans**—Combination of polyfunctional and bi-functional reagents glutaraldehyde and diepoxybutane was used to increase the efficiency of glycans cross-linking. The dried purified protein-free g200 glycans (500 μg) were dissolved in 10 μl of 0.3% glutaraldehyde in 0.1 M NaHCO₃, pH 9.0 (preincubated for 30 min at room temperature), and cross-linking was allowed to proceed for 12 h at room temperature. Subsequently 2 μl of 50% diepoxybutane in 0.1 M NaHCO₃, pH 9.0, was added and the mixture was incubated for 1 h at room temperature. Polymers with Mₓ > 15 × 10⁶ were separated from smaller molecules by gel filtration on a Bio-Gel A-15m column (1 × 41 cm) (Misevic and Burger, 1990b). In order to label cross-linked g200 polymers through unreacted groups of covalently linked glutaraldehyde and/or diepoxybutane, 50 μl of L-[¹⁴C]tyrosine (1 mCi/ml, 100 Ci/mmol) was used. Upon 1 h labeling, residual aldehyde and epoxy groups were quenched with 0.1 M Tris, pH 7.4, and unbound radioactivity was separated by gel filtration.

**Monoclonal Antibodies**—BALB/c mice were immunized with AP as described previously. Monoclonal antibodies were prepared from culture supernatants of AP-positive clones obtained after fusion of mice spleen cells with the FO myeloma line and were prepared from culture supernatants of AP-positive clones obtained after fusion of mice spleen cells with the FO myeloma line and were thus termed Block antigens. The purified Fab fragments from this antibody were assayed for their ability to inhibit AP-promoted cell-cell adhesion. Dissociated cells depleted of their endogenous AP were incubated with a constant amount of the purified AP in the presence or absence of the isolated Fab fragments from clone 17. As shown in Fig. 1, these Fab fragments blocked cell reaggregation in a concentration-dependent manner and were thus termed Block 2.

**RESULTS**

**Functional Characterization of the Cell Adhesion Blocking Anti-proteoglycan Antibody**—Using ELISA and immunodot assays, the monoclonal antibody isolated from clone 17 was shown to be directed against the M. prolifera sponge adhesion proteoglycan (Table I). The purified Fab fragments from this antibody were assayed for their ability to inhibit AP-promoted cell-cell adhesion. Dissociated cells depleted of their endogenous AP were incubated with a constant amount of the purified AP in the presence or absence of the isolated Fab fragments from clone 17. As shown in Fig. 1, these Fab fragments blocked cell reaggregation in a concentration-dependent manner and were thus termed Block 2.

**Polyacrylamide Gel Electrophoresis, Immunoblotting, and Immunobinding Retardation Assay**—Polyacrylamide gel electrophoresis of glycans was performed according to Lampson and Gallagher (1984) with some modification (Misevic, 1989). Immunoblotting on DEAE-nitrocellulose and immunobinding retardation assays were done as described previously (Misevic, 1989). Blockage of gel pores by excess antibody was minimized by employing less than 1 μg of glycans together with saturating levels of antibody. Gels were stained with an Alcian blue-enhanced silver method (Min and Cowman, 1986).

**Functional Assays**—Assays of cell aggregation and inhibition of AP-promoted cell adhesion were performed as previously described (Misevic et al., 1987) with fixed M. prolifera cells in 0.4 ml of seawater (2 × 10⁶ cells/ml). Equivalent results were obtained with living cells. The binding of radiolabeled AP, monomeric glycans, and cross-linked glycans to fixed cells (2 × 10⁵ cells) was assayed in 130 μl of CSW (Misevic and Burger, 1990b).

The self-binding assay with ¹²⁵I-AP, H-g200, and H-xg200 was performed in 100 μl of CSW containing 0.5% BSA and with inclusion of 2 or 10 mM CaCl₂. After incubation for 20 min at room temperature, samples were centrifuged for 10 min at 10,000 × g and the radioactivity in supernatants and redissolved pellets was determined (Misevic et al., 1987).

AP was covalently coupled to agarose beads as previously described (Johansen et al., 1980). AP and g200 were adsorbed to aminopropyl glass beads (50 μm in diameter, 75-Å pore size, 231 μm amine content/g; Sigma) in CSW. Saturable coating of beads with a similar number of g200 molecules per bead was reached after addition of 100 μg of g200, or 10 μg of the intact AP per 10 μl of dry beads. After washing of beads with CSW, adhesion of both g200-beads and AP-beads was monitored in the presence of 2 and 10 mM CaCl₂.

**Enzymatic Treatment of g200**—In a typical experiment 10 μg of g200 was treated with 0.1 unit of chondroitinase ABC (EC 4.2.2.4), pH 8.0, heparinase (EC 4.2.2.7), pH 7.0, heparinase (EC 4.2.2.8), pH 7.0, hyaluronidase (EC 4.2.2.1), pH 6.0, and keratinase (EC 3.2.19-5), pH 7.4. All enzymes were purchased from Seikagaku Kogyo Corp. After a 6-h digestion at 37 °C samples were analyzed either by gel electrophoresis on polyacrylamide gels stained by Alcian blue, or by high frequency liquid chromatography gel filtration (Bio-Rad) on a TSK 400 XL (300 × 7.8 mm) column with continuous monitors of absorption at 206, 222 nm, and the refractive index.

**Analytical Methods**—Carbohydrate analysis of AP glycans was performed according to Chaplin (1982) after methanolysis and trimethylsilylation either in a Hewlett-Packard gas chromatograph 7620A using a glass column (2 mm × 2 m) packed with 4% SE-30 on 100/120 Supelcoport, or in a Shimadzu GC-RIA gas chromatograph using a fused silica SPB-1 Supelco capillary column (0.53 mm × 15 m). Amino acid analysis was done following hydrolysis of samples with ¹M HCl using the Pico-Tag method (Bidelmgreen et al., 1984; Henrikson and Meredith, 1984). Colorimetric reactions for neutral hexose, uronic acid, sulfate, and phosphate were conducted as previously described (Shields and Burnett, 1960; Duche, 1947; Spencer, 1960; Stewart, 1974).
iodinated AP to homotypic cells, even when the molar ratio of Fab to AP was 5000:1 (Table II). The Block 2 monoclonal antibody therefore appears to preclude cell adhesion through a direct inhibition of AP self-interaction rather than inhibition of AP-cell binding.

Adhesion Epitope of AP Is a Novel Carbohydrate Structure Localized in the g200 Glycan—The ability of the Block 2 antibody to inhibit the self-interaction of AP enabled its use in determining whether this functional domain is localized in the carbohydrate or protein part of AP. For this purpose, purified AP was first delipidated to remove possible contaminating lipids and then extensively treated with Pronase to digest the protein part of the proteoglycan molecule. The amino acids and small peptides were separated from the glycans by gel filtration (see “Materials and Methods”). This protein-free carbohydrate fraction of AP was then electrophoresed on a preparative 7.5–20% linear polyacrylamide gel (Fig. 2A, lane a). Two major acidic glycans were identified by Alcian blue staining (Fig. 2A, a–c). According to hyaluronic acid and chondroitin sulfate standards, the larger glycan had apparent $M_r = 200 \times 10^3 \pm 40 \times 10^3$ (g200). The smaller glycan of $M_r = 6 \times 10^4$ (g6) has been previously described (Misevic and Burger, 1990a, 1990b). Colorimetric assay procedures and gas chromatography carried out on the gel-eluted g200 glycan enabled us to determine that it contains 37% of the total AP carbohydrate. Of this amount 75% represented neutral hexose and 25% uronic acid. Taken together with the results of Alcian blue-stained gels, these findings suggest that g200 is the major acidic polysaccharide of AP (Fig. 2A, lanes a–c).

To examine whether acidic polysaccharides of AP are recognized by the Block 2 antibody, an immunobinding gel electrophoresis retardation assay was performed. The total AP glycans were mixed with the Block 2 antibody (5 and 20 $\mu$g), incubated for 2 h, and applied to a 7.5–20% polyacrylamide gel. As shown in Fig. 2B, Block 2 was bound to g200 but not g6 as inferred from standard electrophoretic mobility of the g6 glycan (non-bound) coupled with the lack of migration of the g200-Block 2 complex into gel. This selectivity of Block 2 for g200 was confirmed by immunoblots carried out on electrophoretically separated AP glycans (Fig. 2A, lanes d and e).

**TABLE I**

| 125I Antigen | No. of binding sites | $K_s$ | Inhibition of 125I Block 2 binding to AP
|---------------|----------------------|-------|---------------------------------|
|               | mol/mol               | $M$   | $\mu$                           | $\mu$ |
| Block 2 IgG6  | 600 1.0 $\times 10^6$ | 3–5   | 3–5                             | 4–6   |
| Block 2 Fab   | 2500 2.1 $\times 10^6$ | ND    | ND                             | ND    |

a Range of the amount of ligand needed to cause 100% inhibition of sub-saturating 0.5 $\mu$g of 125I-labeled Block 2 binding to 40 ng of AP.

1 Amount of AP carbohydrate added per well.

2 Amount of AP carbohydrate added per well.

3 ND, not determined.

Two different experiments were performed to determine antibody specificity and to characterize the structure of the Block 2 antibody recognition site on the g200 carbohydrate. In the first assay different types of carbohydrate structures were tested as putative inhibitors of the Block 2-AP binding. They include chondroitin sulfate A, B, and C, heparan sulfate, keratan sulfate, hyaluronic acid, and heparin at concentrations of 10 mg/ml, fetuin glycopeptides at concentrations of 10 mg/ml, and various monosaccharides (L-fucose, d-mannose, d-galactose, N-acetyl-d-glucosamine, N-acetyl-d-galactosamine, d-glucuronic acid, and d-galacturonic acid) at a concentration of 0.5 M. None of these compounds were able to inhibit the binding of 0.5 $\mu$g of 125I-labeled Block 2 to 40 ng of AP. In contrast, native AP, protein-free glycans isolated from AP, and purified g200 completely inhibited the binding of the antibody to AP (Table I). In the same study it was shown that the smaller AP glycan g6 was non-inhibitory (Table I). In a second series of experiments, immunodot analyses with the above-mentioned glycosaminoglycans confirmed that Block 2 selectively recognizes only acidic glycans isolated from AP (not shown). These immunological data strongly suggest that the structure of the carbohydrate epitope for the Block 2 antibody is different from that of previously characterized glycosaminoglycans, N-linked mannos core oligosaccharides, and common monosaccharide ligands.

Characterization of the g200 Glycan—The g200 glycan was isolated by elution after electrophoretic separation of AP glycans on a 7.5–20% polyacrylamide gel (Fig. 2A). Recovery was 80–90% as determined by either the carbohydrate content or the intensity of Alcian blue staining of the eluted glycan (Fig. 2). The purity of such electrophoretically isolated g200 was analyzed by gel filtration on a TSK 40 XL column equipped with a TSK SW precolumn. Continuous measurements of absorbance at 206 nm and the refractive index showed that g200 elutes as a single peak with $K_w = 0.20$ (Fig. 3). The apparent molecular mass was estimated to be 150 $\times 10^3$ ± 50 $\times 10^3$ daltons according to the hyaluronic acid, chondroitin sulfate, and heparin standards. This is likely to be an underestimate because glycosaminoglycan standards possess a higher net charge than the sponge glycan. Therefore, we have adopted the higher molecular mass of 200 $\times 10^3$ daltons as a more realistic value. Sequence analyses of this molecule are planed and will enable precise measurements of its size. Colorimetric measurements of uronic acid and neutral hexose content of the g200 peak demonstrated that more than 90% of the material was recovered in these fractions. The homogeneity of the g200 glycan was also examined by ion exchange chromatography on a DEAE-Sephacel column. Over 95% of the carbohydrate mass (uronic acid and neutral hexose) was recovered in a single peak eluting at a pyridine acetate concentration of 0.7 M, pH 5.3 (Fig. 4). Electrophoretic and chromatographic separation techniques indicated that the g200 glycan is a single molecular species with possible charge and size microheterogeneities.

The immunological data indicated that the structure of the g200 epitope recognized by Block 2 is not present within previously characterized classes of glycosaminoglycans. However, the possibility that the g200 molecule contains some structural features of the classical glycosaminoglycans apart from the Block 2 epitope site could not be excluded. To examine this possibility g200 was subjected to treatment by chondroitinase ABC, heparinase, heparitinase, hyaluronidase, and keratinase each at concentrations and under conditions which result in total degradation of their normal substrates. No detectable degradation of g200 by any of the tested enzymes was observed as assayed by gel electrophoresis and
prolifera, C. celata, and were taken with 10 of the Block 2 monoclonal antibody Fab fragments. After raising the Ca²⁺ concentration to 10 mM, beads were gently rotated by hand for 10 min in a moist chamber. Microphotographs were taken with 10 cells/ml was assayed in 100 μl of CSW buffered with 20 mM Tris, or presence of 20 pg of Block Fab, 0.1 pg of 3H-labeled g200 was measured in either absence or presence of 20 μg of Block Fab, 0.1 μg of ²H-labeled xg200 (7 × 10⁶ cpm/μg) and 10 μg of ²H-labeled g200 (5 × 10⁶ cpm/μg) to M. prolifera, C. celata, and H. occulata glutaraldehyde fixed cells (2 × 10⁸ cells/ml) was assayed in 100 μl of CSW buffered with 20 mM Tris, pH 7.4. After a 20-min incubation, cells were washed and bound radioactivity was measured. The variation from two experiments using the same batch of cells was less than 8% of each value.

### Table II

| % bound to       | M. prolifera | C. celata | H. occulata |
|------------------|--------------|-----------|-------------|
| AP               | 23.3         | 2.1       | 1.9         |
| AP + Block 2 Fab | <3.0         | <3.0      | <3.0        |
| xg200            | <5.0         | <5.0      | <5.0        |

HPLC gel filtration (not shown).

The carbohydrate composition of the g200 glycan was determined by gas chromatography. The results showed that g200 contains about 60% fucose in addition to glucuronic acid, galactose, N-acetylgalcosamine, and mannose (Table III). Sulfate and phosphate content could not be demonstrated in g200 by specific colorimetric reactions. The amino acid composition of g200 was analyzed by the Pico-Tag method. As shown in Table III the only amino acid found was asparagine. Since 1 mol of glycan had approximately 1 mol of asparagine, g200 is most likely N-linked to the AP protein core. However, extensive digestion of AP with glycopeptidase N-glycosidase F, which completely removes g6 from the AP protein core (Misevic and Burger, 1990a), was incapable of releasing g200 (not shown). The compositional analysis as well as the enzymological, immunological, and biochemical evidence suggests that the g200 glycan is a highly fucosylated, acidic, N-linked glycan with a novel structure distinct from other characterized glycosaminoglycans.

**Adhesion Epitope of the g200 Glycan Is Highly Repetitive in AP**—Binding of iodinated Block 2 Fab fragments to AP-coated ELISA wells enabled us to determine the number of epitope sites present per AP molecule. As shown in Table I, one AP has approximately 2500 binding sites. All 2500 sites on each AP must interact with the Block 2 antibody in order to produce full inhibition of AP-bead or AP cell-promoted agglutination as judged from the data summarized in Fig. 1. These results support the hypothesis that the high polyvalency of the g200 epitope site is a significant factor promoting the AP-AP binding.

The number of g200 copies per AP was determined from the mass of total carbohydrate recovered in g200 fractions either after gel electrophoresis or gel filtration on a P-60 column. Since 37% of the total AP carbohydrate content (70% of AP mass is carbohydrate, AP has M₉ = 2 × 10⁷) was found in g200, it was calculated that one AP has 26 copies of this glycan. Thus, each g200 polysaccharide should have 96 epitope sites.

**Polyvalent Homophilic Carbohydrate-Carbohydrate Interactions of g200 Are the Basis for AP Self-Binding**—Functional experiments using inhibition of cell and AP-bead aggregation by the Block 2 antibody together with the binding studies suggest that, 1) the large g200 glycan carries the adhesion self-binding domain of AP; and 2) the high repetitivity of adhesion epitope (2500 sites) may be essential for establishing strong AP-AP binding mediated cell aggregation. To obtain
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FIG. 2. Separation of AP glycans by electrophoresis on polyacrylamide gel, immunoretardation, and immunoblotting of g200 by Block 2. A, 5 μl of AP glycan samples were applied to a linear 7.5–20% polyacrylamide gel. After electrophoresis gels were either stained with Alcian blue (a-c) or electroblotted to DEAE-nitrocellulose paper and decorated with antibodies (d and e). a, 10 μg of the total AP glycans; b, 5 μg of the g200 glycan; c, 10 μg of the g6 glycan; d, 10 μg of the total AP glycans blotted onto a DEAE paper and decorated with 2 μg of Block 2 antibody; and e, 10 μg of the total AP glycans decorated with rabbit anti-mouse peroxidase-conjugated antibody. B, total protein-free AP glycans were incubated with the Block 2 antibody for 2 h at room temperature in phosphate-buffered saline. Samples were then electrophoresed on a 7.5–20% linear polyacrylamide gel which was subsequently stained with the Alcian blue-silver enhancement method (see "Materials and Methods"). a, 30 ng of total AP glycans with 20 pg of Block 2; b, 30 ng of total AP glycans with 5 pg of Block 2; c, 30 ng of total AP glycans. HA 2000 is hyaluronic acid of $M_r = 2 \times 10^6$ and CS 150 is chondroitin sulfate of $M_r = 1.5 \times 10^5$, both obtained from Sigma.

FIG. 3. Gel filtration of the g200 glycan on a TSK 40 XL column. 10 μg of the g200 glycan isolated by gel filtration and gel electrophoresis were applied to a TSK 40 XL column (800 × 7.8 mm) equipped with a precolumn (40 × 8 mm). Elution was carried out with CSW buffered with 7 mM Tris, pH 7.4, with a flow rate of 0.3 ml/min. Optical density at 206 nm (---) and refractive index (-----) were continuously recorded by a Bio-Rad high resolution liquid chromatography system. As molecular weight standards hyaluronic acid of $M_r = 2.25 \times 10^3$ obtained from Sigma; chondroitin sulfate of $M_r = 1.01 \times 10^3$, $7.6 \times 10^2$, $4.3 \times 10^2$, $2.9 \times 10^2$, $20 \times 10^0$, obtained from N. K. Karamanos and A. J. Aletras, University of Patras, Greece; and heparin of $M_r = 1.1 \times 10^3$, $8 \times 10^2$, and $4 \times 10^2$, obtained from A. Lustig, Biozentrum, University of Basel, Switzerland, were used.

Direct evidence for the functional activity of the g200 epitope site, the g200 polysaccharide was cross-linked by glutaraldehyde and diepoxybutane to form polymers containing a similar number of g200 repeats to that in the native AP. The cross-linked g200 polymers of $M_r > 15 \times 10^6$ (xg200) were separated from the smaller polymers by gel filtration on a Bio-Gel A-15m column and were then labeled with [3H] tyrosine (for details, see "Materials and Methods") (Misevic et al., 1987). The content of cross-linking reagents was estimated to be 30–40% of the total dry weight of xg200 using gas chromatography. Therefore, the xg200 polymer contains about 40–50 copies of the protein-free g200 glycan. The gas chromatography and amino acid analyses of xg200 also showed that only <10% of fucose and >80% of Asp were modified by cross-linking.

The functional assays for the self-interaction activity were performed with radiolabeled g200 and xg200 in the presence of 2 and 10 mM CaCl$_2$. After 20 min at room temperature samples were centrifuged for 10 min at 10,000 × g and the amount of coaggregated molecules was determined in pellets by counting radioactivity. The formation of large coaggregates occurred only in the case of the xg200 glycan and only at 10 mM CaCl$_2$ (Table IV). This coaggregation was similar to that of the native AP. In a control experiment polymerized and
on a DEAE-Sephacel column. 500 pg of g200 isolated after gel filtration on a Bio-Gel P-60 column were applied to a DEAE-Sephacel column (1.5 x 10 cm) which was equilibrated with 0.05 M pyridine acetate buffer, pH 5.3. Elution with 20 ml of the equilibrating buffer was followed with a linear gradient from 0.05 to 1 M pyridine acetate, pH 5.3. Fractions of 2 ml were collected and aliquots of 400 pl were used for neutral hexose (O-O) and uronic acid determination (x-x).

**TABLE III**

Carbohydrate and amino acid composition of the g200 glycan

Carbohydrate analysis of the g200 glycan was performed after methanolysis by gas chromatography and amino acid analysis after HCl hydrolysis using the Pico-Tag method (see "Materials and Methods"). Within one preparation of g200 the variation was less than 10% of each value. Amino acids other than asparagine were not detected (less than 0.1 mol/mol of g200).

|        | mol/mol g200 |
|--------|--------------|
| Fuc    | 68           |
| Man    | 2            |
| Gal    | 18           |
| GlcNAc | 19           |
| GlcUA  | 32           |
| Asn    | 1            |

labeled cross-linking reagents were inactive in the self-association assay (not shown). These results indicate that the presence of cross-linking reagents in xg200 does not influence the self-interaction activity. Gel filtration analysis of g200 in the presence of 10 mM CaCl2 indicated that this molecule does not even undergo detectable dim- or tetramerization, probably because of its low self-binding affinity (not shown). Recovery of the self-interaction activity in the cross-linked g200 polymers indicated that highly polyvalent and Ca2+-dependent g200 glycan-glycan binding is essential for high affinity AP-AP interactions.

**FIG. 4.** Ion-exchange chromatography of the g200 glycan on a DEAE-Sephacel column. 500 pg of g200 isolated after gel filtration on a Bio-Gel P-60 column were applied to a DEAE-Sephacel column (1.5 x 10 cm) which was equilibrated with 0.05 M pyridine acetate buffer, pH 5.3. Elution with 20 ml of the equilibrating buffer was followed with a linear gradient from 0.05 to 1 M pyridine acetate, pH 5.3. Fractions of 2 ml were collected and aliquots of 400 pl were used for neutral hexose (O-O) and uronic acid determination (x-x).

**TABLE IV**

Homophilic carbohydrate-carbohydrate binding of the g200 glycan

Radioiodinated AP (1 pg; 2.2 x 10⁶ cpm/pg), 3H-labeled xg200 (0.1 pg; 7 x 10⁶ cpm/pg), and 3H-labeled g200 (10 pg; 5 x 10⁶ cpm/pg), were incubated with 2 or 10 mM CaCl2 in 100 µl of CSW containing 0.5% of BSA. After 20 min at room temperature the percentage of self-congregated molecules was measured in pellets obtained by 10 min centrifugation at 10,000 x g. The standard error from duplicate experiments was less than 10% of each value.

|        | Coaggregation |
|--------|---------------|
| 2 mM Ca++ | 10 mM Ca++   |
| %      | %             |
| Sponge proteoglycan AP | 4.8 | 61.0 |
| Monomeric g200 | <1* | <1* |
| Cross-linked polymeric xg200 | <1 | 23.6 |

*Dimers or multimers could not be detected neither in 2 nor 10 mM CaCl2 using HPLC gel filtration.

The binding of 3H-labeled xg200 polymer to homotypic cells was carried out to test cell-binding domain activity. Neither xg200 nor monomeric g200 were able to bind to M. prolifera cells (Table II). We conclude that g200 carries only the self-interaction site.

**Homophilic Carbohydrate-Carbohydrate Interactions of the g200 Glycan Mediate Bead Adhesion**—To provide direct evidence that g200-g200 binding can promote adhesion, glass aminopropyl beads (50 µm in diameter) were coated with either g200 or AP and their aggregation monitored following addition of a physiological concentration of CaCl2 (10 mM). Aggregation of both g200- and AP-beads occurred as AP-promoted cell aggregation in the presence of 10 mM CaCl2 but not with 2 mM CaCl2 (Fig. 5). Such calcium-dependent aggregation of g200 beads suggests that the g200 glycan is capable of mediating adhesion exclusively through homophilic carbohydrate-carbohydrate interactions. Thus, highly polyvalent g200-g200 binding represents the basis for AP-AP association, which by itself promotes cell aggregation (Fig. 6).

**DISCUSSION**

In this report we have presented biochemical and immunological evidence that carbohydrate-carbohydrate binding provides the essential strength for cell-cell adhesion in the marine sponge *M. prolifera*. The carbohydrate fundamental for this function has been characterized as a novel highly fucosylated g200 acidic glycan. An indirect immunological approach combined with direct *in vitro* studies using purified glycans established that the molecular mechanism for such carbohydrate-carbohydrate interactions is based on a Ca2+-dependent highly polyvalent associations of a acidic glycan epitope localized in the g200 polysaccharide.

AP-AP association mediates cellular aggregation only when AP is anchored to the plasma membrane through the Ca2+-independent AP-cell receptor (Jumblatt et al., 1980). The AP cell-binding activity alone is also unable to promote cell adhesion without the Ca2+-dependent AP-AP association (Jumblatt et al., 1980; Misevic and Burger, 1990a, 1990b). Thus, aggregation of the marine sponge *M. prolifera* cells is only achieved through the combined functioning of both the cell-binding and the self-interaction domains of AP (Fig. 6). The observation that the self-interaction g200 glycan was unable to bind to cells in its monomeric and polymeric cross-linked form provides evidence that g200 does not contain the cell-binding site. It is therefore not surprising that neither the monomeric nor the polymeric g200 glycan could induce aggregation of AP-depleted cells (not shown). It has been previously shown that the small g6 glycan of AP is involved in AP binding to the cell-surface (Misevic and Burger, 1990a,
We have demonstrated that the reconstituted cross-linked polymers of g6 cell-binding glycan are also unable to promote cell adhesion, in spite of strong polyvalent and species-specific binding to cells (Misevic and Burger, 1990a, 1990b). These data confirm that two AP functional sites are specifically localized in two different polysaccharides and that both are necessary for cell aggregation (Fig. 6).

Direct cross-linking studies and assays using specific antibodies have shown that highly polyvalent g200-g200 glycan binding (2500 epitope sites per AP molecule) mediates self-interaction of AP. As indicated in Table IV, self-association of the cross-linked g200 was not as strong as that of the native AP in spite of a somewhat higher valency presumably because, 1) the three-dimensional organization of the g200 cannot be reconstituted to exactly resemble the native AP due to random orientation and different spacing between the cross-linked polysaccharides in artificial polymers of g200, and 2) trauma to active sites during the cross-linking procedure. Nevertheless the self-binding activity of the cross-linked polymers in the presence of 10 mM Ca^{2+} increased from undetectable coaggregation for single g200 molecules to coaggregation of about 40% of that for the native AP.

Our studies with the simple sponge model system provide direct evidence for two new molecular mechanisms favoring cellular interactions. First, cell recognition and cell adhesion are both mediated through highly polyvalent (>1000 sites) but low, experimentally unmeasurable affinity of a single carbohydrate ligand which binds to a surface receptor (Misevic and Burger, 1990a, 1990b). Second, carbohydrate-carbohydrate associations of a high affinity complex generate cell-cell adhesion (Fig. 6). Both types are distinct from the higher affinity low valency protein-protein or protein-carbohydrate binding described for lectin-carbohydrate, integrin-extracellular matrix, immunoglobulin-immunoglobulin, and cadherin-cadherin adhesion molecules. Carbohydrate-carbohydrate interactions have also been proposed as a force mediating cell adhesion in two other instances; Le"-Le" interaction in the case of teratocarcinoma cells (Eggens et al, 1989a, 1989b) and G_{ Ga}G_{ GM} ganglioside association in the case of lymphoma and melanoma cells (Kojima and Hakomori, 1989). The presence of glucuronic acid in sponge g200 is unique and indicative of structural differences from Le", G_{ Ga}, and G_{ GM}. The question whether these adhesion polysaccharides operate via the same mechanism can only be answered by comparative studies on valency and carbohydrate affinities.

Although the primary structure of the g200 glycan remains to be determined, the immunological, enzymological, and compositional data indicate that this N-linked highly fucosylated and glucuronic acid-rich polysaccharide belongs to a new class of acidic glycans distinct from the classical glycosaminoglycans. Nevertheless, g200 belongs to the large family of acidic polysaccharides, and therefore it would be of interest to eval-

**Fig. 5.** Homophilic g200-g200 glycan binding mediates g200 glycan-coated bead aggregation. 10 µl of dry glass aminopropyl beads (50 µm in diameter) were coated with 100 µg of the g200 glycan or 10 µg of AP (see "Materials and Methods"). After washing with CSW the bead suspension was gently agitated in a moist chamber for 10 min in the presence of 2 and 10 mM Ca^{2+} and was microscopically examined for the presence of bead aggregation. Microphotographs were taken with 10 X objective. A, AP-coated beads with 10 mM Ca^{2+}; B, AP-coated beads with 2 mM Ca^{2+}; C, g200-coated beads with 10 mM Ca^{2+}; and D, g200-coated beads with 2 mM Ca^{2+}.
whether such an interaction provides the degree of specificity required for cell recognition. Our knowledge of noncovalent ionic and hydrogen bonding suggests that the following parameters may determine selectivity in binding adjacent carbohydrate structures: 1) differences in the primary structure of glycans; 2) spacing between charged groups within one glycan molecule; 3) variability in the type of these charged groups; 4) degree of glycan hydrophobicity; 5) degree of repetitiveness of functional glycan epitopes within the glycan structure as well as the repetitiveness of the glycan molecule on the protein core; and 6) the distances between glycan chains attached to the protein core. Future studies using a similar approach to ours may demonstrate whether carbohydrate-carbohydrate binding mediates cell adhesion and recognition during multistep processes of cell-cell or cell-extracellular matrix interactions.

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FIG. 6. Model of g200 carbohydrate-carbohydrate interaction-mediated cell adhesion of sponge cells. Cell adhesion and recognition in the marine sponge M. prolifera is mediated through a Ca2+-dependent highly polyvalent homophilic carbohydrate-carbohydrate interaction of g200 glycan and Ca2+-independent binding of g6 glycan to its cell-surface receptor. The model does not account for the stoichiometry and the size of glycans attached to the protein core of AP. PMR, plasma membrane receptors; PC, protein core.

Carbohydrate-Carbohydrate Interactions Mediate Cell Adhesion