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Isolation and Characterization of a New Class of Acidic Glycans Implicated in Sea Urchin Embryonal Cell Adhesion

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Abstract Three major glycan fractions of 580 kDa (g580), 150 kDa (g150), and 2 kDa (g2) were isolated and purified from *Lytechinus pictus* sea urchin embryos at the mesenchyme blastula stage by gel filtration and high pressure liquid chromatography. Chemical analysis, by gas chromatography, revealed that g580 is highly sulfated and rich in *N*-acetylglucosamine, *N*-acetylgalactosamine, glucuronic acid, and fucose. The g150 fraction is less acidic than g580 and contains high amounts of amino sugars, xylose, and mannose. The g2 fraction is neutral, rich in *N*-acetylglucosamine, mannose, and galactose. The g580 and g150 fractions are resistant to glycosaminoglycan-degrading enzymes, indicating that they are distinct from the glycosaminoglycans. The g580 fraction resembles, with respect to chemical composition, a previously characterized 200 kDa sponge adhesion glycan (g200). The binding of the monoclonal antibody Block 2, which recognizes a repetitive epitope on g200, as well as of the anti-g580 polyclonal antibodies to both g580 and g200 indicated that these two glycans share similar antigenic determinants. The Fab fragments of the Block 2 antibody, which previously have been shown to inhibit cell adhesion in sponges, also blocked the reaggregation of dissociated sea urchin mesenchyme blastula cells. These results indicate that g580 carries a carbohydrate epitope, similar to the sponge adhesion epitope of g200, which is involved in sea urchin embryonal cell adhesion.

Key words: acidic glycans, carbohydrates, sea urchin embryos, cell adhesion, cell reaggregation

Cell–cell interactions, such as recognition and adhesion, play an important role in a variety of biological processes including fertilization [Vacquier and Moy, 1977], embryogenesis [Ruiz-Bravo et al., 1986; Simmons et al., 1988; Staunton et al., 1989], immune defense [Liotta et al., 1986], and some pathological organ-specific metastasis. Several families of adhesion receptors mediate cell–cell and cell–substratum interactions such as the integrins [Ruoslahti, 1991], the cadherins [Takeichi, 1988], the selectins [Sharon and Lis, 1989], and the adhesion molecules of the immunoglobulin superfamily [Springer, 1990]. Carbohydrates are another class of molecules involved in cellular interactions as shown by experimental evidence regarding the homing of lymphocytes [Yednock et al., 1987; Brandley et al., 1990; Lowe et al., 1990; Phillips et al., 1990], mouse embryo compaction and the reaggregation of teratocarcinoma cells by the Le^x antigen [Bird and Kimber, 1984; Eggins et al., 1989], the binding of laminin, thrombospondin, and von Willebrand factor to sulfated glycolipids [Roberts et al., 1986], as well as the reaggregation of marine sponge cells by a new class of acidic glycans [Misevic and Burger, 1990, 1993; Misevic et al., 1987].

Several lines of evidence indicate that carbohydrates also play an important role during sea urchin embryonal development. It has been reported that wheat germ agglutinin binds exclusively to primary mesenchyme cells when microinjected into the blastocoels of living *Lytechinus pictus* embryos [DeSimone and Spiegel, 1986]. When embryos are cultured in the presence of

Abbreviations used: AP, adhesion proteoglycan; mAb, monoclonal antibody; ASW, bicarbonate-buffered artificial seawater, pH 7.4; ddH_2O, double-distilled water; HPLC, high pressure liquid chromatography; CSW, Ca^{2+}, Mg^{2+}–free seawater supplemented with 2 mM $CaCl_2$ buffered with 7 mM Tris, pH 7.4; CMFSW, Ca^{2+}, Mg^{2+}-free seawater buffered with 0.18 g/l of $NaHCO_3$; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline (200 mM NaCl, 50 mM Tris–HCl, pH 7.4); AUC, area under curve.

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tunicamycin, an inhibitor of *N*-linked glycosylation, primary mesenchyme cells do not develop binding sites to wheat germ agglutinin [DeSimone and Spiegel, 1986], they do not migrate into the blastocoel, and gastrulation is inhibited [Schneider et al., 1978]. Furthermore, microinjection of Concanavalin A into midgastrula stage embryos causes detachment of the secondary mesenchyme cells from the blastocoel wall, possibly by interfering with the cell surface carbohydrate molecules responsible for cell adhesion in the embryo [Spiegel and Burger, 1982]. It has also been observed that the blastocoel of mesenchyme blastula stage sea urchin embryos contains sulfated acidic mucopolysaccharides [Karp and Solursh, 1974; Solursh and Katow, 1982]. Incorporation and autoradiographic studies have shown that primary mesenchyme cells incorporate $(^{35}S)O_4$ and that the label strongly associates with these cells when they migrate during development [Immers, 1961; Sugiyama, 1972]. When embryos are cultured in the absence of sulfate, primary mesenchyme cell migration and gastrulation are inhibited [Akasaka et al., 1980; Katow and Solursh, 1981].

In the marine sponge *Microciona prolifera,* species-specific recognition and adhesion of dissociated cells are controlled by an adhesion proteoglycan (AP) via two highly polyvalent functional domains, a cell-binding domain and a self-interacting domain [Wilson, 1907; Cauldwell et al., 1973; Henkart et al., 1973; Jumblatt et al., 1980; Misevic et al., 1982]. The monoclonal antibody (mAb) Block 2 specifically recognizes a highly repetitive epitope, located on a 200 kDa glycan (g200), and has been shown to inhibit cell adhesion in sponges. The molecular mechanism of this inhibition was shown to be mediated by the blockage of the carbohydrate–carbohydrate self-binding activity of the sponge AP [Misevic and Burger, 1993]. In the present study, we show that the Fab fragments of the mAb Block 2 completely inhibit the reaggregation of dissociated cells obtained from mesenchyme blastula stage embryos of the sea urchin *Lytechinus pictus*. This experimental evidence indicated that a carbohydrate epitope, similar to the one which mediates cell adhesion in sponges, is implicated in cell adhesion at the mesenchyme blastula stage of sea urchin embryos. In order to locate this epitope we isolated the three major glycan fractions from the mesenchyme blastula stage embryos of the sea urchin *Lytechinus pictus*. Biochemical, chemical, and enzymological characterization of the purified fractions revealed that the 580 kDa fraction (g580) and the 150 kDa fraction (g150) are highly acidic but distinct from the previously described glycosaminoglycans. The larger glycan fraction, g580, is similar to the sponge adhesion glycan g200 as demonstrated by chemical composition, resistance to glycosaminoglycan-degrading enzymes, and binding of anti-g580 polyclonal antibodies and of the mAb Block 2 to both g580 and g200 glycans. These results indicate that the glycan fraction g580 carries a functional carbohydrate epitope, similar with the sponge adhesion epitope, involved in cell adhesion during sea urchin embryonal development.

MATERIALS AND METHODS

Sea Urchins

Adult *Lytechinus pictus* were purchased from Marinus Inc., Westchester, CA, and maintained in aquariums supplied with a constant flow of seawater, precooled to 12°C.

Handling of Gametes and Embryos

Gametes were obtained from gravid *Lytechinus pictus* adults by intracoelomic injection of 0.5 M KCl [Tyler, 1949]. Sperm was collected in sterile Petri dishes and eggs in 30 ml of freshly prepared and sterilized bicarbonate-buffered artificial seawater, pH 7.4 (ASW), which was made according to Marine Biological Laboratory Woods Hole standards [Cavanaugh, 1956]. Eggs were passed through an 80 μm nylon mesh to remove debris, broken spines, and fecal pellets. The egg suspension was allowed to sediment for 2 min, the supernatant was removed, and eggs resuspended in 30 ml ASW. This procedure was repeated twice. The final egg suspension was then inseminated, with gentle mixing, with a dilute sperm solution at a ratio of about 50 sperms to one egg. Fertilized eggs were allowed to sediment and excess sperm was removed by aspiration. Only batches of eggs with 95–100% fertilization and subsequent normal development were used. Embryos were diluted to a concentration of about 10^4 embryos/ml in ASW containing 50 U/ml penicillin and 50 μg/ml streptomycin and allowed to grow in 100 ml flat glass bowls at 16°C.

Isolation and Purification of Glycans

Twenty-five hours after initiation of fertilization, embryos developed to the stage of mesen-

chyme blastula. Embryos were concentrated (3×10^6 in 10 ml of ASW) by centrifugation at $170 \times g$ for 5 min and delipidated with 4 vol of a 1:2 mixture of chloroform/methanol (v/v) according to Svennerholm and Fredman [1980]. Centrifugation was carried out at $700 \times g$ for 10 min and the organic solvents were removed from the resulting pellet by the addition of 10 ml of ethanol. The mixture was then centrifuged at $700 \times g$ for 10 min and the pellet dried at 40°C for 4 h. The pellet was weighed and resuspended in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM $CaCl_2$. Protein digestion of the pellet was carried out using 1.5 ml of 10 mg/ml Pronase (protease from *Streptomyces griseus,* Calbiochem), dissolved in the same buffer and preincubated for 30 min at 60°C in order to eliminate any glycosidase activity. Digestion was carried out as previously described [Finne and Krusius, 1982] for 72 h at 60°C, by adding equal amounts of Pronase at 24 h intervals. Glycans were then separated from amino acids and small peptides by gel filtration on a Sephadex G-25 fine (Pharmacia) column (0.75 × 110 cm), by elution with 10 mM pyridine acetate buffer, pH 5.0. Two milliliter fractions were collected at a flow rate of 1.75 ml/min. The UV absorbance of the fractions was recorded continuously at 280 nm using a Pharmacia UV monitor. Colorimetric determinations of neutral hexoses, uronic acids, and protein were performed on every second fraction according to Shields and Burnett [1960], Dische [1947], and Lowry et al. [1951], respectively. Fractions containing neutral hexoses and uronic acids were pooled, lyophilized twice to ensure the complete removal of pyridine acetate, and dissolved in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 10 mM $MgCl_2$. DNA digestion was accomplished by adding 1 ml of 1 mg/ml DNase I (Sigma type IV, 2,200 KU/mg protein), which was dissolved in the same buffer and incubated for 8 h at 37°C. After the incubation period the $CaCl_2$ concentration was adjusted to 1 mM and the reaction was stopped by adding 1 mg of Pronase, dissolved as above, and incubating the mixture at 60°C for 24 h. The glycans were then treated with 10 mM NaOH pH 10.0–11.0, in the presence of 1 M $NaBH_4$ at 45°C for 16 h [Roden et al., 1972]. At the end of the incubation period, samples were neutralized with 50% (v/v) acetic acid and glycans were separated from digestion products by gel filtration on a Sephadex G-25, as described above. Fractions containing neutral hexoses and uronic acids were identified as above, pooled, and lyophilized. Samples were then dissolved in 5 ml of ddH_2O, applied on a Superose 6 column (Pharmacia) (0.75 × 93 cm), and eluted with 50 mM pyridine acetate, pH 5.0. Fractions of 1.5 ml were collected at a flow rate of 0.75 ml/min. Those containing glycans were determined as described above, pooled, and lyophilized twice to ensure complete removal of pyridine acetate. Further fractionation of the glycans was achieved by high pressure liquid chromatography (HPLC) (Bio Rad) using a Bio-gel TSK 40XL gel filtration column (300 × 7.8 mm), equipped with an MXGL 0035 precolumn (40 × 7.8 mm). Elution was carried out with Ca^{2+}, Mg^{2+}–free seawater supplemented with 2 mM $CaCl_2$, buffered with 7 mM Tris, pH 7.4 (CSW) [Cavanaugh, 1965], at a flow rate of 0.3 ml/min. The apparent molecular weight of the glycans was estimated using the following molecular weight markers: hyaluronic acid of 225 kDa (Sigma), chondroitin sulfates of 19.7–101.6 kDa (donated by Dr. A. Aletras and Dr. N. Karamanos, Department of Biochemistry, University of Patras, Greece), and heparins of 4.44–11 kDa (donated by A. Lustig, Biocenter, University of Basel, Switzerland). Ion exchange chromatography of the purified glycans was performed by HPLC on a Bio-gel MA7P anion exchange column (50 × 7.8 mm) equipped with a Carbo-C cartridge precolumn. The column was equilibrated with 10 mM phosphate buffer, pH 6.0, and elution was carried out in a gradient of 10 mM to 100 mM phosphate buffer, pH 6.0, at a flow rate of 1.2 ml/min. Fractions were collected every 30 s and further analyzed for neutral hexose and uronic acid content. During the HPLC gel filtration and ion exchange analyses the absorbance was monitored at 206 or 232 nm using a UV/VIS monitor (Bio Rad, model 1706) and a refractive index monitor (Bio Rad, model 1755). The purified glycans were dissolved in ddH_2O and stored at 4°C.

Ultracentrifugation Analysis

Sedimentation equilibrium and sedimentation velocity analyses were performed in a Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference and schlieren optics. In both cases an interference-double-sector cell 12 mm Epon-filled–charcoal and an An-D rotor were used. Dried samples were dissolved in CSW buffered with 7 mM Tris, pH 7.4. For sedimentation equilibrium analysis, a range of speeds from 4,800 to 8,000 rpm was used. The speed was

adapted for different molecular masses and concentrations. When samples with molecular masses smaller than 5×10^3 were to be analyzed speeds of 34,000 and 44,000 rpm were used according to the "midpoint mode" method of Yphantis [1960]. The initial concentration of glycans with molecular masses smaller than 5×10^3 was determined in a separate run in a synthetic-boundary Capillary-type. Sedimentation velocity analysis was performed at 34,000 and 40,000 rpm using interference optics. All runs were performed at 20°C. The molecular weights were calculated using a linear regression computer program to obtain the best linear fit of concentration versus r^2 (r = radial distance).

Polyacrylamide Gel Electrophoresis (PAGE)

Linear gradient 5–17% polyacrylamide gels, 0.8 mm × 12 cm × 12 cm, were prepared as previously described [Misevic, 1989]. Gels were stained with a solution of 0.5% (w/v) Alcian blue (Fluka) dissolved in 25% (v/v) isopropyl alcohol and 1% (v/v) acetic acid for 12 h. The same solution without the dye was used for destaining.

Carbohydrate Analysis

Dried glycan samples (50 μg) were dissolved in 80 μl of 0.625 M HCl in methanol to which 20 μl of absolute methyl acetate was added. Methanolysis was carried out for 17 h at 70°C [Chaplin, 1982]. Absolute t-Butyl alcohol (20 μl) was then added to each sample and evaporated using a stream of nitrogen at room temperature. Dry methanol (100 μl), pyridine (10 μl), and acetic anhydride (10 μl) were added successively with intermediate mixing in order to re-*N*-acetylate the amino sugars [Kozulic et al., 1979]. Solutions were left at room temperature for 15 min and then evaporated to dryness, initially under a nitrogen stream and then by vacuum. After thorough drying, 40 μl of the silylation reagent Sylon TP (Supelco) was added and samples were vortexed and left for 1 h at room temperature. The derivatized samples were re-evaporated under a nitrogen stream, dissolved immediately in 50 μl hexane, and centrifuged at 4,000 × *g* for 5 min. The sugar composition was determined in 40 μl aliquots of the supernatant on a silica glass column (12.5 m × 0.2 mm) coated with 0.33 μm methyl-silicon gum (Hewlett-Packard) in a Hewlett-Packard gas chromatograph 7620 A equipped with a 5970 Mass selective detector. Inositol, which was shown to be absent in the sea urchin glycans, was used as the internal standard.

The sulfate content of the purified glycans was determined colorimetrically [Spencer, 1960].

Amino Acid Analysis

The dried glycan samples (50 μg) were hydrolyzed in 6 M HCl at 110°C in vacuum-sealed glass tubes for 16 h. Their amino acid composition was determined using the Pico-Tag method [Bidlingmeyer et al., 1984; Heinrikson and Meredith, 1984] on a Waters HPLC system.

Treatment of the Purified Glycans With Glycosaminoglycan-Degrading Enzymes

Lyophilized glycans (100 μg) were treated in a final volume of 100 μl as follows: (1) Treatment with heparinase: samples dissolved in 0.1 M Tris-HCl buffer, pH 7.0, containing 1 mM $CaCl_2$ and 0.1 M NaCl were treated with 4×10^{-4} U of heparin lyase (Seikagaku 100700) at 35°C for 15 h. (2) Treatment with heparitinase: samples dissolved as above were treated with 4×10^{-4} U of heparan sulfate lyase (Seikagaku 100703) at 43°C for 16 h. (3) Treatment with chondroitinase ABC: samples dissolved in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.05 M sodium acetate were treated with 4×10^{-4} U of chondroitin ABC lyase (Seikagaku 100330) at 37°C for 16 h. (4) Treatment with keratanase: samples dissolved in 50 mM Tris-HCl buffer, pH 7.4, were treated with 0.1 U of keratan sulfate 1,4-β-D-galactanohydrolase (Seikagaku 100810) at 37°C for 5 h. (5) Treatment with hyaluronidase: samples dissolved in 0.02 M sodium acetate containing 0.15 M NaCl, buffered with acetic acid to pH 6.0, were treated with 5 U of hyaluronate lyase (Seikagaku 100740) at 60°C for 14 h.

The standard glycosaminoglycans (100 μg)—chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, hyaluronic acid, keratan sulfate, heparan sulfate (all from Sigma), and heparin of 11 kDa (Roche)—were also treated with the above mentioned glycosidases following appropriate incubation procedures. Incubation times and enzyme concentrations used were those required for the complete degradation of their respective standard substrates, as estimated by preliminary investigation. Substrates incubated separately with the respective buffers served as controls. Digestion was initially evaluated by PAGE and then by HPLC gel filtration analysis with continuous recording of the absor-

bance at 206 and 232 nm and of the refractive index.

Preparation of the Antibodies

BALB/c mice were immunized by an intraperitoneal injection of an emulsified mixture containing 300 μl of 1.0 mg/ml of purified g580 glycan in ddH_2O and 300 μl of Freund's complete adjuvant. The injection was repeated twice at 3–4 week intervals. Ten days after the last injection sera was taken from the tail vein and tested by immunodot assay. The mAb Block 2 was prepared as previously described [Misevic et al., 1987; Misevic and Burger, 1993].

The Fab fragments of the mAb Block 2 were prepared by papain digestion [Malinowski and Manski, 1981] of the antibody and then purified by gel filtration on an ACA 44 LKB column (1.8 × 66 cm) followed by a passage through a 1 × 6 cm column of protein A-Sepharose (Pharmacia P-L Biochemicals) as previously described [Misevic and Burger, 1993].

Immunoblot Assays

Glycans were subjected to PAGE on 5–17% linear gradient gels and then blotted onto DEAE-cellulose sheets (NA45 membranes, Schleicher and Schuell, Keene, NH) as previously described [Misevic, 1989]. DEAE-cellulose sheets were rinsed once in Tris-buffered saline (TBS: 200 mM NaCl, 50 mM Tris-HCl, pH 7.4), preincubated for 30 min at room temperature with 1% bovine serum albumin solution in TBS (blocking buffer), and incubated for an additional 2 h in blocking buffer containing 1 μg/ml of the mAb Block 2 at room temperature with constant shaking. Incubations in blocking buffer without the mAb served as controls. DEAE-cellulose sheets were then washed four times using 10 ml of TBS and incubated for 2 h in the blocking buffer containing 10 μg/ml of peroxidase-conjugated goat antimouse antibody (Cappel) under the same incubation conditions. DEAE-cellulose sheets were again washed four times with TBS and assayed for peroxidase reaction products using as a substrate 0.6 ml of 0.3% (w/v) chloronaphthol solution in methanol, diluted with 9.4 ml TBS and 3 μl of 30% H_2O_2.

Competition studies for the binding of the mAb Block 2 to g580 or to the sponge glycans using various carbohydrate ligands were performed by an immunodot assay. Glycan solution (0.5 μl of 1 mg/ml dd H_2O) was spotted onto DEAE-cellulose sheets. In order to neutralize the residual charges, the DEAE-cellulose sheets were incubated with 50 μl of blocking buffer containing 0.4 mg/ml of each of the glycosaminoglycans (chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, heparin, heparan sulfate, keratan sulfate, and hyaluronic acid) for 30 min at room temperature. Subsequently, 50 μl of blocking buffer containing 0.02 mg/ml of the mAb Block 2 were added. The binding of the mAb Block 2 to g580 or to the sponge glycans was assessed after preincubation of the antibody for 30 min at room temperature with either: (1) Serial dilutions of sponge glycans. This is an equimolar mixture of g200 and g6 glycans from which only the g200 is recognized by the mAb Block 2, as shown by Misevic et al., 1987, and Misevic and Burger, 1993; (2) Serial dilutions of g580; (3) A 20-fold weight excess of g2; or (4) A mixture containing a 20-fold weight excess of each of the glycosaminoglycans: chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, heparin, heparan sulfate, keratan sulfate, and hyaluronic acid. From this step, the same procedure as described above, after the addition of the mAb, was followed.

Immunodot assays with the mouse antisera were performed after spotting 0.5 μl of 1 mg/ml glycan solution in ddH_2O onto DEAE-cellulose sheets and incubating with 3 μl/ml mouse antisera in blocking buffer, following the same procedure as described above. Incubations in blocking buffer without serum as well as in preimmune serum served as controls.

Reaggregation Studies

Embryos obtained at the mesenchyme blastula stage were washed twice with ASW and once with Ca^{2+}- and Mg^{2+}-free sea water (CMFSW). They were then dissociated in CMFSW by repeated pipetting with a polished glass Pasteur pipette. Dissociated cells were washed twice in CMFSW by centrifugation at 1,000 × *g* for 1 min and were finally resuspended in ASW.

For the reaggregation studies, 30 μl of a solution containing 10^6–10^7 dissociated cells/ml in ASW were placed in a small chamber prepared by sealing a rubber ring (1 cm in diameter and 0.2 cm high) onto a glass coverslip. The cells were then incubated at room temperature, in a moist chamber under gentle rotation in the presence or absence of 100 μg/ml of the Fab fragments of the mAb Block 2.

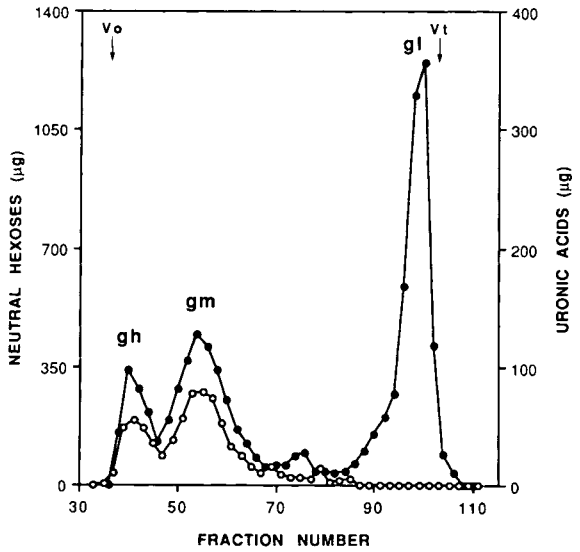

Fig. 1. Gel filtration of total glycans on a Superose 6 column. Lyophilized glycans after G-25 gel filtration (14 mg of neutral hexoses and 1.5 mg of uronic acids) dissolved in 5 ml of ddH_2O and applied to a Superose 6 column (0.75 × 93 cm). Elution was carried out with 50 mM pyridine acetate buffer, pH 5.0, at a flow rate of 0.75 ml/min. Fractions of 1.5 ml were collected and analyzed for the neutral hexose (●) and the uronic acid (○) content. Fractions pooled: gh (35–45), gm (46–66), gl (96–100). Vo and Vt are the void volume and the total volume, respectively.

RESULTS

Isolation of Total Glycans From Mesenchyme Blastula Stage Embryos

Lytechinus pictus embryos (3×10^6), at the mesenchyme blastula stage, were delipidated and treated extensively with pronase, DNase, and alkali. Glycans were separated from digestion products on a Sephadex G-25 column as a single peak. Amino acid analysis and measurement of the absorbance at 260 and 280 nm confirmed that the isolated glycans were free of nucleic acids and proteins. The neutral hexose and the uronic acid content of the peak fractions was found to be 14 mg and 1.5 mg, respectively, indicating a 90% recovery.

Fractionation of Total Glycans

Total glycans were fractionated according to their molecular size by gel filtration on a Superose 6 column, with a recovery of 97%. Three distinct molecular size species were identified by measuring the neutral hexose and the uronic acid content of the fractions (Fig. 1). The first peak, gh (glycan of high molecular weight), was eluted in the void volume and contained 1.7 mg of neutral hexoses (12.1% of neutral hexoses in starting material) and 0.35 mg of uronic acids (23.3% of uronic acids in starting material). The second peak, gm (glycan of middle molecular weight), was eluted with K_{av} = 0.22 and contained 4.7 mg (33.6%) of neutral hexoses and 0.8 mg (53.3%) of uronic acids. The third peak, gl (glycan of low molecular weight), was eluted with a K_{av} = 0.93 and contained 5.0 mg (35.7%) of neutral hexoses; uronic acids could not be detected. The gh, gm, and gl fractions corresponded to 84% of the total glycans which were eluted after the Superose 6 gel filtration. The remaining 16% was distributed among minor glycan populations which eluted between gm and gl peaks and were not further investigated.

The gh, gm, and gl fractions were analyzed by HPLC gel filtration. The apparent molecular weight of the glycans was estimated using chondroitin sulfates of 19.7, 28.8, 43.6, 75.8, and 101.6 kDa, heparins of 4.44, 8, and 11 kDa, and hyaluronic acid of 225 kDa as molecular weight markers. The gh fraction was eluted following HPLC gel filtration as a single peak at the void volume (Fig. 2A). The apparent molecular mass of this glycan fraction was estimated to be greater than 550 kDa (g580, see also ultracentrifugation analysis below). The gm fraction was eluted as two peaks; the first peak (g580-c, c = contaminant) was eluted at the void volume and the second peak with K_{av} = 0.20. The apparent molecular mass of the second peak was estimated to be 150 kDa (g150) (Fig. 2B). The high refractive index of g150 indicated that it corresponds to the major fraction (about 95%) of gm. The g580-c fraction is a gh contaminant of gm on the Superose 6 column, since (1) the ratios of the area under curve (AUC) of the absorbance at 206 nm over the AUC of the refractive index of g580 and g580-c are almost identical, (2) the % content of neutral hexoses, uronic acids, and sulfate of g580 and g580-c are also identical within the limitations of the colorimetric methods used, and (3) carbohydrate analysis of g580 and g580-c showed that they have the same chemical composition (data not shown). The gl fraction was eluted following HPLC gel filtration as a single peak with K_{av} = 0.83 (Fig. 2C). The apparent molecular mass of this glycan fraction was estimated to be 2 kDa (g2).

Ultracentrifugation Analysis

The molecular weight of the isolated glycans was also analyzed by ultracentrifugation. A partial specific volume of 0.56 cm^3/g was used for

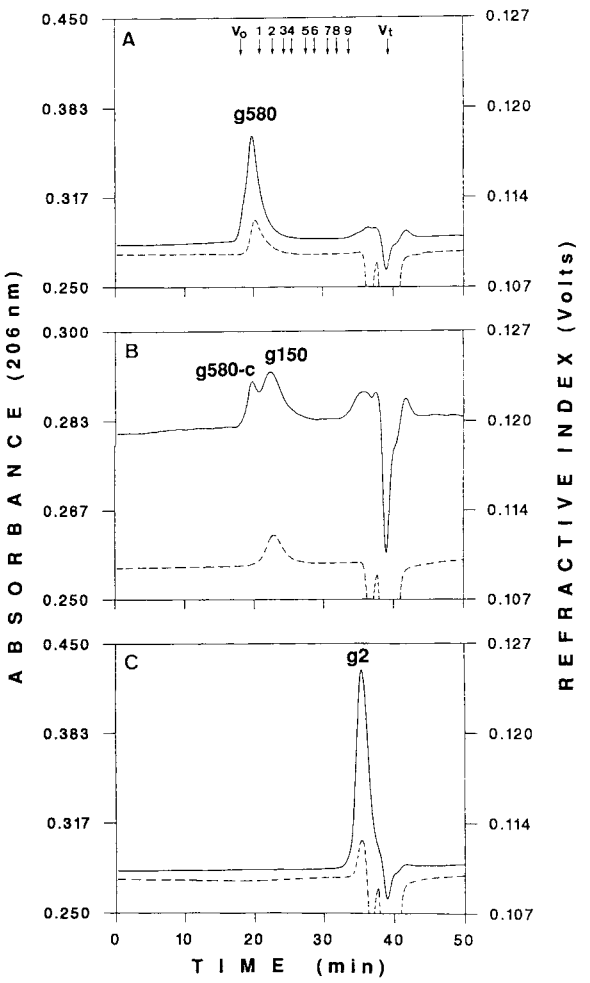

Fig. 2. HPLC gel filtration of gh, gm, and gl on a TSK 40XL column. Lyophilized gh, gm, and gl were dissolved in 20 μl of ddH_2O and applied to a TSK 40XL column (300 × 7.8 mm) equipped with a precolumn (40 × 7.8 mm). Elution was carried out with CSW buffered with 7 mM Tris, pH 7.4, at a flow rate of 0.3 ml/min. Absorbance at 206 nm (——) and refractive index (---) were continuously recorded by a Bio Rad HPLC system. **A:** 17 μg from gh; **B:** 50 μg from gm (a fourfold scale of absorbance is shown); **C:** 15 μg from gl. Molecular weight markers used are [1 to 9]: Hyaluronic acid of 225 kDa [1], chondroitin sulfates of 101.6 kDa [2], 75.8 kDa [3], 43.6 kDa [4], 28.8 kDa [5], 19.7 kDa [6], and heparins of 11 kDa [7], 8 kDa [8], and 4.44 kDa [9]. The molecular weight of the markers was determined by ultracentrifugation. Vo and Vt are the void volume and the total volume, respectively.

g580 and of 0.57 cm^3/g for g2, according to Perkins et al. [1981]. Sedimentation velocity analysis for g580 was performed at 34,000 rpm. Due to the relatively low diffusion constant it was possible to distinguish the fringe shift of the sedimenting front and determine the exact sample concentration, which was 0.03% (w/v). Using interference optics (Fig. 3A), a sedimentation coefficient corresponding to 8.5 S was determined. Sedimentation equilibrium analysis for g580 was performed at 4,800 rpm for a glycan solution of 0.06% (w/v) using interference optics (Fig. 3B). The molecular mass was determined to be 580 kDa. Sedimentation equilibrium analysis of g150 was also performed under the same conditions. However, the sedimentation rates were very high even when very low concentrations were used. The molecular masses obtained were 300, 600, and 1,200 kDa indicating that g150 self-interacts under the experimental conditions adopted to form larger aggregates which sediment at higher rates. The same results were obtained even when Ca^{2+} was excluded from the CSW buffer or when EDTA was added to CSW (data not shown). The mechanism of this self-aggregation of g150 remains to be elucidated. For g2, the initial sample concentration was 0.3% (w/v) as estimated by sedimentation equilibrium analysis at 34,000 rpm using schlieren optics (Fig. 3C). Sedimentation equilibrium analysis was also performed at 44,000 rpm using schlieren optics (Fig. 3D). The molecular mass of g2 was estimated to be 2 kDa, as also determined by HPLC gel filtration analysis.

Chemical Characterization of g580, g150, and g2

Carbohydrate analysis of g580, g150, and g2 by gas chromatography revealed that g580 and the g150 are highly acidic glycans containing mainly *N*-acetylglucosamine, *N*-acetylgalactosamine, and glucuronic acid (Table IA). In addition to these components, g580 contains high amounts of fucose, which accounts for 10% of total sugar content. The g150 fraction contains unusually high amounts of xylose, accounting for 13.1% of the total sugar content, as well as high amounts of mannose. The g2 fraction is a neutral glycan rich in *N*-acetylglucosamine, mannose, and galactose. Both g580 and g150 are highly sulfated (Table IB). The g580 fraction contains approximately 1 mol sulfate per mol of disaccharides and g150 contains about 0.5 mol sulfate per mol of disaccharides. The g2 fraction is the least sulfated, having only 0.06 mol sulfate per mol of disaccharides. The sugar composition of g580 and g150 indicated that these acidic glycans are different from the classical glycosaminoglycans. All three glycan fractions differ in their molecular composition and this was reflected in the different ratios of their absorbance at 206 nm to their refractive index (Table ID).

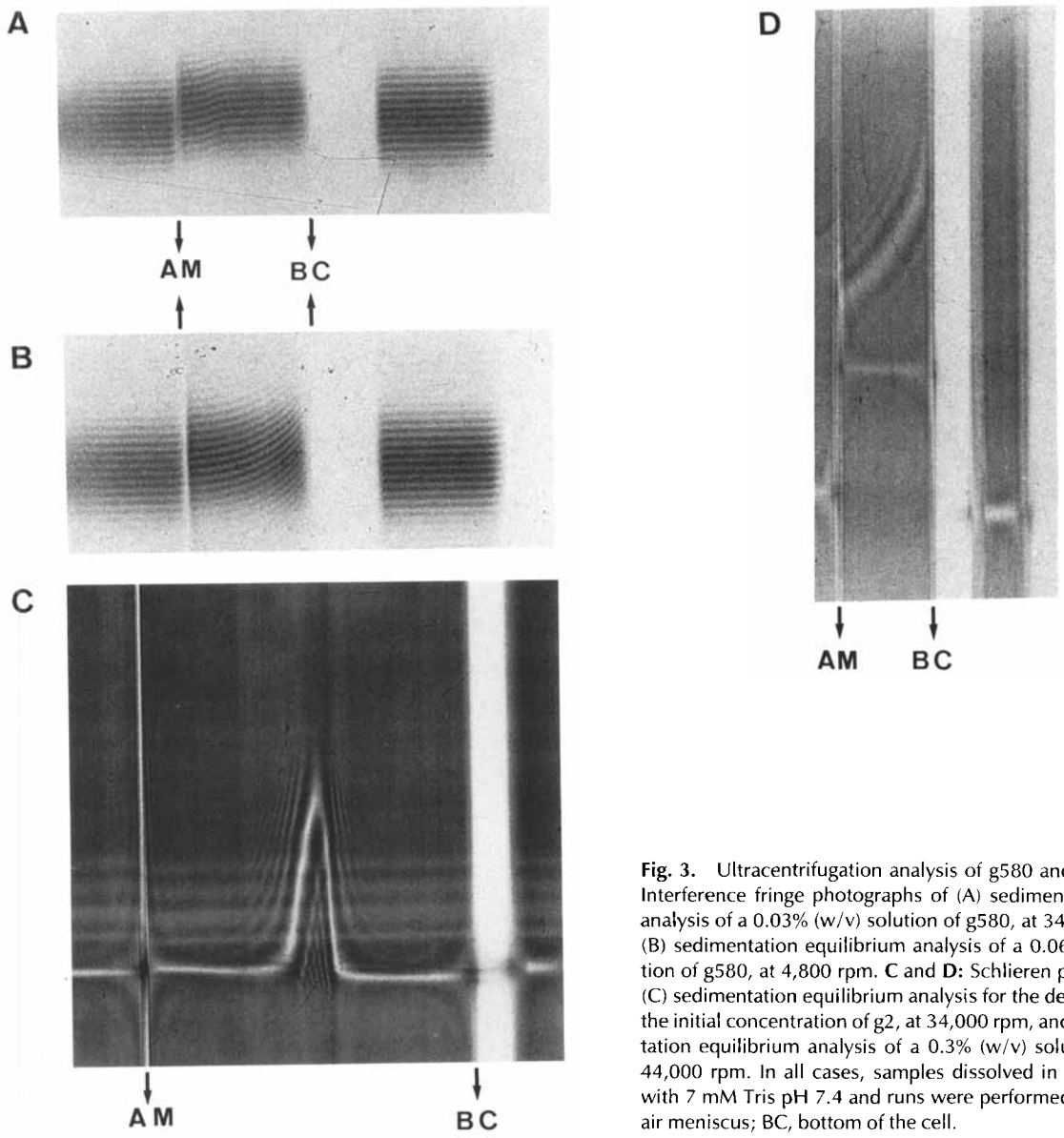

Fig. 3. Ultracentrifugation analysis of g580 and g2. **A** and **B:** Interference fringe photographs of (A) sedimentation velocity analysis of a 0.03% (w/v) solution of g580, at 34,000 rpm, and (B) sedimentation equilibrium analysis of a 0.06% (w/v) solution of g580, at 4,800 rpm. **C** and **D:** Schlieren photographs of (C) sedimentation equilibrium analysis for the determination of the initial concentration of g2, at 34,000 rpm, and (D) sedimentation equilibrium analysis of a 0.3% (w/v) solution of g2, at 44,000 rpm. In all cases, samples dissolved in CSW buffered with 7 mM Tris pH 7.4 and runs were performed at 20°C. AM, air meniscus; BC, bottom of the cell.

In order to confirm that the purified glycans were free of protein and to identify the nature of the glycan–protein linkage, amino acid analysis of g580, g150, and g2 was performed. The results obtained showed that the purified glycans were free of amino acids (Table IB). Amino acid analysis of the purified glycan fractions was also performed when the stage of alkali treatment was omitted (Table IC). In this case, g580 contained about 2 mol of serine per mol of glycan, as the sole amino acid. The complete removal of serine from g580 by alkali treatment indicated that g580 is *O*-linked with the core protein. The g150 fraction contained 1 mol of asparagine and 1 mol of serine per mol of glycan, indicating a mixture of *O*- and N-linked glycans. The g2 fraction contained 1 mol of asparagine and less than 1 mol of serine per mol of glycan. The content of other amino acids present in the purified glycans was two to three times lower than that of serine or asparagine.

PAGE of g580, g150, and g2

The electrophoretic mobility of g580, g150, and g2 was analyzed by PAGE on a 5–17% linear gradient gel which was stained with Alcian blue (Fig. 4). The g580 fraction stained more intensely than g150, since it is more acidic. The g150 fraction migrated as a broader band compared with g580, implying a more heteroge-

TABLE I. Carbohydrate, Amino Acid, and Sulfate Content of g580, g150, and g2

		g580	g150	g2
			mol%	
A	GalNAc	30.0	30.5	2.0
	GlcNAc	27.0	20.3	33.6
	GlcA	26.3	14.5	0.0
	Fuc	10.5	0.5	0.9
	Man	0.0	11.1	36.4
	Gal	2.6	4.7	18.2
	Glc	3.4	3.6	9.1
	Xyl	<0.1	14.5	0.0
			mol/mol	
B	Sulfate	1,600.0	233.0	0.3
	Ser	0.0	<0.5	<0.1
	Asn	0.0	<1.0	<1.0
			mmol/mol	
C	Ser	2.0	1.0	<0.5
	Asn	0.0	1.0	1.0
D	AUC A_{206}/AUC RI	8.0	1.0	22.6

A: Carbohydrate composition of g580, g150, and g2 determined by gas chromatography; values represent mol % of total carbohydrates. **B:** Amino acid analysis, after alkali treatment; values represent mol of amino acid per mol of glycan. The content of sulfate was determined colorimetrically; values represent mol of sulfate per mol of glycan. **C:** Amino acid analysis, prior to alkali treatment; values represent mol of amino acid per mol of glycan. **D:** The AUC of the absorbance at 206 nm and of the refractive index for each glycan was measured by integration analysis using the BioRad HPLC, Version 2.3, software system. The value for g150 was set to 1.0. Values shown (A–D) are the means of two determinations from three different preparations. The maximal error was always less than 20%, 15%, 15%, and 10% for A, B, C, and D, respectively. GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; Fuc, fucose; Xyl, xylose.

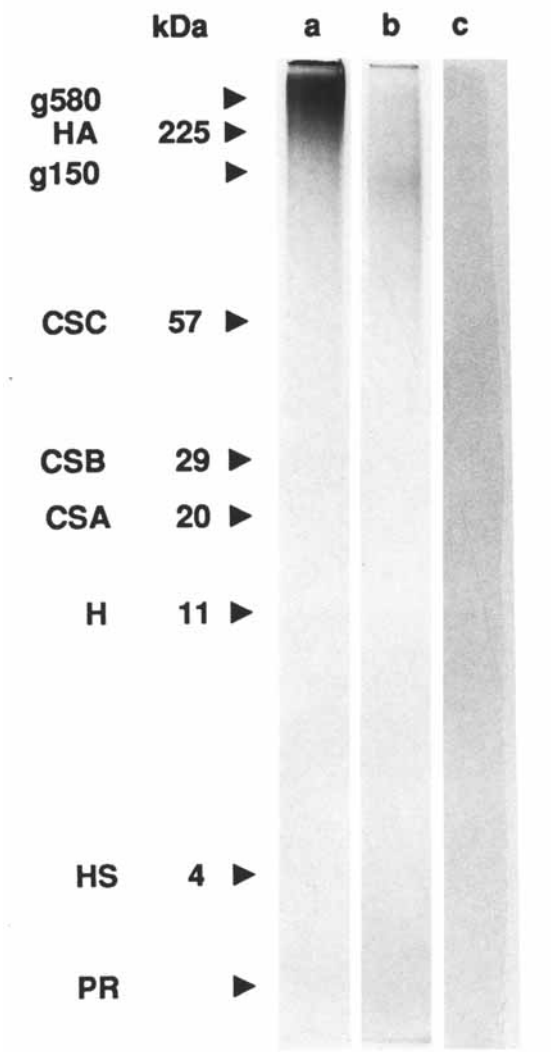

Fig. 4. PAGE of g580, g150, and g2 on a 5–17% linear gradient gel. **Lane a:** 10 μg of g580; **lane b:** 20 μg of g150; **lane c:** 20 μg of g2. After electrophoresis, gels were stained with Alcian blue. CSA, chondroitin sulfate A; CSB, chondroitin sulfate B; CSC, chondroitin sulfate C; HA, hyaluronic acid; H, heparin; HS, heparan sulfate; PR, phenol red.

neous structure. The electrophoretic mobilities of g580 and g150 correspond to molecular sizes which correlate with those obtained from HPLC gel filtration analysis. The g2 fraction, due to lack of charge, could not be analyzed by gel electrophoresis.

Treatment of g580, g150, and g2 With Glycosaminoglycan-Degrading Enzymes

In order to examine whether the isolated acidic glycans share common structural features with other well characterized glycosaminoglycans, enzymatic treatment with specific glycosaminoglycan-degrading enzymes was performed. Digestion of g580 and g150 was initially evaluated by PAGE (data not shown) and then by HPLC gel filtration analysis under continuous measurements of absorbance at 206 and 232 nm and of refractive index (Table II). The g580 fraction could be partially (12%) digested only by heparinase, indicating that this glycan fraction includes a minor amount of a heparin-like structure which is accessible to this enzyme. None of the enzymes used could digest g150. The g2 fraction was also treated with the same enzymes and its digestion was monitored by HPLC gel filtration analysis. As expected from its chemical composition, g2 fraction could not be digested with any of the glycosidases used. Enzyme activ-

TABLE II. Enzymatic Digestion of g580, g150, and g2*

	Ch-ase ABC	Heparin-ase	Hepari-tinase	Hyaluron-idase	Kera-tanase
	% digestion				
g580	0	12	0	0	0
g150	0	0	0	0	0
g2	0	0	0	0	0
CSA	82	0	0	0	0
CSB	100	5	0	0	0
CSC	100	0	0	0	0
H	0	100	5	0	0
HA	30	0	0	84	40
HS	0	90	95	0	0
KS	0	0	0	0	100

*The purified sea urchin glycans or standard glycosaminoglycans (100 μg) were digested with various glycosaminoglycan-degrading enzymes as described under "Materials and Methods." The digestion was monitored by HPLC gel filtration analysis. Absorbance at 206 and 232 nm and refractive index were continuously recorded and their AUC was measured by integration analysis using the Bio-Rad HPLC, Version 2.3, software system. Digestion (%) was calculated from the ratios of AUC of buffer-treated substrates over AUC of enzyme-treated substrates of (1) the absorbance at 206 nm, (2) the absorbance at 232 nm, and (3) the refractive index. In all cases (1–3), the % digestion calculated was identical. The results shown are the means of two determinations from three different preparations; the maximal error was less than 10% of each value. CSA, chondroitin sulfate A; CSB, chondroitin sulfate B: CSC, chondroitin sulfate C; HA, hyaluronic acid; H, heparin; HS, heparan sulfate; KS, keratan sulfate; Ch-ase ABC, chondroitinase ABC.

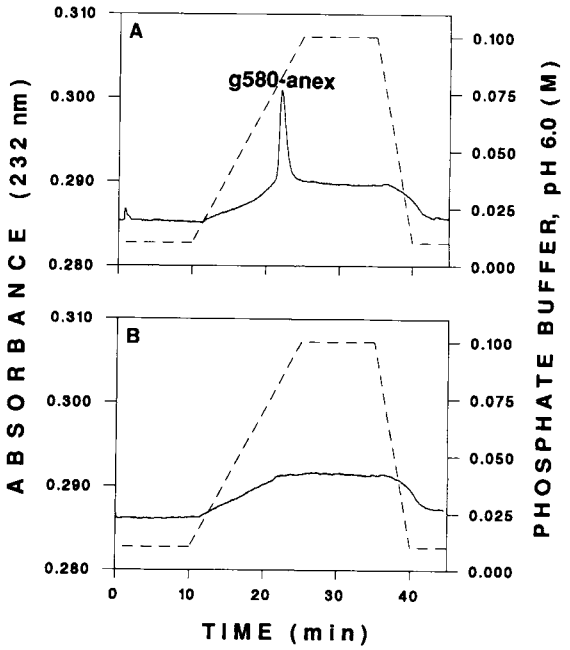

Fig. 5. Anion-exchange chromatography of g580 on a Bio gel MA7P column. Lyophilized g580 was dissolved in ddH_2O and applied on a Bio gel MA7P column (50 × 7.8 mm) equipped with a Carbo-C cartridge precolumn. The column was previously equilibrated in 10 mM phosphate buffer, pH 6.0. Elution was carried out using a linear gradient of 10 to 100 mM phosphate buffer, pH 6.0, with a flow rate of 1.5 ml/min. Optical density at 232 nm (——) was continuously recorded by a BioRad HPLC system. **A:** 22 μg of g580; **B:** baseline obtained when ddH_2O was applied to the column.

ity and purity of substrates was investigated following the same incubation conditions. All enzymes used could completely digest their respective specific substrates, with the exception of hyaluronic acid which was partially digested (84%) by hyaluronidase. Hyaluronic acid was also partially digested by keratanase (40%) and chondroitinase ABC (30%), indicating that this glycosaminoglycan may contain some impurities of keratan and chondroitin sulfates. Chondroitin sulfate A also contains a small impurity (18%) which was not accessible to chondroitinase ABC or to any other of the enzymes used (Table II).

Ion Exchange Chromatography for g580

Charge homogeneity of g580 was examined by anion exchange chromatography. Continuous recording of the absorbance at 232 nm showed that g580 elutes as a single peak (g580-anex, anex = anion exchange) at 87 mM phosphate buffer, pH 6.0 (Fig. 5), indicating that g580 is homogeneous with respect to overall charge. Measurements of the neutral hexose and the uronic acid content of g580-anex revealed a recovery higher than 90%, while less than 5% of the material applied on the column was eluted with the equilibration buffer.

Immunological Characterization of g580

The chemical composition of g580 as well as its resistance to glycosaminoglycan-degrading enzymes indicated that this glycan closely resembles the 200 kDa sponge adhesion glycan (g200) [Misevic and Burger, 1993]. In order to further investigate structural similarities shared by these two glycans, we tested the binding of mouse polyclonal antisera, raised against purified g580, to the sponge glycan g200 and to g580 by an immunodot assay. As shown in Figure 6, this polyclonal antisera binds to g580 and to g200 but not to a glycan fraction, with the same molecular weight as g580, isolated from fertilized sea urchin eggs. There was also no binding

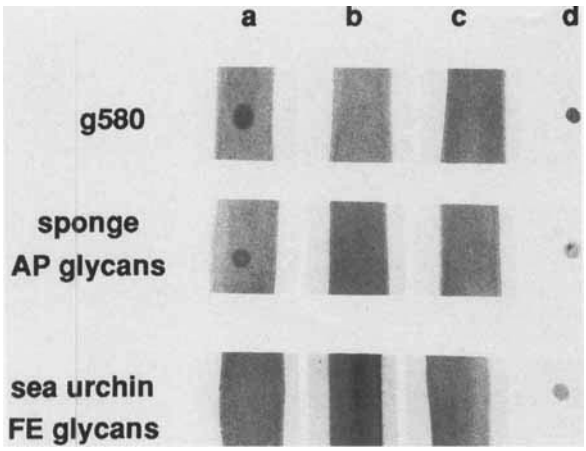

Fig. 6. Immunodot assay with anti-g580 polyclonal antibodies. From 1 mg/ml solution of g580, g200, or of a glycan fraction isolated from sea urchin fertilized eggs (gFE) 0.5 μl were spotted onto DEAE-cellulose sheets and assayed for cross reactivity with mouse polyclonal antisera, raised against purified g580, or stained with Alcian blue. **a:** Blots assayed with mouse anti-g580 antisera, **b:** blots assayed with pre-immune serum, **c:** control, only second antibody, **d:** blots stained with Alcian blue.

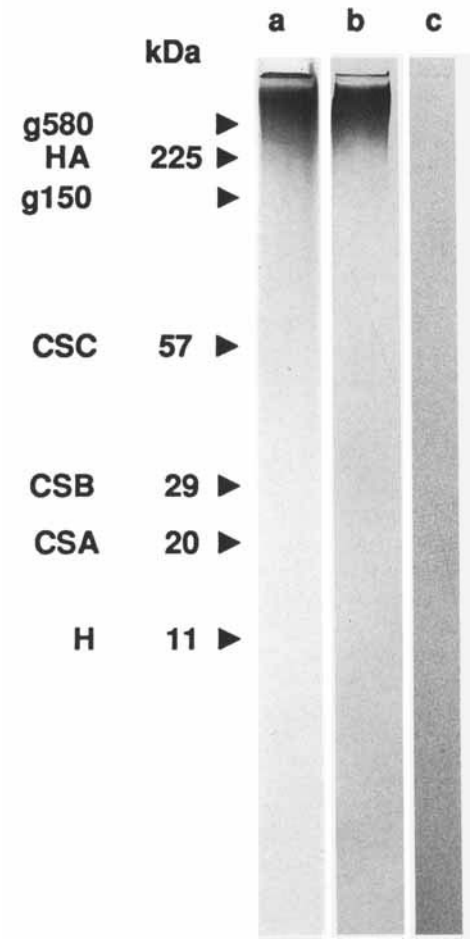

Fig. 7. Immunoblotting of g580 with mAb Block 2. Gel electrophoresis of 10 μg of g580 and blotting onto DEAE-cellulose sheets was performed as described under "Materials and Methods." Blots were either stained with Alcian blue or assayed for cross reactivity with the mAb Block 2. **a:** Blot stained with Alcian blue; **b:** blot assayed with mAb Block 2; **c:** control, blot assayed with only the second antibody. CSA, chondroitin sulfate A; CSB, chondroitin sulfate B; CSC, chondroitin sulfate C; HA, hyaluronic acid; H, heparin.

of any of the glycans tested with preimmune serum or with an unrelated antibody.

The g200 sponge glycan carries a highly repetitive epitope, mediating cell adhesion through carbohydrate–carbohydrate interactions in marine sponges [Misevic and Burger, 1993]. This epitope is specifically recognized by the mAb Block 2. We showed, by immunoblot assays, that g580 strongly cross reacts with the mAb Block 2 (Fig. 7). Alcian blue staining of the DEAE-cellulose sheets and the gels after blotting showed that more than 90% of the glycan material was transferred on DEAE-cellulose sheets (data not shown).

Competition studies were performed for the binding specificity of the mAb Block 2 to g580 using different concentrations of (1) purified g580 and (2) the sponge AP total glycans. This is an equimolar mixture of g200 and g6 glycans from which only the g200 is recognized by the mAb Block 2, as shown by Misevic et al. [1987] and Misevic and Burger [1993]; (3) of the g2 neutral glycan fraction; and (4) of a mixture of glycosaminoglycans (chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, heparin, heparan sulfate, keratan sulfate, and hyaluronic acid). As shown in Figure 8 (columns 1–3), complete inhibition of the binding of 1 μg of the mAb Block 2 to 0.5 μg of g580 was achieved by 10–20 μg of g580 and by 5 μg of g200. The neutral glycan fraction g2 as well as the mixture of the glycosaminoglycans could not inhibit the binding of the mAb to g580 even at a 20-fold weight excess. A competition assay for the binding of the mAb Block 2 to g200 was also performed using the same compounds. The results shown in Figure 8 (columns 4–6) indicated that 20 μg of g580 and 5–10 μg of g200 could completely inhibit the binding of 1 μg of the mAb Block 2 to 0.25 μg of g200. Neither the g2 fraction nor the mixture of the glycosaminoglycans were able to block the binding of the mAb to the sponge glycans. These results showed that g580 bears a carbohydrate epitope similar to the one reported

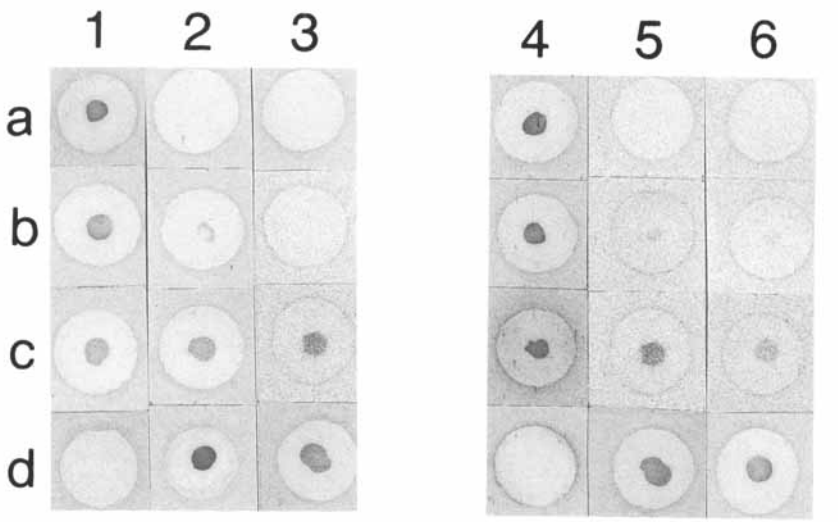

Fig. 8. Binding competition of g580 and of sponge AP total glycans to mAb Block 2. From 1 mg/ml solution of g580 (columns 1–3) or of sponge AP total glycans (containing an equimolar mixture of g6 and g200 glycans where only g200 binds to the mAb Block 2, columns 4–6) 0.5 μl were spotted on DEAE-cellulose sheets and assayed for the binding of the mAb Block 2. **1a, 4a,** mAb Block 2; **1b, 4b,** mAb Block 2 preincubated with a 20-fold weight excess of g2; **1c, 4c,** mAb Block 2 pre-incubated with a 20-fold weight excess of a mixture of glycosaminoglycans; **1d, 4d,** control, only second antibody; **2a–d, 5a–d,** mAb Block 2 preincubated with 20, 10, 1, and 0.1-fold weight excess of g580, respectively; **3a–d, 6a–d,** mAb Block 2 preincubated with 20, 10, 1, and 0.1-fold weight excess of sponge AP total glycans, respectively.

to mediate cell adhesion through carbohydrate–carbohydrate interactions in marine sponges.

Reaggregation Studies

In order to investigate whether the g580 carbohydrate epitope recognized by the mAb Block 2 has a function in sea urchin embryonal cell adhesion, the reaggregation of dissociated mesenchyme blastula cells was followed in the presence or absence of monovalent Block 2 Fab fragments. As shown in Figure 9, the naturally occuring adhesion was completely inhibited by the addition of 100 μg/ml of the Fab fragments. These results indicated that the carbohydrate epitope of g580, recognized by the mAb Block 2, is involved in sea urchin embryonal cell adhesion.

DISCUSSION

In the present study we report the isolation and characterization of two novel acidic glycan fractions, g580 and g150, from mesenchyme blastula sea urchin embryos, as well as the potential involvement of g580 in sea urchin embryonal cell adhesion. Both glycan fractions have a unique chemical composition that is distinct from the glycosaminoglycans. The g580 fraction is highly sulfated and contains high amounts of *N*-acetylglucosamine, *N*-acetylgalactosamine, glucuronic acid, and fucose. The g150 fraction is less acidic than g580 and contains both amino sugars, mannose, and remarkably high amounts of xylose. These high amounts of xylose have not previously been reported for eukaryotes. The complete removal of serine from g580 after alkali treatment suggested that this glycan fraction was *O*-linked to the core protein. Amino acid analysis of g150, prior to alkali treatment, showed the presence of both serine and asparagine, indicating that g150 fraction is more heterogeneous with respect to the linkage with the core protein.

The chemical composition of g580 and g150 as well as their resistance to glycosaminoglycan-degrading enzymes, such as chondroitinase ABC, heparinase, heparitinase, keratanase, and hyaluronidase, indicates that they differ from the glycosaminoglycans. They are also different from glycans isolated from several invertebrate tissues, such as the body wall of sea cucumber [Vieira and Mourao, 1988], the tunic of the ascidia *Styela plicata* [Vieira and Mourao, 1986], and the skin of squid [Karamanos et al., 1990]. Compared with the 35 kDa chondroitin sulfate of sea cucumber, sea urchin glycans are different because they contain *N*-acetylglucosamine and lower amounts of fucose. The presence of glucuronic acid in g580 and g150 sea urchin glycans also distinguishes them from the ascidian glycans. Furthermore, g150 has high

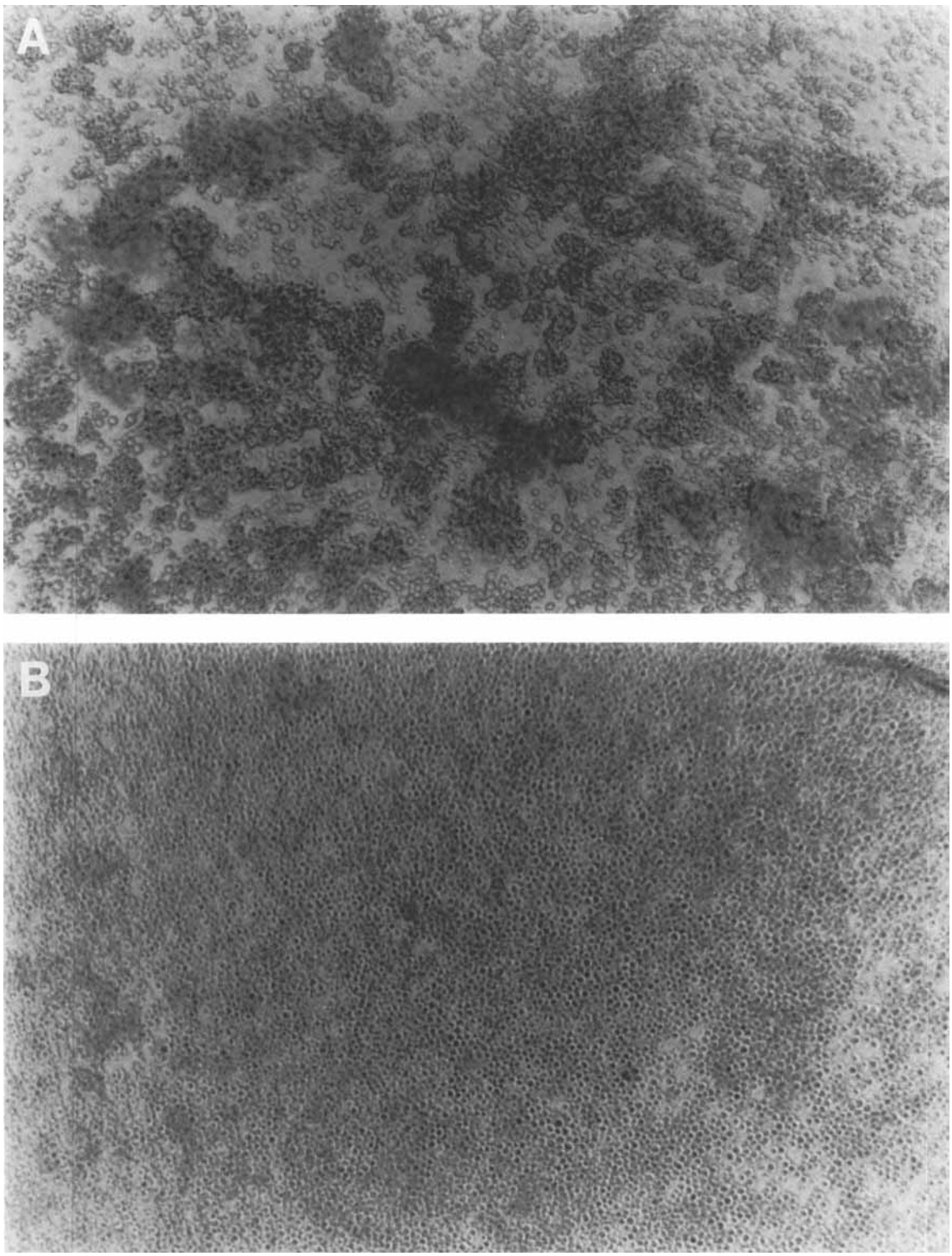

Fig. 9. Reaggregation studies. Thirty microliters of ASW containing 10^6–10^7 dissociated mesenchyme blastula cells per ml were placed in a glass chamber. The cells were then incubated at room temperature, under gentle rotation in the absence (**A**) or presence (**B**) of 100 μg/ml of the mAb Block 2 Fab fragments. After 20 min photographs were taken using a 10× objective.

amounts of xylose which are not found in sea cucumber or ascidian glycans. The sea urchin glycans also differ from the oversulfated chondroitin sulfate chains present in squid skin because the former are less sulfated and they contain different proportions of neutral sugars.

The g580 fraction appears to be related to a recently described glycan, g200, from the marine sponge *Microciona prolifera,* which belongs to a new class of acidic carbohydrates [Misevic and Burger, 1993]. Both g200 and g580 contain high amounts of *N*-acetylglucosamine, glucuronic acid, and fucose. The g200 and g580 are resistant to glycosaminoglycan-degrading enzymes, indicating that they are distinct from the glycosaminoglycans. The binding of polyclonal antibodies, prepared against purified g580, to g200 and to g580 indicated that they carry similar antigenic determinants. It was shown that g200 carries a highly repetitive epitope which mediates cell adhesion via carbohydrate–carbohydrate interactions in marine sponges. This epitope is specifically recognized by the mAb Block 2 [Misevic and Burger, 1993]. In this report, we showed by immunoblotting that g580 specifically cross reacts with the mAb Block 2. Competition studies using the sponge AP total glycans, the known glycosaminoglycans, as well as the neutral glycan fraction g2, provided evidence for the specificity of binding of the mAb Block 2 to g580 and to sponge glycans. Since the sponge AP consists of an equimolar mixture of g200 and g6 glycans, and since the mAb Block 2 recognizes only the g200 glycan [Misevic et al., 1987; Misevic and Burger, 1993], the binding inhibition obtained with the total AP glycans can be attributed only to the binding of g200 to the mAb Block 2.

It was previously shown that the mAb Block 2 inhibits cell adhesion in sponges [Misevic and Burger, 1993]. In the present study we demonstrated that the Fab fragments of the mAb Block 2 completely inhibited the reaggregation of dissociated mesenchyme blastula cells, indicating that the g580 carbohydrate epitope recognized by the mAb Block 2 is involved in sea urchin embryonal cell adhesion.

Cell adhesion in sponges is mediated by multiple, low affinity carbohydrate–carbohydrate interactions of the epitope sites which are recognized by the mAb Block 2 [Misevic and Burger, 1993]. This allows for specific but reversible cell–cell or cell–extracellular matrix attachment and in addition facilitates modulation of the specificity and strength of binding by small structural changes of the active sites. In this way, polyvalent carbohydrates could complement the function of other cell–cell and cell–substratum adhesion molecules in the multistep processes of cellular interactions during embryonal development. Sea urchin embryos also contain a carbohydrate adhesion epitope recognized by the mAb Block 2 which resembles the sponge epitope. It remains to be elucidated whether the same adhesion mechanism, via carbohydrate–carbohydrate interactions, is shared by the evolutionary more advanced sea urchins.

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