

Immunological Characterization of a Basement Membrane-specific Chondroitin Sulfate Proteoglycan

Kevin J. McCarthy, Mary Ann Accavitti,* and John R. Couchman

Departments of Cell Biology and Anatomy and *Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract. Reichert's membrane, an extraembryonic membrane present in developing rodents, has been proposed as an *in vivo* model for the study of basement membranes. We have used this membrane as a source for isolation of basement membrane proteoglycans. Reichert's membranes were extracted in a guanidine/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate buffer followed by cesium chloride density-gradient ultracentrifugation under dissociative conditions. The proteoglycans were subsequently purified from the two most dense fractions (>1.3 g/ml) by ion-exchange chromatography. Mice were immunized with the proteoglycan preparation and four mAbs recognizing the core protein of a high-density, buoyant chondroitin sulfate proteoglycan were raised.

Confirmation of antibody specificity was carried out by the preparation of affinity columns made from each of the mAbs. Chondroitin sulfate proteoglycans (CSPGs) were purified from both supernatant and tis-

sue fractions of Reichert's membranes incubated in short-term organ culture in the presence of radiolabel. The resultant affinity-purified proteoglycan samples were examined by gel filtration, SDS-PAGE, and immunoblotting. This proteoglycan is of high molecular weight ($M_r = 5-6 \times 10^5$), with a core protein of $M_r = \sim 1.5-1.6 \times 10^5$ and composed exclusively of chondroitin sulfate chains with an average $M_r = 1.6-1.8 \times 10^4$. In addition, a CSPG was purified from adult rat kidney, whose core protein was also $M_r = 1.6 \times 10^5$. The proteoglycan and its core protein were also recognized by all four mAbs. Indirect immunofluorescence of rat tissue sections stained with these antibodies reveal a widespread distribution of this proteoglycan, localized specifically to Reichert's membrane and nearly all basement membranes of rat tissues. In addition to heparan sulfate proteoglycans, it therefore appears that at least one CSPG is a widespread basement membrane component.

BASEMENT membranes are highly specialized extracellular matrices present at the interface between the parenchymal and mesenchymal compartments of tissues. A number of macromolecules have been isolated from basement membranes; some of the most abundant and well characterized include laminin, entactin, fibronectin, type IV collagen, and heparan sulfate proteoglycans (23, 24, 41, 42, 44, 46, 54). However, although it is clear that basement membranes provide molecular information to tissues, the physical, biochemical, and biological properties of their component molecules are not well understood.

We are currently concerned with the isolation, characterization, and determination of function of the proteoglycan component of basement membranes. Unlike other basement membrane components, proteoglycans are hypervariable molecules resulting from variation in glycosaminoglycan chain number, length, sulfation, and uronic acid epimerization as well as the presence of a number of distinct core proteins (11,

23). These possibilities for structural modification may, in turn, be reflected in a wide range of functions for basement membrane proteoglycans. At present postulated functions include a structural role in maintaining tissue histoarchitecture (41, 46), selective filtration properties (17), sequestration of growth factors (26, 47) and extracellular ions (1, 37), and regulation of cellular differentiation (2, 8, 38).

Basement membrane proteoglycans have primarily been isolated either from *in vivo* sources such as glomerular basement membrane, placenta, tumors such as the Englebreth-Holm-Swarm (EHS) tumor, or *in vitro* sources such as the PYS-2 and L-2 yolk sac tumor cell lines (9, 19, 21, 24, 31, 43, 44, 46). Reichert's membrane, also known as the parietal yolk sac, has been proposed both as a source for basement membrane components and as a model for the study of basement membrane structure and function (25). It is a transient extraembryonic membrane surrounding the amniotic sac of the developing rodent fetus, first appearing on approximately day 8 of gestation and disappearing ~ 9 d later (6). The extracellular matrix of Reichert's membrane is mainly a product of the adherent parietal endoderm cells (6, 7), and has similarities to other basement membranes in that laminin, type IV collagen, fibronectin, and heparan sulfate proteoglycans all have been demonstrated in Reichert's membrane ei-

1. *Abbreviations used in this paper:* CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; CSPG, chondroitin sulfate proteoglycan; DTE, dithioerythritol; EHS, Englebreth-Holm-Swarm tumor; GAG, glycosaminoglycan; NEM, *N*-ethylmaleimide; TBS, Tris-buffered saline; TTBS, TBS with 0.5% Tween-20.

ther biochemically or immunohistochemically (7, 18, 25, 28). At the same time, it differs ultrastructurally from most basement membranes in that it has a structure distinct from the "prototypical" lamina rara and densa-type organization (27).

In this study we have examined whether a basement membrane-specific chondroitin sulfate proteoglycan (CSPG) could be demonstrated with core protein-specific antibodies, following earlier work which showed a widespread distribution of the carbohydrate chondroitin 6-sulfate in basement membranes (10). We now report a panel of four mAbs that all recognize the core protein of a CSPG present in Reichert's membrane. Immunostaining of tissue sections with the mAbs show that this antigen is located specifically in Reichert's membrane and, more importantly, it is present in the basement membrane of almost every tissue so far examined.

Materials and Methods

Materials

All chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Sepharose CL-2B or -4B, DEAE-Sephacel, Mono-Q HR, Sephadex G-25 Superfine or G-50, and Sephacryl S-300 were all from Pharmacia Fine Chemicals (Piscataway, NJ). EDTA was from Fisher Scientific (Pittsburgh, PA).

Isolation of Proteoglycans from Rat Reichert's Membrane and Kidney

Reichert's Membrane. 20 timed pregnant Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) were killed on day 12 of gestation by lethal injection of pentobarbital. The uteri were removed, washed with PBS and opened and the fetus, placenta, and associated membranes were removed. The Reichert's membranes were then dissected free from the placenta and immediately placed in 100 ml of lysis buffer (50 mM Tris pH 7.0, 0.1% Triton X-100, 10 mM EDTA, 100 mM epsilon-aminocaproic acid, 5 mM benzamidine HCl, 5 mM *N*-ethylmaleimide (NEM), 0.01 mM pepstatin, and 0.005 mM leupeptin). The membranes were gently agitated in the buffer, and then sedimented under low-speed centrifugation (1,000 rpm). The supernatant was discarded and the membranes were resuspended in lysis buffer for two further agitation/centrifugation cycles. After a final buffer wash, the membranes were resuspended in buffer (as above with 4.2 mM MgSO₄ and lacking 10 mM EDTA). Deoxyribonuclease I was added (final concentration of 20 µg/ml), and the membranes were incubated at 25°C for 4 h. The membranes were rinsed, then suspended in 30 ml extraction buffer (150 mM sodium acetate pH 5.8, containing 6 M guanidine, 1 mM dithioerythritol (DTE), 10 mM EDTA, 0.2 mM PMSF, 100 mM epsilon-aminocaproic acid, 4% 3-(3-cholamidopropylammonio)-1-propanesulfonate (CHAPS) (Pierce Chemical, Rockford, IL), briefly homogenized, and then extracted at 4°C on an end-over-end mixer for 48 h. The extracts were centrifuged at 15,000 rpm (SA-600 rotor, Sorvall Superspeed Centrifuge, Sorvall Instruments Div. Dupont Co., Wilmington, DE) for 15 min and the pellet was discarded. The guanidine concentration of the supernatant was adjusted to 4 M and solid cesium chloride added to give a starting density of 1.2 g/ml before centrifugation for 40 h at 100,000 g (Ti 50.2 rotor, Sorvall RC-60 Ultracentrifuge). The contents of the tubes were divided into five fractions and their densities were measured. The two most dense fractions, (4 and 5: buoyant density 1.30 and 1.35 g/ml, respectively) were separately dialyzed into 50 mM Tris pH 8.0, containing 8 M urea, 0.2 M NaCl, 1 mM DTE, 10 mM EDTA, 0.2 mM PMSF, 0.1% Triton X-100 and loaded onto 3 × 1 cm columns of DEAE-Sephacel preequilibrated with the same buffer. The columns were washed sequentially with 100 ml of buffer, 50 ml of 50 mM sodium acetate pH 4.0, containing 8 M urea, 0.2 M NaCl, 1 mM DTE, 10 mM EDTA, 0.2 mM PMSF, 0.1% Triton X-100, and 50 ml of the same buffer without Triton X-100, and then eluted in 1-ml fractions with 50 mM sodium acetate pH 4.0, containing 8 M urea, 1.5 M NaCl, 1 mM DTE, 10 mM EDTA, 0.2 mM PMSF. Protein concentration of each fraction was checked by reading UV absorbance at 280 nm and constituents assayed by 3–15% SDS-PAGE followed by silver staining (9). The presence of uronic acid in each fraction was determined by the method of Bitter and Muir (3).

Kidney. The extraction scheme for isolation of proteoglycans from rat

kidney was similar to that described above for Reichert's membrane, with the following changes. Kidneys were removed from 150 young adult rats, minced into 5 × 5 mm pieces, put into cell lysis buffer (see above) chilled to 4°C, homogenized in a rotary mill homogenizer (Yamato Scientific, Tokyo, Japan); the homogenate was then processed for DNase digestion as above. Afterwards, the mixture was repelleted by low-speed centrifugation (1,000 rpm), resuspended in 20 vol of extraction buffer, extracted for 24 h, and processed through dissociative cesium chloride density gradient centrifugation. The resultant two most dense fractions (buoyant density 1.40 and 1.45 g/ml, respectively) were then dialyzed into 50 mM Tris pH 8.0, containing 4 M urea (Ultrapure; Bethesda Research Laboratories, Bethesda, MD), 0.2 M NaCl, 1 mM DTE, 10 mM EDTA, 0.2 mM PMSF, and 0.1% Triton X-100 and loaded at 0.25 ml/min onto an HPLC (Gilson Co., Worthington, OH) equipped with a Mono-Q ion exchange column preequilibrated in the same buffer. The column was then washed with buffer at 0.5 ml/min for 1 h, and then with 50 mM sodium acetate pH 4.0, containing 4 M urea (Ultrapure, Bethesda Research Laboratories), 0.2 M NaCl, 1 mM DTT, 10 mM EDTA, 0.2 mM PMSF, 0.1% Triton X-100 for an additional hour. The column was then eluted with a 20-ml, 0.2–1.5 M sodium chloride gradient in the acetate buffer (pH 4.0) at a flow rate of 0.25 ml/min. 1-ml fractions were collected, and the composition of each fraction was assayed by SDS-PAGE and immunoblotting with R44 antiserum after chondroitinase ABC treatment (see below). Those fractions found to contain CSPG were pooled, dialyzed into Tris-buffered saline (TBS) with protease inhibitors (20 mM Tris [pH 7.5], 500 mM NaCl, 5 mM benzamidine HCl, 0.2 mM PMSF). The glycosaminoglycan (GAG) concentration for each sample was estimated by the method of Farndale et al. (16).

mAb Production

BALB/c mice (Charles River Breeding Laboratories, 8–12 wk old) were immunized according to the procedure of Lieberman et al. (39) with slight modifications. Each mouse was injected with 200 µg of purified Reichert's membrane proteoglycan emulsified with an equal volume of complete Freund's adjuvant, distributing the antigen subcutaneously into the rear footpads and inguinal region. Using antigen in physiological saline, we repeated this procedure four times at 3–4 d intervals. 1 d after the last injection, the local lymph nodes draining the subcutaneous areas of immunization were removed, prepared as a cell suspension, and fused with the nonsecreting mouse myeloma line P3X63AB.653 according to established methods (34). 14 d after the fusion, tissue culture supernatants were screened by indirect immunofluorescence on frozen tissue sections of Reichert's membrane, skeletal muscle, and duodenum. Those clones whose supernatants were positive in the screening procedure were subcloned by limiting dilution, expanded to high density, and injected into pristane-primed BALB/c mice for the production of ascites fluid. The determination of the subclass of the resultant mAbs was established by Ouchterlony plate assay using Ig-subclass-specific antibodies (Zymed, South San Francisco, CA).

Purification of IgG Fractions from Ascites Fluid

Solid calcium chloride was added to the ascites sample to give a final concentration of ~1 mM and the sample was incubated at room temperature for 2 h. Any clots were removed. After overnight incubation at 4°C, further clots were removed and the ascites was centrifuged at 11,500 rpm (Sorvall SA-60 rotor) for 60 min at 4°C. The supernatant was diluted 1:2 with binding buffer (pH 9.3) (Bio-Rad Laboratories, Richmond, CA) or with an alternate binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9). Five milliliters of the clarified ascites fluid was loaded at 5 ml/min onto a Protein A-MAPS preparative cartridge (Bio-Rad Laboratories) equilibrated with either binding buffer. The column was eluted at 5 ml/min with either elution buffer (pH 3.0) (Bio-Rad Laboratories), the pH of the eluent being adjusted immediately to 7.0, or 0.1 M citric acid, pH 6.0. The eluted antibodies were immediately dialyzed into coupling buffer (0.5 M NaCl, 0.1 M NaHCO₃, pH 8.0).

Organ Culture of Reichert's Membranes and Placentae

Reichert's membranes and placentae were removed from the fetuses of four dams, pooled separately, and placed into 35-mm tissue-culture dishes (Becton Dickinson & Co., Oxnard, CA) with 3 ml of DME medium (Mediatech, Herndon, VA) supplemented with 10% dialyzed FBS (Flow Laboratories, McLean, VA) glutamine, and penicillin/streptomycin. Radioactive inorganic sulfate was added to the cultures at a concentration of 250 µCi/ml (Amersham Corp., Arlington Heights, IL, SJS.1A, 1,103 Ci/mmol) and the

cultures incubated overnight at 37°C with 10% CO₂. After 1 d, the tissues were removed from the culture medium, rinsed briefly in unlabeled medium, and processed as above for proteoglycan isolation. The culture supernatants were collected and dialyzed into TBS with protease inhibitors (20 mM Tris pH 7.5, containing 500 mM NaCl, 5 mM benzamidine HCl, and 0.2 mM PMSF).

Preparation of Affinity Matrices and Affinity Chromatography

Affinity columns (2 ml volume, 2 mg IgG/ml gel) were made by covalently coupling (40) the four mAbs designated 2B5, 2D6, 5A3, and 4D5 or a nonimmune mouse IgG (Jackson Immunoresearch Labs, West Grove, PA) to Sepharose CL-2B. Affinity columns were equilibrated with immunoaffinity buffer (0.01 M Tris [pH 7.0] containing 0.5 M NaCl, 3 mM calcium acetate, 0.2 M PMSF, 5 mM benzamidine HCl, 1% Tween 20, and 5% 1-methyl-2-pyrrolidone). Aliquots of either ³⁵S-labeled supernatant, tissue proteoglycan fractions from organ cultures, or purified kidney CSPGs (see above) were gently mixed with the affinity matrices for 16 h. The columns were then drained, saving the flow through for future use, and washed thoroughly with immunoaffinity buffer until the radiolabel content of the wash was close to background. Radiolabeled proteoglycans were eluted from the columns with 3 ml of 3 M sodium thiocyanate, and the columns were regenerated by washing the columns with 10 ml of immunoaffinity buffer. Alternatively, antibodies were coupled to Nalgene affinity chromatography units, which couple proteins to inert support matrices via an aldehyde linkage (Nalgene, Rochester, NY), according to the methods described by the manufacturer and used in a manner similar to that described above.

Characterization of Affinity-purified Proteoglycans

The approximate mass of the ³⁵S-labeled affinity-purified proteoglycan was estimated by gel filtration on 0.5 × 104 cm Sepharose CL-4B columns equilibrated in either PBS; 50 mM Tris (pH 8.0), containing 4 M guanidine, 0.2 mM PMSF, 5 mM NEM, 10 mM EDTA, 0.1% Triton X-100; or 50 mM Tris (pH 8.0) containing 0.35 M NaCl, 0.1% SDS, 0.02% sodium azide, 0.2 mM PMSF, 5 mM NEM, 10 mM EDTA at a flow rate of 1 ml/h. The column was calibrated with blue dextran and dinitrophenol-alanine as standards. 80 300- μ l fractions were collected and assayed for radiolabel. Some samples were reduced and alkylated in the presence of SDS before chromatography (13). Further samples were dialyzed into distilled water and then subjected to alkaline elimination (13). The free GAG chains were treated overnight with nitrous acid (49), or dialyzed into 50 mM Tris (pH 8.0), 30 mM sodium acetate, 10 mM NEM, 0.2 mM PMSF, 0.02% sodium azide for overnight digestion with 0.1 U/ml chondroitinase AC II or ABC (ICN Biochemicals, Lisle, IL). Aliquots of the untreated, nitrous acid-treated, and chondroitinase ABC-digested samples were chromatographed on a 0.5 × 31 cm Sephadex G-50 column equilibrated in 20 mM Tris (pH 7.4), containing 1.0 M NaCl, 0.02% sodium azide, and 350- μ l fractions were collected. Aliquots of the chondroitinase AC II- and ABC-digested material were also chromatographed on a 0.5 × 31 cm Sephadex G-25 Superfine column in the same buffer and 250 μ l fractions were collected. The fractions were analyzed for radiolabel content.

Bovine tracheal cartilage chondroitin sulfate and heparin (molecular weight 4–6,000; Sigma Chemical Co.) GAGs were used as HPLC standards. The chondroitin sulfate GAG standards were prepared by chromatography on a Sephacryl S-300 column equilibrated in 0.2 M ammonium bicarbonate, collecting 1.5-ml fractions, and detecting the GAGs in each fraction by absorbance at 535 nm (16) after mixing 250 μ l aliquots with an equal volume of dimethylmethylene blue (Serva Fine Biochemicals, Garden City Park, NY) in formate buffer (pH 4.0) (16). Five consecutive fractions adjacent to (on both sides) and including the peak were pooled as separate groups and lyophilized. These were then rehydrated, and aliquots chromatographed over a Sephadex G-200 column (1 × 100 cm) equilibrated in 50 mM Tris pH 7.4, containing 0.2 M NaCl, 0.02% sodium azide at a flow rate of 1 ml/h. 80 2-ml fractions were collected, the presence of GAGs detected by the dimethylmethylene blue colorimetric assay (see above), and the molecular weight estimated from the work of Wasteson (56). Pool 1 (K_{av} = 0.11, estimated M_r = 35,000) and pool 3 (K_{av} = 0.28, estimated M_r = 19,000) GAGs were used as HPLC standards.

GAG chain size of alkaline borohydride-treated ³⁵S-labeled material was estimated by HPLC (Gilson Co.) using a Bio-Sil TSK-400 column (Bio-Rad Laboratories) that had been calibrated using the GAG standards prepared above. The column was equilibrated in 0.02 M sodium phosphate (pH 6.8),

containing 0.3 M sodium sulfate, 5% DMSO and run at 1 ml/min. Absorbance was read at 240 nm, and 250- μ l fractions of the eluent collected into tubes containing 250 μ l of dimethylmethylene blue to detect the GAG standards (16). The radiolabel content of the fractionated ³⁵S-labeled material was estimated by liquid scintillation counting.

Preparation of Rat Laminin

Rat laminin was prepared by affinity chromatography. The rat cell line L-2 (a gift from Dr. Ulla Wewer, National Institutes of Health) was grown in roller bottles (Corning Glass, Corning Medical, Corning, NY) in DME with 10% FBS. The medium was harvested at regular intervals, centrifuged to remove cellular debris, then protease inhibitors were added (0.2 mM PMSF, 10 mM NEM) and the medium stored at -20°C. 10 liters of medium was concentrated to ~200 ml in an ultrafiltration cell (Amicon Corp., Danvers, MA), and centrifuged. The supernatant was discarded and the pellet was redissolved in 30 ml of a 6 M guanidine/CHAPS buffer (see above) before exhaustive dialysis into immunoaffinity buffer (see above). This was repeatedly passed over a column of Sepharose 2B to which affinity-purified rabbit antibodies against murine laminin were coupled (40). The column was then washed with immunoaffinity buffer until the A₂₈₀ was at background, before elution with 3 M sodium thiocyanate. The eluent was dialyzed into PBS, and the protein concentration adjusted to 0.5 mg/ml. The composition of the eluent was checked by SDS-PAGE followed by both Coomassie brilliant blue and silver staining of the gel.

Gel Electrophoresis and Immunoblotting

5- μ l aliquots of ion-exchange chromatography-purified kidney proteoglycan fractions were diluted in 100 μ l of 50 mM Tris (pH 8.0), 30 mM sodium acetate, 10 mM NEM, 0.2 mM PMSF, 0.02% sodium azide and either kept untreated or digested overnight with chondroitinase ABC (0.03 U/100 μ l). Affinity-purified CSPGs isolated from Reichert's membrane or kidney preparations were also treated as above then concentrated to ~50 μ l in a Speed-vac (Savant Instruments, Inc., Farmingdale, NY) centrifuge. Samples were reduced with DTE (13) and run on 3–15% gradient SDS-PAGE (9) minigels. Gels were then fixed and processed for either silver (9) or alcian blue staining (36), or the material was electrophoretically transferred to nitrocellulose or cationized nylon (Bio-Rad Laboratories) for immunoblotting. For nitrocellulose, transfer was achieved over 3 h at 50 V using the buffer system described by Towbin (55); transfer conditions for cationized nylon were as previously described (9). The membranes were blocked overnight with a solution of TBS/milk (TBS with 0.5% powdered milk) and probed with either a polyclonal antibody, R44, that recognizes the remaining carbohydrate stubs of chondroitinase ABC-digested CSPGs (13); or R48, a polyclonal antibody recognizing heparan sulfate proteoglycan (9). The membrane was incubated with a 1:100 dilution of the antibody in TBS/milk for 5 h, followed by three 15-min washes with TTBS, incubation for 45 min with a 1:1,000 dilution of horseradish peroxidase conjugated goat anti-rabbit (Bio-Rad Laboratories) in TBS/milk, and washed in TTBS (3 × 15 min). Color development was completed using 4-chloro-1-naphthol (Bio-Rad Laboratories) as a substrate, according to the manufacturer's instructions. For some blots, a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, South San Francisco, CA) was diluted in TBS/milk and followed by detection with a streptavidin/biotin-alkaline phosphatase complex (Vector Laboratories) and color-developed using an appropriate substrate according to the manufacturer's instructions.

Dot Blot and ELISA Assays

Ion exchange-purified kidney material (2 μ g/well) or 2D6 antibody affinity-purified kidney CSPG (1 μ g/well) were applied to cationized nylon (Bio-Rad Laboratories) in a dot-blotting apparatus (Bio-Rad Laboratories). The samples were allowed to interact with the nylon for 5 h, after which the wells were rinsed with TBS then blocked overnight with TBS/milk. Some of the wells were additionally treated with 0.1 U/ml of chondroitinase ABC for 2 h. Afterwards, all the wells were rinsed with three 10-min changes of TTBS and the primary antibodies consisting of either the anti-CSPG mAbs 2B5, 2D6, 5A3, 4D5, or a chondroitin-6-sulfate-specific mAb 3B3 (5) or the chondroitin-4-sulfate-specific mAb 2B6 (5) applied to the wells at concentrations of 1, 5, and 10 μ g protein/well. A commercially available control mouse IgG was used at the same concentrations. Chondroitinase ABC activity was confirmed by the presence of reaction product in enzyme-treated wells with the anti-C-4 or -6 sulfate mAbs (5) (data not shown). The primary antibodies were allowed to interact with the proteoglycans for 5 h, af-

ter which the wells were rinsed with three 10-min changes of TTBS. A 1:1,000 dilution in TBS/milk of secondary antibodies consisting of either peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories) (for mAbs 2B5, 2D6, 5A3, 4D5, and control IgG) and/or peroxidase conjugated goat anti-mouse IgM (Organon/Technika, West Chester, PA) (for mAbs 3B3 and 2B6) was applied to the wells and incubated for 1 h. Afterwards, the wells were washed with three 10-min changes of TTBS. Color development was completed using 4-chloro-1-naphthol as a substrate.

Rat laminin, rat chondrosarcoma proteoglycan, and kidney proteoglycans in 20 mM sodium bicarbonate, 20 mM sodium carbonate buffer, pH 9.2 were adsorbed (or in the case of the CSPG, dried) overnight at 4°C onto a 96-well plate (Flow Laboratories). The wells were washed with PBS, blocked with 1% heat-treated BSA and 1% normal goat serum, and washed with Tween-PBS (TPBS, PBS with 0.05% Tween 20). Anti-CSPG mAbs, polyclonal antilaminin serum (1:200 dilution), anti-bovine nasal cartilage proteoglycan antiserum (13) (1:200) or preimmune rabbit serum (from the rabbit used to make the antilaminin polyclonal antibodies) (1:200) diluted with TPBS, were added to the wells and incubated at 37°C for 1 h. The wells were washed with TPBS, and then a 1:2,000 dilution of either alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Bio-Rad Laboratories), was added and the plate incubated for a further 1 h at 37°C. The plate was washed with both TPBS and PBS, color developed with *p*-nitrophenol phosphate per the manufacturer's instructions, and read on a microtiter plate reader (Molecular Devices, Menlo Park, CA) at 405 nm.

Indirect Immunofluorescence Microscopy

Immunostaining of frozen tissue sections with hybridoma tissue culture supernatants was performed as previously described (9). In some cases, tissue sections were immunostained after a prior 1-h digestion with a 0.1-U/ml solution of chondroitinase ABC. Enzyme activity was confirmed by immunostaining some tissue sections with mAb 3B3 (10), which recognizes CSPGs after prior digestion with chondroitinase ABC. Primary antibody localization was detected using FITC-conjugated goat anti-mouse IgG (no cross reactivity to rat IgG, Organon/Technika) as a secondary antibody (1:50 dilution). For comparison, some tissue sections were double-labeled with both anti-CSPG mAb 2D6 and antiserum against a small heparan sulfate proteoglycan (9) used at a 1:50 dilution. Primary antibody localization for the anti-CSPG antibody was detected as above; the anti-heparan sulfate proteoglycan antibody localization was detected by TRITC-conjugated goat anti-rabbit IgG (Organon/Technika). In all circumstances, the mAb was applied first in the double staining protocol.

For single-label studies, negative controls included omission of the primary antibody, or nonimmune mouse IgG in place of the mAb and the use of preimmune serum from the rabbit used to raise the antiproteoglycan serum. These controls gave background levels of fluorescence in each case. Controls for double labeled studies included tissue sections stained with (a) single primary antibodies followed by inappropriate secondary antibodies; (b) one primary antibody with both secondary antibodies; (c) secondary antibodies alone. Control *b* was positive only for the appropriate secondary antibody. Controls *a* and *c* were negative.

All tissue sections were viewed on a Nikon Optiphot microscope equipped with epi-illumination optics and appropriate filters. Photomicrographs were taken on Ilford HP-5 or Kodak Ektachrome-400 film.

Results

Isolation of Proteoglycans from Reichert's Membrane

Tissue extracts from ~250 Reichert's membranes were processed through cesium chloride density-gradient ultracentrifugation under dissociative conditions. The two most dense fractions, having a buoyant density of 1.30 (fraction 4) and 1.35 g/ml (fraction 5) contained 0.052 and 0.041 mg/ml uronic acid, respectively. Fig. 1 shows a silver-stained 3–15% SDS-PAGE gel of the material present in fractions 4 and 5. The composition of fraction 4 is relatively homogeneous, whereas the material found in fraction 5 shows the presence of many polypeptides. These fractions were separately processed during the rest of the isolation protocol. DEAE-ion exchange chromatography was used in attempts to further purify and concentrate both fractions, but the SDS-PAGE

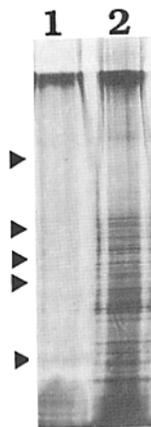


Figure 1. Silver-stained 3–15% SDS-PAGE of cesium chloride density gradient fractions 4 (lane 1) and 5 (lane 2) from a rat Reichert's membrane preparation. The densities for each fraction were 1.30 and 1.35 g/ml, respectively. Fraction 4 consists primarily of one high-molecular weight protein species, fraction 5 has a more heterogeneous protein composition. Molecular mass markers (from top to bottom): 200 kD, 116 kD, 94 kD, 68 kD, 43 kD.

profiles of the eluted fractions remained virtually unchanged (data not shown).

mAb Production and Screening

Fractions 4 and 5 were separately used as antigens to immunize mice for the production of mAbs. The resultant fusions yielded ~650 clones, whose activities were screened by indirect immunofluorescence microscopy. Using specific immunostaining of basement membranes of frozen tissue sections as a selection criterion, we were able to select twelve clones, the characterization of four of which is presented below. All four clones were from a mouse injected with the more homogeneous fraction 4 material.

Affinity Chromatography

Affinity columns were made by coupling the four immune and control mouse IgG preparations to Sepharose CL-2B. Table I shows the results of two typical affinity purification procedures made with two different sets of affinity columns. Approximately 710,000 cpm of ³⁵S-labeled supernatant proteoglycans or 71,000 cpm of ³⁵S-labeled tissue extract-derived proteoglycans (buoyant density, 1.45 g/ml on cesium chloride density-gradient centrifugation) obtained from short-term organ cultures of Reichert's membranes were loaded onto the affinity columns. As Table I indicates, the monoclonal affinity columns were able to specifically bind ³⁵S-la-

Table I. Affinity Purification of ³⁵S-labeled Material

	Supernatant*	Membrane†‡
	³⁵ S-associated cpm	
2B5	14,990	3,890
2D6	55,970	12,870
5A3	59,160	11,250
4D5	27,640	6,700
IgG	45	ND§

Affinity purification with the mAbs covalently coupled to Sepharose CL-2B. The columns bind [³⁵S]-labeled macromolecules from both medium and cesium chloride density-gradient material obtained from Reichert's membrane in short-term organ culture with [³⁵SO₄]. A nonimmune mouse IgG (column 1) did not bind ³⁵S-labeled material.

* 710,000 cpm loaded.

† 71,000 cpm loaded.

‡ Not done.

§ The proteoglycan from Reichert's membrane was taken from a cesium chloride density gradient centrifugation fraction of 1.45 g/ml buoyant density.

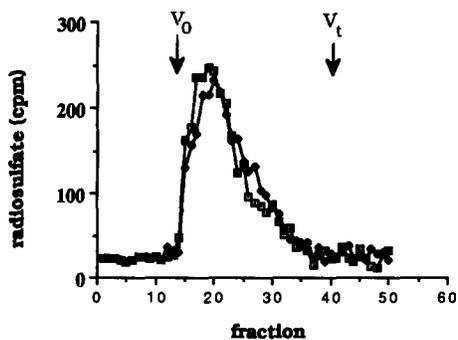


Figure 2. Gel chromatography on a column of Sepharose CL-4B of ^{35}S -labeled, affinity purified CSPG from rat Reichert's membrane organ culture supernatant, before (open squares) and after (closed diamonds) reduction and alkylation. The material was purified with 2D6 antibody, and chromatographed in 0.1% SDS, 0.35 M NaCl, 0.05 M Tris HCl, pH 8.0, and protease inhibitors.

beled proteoglycans from both the supernatant and tissue extract-derived material isolated from organ cultures. The relative amounts of bound proteoglycan were different for each antibody, yet constant between different batches of affinity matrix for the same antibody. These results probably arise from differences in the affinity of the antibodies for proteoglycan, because the antibody loading on to each affinity matrix was fairly constant. Antibodies were also coupled to membrane affinity matrices (Nalgene), a very different solid-phase support. Similar results in terms of ^{35}S -labeled macromolecular binding were seen using these supports. Further evidence for the specificity of the antibodies was provided by the fact that all the ^{35}S label bound to the affinity column was CSPG, which we have found accounts for only 44% of the total macromolecular radiolabel applied to the column. Additionally, radiolabeled GAG chains, released by alkaline borohydride treatment of affinity-purified proteoglycans also used to test the specificity of binding, were found not to be retained by the affinity columns. (McCarthy, K. J., and J. R. Couchman, unpublished results.)

Characterization of Affinity-purified Proteoglycan

To determine the approximate mass of the proteoglycan recognized by the antibodies, ^{35}S -labeled affinity-purified proteoglycan from Reichert's membrane was subjected to gel filtration on a column of Sepharose CL-4B in the presence of SDS. Fig. 2 shows that the proteoglycan eluted from the column with a $K_{av} = 0.16$, giving an $M_r = \sim 5\text{--}6 \times 10^5$ for the intact proteoglycan. The elution pattern was not altered by prior reduction and alkylation in the presence of SDS. The proteoglycan also eluted with the same K_{av} from CL-4B columns equilibrated in either PBS or 4 M guanidine and 0.5% Triton X-100 (not shown). These results indicate that the proteoglycan does not exist as a multimolecular aggregate either through covalent or noncovalent associations. The proteoglycan was further characterized by testing its susceptibility to either nitrous acid or chondroitinase ABC, followed by chromatography of the digests over a Sephadex G-50 column. The results (data not shown) indicated that the GAG chains attached to the core protein were chondroitinase ABC sensitive. In a further analysis, ^{35}S -labeled GAG chains were released from the core protein by alkaline elimination

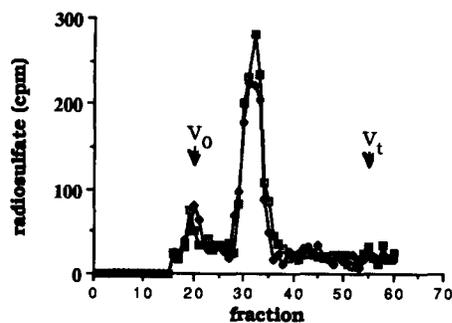


Figure 3. Glycosaminoglycan chains were prepared from 2D6 antibody-purified CSPG from ^{35}S -labeled rat Reichert's membrane organ culture supernatants, subjected to alkaline elimination and chromatographed on a column of Sephadex G-25 Superfine after chondroitinase ABC (open squares) or chondroitinase AC II treatment (closed diamonds). Identical elution profiles after both enzyme treatments indicate the absence of iduronic acid-rich regions in the GAG chains.

and digested overnight with either chondroitinase AC II or ABC, followed by gel filtration on a column of Sephadex G-25 Superfine. Fig. 3 shows that the glycosaminoglycan chains were completely susceptible to both enzymes, indicating that chondroitin sulfate is the predominant GAG, with undetectable levels of dermatan sulfate.

GAG chain size was estimated by HPLC gel filtration of ^{35}S -labeled GAG on a TSK-400 column and compared with the elution profiles of glycosaminoglycan standards of known molecular size. Fig. 4 shows that the chondroitin chains derived from the Reichert's membrane proteoglycan eluted as a single broad peak later than the chondroitin sulfate GAG chains of $M_r = 3.5 \times 10^4$, almost coincident with GAG chains of $M_r = 1.9 \times 10^4$, and earlier than the heparin GAG chains (estimated $M_r = 4\text{--}6 \times 10^3$). From the elution profile we estimate the GAG chains to have an $M_r = \sim 1.6\text{--}1.8 \times 10^4$.

Identification of the Basement Membrane CSPG Core Protein

Although Reichert's membrane was the source of the antigen used to raise mAbs against basement membrane CSPG and short-term organ cultures provided adequate quantities of

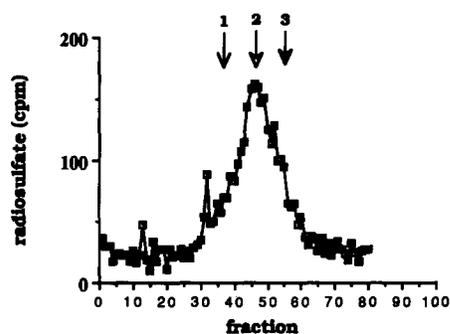


Figure 4. HPLC gel filtration on a TSK-400 column of alkaline borohydride released glycosaminoglycan chains from ^{35}S -labeled, affinity purified Reichert's membrane CSPG. The arrows indicate the elution peaks of glycosaminoglycan standards; $M_r = 35,000$ (1), 19,000 (2), and 4-6,000 (3), respectively.

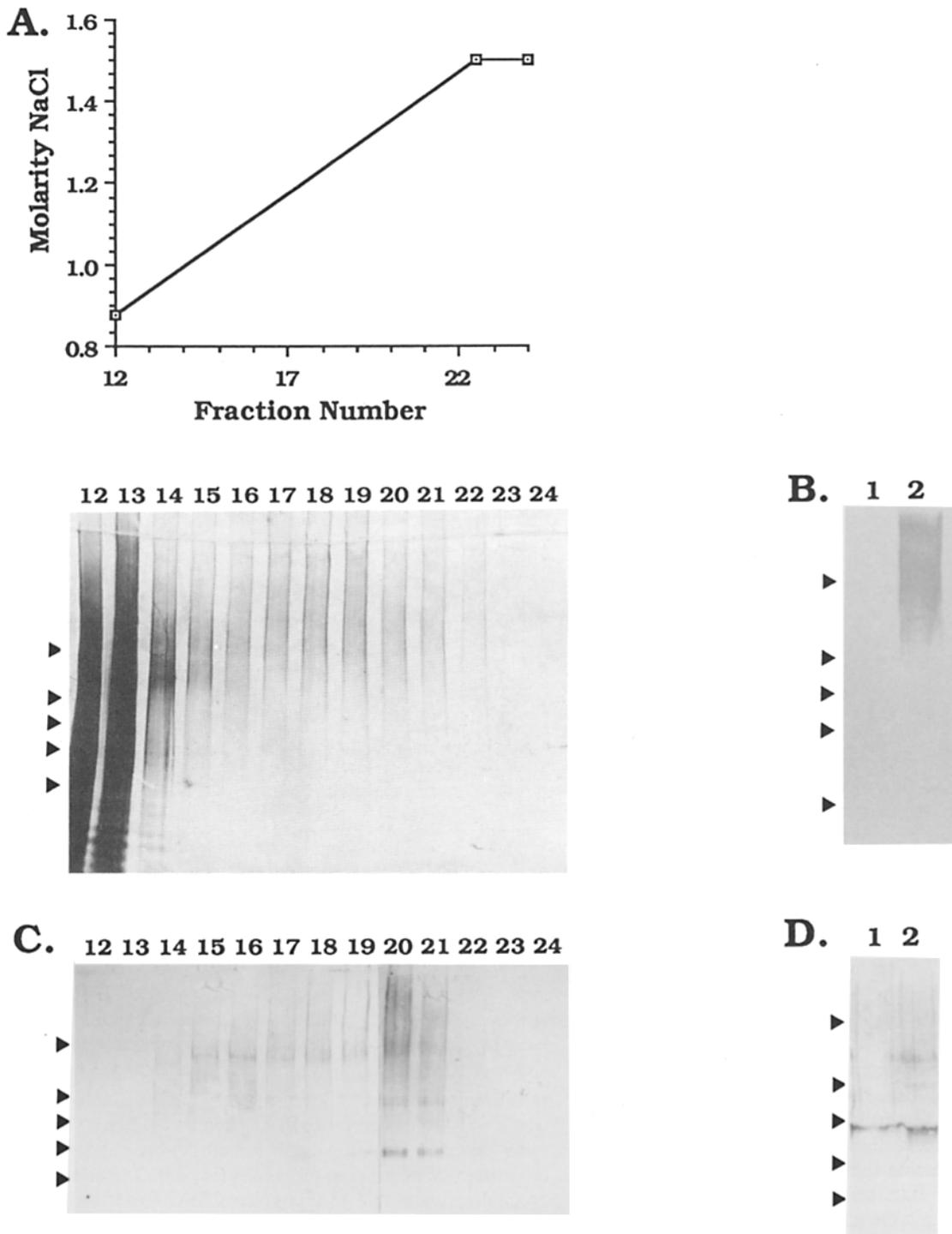


Figure 5. (A) Silver-stained polyacrylamide gel showing the composition of fractions 12-24 of high buoyant density (>1.45 g/ml) kidney proteoglycans eluted from a Mono Q column. Equal volumes ($5 \mu\text{l}$) of each fraction were loaded into the wells of a 3-15% gradient SDS-PAGE. Fractions 15-24 show the presence of heterogeneous material typical of proteoglycan and virtually no other peptides. In contrast, fractions 12-14 contain a large number of polypeptides. The graph above the gel represents salt gradient used to elute the anion exchange column. (B) Immunoblot of a 3-15% gradient SDS-PAGE of cartilage chondroitin sulfate proteoglycan before (lane 1) or after (lane 2) digestion with chondroitinase ABC. The blot was probed with antibodies which recognize the carbohydrate stubs left on the core protein after digestion with chondroitinase ABC. Lane 1 shows no reaction product. The cartilage proteoglycan core proteins in lane 2, were detected by the anticarbohydrate antibodies. The lack of clear resolution of a core protein is probably a result of variable amounts of keratan sulfate chains remaining on the core protein. This experiment served as a positive control for the experiment shown in C. (C) Immunoblot of a 3-15% gradient SDS-PAGE of the material shown in figure 5A run after prior digestion with chondroitinase ABC and probed with R44. Lanes 14-22 reveal the core protein of a major CSPG present in the kidney preparation, with an $M_r = 1.6 \times 10^5$. Note that no CSPG core protein is present in fractions 12 and 13. A second blot of the same material but probed with an anti-HSPG antiserum (see text) showed HSPG to be absent in this preparation (data not shown). (D) Immunoblot of a 3-15% gradient SDS-PAGE of antibody 2D6

affinity-purified ^{35}S -labeled proteoglycan to supply radiolabeled GAG for carbohydrate analysis, we were unable to label the core protein of the proteoglycan with radiolabeled amino acids using this technique. Furthermore, the yield of core protein from this tissue source was relatively low, due in part to persistent contaminating proteins that were difficult to remove and resulted in high losses. Immunostaining of adult rat kidneys with the anti-CSPG mAbs (see below) showed the antigen to be relatively abundant in this organ. In later studies it was decided to use this tissue as the source of purified CSPG.

Kidney proteoglycans harvested from the most dense fractions of a cesium chloride density-gradient ultracentrifugation preparation (>1.45 g/ml) were separated further by gradient anion exchange chromatography. As shown in Fig. 5 *a*, CSPGs eluted from the column at salt concentrations between 1.0 and 1.5 M. No basement membrane HSPG was detectable by immunoblotting of these fractions with a polyclonal antiserum (9). After chondroitinase ABC treatment, however, a major pool of CSPG core protein of $M_r = 1.6 \times 10^5$ was detectable on immunoblots with R44 serum (Fig. 5 *c*). This antiserum (13) detects all CSPGs and DSPGs after chondroitinase digestion, by virtue of reaction with the carbohydrate stubs, containing unsaturated uronic acid. Undigested CSPG is not detectable with this antiserum, but core proteins can be readily resolved once chondroitinase treatment has gone to completion. As can be seen (Fig. 5 *c*), only fractions 14–24 of the salt gradient elution from the ion-exchange column contain CSPG, seen on the immunoblot as core proteins detected by R44 after chondroitinase digestion. No polypeptides were detected on a blot where samples were not chondroitinase ABC pretreated (data not shown).

Because repeated attempts, using a variety of methods, to immunoblot with the four mAbs were unsuccessful, we used the same procedure to confirm the identity of the CSPG core protein, after affinity purification from pooled kidney material (fractions 15–24) with one of the mAbs (2D6). The core protein of the affinity-purified CSPG had an estimated M_r of 1.6×10^5 (Fig. 5 *d*). This is similar to the size of the CSPG core protein found in Reichert's membrane ([28], see Fig. 6), which could also be affinity purified with our mAbs (data not shown).

We investigated the possibility that the core protein of rat Reichert's membrane CSPG might be antigenically related to that of basement membrane heparan sulfate proteoglycans. An immunoblot of material prepared from fraction 5 (1.45 g/ml) of a cesium chloride density-gradient centrifugation of Reichert's membrane material was probed with a polyclonal antiserum recognizing large and small basement membrane heparan sulfate proteoglycans (9). Fig. 6 shows that the CSPG was not recognized by this antibody, either before or after chondroitinase ABC digestion.

The Anti-CSPG Antibodies Recognize the Core Protein of the Same Proteoglycan

Dot blot assays were used to verify that all four monoclonal

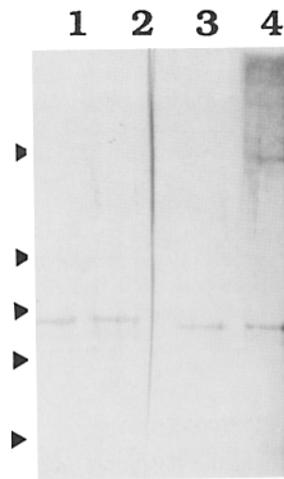


Figure 6. Immunoblot of a 3–15% SDS-PAGE of rat Reichert's membrane proteoglycans isolated from a cesium chloride density gradient fraction of >1.45 g/ml density, untreated (lanes 1 and 3) or chondroitinase ABC treated (lanes 2 and 4). The blot was probed with R44 (lanes 3 and 4) and a polyclonal antibody recognizing basement membrane HSPG (lanes 1 and 2). The results indicate that although a CSPG is present in this fraction, the core protein was not recognized by antiserum against HSPG. The polypeptides of $M_r = 5.3 \times 10^4$ and 6.3×10^4 are nonspecifically recognized by the antisera. Molecular mass markers are the same as in Fig. 5.

antibodies recognize the core protein of the same proteoglycan. Fig. 7 is a dot blot of 2D6 affinity-purified material (Fig. 5 *D*) probed with the anti-CSPG antibodies (lanes *a–d*) or a commercially available mouse IgG (lane *e*). All of the anti-CSPG antibodies recognized the affinity-purified material. Surprisingly, antibody 2B5 had the best performance in this and other dot blot assays, although it was not the antibody used in the affinity-purification process (see above). Fig. 8 *a* is a dot blot of pooled kidney material (from fractions 15–24, see above), before (rows 3 and 4) and after (rows 1 and 2) digestion with chondroitinase ABC, probed with either the anti-CSPG antibodies (lanes *a–d*) or a commercially available mouse IgG (lane *e*). The anti-CSPG antibodies are able to detect antigen either before (rows 3 and 4) or after (rows 1 and 2) digestion with chondroitinase ABC. Chondroitinase ABC digestion appeared to enhance the ability of some of the antibodies to recognize antigen bound to the membrane. We have noted a similar trend on tissue sections digested with the enzyme prior to immunostaining (see below).

ELISAs were performed with the anti-CSPG mAbs against a variety of antigens including rat kidney CSPG (Fig. 8 *b*), rat laminin, and rat chondrosarcoma CSPG. Laminin was tested because it is enriched in Reichert's membrane (25) and other basement membranes. It is both highly antigenic and has proteoglycan-binding properties (14, 48). The anti-CSPG antibodies were able to recognize CSPG from the kidney preparation, but no reactivity of any of the four anti-CSPG antibodies was seen against rat laminin. A strong response was seen with antilaminin serum (data not shown). Because one antibody (4D5, see below) had the capacity to stain rat tracheal cartilage, we also tested activity of the antibodies against the large rat chondrosarcoma CSPG. These assays were also negative, although a positive control antiserum gave a strong reaction (data not shown).

affinity-purified kidney CSPG isolated from pooled fractions 15–24, before (lane 1) and after (lane 2) digestion with chondroitinase ABC. R44 antiserum was used to detect the core protein of the affinity-purified CSPG which has an $M_r = 1.6 \times 10^5$. The smaller peptide (~ 100 kD) may be a degradation product of the larger core protein. A nonspecific peptide is present in both lanes. Molecular mass markers (from top to bottom), 200 kD, 94 kD, 68 kD, 43 kD, 25 kD.

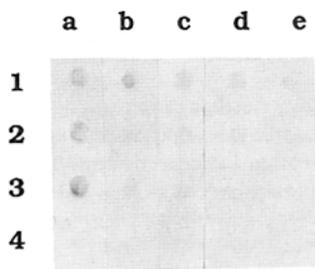


Figure 7. Dot blot of 2D6-affinity-purified proteoglycans probed with the anti-CSPG antibodies. 1 μg per well of affinity-purified kidney CSPG was loaded into a dot-blotting apparatus and probed with anti-CSPG antibody 2B5 (lane a), 2D6 (lane b), 5A3 (lane c), and 4D5 (lane d) or a control mouse IgG (lane e). Rows 1,

2, and 3 correspond to 10, 5, and 1 μg /well of immunoglobulin. Row 4 was probed with secondary antibody only. Note that all the anti-CSPG antibodies are capable of recognizing the same 2D6 affinity-purified material.

Indirect Immunofluorescence Microscopy

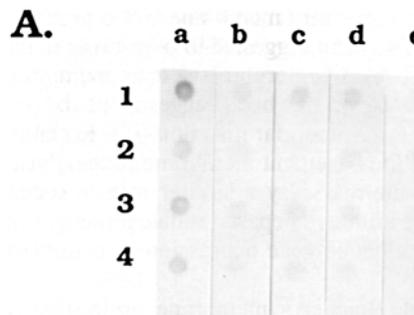
All four of the mAbs strongly stained the basement membranes of most rat tissues examined. Fig. 9 shows examples of immunostaining with the mAbs on frozen tissue sections. In some cases, the same sections were also stained with anti-HSPG antiserum. The pattern of basement membrane staining among the antibodies was essentially the same, but two differences were noted. mAb 5A3 weakly stained the basement membrane of skeletal muscle to the point that it was difficult to detect. The other three antibodies stained these basement membranes strongly. mAb 4D5 stained the matrix immediately adjacent to chondrocytes in tracheal cartilage (Fig. 9 H) but the other monoclonal antibodies did not. The results of ELISAs above, however, showed that none of the four mAbs recognized a large rat chondrosarcoma CSPG. One notable exception to the widespread distribution of the CSPG was rat kidney glomerular basement membranes (Fig. 9 E), where a total absence of staining was apparent with all the mAbs in contrast to anti-HSPG serum (Fig. 9 F), which strongly stained these basement membranes. The adjacent mesangia, Bowman's capsula, and tubular basement membranes were, in contrast, strongly stained by all the mAbs. A detailed study of the tissue distribution of these proteoglycans, both at the light microscopy level and at the ultrastructural level is currently in progress. Our preliminary observations are that all four anti-CSPG mAbs react solely with rat tissues.

The specificity of the mAbs for the CSPG core protein was also shown by the persistence of basement membrane-specific immunostaining of tissue sections after prior digestion with chondroitinase ABC. In the case of one antibody, 5A3, immunostaining was intensified, suggesting that epitopes recognized by 5A3 might be unmasked by chondroitinase ABC digestion (data not shown).

Discussion

Basement membranes were originally thought to serve solely as a support for tissues and a physical barrier separating the epithelial and mesenchymal compartments. In 1967, Grobstein (22) proposed that extracellular matrix molecules might influence the behavior of cells with which they are associated. From that initial hypothesis, others have shown that component molecules of the basement membrane can influence cell shape and polarity (52, 58), differentiation and tissue morphogenesis (2, 15, 42), and movement (42, 53).

The functions of proteoglycans within basement membranes and the effect of basement membrane proteoglycans on cellular behavior are not well understood. Proteoglycans are integrated into the matrices of basement membranes, of-



B.

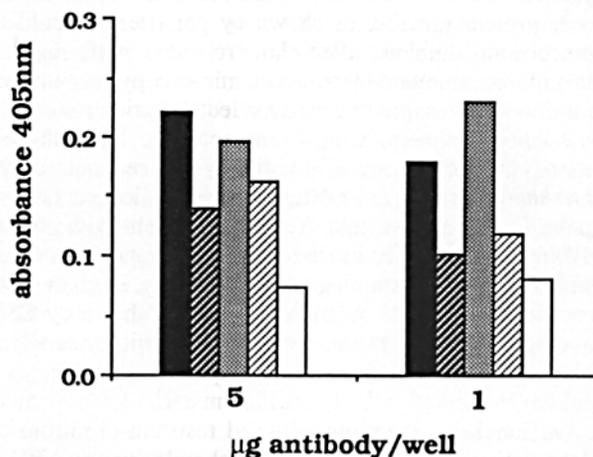


Figure 8 (A) Dot blot of ion-exchange-purified kidney CSPG probed with the anti-CSPG antibodies. 2 μg per well of kidney CSPG were probed with anti-CSPG antibody 2B5 (lane a), 2D6 (lane b), 5A3 (lane c), and 4D5 (lane d) or a control mouse IgG (lane e). The material in rows 1 and 2 was also digested with chondroitinase ABC before application of the primary antibodies. The material in rows 3 and 4 was left in the native state. Rows 1 and 3 and 2 and 4 correspond to 10 and 5 μg /well of immunoglobulin, respectively. The antibodies not only recognize material before but also after chondroitinase ABC digestion, demonstrating that the epitopes recognized by the antibodies are present on the core protein of the molecule. In addition, in some cases, chondroitinase digestion markedly improved the ability of the antibody to bind the antigen. **(B)** ELISA of kidney proteoglycan purified by dissociative cesium chloride density gradient ultracentrifugation followed by ion-exchange chromatography (see Fig. 5 a). Kidney CSPG (2 μg /well) was probed with either 5 or 1 μg /well of anti-CSPG antibodies or with a nonimmune mouse IgG. The anti-CSPG antibodies are able to recognize the purified kidney CSPG. 2B5, ■; 2D6, ▨; 5A3, ▩; 4D5, ▨; IgG, □.

ten requiring extensive chaotrope treatment for extraction and are present in small quantities within these structures (21, 46), thereby making it difficult to isolate chemical quantities of these molecules. However, some information about their function has become known in recent years. Basement membrane proteoglycans are known to interact with other matrix components suggesting a role in the assembly of basement membranes (21). Basement membrane GAGs probably proteoglycans, have also been suggested to play a role in tissue morphogenesis (2, 8). Glomerular basement membrane heparan sulfate proteoglycan has been suggested as the primary anionic barrier for glomerular filtration (17). Recently, it has been proposed the basement membrane proteoglycan of Reichert's membrane may play a similar role in rodent placentation (29). In addition, a heparan sulfate proteoglycan has been shown to influence gene expression in mammary epithelial cells (38).

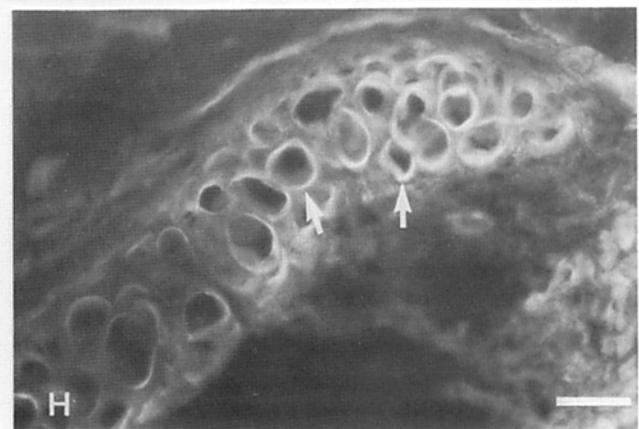
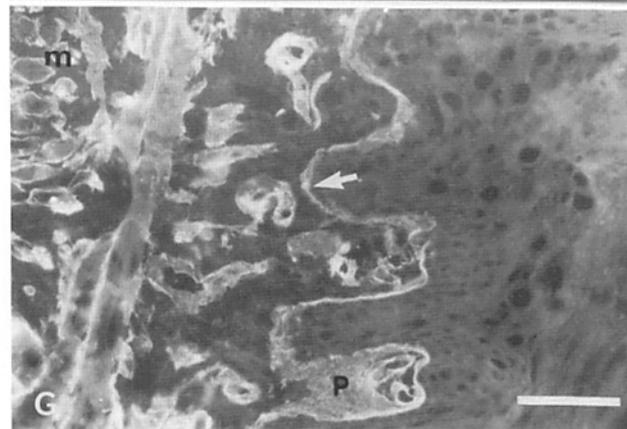
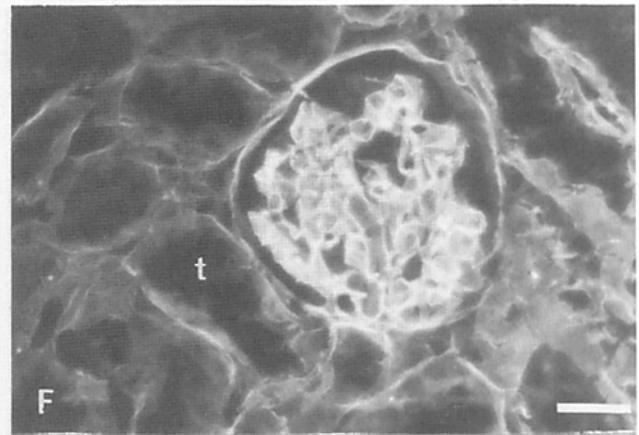
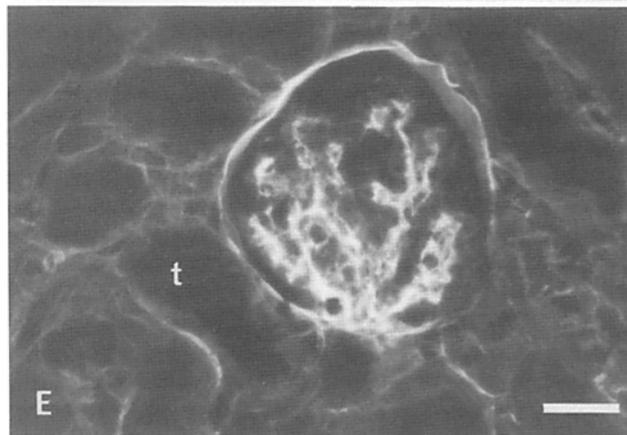
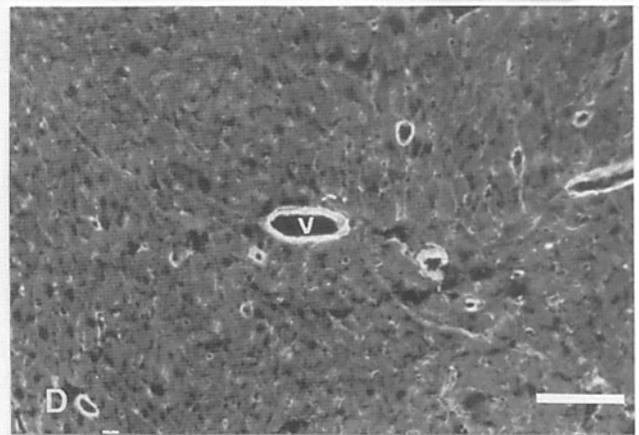
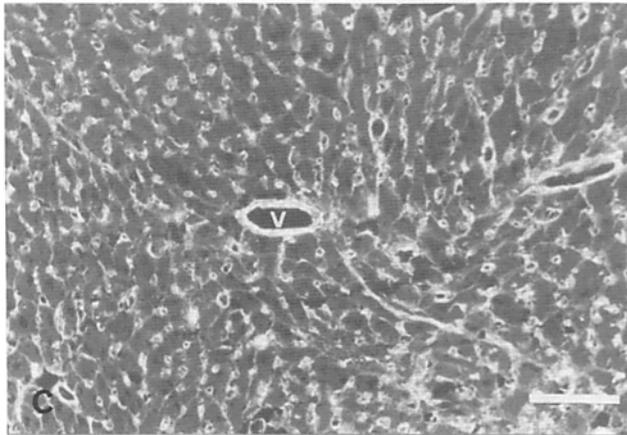
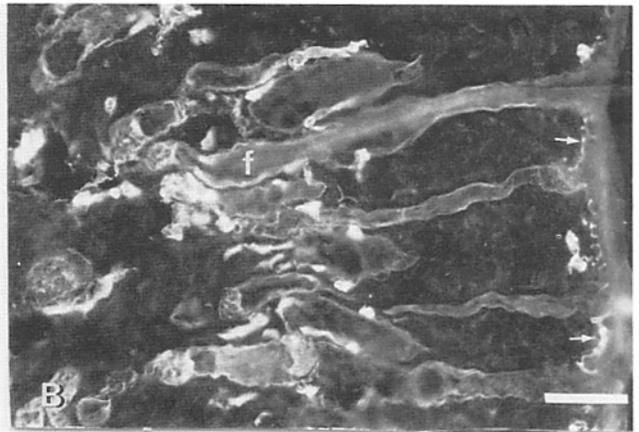
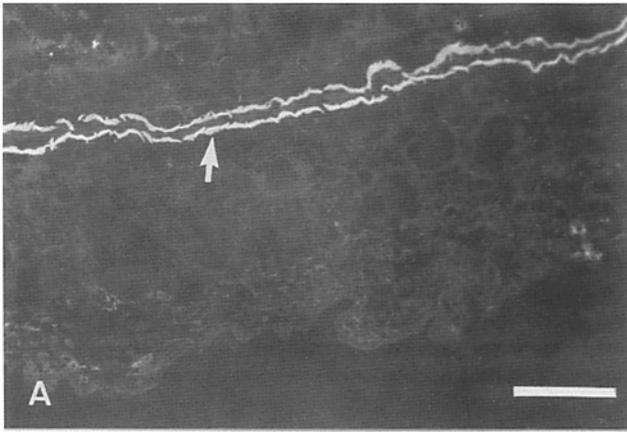
We have used both Reichert's membrane proteoglycans and those of adult rat kidney in the analysis of our mAbs, for several reasons. Although the former was the source of the antigen, the CSPG and its core protein proved difficult to purify to homogeneity in sufficient quantities. Immunofluorescent and immunochemical analysis indicated, however, that adult rat kidney was a rich source of the CSPG, which proved to be amenable to purification. In addition, as a result of our experiments, we have shown that adult, as well as embryonic basement membranes contain a CSPG, with a similar core protein of $M_r = \sim 1.5\text{--}1.6 \times 10^5$. All four antibodies were core protein specific, as shown by persistent or enhanced reaction on dotblots, after chondroitinase ABC digestion. In addition, immunofluorescence microscopy was not weakened, and was sometimes intensified, by prior chondroitinase ABC treatment of rat tissue sections. Because CSPG affinity-purified by the 2D6 antibody was recognized by all four antibodies, we infer that all the antibodies react with the same CSPG core protein. This is consistent with only one major CSPG species in Reichert's membrane (Fig. 6), and with similar tissue staining patterns. Finally, ELISAs showed reactivity of the monoclonal antibodies with kidney CSPG, but not with rat laminin, a highly antigenic proteoglycan-binding basement membrane glycoprotein (14, 48).

The ^{35}S -labeled, affinity-purified material from organ cultured Reichert's membrane proved resistant to nitrous acid degradation but sensitive to both chondroitinase ABC and AC II digestion, confirming the presence of a CSPG. Gel filtration analysis showed the intact proteoglycan to have an $M_r = 5\text{--}6 \times 10^5$, with an average glycosaminoglycan chain size of $1.6\text{--}1.8 \times 10^4$. Immunoblotting of the chondroitinase-digested, affinity-purified kidney proteoglycan with a polyclonal antibody showed the core protein to have an $M_r = \sim 1.6 \times 10^5$. This is the same size core protein seen on immunoblots of affinity-purified Reichert's membrane CSPG (data not shown). On the basis of these characteristics, we believe this CSPG to be identical to that previously described from rat Reichert's membrane by Iozzo and Clark (28). The CSPG has an unusual structure, with perhaps as many as 13–22 chondroitin chains on each molecule, based on our preliminary analysis. At this point, however, we do not know if the molecule is additionally substituted with *N*- or *O*-linked oligosaccharides, which would influence the apparent core protein size. However, a number of heavily substituted CSPGs have been described from rat L-2 cells (4, 43), murine PYS-

2 cells (13) and secretory granules (50). However, in these cases, the core proteins are much smaller than that described here for the Reichert's membrane CSPG. It will be interesting in the future to ascertain the possible core protein relationships between this group of CSPGs. We have noted as others have previously (28, 29), that the core protein of this CSPG, once prepared by chondroitinase ABC treatment is highly insoluble with a tendency to aggregate, rendering further analysis very difficult.

A number of tissue basement membrane proteoglycans have now been isolated from the EHS tumor and other sources (21, 24, 30, 31, 33, 35, 44, 51), but none has so far been a CSPG. However, both large and small proteoglycans of basement membrane type have been isolated, bearing both heparan and dermatan sulfate or heparan and chondroitin sulfate chains, the former from human placenta (30) the latter from the EHS tumor (33). We believe for several reasons that the CSPG identified here as a basement membrane component does not share the same core protein as these previously described. First, the core protein was not recognized in immunoblots by a polyclonal antiserum recognizing large and small heparan sulfate proteoglycans, which has cross reactivity with rat basement membrane proteoglycans (9). Second, this CSPG has a core protein of quite different size to those previously reported and is heavily substituted with chondroitin sulfate chains. In addition, a dermatan sulfate proteoglycan synthesized by murine PYS-2 cells (13), has a core protein that is not recognized by antibodies against PYS-2 derived or EHS tumor-derived heparan sulfate proteoglycans (9, 12, 33). The PYS-2 dermatan sulfate proteoglycan may be related to the CSPG discussed here since both core proteins are heavily substituted with glycosaminoglycan chains, and the PYS-2 cell line has affinities with parietal endoderm, these cells being mostly responsible for synthesis of Reichert's membrane in the rodent embryo (6, 7). Finally, the absence of staining in the glomerular basement membrane is quite unlike that recorded with antibodies against large and small basement membrane heparan sulfate proteoglycans (9, 12, 33, 51). Our antiserum against HSPG does stain rat Reichert's membrane, indicating the presence of one or more HSPGs in this tissue, consistent with data from the corresponding mouse basement membrane (45).

The presence of CSPG as a constitutive part of basement membranes has, however, been long suspected, based on the results of earlier histochemical, biochemical, and immunohistochemical studies. Investigators have shown chondroitin sulfate to be present in varying quantities in the kidney mesangium (31, 32, 44), developing submandibular gland (8), and blood vessels (57). Recently, using a mAb that recognized chondroitin 6-sulfate we provided further evidence for a more global tissue distribution of CSPGs by showing staining of the basement membrane zones of nerve fibers, blood vessels, dermal-epidermal junction, striated muscle, kidney tubules, and glomerular mesangium (10, 20). We now show that four mAbs with anti-CSPG core protein specificity immunostain the basement membranes of various rat tissues. That all four of the mAbs preferentially stain kidney mesangium and not glomerular basement membrane supports previous data that showed that CSPG is the predominant proteoglycan within the mesangium (10, 32), but with relatively little in the glomerular basement membrane (31, 32, 44). Basement membranes associated with smooth muscle of



both large and small blood vessels were particularly highlighted in all tissue sections examined with these antibodies. This may also be a reflection of the fact that these areas are known to be rich in CSPGs (57).

Although all four of our mAbs recognized the same antigen, there were differences in their immunostaining patterns, suggesting that they might recognize different epitopes on the same molecule. Unlike the other mAbs, 5A3 showed little or no staining of skeletal muscle basement membrane; however, it did strongly stain the muscle vasculature in the same tissue section. Another antibody, 4D5, immunostained the immediate lacunar matrix surrounding chondrocytes of tracheal cartilage, but none of the other mAbs shared this property. The nature of the antigen recognized by 4D5 in cartilage is presently unknown, but seems not to be the large CSPG, based on ELISA with rat chondrosarcoma CSPG. It is possible that 4D5 recognizes an oligosaccharide epitope shared with a cartilage matrix component and this is currently under investigation. In addition, differences in tissue staining might be due to either differential masking of particular epitopes, or the absence of that particular epitope in specific matrices.

Currently, the function of this CSPG in basement membranes is unknown, but our results show that it is a widespread component, as are a large heparan sulfate proteoglycan, laminin, entactin, and type IV collagen (24, 41, 42, 46, 54). For this reason, it may be that it has a structural role in the basement membrane matrix. However, if our subsequent studies confirm that this CSPG is absent from kidney glomerular basement membrane, it is clearly not essential for the assembly or function of this highly specialized matrix. The work of Bernfield and co-workers (2, 8) raised the possibility of variable amounts of basement membrane-associated CSPG in concert with the development of branching morphology in the mouse salivary gland. This suggests an influence of basement membrane CSPG on cell behavior, an area we shall investigate in our future work. It is clear, however, that just as in many other extracellular matrices such as cartilage, bone and loose connective tissue, CSPGs are a constituent of a large number of basement membranes in the mammal.

We should like to thank Sheila Bright, Timothy Tech, and Fran Allen for their assistance; Dr. Dale Abrahamson for his generous gift of laminin; Dr. John Baker for his generous gift of the rat chondrosarcoma proteoglycan; and Dr. Anne Woods for her critical review of this manuscript.

This work was supported by National Institutes of Health (NIH) grants AR-36457, HL-34343, and HL-07631 and from The Upjohn Company (Kalamazoo, MI). The Hybridoma Core Facility of the Multi-purpose Arthritis Center at University of Alabama-Birmingham is supported in part by NIH grant AR-20614.

References

1. Arnott, S., and A. K. Mitra. 1984. X-ray diffraction analyses of glycosaminoglycans. *Molecular Biophysics of the Extracellular Matrix*. S. Arnott, D. A. Rees, and E. R. Morris, editors. Humana Press, NJ. 41-67.
2. Bernfield, M., S. D. Banerjee, J. E. Koda, and A. C. Rapraeger. 1984. Remodelling of the basement membrane: morphogenesis and maturation. *Ciba Found. Symp.* 108:179-196.
3. Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4:330-334.
4. Bourdon, M. A., M. Shiga, and E. Ruoslahti. 1986. Identification from cDNA of the precursor form of a chondroitin sulfate proteoglycan core protein. *J. Biol. Chem.* 261:12534-12537.
5. Caterson, B., J. E. Christner, J. R. Baker, and J. R. Couchman. 1985. Production and characterization of monoclonal antibodies directed against connective tissue proteoglycans. *Fed. Proc.* 44:386-393.
6. Clark, C. C., R. R. Minor, T. R. Koszalka, R. L. Brent, and N. A. Kefalides. 1975. The embryonic rat parietal yolk sac. Changes in the morphology and composition of its basement membrane during development. *Dev. Biol.* 46:243-261.
7. Clark, C. C., E. A. Tomich, T. R. Koszalka, R. R. Minor, and N. A. Kefalides. 1975. The embryonic rat parietal yolk sac. The role of the parietal endoderm in the biosynthesis of basement membrane collagen and glycoprotein in vitro. *J. Biol. Chem.* 250:5259-5267.
8. Cohn, R. H., S. D. Banerjee, and M. R. Bernfield. 1977. Basal lamina of salivary epithelia. Nature of glycosaminoglycan and organization of extracellular matrix. *J. Cell Biol.* 73:464-478.
9. Couchman, J. R. 1987. Heterogenous distribution of a basement membrane heparan sulfate proteoglycan in rat tissues. *J. Cell Biol.* 105:1901-1916.
10. Couchman, J. R., B. Caterson, J. E. Christner, and J. R. Baker. 1984. Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. *Nature (Lond.)*. 307:650-652.
11. Couchman, J. R., and M. Hook. 1988. Proteoglycans and wound repair. *In The Molecular and Cellular Biology of Wound Repair*. R. A. F. Clark and P. M. Henson, editors. 1988. Plenum Press, New York. 437-470.
12. Couchman, J. R., and A. V. Ljubimov. 1989. Mammalian tissue distribution of a large heparan sulfate proteoglycan detected by monoclonal antibodies. *Matrix*. 9:311-321.
13. Couchman, J. R., A. Woods, M. Hook, and J. E. Christner. 1985. Characterization of a dermatan sulfate proteoglycan synthesized by murine parietal yolk sac (PYS-2) cells. *J. Biol. Chem.* 260:13755-13762.
14. Del Rosso, M., R. Cappelletti, M. Viti, S. Vannucchi, and V. Chiarugi. 1981. Binding of the basement-membrane glycoprotein laminin to glycosaminoglycans. *Biochem. J.* 199:699-704.
15. Ekblom, P., D. Vestweber, and R. Kember. 1986. Cell-matrix interactions and cell adhesion during development. *Annu. Rev. Cell Biol.* 2:27-47.
16. Farndale, R. W., C. A. Sayers, and A. J. Barrett. 1982. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. *Connect. Tissue Res.* 9:247-248.
17. Farquhar, M. G. 1981. The glomerular basement membrane: a selective macromolecular filter. *In Cell Biology of Extracellular Matrix*. E. D. Hay, editor. Plenum Press, New York. 335-378.
18. Fatemi, S. H., and C. P. Leblond. 1985. Sulfation and transport of basement membrane proteoglycans as visualized by ³⁵S-sulfate radioautography in the endodermal cells of the rat parietal yolk sac. *Am. J. Anat.* 173:127-145.
19. Fenger, M., U. Wewer, and R. Albrechtsen. 1984. Basement membrane heparan sulfate proteoglycan from the L2 rat yolk sac carcinoma. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 173:75-79.
20. Fine, J. D., and J. R. Couchman. 1988. Chondroitin-6-sulfate-containing proteoglycan: a new component of human skin dermoepidermal junction. *J. Invest. Dermatol.* 90:283-288.
21. Fujiwara, S., H. Wiedeman, R. Timpl, A. Lustig, and J. Engel. 1984. Structure and interactions of heparan sulfate proteoglycans from a mouse tumor basement membrane. *Eur. J. Biochem.* 143:145-157.
22. Grobstein, C. 1967. Mechanisms of organogenetic tissue interaction. *Natl. Cancer Inst. Monogr.* 26:279-299.

Figure 9. Micrographs of rat tissues stained with the monoclonal antibodies recognizing the rat Reichert's membrane CSPG. (A) Reichert's membrane, folded over upon itself, stained with mAb 2D6. The arrowhead indicates intense staining of the basement membrane of Reichert's membrane, the rest of the tissue being relatively unstained. (B) A section of rat skin stained with mAb 2B5. Note the staining of the dermal-epidermal junction (*small arrows*) and of the basement membranes and dermal papillae of hair follicles. (C and D) A section of rat heart muscle double stained with anti-CSPG (2D6) (C) and anti-HSPG (D) antibodies. The anti-CSPG stains both the basement membranes of cardiac muscle and blood vessels associated with the muscle; the anti-HSPG stains primarily the blood vessels of the muscle, the muscle basement membrane being negative for the proteoglycan. (E and F) Section of rat kidney double stained with anti-CSPG (2D6) (E) and anti-HSPG (F) antibodies. The anti-CSPG stains the mesangium and Bowman's capsule but does not stain the glomerular basement membrane. The anti-HSPG stains all glomerular basement membranes. (G) A section of rat tongue stained with monoclonal 4D5. Note that the basement membrane of skeletal muscle (*M*), blood vessels, and epithelium (*arrow*) all stain positive with this antibody. (H) Rat tracheal cartilage stained with monoclonal 4D5. Note the immunostaining around the chondrocyte lacunae (*arrows*). Bar in A, 200 μ m; Bars in B, C, D, and G, 100 μ m; Bars in E, F, and H, 30 μ m.

23. Hassell, J. R., J. H. Kimura, and V. C. Hascall. 1986. Proteoglycan core protein families. *Annu. Rev. Biochem.* 55:539-567.
24. Hassell, J. R., P. G. Robey, H. J. Barrach, J. Wilczek, S. I. Rennard, and G. R. Martin. 1980. Isolation of a heparan sulfate-containing proteoglycan from basement membrane. *Proc. Natl. Acad. Sci. USA.* 77:4504-4508.
25. Hogan, B. L. M., D. P. Barlow, and M. Kurkinen. 1984. Reichert's membrane as a model for studying the biosynthesis and assembly of basement membrane components. *Ciba Found. Symp.* 108:60-74.
26. Ingber, D. E., J. E. Madri, and J. E. Folkman. 1987. Endothelial growth factors and extracellular matrix regulate DNA synthesis through modulation of cell and nuclear expansion. *In Vitro (Rockville).* 23:387-394.
27. Inoue, S., C. P. LeBlond, and G. W. Laurie. 1983. Ultrastructure of Reichert's membrane, a multilayered basement membrane in the parietal wall of the rat yolk sac. *J. Cell Biol.* 97:1524-1537.
28. Iozzo, R. V., and C. C. Clark. 1986. Biosynthesis of proteoglycans by rat embryo parietal yolk sacs in organ culture. *J. Biol. Chem.* 261:6658-6669.
29. Iozzo, R. V., and C. C. Clark. 1987. Chondroitin sulfate proteoglycan is a constituent of the basement membrane in the rat parietal yolk sac. *Histochemistry.* 88:23-29.
30. Isemura, M., N. Sato, Y. Yamaguchi, J. Aikawa, H. Munakata, N. Hayashi, Z. Yosizawa, T. Nakamura, A. Kubota, M. Arakawa, and C. Hsu. 1987. Isolation and characterization of fibronectin-binding proteoglycan carrying both heparan sulfate and dermatan sulfate chains from human placenta. *J. Biol. Chem.* 262:8926-8933.
31. Kanwar, Y. S., V. C. Hascall, and M. G. Farquhar. 1981. Partial characterization of newly synthesized proteoglycans from the glomerular basement membrane. *J. Cell Biol.* 90:527-592.
32. Kanwar, Y. S., L. J. Rosenzweig, and M. J. Jakubowski. 1983. Distribution of de novo synthesized sulfate glycosaminoglycans in the glomerular basement membrane and mesangial matrix. *Lab. Invest.* 50:216-225.
33. Kato, M., Y. Koike, Y. Ito, S. Suzuki, and K. Kimata. 1987. Multiple forms of heparan sulfate proteoglycans in the Englebreth-Holm-Swarm tumor: the occurrence of high density forms bearing both heparan sulfate and chondroitin sulfate side chains. *J. Biol. Chem.* 262:7180-7188.
34. Kearney, J. F. 1984. Hybridomas and monoclonal antibodies. In *Fundamental Immunology*. W. E. Paul, editor. Raven Press, New York. 751-766.
35. Klein, D. J., D. M. Brown, T. R. Oegema, P. E. Brenchley, J. C. Anderson, M. A. J. Dickinson, E. A. Horigan, and J. R. Hassell. 1988. Glomerular basement membrane proteoglycans are derived from a large precursor. *J. Cell Biol.* 106:963-970.
36. Krueger, R. C., and N. B. Schwartz. 1987. An improved method of sequential alcian blue and ammonical silver staining of chondroitin sulfate proteoglycan in polyacrylamide gels. *Anal. Biochem.* 167:295-300.
37. Lerner, L., and D. A. Torchia. 1986. A multinuclear study of the interactions of cations with proteoglycans, heparin, and ficoll. *J. Biol. Chem.* 261:12706-12714.
38. Li, M. L., J. Aggeler, D. A. Farson, C. Hatier, J. Hassell, and M. J. Bissell. 1987. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc. Natl. Acad. Sci. USA.* 84:136-140.
39. Lieberman, R., M. Potter, W. Humphrey, Jr., E. B. Mushinski, and M. Vrana. 1975. Multiple individual and cross-specific idiotypes on 13 levan-binding myeloma proteins of BALB/c mice. *J. Exp. Med.* 142:106-119.
40. March, S. C., I. Parikh, and P. Cuatrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* 60:150-152.
41. Martin, G. R., H. K. Kleinman, V. P. Terranova, S. Ledbetter, and J. R. Hassell. 1984. The regulation of basement membrane formation and cell-matrix interactions by defined supramolecular complexes. *Ciba Found. Symp.* 108:197-212.
42. Martin, G. R., and R. Timpl. 1987. Laminin and other basement membrane components. *Annu. Rev. Cell Biol.* 3:57-85.
43. Oldberg, A., E. G. Hayman, and E. Ruoslahti. 1981. Isolation of a chondroitin sulfate proteoglycan from a rat yolk sac tumor and immunochemical demonstration of its cell surface localization. *J. Biol. Chem.* 256:10847-10852.
44. Parthasarathy, N., and R. G. Spiro. 1984. Isolation and characterization of the heparan sulfate proteoglycan of the bovine glomerular basement membrane. *J. Biol. Chem.* 259:12750-12755.
45. Paulsson, M., M. Dziadek, C. Suchanek, W. B. Huttner, and R. Timpl. 1985. Nature of sulphated macromolecules in mouse Reichert's membrane. Evidence for tyrosine-O-sulphate in basement membrane proteins. *Biochem. J.* 231:571-579.
46. Paulsson, M., S. Fujiwara, M. Dziadek, R. Timpl, G. Pejler, G. Backstrom, U. Lindahl, and J. Engel. 1986. Structure and function of basement membrane proteoglycans. *Ciba Found. Symp.* 124:189-203.
47. Roberts, R., J. Gallagher, E. Spooncer, T. D. Allen, F. Bloomfield, and T. M. Dexter. 1988. Heparan sulfate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature (Lond.)* 332:376-378.
48. Sakashita, S., E. Engvall, and E. Ruoslahti. 1980. Basement membrane glycoprotein laminin binds to heparin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 116:243-246.
49. Shively, J. E., and H. E. Conrad. 1976. Formation of anhydrosugars in chemical depolymerization of heparin. *Biochemistry.* 15:3932-3942.
50. Stevens, R. L. 1987. Intracellular proteoglycans in cells of the immune system. In *Biology of Proteoglycans*. T. N. Wight and R. P. Meecham, editors. Academic Press, New York. 367-387.
51. Stow, J. L., H. Sawada, and M. G. Farquhar. 1985. Basement membrane heparan sulfate proteoglycans are concentrated in the lamina rarae and in podocytes of the rat renal glomerulus. *Proc. Natl. Acad. Sci. USA.* 82:3296-3300.
52. Sugrue, S. P., and E. D. Hay. 1981. Response of the basal epithelial cell surface and cytoskeleton to solubilized extracellular matrix molecules. *J. Cell Biol.* 91:45-54.
53. Thiery, J. P., J. L. Duband, and G. C. Tucker. 1985. Cell migration in the vertebrate embryo. *Annu. Rev. Cell Biol.* 1:91-113.
54. Timpl, R., S. Fujiwara, M. Dziadek, M. Aumailley, S. Weber, and J. Engel. 1984. Laminin, proteoglycan, nidogen, and collagen IV: structural models and molecular interactions. *Ciba Found. Symp.* 108:24-43.
55. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
56. Wasteson, A. 1971. A method for the determination of the molecular weight and the molecular weight distribution of chondroitin sulfate. *J. Chromatogr.* 59:87-97.
57. Wight, T. N., M. W. Lark, and M. G. Kinsella. 1987. Blood vessel proteoglycans. In *Biology of Proteoglycans*. T. N. Wight and R. P. Meecham, editors. Academic Press, New York. 267-300.
58. Woods, A., and J. R. Couchman. 1988. Focal adhesions and cell-matrix interactions. *Collagen Relat. Res.* 8:152-182.