Isolation and Characterization of Chondroitin Sulfate Proteoglycans from Embryonic Quail That Influence Neural Crest Cell Behavior

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The movement of neural crest cells is controlled in part by extracellular matrix. Aggrecan, the chondroitin sulfate proteoglycan from adult cartilage, curtails the ability of neural crest cells to adhere, spread, and move across otherwise favorable matrix substrates in vitro. Our aim was to isolate, characterize, and compare the structure and effect on neural crest cells of aggrecan and proteoglycans purified from the tissues through which neural crest cells migrate. We metabolically radiolabeled proteoglycans in E2.5 quail embryos and isolated and characterized proteoglycans from E3.3 quail trunk and limb bud. The major labeled proteoglycan was highly negatively charged, similar in hydrodynamic size to chick limb bud versican/PG-M, smaller than adult cartilage aggrecan but larger than reported for embryonic sternal cartilage aggrecan. The molecular weight of the iodinated core protein was about 400 kDa, which is more than reported for aggrecan but less than that of chick versican/PG-M. The proteoglycan bore chondroitin sulfate glycosaminoglycan chains of 45 kDa, which is larger than those of aggrecan. It lacked dermatan sulfate, heparan sulfate, or keratan sulfate chains. It bound to collagen type I, like aggrecan, but not to fibronectin (unlike versican/PG-M), collagen type IV, or laminin-1 in solid-phase assays and it bound to hyaluronate in gel-shift assays. When added at concentrations between 10 and 30 \( \mu \)g/mL to substrates of fibronectin, trunk proteoglycan inhibited neural crest cell spreading and migration. Attenuation of cell spreading was shown to be the most sensitive and titratable measure of the effect on neural crest cells. This effect was sensitive to digestion with chondroitinase ABC. Similar cell behavior was also produced by aggrecan and the small dermatan sulfate proteoglycan decorin; however, 30-fold more aggrecan was required to produce an effect of similar magnitude. When added in solution to neural crest cells which were already spread and migrating on fibronectin, the embryonic proteoglycan rapidly and reversibly caused complete rounding of the cells, being at least 30-fold more potent than aggrecan in this activity.

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INTRODUCTION

Neural crest (NC) \(^1\) cells undergo a critical migratory period to colonize specific areas of the developing embryo and later form the craniofacial and cervical architecture, adrenal medulla, and autonomic and dorsal root ganglia (Le Douarin, 1982). Migratory cues that help guide cells are supplied in part by the microenvironment of the cells (Bronner Fraser and Stern, 1991; Keynes and Stern, 1984) and this includes molecules found in the extracellular matrix (ECM). This idea was based first on the spatiotemporal relationship of ECM molecules to NC cell migration paths and second on the influence of ECM on the behavior of NC cells in vivo and in vitro (reviewed by Newgreen, 1992).

Fibronectin, laminin, and collagen types I and IV are found in NC cell migration paths (Newgreen and Thiery, 1980; Duband and Thiery, 1987). These molecules encourage and guide NC cell movement in vitro (Newgreen et al., 1982; Rovasio et al., 1983; Newgreen et al., 1984; Perris et al., 1989). A postulated mechanism for positive control of migratory behavior involves these molecules acting as adhesive ligands for cell surface integrin receptors, which permit or promote cell movement (Gumbiner, 1996). This adhesive interaction and cell migration in vivo and in vitro may be perturbed by addition of blocking antibodies to either ligand

\(^{1}\) Abbreviations used: BSA, bovine serum albumen; CHAPS, 3-(\(N\)-cholamidopropyl)dimethylammonio\)-1-propane-sulfonate; CS, chondroitin sulfate; DEAE, diethylaminoethyl; DS, dermatan sulfate; E, embryonic day; ECM, extracellular matrix; GAG, glycosaminoglycan; HH, Hamburger and Hamilton stages; KS, keratan sulfate; MOPS, 3-(N-morpholino)propanesulfonic acid; NC, neural crest; PEG, polyethylene glycol; PG, proteoglycan; Tris, tris(hydroxymethyl)aminomethane.
In the first paper to describe the positive association of a specific ECM molecule, fibronectin, with NC cell migration paths (Newgreen and Thiery, 1980), it was pointed out that the distribution of NC cells in vivo could not be accurately modeled using positive adhesive control molecules alone. Certain regions in the embryo (subepidermal space and the perinotochordal zone) are rich in fibronectin and other permissive ECM molecules, yet NC cells avoid these areas. Newgreen et al. (1982) used electron microscopy to demonstrate that these areas are also rich in proteoglycans (PG). Newgreen et al. (1986) showed that cultured notochords inhibited NC cell movement in an area of otherwise permissive ECM close to the notochord. This effect was sensitive to digestion with chondroitinase ABC but not Streptomyces hyaluronidase, suggesting the involvement of chondroitin sulfate (CS) PG or an associated molecule. An anti-adhesive and anti-migratory effect could be produced by the addition of aggrecan, a large aggregating CSPG extracted from juvenile or adult cartilage, to the ECM in vitro (Newgreen, 1982; Perris and Johansson, 1987). These approaches pointed to high levels of CSPG in areas avoided by NC cells and suggested that large CSPGs can act as negative migration control elements: a new and distinctive class of morphogenetic molecule. CSPGs play an important, directive developmental role; specific enzymatic degradation of CSPGs leads to aberrant cell localization in amphibia (Tucker, 1986) and rats (Morriss-Kay and Tucker, 1989). Also, abnormal expression of CSPGs is associated with spontaneous neural tube defects in quail (Newgreen et al., 1997). These data suggested a two-branched model of NC cell migration control: coordinated expression of positive and negative control molecules maps out areas that will support NC cell migration to varying degrees. Other negative control or barrier molecules that use contact-mediated or diffusion-based mechanisms to guide the development of the nervous system have subsequently been isolated, characterized, and cloned (reviewed by Varola-Echvarria and Guthrie, 1997).

Later investigation of the guidance activities of CSPGs has focused on further identification of the molecules involved and modeling the mechanism of action. Perris et al. (1991) used monoclonal antibodies to map sulfation isotypes of CS and keratan sulfate (KS) glycosaminoglycan (GAG) in areas avoided by NC cells. Other workers have used core protein-specific antibodies to localize PG expression. A cytotactin-binding CSPG that bears the HNK-1 epitope is expressed around the notochord, neural tube, and caudal sclerotome in E2.5 chicks (Tan et al., 1987). Versican isotypes V0 and V1 have been detected in the caudal sclerotome, early subectodermal tissue, and perinotochordal mesenchyme (Landolt et al., 1995). A notochord-specific variant of aggrecan is first detected in E2.5 chick embryos; this expression increases throughout the period of NC cell outgrowth and sclerotomal differentiation (Domowicz et al., 1996).

MATERIALS AND METHODS

Radioactive Metabolic Labeling

Labeling in ovo. Fertile Japanese quail eggs (Coturnix coturnix japonica) from Lago Game (Thomastown, Victoria, Australia) were incubated at 38°C to E2.5 in a forced draught incubator. The eggs were windowed and the embryos staged using HH stages (Hamburger and Hamilton, 1951). Fifty microcuries of Na\(^{35}\)SO\(_4\), \([^{14}\text{C}]\)glutamic acid, or \([^{3}\text{H}]\)leucine (NEN duPont, Australia) in 50 to 100 \(\mu\)l Ham's F-12 medium was injected under the vitelline membrane. The eggs were rescaled and incubated for 16–22 h. Embryos were harvested and stored in ice-cold Hepes-buffered Eagle's medium or Ham's F-12, then staged and inspected for gross deformities. Only normal HH 21 or 22 embryos (nominally E3.3) were used. Fertile chicken eggs (Gallus domesticus) from Research Hatcheries, Victoria, were windowed at E14 and labeled as above for 3 consecutive days. The sternal cartilage was used at E17 as a source of avian cartilage aggrecan.

Labeling in vitro. E2.5 quail embryos were dissected to provide a trunck tissue piece that consisted of the neural tube, notochord, somites, ectoderm, and endoderm from between the fore and hind limb buds. This tissue was explanted onto 1.5% low melting point agarose (FMC BioProducts, Rockland, ME) in Ham's F-12 containing 3% heat-inactivated fetal calf serum and 20 \(\mu\)Ci of Na\(^{35}\)SO\(_4\) for 16–24 h.

Autoradiography. To reveal which tissues incorporated the injected Na\(^{35}\)SO\(_4\), sections of quail embryos were autoradiographed. Briefly, labeled embryos were rinsed in PBS and fixed in Saint Marie's fixative (99.5% ethanol, 0.5% acetic acid) for 2 h at room temperature. The fixed embryos were rinsed in PBS then taken through a series of 50, 70, and 100% polyethylene glycol (PEG) 200 (30 to 60 min each step), then similarly with PEG 1000 at 45°C, and finally with 50% PEG 1500 and two changes of 100% PEG 1500 at 45°C. The specimens were embedded in 100% PEG 1500 and 10-\(\mu\)m transverse sections were cut between the fore and hind.
limb buds and mounted on washed glass slides. Autoradiography was done using Kodak L4 emulsion for 48 h at 4°C in the dark, followed by D19 developer (Earle and Choo, 1994).

**Purification Procedures**

**Extraction of labeled molecules.** Pieces of trunk between the fore and hind limb bud, including the neural tube, dermomyotome, and sclerotome but excluding the developing kidney and other more lateral tissues, were microdissected from in ovo-labeled embryos and pooled and frozen in liquid nitrogen. Explants in vitro were similarly pooled. Fore and hind limb buds were harvested as a source of versican/PG-M (Kimata et al., 1986). Pooled tissue was pelleted, weighed, and stored in ice-cold 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.4, 5 mM Na2SO4, containing a protease inhibitor cocktail of 100 mM EDTA, 300 mM 6-aminohexanoic acid, 30 mM N-ethylmaleimide, 15 mM benzamidine HCI (Sigma, St. Louis, MO), and 2 mM 4-(2-aminoethyl)benzenesulfonylfluoride HCl (ICN Biomedicals, Irvine, CA). Tissue was frozen in dry ice and stored at −80°C. Frozen pooled tissue was quickly thawed and transferred to 10 volumes of chilled extraction buffer, containing 7 M urea, 20 mM MOPS, pH 7.4, 0.5% 3-(3-cholamidopropyl)dimethylammonio) propane-sulfonate (CHAPS, Sigma), and the above protease inhibitor cocktail. The tissue was homogenized by expulsion through a 5-ml syringe followed by passage through 18-, 21-, and 23-G hypodermic needles and left on ice for 2 to 4 h. The homogenate was clarified by centrifugation (1800 g for 30 min at 4°C) and the pellet reextracted in 10 volumes of the same buffer. The supernatant was precipitated in 4 volumes of acetone at 4°C for 2 to 4 h then clarified as above.

**Anion-exchange chromatography.** The pellet was resuspended in 10 ml of anion-exchange buffer (7 M urea, 20 mM MOPS, pH 7.4, 0.05% CHAPS) and loaded onto a DEAE Sephadex column (Pharmacia, Australia) equilibrated in the same buffer. A linear gradient of 0 to 1 M NaCl in anion-exchange buffer was pumped across the column. Outflow was monitored for chloride content using an on-line ion probe connected to a pH/ion conductivity meter (IBF Biotechnics, France) and recorded on a twin-pen chart recorder. One-milliliter fractions were collected and 100 µl was used for liquid scintillation counting in 20 volumes of ReadySafe counting cocktail (Beckman, Fullerton, CA) using a Beckman LS 3801 liquid scintillation counter.

**Size-selection chromatography.** Fractions of interest from the anion-exchange profile were pooled and the buffer was exchanged into size exclusion buffer (7 M urea, 100 mM sodium acetate, 25 mM EDTA, pH 6.0, 0.05% CHAPS) using Centricon 30 (Amicon Corp., U.S.A.), 1800g at 4°C. The concentrated sample was chromatographed on a 75-ml bed of either Sepharose CL-2B or Sephacryl S-500 equilibrated in size selection buffer. Two-milliliter samples were collected under gravity or using a peristaltic pump and 400-µl aliquots were taken for scintillation counting as before. Pig aggrecan (gift from Dr. A. J. Fosang, Department of Orthopaedic Research, Royal Children's Hospital, Parkville, Vic, Australia) was used as an elution volume standard. Void and total volume were estimated using salmon sperm DNA and N-acetyl, respectively. Tubes of interest were pooled, dialyzed against three changes of 100 volumes of deionized water or 50 mM ammonium acetate, pH 6 or 7.3, lyophilized, and resuspended in either deionized water or PBS.

**Purification of decorin.** Decorin was purified from bovine fetal nuchal ligament by octyl-Sepharose affinity chromatography, using an elution gradient of 2 to 6 M guanidine hydrochloride in sodium acetate, pH 6.3 (Choi et al., 1989) and was a gift from Dr. E. G. Cleary and Ms. B. Reinboth, Department of Pathology, University of Adelaide, Australia.

**Quantitation of yield.** The standard methods of Blumenkrantz and Asboe-Hansen (1973) and Farndale et al. (1986) were not sensitive enough to be used. Instead, the detection method of Barthold and Page (1985) was modified to increase its sensitivity and to enable it to be used quantitatively. Briefly, duplicate samples were spotted onto cellulose acetate membrane and air dried. The membrane was stained with 0.2% alcan blue 8GX (Aldrich, St. Louis, MO) in 50 mM MgCl2, 25 mM sodium acetate, 50% ethanol (v/v) for 30 min at room temperature and rinsed 3 × 15 min in 50 mM MgCl2, 25 mM sodium acetate, 50% ethanol (v/v). The sample spots were cut out, dried, and dissolved in 100% dimethyl sulfoxide (BDH, Australia). The sample unknowns were determined according to a standard curve generated using pig aggrecan—which had been quantitated using the method of Blumenkrantz and Asboe-Hansen (1973)—after reading the spectrophotometric absorbance of samples at 678 nm.

**Analytical Procedures**

**Electrophoresis.** Fractions of interest were analyzed by composite agarose/acylamide electrophoresis (McDevitt and Muir, 1973). Briefly, digests were loaded onto a 1.2% acrylamide/0.6% agarose gel in 10 mM Tris-acetate, pH 6.8, 0.25 mM Na2SO4 over a 10% acrylamide gel plug. The gel was run at 70 V until the sample had entered the gel, then increased to 110 V and stopped when the sample buffer dye had run 3 cm into the gel. The gel was fixed in 150 mM sodium acetate in 100% ethanol then autoradiographed at −70°C using Hyperfilm β-max (Amersham, Arlington Heights, IL).

**Analysis of core protein.** Analysis of the molecular weight of the core protein of the CSPG from the trunk and the limb bud of HH stage 21–22 quail and of aggrecan was done by labeling the molecules with 125Ι, removing the GAG chains with chondroitinases, and separating the digested components on 3–15% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) slab gels (Laemmli 1970). Iodination of 9.0 µg of trunk CSPG, 5.2 µg of limb bud CSPG, and 800 µg of pig aggrecan was done using the method of Bolton and Hunter (1973) by Austin Biomedical Services, Australia. Unbound radioactivity was removed by filtration on Bio-Gel P6 (BioRad, Richmond, CA) in PBS with 0.1% human serum albumin. Peak fractions were monitored using γ counting, pooled, and stored at 4°C. Incorporation was 4.75, 5.4, and 5.4 µCi/µg of each CSPG, respectively. Digestion of GAG chains was done using chondroitinases ABC or ACII for 4 h or overnight at 37°C as detailed below. The digests were resolved on 3–15% SDS–PAGE slab gels run at 30 mA at 4°C. The gel was fixed in 40% ethanol/10% acetic acid, dried, and autoradiographed as above.

**Glycosaminoglycan subtyping.** Subtyping of GAG chains attached to PG was performed using digestions with specific glycosaminoglycans at 37°C (all from Seikagaku, Japan or ICN Biomedicals, Seven Hills, NSW, Australia). Chondroitinase ABC digestions (10–50 µmol/ml) were done in 50 mM Tris–HCl, pH 6.0, 10 mM EDTA, 10 mM N-ethylmaleimide. 1 mM pepstatin A, plus 0.01% bovine serum albumin (BSA); Fraction V, Sigma) or ovalbumin. Chondroitinase ACII digestions (10–50 µmol/ml) were done in 20 mM sodium acetate, pH 6.0, plus 0.01% BSA or ovalbumin. Chondroitinase ACII digestions (10–50 µmol/ml) were done in 50 mM Tris–acetate, pH 7.3, plus 0.01% BSA. Chondroitinase ACI/keratanase digestions (30–60 µmol/ml for both enzymes) were done in 50 mM Tris–acetate, pH 7.3, plus 0.01% BSA. Heparitinase I digestions (20–40 µmol/ml) were done in 50 mM Tris–acetate, pH 7.5, plus 0.01% BSA.
Functional Procedures

Binding of proteoglycans to extracellular matrix components. The binding of PGs to other ECM molecules was assayed using a solid-phase affinity system. Aliquots of collagen type I (rat tail; Boehringer Mannheim), fibronectin (from human plasma) purified by the method of Miekka (1982) or purchased from Boehringer Mannheim, laminin-1 (EHS sarcoma; Boehringer Mannheim), and collagen type IV were adsorbed at 0.45 μg protein/mm² onto 12 × 12-mm squares of wetted nitrocellulose membrane (Schleicher and Schuell). BSA was absorbed similarly at 2.77 μg/ml. The membrane was blocked in 0.5% casein in PBS and incubated with radiolabeled PG in a sealed polyurethane bag for 120 min at room temperature. The unbound PG was removed and the membrane was extensively washed in PBS. Each square of membrane was dried and cut into 12 × 4-mm strips, which were autoradiographed as before.

Binding of CSPG to hyaluronate was assayed by a codialysis assay in the liquid phase. Radiolabeled limb bud CSPG was mixed with a concentration series of unlabeled hyaluronate (2000–0.2 pg/200 μl hyaluronate; Sigma) in 4 M guanidinium hydrochloride, 100 mM sodium acetate, pH 5.8, in microdialysis chambers made using the tops of Eppendorf tubes and dialysis tubing (Spectra, Germany) with a 7-kDa cutoff. The chambers were dialyzed against three changes of 100 volumes of 100 mM sodium acetate, pH 5.8, at 4°C. Samples of each microdialysis chamber were taken and subjected to composite agarose/polyacrylamide electrophoresis, fixed, and autoradiographed as outlined above. Binding was indicated by a retardation in migration of the labeled PG, when compared to a control lacking hyaluronate.

Cell behavior assays. E2.0–2.5 quail were harvested and the axial tissue adjoining the six most caudal somites was dissected out using electrolytically sharpened tungsten needles, under a dissecting microscope. This tissue consisted of the neural tube containing migratory NC cells, notochord, somites, ectoderm, and endoderm. The neural tube was isolated from the tissue complex after digestion with Dispase II (Boehringer Mannheim), 2 mg/ml in Ham’s F-12, and gentle manipulation with tungsten needles. The neural tube was incubated at 37°C in a growth medium of 3% fetal calf serum in Hepes-buffered Ham’s F-12 or Hepes-buffered Eagle’s medium for 1–2 h before being explanted onto ECM substrates.

PGs were presented to cells as part of their substrate. Molecules were adsorbed to bacteriological grade petri dishes (Disposable Products, Australia) as a standing drop. Human plasma fibronectin (20 μg/ml) in PBS was absorbed at 37°C for 30 to 60 min onto the petri dish, which was then rinsed in three changes of PBS followed by absorption of aggrecan from pig articular cartilage, CSPG from quail embryo trunk, or decorin from bovine nuchal ligament for 60–150 min followed by rinsing and blockade of any free binding sites with 1% BSA in Ham’s F-12.

The neural tubes with attached notochord were stranded on the substrate in 500 μl of growth medium and incubated at 37°C for 60 min to allow attachment of the explant to the substrate. The explant was then covered in growth medium and incubated as before. In order to determine which parameters of the NC cell outgrowth were most affected by the PGs, outgrowth, spreading, and movement were observed 4 h after start of culture using a Zeiss IM-35 phase-contrast microscope and photographed using a Nikon FSE camera with Kodak Technical Pan film. Numbers of NC cells on the substrate were counted and the cells were scored as spread (flat cells with stellate lamellae processes), part-spread (flat cells with short and narrow processes), or round (cells with a pronounced phase halo, no obvious polarity, and no processes other than blebs). Examples of these three distinct morphologies are given in Fig. 11a. The degree of movement of the NC cell outgrowths on specific substrates was assayed by deriving the median migratory distance, which was calculated by measur-
FIG. 2. Anion-exchange profiles of in vivo-labeled molecules extracted from pieces of E3.3 quail. (a) Na$_{35}^2$SO$_4$-labeled molecules extracted from trunk. (b) Na$_{35}^2$SO$_4$-labeled molecules extracted from limb bud. The profiles of these tissues were identical when labeled in vivo or in vitro. Peaks A, B, and C are indicated. Note the slight difference in elution position of peak C in a and b. (c) [14C]Glutamic acid-labeled molecules extracted from trunk. Note the labeled peak corresponding to peak C in a. (d) Na$_{35}^2$SO$_4$-labeled molecules extracted from E17 chick sternal cartilage. Note that this elutes at the same position as peak C in a.

ing the distance between the dorsal aspect of the neural tube (taken as the point of origin) and the median cell in a contiguous population of cells extending away from the dorsal neural tube. This measure is influenced minimally by NC cells that have detached from the neural tube during alignment in the culture dish and have drifted away and attached to distant substrate.

Alternatively, to produce conditions in which soluble PG was presented to NC cells independent of adhesive ECM molecules, neural tubes were explanted into fibronectin and BSA-coated wells of bacteriological grade Terasaki plates (Disposable Products) in the same growth medium, and NC cells were allowed to adhere, spread, and migrate for 18-24 h at 37°C. At this time, a concentration series of trunk CSPG or aggrecan was added to the growth medium and the cells were monitored microscopically at 15-min intervals for 1 h, then the PG was removed by flooding the entire Terasaki plate with three changes of growth medium. The NC cells were monitored again at 15-min intervals for 1 h. The cells were scored as spread, part-spread, or round using the three morphologies defined in Fig. 11a. Statistical analyses of at least four measurements of each index of cell behavior per group were done using Student’s t test.

RESULTS

Labeling and Autoradiography of Embryos

Injection of 50 μCi Na$_{35}^2$SO$_4$ in Ham's F-12 into the extraembryonic space beneath the vitelline membrane...
FIG. 3. (Top) Anion-exchange profile of in vitro Na$^{35}$SO$_4$-labeled molecules extracted from quail trunk. Note the similarity to the in vivo-labeled tissue (Fig. 2a). (Bottom) Fractions from above electrophoresed on a composite agarose/polyacrylamide gel. Peaks are defined in Fig. 2. Fractions from peak C were diluted 1 in 5 relative to peak B. Note that peak B includes three sulfated molecules and peak C, which contains most of the counts, has a single component.

Anion-Exchange Chromatography

The anion-exchange profiles of $^{35}$SO$_4$-labeled molecules extracted from the trunk and limb bud are shown in Figs. 2a and 2b, respectively. The profiles of the in ovo and in vitro-labeled trunks were almost identical. In Fig. 2a, peak A contained a mixture of molecules with a molecular weight less than 200 kDa on SDS-PAGE, which were completely de-
FIG. 4. Pools of fractions from peaks B and C of the anion exchange of $^{35}$SO$_4$-labeled trunk tissue were incubated in the presence of chondroitinase ABC (chABC), ACII (chACII), heparitinase (Htase), keratanase (Kase) or no enzyme (−), then electrophoresed on a composite agarose/polyacrylamide gel. The results show that peak B is resistant to ABC digestion, whereas peak C is totally digested by chondroitinases, but not heparitinase or keratanase.

graded by sequential treatment with alkali and Pronase, suggesting that no GAG-bearing material was present (data not shown). Peak B contains molecules that ran as three bands on composite agarose/acylamide gels (Fig. 3), were not sensitive to digestion with chondroitinase ABC or ACII (Fig. 4), and are not analyzed further in this paper. Peak C contained most of the $^{35}$SO$_4$-labeled material and was sensitive to digestion with chondroitinase ABC and ACII (Fig. 4). The anion-exchange elution profile of $[^{14}$C]glutamic acid-labeled trunk tissue contained a peak that eluted at the same salt concentration as peak C (Fig. 2c), whereas the anion-exchange profile of the same tissue labeled with $[^{3}$H]leucine did not show a peak at or near this salt concentration (data not shown). The limb bud peak C eluted at a fractionally lower salt concentration than the trunk peak C. Chick E17 sternal cartilage PG eluted from the anion-exchange column at the same anion concentration as trunk peak C (indicated in Fig. 2d).

### Size-Selection Chromatography

The profiles of pooled anion-exchange peak C from trunk and limb buds, buffer exchanged then chromatographed on Sepharose CL-2B in size-selection buffer, are shown in Fig. 5. Each species elutes as a single peak at $K_{av} = 0.13$. Chromatography of trunk and limb bud material on the more robust Sephacryl S-500 medium in size-selection buffer is presented in Fig. 6. Both molecules have a $K_{av} = 0.15$ on this medium. Pig cartilage aggrecan was also chromatographed on S-500 under these conditions and eluted with a $K_{av} = 0.10$, which is shown by the arrowhead A in Fig. 6.

### Analysis of Core Protein

Iodinated trunk and limb bud CSPGs were digested with chondroitinase ABC and ACII and electrophoresed alongside undigested samples on discontinuous 3-15% gradient SDS-PAGE. The undigested CSPGs did not enter the sepa-

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Embryonic Cell Migration-Inhibiting CSPG

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droitinase ABC and ACII produced a high molecular weight smear which required combined digestion with both a chondroitinase (ACI) and keratanase to be resolved as a discrete band at about 220 kDa (Fig. 7b).

Glycosaminoglycan Subtyping

The GAG chain subtyping was performed using specific glycosidases. The reporting of the results of the digestion was done in two ways. The results of the first screening of trunk CSPG on composite agarose/acrylamide gels are summarized in Fig. 4. Only chondroitinase ABC and ACII degraded the PG, suggesting the presence of CS chains. This was investigated further by size-selection chromatography on Sephadex G-50 on a second round of digests. Figures 8a and 8b show an identical shift in the elution volume of the $^{35}$SO$_4$ from $V_o$ to $V_t$ after digestion with either chondroitinase ABC or ACII. This shift represents production of disaccharide products by these two enzymes and supports the findings of the first screening assay. The PG was not sensitive to digestion with keratanase, as no radioactive product was seen at $V_t$. Also, sequential digestion with chondroitinase ABC and keratanase did not increase the yield of sulfated disaccharide (relative to chondroitinase ABC alone) when analyzed by this approach (not shown).

Glycosaminoglycan Sizing

The GAG liberated from the core protein of the trunk and limb bud CSPG was chromatographed on a bed of Sephacryl S-400 under associative conditions and eluted at $K_{av} = 0.42$, as shown in Fig. 9a. A standard curve of $K_{av}$ was constructed using CS GAG pools taken from bovine tracheal cartilage that were calibrated against a series of standards of which molecular weights were calculated based on intrinsic velocity measurements (Wastesen, 1971). The standard curve is shown in Fig. 9b. Thus, the molecular weight of the GAG chains from quail trunk CSPG can be deduced as about 45 kDa.

Binding of Proteoglycan to Extracellular Matrix Components

The trunk CSPG bound to immobilized collagen I but not to fibronectin, laminin-1, or collagen type IV, as revealed by autoradiography of the solid-phase binding assay (Fig. 10a). Codialysis of hyaluronate with samples of limb bud CSPG in steadily decreasing concentrations of guanidinium hydrochloride induced retardation of migration in subsequent composite gel electrophoresis (Fig. 10b), which suggested that this PG can interact with hyaluronate. This retardation was less apparent with lesser amounts of hyaluronate in the dialysis chamber.

Cell Spreading and Movement Assays

Each series of experiments is presented with its parallel fibronectin control, to accommodate variations due to fibronectin batch and precise embryonic donor age and duration in culture.
**Substrates of fibronectin and large CSPG.** The behavior of NC cells on substrates of fibronectin and large CSPG was dependent on the concentration of the solution of CSPG that was used in the absorption step. The number of NC cells attached to the substrate was the least sensitive measure of the influence of CSPGs on NC cell behavior. The most sensitive and concentration-dependent measure of the response of NC cells to differences in their substrate lay in the morphology of the NC cells when their spreading response was assessed, which was corroborated by the me-

![Diagram](image-url-a)

![Diagram](image-url-b)

**FIG. 9.** Chromatography of GAG preparations on Sephacryl S-400 in 250 mM sodium chloride, 100 mM sodium acetate, pH 5.8. (a) Trunk CSPG elutes at $K_{av} = 0.42$. (b) The standard curve of elution values of sized CS chains (dotted line) with line of best fit (solid line) and equation describing line of best fit (correlation $R^2 = 0.993$). A $K_{av} = 0.42$ corresponds to a molecular weight of 45 kDa.

**FIG. 8.** Chromatography of $^{35}$SO$_4$-labeled trunk material on Sephadex G-50 in 250 mM sodium chloride, 100 mM sodium acetate, pH 5.8, or 100 mM ammonium acetate, pH 5.8, after incubation with (a) no enzyme or chondroitinase ACII or (b) chondroitinase ABC or keratanase. The shift in elution position from $V_o$ to $V_t$ after incubation with both chondroitinase ABC and ACII but not keratanase suggests that the material is CSPG.

Median migratory distance. For example, the NC cell outgrowth on fibronectin/aggrekan absorbed at 150 $\mu$g/ml/BSA versus fibronectin/BSA control cultures had the following mean scores: 292 ± 34.51 cells in the outgrowth (control, 240.5 ± 56.3), 25 ± 1.73% round cells (control, 14.25 ± 3.5%), 38.67 ± 7.37% part-spread cells (control, 31.0 ± 5.7%), 36.33 ± 8.08% spread cells (control, 54.75 ± 5.0%), 103 ± 29 $\mu$m from point of origin (control, 154 ± 29 $\mu$m).
Comparison of these results to control cultures on fibronectin/BSA using Student’s t test demonstrates that the differences in cell number are not significant (P = 0.255). However, the differences in the percentages of round and spread cells are significant (P = 0.005 and 0.013, respectively). The median migratory distance is also significantly less than control (P = 0.001). Similar behavior was observed by NC cells on substrates of fibronectin/trunk CSPG/BSA.

Based on these data, the most useful reflection of differences in this system is the alteration in the spreading response of the migratory cells. Examples of the different morphologies in each score class are shown in Fig. 11a. Both aggrecan and trunk CSPG reduced cell spreading in a concentration-dependent manner (Figs. 11b and 11c, respectively) and this response was abolished by digestion with chondroitinase ABC (Fig. 12). Nevertheless, comparison of responses revealed that to produce a similar reduction of NC cell spreading, the aggrecan needed to be absorbed to the substrate at 15–40 times the concentration of trunk CSPG (Figs. 11b and 11c). Similar results were seen at concentrations of 10–30 μg/ml with limb bud CSPG and decorin (not shown).

Soluble CSPG added to established NC cell cultures on fibronectin. A rapid inhibition of NC cell spreading was also seen when the PG was applied in solution to the medium of NC cells which had migrated from neural tubes in culture for 18–24 h. The cell morphology suggested that the cells transiently passed through a part-spread stage defined by the presence of very narrow processes, before rounding up and blebbing (Fig. 13). This occurred in less than 15 min, after which there was little further change over 60 min. Although rounded, these cells were still adherent after 60 min, with 92% (mean of five measurements) of cells still attached to the substrate 45 min after medium exchange. A similar effect was produced by aggrecan but the inhibitory activity of the trunk CSPG was 30- to 60-fold higher than that of aggrecan (see Fig. 14).

### DISCUSSION

Large CSPGs have been proposed as key members of the group of negative migration control molecules for NC cells, yet the probes used to identify these molecules or the CSPGs used in analyzing the effect on embryonic cells have been derived from other nonembryonic biological systems. Here we describe the labeling and purification of a molecule extracted from the appropriate developing tissue and explore its functional properties.

**Sulfate Is Incorporated Chiefly into Appropriately Localized GAGs in Tissues**

The heavy incorporation of $^{35}$SO$_4$ delivered either in vivo or in vitro as seen by autoradiography of transverse sections shows that radioisotope has been incorporated in a pattern that is consistent with the localization of GAG epitopes reported by Perris et al. (1991). We conclude that the metabolically labeled molecules are a representative sample of total GAG-containing molecules and are mostly derived from the perinotochordal region.

**The Purified Labeled Molecules Are Large Proteoglycans**

Large CSPGs show the extreme of sulfation, negative charge, and size. The $^{35}$SO$_4$ incorporation and high negative charge of the trunk and limb bud anion-exchange peak C are suggestive of a PG, especially since E17 chick sternal cartilage aggrecan, which is highly negatively charged (Swann et al., 1984), elutes at a similar chloride concentration. The very large hydrodynamic size of the trunk material is shown by the size-selection chromatography data on CL-2B ($K_w = 0.13$) and S-500 ($K_w = 0.15$). Limb bud PG also eluted from CL-2B at $K_w = 0.13$, showing that the two PGs...
FIG. 11. (a) Examples of each of the three distinct morphologies that were scored for Figs. 11–14. Round (R), part-spread (P), and spread (S) are indicated by letters and arrowheads. (b) Histogram of NC cell spreading with increasing concentrations of aggrecan absorbed to a fibronectin substrate. Note the dramatic and progressive increase in the percentage of round cells at the expense of percentage of spread cells and, at higher concentrations, percentage of part-spread cells with the increase of aggrecan. (c) Histogram of NC cell spreading with increasing concentrations of trunk CSPG (CSPGtr) absorbed to a fibronectin substrate. Note similarities in behavior of the NC cells influenced by these two CSPGs, but at dramatically different ranges of concentrations. Round cells are presented on the bottom, part-spread cells in the middle, and spread cells on the top of each column. Statistical analysis by Student's t test is shown. * P ≤ 0.05, ** P ≤ 0.01, which applies for Figs. 11–14.
are of a similar size. In comparison, Kimata et al. (1986) showed that embryonic versican/PG-M eluted at $K_{av} = 0.12$ and embryonic chick sternal aggregan/PG-H eluted at $K_{av} = 0.26$ under similar conditions. We found that adult pig articular cartilage aggregan eluted from S-500 at $K_{av} = 0.10$, relative to $K_{av} = 0.15$ for the embryonic CSPGs. The most specific evidence for the trunk material being a PG is its sensitivity to chondroitinasases (Figs. 4 and 8), since their GAG targets occur only on PGs (Gallagher, 1989).

After anion exchange and size selection, we conclude that the preparations from E3.3 quail trunk and limb bud are highly negatively charged large PGs of uniform size, similar in size to versican and intermediate in size between embryonic and mature cartilage aggregan. The lack of contamination by proteins unrelated to CSPGs after anion-exchange and size-selection chromatography is suggested by the lack of distinct iodinated bands on SDS-PAGE after the indiscriminant labeling of all proteins in the PG preparations by the Bolton and Hunter method.

The Large Proteoglycans Have Large Glutamic Acid-Rich Core Proteins

The size of the core protein of the trunk and limb bud CSPGs was determined by iodination of each of the molecules, complete with GAG chains, using the Bolton and Hunter method (1973), followed by the removal of the GAG chains with chondroitinasase ABC or ACII and separation of the components. To enable an accurate determination of molecular weight, a ladder of $^{14}$C-labeled molecular weight markers was run, as well as laminin-1. We also used pig aggregan, digested with chondroitinasase ABC or ACII or ACI relative to $K_{av}$ Å 0.15 for the embryonic CSPGs. The most specific evidence for the trunk material being a PG is its sensitivity to chondroitinasases (Figs. 4 and 8), since their GAG targets occur only on PGs (Gallagher, 1989).
FIG. 12. Spreading of NC cells is determined by the CS chains on the CSPG. Digestion of the CSPG with chondroitinase ABC allows a similar percentage of NC cells to maintain the fully spread phenotype, when compared to control.

The anion-exchange profile for trunk samples labeled with [¹⁴C]glutamic acid showed a distinct peak at the elution position of peak C, which was not apparent when another amino acid, [³H]leucine, was used for labeling. We conclude that the core protein of the embryonic CSPGs are rich in glutamic acid, as are the core proteins of aggrecan and versican (Ayad et al., 1994).

The Trunk Proteoglycan Has Large Monotypic Chondroitin Sulfate Chains

The trunk PG yields equivalent amounts of sulfated disaccharides after digestion with chondroitinase ABC and ACII. As both of these enzymes cleave exolytically from the nonreducing terminal of the GAG chain, and ACII but not ABC is inhibited by dermatan sulfate (DS) (Yamagata et al., 1968), we conclude that the GAG chains lack DS. The trunk PG resisted keratanase and heparitinase treatment, implying that the molecule does not bear KS or heparan sulfate chains. We conclude that the sole GAG type on the trunk and limb bud PG is CS. Kimata et al. (1986) reported that versican/PG-M is predominantly CS, with some 4-8% of disaccharides produced by a chondroitinase ABC digest featuring iduronic acid, which is unique to DS chains. No work has been reported to verify the presence of DS chains on the KS-deficient aggrecan from notochord or the large CSPG from embryonic chick brain (Krueger et al., 1992). The expression of GAGs on other large neural CSPGs such as T antigen (Iwata and Carlson, 1993) is not known at these early stages.

Additional information was derived from the size of the GAG chains. The GAG chains were liberated from the trunk PG and sized on a column of Sephacryl S-400 in associative conditions. The elution of a single sharp peak from the column suggests that the trunk PG only has one size class of GAG chain. The comparison of the elution position of the trunk GAG to the elution positions of a set of calibrated, highly defined set of CS GAG standards allowed a very accurate determination of the size of the GAG chains. We conclude that the trunk and limb bud PG has monotypic CS GAG with a size of about 45 kDa. A GAG size of 45 kDa is similar to the 50-kDa value reported for versican/PG-M (Kimata et al., 1986) but far larger than reported for aggrecan (20–25 kDa) from embryonic or mature tissue (Gallagher, 1989; Kimata et al., 1986; Swann et al., 1984).

The Proteoglycan Binds to Collagen I and to Hyaluronate

We conclude from solid-phase binding assays that the trunk CSPG binds to immobilized collagen type I but not...
FIG. 13. Time course of spreading of 1-day cultures of NC cells on fibronectin after addition of 0.5 μg/ml CSPGtr to the culture medium. A rapid response was observed within 15 min of incubation at 37°C. The rounding response did not include detachment after challenge by medium exchange (wash out) after 60 min. Most cells have respread by the last time point.

to collagen type IV, fibronectin, laminin-1, or BSA. Aggrecan also binds to collagen type I (Perris et al., 1996), and the chick embryo versican/PG-M binds both collagen type I and fibronectin (Kimata et al., 1986). The codalysis assay presented here is designed such that the hyaluronate and CSPG are placed together under conditions that do not allow molecular interaction (dissociative conditions: 4 M guanidinium hydrochloride, 100 mM sodium acetate, pH 5.8) which are then steadily altered by dialysis to conditions that will allow molecular interaction (so-called associative conditions: 100 mM sodium acetate, pH 5.8). We conclude from this codalysis assay that labeled limb bud CSPG interacts with hyaluronate. This interaction with hyaluronate is a property that is shared by the large CSPGs versican (LeBaron et al., 1992) and aggrecan (Gallagher, 1989).

The Large CSPGs Interfere with NC Cell Migration on ECM

PGs are a very large, structurally and functionally diverse group (Gallagher, 1989). Aggrecans interfere with NC cell adhesion both as bound molecules and in solution (Newgreen, 1982; Perris and Johansson, 1987; Perris et al., 1996), but are of unknown spatiotemporal relevance to NC cell migration pathways, with the exception of the unusual KS-deficient aggrecan (Domowicz et al., 1995) which has not been functionally tested in this manner. Versican/PG-M purified from cultured embryonic fibroblasts and bound to culture surfaces inhibits the attachment of embryonic fibroblasts, baby hamster kidney, and Chinese hamster ovary cell lines and a metastatic melanoma cell line in vitro (Yamagata et al., 1989). However, these cell types are either unrelated to NC cells and are nonmotile or have undergone transformation. Embryonic fibroblasts do not respond to migratory cues if grafted into NC cell migratory routes in vivo (Erickson et al., 1980). Thus, it may be inappropriate to extrapolate these findings to NC cells which have a distinctly different cytoskeletal organization and motile behavior (Duband et al., 1988). Versican/PG-M, when substrate bound, inhibits NC cell attachment, but has little effect (in contrast to aggrecan) when free in the culture medium (Perris et al., 1996).

Substrates of fibronectin and large CSPG. This study investigated three measures of NC cell behavior when confronted by CSPGs. The number of cells attached to the substrate, the spreading response, and the median migratory distance which represent the ability of NC cells to adhere, spread, and move were all decreased when NC cells were plated on substrates of fibronectin and CSPGs, compared to fibronectin alone. This was particularly evident at high concentrations (375 or 3000 μg/ml) of aggrecan. However, attenuation of the spreading response of the cells was by far the most sensitive and titratable measure of NC cell
Behavior with both CSPGs tested in this assay. The effect of the CSPGs on the spreading response was abolished by digestion with chondroitinase ABC, indicating that the CS chains played a central part in attenuation of the spreading response. This is consistent with results reported by Newgreen et al. (1986) using a different assay system. However, the trunk CSPG was much more effective in attenuating the spreading response of NC cells than aggrecan, as similar percentages of cell rounding are seen with approximately 40 times more aggrecan than trunk CSPG. Given the similar composition and charge density, we can expect that absorption of aggrecan and the embryonic trunk PG to be similar. Similar results at similar absorption concentrations were seen with limb bud CSPG and the small DS PG, decorin. Recently, Ring et al. (1996) showed that collagen type IX, which is substituted with CS chains, also inhibits NC cell adhesion.

We conclude that the inhibition in these assays requires CS or DS GAGs but is not specific as to the core protein or to the number or length of the GAG chains bound to the core protein. This is consistent with the PGs imposing a steric hindrance on interactions between the NC cell membranes and coabsorbed ECM molecules, although more specific effects are also likely (see below).

**Soluble CSPG added to established NC cell cultures on fibronectin.** A rapid, reversible inhibition of NC cell spreading was also seen when the CSPG was applied in solution to an explant culture. In this assay, the inhibitory activity of the trunk CSPG was around 30 times greater than that of aggrecan. We conclude that an additional inhibitory modality is operating that does not require juxtaposition of the adhesive ECM on the substrate and the CSPG, since we showed that the CSPGs did not bind to fibronectin substrates. This inhibitory effect shows some specificity between similar large CSPGs and is rapid and reversible. Given that the response of the cells to the CSPG is rounding up rather than detachment, we propose that the large CSPGs, at sites on the cell surface separate from integrin/ECM sites, initiate signal alterations that reduce integrin-dependent cell spreading and movement.

Similar results have been reported by Perris et al. (1996) with a series of large CSPGs. In this case, the inhibitory ability, as measured by cell attachment or spreading, of the versican/PG-M was minimal in solution, whereas aggrecans from several nonembryonic sources were potent. Inhibitory activity was deduced (Perris and Johansson, 1989; Perris et al., 1996) to depend on the hyaluronic acid binding region of the core protein working in synergy with the CS and, more importantly, the KS chains. The model presented by Perris et al. (1996) proposed that the entire aggrecan molecule anchors to the cell surface hyaluronate and signals to the cell to downregulate motility.

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The CSPG that we have isolated from embryonic quail trunk has physical properties similar to those of versican/PG-M but has several distinct functional properties: It does not bind to fibronectin and it shows a high inhibitory activity for NC cells both when in solution and when substrate bound. It also differs from aggregan in possessing larger CS GAGs and no KS. The inhibitory activity of trunk CSPG may operate via a distinct mechanism from that suggested for aggregan (Perris et al., 1996) as we show that it is not substituted with KS chains, only CS chains. Moreover, the requirement of the hyaluronic acid binding region for the inhibitory activity suggested by Perris and Johannesson (1987) may not be necessary for some PGs, since the inhibitory effect of CSPG released into the environment by the notochord in vitro is completely unchanged by high concentrations of specific hyaluronidase (Newgreen et al., 1986).

In conclusion, embryonic CSPGs can inhibit NC cell migration, as assessed by cell spreading and movement in vitro, when presented as a substrate or in solution. When attached to substrates, the effect may be in part via a relatively nonspecific biophysical mechanism such as steric hindrance of adhesive ECM molecules by the much larger CSPG. The importance of such nonspecific effects in vivo cannot be dismissed, given the prevalence of PG-binding structural motifs that form the NC cell migration routes in vivo. However, soluble CSPGs also inhibit NC cell migration and this effect is relatively specific for the PG type. A full understanding of this interaction requires a detailed knowledge of the identity, diversity, structure, and properties of PGs of the appropriate regions. We suggest that a large range of CSPGs will produce a range of specific modulatory roles for NC cells and other cell lineages in development. These will form a major and specific group of functions separate from the more familiar roles of CSPGs in maintaining the biophysical properties of various tissues.

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