Isolation of Three Species of Proteoglycan Synthesized by Cloned Bone Cells†

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ABSTRACT: Three proteoglycan fractions have been isolated from clonal populations of osteoblast-like cells derived from fetal rat calvaria. One of these is secreted into the culture medium, is of apparent M_r 350 000, and has a glycosaminoglycan (GAG) composition of 77% chondroitin sulfate (CS) and 20% dermatan sulfate (DS). The remaining two proteoglycan fractions are associated with the cell layer. One of these has an apparent molecular weight of approximately 250 000 and a GAG composition of 54% CS and 40% DS. Both this species and the secreted proteoglycan have GAG chains of M_r 25 000. The other cell-associated proteoglycan contains heparan sulfate (HS), is solubilized by detergents, and appears to be contaminated with a CS proteoglycan. This HS-containing species may be similar to plasma membrane proteoglycans that have been isolated from several other cell types. Rat calvarial clones also synthesize hyaluronic acid and a number of glycoproteins.

Proteoglycans are important structural components of the extracellular matrix of many tissues (Hascall & Hascall, 1982). These molecules consist of glycosaminoglycan (GAG) chains covalently attached to serine or asparagine residues of a core protein. In cartilage, in which the proteoglycan component has been extensively characterized, the major proteoglycan contains both chondroitin sulfate (CS) and keratan sulfate (KS) chains and has a molecular weight of approximately 2–3 × 10^6. Cartilage proteoglycan monomers associate with hyaluronic acid and two link proteins to form large aggregates of approximately 2 × 10^8 daltons.

Proteoglycans in other tissues are less well characterized. Chondroitin sulfate rich proteoglycans have been isolated from aorta (Salisbury & Wagner, 1981; Kapoor et al., 1981), corneal stroma (Hassell et al., 1979), ovarian follicular fluid (Yanagishita et al., 1979), and several other tissues. These molecules are generally smaller than cartilage proteoglycans and vary widely in protein/carbohydrate ratio, GAG composition, buoyant density, and other physicochemical properties. Proteoglycans containing heparan sulfate (HS) as the major GAG component have been isolated from basement membranes (Hassell et al., 1980; David & Bernfield, 1981) and from plasma membranes (Kjellen et al., 1981). In general, chondroitin sulfate type proteoglycans may be specialized for secretion from the cell, and heparan sulfate type proteoglycans may be normally associated with the pericellular region of the extracellular matrix.

Since the initial observation by Herring that bovine cortical bone contains protein-bound chondroitin sulfate (Herring, 1968), the difficulty of extracting matrix components from mineralized tissues has deterred further investigation. However, synthesis of bone proteoglycans has been studied during the process of matrix-induced endochondral bone formation (Reddi, 1981). In this system, in which ectopic bone is induced to form around a cartilaginous model by subcutaneous implantation of demineralized bone matrix, a proteoglycan smaller than the cartilage species but with larger chondroitin sulfate chains is produced (Reddi et al., 1978). Bone proteoglycans are also amenable to extraction prior to the onset of mineralization in vitro. Embryonic chick calvaria have been reported to contain a small (M_r 70 000) proteoglycan, in which the major GAG is chondroitin sulfate (Sugahara et al., 1981).

In the present study, synthesis of bone proteoglycans has been investigated in vitro by using clonal populations of cells from fetal rat calvaria. Such clones have previously been shown to respond to parathyroid hormone with an increase in intracellular cyclic AMP, to synthesize collagen types I, III, and V, and to produce the GAG's[1] chondroitin sulfate, dermatan sulfate, heparan sulfate, and hyaluronic acid (Aubin et al., 1982). Three species of proteoglycan have now been demonstrated to be synthesized by clones of calvarial cells; one of these is secreted into the culture medium, and the remaining two are associated with the cell layer.

Experimental Procedures

Materials. Ultrapure guanidine hydrochloride (Gdn·HCl) was obtained from Schwarz/Mann and used for extraction solutions. Sigma type I guanidine hydrochloride was used for chromatography. Sepharose CL-2B, CL-4B, and CL-6B were obtained from Pharmacia. Diethylaminoethylcellulose (DEAE-cellulose; DE-52) was obtained from Whatman Laboratories. Pronase E (protease type XIV), chondroitinase ABC (EC 4.2.2.4), and chondroitinase AC (EC 4.2.2.5) were obtained from Sigma Chemical Co. *Streptomyces* hyaluronidase (EC 4.2.99.1) was obtained from Calbiochem.

Radiolabeling of Rat Calvaria Cells and Proteoglycan Extraction. Cells used in these experiments were of clone RCX1.10, derived from primary cultures of fetal rat calvaria cells by limiting dilution, as described by Aubin et al. (1982). RCX1.10 cells were shown to be osteoblast-like by a number of criteria, including morphology, synthesis of type I and type III collagen, and stimulation of intracellular cyclic AMP in response to parathyroid hormone (not shown). Cultures were grown in α minimal essential medium (α-MEM) containing 15% fetal calf serum and antibiotics (100 μg/mL penicillin G, 50 μg/mL gentamicin, and 0.3 μg/mL amphotericin B). All experiments described here were performed between subculture numbers 80 and 100.

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[1] Abbreviations: GAG, glycosaminoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; Gdn·HCl, guanidine hydrochloride; DEAE-cellulose, diethylaminoethylcellulose; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; CPC, cetylpyridinium chloride; $NaDodSO_4$, sodium dodecyl sulfate.

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Confluent cultures of RCX1.10 cells in 75-cm^2 flasks were radiolabeled by incubation for 16 h in α-MEM containing 5% dialyzed fetal calf serum and either 100 μCi/mL [^{35}S]sulfate (25–40 Ci/mg, SJS-1, Amersham) or 50 μCi/mL [^{35}S]sulfate plus 5 μCi/mL [^{3}H]glucosamine (10–30 Ci/mmol, NET-190, New England Nuclear). At the end of the labeling period, medium was collected into an equal volume of 8 M Gdn·HCl–100 mM sodium acetate, pH 6.8, containing the following proteolytic enzyme inhibitors: 20 mM EDTA, 10 mM benzamidine, and 0.2 M 6-aminohexanoic acid. The cell layer was washed once with phosphate-buffered saline, the wash solution was discarded, and the cells were extracted by gentle agitation at 4 °C for 6 h in 4 M Gdn·HCl–50 mM sodium acetate, pH 6.8, containing half the above concentrations of proteinase inhibitors. This procedure solubilized more than 90% of the radioactivity in the cell layer.

All the following procedures were performed at 4 °C unless otherwise stated. Both medium and cell extracts were made 70% (v/v) ethanol by the addition of absolute ethanol. After standing overnight, ethanol precipitates were collected by centrifugation at 10000*g* for 10 min. Pellets were washed once with 70% (v/v) ethanol and then redissolved in 4 M Gdn·HCl–50 mM sodium acetate plus proteinase inhibitors. Extracts were stored at −20 °C until used.

Gel Permeation Chromatography of Guanidine Extracts. Sepharose CL-2B and Sepharose CL-4B gel chromatography was performed by using 90 × 1.5 cm columns, preequilibrated and eluted with 4 M Gdn·HCl–50 mM sodium acetate, pH 6.8, at a flow rate of 7 mL/h. Fractions of 1.5 mL were collected, and aliquots of 100 or 200 μL were adjusted to 0.5 mL with distilled water and counted in 6 mL Aquasol (New England Nuclear) on a Searle Analytic Mark III liquid scintillation counter. Void volume (V_0) and total volume (V_t) were calculated with Dextran Blue and [^{3}H]hydroxyproline, respectively. Recovery of applied radioactivity on gel chromatography was routinely approximately 70%.

Sepharose CL-2B columns were calibrated with two proteoglycans of known molecular weight. BM-1 proteoglycan from EHS sarcoma was a kind gift of Dr. S. Ledbetter and Dr. J. Hassell, National Institutes of Health. Cartilage proteoglycan was prepared from ^{35}S-labeled femoral epiphyseal cartilage of fetuses from term-pregnant rats.

Ion-Exchange Chromatography. Peaks from Sepharose columns were pooled, dialyzed against distilled water, and lyophilized. Dried residues were redissolved in 6 M urea–0.1 M sodium acetate, pH 5.8, and chromatographed on DEAE-cellulose by using a linear gradient of 0.1–1.0 M sodium acetate, pH 5.8 (30 mL of each buffer). Column volume was 7 mL, flow rate was 15 mL/h, and fractions of 1.1 mL were collected. Aliquots of 100 μL of each fraction were adjusted to 0.5 mL with distilled water and counted as above. In some experiments, a further step of one column volume of 2.0 M sodium acetate was performed, and this failed to elute any significant quantity of radioactivity.

Preparation of Free GAG Chains and Chromatography on Sepharose CL-6B. GAG chains were released from ion-exchange-purified proteoglycans by Pronase digestion (Merrilees et al., 1977) or by alkaline hydrolysis using the method of Carlson (1968) modified according to Oldberg et al. (1981). In the former method, lyophilized proteoglycans were dissolved in 5 mL of 0.2 M Tris-HCl, pH 7.4, and incubated at 56 °C for 24 h with 10 mg of Pronase. The reaction was terminated by addition of sodium chloride and acetic acid and heating at 100 °C for 10 min. After centrifugation, the supernatant was dialyzed against distilled water and lyophilized. For alkaline hydrolysis, proteoglycans were dissolved in 0.1 M sodium borohydride in 0.5 M sodium hydroxide and incubated at room temperature for 24 h. The solution was neutralized with 10% (v/v) acetic acid, diluted 1:10 with distilled water, and lyophilized.

Free GAG chains were dissolved in 0.2 M pyridinium acetate, pH 5.0, and chromatographed on a column of Sepharose CL-6B equilibrated and eluted with the same buffer. Column dimensions were 50 × 1.5 cm, flow rate was 7 mL/h, and fractions of 1.1 mL were collected. Aliquots of 0.5 or 1.0 mL were solubilized with 6 mL of Aquasol and counted.

Glycosaminoglycan Analysis. Hyaluronic acid was quantitated by digestion with *Streptomyces* hyaluronidase. Freeze-dried samples were dissolved in 1 mL of 0.15 M NaCl–0.01 M sodium acetate, pH 5.0, and incubated at 37 °C for 24 h with 5 turbidity-reducing units (TRU) of hyaluronidase. Reaction was terminated by heating at 100 °C for 10 min, and undigested GAG's precipitated by the addition of 30 μL of 10% (w/v) cetylpyridinium chloride (CPC) and 50 μg each of hyaluronic acid and chondroitin 4-sulfate as carrier. Precipitates were collected by centrifugation, washed once, and dissolved in 1 mL of methanol for scintillation counting.

For analysis of sulfated GAG's, GAG chains were released from proteoglycans as described above. For quantitation of CS and DS, freeze-dried residues were dissolved in 1 mL of 0.15 M NaCl–0.01 M Tris-HCl, pH 8.0, and incubated at 37 °C for 24 h with 0.25 unit of chondroitinase ABC or chondroitinase AC. Reaction was terminated by heating at 100 °C for 10 min, and undigested GAG's were quantitated as above.

Heparan sulfate was quantitated by nitrous acid digestion (Cifonelli, 1968). Freeze-dried GAG's were dissolved in 50 μL of distilled water and added to 50 μL of 33% (v/v) acetic acid and 50 μL of 5% (w/v) sodium nitrite. After incubation at room temperature for 3 h, reaction was terminated by lyophilization. Residues were redissolved in 1 mL of 0.05 M Tris-HCl, pH 7.4, and CPC precipitated as above.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. [^{3}H]-Glucosamine-labeled glycoprotein fractions were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis, essentially by the method of Laemmli (1970). Putative glycoprotein peaks from ion-exchange chromatography were dialyzed against distilled water and lyophilized. Aliquots corresponding to 10^4 cpm were dissolved in NaDodSO$_4$–mercaptoethanol solution and electrophoresed on 7.5% polyacrylamide gels containing 2 M urea. After colorimetric staining with Coomassie Brilliant Blue R-250, gels were processed for fluorography by the method of Bonner & Laskey (1974).

Results

Gel Chromatography of Calvarial Medium and Cell Extracts. Cultures of cloned rat calvarial cells were radiolabeled with [^{3}H]glucosamine and [^{35}S]sulfate, and then the medium and cell layer were extracted separately with 4 M Gdn·HCl. When the cell culture medium extract was analyzed by gel chromatography on Sepharose CL-2B in the presence of 4 M Gdn·HCl, the elution profile shown in Figure 1 was obtained. Three major peaks could be discerned: a peak comprising mainly ^{3}H label coeluting with the void volume (V_0) of the column (designated peak I), a broad included peak of ^{3}H and ^{35}S label centered around K_{av} = 0.7 (peak II), and a peak of ^{3}H and ^{35}S label coeluting with the total volume (V_t) of the column (peak III). The vertical arrows represent the elution positions on the column of cartilage proteoglycan and the BM-1 basement membrane proteoglycan from EHS sarcoma.

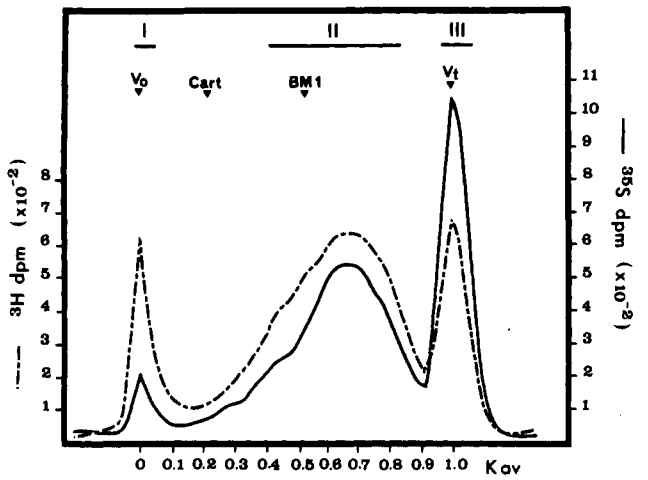

FIGURE 1: Chromatography on Sepharose CL-2B of RCX1.10 culture medium extract. Horizontal bars represent fractions pooled into peaks I, II, and III for further analysis. V_0, void volume; V_t, total volume; Cart, elution position of cartilage proteoglycan; BM-1, elution position of BM-1 proteoglycan from EHS sarcoma.

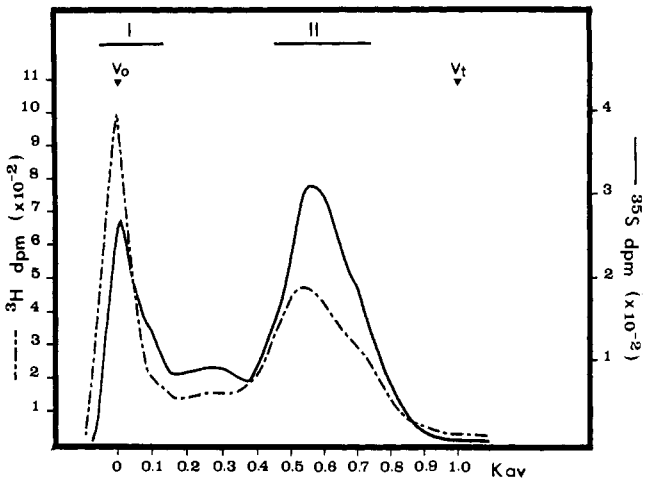

FIGURE 2: Chromatography on Sepharose CL-4B of RCX1.10 cell layer extract. Horizontal bars represent fractions pooled into peaks I and II for further analysis. V_0, void volume; V_t, total volume.

The three peaks were pooled as indicated by the horizontal bars (Figure 1) and dialyzed against distilled water. Peak III was largely lost upon dialysis, probably because it comprised mainly residual free sulfate and glucosamine or their low molecular weight metabolites, and was not further characterized. Peaks I and II were lyophilized and dissolved in 6 M urea for further analysis by ion-exchange chromatography (see below).

The guanidine extract of the cell layer, containing radiolabeled macromolecules too small to be fractionated by Sepharose CL-2B, was chromatographed instead on Sepharose CL-4B (Figure 2). Two major peaks of ^3H and ^{35}S label were routinely resolved: one eluting at or near the V_0 of the column (peak I) corresponding to approximately 30% of the recovered ^{35}S activity and a well-defined peak at approximately K_{av} = 0.6 (peak II) corresponding to approximately 70% of the recovered ^{35}S activity. These peaks were pooled as indicated by the horizontal bars for further analysis by ion-exchange chromatography.

Ion-Exchange Chromatography of Sepharose Fractions of Calvarial Medium and Cell Extracts. Peaks I and II from the medium extract and peaks I and II from the cell layer extract were rechromatographed on DEAE-cellulose in the presence of 6 M urea. Peak I from the medium extract eluted as a single peak at 0.35 M salt on ion-exchange chromatography (not shown). When this material was pooled, dialyzed into distilled water, and digested with *Streptomyces* hyaluronidase, 99% of the ^3H radioactivity was released (Table I), demonstrating that medium peak I consisted of hyaluronic acid. Peak II from the culture medium resolved into three components on ion-exchange chromatography (Figure 3A). A large peak, of primarily ^3H counts, did not adsorb to the ion-exchange resin and was eluted with 0.1 M sodium acetate (peak M/II/0.1). This peak was identified as glycoprotein by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4). The reproducible presence of small amounts of ^{35}S radioactivity in this peak may indicate the synthesis by bone cells of sulfur-containing proteins (Liau & Horowitz, 1982). A second peak of ^3H radioactivity, eluting from DEAE-cellulose at 0.35 M salt, was shown to be hyaluronic acid by digestion with *Streptomyces* hyaluronidase (not shown). The third component was a broad peak containing both ^3H and ^{35}S and eluting at 0.8 M salt (peak M/II/0.8), and this putative proteoglycan peak was further analyzed as described below.

Peak II from the Sepharose CL-4B profile of the cell extract gave a similar profile to peak M/II on ion-exchange chromatography (Figure 3B), except that the 0.35 M peak corresponding to hyaluronic acid appeared to be absent. Peak C/II/0.1 was identified as glycoprotein by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4). Peaks corresponding to glycoprotein (C/II/0.1) and proteoglycan (C/II/0.8) were pooled and analyzed further as described below. Peak I from the Sepharose CL-4B profile of the cell extract behaved anomalously on ion-exchange chromatography. A peak of ^3H was eluted at 0.35 M salt (hyaluronic acid), but ^{35}S was recovered only with low efficiency. For clarification of this result, cultures were single labeled with [^{35}S]sulfate (see Experimental Procedures). When the ^{35}S-labeled peak I material from the cell extract was chromatographed on DEAE-cellulose, a small broad peak of radioactivity eluted at 0.8 M salt (Figure 5). This corresponded to only 30% of the total applied radioactivity. Only a small amount of radioactivity eluted with 2 M sodium acetate, but a subsequent wash with 1% (v/v) Triton X-100 eluted a sharp peak of counts corresponding to a further 35% of applied radioactivity. The material eluting at 0.8 M salt (peak C/I/0.8) and the detergent-solubilized material (peak C/I/TX) were pooled separately for further analysis.

GAG Composition of Proteoglycan Fractions from Culture Medium and Cells. GAG chains were released from proteoglycan peaks of culture medium and cell layer extracts and characterized by specific enzymatic and chemical treatments. Peak M/II/0.8 from culture medium had a GAG composition of 77% chondroitin sulfate (CS) and 20% dermatan sulfate (DS) (Table I). Quantitatively similar results were obtained by cellulose acetate electrophoresis of free GAG chains (not shown). Peak C/II/0.8 from the cell layer extract had a GAG composition of 54% CS and 40% DS (Table I). Approximately 5% of this fraction was reproducibly resistant to chondroitinase ABC, nor was this material degraded by treatments specific for heparan sulfate (HS) (Table I). Peak C/I from the cell layer contained 45% HS (Table I). Because fractionation of this material on DEAE-cellulose resulted in two components, peak C/I/0.8 and peak C/I/TX, these were analyzed separately for GAG composition. The material eluted by Triton X-100 (C/I/TX) had a higher content of HS (54%) than the material eluted at 0.8 M salt (C/I/0.8) (29% HS). The remainder of the GAG in both cases was a mixture of CS and DS. The Triton-eluted peak is therefore enriched in HS

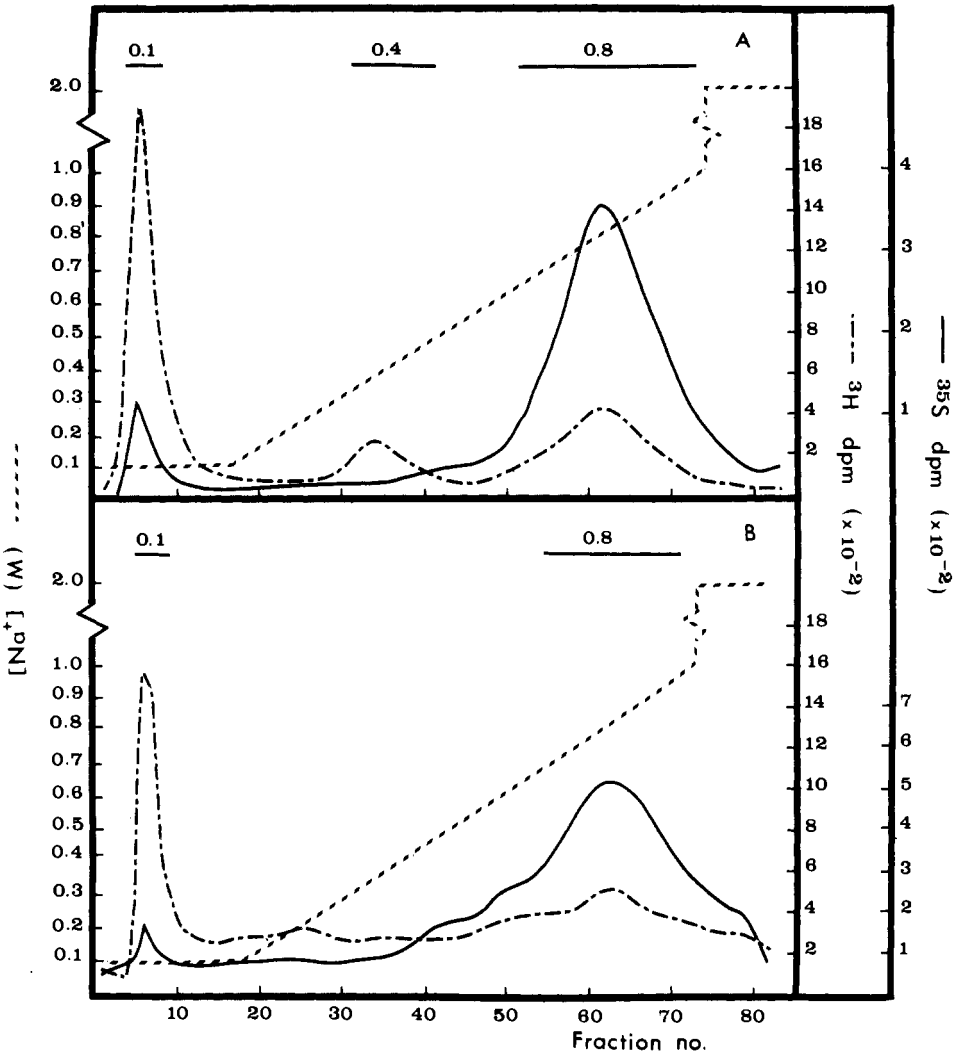

FIGURE 3: DEAE-cellulose chromatography in 6 M urea of peaks M/II and C/II from Sepharose profiles of RCX1.10 extracts. Horizontal bars represent fractions pooled for further analysis. (A) Peak M/II from Sepharose CL-2B profile of RCX1.10 culture medium. (B) Peak C/II from Sepharose CL-4B profile of RCX1.10 cell layer.

Table I: GAG Compositions of Rat Calvarial Cell Secreted and Cell-Associated Proteoglycan Fractions[a]

	composition (% total)			
	HA[b]	CS[c]	DS[c]	HS[c]
medium peak I	99	ND[d]	ND	ND
medium peak II/0.8	ND	77	20	0
cell peak II/0.8	ND	54	40	0
cell peak I	ND	ND	ND	45
cell peak I/0.8	ND	55	16	29
cell peak I/TX	ND	31	18	54

[a] GAG chains from proteoglycan fractions were subjected to specific degradative treatments as described (see Experimental Procedures). Control samples contained only GAGs and buffer. Percentage degradation was determined as 1 − [CPC-precipitable dpm (experimental)/CPC-precipitable dpm (control)] × 100. Values represent the mean of three determinations. [b] % total ^3H dpm. [c] % total ^{35}S dpm. [d] ND, not determined.

compared to the peak C/I material applied to the ion-exchange column.

Fractionation of GAG Chains from Cell-Associated Peak I Proteoglycan. As described above, the proteoglycan fraction C/I from the cell layer contained both CS/DS and HS. So that these components could be analyzed separately, the proteoglycan was incubated with alkaline borohydride reagent to release free GAG chains, followed by chromatography on DEAE-cellulose under nondenaturing conditions (Figure 6). Two approximately equal peaks were resolved, one eluting at 0.4 M NaCl and the other eluting at 0.6 M NaCl. These correspond to the HS chains and the CS/DS chains, respectively, as shown below.

Size Determination of Proteoglycan GAG Chains. GAG chains from the three species of proteoglycan reported here were chromatographed on Sepharose CL-6B in order to determine their size distribution. GAG chains from culture medium proteoglycan M/II/0.8 eluted in a single peak at a K_{av} value of 0.45 (Figure 7A). By the criteria of Wasteson (1971), this corresponds to a molecular weight of approximately 25000. Treatment of this material with chondroitinase ABC resulted in the loss of this peak, with concomitant appearance of a peak near the V_t of the column (Figure 7B), which presumably represents the disaccharide product of the enzyme (Yamagata et al., 1968). GAG chains from the cell-associated proteoglycan C/II/0.8 eluted in a similar position to those from the medium proteoglycan (Figure 7C). This material was also degraded by chondroitinase ABC treatment (Figure 7D). GAG chains from these two species of proteoglycan are therefore all of apparent M_r 25000.

The two GAG-isolated fractions from cell-associated proteoglycan C/I and separated by ion-exchange chromatography (Figure 6) were analyzed similarly. The GAG component displaced from DEAE-cellulose by 0.4 M salt eluted at K_{av} = 0.45, corresponding to a molecular weight of approximately

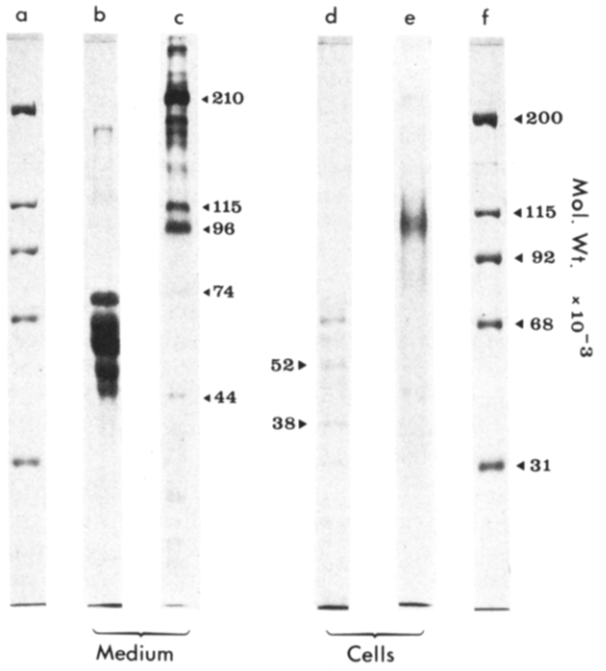

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of glycoprotein fractions from RCX1.10 culture medium and cell layer extracts. (a) (f) Standard marker proteins. (b) Coomassie Blue staining of peak M/II/0.1 from RCX1.10 culture medium. (c) Fluorograph of peak M/II/0.1 from RCX1.10 culture medium. (d) Coomassie Blue staining of peak C/II/0.1 from RCX1.10 cell layer. (e) Fluorograph of peak C/II/0.1 from RCX1.10 cell layer.

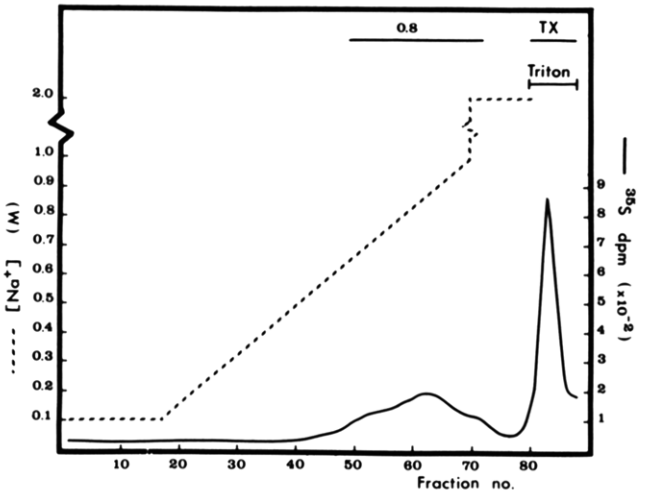

FIGURE 5: Ion-exchange chromatography on DEAE-cellulose in 6 M urea of peak C/I from Sepharose CL-4B profile of RCX1.10 cell layer extract. The column was eluted with a linear gradient of 0.1–1.0 M sodium acetate, a step of 2.0 M sodium acetate, and a step of 1% (v/v) Triton X-100. Horizontal bars represent fractions pooled for further analysis.

25 000 (Figure 8A). Treatment of this material with chondroitinase ABC had no effect (Figure 8B), but incubation with nitrous acid resulted in conversion of the GAG chains to low molecular weight products (Figure 8C). These data indicate that the 0.4 M salt peak from peak C/I consists of heparan sulfate. The GAG component displaced from DEAE-cellulose by 0.6 M salt eluted in a similar position to that of the HS chains on Sepharose CL-6B (Figure 8D) and therefore was of approximately the same molecular weight. However, this material was completely degraded by chondroitinase ABC (Figure 8E) and was not affected by nitrous acid treatment (Figure 8F). This indicates that the 0.6 M salt peak from C/I consists of CS/DS chains. Therefore, the cell-associated proteoglycan fraction C/I contains two classes of GAG chain,

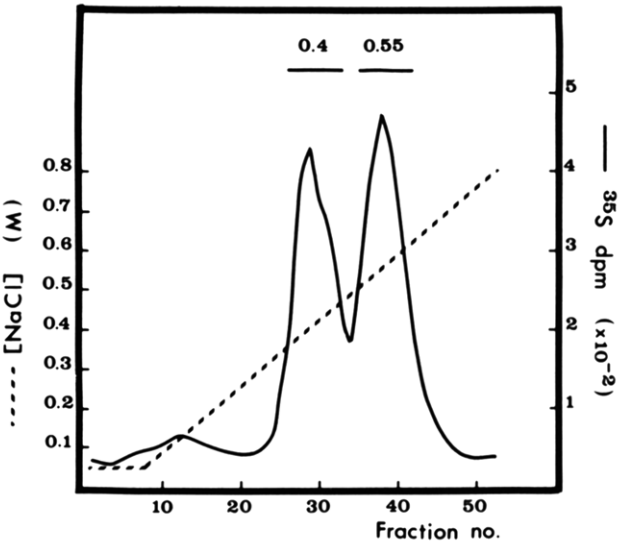

FIGURE 6: Ion-exchange chromatography on DEAE-cellulose of free GAG chains from peak C/I of the Sepharose CL-4B profile of RCX1.10 cell layer extract. Horizontal bars represent fractions pooled for further analysis.

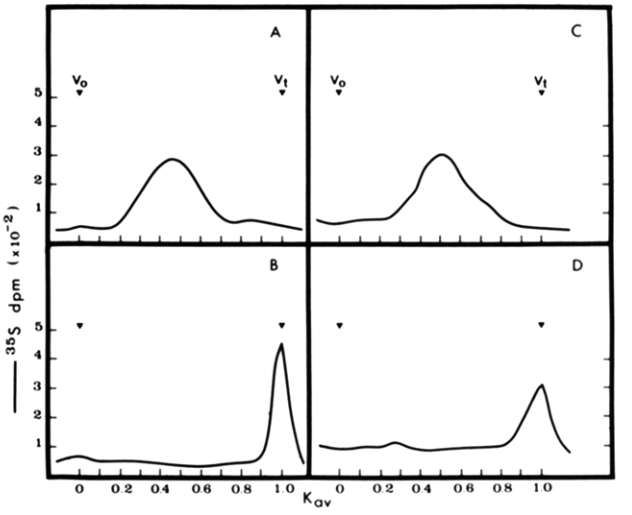

FIGURE 7: Chromatography on Sepharose CL-6B of GAG chains from RCX1.10 culture medium proteoglycan M/II/0.8 and cell layer proteoglycan C/II/0.8. V_0, void volume; V_t, total volume. (A) GAG chains from culture medium proteoglycan M/II/0.8. (B) GAG chains from (A) treated with chondroitinase ABC. (C) GAG chains from cell layer proteoglycan C/II/0.8. (D) GAG chains from (C) treated with chondrotinase ABC.

CS/DS chains of M_r 25 000 and HS chains of M_r 25 000.

Discussion

Clonal populations of osteoblast-like cells provide a useful model system for the study of bone cell biology and biochemistry. The ability of some of these clones to synthesize GAG's and collagen and respond to bone-specific hormones has been previously described (Aubin et al., 1982). Results of the present study indicate that at least three species of proteoglycan are synthesized by fetal rat calvaria clones, two associated with the cell layer and one secreted into the culture medium. Preliminary evidence is also presented for the synthesis of several glycoproteins.

The secreted proteoglycan has the largest hydrodynamic volume, eluting at a K_{av} value of approximately 0.7 on Sepharose CL-2B (Figure 1). The cell-associated CS proteoglycan elutes at K_{av} = 0.8 on Sepharose CL-2B but is better resolved from other components on Sepharose CL-4B, on which it elutes at K_{av} = 0.6 (Figure 2). Approximate mo-

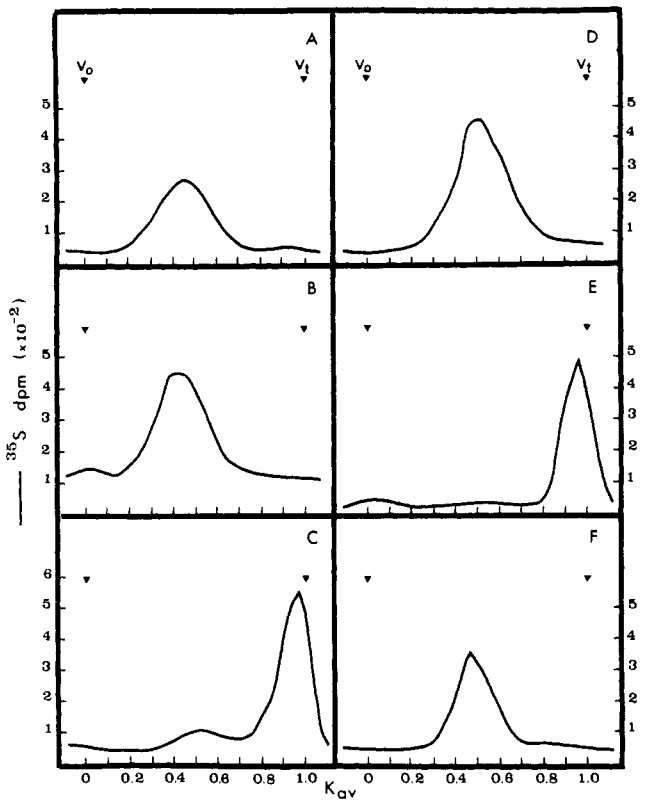

FIGURE 8: Chromatography on Sepharose CL-6B of GAG chains from RCX1.10 cell layer proteoglycan C/I. V_0, void volume; V_t, total volume. (A) GAG chains from DEAE-cellulose peak 0.4. (B) GAG chains from (A) treated with chondroitinase ABC. (C) GAG chains from (A) treated with nitrous acid. (D) GAG chains from DEAE-cellulose peak 0.6. (E) GAG chains from (D) treated with chondroitinase ABC. (F) GAG chains from (D) treated with nitrous acid.

lecular weights for these molecules were derived by calibration of the CL-2B column with cartilage proteoglycan (M_r 2.5 × 10^6; Hascall & Sajdera, 1970) and the BM-1 proteoglycan from EHS sarcoma (M_r 0.75 × 10^6; Hassell et al., 1980). This yields values of approximately 350 000 daltons for the secreted proteoglycan and approximately 250 000 daltons for the major cell-associated proteoglycan. However, as discussed by Hassell et al. (1980), the chromatographic elution properties of proteoglycans are dependent not only upon molecular weight but also upon protein/carbohydrate ratio. Further, the molecular weight of the BM-1 proteoglycan used as a marker has not been determined by physical techniques. Therefore, the molecular weights generated for bone cell proteoglycans in the present study can only be considered rough estimates.

These two bone cell proteoglycans have GAG chains of identical size, corresponding to a molecular weight of approximately 25 000. The size of chondroitin sulfate chains from bone has generally been believed to be larger, approximately M_r 40 000. An early report on the CS chains of dog compact bone indicated a molecular weight distribution of 45 000–56 000 determined by velocity sedimentation (Hjertquist & Vejlens, 1968). However, these figures were based on arbitrary values for the sedimentation constants. More recent studies have used gel chromatography to determine GAG chain molecular weight, as described by Wasteson (1971). In the embryonic chick calvaria, the major proteoglycan has chondroitin sulfate chains of apparent M_r 41 000 (Sugahara et al., 1981). Direct extraction of GAG's from chick calvaria by digestion with proteinase, however, resulted in a chondroitin sulfate chain size of M_r 20 000–25 000 (Sugahara et al., 1981). In matrix-induced bone formation in the rat, a small proteoglycan synthesized during the osteogenic phase has chondroitin sulfate chains of M_r ~50 000 (Reddi et al., 1978).

This variation in reported chain size of bone chondroitin sulfate may be, in part, related to the chromatographic system used. Proteoglycan GAG chains are commonly analyzed on Sephadex G-200. Chromatography of rat calvarial cell CS on this resin gives an apparent molecular weight of 40 000 (not shown). However, this corresponds to a K_{av} value of approximately 0.1, which is on a nonlinear region of the molecular weight calibration curve (Wasteson, 1971). Analysis of this same material on Sepharose CL-6B, as described in the present study, resulted in a peak around the midpoint of the elution profile. It is suggested, therefore, that Sepharose CL-6B may be a more appropriate chromatography resin for the separation of bone GAG chains.

The ratio of CS to DS is significantly different for the secreted proteoglycan and the major cell-associated proteoglycan. Therefore, it appears likely that these two fractions represent distinct species of proteoglycan. Preliminary pulse-labeling experiments failed to demonstrate any precursor–product relationship between these species (G. K. Hunter, J. N. M. Heersche, and J. E. Aubin, unpublished results). However, analysis of the core proteins will be necessary to determine whether these proteoglycans share any relationship at the genetic level.

The third proteoglycan fraction isolated from cloned calvarial cells has very different properties. It elutes in the void volume of Sepharose CL-4B, contains HS as well as CS and DS, and is only eluted from DEAE-cellulose with detergents. This latter property may indicate that this proteoglycan is normally associated with the plasma membrane. Cell-surface HS proteoglycans have been isolated from rat liver cells (Kjellen et al., 1981) and several other cultured cell types (Norling et al., 1981). Whereas these species contain HS as their sole GAG component, the cell-associated HS proteoglycan isolated in the present study (peak C/I) also contains CS/DS chains. One explanation of these data is that the peak C/I proteoglycan is contaminated with a fraction of the other cell-associated proteoglycan. Consistent with this is the observation that the fraction of this material solubilized with detergent is enriched for HS compared to the salt-eluted fraction (54% as compared to 29%), indicating that the contaminating CS proteoglycan may be selectively eluted by the salt gradient. In what may be a similar finding, an HS-containing proteoglycan isolated from glomerular basement copurified with a small amount of CS proteoglycan (Kanwar et al., 1981). However, an alternative possibility that the peak C/I fraction represents a hybrid proteoglycan containing both HS and CS/DS chains cannot be excluded at the present level of analysis.

The presence of membrane-associated HS proteoglycans in liver cells (Norling et al., 1981), glial cells, endothelial cells, and fibroblasts (Kjellen et al., 1981), as well as bone cells in the present study, may indicate that HS proteoglycans are ubiquitous components of mammalian cell surfaces. The two CS proteoglycans identified in calvarial cell cultures, however, may be specific for bone. Radiolabeling of fetal rat calvarial proteoglycans in utero has shown that two CS proteoglycans are synthesized in mineralizing bone (G. K. Hunter, J. N. M. Heersche, and J. E. Aubin, unpublished results). These have similar Sepharose CL-2B elution profiles to those synthesized by bone cells in vitro and also have GAG chains of M_r 25 000. The CS/DS ratios, however, are different for the proteoglycans synthesized in vivo, indicating that postsynthetic modification

events may be altered in cultured cells. Clonal populations of bone cells therefore represent a convenient system for the study of bone matrix macromolecules and are amenable for investigation of the effects mediated by bone-specific hormones.

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Diffusion Potential Cascade. Convenient Detection of Transferable Membrane Pores†

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ABSTRACT: A valinomycin-mediated K^+ diffusion potential across the membrane of multilamellar liposomes is stable for longer than 30 min and can be collapsed by a nonselective channel such as gramicidin. The kinetics of the potential collapse are complex but can be qualitatively broken down into a series of processes involving (1) binding of the gramicidin to the outer membrane, (2) dimerization to form a functional channel, (3) the flow of ions through the channel, (4) the establishment of a new diffusion potential on the next bilayer within the multilamellar liposome, and (5) the dissociation of gramicidin from the outer bilayer into the adjacent internal aqueous space. These processes are then repeated, in turn, for all the internal bilayers until the K^+ concentration gradient (and membrane potential) is completely dissipated. Process 5 appears to be rate limiting at high gramicidin concentrations, but ion flux, process 3, becomes slower at low gramicidin concentrations where the collapse of the K^+ gradient displays voltage dependence. Of course the rates of these processes can also be manipulated by changing the composition or size of the liposome and by varying the ion concentrations. Since the diffusion potential can be conveniently monitored with a voltage-sensitive fluorescent dye, 3,3′-diethylthiodicarbocyanine iodide [diS-C_2-(5)], a simple method for the detection and partial characterization of membrane pores emerges from this investigation.

The maintenance or dissipation of electrolyte concentration gradients across cell memranes is mediated by protein pores, a term by which we intend here to encompass both channels and carriers. A wide variety of toxins and antibiotics have been identified which behave as transferable pores; a subclass of these, the ionophores, are low molecular weight molecules which are freely soluble in detergent-free aqueous solutions. The detection and characterization of pores have relied heavily upon ion current measurements through voltage-clamped planar lipid bilayer membranes (Meuller & Rudin, 1969; Montal & Meuller, 1972) into which the pores have been

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