Immortalized, cloned mouse chondrocytic cells (MC615) produce three different matrix proteoglycans with core-protein-specific chondroitin/dermatan sulphate structures¹

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Cloned immortalized MC615 mouse chondrocytic cells were used to examine their capability to produce multiple types of matrix proteoglycans. Immunofluorescence staining indicated a uniform expression of aggrecan, biglycan and decorin by all cells. After culture with [³⁵S]sulphate, proteo[³⁵S]glycans secreted by the cells were found to elute in two peaks from a Sepharose CL-4B column. The first peak, at the void volume of the column, contained a large proteoglycan with an estimated average hydrodynamic mass of 10³ kDa. The glycosaminoglycan chains of this proteoglycan had an average hydrodynamic size of 17 kDa, estimated by Sepharose CL-6B chromatography, indicating the presence of 30–70 glycosaminoglycan chains per core protein, which was consistent with the characteristics of aggrecan. Biglycan and decorin were immunisolated from the second Sepharose CL-4B peak, and had average glycosaminoglycan hydrodynamic sizes of approx. 25 kDa and 32 kDa respectively. Glycosaminoglycan chains of the aggrecan, biglycan and decorin were treated with chondroitin ABC lyase, chondroitin AC lyase

and chondroitin B lyase to determine the positions of sulphation and the degree of uronic acid epimerization. The aggrecan glycosaminoglycan chains were found to contain a 4-sulphate/6-sulphate ratio of 7:3, with no epimerization of glucuronic acid to iduronic acid. The biglycan glycosaminoglycan chains were found to contain a similar ratio of 4-sulphate/6-sulphate, but with approx. 40–45% of the glucuronic acid epimerized to iduronic acid. The decorin glycosaminoglycan chains were found to contain 4-sulphate but no detectable 6-sulphate, and approx. 30–35% epimerization of the glucuronic acid to iduronic acid. The results, using these cloned cells, indicated that a single MC615 cell is able to make all three proteoglycans with distinctive differences between the glycosaminoglycans of aggrecan, biglycan and decorin. These data indicate that a mechanism must exist for a single MC615 cell to regulate the sizes and fine structures of glycosaminoglycans on simultaneously produced, different proteoglycans in a core-protein-specific manner.

INTRODUCTION

It is well established that proteoglycan core proteins constitute several distinct families, and that most tissues contain representatives of more than one core protein family for chondroitin/dermatan sulphate matrix proteoglycans [1]. The proteoglycans of cartilage have been the most studied and include aggrecan, which represents the large aggregating core protein family, and biglycan and decorin representing the small leucine-rich core protein family [2]. Aggrecan generally has relatively short chondroitin sulphate chains with 4-sulphation and 6-sulphation, but without iduronate. Biglycan and decorin generally have relatively long chondroitin/dermatan sulphate chains with both 4-sulphation and 6-sulphation or only 4-sulphation and high iduronate content [3–6]. Thus members of these two core protein families have glycosaminoglycans with differences in hydrodynamic size and in sulphation and epimerization patterns.

It has not been shown definitively whether a single population of differentiated chondrocytes is able to synthesize more than a single species of matrix proteoglycan such as aggrecan, biglycan or decorin with their respective glycosaminoglycan structures or whether there are specialized chondrocyte populations, each

limited to making only one species of matrix proteoglycan with a single glycosaminoglycan structure. In this regard, immunohistochemical studies of cartilage tissue have shown the presence of aggrecan and decorin deposited in similar areas around groups of chondrocytes [7,8] suggesting that each cell may be able to make both of these proteoglycans. However, the distribution of aggrecan and decorin have been shown to differ in various layers and zones of cartilage [9] suggesting the alternative, that there might be variable proportions of separate types of chondrocytes specializing for the production of each of these proteoglycans.

Cloned immortalized chondrocytes would offer an ideal model system to determine whether a single chondrocyte is capable of making more than one type of matrix proteoglycan and, if so, to investigate the mechanisms of core-protein-specific glycosaminoglycan formation. Systems of cloned immortalized cell lines have been developed from rat cartilage [10] and from human [11] and rat [12] chondrosarcomas. However, the multiple proteoglycans produced by these cloned cells have not been well characterized and the cells express little or none of the cartilage-specific types II, IX and XI collagens, indicating that these cells appeared to have lost most of the chondrocytic phenotype markers. In

Abbreviations used: Δ Di-4S, 2-acetamido-2-deoxy-3-O-(β -D-gluc-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose; Δ Di-6S, 2-acetamido-2-deoxy-3-O-(β -D-gluc-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose; Δ Di-4,6-diS, 2-acetamido-2-deoxy-3-O-(β -D-gluc-4-enepyranosyluronic acid)-4,6-di-O-sulpho-D-galactose.

¹ Dedicated to Roger W. Jeanloz on the occasion of his eightieth birthday. His contributions have been fundamental to the structural determination of complex carbohydrates, and have provided a pattern for two generations of scientists in glycobiology. He also deserves the credit for nomenclature which released investigators from the term 'mucopolysaccharide' by proposing the terms glycosaminoglycan, dermatan, heparan and keratan, leading to the term 'proteoglycan'.

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contrast, a clonal mouse chondrocytic cell line (MC615) has been shown to express aggrecan, type II, IX and XI collagens as well as link proteins, and has been found to retain the chondrocytic phenotype for at least 20 passages in culture [13]. These cells have also been shown to be genotypically homogeneous as indicated by the presence of a single genomic insertion site for the SV40 large T-antigen DNA [13]. Because of the cellular homogeneity of the MC615 cultures, the results from this model system can be considered to be indicative of the biosynthetic capability of a single chondrocyte.

We have now examined these cells for their capacity to produce three matrix-specific proteoglycans found in cartilage. Our results demonstrated that these cells make aggrecan, biglycan and decorin, and that the hydrodynamic size, sulphation and epimerization patterns of their glycosaminoglycan substituents were specific for each of the three core proteins. Thus a single cell must contain a mechanism for regulating the biosynthesis of the three different glycosaminoglycan fine structures specific for each of these three different core protein species.

EXPERIMENTAL

Materials

Carrier-free $\text{H}_2^{35}\text{SO}_4$, $[^3\text{H}]$ leucine and $[^3\text{H}]$ glucosamine were from New England Nuclear (Boston, MA, U.S.A.). Sepharose CL-2B, CL-4B, CL-6B, Sephadex G-50 and DEAE-Sephacel were from Pharmacia LKB (Piscataway, NJ, U.S.A.). All glycosaminoglycan-degrading enzymes, the 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-4-*O*-sulpho-D-galactose ($\Delta\text{Di-4S}$), 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-6-*O*-sulpho-D-galactose ($\Delta\text{Di-6S}$) and 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-4,6-di-*O*-sulpho-D-galactose ($\Delta\text{Di-4,6-diS}$) standards were from Seikagaku America (Rockville, MD, U.S.A.). Immobilon N-membrane was from Millipore (Bedford, MA, U.S.A.). Alkaline phosphatase and fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies were from Zymed Laboratories (South San Francisco, CA, U.S.A.). Buffers and chemicals for colour detection of alkaline phosphatase-conjugated secondary antibodies on immunoblots were from Bio-Rad (Hercules, CA, U.S.A.). Chondroitin sulphate A and C, Protein A-agarose and all other chemicals and were from Sigma (St. Louis, MO, U.S.A.). The aggrecan-specific anti-ATEGQV polyclonal antibody was generously given by Dr. John D. Sandy (Shriners Hospital for Crippled Children, Tampa, FL, U.S.A.) [14]; the aggrecan-specific polyclonal antibody was generously given by Dr. Dick Heinegard (University of Lund, Lund, Sweden). The decorin-specific polyclonal antibody J5 was generously given by Dr. J. Frederick Woessner, Jr. (University of Miami, Coral Gables, FL, U.S.A.) [15]. The versican-specific polyclonal antibody was generously given by Dr. George Perides (New England Medical Center, Boston, MA, U.S.A.) [16,17]. The biglycan-specific anti-peptide polyclonal antibody LF-106 was generously given by Dr. Larry W. Fisher (National Institutes of Health, Bethesda, MD, U.S.A.) [18]. X-ray film was from Fuji (Tokyo, Japan).

Labelling of cell cultures

MC615 cells (generously given by Dr. Bjorn R. Olsen, Harvard Medical School, Boston, MA, U.S.A.) were received at the ninth passage and cells at passage 12 were used for experiments. Some experiments were repeated at passage 20. The cells were cultured in Dulbecco's modified Eagle's medium-Ham's F12 medium containing 10% (v/v) fetal bovine serum and 200 $\mu\text{g}/\text{ml}$ geneticin [13]. The medium was replaced every 48 h and the cells were

maintained at 60–80% confluency. Metabolic labelling was accomplished by adding 100 μCi of carrier-free $\text{H}_2^{35}\text{SO}_4/\text{ml}$ or 25 μCi of $[^3\text{H}]$ leucine/ml to freshly plated, 30–50% confluent cultures, which were then incubated for 24 h. The conditioned medium was removed, the cells were washed 3 times with ice-cold PBS and extracted as previously described [19]. The conditioned medium was stored at -20°C until purification.

Partial purification of proteoglycans

All purification steps were carried out at 4°C . Conditioned medium was centrifuged at 5000 *g* for 10 min and the supernatant was adjusted to 0.05 M sodium acetate buffer, pH 4.5/6 M urea/1% (v/v) Triton X-100. DEAE-Sephacel (1 ml bed volume for every 50 ml of conditioned medium) was equilibrated with the same buffer containing 0.15 M NaCl and was mixed with the adjusted conditioned medium for 2 h. The slurry was poured into a column, washed with 10 bed volumes of the same buffer, followed by 5 bed volumes of the same buffer but with 0.1% (v/v) instead of 1% (v/v) Triton X-100, and was batch-eluted with 1 M NaCl in the same buffer. The eluate was dialysed against water (3 changes in 36 h), lyophilized and redissolved in 0.05 M Tris/HCl buffer, pH 7.4/4 M guanidine HCl.

Caesium chloride density gradient and immunodot-blot analysis

Proteoglycans from unlabelled cultures were partially purified on DEAE-Sephacel, dissolved in 4 M guanidine HCl and the density was adjusted to 1.47 g/ml by adding 0.55 g CsCl per g of solute. The sample was centrifuged at 120000 *g* in an SW 60 rotor for 72 h at 10°C [20] and fractions were collected from the bottom of the tube. The densities of the fractions were determined by weighing 10 μl on an analytical balance and each fraction was desalted on a Sephadex G-50 column previously equilibrated with 0.5 M ammonium bicarbonate, lyophilized and redissolved in PBS. Aliquots of the fractions were transferred on to an Immobilon N membrane using a dot-blot apparatus (Bio-Rad), the membrane was treated with 0.0025% glutaraldehyde for 5 min, followed by 0.1 M Tris/HCl buffer, pH 7.4, for 5 min and was then incubated with Blotto [10% (w/v) Carnation non-fat dried milk in Tris-buffered saline (0.05 M Tris/HCl buffer, pH 7.4/0.15 M NaCl)] at room temperature for 1 h. The membrane was incubated overnight at 4°C with either a 1:250 dilution of aggrecan-specific antibody or a 1:500 dilution of decorin-specific antibody in Blotto. The membrane was then washed with 0.3% (v/v) Tween-20 in Tris-buffered saline, incubated at room temperature for 30 min with alkaline phosphatase-conjugated goat anti-rabbit antibody and washed again in the same buffer. The bound antibodies were revealed by adding a mixture of Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad) in the buffer provided by the manufacturer and developed until visible dots appeared (1–15 min).

Molecular-sieve chromatography

For hydrodynamic-size estimation and separation of proteoglycans, Sepharose CL-2B and CL-4B (1 cm $\times$ 47 cm columns) were eluted with 0.05 M Tris/HCl buffer, pH 7.4/4 M guanidine HCl. For hydrodynamic-size estimation of glycosaminoglycans and degradation products, Sepharose CL-6B and Sephadex G-50 (1 cm $\times$ 47 cm columns) were eluted with 0.5 M ammonium bicarbonate. Void volume and total volume were determined by the elution positions of Blue Dextran and $[^3\text{H}]$ glucosamine respectively.

Western blots

Unlabelled, partially purified proteoglycans were chromatographed on a Sepharose CL-4B column and fractions corresponding to peaks I and II were desalted on a Sephadex G-50 column using 0.5 M ammonium bicarbonate as eluent and were then lyophilized. The residue was dissolved in Tris-buffered saline containing 0.1 % (v/v) Triton X-100 and aliquots were incubated with chondroitin ABC lyase. The enzyme digests were subjected to SDS/PAGE (3–10 % gradient gel) for determining the presence of versican or to SDS/PAGE (10 % gel) for the detection of biglycan and decorin. Proteins were transferred electrophoretically (400 mA for 90 min) on to an Immobilon-N membrane in a semi-dry transfer apparatus (Bio-Rad) and the membrane was processed for the immunodot-blot procedure, as described above, except that the following primary antibodies were used: a 1:500 dilution of versican-specific antibody or a 1:400 dilution of biglycan-specific antibody or a 1:500 dilution of decorin-specific antibody.

Immunoprecipitation

Analytical immunoprecipitation

Pooled fractions from the Sepharose CL-4B column were dialysed against 3 changes of distilled water, lyophilized and dissolved in a small volume of PBS containing 1 mg/ml BSA. The samples were precleared by incubation with Protein A-agarose (50 μ l of the slurry to 500 μ l of sample) for 1 h at room temperature and the supernatant was obtained by brief centrifugation. Antisera (1:50 dilution) or normal rabbit IgG, as a control (0.2 mg/ml final concentration in PBS), were added to the precleared supernatant and the mixtures were rocked overnight at 4 °C. Protein A-agarose (previously equilibrated with unlabelled MC615 conditioned medium) was then added (50 μ l of the slurry to 250 μ l of sample) and mixed for 1 h at room temperature. The beads were washed 3 times with 1 mg/ml BSA in PBS and once with PBS containing 0.5 % (v/v) Nonidet P40. Aliquots of the agarose beads were transferred to scintillation fluid and radioactivity was estimated using scintillation spectrometry.

Preparative immunoprecipitation

Conditioned medium from radiolabelled cells was centrifuged at 14000 *g* for 10 min to remove particulate material and the supernatant was precleared and processed as described in the analytical procedure. An aliquot of the immunoprecipitated material was subjected to SDS/PAGE (7.5 % gel), dried and exposed to X-ray film for 2 days. Other aliquots were used for the characterization of the glycosaminoglycans.

Immunofluorescence microscopy

MC615 cells were plated on to glass coverslips at 30 % confluency and grown to near confluency. The medium was removed, the coverslips were rinsed twice with PBS and the cells were fixed with 3 % (w/v) paraformaldehyde in PBS for 10 min at room temperature. The coverslips were then washed twice with Tris-buffered saline, the cells were permeabilized with 0.2 % (v/v) Triton X-100 in Tris-buffered saline for 10 min. The cells on some coverslips were subjected to reduction and alkylation by including 10 mM dithiothreitol in the solutions used for fixation and permeabilization and then alkylating the reduced cells with 25 mM iodoacetamide for 30 min at room temperature, followed by two washes with 0.1 M dithiothreitol. All coverslips were washed three times with Tris-buffered saline, incubated with 10 % (v/v) goat serum in Tris-buffered saline for 30 min and

individual coverslips were reacted with proteoglycan-specific primary antibodies at 1:50 dilution in 10 % (v/v) goat serum in Tris-buffered saline overnight at 4 °C. The antibodies used were: aggrecan-specific antibody, biglycan-specific antibody or decorin-specific antibody. After incubation with primary antibodies the coverslips were washed three times with Tris-buffered saline, incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG at 1:50 dilution in Blotto for 30 min at room temperature, washed three times with Tris-buffered saline and mounted in glycerol. The coverslips were sealed with nail polish and the fluorescence pattern was examined using an Axiophot microscope (Zeiss) using a $\times 40$ objective. Photographs were taken using TMAX 400 Kodak film. Control coverslips were treated similarly except incubation with the primary antibody was omitted.

Reverse transcriptase PCR

Micro Poly (A) Pure kit (Ambion, Austin, TX, U.S.A.) was used according to manufacturer's recommendation to isolate total and mRNA from MC615 cells. First-strand cDNA was generated using the Superscript Preamplification kit (Gibco BRL; Gaithersburg, MD, U.S.A.) according to manufacturer's recommendation. PCR was performed using Vent DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.) on 10 % of the first strand reaction mixture. The following primer pairs (forward and reverse) were used: 5'-GAGACACTTCAAGAAATCGA-ATCC-3' and 5'-CTCAGCATCCTGGGCACATTATGG-3' for amplification of mouse aggrecan cDNA [21]; 5'-GCAGCC-TGCACCTGGCTGCTTGTC-3' and 5'-GCCTCTACTTCTT-ATAATTTCCAA-3' for amplification of mouse biglycan cDNA [22]; 5'-GACTTCATGCTAGAAGATGAGGC-3' and 5'-CTTGTAAGTTTCCAAGTTGAATGG-3' for amplification of mouse decorin cDNA [23]; 5'-CCTGATCTCTGCAAAACAA-ACCCA-3' and 5'-TTGCAGTGCAGTCCGTCAGTCCA-3' for amplification of mouse versican cDNA [24]. The amplified DNA was subcloned into pCR-Script vector (Stratagene; La Jolla, CA, U.S.A.) and sequenced by thermal-cycle sequencing using the CircumVent kit (New England Biolabs).

Alkali elimination

Samples from pooled column fractions or from immunoprecipitations were incubated in 0.5 M NaOH for 10 h at room temperature, diluted with column buffer and applied directly to the column.

Enzymic degradation of glycosaminoglycans

Samples were treated with 0.025 unit of chondroitin ABC or AC lyases/sample for 30 min at 37 °C, as described previously [25]. Samples were treated with 0.02 unit of chondroitin B lyase/sample for 45 min at 30 °C, according to manufacturer's recommendation.

Paper chromatography

Samples were mixed with 0.5 mg of unlabelled chondroitin sulphate A and C (internal control), digested with 0.025 unit of chondroitin ABC lyase for 30 min and the digests were applied directly to Whatman 1 chromatography paper. The chromatograms were developed with acetic acid/butanol/ammonium hydroxide (3:2:1, by vol.) [25]. The position of the unlabelled degradation products were identified under UV light, then the paper was dried and cut into 1 cm strips which were assayed for radioactivity using scintillation spectrometry.

RESULTS

Characterization of proteoglycans

Since 95 % of the proteo[³⁵S]glycans produced by subconfluent MC615 cells (passage 12) were secreted into the conditioned medium, the cell-associated proteoglycans (5 % of total) were not examined. Initially we studied unlabelled, secreted proteoglycans after separation on a dissociative CsCl density gradient. The dot-blot immunoassay detected aggrecan only in the high-density fractions (1.54–1.68 g/ml) (Figure 1), while decorin was detected only in the medium-density fractions (1.42–1.54 g/ml). These results indicated that MC615 cells synthesize aggrecan and

Figure 1 Immuno-dot-blot identification of proteoglycans separated by CsCl density gradient

Proteoglycans fractionated on a dissociative CsCl gradient were identified as aggrecan and decorin by the use of aggrecan-specific anti-ATEGQV and decorin-specific antibodies on dot blots, as described in the Experimental section.

Figure 2 Sepharose CL-4B chromatography of proteo[³⁵S]glycans

Proteo[³⁵S]glycans were chromatographed on a Sepharose CL-4B column (1 cm × 47 cm) as described in the Experimental section. V_0 , void volume; V_t , total volume.

Figure 3 Sepharose CL-2B chromatography of peak I

Fractions 15–18 from peak I (see Figure 2) were pooled and chromatographed on a Sepharose CL-2B column (1 cm × 47 cm) as described in the Experimental section. V_0 , void volume; V_t , total volume.

decorin and that these proteoglycans have biophysical properties similar to aggrecan and decorin isolated from bovine or human cartilage.

Proteo[³⁵S]glycans were found in two major peaks when chromatographed under dissociative conditions on a Sepharose CL-4B column (Figure 2). Approx. 10 % was excluded from the column (peak I) indicating the presence of large proteoglycans, while the remaining 90 % (peak II) was eluted as a broad peak with a $K_{av} = 0.5$, indicating the presence of small proteoglycans. Although the small proportion of the large proteoglycans was somewhat unexpected for cartilage, elution profiles with similar low amounts of aggrecan have been obtained from metabolically-labelled adult cartilage explants [26], from unlabelled juvenile human cartilage [27] and from cultures of chondrocytes obtained from areas near the surface of bovine articular cartilage [28].

Peak I proteoglycans in fractions 15–18 were pooled and aliquots were rechromatographed on a Sepharose CL-2B column under dissociative conditions (Figure 3), resulting in a broad peak with the average elution position of $K_{av} = 0.3$, corresponding to an estimated average hydrodynamic size of 10^3 kDa, similar to that of the aggrecan monomer from bovine cartilage [29], rat cartilage [30], chicken primary chondrocyte cultures [31] or cultured explants of bovine cartilage [32]. Immunoprecipitation with the aggrecan-specific antibody did not yield enough proteoglycan for characterization (confirming a relatively weak reactivity of this antibody with the mouse aggrecan on the dot blots). Therefore, pooled peak I material was used directly for the analysis of the aggrecan glycosaminoglycans. No versican, biglycan or decorin was detected in peak I by immunoprecipitation or by Western blotting (results not shown). PCR amplification of MC615-derived cDNA with aggrecan-specific primers resulted in DNA fragments encoding for the C-terminus of aggrecan, determined by DNA sequencing, thus providing additional evidence that the proteoglycan in peak I is aggrecan. In contrast, amplification with versican-specific primers did not

Figure 4 Detection of biglycan and decorin core proteins

(A) [^3H]Leucine-labelled proteoglycans immunoprecipitated with antisera as described in the Experimental section, were subjected to SDS/PAGE (7.5% gel) and autoradiographed. Lane 1, biglycan; lane 2, biglycan after treatment with chondroitin ABC lyase; lane 3, decorin; lane 4, decorin after treatment with chondroitin ABC lyase; lane 5, control immunoprecipitation without primary antibody. Proteoglycans are indicated by square brackets; core proteins are marked with arrows. (B) Chondroitin-ABC-lyase-treated, pooled fractions 23–35 from peak II (see Figure 2) were subjected to SDS/PAGE (10% gel) and analysed by Western blotting using antisera, as described in the Experimental section, to detect biglycan (lane 1) or decorin (lane 2). The positions of prestained molecular-mass markers (kDa) for both (A) and (B) are indicated on the right.

yield any product, confirming that versican is not made by these cells.

Fractions 23–35 (peak II) from the Sepharose CL-4B column were pooled, dialysed and immunoprecipitated with the biglycan-specific and decorin-specific antibodies, resulting in an initial precipitation of 16% and 4% of total peak II proteo[^{35}S]glycans respectively. Repeated immunoprecipitations were used to deplete biglycan or decorin totally from the samples, demonstrating that approx. 25% and 10% of the peak II proteo[^{35}S]glycans were biglycan and decorin respectively. Control immunoprecipitations with non-immune rabbit IgG resulted in less than 0.5% of the radioactivity being precipitated with the immune sera. Amplification of MC615-derived cDNA with biglycan-specific or decorin-specific primers resulted in DNA fragments encoding for full-length biglycan and decorin DNAs, determined by DNA sequencing. The remaining proteoglycans in peak II were not examined but possibly included type IX and type XI collagens, which had been shown to be produced by these cells previously [13].

In order to define the specificities of the antibodies, immunoprecipitations were performed from [^3H]leucine-labelled conditioned medium. An aliquot of the immunoprecipitated material was treated with chondroitin ABC lyase and then the treated and untreated samples were subjected to SDS/PAGE (7.5% gel). The autoradiogram showed that the biglycan-specific antibody precipitated a proteoglycan with a molecular mass > 200 kDa (Figure 4A, lane 1) and that treatment of this proteoglycan with chondroitin ABC lyase resulted in a closely spaced core protein doublet with an approx. molecular mass of 46 kDa (Figure 4A, lane 2). The decorin-specific antibody precipitated a proteoglycan with an approx. molecular mass of 80–120 kDa (Figure 4A, lane 3) and treatment of this proteoglycan with chondroitin ABC lyase resulted in a core protein with the approx. molecular mass of 45 kDa (Figure 4A, lane 4). These core protein masses are consistent with values reported previously [33].

In separate confirmatory experiments, aliquots of unlabelled peak II material were treated with chondroitin ABC lyase,

subjected to SDS/PAGE (10% gel) and analysed on Western blots using the biglycan-specific or decorin-specific antibodies. Biglycan core protein was detected as a doublet of approx. 46 kDa (Figure 4B, lane 1). Decorin core protein was detected as a major band of approx. 45 kDa, representing the intact core protein, and a much lighter band of 33 kDa, probably representing a proteolytic degradation product (Figure 4B, lane 2).

At passage 20 (results not shown) the proportions of secreted aggrecan, biglycan and decorin were essentially identical with the proportions found at passage 12, indicating that the MC615 cells were stable with regard to proteoglycan synthesis.

Intracellular localization of proteoglycans

Immunofluorescence microscopy was used to assess the intracellular localization of aggrecan, biglycan and decorin. All cells on the coverslips stained uniformly with each of the antibodies, consistent with the production of aggrecan, biglycan and decorin by all cells (Figure 5). Using traditional fixation and permeabilization, the aggrecan-specific anti-ATEGQV antibody produced a diffuse staining pattern, most dense over the entire nucleus and less dense over the cytoplasm. In addition, there were multiple intensely stained, small round spots over the nucleus. The same diffuse staining pattern, most dense over the nucleus, was obtained when the cells were reduced and alkylated concomitantly with fixation (a procedure suggested by Dr. John D. Sandy to improve reactivity of the antibody) or when a different anti-aggrecan polyclonal antibody (generously provided by Dr. Dick Heinegard) was used (results not shown). However, the number of intensely stained spots were decreased or eliminated, suggesting that they were artifacts of the anti-ATEGQV antibody. The biglycan-specific and decorin-specific antibodies produced patterns which differed greatly from the dense anti-aggrecan staining over the nucleus. The biglycan-specific antibody produced a pattern that ranged from perinuclear partial caps to circumferential crowns, as well as light staining over the entire nucleus. The decorin-specific antibody produced a pattern that was similar to the biglycan-staining, but the intensity of staining of the perinuclear areas was decreased, while the staining over the nucleus was more intense. Several fields of each coverslip were examined and no differences were found between fields, indicating the homogeneity of MC615 cells for expression of aggrecan, biglycan and decorin.

Hydrodynamic size of glycosaminoglycans

After alkali cleavage of [^{35}S]glycosaminoglycans from the isolated proteo[^{35}S]glycans, their size was determined by chromatography on a Sepharose CL-6B column. [^{35}S]Glycosaminoglycan chains from the aggrecan had an estimated median hydrodynamic size of 17 kDa based on a K_{av} of 0.53 (Figure 6), which was similar to the size reported for glycosaminoglycan chains of aggrecan from cartilage [34–36] and mouse intervertebral disc [37]. Comparison of the hydrodynamic size of [^{35}S]glycosaminoglycan with the hydrodynamic size of proteo[^{35}S]glycan indicated that the aggrecan contained a range of 30–70 glycosaminoglycan chains per core protein. The small hydrodynamic size with relatively narrow distribution, together with the large number of chains per core protein, provided additional evidence that peak I did not contain versican, since versican has been reported to have much larger glycosaminoglycan chains [38–40] and many fewer glycosaminoglycan chains per core protein [41].

[^{35}S]Glycosaminoglycan chains from the immunoprecipitated biglycan eluted at a K_{av} of 0.46 (Figure 6), corresponding to an estimated median hydrodynamic size of 25 kDa, somewhat smaller than that reported for glycosaminoglycan chains of

Figure 5 Intracellular localization of proteoglycans

Fixed, permeabilized MC615 cells were reacted with proteoglycan-specific primary antibodies followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. (A) Aggrecan-specific antibody; (B) biglycan-specific antibody; (C) decorin-specific antibody; (D) control without primary antibody.

biglycan from bone [42] or the temporo-mandibular joint [43]. [^{35}S]Glycosaminoglycan chains of immunoprecipitated decorin were eluted from the Sepharose CL-6B column (Figure 6) at a K_{av} of 0.42, corresponding to an estimated median hydrodynamic size of 32 kDa. This is in good agreement with values reported previously for decorin glycosaminoglycan from other tissues [33].

Iduronate content of glycosaminoglycans

[^{35}S]Glycosaminoglycans from the aggrecan and from the immunoprecipitated biglycan and decorin proteo[^{35}S]glycans were digested with chondroitin AC I lyase, in order to cleave $\text{GalNAc}(\text{SO}_4)$ -GlcA linkages while leaving $\text{GalNAc}(\text{SO}_4)$ -IdoA linkages intact. [^{35}S]Glycosaminoglycans derived from the aggrecan were completely degraded to disaccharides (Figure 7A), indicating that there had been no epimerization of the uronic acid residues to iduronate. In contrast, [^{35}S]glycosaminoglycan chains of biglycan and decorin were partially resistant to the enzyme (Figures 7B and 7C), resulting in oligosaccharides and disaccharides. Increasing the reaction time and the addition of fresh enzyme did not change the elution

profile, indicating that, in all cases, reaction with 0.025 units of the enzyme for 30 min resulted in complete degradation. These elution profiles indicated that approx. 40–45% and 30–35% of the uronic acid in the glycosaminoglycan chains of the biglycan and decorin respectively was iduronic acid.

In separate experiments [^{35}S]glycosaminoglycans from the aggrecan, biglycan and decorin were digested with chondroitin B lyase in order to cleave $\text{GalNAc}(\text{SO}_4)$ -IdoA linkages while leaving $\text{GalNAc}(\text{SO}_4)$ -GlcA linkages intact. [^{35}S]Glycosaminoglycan derived from aggrecan was completely degraded (Figure 8A), confirming the absence of iduronate, while [^{35}S]glycosaminoglycan chains of biglycan and decorin were partially degraded (Figures 8B and 8C). A larger proportion of the smaller oligosaccharides among the degradation products of biglycan indicated a higher degree of epimerization of the GlcA residues than in decorin, confirming the results obtained with chondroitin AC I lyase.

Position of *O*-sulphate on *N*-acetylgalactosamine residues

The relative amounts of 4-sulphate and 6-sulphate on the [^{35}S]glycosaminoglycan chains were determined by paper

Figure 6 Sepharose CL-6B chromatography of [^{35}S]glycosaminoglycans

Proteo[^{35}S]glycans were treated with alkali and the resulting [^{35}S]glycosaminoglycans derived from aggrecan (■), biglycan (□) and decorin (●) were chromatographed on a Sepharose CL-6B column (1 cm $\times$ 47 cm) as described in the Experimental section.

chromatography of degradation products after treatment with chondroitin ABC lyase (Figure 9). Aggrecan was found to have glycosaminoglycans with approx. 70% of the ^{35}S label in 4-sulphate and 30% in 6-sulphate (Figure 9A). Similar findings have been reported for porcine aggrecan [4], while human aggrecan has been shown to contain mostly 6-sulphate [5]. Biglycan [^{35}S]glycosaminoglycans had a similar 7:3 proportion of 4-sulphate to 6-sulphate (Figure 9B), and differed from bovine cartilage biglycan, which has been reported to have no 6-sulphate [6]. Decorin was found to have [^{35}S]glycosaminoglycans with no 6-sulphate, but approx. 10% of the sulphated degradation products co-migrated with a $\Delta\text{Di-4,6-diS}$ standard (Figure 9C). However, treatment with chondro 6-sulphatase produced no change in the migration pattern of this radiolabelled material, indicating that it contained no 6-sulphate. This disaccharide was not characterized further. Decorin from bovine cartilage has been reported to lack 6-sulphate [6], in contrast with human and porcine cartilage decorin which have been reported to have some 6-sulphate [4].

DISCUSSION

Our results provide direct evidence that the immortalized cloned mouse chondrocytes make aggrecan, biglycan and decorin with glycosaminoglycan substituents similar to those of the aggrecan, biglycan and decorin in cartilage tissue, cartilage explant cultures or primary chondrocyte cultures from other species. Moreover, our data show that the MC615 cells are able to synthesize these chondroitin sulphate and dermatan/chondroitin sulphate matrix proteoglycans simultaneously, with glycosaminoglycan chains having core-protein-dependent hydrodynamic size, sulphation and epimerization. Since the MC615 cell line is made up of phenotypically and genotypically homogeneous cells, we consider these results to be representative of the actions of a

Figure 7 Sephadex G-50 chromatography of chondroitin AC I lyase degradation products

[^{35}S]Glycosaminoglycans derived from (A) aggrecan, (B) biglycan or (C) decorin were degraded with chondroitin AC I lyase and the degradation products were chromatographed on a Sephadex G-50 column (1 cm $\times$ 47 cm) as described in the Experimental section. V_0 , void volume; V_t , total volume.

single cell. Thus each MC615 cell has the enzymes both for 4- and 6-sulphation of *N*-acetylgalactosamine and for epimerization of glucuronic acid. Nevertheless, the action of these various enzymes is restricted to glycosaminoglycans on selected core proteins. Consequently, our data demonstrate that a cellular mechanism must exist by which a single differentiated cell is able to regulate the length and fine structure of glycosaminoglycans on matrix proteoglycans in a core-protein-dependent manner.

It is unknown what the nature of the regulatory mechanisms might be, but recent studies have implicated intracellular trafficking as an important determinant in the synthesis of glycosaminoglycans with different fine structure. It is well established that core proteins are transported from the endoplasmic reticulum to Golgi compartments for the formation of the linkage region [44–47] and for the formation and sulphation of the glycosaminoglycan chains [44–46, 48–50]. Experiments with brefeldin A or xylosides using articular-cartilage chondrocytes and embryonic-chick muscle cells have indicated that the intracellular compartments into which decorin core protein is transported has characteristics different from those to which aggrecan [51] or versican [52] core proteins are transported. These results can be interpreted to indicate a restricted access of core proteins to intracellular compartments carrying a particular organization and/or set of glycosaminoglycan-synthesizing

Figure 8 Sephadex G-50 chromatography of chondroitin B lyase degradation products

[³⁵S]Glycosaminoglycans derived from (A) aggrecan, (B) biglycan or (C) decorin were degraded with chondroitin B lyase and the degradation products were chromatographed on a Sephadex G-50 column (1 cm × 47 cm) as described in the Experimental section. V_0 , void volume; V_t , total volume.

enzymes. Thus the ability of MC615 cells to form three proteoglycan species with core-protein-specific glycosaminoglycans would be consistent with the transport of core proteins to three separate intracellular compartments for the synthesis of core-protein-specific glycosaminoglycans. Aggrecan core protein would be transported to a compartment containing both 4- and 6-sulphotransferases, but no epimerase. Biglycan core protein would be transported to a compartment containing both 4- and 6-sulphotransferases and the epimerase. Decorin core protein would be transported to a compartment containing 4-sulphotransferase and the epimerase, but no 6-sulphotransferase. Our photo-micrographs of intracellular proteoglycan staining with specific antibodies, which exhibit different staining patterns, reinforce this concept.

The stable culture phenotype, the presence of both sulphotransferases and epimerases, and the ability to synthesize core-protein-specific glycosaminoglycans make the MC615 cell line a valuable model system *in vitro* for studies related to the regulation of glycosaminoglycan biosynthetic processes.

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Figure 9 Paper chromatography of disaccharides generated by chondroitin ABC lyase

[³⁵S]Glycosaminoglycans derived from (A) aggrecan; (B) biglycan; (C) decorin together with unlabelled chondroitin sulphate A and chondroitin sulphate C standards were degraded with chondroitin ABC lyase and the degradation products were chromatographed on Whatman paper as described in the Experimental section. The positions of the unlabelled disaccharides (Δ Di-6S and Δ Di-4S) derived from the degradation of the internal control chondroitin sulphate are indicated in (A).

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