Inventory of human skin fibroblast proteoglycans

Identification of multiple heparan and chondroitin/dermatan sulphate proteoglycans

Artur SCHMIDTCHEN, Ingemar CARLSTEDT, Anders MALMSTRÖM and Lars-Åke FRANSSON

Department of Physiological Chemistry 2, University of Lund, P.O. Box 94, S-221 00 Lund, Sweden

Heparan sulphate and chondroitin/dermatan sulphate proteoglycans of human skin fibroblasts were isolated and separated after metabolic labelling for 48 h with $^{35}\text{SO}_4^{2-}$ and/or $[^3\text{H}]$ leucine. The proteoglycans were obtained from the culture medium, from a detergent extract of the cells and from the remaining 'matrix', and purified by using density-gradient centrifugation, gel and ion-exchange chromatography. The core proteins of the various proteoglycans were identified by electrophoresis in SDS after enzymic removal of the glycosaminoglycan side chains. Skin fibroblasts produce a number of heparan sulphate proteoglycans, with core proteins of apparent molecular masses 350, 250, 130, 90, 70, 45 and possibly 35 kDa. The major proteoglycan is that with the largest core, and it is principally located in the matrix. A novel proteoglycan with a 250 kDa core is almost entirely secreted or shed into the culture medium. Two exclusively cell-associated proteoglycans with 90 kDa core proteins, one with heparan sulphate and another novel one with chondroitin/dermatan sulphate, were also identified. The heparan sulphate proteoglycan with the 70 kDa core was found both in the cell layer and in the medium. In a previous study [Fransson, Carlstedt, Cöster & Malmström (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5657–5661] it was suggested that skin fibroblasts produce a proteoglycan form of the transferrin receptor. However, the core protein of the major heparan sulphate proteoglycan now purified does not resemble this receptor, nor does it bind transferrin. The principal secreted proteoglycans are the previously described large chondroitin sulphate proteoglycan (PG-L) and the small dermatan sulphate proteoglycans (PG-S1 and PG-S2).

INTRODUCTION

Proteoglycans are complex macromolecules which consist of a core protein substituted with sulphated glycosaminoglycans, such as keratan sulphate, chondroitin sulphate (CS), dermatan sulphate (DS), heparan sulphate (HS) or heparin. CS/DS-containing proteoglycans are mainly found in cartilage and fibrous connective tissues. The large cartilage CS-proteoglycan forms aggregates with hyaluronan, and a small DS-proteoglycan (decorin) interacts with the surface of collagen fibrils (for reviews, see Hassell *et al.*, 1986; Poole, 1986; Heinegård & Sommarin, 1987; Ruoslahti, 1988). HS-proteoglycans are associated with cell surfaces, pericellular matrices and basement membranes. Their HS-chains may sequester growth factors, immobilize lipoprotein lipase, interact with fibronectin, collagens and laminin (for reviews see Gallagher *et al.*, 1986; Fransson, 1987), and affect the reactivity of cell-adhesion molecules (Cole & Glaser, 1986). Composite proteoglycans carrying both HS and CS chains have been isolated from placenta (Isemura *et al.*, 1987), simple and stratified epithelia (denoted syndecan; Sanderson & Bernfield, 1988) and from mouse mammary epithelial cells (David & Van den Berghe, 1989). The type III receptor for transforming growth factor- β , which has been detected in a variety of cell types, has also been proposed to be a composite proteoglycan (Cheifetz *et al.*, 1988; Segarini & Seyedin, 1988).

Cultured cells can synthesize and secrete various forms

of proteoglycans. Chondrocytes (Heinegård & Sommarin, 1987) and skin fibroblasts (Cöster *et al.*, 1984; Glössl *et al.*, 1984) are high producers of CS/DS-proteoglycans. Synthesis and secretion of HS-proteoglycans have been studied in many different cell types, e.g. rat hepatocytes, various tumour cells, human endothelial and glial cells (for review see Höök *et al.*, 1984), bovine arterial smooth-muscle cells (Schmidt & Buddecke, 1988), as well as fibroblasts from rat embryos (Woods *et al.*, 1985), human skin (Carlstedt *et al.*, 1983; Cöster *et al.*, 1986) and human lung (Lories *et al.*, 1987; Heremans *et al.*, 1988).

In previous studies from our laboratory the major HS-proteoglycan of human skin fibroblasts yielded core proteins that were sensitive to reduction of disulphide bonds and had affinity for transferrin, and it was suggested that fibroblasts could synthesize a proteoglycan form of the transferrin receptor (Cöster *et al.*, 1983; Fransson *et al.*, 1984; Fransson, 1987). In the present work we have re-examined the HS- as well as the CS/DS-proteoglycans made by human skin fibroblasts. Radiolabelled proteoglycans have been purified from the culture medium, from a detergent extract of the cell layer and from the remaining 'matrix' by using density-gradient centrifugation, gel and ion-exchange chromatography. The proteoglycans were identified by the size of their core proteins and the nature of their side chains. Certain proteoglycans are restricted to the medium or to the cell layer, whereas others are found in both compartments. The major cell-bound HS-

Abbreviations used: Gdn, guanidinium chloride; CS, chondroitin sulphate; DS, dermatan sulphate; HS, heparan sulphate; TGF, transforming growth factor.

proteoglycan has a single-chain core protein of 310–350 kDa and showed no affinity for transferrin. Two of the proteoglycans have not previously been detected in skin fibroblasts, i.e. one HS-proteoglycan from the culture medium with a 250 kDa core protein and one cell-associated CS/DS proteoglycan with a 90 kDa core protein.

EXPERIMENTAL

Materials

[³H]Leucine (50 Ci/mmol) and ³⁵SO₄²⁻ (1310 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Heparan-sulphate lyase and chondroitin ABC lyase were products of Seikagaku Kogyo Co., Tokyo, Japan. DE-53 DEAE-cellulose was from Whatman, and Sephacryl S500 HR, Sepharose CL-2B and Mono Q HR were from Pharmacia. Mulgophene [tridecyloxypoly(ethyleneoxy)ethanol] was purchased from GFA Corp., Johanneshov, Sweden. Heparan sulphate (HS 3-5) was prepared from the heparin by-products of bovine lung (Fransson *et al.*, 1981). Other chemicals were obtained from sources listed previously (Carlstedt *et al.*, 1983).

Solvent compositions

(a) Phosphate-buffered saline contained 0.137 M-NaCl, 3 mM-KCl, 8 mM-Na₂HPO₄ and 2 mM-KH₂PO₄, pH 7.4; (b) Triton X-100 extractant was composed of 2% (v/v) Triton X-100, 0.15 M-NaCl, 10 mM-EDTA and 10 mM-Na₂HPO₄, pH 7.4; (c) urea solutions, pH 5.8, consisted of 6 M-urea, 0.1% Triton X-100, 5 µg of ovalbumin/ml and 0.2 M- or 0.5 M-sodium acetate, pH 5.8; (d) urea solution, pH 8.0, contained 7 M-urea, 0.1% Mulgophene and 10 mM-Tris/HCl, pH 8.0; (e) 6 M-guanidinium chloride (Gdn) extraction was performed with 6 M-Gdn containing 1% Triton X-100, 10 mM-EDTA, 5 µg of ovalbumin/ml and 50 mM-sodium acetate, pH 5.8; (f) 4 M-Gdn solvent contained 4 M-Gdn, 0.2% Triton X-100, 5 µg of ovalbumin/ml and 50 mM-sodium acetate, pH 5.8; (g) chondroitin ABC lyase buffer was composed of 0.1% Triton X-100, 10 mM-EDTA and 10 mM-Tris/HCl, pH 7.3; (h) heparan-sulphate lyase buffer consisted of 0.1% Triton X-100, 3 mM-calcium acetate and 10 mM-Hepes, pH 7.0. The proteinase inhibitors *N*-ethylmaleimide and di-isopropyl phosphorofluoridate were added from stock solutions to final concentrations of 10 mM and 1 mM respectively.

Cell culture and incorporation of radioactive precursors

Culture of human embryonic skin fibroblasts were established and grown as monolayers on 175 cm² plastic dishes as described by Malmström *et al.* (1975). Cells were grown to confluence in Earle's essential medium supplemented with 10% (v/v) donor calf serum, 2 mM-L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml) and fungizone (2.5 µg/ml). Incorporation of radioactive precursors was performed in low-sulphate and low-leucine medium for 48 h either with L-[³H]leucine (20 µCi/ml) and ³⁵SO₄²⁻ (50 µCi/ml), or with L-[³H]leucine (20 µCi/ml) only (Cöster *et al.*, 1984). This medium does not markedly influence either proteoglycan synthesis or sulphation (A. Malmström, unpublished work).

Extraction and isolation of proteoglycans

After incubation with radioactive precursors, the culture medium was decanted and saved (medium fraction, M). The cell layer was washed with 3 × 20 ml of ice-cold phosphate-buffered saline/dish and then extracted with 25 ml of 2% Triton X-100 (pH 7.5) containing proteinase inhibitors/dish, at 4 °C for approx. 3 h. This step was repeated overnight. The two extracts were collected, centrifuged (1500 g for 30 min) and the supernatants were combined (Triton extract, TX). The cell layers were then rinsed with 50 ml of ice-cold phosphate-buffered saline. The pellet from the Triton extract and the washed and Triton-extracted cell layer were finally solubilized in 15 ml of 6 M-Gdn, pH 5.8, containing proteinase inhibitors/dish, at 4 °C overnight (guanidinium chloride extract, GX).

To recover proteoglycan material (i.e. polyanions) from the culture medium (M) and the Triton extract (TX), the solutions were adjusted to pH 5.8 and applied to columns containing 20 ml of DE-53 DEAE-cellulose equilibrated in 6 M-urea, pH 5.8, containing 0.2 M-sodium acetate/10 mM-*N*-ethylmaleimide. The columns were washed with 5 bed vol. of the equilibrating buffer, followed by the same buffer containing 0.5 M-sodium acetate. Proteoglycan material was finally eluted with 5 × 1 bed vol. of 6 M-Gdn containing 10 mM-*N*-ethylmaleimide.

Density-gradient ultracentrifugation in CsCl

The crude proteoglycan material derived from the extracts M and TX (see above), as well as the entire GX extract, were adjusted to a density of 1.35 g/ml in 4 M-Gdn/1% Triton X-100 by adding solid CsCl, Triton X-100 and water (Carlstedt *et al.*, 1983). Centrifugation was performed at 36000 rev./min for 65 h at 15 °C, in a Beckman model L8 60 M ultracentrifuge with a Ti 50.2 rotor. Tubes were emptied from the bottom and collected in 2 ml fractions. The density was determined by weighing 100 µl portions of the fractions. The material obtained from GX was dialysed against 4 M-Gdn buffer before determination of radioactivity.

Gel-permeation chromatography

Proteoglycan fractions from the CsCl density gradient were subjected to preparative gel-permeation chromatography at room temperature on a column (5 cm × 100 cm) of Sepharose CL-2B, or on columns (1 cm × 100 cm) of Sephacryl S-500 HR. Both columns were eluted with the 4 M-Gdn buffer, at flow rates of 40 ml/h and 5 ml/h respectively. Recovery was 85% or better in all cases. Before chromatography, samples were concentrated by ultrafiltration in a Novacell 10 K (Filtron) or by binding to DE-53 DEAE-cellulose columns (0.5 ml) equilibrated with 6 M-urea as described above. In the latter case, samples were first dialysed against the equilibrating buffer and then applied to the columns, which were subsequently washed with the same buffer. Bound material was eluted in a small volume (< 2 ml) of the 4 M-Gdn buffer.

Ion-exchange chromatography on Mono Q HR

Ion-exchange chromatography was performed on a Mono Q HR 5/5 column connected to an f.p.l.c. system (LKB) as described by Lindblom *et al.* (1989). Samples (containing 20 µg of heparan sulphate carrier) were

dialysed against the equilibrating buffer, containing 0.5 M-NaCl, and centrifuged at 3000 *g* for 30 min before application. Recovery from Mono Q was 70 % or better. Pooled material was diluted 4-fold with 6 M-urea containing 5 μ g of ovalbumin/ml, and recovered by passage through DEAE-cellulose as described above. Recovery after this step was 90 % or better. Purified proteoglycans were stored in the 4 M-Gdn solvent at 4 °C.

Degradative methods

Digestions with heparan-sulphate lyase or chondroitin ABC lyase were performed in the presence of proteinase inhibitors as described by Lindblom *et al.* (1989). Purified proteoglycans (10–100 μ l) were precipitated with at least a 9-fold excess of 95 % ethanol at 4 °C overnight, and recovered by centrifugation at 4000 *g* for 60 min. The pellets were air-dried for 30 min and then solubilized in 300 μ l of the appropriate digestion buffer. Digestions were performed at 37 °C for 2–3 h. The glycan side chains were released from the proteoglycans by digestion with papain (Carlstedt *et al.*, 1983).

SDS/polyacrylamide-gel electrophoresis

This was performed on 3–12 % polyacrylamide gradient gels (T/C = 30/0.8), with a 3 % stacking gel, in the buffer system of Laemmli (1970) as described by Lindblom *et al.* (1989). Digested samples were precipitated with a 9-fold excess of 95 % ethanol, centrifuged (4000 *g* for 60 min) and dried. Recovery was 90 % or better. Samples were dissolved in 60 μ l of 5 % (w/v) SDS/20 % (v/v) glycerol/4 mM-EDTA/0.04 % Bromophenol Blue/125 mM-Tris/HCl, pH 6.8. To reduce disulphide bonds, β -mercaptoethanol was added to a final

concentration of 10 % (v/v). Samples were boiled for 3 min and electrophoresed for approx. 30 h at a constant current of 10 mA. Approx. 30 000–40 000 d.p.m. of [³H]leucine-labelled material was applied in each lane. After electrophoresis, gels were stained with 0.25 % Coomassie Brilliant Blue R-250, destained and soaked in Amplify NAMP-100 (Amersham). Fluorography was performed at –60 °C for 3–7 days, with Kodak XAR-5 film.

Assay for transferrin binding

Binding to transferrin was tested on transferrin coupled to anti-transferrin and Protein A-agarose as described elsewhere (Cöster *et al.*, 1986).

Radioactivity determination

This was done by liquid scintillation in an LKB–Wallach RackBeta counter with automatic quench correction, with a 10-fold excess of Ready-Safe (Beckman) as scintillator. All ³⁵S radioactivity data were corrected for decay.

RESULTS

Purification of proteoglycans

Human embryonic skin fibroblasts in confluent cultures were metabolically labelled for 48 h with ³⁵SO₄²⁻ and [³H]leucine or with [³H]leucine alone. ³⁵S- and ³H-proteoglycans as well as ³H-proteoglycans were isolated in parallel from the spent culture medium (M), from a Triton X-100 extract of the cell layer (TX) and from a guanidinium chloride extract (GX) of the detergent-insoluble residue and purified in accordance with Scheme

Scheme 1. Procedure for the isolation of proteoglycans from human skin fibroblasts

Proteoglycans were purified from the culture medium (M), a detergent extract of the cells (TX) and a Gdn extract of the matrix (GX). The first purification step was isopycnic density-gradient centrifugation, which yielded proteoglycan populations of high (HD) and medium density (MD). In the second step, proteoglycans were separated into large (I) and small forms (II) by gel-permeation chromatography. Finally, separate HS- and CS/DS-proteoglycans were obtained by ion-exchange h.p.l.c. Proteoglycan core proteins were identified by SDS/polyacrylamide-gel electrophoresis (PAGE) after enzymic removal of the glycosaminoglycan side chains.

Fig. 1. Isopycnic density-gradient centrifugation in CsCl/4 M-Gdn/1% Triton X-100 of proteoglycans from (a) the culture medium, (b) the detergent extract of the cells, and (c) the Gdn extract of the cell residue

The culture medium and the detergent extract were passed over DE-53 DEAE-cellulose columns to isolate polyanionic material, whereas the Gdn extract was centrifuged directly as described in the Experimental section. After centrifugation (34000 rev./min; 65 h) 3 ml fractions were collected and pooled as indicated above the panels (HD, high density; MD, medium density; LD, low density). Samples (20 μ l) were analysed for radioactivity ($\blacksquare$, ^{35}S ; $\square$, ^3H leucine); $\circ$, density.

1. The first purification step was isopycnic density-gradient centrifugation in CsCl/4 M-Gdn/1% Triton X-100 (Fig. 1). Material from each extract (M, TX and GX) was pooled as a 'high-density' fraction (HD; density > 1.40 g/ml), a 'medium-density' fraction (MD; 1.40 g/ml > density > 1.30 g/ml) and a 'low-density' fraction (LD; density < 1.30 g/ml). In M (Fig. 1a) and TX (Fig. 1b) most of the ^{35}S -labelled material was found in the HD fraction (83% and 71% respectively), and no distinct peak was seen in the MD region, although 11% and 17% of the ^{35}S radioactivity respectively was present in this fraction. In contrast, GX (Fig. 1c) contained two distinct populations of ^{35}S -proteoglycans: 32% in the HD fraction and 43% in the MD one, the latter appearing at a density of approx. 1.35 g/ml. The LD fractions (Figs. 1a-1c) were dominated by ^3H -labelled material.

After density-gradient centrifugation of GX, a sharp band at density of approx. 1.25 g/ml accounted for approx. 25% of the total ^{35}S -labelled material (Fig. 1c). When examined by gel chromatography on Sephacryl S-500 and by ion-exchange chromatography on Mono Q (before and after digestion with papain), the material was shown to contain proteoglycans of different sizes and charges (results not shown). Hence some of the ^{35}S -proteoglycans from GX (Fig. 1c) were probably adsorbed to or entrapped by a proteinaceous precipitate.

The second purification/separation step was gel-permeation chromatography. The results of analytical runs of the HD and MD fractions on Sephacryl S-500 HR are shown in Fig. 2. Two different size classes of proteoglycans were generally observed, a large one (I; K_{av} 0.2-0.3) and a small one (II; K_{av} 0.6-0.7). In the two

Fig. 2. Gel-permeation chromatography on Sephacryl S-500 HR of fractions obtained after CsCl-density-gradient centrifugation

Proteoglycans from the culture medium (M), the detergent extract (TX) and the Gdn extract (GX) were fractionated by isopycnic density-gradient centrifugation into populations of high density (HD) and medium density (MD) as described in Fig. 1. Samples of fractions (a) M.HD, (b) M.MD, (c) TX.HD, (d) TX.MD, (e) GX.HD and (f) GX.MD were chromatographed on a column of Sephacryl S-500 HR as described in the Experimental section. Fractions (1 ml) were collected and assayed for radioactivity. The elution positions of (I) large and (II) small proteoglycans are indicated in the Figure. Fraction III in (c) consists of HS-oligosaccharides (Cöster *et al.*, 1986).

fractions from TX (Figs. 2c and 2d) the material eluted at K_{av} 0.7 or higher (pool III) consisted of oligosaccharides and contaminating proteins (see Cöster *et al.*, 1986). Fractions M.HD, M.MD, TX.HD and GX.HD, which contained both proteoglycan populations I and II (Fig. 2), were subjected to preparative gel chromatography. The first three were chromatographed on Sephacryl S-500, whereas fraction GX.HD was chromatographed on Sepharose CL-2B in order to separate proteoglycans from nucleic acids, which are eluted in the void volume of this gel (results not shown). Fractions GX.MD and TX.MD were not subjected to preparative gel chromatography, as they contained only one size class of proteoglycans (type I in GX.MD and type II in TX.MD).

The third and final purification/separation step was ion-exchange chromatography on Mono Q (Fig. 3). Besides further removal of contaminating proteins, the proteoglycans separated into two populations (denoted A and B) on the basis of charge density. The proteoglycans in peak A were sensitive to heparan sulphate lyase, whereas those in peak B were sensitive to chondroitin ABC lyase (results not shown).

The yields of the various proteoglycan fractions isolated after ion-exchange chromatography on Mono Q are summarized in Table 1. The data represent a typical experiment. Results obtained with the same purification procedure or variations thereof (six in all) did not differ significantly from the given data. The results are presented as the percentage both of total ^{35}S and of total ^3H incorporated into proteoglycan. The latter value is proportional to the amount of proteoglycan core protein, whereas ^{35}S is a measure of the glycosaminoglycan content. In any case, the two principal proteoglycan fractions from the medium were M.HD.I.B and M.HD.II.B, both of which contained CS/DS-proteoglycans. The major HS-containing proteoglycan fraction in the medium was M.HD.II.A. Fraction M.MD.II.A contained mostly non-proteoglycan proteins. From the detergent extract (TX), five proteoglycan fractions of similar yield were obtained, two HS-containing ones (TX.HD.II.A and TX.MD.II.A) and three CS/DS-containing ones (TX.HD.I.B, TX.HD.II.B and TX.MD.II.B). The matrix extract (GX) afforded large amounts of HS-proteoglycan (GX.MD.I.A) and minor amounts of CS/DS-

Fig. 3. Ion-exchange h.p.l.c. on Mono Q of proteoglycan populations isolated after density-gradient centrifugation followed by gel chromatography

Proteoglycans were isolated from the culture medium (M), a detergent extract of the cells (TX) and a Gdn extract of the cell residue (GX), separated into high-density (HD) and medium-density (MD) populations (Fig. 1) and then, when required, into large (I) and small (II) proteoglycans by gel chromatography. The various proteoglycan preparations are indicated in the panels. Fractions (A and B) were pooled as indicated by the bars. For further details, see the Experimental section.

proteoglycans, one larger (GX.HD.I.B) and one smaller (GX.HD.II.B).

Identification of core proteins

Samples of the purified [^3H]leucine-labelled proteoglycans were treated with heparan-sulphate lyase (A peaks) or chondroitin ABC lyase (B peaks) in the presence of proteinase inhibitors and subjected to SDS/polyacrylamide-gel electrophoresis on 3–12% gels in the absence or presence of mercaptoethanol. Results obtained with CS/DS-proteoglycans from the culture medium are shown in Fig. 4. Fraction M.HD.I.B (Fig. 4, lanes a–d) contained a large proteoglycan (probably exceeding 700–800 kDa) which yielded two core protein bands with apparent molecular masses 530 and 400 kDa respectively (Fig. 4, lane b). The proteoglycan, which was not sensitive to reduction of disulphide bonds (Fig. 4, lanes c and d), corresponds to the previously described large fibroblast CS/DS-proteoglycan (Cöster *et al.*, 1984), which could have a heterogeneous core protein (Krusius *et al.*, 1987). A small amount (Table 1) of this proteoglycan appeared in fraction M.MD.I.B (Fig. 4, lanes i–l). Small CS/DS-proteoglycans from the culture medium appeared in the fractions of both high and

medium density (see M.HD.II.B and M.MD.II.B in Table 1 and in Fig. 4, lanes e–h and m–p respectively). Both migrated as broad bands on electrophoresis (Fig. 4, lanes e and g, and m and o, respectively). The 'heavier' proteoglycan appeared to be of 200 kDa or higher, whereas the 'lighter' one also contained smaller species. Both proteoglycan preparations afforded core proteins of apparent molecular mass 40–50 kDa. The 'heavier' one had an additional faint band at 35–40 kDa (Fig. 4, lanes f and h), whereas the 'lighter' one had a minor band at 50 kDa which was best resolved in the reduced lane (Fig. 4, lane p). These small medium proteoglycans with core proteins of 45–50 kDa correspond to the iduronate-rich CS/DS-proteoglycans described previously (Cöster *et al.*, 1984). The 'lighter' of the two proteoglycan preparations also contained significant protein contaminants of apparent molecular mass 90–200 kDa, which were detected after digestion with chondroitin ABC lyase (Fig. 4, lanes n and p). Fraction M.MD.II.A (Fig. 4, lane r) contained most of these proteins which were sensitive neither to reduction nor to digestion with chondroitin ABC lyase (Fig. 4, lane s). Furthermore, upon rechromatography on Mono Q these proteins emerged in the same position, suggesting that

Table 1. Yields and composition of proteoglycans in the medium and cell layer fractions after a labelling period of 48 h

Radiolabelled proteoglycans were isolated from the culture medium (M), a detergent extract of the cells (TX) and the remaining pericellular matrix (GX). The first purification step (see Scheme 1) afforded proteoglycan populations of high (HD) and medium density (MD). The amounts obtained in these fractions were regarded as the starting material (100%). In the next step, proteoglycans were sorted according to size by gel chromatography; recovery was 85% or better in each case. In the final step, separate HS- and CS/DS-proteoglycans were obtained by ion-exchange h.p.l.c.; recovery was 70% or better. The amounts of radiolabelled proteoglycan in each of the final preparations were calculated by integration of the respective peaks. Yields of different proteoglycan preparations are expressed as the percentage of total proteoglycan in the culture medium (M) or the cell layer (TX and GX) respectively. The size of the core proteins was determined by SDS/polyacrylamide-gel electrophoresis after enzymic degradation of the glycosaminoglycan side-chains.

Fractions	Yield		HS-core proteins (kDa)	CS/DS-core proteins (kDa)
	as ³⁵ S (%)	as ³ H (%)		
Medium				
M.HD.I.A	4	5	250	
M.HD.I.B.	52	38		530, 400
M.HD.II.A	12	8	250, 130, 70, 45, 35	
M.HD.II.B	21	25		40–50
M.MD.I.A.	< 1	3	310, 250	
M.MD.I.B	2	3		530, 400
M.MD.II.A	2	5	—*	—*
M.MD.II.B	7	13		40–50
‘Membrane’				
TX.HD.I.A	< 1	< 1	—†	—†
TX.HD.I.B	9	7		530, 400
TX.HD.II.A	8	4	130, 90, 70, 45	
TX.HD.II.B	23	10		90, 45
TX.HD.III	22	0	—†	—†
TX.MD.II.A	6	7	130, 90, 70, 45	
TX.MD.II.B	8	10		90, 45
‘Matrix’				
GX.HD.I.A	< 1	< 1	—†	—†
GX.HD.I.B	4	3		530, 400
GX.HD.II.A	2	2	70, 45	
GX.HD.II.B	3	3		40–50
GX.MD.I.A	12	51	310	
GX.MD.II.B	1	3	—†	—†
* Non-proteoglycan protein fraction. † HS-oligosaccharide fraction. ‡ Not examined.				

* Non-proteoglycan protein fraction.

† HS-oligosaccharide fraction.

‡ Not examined.

they are very acidic (results not shown). No effects were observed after digestion with heparan-sulphate lyase (results not shown).

The detergent extract of the cell layer (TX) also contained CS/DS-proteoglycans corresponding to those in the culture medium (Fig. 5). Fraction TX.HD.I.B contained minor quantities (Table 1) of the large CS/DS-proteoglycan (cf. Fig. 4, lanes a–d, with Fig. 5, lanes a–d), but considerable amounts of small CS/DS-proteoglycans were found in both the 'high-density' (TX.HD.II.B) and the 'medium-density' (TX.MD.II.B) fractions (Table 1). Proteoglycans from the latter fraction appeared to be the smallest ones (Fig. 5, lanes i and k). Digestion of the small CS/DS-proteoglycans with chondroitin ABC lyase yielded two core proteins of apparent molecular mass 45 kDa and 90 kDa (Fig. 5, lanes f, h, j and l). The 90 kDa core protein was most prominent in fraction TX.MD.II.B, and neither proteoglycan was sensitive to reduction of disulphide bonds (Fig. 5, lanes g, h, k and l). A CS/DS-proteoglycan with a 90 kDa core protein has not obviously been detected in skin fibroblasts.

The guanidinium chloride extract of the matrix (GX) contained small amounts of CS/DS-proteoglycans (Table 1). In fraction GX.HD.I.B the large proteoglycan with cores of 530 and 400 kDa, and possibly also smaller ones, was identified (Fig. 5, lanes m–p). Fraction GX.HD.II.B contained the small proteoglycans with a 45 kDa core (results not shown).

HS-proteoglycans (A peaks from the ion-exchange chromatography in Fig. 3) were treated with heparan-sulphate lyase, and the resulting core proteins were analysed by SDS/polyacrylamide-gel electrophoresis under reducing or non-reducing conditions. The results obtained with HS-proteoglycans derived from the culture medium are shown in Fig. 6. There were two major HS-containing fractions in the medium (M.HD.I.A and M.HD.II.A; Table 1). The large HS-proteoglycan of high density (M.HD.I.A) gave one major core protein of apparent molecular mass 250 kDa (Fig. 6, lanes b and d), which was not sensitive to reduction of disulphide bonds (Fig. 6, lanes c and d). The small HS-proteoglycans of high density (M.HD.II.A) gave a broad band with an apparent molecular mass around 300 kDa (Fig. 6, lanes e

Fig. 4. SDS/polyacrylamide-gel electrophoresis of CS/DS-proteoglycans from the medium before and after digestion with chondroitin ABC lyase

The fractions analysed (see Table 1) and the treatments performed (+ / -) are indicated above the lanes. The migration positions of the protein standards are indicated on the left. Abbreviations: ABC, chondroitin ABC lyase; SH, β -mercaptoethanol; O, start; F, front.

Fig. 5. SDS/polyacrylamide-gel electrophoresis of CS/DS-proteoglycans from the cell layer before and after digestion with chondroitin ABC lyase

The fractions analysed and the treatments performed are indicated above the lanes; see Fig. 4 for abbreviations.

Fig. 6. SDS/polyacrylamide-gel electrophoresis of HS-proteoglycans from the medium before and after digestion with heparan-sulphate lyase

The fractions analysed (see Table 1) and the treatments performed are indicated above the lanes. HS'ase, heparan-sulphate lyase; for other abbreviations see Fig. 4. In lanes b, d and j there is some undigested proteoglycan material which had not been completely solubilized in the digestion buffer.

and g). These proteoglycans comprised species with core proteins of apparent molecular mass 250, 130, 70, 45 and 35 kDa (Fig. 6, lanes f and h). A 35 kDa component also appeared after reduction of the intact proteoglycans (Fig. 6, lane g). Upon reduction of the core-protein preparation, the 70 kDa component was slightly diminished and the 35 kDa component became more prominent (Fig. 6, lane h). The culture medium contained small quantities of medium-density HS-proteoglycan (M.MD.I.A; Table 1). This proteoglycan was large (Fig. 6, lanes i and k) and yielded a core protein of apparent molecular mass 310 kDa before and 330 kDa after reduction (Fig. 6, lanes j and l). There was also a minor 250 kDa core, possibly derived from contaminating high-density HS-proteoglycans.

The analyses of core proteins from HS-proteoglycans derived from the cell layer are shown in Fig. 7. The detergent extract (TX) contained only small HS-proteoglycans, which were found both in the HD and MD density fractions. The 'heavier' proteoglycans had apparent molecular masses of 200 kDa or higher (Fig. 7, lanes a and c), whereas the 'lighter' ones were smaller (Fig. 7, lanes e and g). Both preparations afforded core proteins of apparent molecular masses 130, 90, 70 and 45 kDa (Fig. 7, lanes b and f), the 70 kDa band being the most prominent one. A 35 kDa component was released upon reduction of disulphide bonds in the intact

proteoglycans (Fig. 7, lanes c and g). Reduction of disulphide bonds in the core-protein preparations also resulted in the appearance of the 35 kDa component (Fig. 7, lanes d and h). This was accompanied by a comparative decrease in the intensity of the 70 kDa band.

The guanidinium chloride extract of the cell layer (GX) contained a major HS-proteoglycan of medium buoyant density (see fraction GX.MD.I.A in Table 1). As shown in Fig. 7 (lanes i-l), this proteoglycan was large (apparent molecular mass above 600 kDa) and contained a single core protein of apparent molecular mass 310 kDa. After reduction of disulphide bonds, the core-protein band corresponded to an apparent molecular mass of 350 kDa (Fig. 7, lane l). This highly purified major cell-associated HS-proteoglycan did not bind to transferrin (results not shown).

Minor quantities of HS-proteoglycans were also present in the HD fraction from GX (GX.HD.I.A and GX.HD.II.A; Table 1). The proteoglycans contained the 250, 70 and 45 kDa core proteins, but not the 90 kDa component (results not shown). The detergent-released proteoglycans with small core proteins could be degradation products. However, the various core proteins described above were also identified in proteoglycans obtained from a direct Gdn/detergent extract of the cell layer (results not shown).

Fig. 7. SDS/polyacrylamide-gel electrophoresis of HS-proteoglycans from the cell layer before and after digestion with heparan-sulphate lyase

The fractions analysed (see Fig. 3) and the treatments performed are indicated above the lanes; see also Figs. 4 and 6.

DISCUSSION

We have made an inventory of proteoglycans synthesized by cultured human skin fibroblasts during a steady-state condition. The proteoglycans have been purified by using a three-step procedure consisting of density-gradient centrifugation, gel chromatography and ion-exchange h.p.l.c. The results show that all three steps are necessary in order to obtain sufficiently pure proteoglycans. This procedure should be particularly useful for the isolation of proteoglycans after metabolic labelling with precursors not unique to proteoglycans.

Fibroblasts secrete or shed some of the proteoglycans into the culture medium, whereas others are retained in the cell layer as either membrane- or matrix-bound species. The proteoglycans vary considerably in size and, in general, smaller proteoglycans could be degradation products of larger forms. Extraction of the cell layer with detergent releases proteoglycans that are considered to be bound to the plasma membrane, as either integral or peripheral components. Considerable amounts of proteoglycans are also present in the remaining 'matrix' which is solubilized by Gdn. The major HS-proteoglycan of the cell layer was present in the Gdn extract of that matrix and accounted for 80% of all [3 H]leucine incorporated into cell-associated HS-proteoglycans. Its overall size was approx. 600 kDa, with a core protein of 310–350 kDa and a buoyant density of 1.35 g/ml, suggesting that the HS side chains amount to about the same total molecular mass as the core protein. This proteoglycan was not found in the detergent extract of

the cells, and only minor quantities were detected in the culture medium. Furthermore, this proteoglycan must correspond to the major HS-proteoglycan preparation studied previously (Carlstedt *et al.*, 1983). That proteoglycan, however, was sensitive to reduction of disulphide bonds and yielded core polypeptides similar in size to those of the transferrin receptor (Cöster *et al.*, 1983). Binding of transferrin to this preparation made us postulate a proteoglycan form of the transferrin receptor (Fransson *et al.*, 1984; Fransson, 1987). In contrast, the HS-proteoglycan preparation described here has a single-chain core protein (310–350 kDa) and no affinity for transferrin. After reduction of disulphide bonds, the apparent molecular mass of the core protein increased (see Fig. 7, lanes i–l), possibly owing to expansion of the molecule when intra-chain disulphide bonds are broken. Large 'low-density' HS-proteoglycans have also been isolated from colon carcinoma cells (Iozzo, 1984), human lung fibroblasts (Heremans *et al.*, 1988), mouse mammary epithelial cells (Jalkanen *et al.*, 1988), human vascular endothelial cells (Lindblom *et al.*, 1989) and basement membranes (Paulsson *et al.*, 1987; Noonan *et al.*, 1988). The latter proteoglycan consists of a large core protein (400 kDa) with several intra-chain disulphide bonds. It seems likely that the matrix-associated HS-proteoglycan from skin fibroblasts is related to the basement-membrane-derived forms.

Previous studies have shown that skin fibroblasts also produce small cell-surface-associated HS-proteoglycans (Cöster *et al.*, 1983, 1986). In the present study these were purified from the detergent extract of the cells and

comprised several different species, with core proteins of 130, 90, 70 and 45 kDa. Some of the 70 kDa core proteins gave a 35 kDa component upon reduction, suggesting that they consisted of two 35 kDa regions also connected by interchain disulphide bonds. The HS chains are likely to be located to one of these 35 kDa regions, as reduction alone was sufficient to release a 35 kDa component from this proteoglycan. Reduction did not completely convert the 70 kDa core protein into 35 kDa subunits, and it is therefore possible that there are two separate core proteins with apparent molecular mass of 70 kDa. The small detergent-released HS-proteoglycans appeared both in the high-density and the medium-density fractions, and gave broad bands upon electrophoresis, suggesting that the number of HS side chains and/or their chain length may vary considerably. Structural heterogeneity among hydrophobic HS-proteoglycans from human lung fibroblasts has been observed by Lories *et al.* (1987), who detected core proteins of apparent molecular mass 125, 90, 64, 48 and 35 kDa. The 35 kDa component appeared to be a subunit of the 64 kDa core protein. The 125, 64 and 48 kDa core proteins are probably derived from distinct proteoglycans, whereas the 90 and 48 kDa species seem to be structurally related (Lories *et al.*, 1989). A partial cDNA sequence has recently been obtained for the 48/90 kDa proteoglycans (Marynen *et al.*, 1989). Hence, skin and lung fibroblasts produce very similar patterns of HS-proteoglycan. In contrast, confluent cultures of vascular endothelial cells synthesize very little of these small HS-proteoglycans (Lindblom *et al.*, 1989).

In the present study a CS/DS-proteoglycan with a 90 kDa core protein was detected in the detergent extract of the cells. This component is very similar to the membrane-associated CS/DS-proteoglycan with a 90 kDa core recently identified by David *et al.* (1989). Thus skin and lung fibroblasts contain two proteoglycans with 90 kDa cores, one with HS and another with CS/DS, and it is possible that the two cores are derived from the same gene. As mentioned above, the type III receptor for transforming growth factor β (TGF- β) is a composite HS/CS-proteoglycan, and affinity-labelling of this receptor with ^{125}I -TGF- β results in the formation of a complex with apparent molecular mass 250–350 kDa before and 110–130 kDa after deglycosylation (Cheifetz *et al.*, 1988). As the molecular mass for TGF- β is 25 kDa, the core protein of the type III receptor should be 85–105 kDa. Although skin fibroblasts do not seem to produce a composite HS/CS-proteoglycan with a 90 kDa core, the two separate fibroblast proteoglycans with 90 kDa core proteins could be related to the type III receptors.

The culture medium contained both large and small HS-proteoglycans. The former had a single-chain core protein of apparent molecular mass 250 kDa, and it has not previously been detected in fibroblast cultures. As it was virtually absent from the cell layer, it may be a degradation product of the matrix-associated HS-proteoglycan with a 350 kDa core. Alternatively, it may be a separate gene product, and further studies are required to distinguish between these possibilities. In the spent medium of vascular endothelial cells, HS-proteoglycans with core proteins of molecular mass 300–350 kDa as well as 210 kDa have been found (Lindblom *et al.*, 1989). The small HS-proteoglycans in the culture medium of skin fibroblasts consisted of several

species with core proteins of apparent molecular mass 130, 70, 45 and 35 kDa, i.e. similar to the proteoglycans in the detergent extract. However, the HS-proteoglycan with a 90 kDa core could not be detected. Also, the CS/DS-proteoglycan with a 90 kDa core protein was absent from the medium. Thus both proteoglycans with a 90 kDa core seem to be exclusively cell-associated. An HS-proteoglycan with a 90 kDa core protein has been obtained from smooth-muscle cells (Schmidt & Buddecke, 1988). This proteoglycan appears to be partly shed into the medium. Other HS-proteoglycans in the culture medium of skin fibroblasts could be degradation products of larger cell-associated forms. However, the proteoglycan with a 70 kDa core may correspond to the lung fibroblast HS-proteoglycan that has an immunologically distinct 64 kDa protein (De Boeck *et al.*, 1987; Lories *et al.*, 1989) and thus be a genuine gene product. The skin fibroblast HS-proteoglycan with a 70 kDa core was detected both in the culture medium and in the cell layer. The medium form was larger, had a higher buoyant density and was less affected by reduction of disulphide bonds.

The culture medium also contained the previously described (Cöster *et al.*, 1984) large (PG-L) and small (PG-S) CS/DS-proteoglycans. The former proteoglycan was mainly secreted into the culture medium, whereas the latter was also present in the detergent extract of the cells. The small proteoglycans had core proteins with apparent molecular mass in the range 40–50 kDa. Different forms of these core proteins have been observed in other cells and tissues, and there appear to be two distinct but similar proteoglycans, PG-S1 and PG-S2, also denoted biglycan and decorin respectively (Krusius & Ruoslahti, 1986; Heinegård & Sommarin, 1987; Cöster *et al.*, 1987; Fisher *et al.*, 1987, 1989; Ruoslahti, 1988). The core proteins may contain variable amounts of *N*-glycosidically linked oligosaccharides (Glössl *et al.*, 1984; Poole, 1986; Larjava *et al.*, 1988), and there may be one or two DS side chains; e.g. PG-S1 (biglycan) is supposed to carry two side chains. We obtained both large (molecular mass above 200 kDa) and small forms of these proteoglycans, and both gave core proteins of about 40–50 kDa, suggesting the presence of proteoglycan species with either one or two glycosaminoglycan chains. The amino acid sequence for PG-S2 (Krusius & Ruoslahti, 1986) does not contain a putative membrane-spanning segment. Therefore the presence of small CS/DS-proteoglycans in the detergent extract of skin fibroblasts is probably due to a peripheral association.

In summary, we have isolated and identified HS-proteoglycans with core proteins of apparent molecular mass 350, 250, 130, 90, 70, 45 and possibly 35 kDa from human skin fibroblasts. The proteoglycan with the largest core protein is also the major one, and it is principally located to the pericellular matrix, whereas the proteoglycan with a 250 kDa core is mainly secreted or shed into the culture medium. The smallest proteoglycans are found in the detergent extract of the cells and, partly, in the medium. A CS/DS-substituted 90 kDa core was also detected. Both the HS- and the CS/DS-carrying 90 kDa cores are exclusively cell-associated.

This work was supported by grants from the Swedish Medical Research Council, National Board for Technical Development, and Cancer Trust, the J. A. Persson and

Österlund Foundations, Gustaf V:s 80-års fond, and the Medical Faculty, University of Lund. We are grateful to Dr. Anders Lindblom for valuable advice and discussion, to Lena Åberg and Birgitta Havsmark for technical assistance, to Birgitta Jönsson for art-work and to Inger Carlsson for secretarial aid.

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Received 22 May 1989/14 August 1989; accepted 18 August 1989