Purification and partial characterization of the major cell-associated heparan sulphate proteoglycan of rat liver

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Heparan sulphate proteoglycans were solubilized from whole rat livers by homogenization and dissociative extraction with 4 M-guanidinium chloride containing Triton X-100 and proteinase inhibitors. The extract was subjected to trichloroacetic acid precipitation and the proteoglycan remained soluble. This was then purified to apparent homogeneity by a combination of (a) DEAE-Sephacel chromatography, (b) digestion with chondroitinase ABC followed by f.p.l.c. Mono Q ion-exchange chromatography, and (c) density-gradient centrifugation in CsCl and 4 M-guanidinium chloride. Approx. 1.5 mg of proteoglycan was obtained from 30 livers with an estimated recovery of 25%. The purified proteoglycan was eluted from Sepharose CL6B as an apparently single polydisperse population with a K_{av} of 0.19 and displayed a molecular mass of ≥ 200 kDa (relative to protein standards) by SDS/PAGE. Its heparan sulphate chains were eluted with a K_{av} of 0.44 and have an estimated molecular mass of 25 kDa. Digestion of the proteoglycan with a combination of heparinases yielded core proteins of 77, 49 and 44 kDa. Deglycosylation using trifluoromethanesulphonic acid, though slightly decreasing the sizes, gave an identical pattern of core proteins. Electrophoretic detergent blotting demonstrated that all of the core proteins were hydrophobic and are probably integral plasma membrane molecules. The peptide maps generated by V_8 proteinase digestion of the two major core proteins (77 and 49 kDa) were very similar, suggesting that these two core proteins are structurally related.

INTRODUCTION

Heparan sulphate proteoglycans (HSPGs) comprise a complex family of diverse structures which can differ in the core protein as well as in the number, size and fine structure of the heparan sulphate (HS) chains [1–3]. Since the isolation and description of syndecan, the mouse mammary epithelial cell HSPG, we now have to consider the added complexity of other glycosaminoglycan chains co-existing with HS chains, thereby forming a composite proteoglycan [4,5].

HSPGs are typically found at the cell surface, or extracellularly in basement membranes and in the pericellular matrix where they are usually in close apposition to cells. Association with the surfaces of cells has been shown to occur by three distinct mechanisms, more than one of which may operate in any particular cell system. HSPGs can be intercalated into the plasma membrane either directly, by virtue of an appropriate hydrophobic protein sequence, e.g. syndecan [6] and fibroglycan [7], or alternatively by possession of a phosphatidylinositol moiety added post-translationally to the C-terminus of a non-hydrophobic protein [8–11]. In addition, proteoglycan may be retained at the cell surface by binding to membrane proteins that interact with HS [12] or with the terminal inositol phosphate remaining after scission of a phosphatidylinositol linkage by a phospholipase C [8].

HSPGs have been implicated in a number of fundamental cellular processes such as control of cell growth and cell–cell matrix adhesion. These are likely to be mediated by a wide range of interactions with matrix and cell surface macromolecules, growth factors, etc. [3,13]. Elucidation of the molecular nature and specificities of these interactions requires the availability of pure HSPG species in quantity, and from normal tissues, with which to test them in the appropriate biological contexts.

Although HSPGs have been purified in relatively large amounts from basement membranes, such as the Engelbreth–Holm–Swarm sarcoma [14,15] or human glomeruli [16], there has been no corresponding purification in quantity of a cell-associated HSPG. This lack has led to the widespread use of commercial heparins as analogues of HS, although there are distinct differences between these molecules [17], with resulting uncertainties of molecular specificity and even biological relevance. Such studies also, by definition, overlook the possible modulation of activity, or even additional specific activities, attributable to the protein core of HSPGs.

We have purified the cell-associated HSPG from rat liver, a relatively rich source [18,19]. Although liver comprises a number of cell types, the most abundant hepatocytes synthesize predominantly HS in culture [18,20–22]. The less abundant non-parenchymal cells also appear to synthesize some HSPG in addition to producing the majority of the hepatic chondroitin/dermatan sulphate proteoglycans (CS/DSPGs) [21–23].

Previous studies of the HSPG extracted from rat liver plasma membranes revealed a population of small proteoglycans (estimated molecular mass of 73 kDa) comprising protein cores of 27–35 kDa [24] and three or four HS chains of 14 kDa [19]. Interestingly, this population can be fractionated into two populations displaying different modes of membrane association. One exhibits the properties expected of an integral molecule, whereas the other is a heparin- or salt-displaceable peripheral molecule, presumably held at the cell surface by a HS-binding protein [12,25–27]. Ishihara *et al.* [8] have demonstrated that, at least in an established hepatocyte cell line, these proteoglycan–membrane interactions may be mediated by inositol phospholipids or inositol phosphate respectively.

In a previous study we demonstrated the presence of a latent endoglycosidase activity intimately associated with isolated rat

Abbreviations used: HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; CS/DSPG, chondroitin sulphate/dermatan sulphate proteoglycan; TFMS, trifluoromethanesulphonic acid; PPO, diphenyloxazole.

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liver plasma membranes which, if activated, can very rapidly degrade HS under physiological conditions [26]. This suggested that an extraction of whole liver under highly dissociative and denaturing conditions may be more preferable for the maintenance of HSPG integrity than a prior subcellular fractionation under relatively mild conditions.

MATERIALS AND METHODS

Materials

Livers were obtained from adult Wistar rats of either sex. Guanidinium chloride from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) was further purified by filtration through activated charcoal. Urea from BDH (Poole, Dorset, U.K.) was de-ionized by treatment with Duolite MB 6113 mixed-bed resin (BDH) before use. DEAE-Sephacel, Sepharose CL6B and Mono-Q were from Pharmacia (Uppsala, Sweden). Trifluoromethanesulphonic acid (TFMS), bovine kidney HS, ovomucoid (chicken egg white trypsin inhibitor) and *Staphylococcus aureus* V₈ proteinase (EC 3.4.21.19) were from Sigma Chemical Co. (Poole, Dorset, U.K.). Acrylamide and *NN'*-methylenebisacrylamide (both Electron Grade) were obtained from BDH. CsCl (molecular biology grade) was from Boehringer (Mannheim, Germany). Heparinase I (from *Flavobacterium heparinum*; EC 4.2.2.7) and chondroitinase ABC (from *Proteus vulgaris*; EC 4.2.2.4) were from Seikagaku Kogyo Co. (Tokyo, Japan), supplied by ICN Biomedicals (High Wycombe, Bucks., U.K.). Heparinases II (*F. heparinum*) and III (*F. heparinum*; specificity apparently identical to that of heparitinase; EC 4.2.2.8 [28,29]) were purchased from Grampian Enzymes (Aberdeen, Scotland, U.K.). ¹⁴C-methylated proteins and [³⁵S]Sulphur Labelling Reagent (specific radioactivity 800 Ci/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). Carrier-free Na₂³⁵SO₄ and ¹²⁵I-labelled Bolton-Hunter reagent (monoiodinated; specific radioactivity 2200 Ci/mmol) were from Du Pont-NEN Research Products (Stevenage, Herts., U.K.). Acrodisc filters (0.22 µm) and Biotrace RP membranes (0.45 µm pore size) were supplied by Gelman Sciences (Northampton, U.K.).

Extraction and purification

Extraction. A total of 30 rat livers were used for each preparation. Three rats were each given an intraperitoneal injection of 0.5 ml of phosphate-buffered saline (0.15 M-NaCl/20 mM-sodium phosphate, pH 7.4) containing 1 mCi of Na₂³⁵SO₄. After 2 h the rats were killed and the livers were quickly excised, together with unlabelled livers from 27 rats (total liver wet wt. approx. 525 g). The livers were rinsed free of extraneous blood with ice-cold phosphate-buffered saline, pH 5.0, cut into small pieces and then homogenized by about 12 strokes of a loose-fitting Dounce homogenizer in an extraction solution containing 4 M-guanidinium chloride, 2% (v/v) Triton X-100, 50 mM-sodium acetate, 0.1 M-6-aminohexanoic acid, 20 mM-benzamidine hydrochloride, 10 mM-EDTA, 5 mM-*N*-ethylmaleimide and 0.5 mM-phenylmethanesulphonyl fluoride, pH 5.0. The homogenate (approx. 80 ml per liver) was stirred at 4 °C for 24 h. Insoluble residues were removed by filtration through a double layer of nylon mesh or by centrifugation at 2000 g. In order to monitor the degree of extraction, the insoluble material was exhaustively digested with papain and chromatographed on DEAE-Sephacel to recover any ³⁵S-labelled glycosaminoglycans.

Precipitation by trichloroacetic acid. Ice-cold 100% (w/v) trichloroacetic acid was added to the extract at 0 °C to give a final concentration of 10% (v/v). After 30 min at 0 °C, the heavy precipitate was centrifuged down at 2000 g for 30 min in a MSE

Mistral 6000 centrifuge at 4 °C. The supernatant was recovered and neutralized to pH 7 by the careful addition of conc. NaOH solution. The sediment was resuspended and washed with a small volume of 4 M-guanidinium chloride/10% (w/v) trichloroacetic acid/2% (v/v) Triton X-100, and then re-centrifuged at 2000 g. The wash supernatant was neutralized, as above, and then pooled with the original supernatant. The recovery of ³⁵S-labelled proteoglycan/glycosaminoglycan was monitored by chromatography of a small portion of extract on DEAE-Sephacel before and after trichloroacetic acid precipitation. The samples (0.5 ml) were diluted with an excess of 8 M-urea/20 mM-Tris/HCl/0.5% (v/v) Triton X-100, pH 8.0, to bring the salt concentration down to below 0.3 M, and then loaded on to a 1 ml column volume of DEAE-Sephacel. After extensive washing with 8 M-urea/0.3 M-NaCl/20 mM-Tris/HCl/0.5% (v/v) Triton X-100, pH 8.0, the bound ³⁵S-labelled macromolecules were step-eluted with 4 M-guanidinium chloride/20 mM-sodium phosphate/0.5% (v/v) Triton X-100, pH 7.0, and quantified. Analyses were performed in duplicate.

DEAE-Sephacel ion-exchange chromatography. The neutralized trichloroacetic acid-soluble extract (approx. 2.5–3 litres) was concentrated to less than one-third of its volume by reverse osmosis against solid carboxymethylcellulose or poly(ethylene glycol). This was then extensively dialysed against 8 M-urea/0.15 M-NaCl/0.5% (v/v) Triton X-100/20 mM-Tris/HCl, pH 8.0, before being batch-adsorbed on to approx. 200 ml of washed and equilibrated DEAE-Sephacel by gentle mixing overnight. The gel was recovered by sedimentation at 300 g for 10 min and the supernatant was removed. The gel was washed with an equal volume of 8 M-urea/0.15 M-NaCl/0.5% (v/v) Triton X-100/20 mM-Tris/HCl, pH 8.0, and re-centrifuged. The washed gel was then resuspended in the 8 M-urea solution and poured into a 2.5 cm × 38 cm column at room temperature. After washing with 2 column vol. of the 8 M-urea solution at pH 8.0, the washing was continued at a lower pH with 6 M-urea/0.15 M-NaCl/0.5% (v/v) Triton X-100/20 mM-piperazine/HCl, pH 5.0, until the dark-coloured front had been eluted from the column. Proteoglycans were then eluted using a gradient of 0.15–1.0 M-NaCl (500 ml total volume) in the 6 M-urea solution (pH 5). Fractions of 5 ml were collected at a flow rate of 30 ml/h and monitored for absorbance at 280 nm. Samples were also counted for their ³⁵S content.

Digestion with chondroitinase ABC. Pooled fractions from the DEAE-Sephacel column were concentrated to approx. 10 ml by reverse osmosis against solid poly(ethylene glycol) or carboxymethylcellulose and then dialysed against 50 mM-NaCl/50 mM-Tris/HCl/0.1% (v/v) Triton X-100/10 mM-6-aminohexanoic acid/10 mM-benzamidine/HCl/10 mM-EDTA/5 mM-*N*-ethylmaleimide, pH 8.0. To this was added 0.1 unit of chondroitinase ABC and sufficient phenylmethanesulphonyl fluoride to give a final concentration of 0.25 mM. After an overnight incubation at 37 °C, a further addition of phenylmethanesulphonyl fluoride and 0.05 unit of enzyme was made, followed by a further 5 h incubation.

F.p.l.c. Mono-Q ion-exchange chromatography. The chondroitinase ABC-treated sample was centrifuged at 3000 g on a bench centrifuge for 10 min followed by filtration through a 0.22 µm pore size Acrodisc filter before being injected on to a Mono Q HR5/5 (1 ml) column linked to a Pharmacia f.p.l.c. system. The column, which had been equilibrated with 6 M-urea/0.15 M-NaCl/0.1% (v/v) Triton X-100/20 mM-Tris/HCl, pH 8, was washed with 1 column vol. of the same solution followed by several volumes of a similar solution containing

0.5% (w/v) CHAPS instead of the Triton X-100. Bound material was then eluted with a 30 ml gradient of 0.15–1.5 M-NaCl in 6 M-urea/0.5% CHAPS/20 mM-Tris/HCl, pH 8.0. Fractions of 0.5 ml were collected at a flow rate of 1.0 ml/min. The absorbance at 280 nm was measured and samples were also analysed for ^{35}S content.

Density-gradient centrifugation. The pooled fractions from Mono Q chromatography were dialysed against 4 M-guanidinium chloride/0.2% (w/v) CHAPS/20 mM-sodium phosphate, pH 7.3, and then adjusted to a final volume of 12 ml and a density of 1.5 g/ml by the addition of 4 M-guanidinium chloride solution and solid CsCl respectively. The sample was then centrifuged at 130000 g_{av} for 66 h at 15 °C on a Beckman L5 ultracentrifuge. The tubes were emptied from the bottom by piercing with a needle, and 1 ml fractions were collected. Densities were determined from the weights of 100 μl samples. Absorbances were measured at 280 nm and samples were counted for ^{35}S content. The appropriate fractions were pooled and proteoglycan was recovered by precipitation with 4 vol. of 95% (v/v) ethanol at –20 °C overnight. The substantial precipitate (mostly CsCl) was recovered by centrifugation at 3000 g for 10 min and then resuspended in 10 ml of fresh 75% (v/v) ethanol at –20 °C to selectively resolubilize the CsCl before re-centrifugation. This procedure was repeated until no further decrease in pellet size was obtained. All ethanol supernatants were monitored for the presence of ^{35}S and no apparent solubilization of proteoglycan occurred. The final pellet was briefly air-dried and then redissolved in 0.15 M-NaCl/20 mM-sodium phosphate, pH 7.2, containing either 0.5% (w/v) CHAPS or 25 mM-octyl glucoside.

Proteoglycan characterization

Radiolabelling of proteoglycan in the core protein. Purified proteoglycan in 0.1 M-sodium phosphate/0.5% (w/v) CHAPS, pH 8.0, was radiolabelled with ^{125}I or ^{35}S by reaction for 30 min at 0 °C with 25 μCi of either ^{125}I -Bolton–Hunter reagent or ^{35}S -Sulphur Labelling Reagent respectively [30].

Gel chromatography. Samples were chromatographed on a Sepharose CL6B column (1.6 cm $\times$ 86 cm) eluted with 4 M-guanidinium chloride/1% (v/v) Triton X-100/20 mM-sodium phosphate/1 mM- Na_2SO_4 , pH 7.0, at a flow rate of 5.4 ml/h. Fractions of 1.08 ml were collected and monitored for ^{35}S content. V_0 and V_T were measured using Dextran Blue and sodium dichromate respectively.

Preparation of core proteins. Proteoglycan radiolabelled in the core protein with ^{125}I or ^{35}S was precipitated from 4 M-guanidinium chloride with ethanol in the presence of unlabelled carrier HS as recommended by Heinegård & Sommarin [31]. This procedure increased the reliability of subsequent polysaccharide lyase digestions proceeding to completion by preventing collapse of the HS chains on to the core protein. Briefly, a portion of radiolabelled HSPG was diluted with an equal volume of 8 M-guanidinium chloride, and 10–20 μg of bovine kidney HS was added. The HSPG was then precipitated by the addition of 9 vol. of 95% (v/v) ethanol at –20 °C. After 2 h the precipitate was recovered by centrifugation at 16000 g for 10 min. The pellet was washed with a small volume of 75% (v/v) ethanol, air-dried and then redissolved in 0.1 M-sodium acetate/0.1 mM-calcium acetate/10 mM-octyl glucoside, pH 7.0, containing proteinase inhibitors [either (a) a mixture of 50 mM-6-aminohexanoic acid, 5 mM-benzamidine HCl and 0.25 mM-phenylmethanesulphonyl fluoride, or (b) 10 μg of ovomucoid/ml [31]]. The radiolabelled HSPG, substantially free of unincor-

porated radiolabel, was then digested with 40 munits of heparinase III/ml (often in combination with 20 munits of heparinase II/ml) at 43 °C for 16 h.

Core proteins were also prepared by chemical deglycosylation with TFMS. ^{35}S -HSPG (note that ^{125}I introduced via the Bolton–Hunter reagent is labile) was dialysed against 1% SDS, lyophilized in a glass hydrolysis tube and treated with TFMS by the method of Herzberg *et al.* [32]. The deglycosylated sample was then extensively dialysed against electrophoresis sample buffer.

SDS/polyacrylamide-gel electrophoresis. Radiolabelled HSPG or core proteins were reduced with β -mercaptoethanol and electrophoresed on 7.5% T/2.67% C isocratic or 4–12% T/2.67% C gradient gels with a 3% T/2.67% C stacking gel using the discontinuous SDS buffer system of Laemmli [33] (where T = total acrylamide and C = cross-linker). [^{14}C]Methylated proteins were run as molecular mass markers and gels were electrophoresed overnight. Gels were fixed in glacial acetic acid, impregnated with 20% (w/v) diphenyloxazole (PPO) in glacial acetic acid and then washed with water to precipitate PPO. The gels were then dried under vacuum and exposed to pre-flashed Kodak X-Omat AR film at –70 °C.

HSPG core proteins were assayed for hydrophobicity by the electrophoretic detergent blotting method of Ito & Akiyama [34]. Heparinase III-generated ^{125}I -core proteins were resolved on a 7.5% T/2.67% C SDS/polyacrylamide gel. The core proteins were then electrotransferred on to a Biotrace RP positively charged nylon membrane via an intervening 10% T/2.67% C polyacrylamide gel containing 2% (v/v) Nonidet P40 in 2.5 mM-Tris/HCl/19.2 mM-glycine, pH 8.3. Electrotransfer was performed at 20 V/cm for 18 h in 2.5 mM-Tris/HCl/19.2 mM-glycine, pH 8.3. After electrotransfer, the Nonidet P40-containing gel was processed for PPO fluorography, and the nylon membrane was sprayed with En 3 Hance (DuPont–NEN Research Products) and then exposed to pre-flashed film.

Peptide mapping. Heparinase III-generated ^{125}I -labelled core proteins were resolved on a 7.5% T/2.67% C SDS/polyacrylamide gel. The core proteins were detected by autoradiography of the frozen gel and then excised. Proteins were eluted from the gel slices by overnight shaking with 0.5 ml of 50 mM- NH_4HCO_3 /0.01% (w/v) SDS/20 μg of BSA/ml. Appropriate quantities of the extracts were lyophilized on a Univap centrifugal evaporator (Uniscience Ltd., London, U.K.) and then redissolved in 25 μl of 0.125 M-Tris/HCl/10% (v/v) glycerol, pH 6.8. V_8 proteinase (1 μg) was added and the samples were incubated at 37 °C for 30 min. The resulting peptide digests were electrophoresed on a 7.5–20% T/2.67% C gradient SDS/polyacrylamide gel, which was then processed for PPO fluorography.

Other analytical procedures

Protein assays, based on the method of Bradford [35], were performed using a Coomassie Blue G-250 kit (Pierce Chemical Co., Rockford, IL, U.S.A.), with BSA as standard.

HS was assayed after exhaustive digestion of unknown samples to their constituent disaccharides using a combination of heparinases I (25 munits/ml), II (50 munits/ml) and III (50 munits/ml) in 0.1 M-sodium acetate/0.1 mM-calcium acetate, pH 7.0, for 16 h at 37 °C. To 10 μl of samples taken both before and after digestion was added 0.49 ml of 30 mM-HCl. The disaccharide content was then quantified from the change in absorbance at 232 nm using a value for the molar absorption coefficient ($\epsilon_{232}^{1\text{ cm}}$) of 5200 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [36]. An average disaccharide molecular mass of 480 Da was then used to calculate the weight of HS present.

Fig. 1. Schematic summary of HSPG purification

RESULTS

Purification

Homogenization and extraction of whole rat liver in 4 M-guanidinium chloride/2% (v/v) Triton X-100/50 mM-sodium acetate, pH 5.0, containing proteinase inhibitors, resulted in > 99% extraction of incorporated ^{35}S radioactivity that was able to bind to DEAE-Sephacel in the presence of 0.3 M-NaCl. The extract was heavily contaminated with nucleic acid and very viscous. However, upon addition to the extract of 10% (w/v) trichloroacetic acid, a bulky precipitate was formed containing a substantial proportion of the protein and nucleic acid content. The majority (75%) of the anionic ^{35}S label remained soluble, and from this the major HSPG component could be purified by the three-stage procedure outlined in Fig. 1.

The supernatant from the trichloroacetic acid precipitation was neutralized and dialysed against 8 M-urea/0.5% (v/v) Triton X-100/0.15 M-NaCl/20 mM-Tris/HCl, pH 8.0. The material was then batch-adsorbed on to DEAE-Sephacel and extensively washed with urea solutions at both pH 8 and pH 5. Upon elution with a NaCl gradient, the bound ^{35}S was recovered as a single peak (at approx. 0.5 M-NaCl) which was partially resolved from the earlier-eluting protein peak (Fig. 2). In order

Fig. 2. DEAE-Sephacel ion-exchange chromatography of trichloroacetic acid supernatant

See the Materials and methods section for experimental details. —, ^{35}S ; ---, A_{280} ; ···, NaCl gradient. The horizontal bar represents pooled fractions.

Fig. 3. Mono Q ion-exchange chromatography of the proteoglycan pool from DEAE-Sephacel after digestion with chondroitinase ABC

See the Materials and methods section for experimental details. —, ^{35}S ; ---, A_{280} ; ···, NaCl gradient. The horizontal bar represents pooled fractions.

to facilitate separation of HSPG from contaminating CS/DSPGs the latter were degraded by exhaustive digestion with chondroitinase ABC in the presence of proteinase inhibitors. The digest was then applied to an f.p.l.c. Mono Q column in 6 M-urea/0.1% (v/v) Triton X-100/0.15 M-NaCl/20 mM-Tris/HCl, pH 8, and extensively washed. Elution with a NaCl gradient gave an asymmetric peak of ^{35}S with the peak maximum being attained at 1.0 M-NaCl (Fig. 3). When the total bound pool of ^{35}S was analysed by chromatography on Sepharose CL6B in 4 M-guanidinium chloride, it was resolved into two distinct components (Fig. 4), a major peak (80%) eluting with a K_{av} of 0.19, which is known from previous work [26] to correspond to HSPG, and a minor peak (20%) with a K_{av} of 0.72, corresponding to a population of large HS fragments. Analysis of specific fractions from the Mono Q profile by chromatography on Sepharose CL6B (results not shown) revealed that the earlier-eluting material (i.e. fractions 26–36 inclusive) contained predominantly the large oligosaccharides, whereas the major peak (i.e. fractions 37–47 inclusive), corresponding to the peak of A_{280} , contained almost solely the high-molecular-mass HSPG. The latter pool was finally purified by density-gradient centrifugation in CsCl/4 M-guanidinium chloride containing 0.2% (w/v) CHAPS at a starting density of 1.5 g/ml. Three peaks of A_{280} were recovered at densities of ≤ 1.33 , 1.42 and 1.60 g/ml (Fig. 5). The first two peaks, which did not contain any appreciable ^{35}S label, probably represented contaminating protein and nucleic acid respectively. The high-density peak was associated with the major peak of ^{35}S label and corresponded to the HSPG. A small

Fig. 4. Sepharose CL6B gel-filtration analysis of the total bound ^{35}S pool from Mono Q

See the Materials and methods section for experimental details. V_0 and V_t are indicated.

Fig. 5. Density-gradient centrifugation in $\text{CsCl}/4\text{ M-guanidinium chloride}$ of the HSPG pool from Mono-Q

See the Materials and methods section for experimental details. —, ^{35}S ; ---, A_{280} ; ····, density. The horizontal bar represents the pooled fractions.

amount of ^{35}S , with no associated absorbance at 280 nm, was recovered at the bottom of the gradient ($\geq 1.71\text{ g/ml}$) and contained a small amount of HS oligosaccharides not completely removed by the prior fractionation on Mono Q. The major ^{35}S peak was pooled and the HSPG was recovered by ethanol

precipitation. Quantification of both protein (0.43 mg) and HS (1.15 mg) gave a yield of 1.58 mg of HSPG (HS/protein ratio of 2.7). The complete purification is summarized in Table 1 and shows a recovery of ^{35}S -HSPG equivalent to 15 % of the initial macromolecular ^{35}S label, with an overall purification of approx. 11000-fold. However, this is clearly an underestimate for the recovery of the HSPG, as not all of the initial ^{35}S label was associated with this molecule. Assuming, for example, estimates of 23 % for the CS/DS content (from the wash-through on Mono Q) and 20 % for the HS oligosaccharide content (from the Sepharose CL6B profile), then the recovery of HSPG would be nearer to approx. 26 %.

Characterization

As mentioned earlier, the HSPG eluted as a single apparently homogeneous peak on Sepharose CL6B, with a K_{av} of 0.19 (see major peak in Fig. 4). The HS chains derived by Pronase digestion or alkaline elimination were previously shown to elute on Sepharose CL6B with a K_{av} of 0.44 [26] corresponding to an approx. molecular mass of 25 kDa by comparison with the published calibration of Wasteson [37]. This finding was confirmed in the present study (results not shown).

The HSPG was labelled in the protein core with either ^{125}I or ^{35}S and analysed by SDS/PAGE. The intact proteoglycan migrated as a smear extending only a short distance into a 7.5 % or a 4–12 % gel with an apparent molecular size exceeding that of myosin (200 kDa), and no significant protein contamination was revealed (Fig. 6, track A). Digestion with a combination of heparinases II and III (or the latter alone) in the presence of proteinase inhibitors yielded a large core protein of 77 kDa and a doublet of 49 kDa (major component) and 44 kDa (Fig. 6, track B). The presence of the latter core protein was found to be somewhat variable between HSPG preparations, and occasionally other smaller minor species were also observed. The material remaining at the top of the gel presumably represents a small proportion of undergraded HSPG, as this was not present in all enzyme digests (or after chemical deglycosylation). The same pattern of core proteins was obtained in the presence or absence of reduction, indicating the lack of inter-chain disulphide bonds. Total chemical deglycosylation with TFMS also generated an identical pattern of core proteins (Fig. 6, track C), though with slightly lower molecular masses of 72, 46 and 40 kDa, demonstrating that, although some *N*- or *O*-linked oligosaccharides may be present, the heterogeneity in core protein sizes is due to actual protein differences rather than to variable levels of glycosylation.

Interestingly, all three core proteins displayed hydrophobic

Table 1. Summary of purification

The final recovery of HSPG (14.7 %) is an underestimate, as the initial ^{35}S pool also contains CS/DS/PG as well as glycosaminoglycan oligosaccharides (see the Results section). These components are mostly removed at the Mono Q step (step 4).

Step	$10^{-6} \times ^{35}\text{S}$ (c.p.m.)	Protein (mg)	$10^{-3} \times ^{35}\text{S}/\text{protein}$ (c.p.m./mg)	^{35}S recovery (%)	Purification (fold)
1. Extract	22.9	32 520	0.704	100.0	1
2. Trichloroacetic acid supernatant	17.2	19 740	1.148	75.1	1.63
3. DEAE-Sephacel	15.4	58	265.5	67.2	377
4. Mono Q	5.6	1.35	4148	24.5	5892
5. Density-gradient centrifugation	3.36	0.43*	7814	14.7	11 099

* Equivalent to 1.58 mg of HSPG.

Fig. 6. SDS/PAGE analysis of radiolabelled HSPG before and after deglycosylation

Samples were electrophoresed on a 7.5% polyacrylamide gel in the presence of SDS after reduction with β -mercaptoethanol. The samples are: track A, intact ^{125}I -HSPG; track B, core proteins after digestion of ^{125}I -HSPG with heparinases II and III; track C, core proteins after deglycosylation of ^{35}S -HSPG with TFMS; track D, ^{14}C -protein markers, with molecular masses indicated by arrowheads.

Fig. 7. Electrophoretic detergent blotting of radiolabelled HSPG core proteins as a test of hydrophobicity

^{125}I -HSPG was digested with heparinases II and III before electrophoresis on an SDS/7.5%-polyacrylamide gel (tracks A). The core proteins were subsequently electrotransferred on to a cationic nylon membrane (tracks B and D) either directly (tracks B), or indirectly (tracks D) via a 10% polyacrylamide gel containing Nonidet P-40 (tracks C). In each pair of tracks, track 1 contains ^{14}C -protein markers and track 2 contains the ^{125}I -HSPG core proteins.

properties consistent with them being integral plasma membrane components (Fig. 7), as demonstrated by the electrophoretic detergent blotting method [34]. The core proteins, separated by SDS/PAGE (Fig. 7, track A2), would electrotransfer to a positively charged nylon membrane (Fig. 7, track B2), but transfer was prevented in the presence of an intervening layer of Nonidet P40 (Fig. 7, compare tracks C2 and D2). In contrast, the hydrophilic molecular mass marker proteins (Fig. 7, track A1) were transferred to the membrane even in the presence of the non-ionic detergent (Fig. 7, track D1), except for myosin, which transfers poorly even under control conditions (Fig. 7, track B1). Preliminary data indicate no observable change in the hydrophobic properties of any of the core proteins after treatment with a phosphatidylinositol-specific phospholipase C. An absence of phosphatidylinositol membrane anchors would confirm the findings of Brandan & Hirschberg [27], who analysed membranes derived from primary hepatocyte cultures.

Fig. 8. Comparative SDS/PAGE peptide mapping of V_8 -proteinase-digested ^{125}I -HSPG core proteins

^{125}I -HSPG was digested with heparinases II and III and the core proteins were resolved on an SDS/7.5%-polyacrylamide gel. The 77 and 49 kDa core proteins were excised, extracted from the gel and digested with 1 μg of V_8 proteinase. The resulting peptides were electrophoresed on a 7.5–20% gradient polyacrylamide gel in the presence of SDS. Track A, 49 kDa core protein; track B, 77 kDa core protein. The arrows indicate the migration positions of the intact core proteins. The arrowheads indicate the migration positions and molecular masses of ^{14}C -protein markers.

In order to investigate any structural similarity between core proteins, the two major components (i.e. 77 kDa and 49 kDa core proteins) were generated by heparinase III digestion and resolved on an SDS/PAGE gel, and their positions were located by autoradiography. The proteins were extracted from the gel and digested with V_8 proteinase. Comparative peptide mapping by SDS/PAGE (Fig. 8) revealed considerable similarity of the larger ^{125}I -peptides, suggesting that these two core proteins are structurally related.

DISCUSSION

We have described a procedure for the extraction and purification of the major cell-associated HSPG from rat liver. This consistently yields approx. 1.5–2 mg of pure HSPG from 30 livers, with a recovery of close to 25%. It can be estimated that the initial tissue content of this molecule would be equivalent to approx. 200 μg of HS per liver. This can be compared with the value for the total HS content of liver of 336 μg determined by Horner [38], who extracted the polysaccharide from a proteinase digest of rat liver and quantified the product by the meta-chromatic binding of 1,9-Dimethylmethylene Blue. This comparison suggests that the cell-associated HSPG purified by the present method is a major HSPG species of rat liver.

The crucial step for the success of the purification is the trichloroacetic acid precipitation of the initial 4 M-guanidinium chloride extract. This extract is highly viscous and totally intractable due primarily to the very high nucleic acid content. The observation that trichloroacetic acid precipitation (in the presence of 4 M-guanidinium chloride) removes a considerable proportion of the nucleic acid and protein content, but leaves the great majority of proteoglycan in solution, meant that the extract could now be dialysed into an 8 M-urea solution and applied, at least in a batch adsorption mode, to DEAE-Sephacel. This had previously been unfeasible and the large volume of the extract had precluded any alternative technique. The non-precipitability of the proteoglycan with trichloroacetic acid presumably reflects the relatively high HS/protein ratio of the proteoglycan and may not be a general characteristic of other proteoglycans. The availability of this procedure allows the extraction of the whole

tissue under highly denaturing and dissociative conditions, thereby obviating the need for either a large-scale and lengthy membrane fractionation (and removal of the nuclei), or the use of milder extraction conditions which, although allowing subsequent use of nucleases, may also allow the activity of endogenous proteinases and endoglycosidases. In a previous study we demonstrated the presence of an endoglycosidase activity associated with isolated rat liver plasma membranes, which under physiological conditions could rapidly and selectively degrade the HS chains of the membrane HSPG [26]. For this reason the homogenization and extraction of the rat liver was also undertaken at pH 5, at which there is no appreciable enzyme activity.

The purified HSPG elutes as a single peak on Sepharose CL6B with a K_{av} of 0.19 (Fig. 4). This compares very well with a K_{av} of 0.21 obtained for the HSPG obtained from isolated rat liver plasma membranes [26] and a K_{av} of 0.22 for the newly synthesized HSPG from rat liver Golgi membranes [39]. This confirms that no aspect of the purification procedure appears to have affected the integrity of the molecule. SDS/PAGE analysis also reveals a single high-molecular-mass smear (apparent molecular mass of ≥ 200 kDa relative to protein standards), with no significant protein contamination. However, both enzymic and chemical deglycosylation gave rise to an identical pattern of multiple core proteins, comprising a 77 kDa protein (72 kDa after TFMS treatment) and a doublet of proteins corresponding to 49 and 44 kDa (46 and 40 kDa after TFMS). Of these, the major component is the 49 kDa protein, whereas the 44 kDa protein is a minor component whose presence varies between preparations.

Interestingly, these core protein sizes are significantly larger than the 35 and 27 kDa proteins previously identified as the integral and peripheral membrane core proteins of the hepatocyte HSPG [24]. From our values for the sizes of the core proteins and the HS chains, together with the measured HS/protein ratio [2,7], it can be estimated that the intact HSPG population has an average molecular mass in the range of 150–200 kDa and is likely to contain an average of four HS chains.

Comparison of the peptide maps generated from the major 77 and 49 kDa core proteins by digestion with V_8 proteinase reveal considerable similarity, suggesting that these two core proteins are structurally related. The minor 44 kDa core protein may result from a further trimming of the 49 kDa protein.

Somewhat surprisingly, all three core proteins behaved as hydrophobic integral membrane proteins. This seemingly conflicts with all previous studies, in which analysis of the HSPG pool associated with either isolated rat liver plasma membranes [25–27] or cultured rat hepatocytes [19,27] revealed the presence of both hydrophobic and non-hydrophobic membrane-associated proteoglycans. It has been suggested from comparison of both Golgi- and plasma-membrane-derived HSPG [27], and from preliminary pulse-chase experiments [24], that the HSPG is transported to the cell surface as a single hydrophobic molecule, which subsequently can lose its hydrophobic anchor, though still being retained at the cell surface. It has also been suggested from incubations of plasma membranes *in vitro* that this conversion is mediated by an endogenous membrane metallo-endopeptidase [24]. It is interesting to speculate that this conversion may be more tightly regulated *in vivo* and may be the prelude to rapid internalization and/or further catabolism, whereas the process of membrane fractionation may activate such a specific proteinase, leading to an uncharacteristic accumulation of the non-hydrophobic HSPG. It may be the case that similar unrestrained proteolytic activity may also account for the significantly lower molecular mass values for the hydrophobic core proteins when plasma membranes are used as a

source for purification. It is clear that much still remains to be done to elucidate the pathways of biosynthesis, processing and degradation of liver HSPG.

Studies on a variety of cell types, such as mammary epithelium [5], fibroblasts [40,41], smooth muscle cells [42], granulosa cells [10] and parathyroid cells [43], have indicated that there exists a number of integral membrane HSPGs (or hybrid HS/CSPG in the case of syndecan) whose relatively small core proteins fall within the molecular mass range 30–90 kDa. It already seems from comparison of the available amino acid sequences of the mammary epithelium HSPG (syndecan) core protein [6,44] and the lung fibroblast HSPG (fibroglycan) 48 kDa core protein [7] that there are noticeable localized sequence similarities. The cytoplasmic and membrane-spanning domains are very similar, though the ectodomains are quite distinct, suggesting the possible existence of a family of related cell-surface HSPGs. Clearly, further characterization and sequence analyses of other such HSPGs will be necessary in order to confirm such proposals.

In conclusion, we have purified to apparent homogeneity and in milligram quantities the major cell-associated HSPG from rat liver. This is a hydrophobic proteoglycan, and upon deglycosylation it reveals up to three core proteins which we believe to be structurally related. Such quantities of a pure cell-surface HSPG have not previously been available from any source, and this will now allow studies to be undertaken on the macromolecular interactions and biological function of the molecules. This will be of particular interest in the case of the liver HSPG because of the number of studies which have implied a role for these proteoglycans in the control of hepatocyte and hepatoma cell growth [8,45–49] as well as its involvement in the maintenance of differentiated functions such as gap junction expression [50].

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