Biochem. J. (1987) 247, 765–771 (Printed in Great Britain)

Identification of a 64 kDa heparan sulphate proteoglycan core protein from human lung fibroblast plasma membranes with a monoclonal antibody

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Human lung fibroblasts produce heparan sulphate proteoglycans (HSPG) that are associated with the plasma membrane. A monoclonal-antibody (Mab)-secreting hybridoma, S1, was produced by fusion of SP 2/0-AG 14 mouse myeloma cells with spleen cells from mice immunized with partially purified cellular HSPG fractions. The HSPG character of the material carrying the epitope recognized by Mab S1 was demonstrated by: (i) the co-purification of the S1 epitope with the membrane HSPG of human lung fibroblasts; (ii) the decrease in size of the material carrying the S1 epitope upon treatment with heparinase or heparitinase, and the resistance of this material to heparinase treatment after *N*-desulphation. The S1 epitope appears to be part of the core protein, since it was destroyed by proteinase treatment and by disulphide-bond reduction, but not by treatments that depolymerize the glycosaminoglycan chains and *N*-linked oligosaccharide chains. Polyacrylamide-gel electrophoresis of non-reduced heparitinase-digested membrane HSPG followed by Western blotting and immunostaining with Mab S1 revealed a single band with apparent molecular mass of 64 kDa. Membrane proteoglycans isolated from detergent extracts or from 4 M-guanidinium chloride extracts of the cells yielded similar results. Additional digestion with *N*-glycanase lowered the apparent molecular mass of the immunoreactive material to 56 kDa, suggesting that the core protein also carries *N*-linked oligosaccharides. Fractionation of ^{125}I-labelled membrane HSPG by immunoaffinity chromatography on immobilized Mab S1, followed by heparitinase digestion and polyacrylamide-gel electrophoresis of the bound material, yielded a single labelled band with apparent molecular mass 64 kDa. Treatment with dithiothreitol caused a slight increase in apparent molecular mass, suggesting that the core protein of this membrane proteoglycan consists of a single subunit containing (an) intrachain disulphide bond(s).

INTRODUCTION

Epithelial and connective-tissue cells contain proteoglycans, predominantly heparan sulphate proteoglycans, that are closely associated with the cell surface (Höök *et al.*, 1984; Gallagher *et al.*, 1986). Some of these HSPG appear to be embedded in the lipid bilayer of the plasma membrane through hydrophobic structures that are part of the non-glycan moiety (Kjellén *et al.*, 1981; Norling *et al.*, 1981; Rapraeger & Bernfield, 1983; Lories *et al.*, 1986). The functions of these integral membrane components are not well established. Largely on the basis of their localization and binding properties, and through interference experiments, a role as transmembrane mediators or modulators of phenomena that involve cell–cell and cell–matrix interactions, such as cell anchorage (Laterra *et al.*, 1983; Koda *et al.*, 1985; Schubert & La Corbière, 1985; Cole *et al.*, 1985; Gill *et al.*, 1986), cell shape (Rapraeger & Bernfield, 1982; Woods *et al.*, 1985, 1986), cell growth (Kawakami & Terayama, 1981; Ratner *et al.*, 1985; Fritze *et al.*, 1985) and matrix assembly (Woods *et al.*, 1984), has been suggested. Other forms, perhaps, may act as receptors for soluble ligands involved in lipoprotein (Cheng *et al.*, 1981; Shimada *et al.*, 1981), iron (Fransson *et al.*, 1984) and proteinase (Marcum & Rosenberg, 1984; Marcum *et al.*, 1986; Farell & Cunningham, 1986) turnover.

We have obtained evidence for the occurrence of multiple forms of hydrophobic HSPG in detergent extracts of human lung fibroblast cultures. After purification and radioiodination, monomeric core proteins of five different sizes (125, 90, 64, 48 and 35 kDa) and a dimeric core protein composed of disulphide-bonded ~ 35 kDa subunits were identified (Lories *et al.*, 1987). Several structurally and functionally distinct core proteins have also been identified in skin fibroblasts (Cöster *et al.*, 1983, 1986). The origin and relevance of this structural heterogeneity are not clear, but the heterogeneity is reminiscent of the multiple processes that are supposed to be modulated by these components.

To obtain tools for the investigation of this heterogeneity of the membrane-associated HSPG of cultured human lung fibroblasts, we have immunized Balb/c mice with liposome-incorporated proteoglycan fractions, and produced a mouse monoclonal antibody that is specific for an epitope carried by a monomeric 64 kDa HSPG core protein. The constancy of the size of the immunoreactive core protein during alternative isolation procedures, whereby care has been taken to minimalize the possibility of proteolytic degradation, implies that the

Abbreviations used: HSPG, heparan sulphate proteoglycan(s); Mab, monoclonal antibody.

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observed heterogeneity of the HSPG core proteins is at least in part genuine.

EXPERIMENTAL

Cell culture and labelling

Normal human embryonic lung fibroblasts were cultured on plastic substrata in Dulbecco's modified Eagle's minimal essential medium (Gibco Europe, Paisley, Scotland, U.K.) supplemented with 10% (v/v) fetal-calf serum. For labelling, confluent monolayers were incubated for 48 h in low-sulphate culture medium (David & Van den Berghe, 1983) containing 100 μCi of carrier-free $H_2{}^{35}SO_4$ (New England Nuclear, Boston, MA, U.S.A.)/ml.

Buffers

Triton X-100 buffer contained 1% (v/v) Triton X-100, 10 mM-Na_2HPO_4 and 2 mM-KH_2PO_4, pH 7.4, and the following proteinase inhibitors: 50 mM-6-aminohexanoic acid, 10 mM-EDTA, 5 mM-*N*-ethylmaleimide, 5 mM-benzamidine hydrochloride, 1 mM-phenylmethanesulphonyl fluoride and 1 μg of pepstatin A/ml.

Urea buffer contained 6 M-urea, 0.5% (v/v) Triton X-100 and 50 mM-Tris/HCl, pH 8.

Guanidinium chloride buffer contained 4 M-guanidinium chloride, 100 mM-6-aminohexanoic acid, 10 mM-EDTA, 10 mM-*N*-ethylmaleimide, 5 mM-benzamidine hydrochloride, 50 μg of bovine serum albumin/ml, 10 μg of heparin/ml, 10 μg of chondroitin sulphate/ml and 50 mM-sodium acetate, pH 5.8.

Extraction and fractionation of human lung fibroblast monolayers

Two methods were used to extract the cells. In a first approach, rinsed human lung fibroblast monolayers were scraped in cold Triton X-100 buffer. The cleared (10000 *g* for 60 min) extract was concentrated by absorption on DEAE-Sepharose Fast Flow and Mono Q (Pharmacia Fine Chemicals, Uppsala, Sweden). Further fractionation of the extract by gel-filtration chromatography over Sepharose CL-4B in 4 M-guanidinium chloride buffer, by ion-exchange chromatography over Mono Q in urea buffer, and by incorporation into liposomes was as reported elsewhere (Lories *et al.*, 1987).

In the alternative approach, rinsed human lung fibroblasts were directly scraped in cold guanidinium chloride buffer supplemented with freshly added phenylmethanesulphonyl fluoride (1 mM), pepstatin A (2.5 μg/ml) and leupeptin (25 μg/ml). After overnight storage at 4 °C, the extract was cleared by centrifugation (30000 *g* for 30 min) and chromatographed in the cold over Sepharose CL-2B in guanidinium chloride buffer. Further fractionation by ion-exchange chromatography on Mono Q in urea buffer was as described above.

Enzymic and chemical treatments

Heparinase and heparitinase digestion. Digestion of the HSPG samples with 0.04 unit of heparinase (heparin lyase, EC 4.2.2.7; Miles Laboratories, Elkhart, IN, U.S.A.)/ml or with 0.04 unit of heparitinase (heparitin sulphate lyase, EC 4.2.2.8; Miles Laboratories) for 3 h at 37 °C were as described elsewhere (Lories *et al.*, 1987).

Proteinase K digestion. Samples were digested with 0.125 mg of proteinase K (EC 3.4.21.14; Merck, Darmstadt, Germany)/ml for 2 h at 5 °C in 50 mM-Tris/HCl buffer, pH 8.0.

Thermolysin digestion. Samples were incubated with 0.125 mg of thermolysin (EC 3.4.24.4; Calbiochem, San Diego, CA, U.S.A.)/ml for 3 h at 54 °C in 50 mM-Tris/HCl buffer, pH 8.0, in the presence of 3.75 mM-$CaCl_2$. Inactivation of the proteinase was achieved by adding EDTA to a final concentration of 10 mM.

***N*-Glycanase treatment.** The HSPG samples were first treated with heparitinase as described above, supplemented with 0.5% SDS, and boiled. Then 0.1 unit of *N*-glycanase (peptide *N*-glycosidase F, EC 3.5.1.52; Genzyme Corp., Boston, MA, U.S.A.) was added (2.5 units/ml) in the presence of 0.5% Triton X-100, and the reaction mixture was incubated overnight at 30 °C.

HNO_2 degradation. HNO_2 degradation at pH 1.5 was performed by the procedure of Shively & Conrad (1976).

***N*-Desulphation with dimethyl sulphoxide.** HSPG samples in buffer containing 1 M-pyridinium chloride, 1 M-guanidinium chloride, 6 M-urea and 10% Triton X-100, pH 5.2, were supplemented with 19 vol. of dimethyl sulphoxide and kept at 37 °C for 4 h (David & Van den Berghe, 1985). Termination of the reaction was achieved by dialysis against urea buffer.

Reduction and alkylation of disulphide bonds. The samples in 4 M-guanidinium chloride (Tris-buffered, pH 8) were made 10 mM with respect to dithiotreitol and kept for 2 h at room temperature in capped and N_2-flushed tubes. Alkylation was performed overnight at room temperature in the presence of 40 mM-iodoacetamide.

Iodination. This was performed by the chloramine-T method with carrier-free $Na^{125}I$ as described elsewhere (Lories *et al.*, 1987).

Preparation of monoclonal antibodies

The material used for immunization was partially purified cellular HSPG, obtained by detergent extraction. A portion of this material (the equivalent of 1.2×10^7 cells) was mixed with Freund's complete adjuvant and injected intraperitoneally into Balb/c mice. The same amount of material, in Freund's incomplete adjuvant, was administered intraperitoneally to the mice three times more, at 10–20-day intervals. The spleens were harvested 4 days after the last injection.

Further procedures and hybridoma selection were as described by Marynen *et al.* (1981). The supernatants of 135 hybridomas were screened by three methods: immunodot assay on Z-probe membrane, reverse immunodot assay on nitrocellulose paper and finally indirect immunoprecipitation (see below).

Immunotechniques

Immunodot assay on Z-probe membrane. Samples to be analysed were spotted on to Zeta-probe membranes with a dot-blot apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Binding sites that had not reacted were blocked by incubation at 37 °C for 1 h with 0.5% (w/v) casein in phosphate-buffered saline (0.15 M-NaCl/0.01 M-

sodium phosphate buffer, pH 7.4) supplemented with NaCl to a final concentration of 0.5 M. The spotted samples were incubated for 1 h at room temperature with hybridoma culture supernatant or with purified Mab S1 diluted (30 μg/ml) in phosphate-buffered saline containing 0.1% casein, washed three times in phosphate-buffered saline containing 0.1% casein, and then incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-(mouse Ig) antibodies (Dakopatts, Glostrup, Denmark) diluted 1:200 in phosphate-buffered saline containing 0.1% casein. The use of (cationized nylon) Z-probe blotting membranes offered distinct advantages. Unlike nitrocellulose paper, it had a high binding capacity for the intact proteoglycan and was able to bind free glycosaminoglycan chains as well as core proteins.

Reverse immunodot assay on nitrocellulose paper. The procedure was as described by Van Leuven *et al.* (1986). The nitrocellulose sheet with the spotted hybridoma supernatants was incubated with [^{35}S]sulphate-labelled HSPG in phosphate-buffered saline containing 0.1% Triton X-100 and 0.1% casein for 1 h at room temperature with shaking. The paper was then washed twice with phosphate-buffered saline containing 0.1% casein and once with phosphate-buffered saline. After drying, the paper was exposed to Kodak XAR-5 film to locate bound proteoglycan.

Indirect immunoprecipitation. [^{35}S]Sulphate-labelled membrane HSPG was mixed with hybridoma culture supernatant and incubated for 4 h at 4 °C. Immune complexes were isolated by adsorption for 4 h at 4 °C on Protein A–Sepharose (Pharmacia) that had been incubated with rabbit anti-(mouse Ig) antibodies (Dakopatts) in 1% Triton X-100/0.15 M-NaCl/0.05 M-Tris/HCl buffer, pH 8. After washing to remove unbound radioactivity, the pellet was suspended in 2% (v/v) SDS and boiled, and the radioactivity was measured by liquid-scintillation counting.

Immunoaffinity chromatography. Mab S1 was coupled to CNBr-activated Sepharose as described elsewhere (Lories *et al.*, 1987). Samples were incubated with a suspension of the gel in 25 mM-octyl glucoside/50 mM-Tris/HCl buffer, pH 8, for several hours at room temperature or overnight at 4 °C. Washing was done with the same buffer containing 0.15 M- or 0.5 M-NaCl. Bound material was eluted in 4 M-guanidinium chloride containing 25 mM-octyl glucoside.

Gel electrophoresis and Western blotting

SDS/agarose-gel electrophoresis was performed in a 4% NuSieve agarose slab gel (FMC Corp., Rockland, ME, U.S.A.) of 3 mm thickness. The tank buffer contained 45 mM-Tris, 45 mM-boric acid, 2 mM-EDTA and 0.1% SDS (pH 8). The buffer in the gel contained 90 mM-Tris, 90 mM-boric acid, 2 mM-EDTA and 0.1% SDS (pH 8). The sample buffer contained 21 mM-Tris/HCl, 1 mM-EDTA, 2% SDS and 10% (v/v) glycerol (pH 6.8) with or without 2% (v/v) 2-mercaptoethanol. The gel was run for 3 h at 100V in a Bio-Rad vertical slab-gel apparatus. Transfer to Z-probe membrane was performed at 80V for 3 h in an LKB Transfor Electroblotting Unit as adapted from Towbin *et al.* (1979).

SDS/polyacrylamide-gel electrophoresis was performed in accordance with Laemmli (1970) on 4–16%-gradient gels; to increase the porosity the acrylamide contained 10% bisacrylamide. The gel was run overnight at 40V in a Bio-Rad vertical slab-gel apparatus. Transfer was as described above, but for 4–5 h.

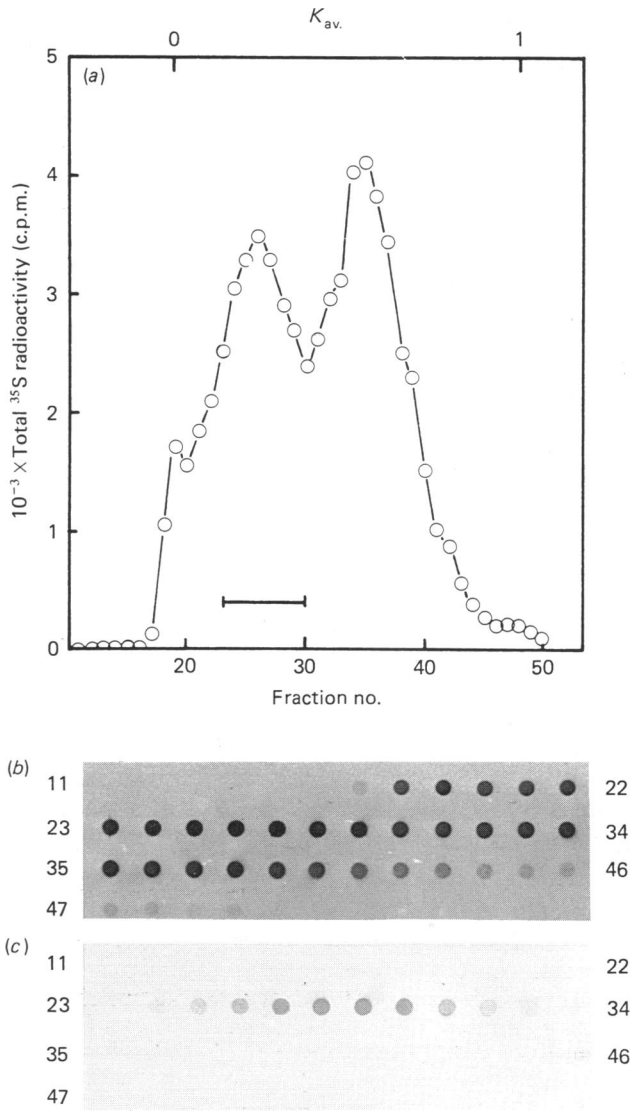

Fig. 1. Co-purification of membrane HSPG and Mab S1-reactive materials during gel filtration of detergent extracts

Metabolically [^{35}S]sulphate-labelled human lung fibroblast monolayers were extracted with Triton X-100 buffer. The proteoglycans in the extract were concentrated in a small volume of guanidinium chloride buffer containing 0.5% Triton X-100 and were applied to Sepharose CL-4B in the same buffer. Under these circumstances membrane HSPG were eluted at $K_{av.} \sim 0.3$. Each eluted fraction was analysed for its total ^{35}S content (*a*). Portions of fractions 11–50 of the gel-filtration profile were dot-blotted on to a Z-probe nylon membrane and binding was confirmed by autoradiography (*b*). Elution of the S1 epitope was detected by immunostaining of the Z-probe sheet with Mab S1 (*c*).

RESULTS

Human lung fibroblasts produce membrane-associated HSPG with core proteins of various sizes (Lories *et al.*,

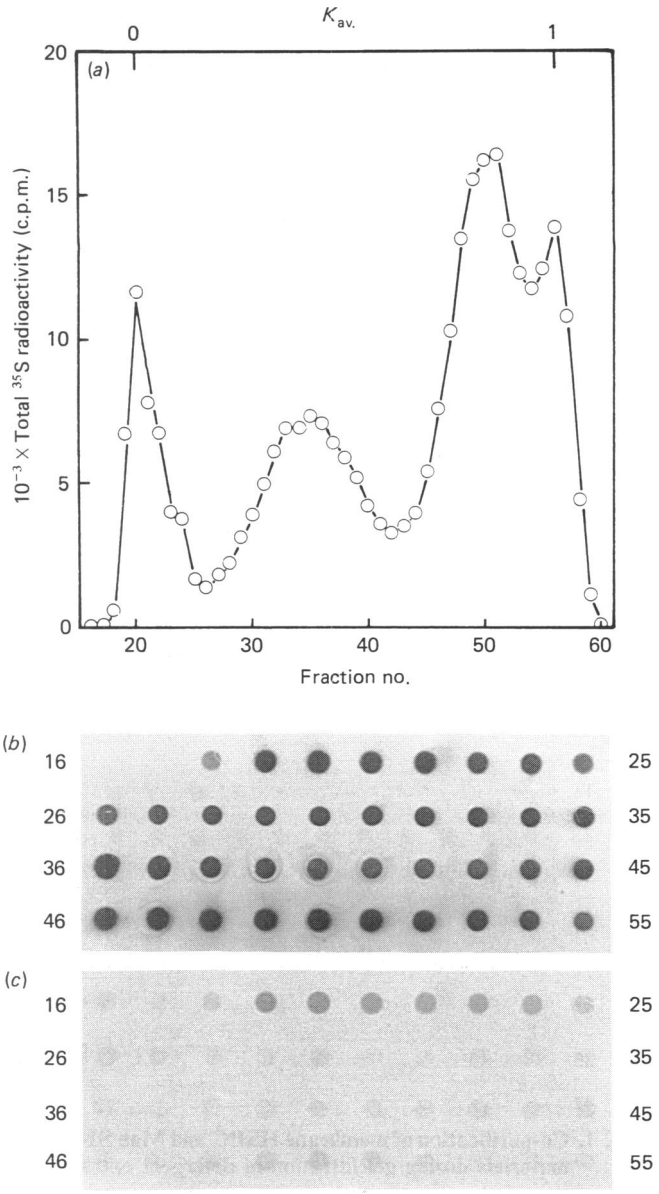

Fig. 2. Co-purification of membrane HSPG and Mab S1-reactive materials during gel filtration of 4 M-guanidinium chloride extracts

Metabolically [^{35}S]sulphate-labelled human lung fibroblast monolayers were extracted in 4 M-guanidinium chloride buffer, without Triton X-100. The extract was chromatographed on Sepharose CL-2B in the same buffer, without Triton X-100. Under these circumstances membrane HSPG were eluted as a micellar aggregate in the void volume of the column. Each fraction was analysed for its total ^{35}S content (*a*). Portions of the eluted fractions were dot-blotted on to a Z-probe membrane; binding was confirmed by autoradiography (*b*), and the elution of the S1 epitope was detected by immunostaining of the Z-probe sheet with Mab S1 (*c*).

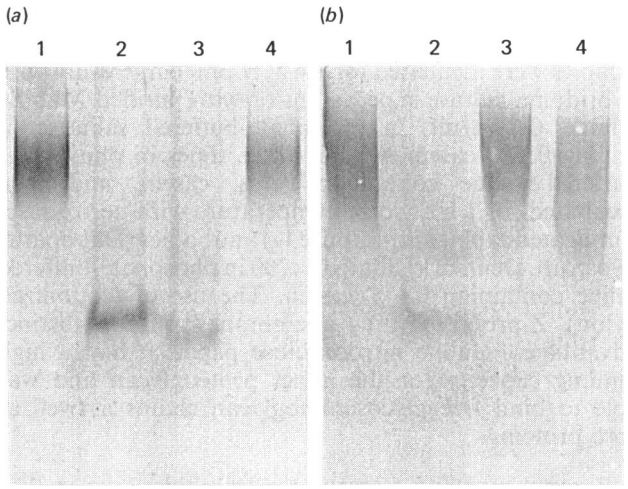

Fig. 3. Identification of the Mab S1-reactive materials as HSPG

(*a*) The material carrying the S1 epitope was decreased in size upon treatment with heparinase or heparitinase: detergent-extracted purified membrane HSPG, mock-treated (lane 1 and 4) or treated with heparinase (lane 2) or with heparitinase (lane 3), were analysed by SDS/agarose-gel electrophoresis, followed by electrotransfer from the slab gels on to Z-probe nylon membrane, and immunostaining with Mab S1. (*b*) Upon *N*-desulphation, the material carrying the S1 epitope became resistant to heparinase: detergent-extracted purified membrane HSPG were either left untreated (lanes 1 and 2) or *N*-desulphated by treatment with dimethyl sulphoxide (lanes 3 and 4), digested with heparinase (lanes 2 and 4) or mock-digested (lanes 1 and 3), and analysed by SDS/agarose-gel electrophoresis, followed by electrotransfer on to Z-probe membrane, and immunostaining with Mab S1.

1987). To explore this structural heterogeneity of the HSPG, hybridomas were raised by immunizing Balb/c mice with the hydrophobic HSPG fraction of cultured human lung fibroblasts. The hybridoma whose conditioned medium was the most effective in immunoprecipitating [^{35}S]sulphate-labelled HSPG was selected for subcloning and ascites production. Its antibody (Mab S1) was used in the characterization of an HSPG that appears to be associated with the membranes of cultured human lung fibroblasts.

S1 epitope is part of a membrane HSPG core protein

To verify the specificity of the monoclonal antibody, metabolically [^{35}S]sulphate-labelled human lung fibroblasts were extracted and fractionated by using two different procedures. The behaviour of Mab S1-reactive material was assessed at each step by the immunodot assay on Z-probe membrane. Like membrane HSPG (Lories *et al.*, 1986), material that carried the S1 epitope was effectively solubilized by extraction with a non-ionic detergent, and bound quantitatively to DEAE-Sepharose or Mono Q during the concentration procedures preceding the gel-filtration step (results not shown). Gel filtration of the concentrated materials on Sepharose CL-4B, in the presence of 4 M-guanidinium chloride and of Triton X-100, yielded the elution profile shown in Fig. 1(*a*). Dot blots of the eluted fractions were subjected to autoradiography, to confirm binding of the proteoglycans (Fig. 1*b*), and immunostained with Mab S1 (Fig. 1*c*). In contrast with the ^{35}S label, the S1-reactive materials were restricted to fractions eluted with $K_{av.} \sim 0.3$, where membrane HSPG are known to be eluted (Lories *et al.*, 1986). These S1-reactive fractions were further analysed by ion-exchange chromatography on

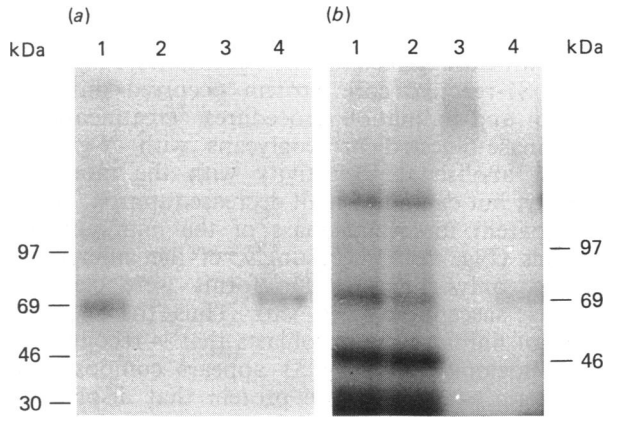

Fig. 4. Immunopurification of a 64 kDa HSPG core protein with Mab S1

(*a*) Detergent-extracted, purified and ^{125}I-labelled membrane HSPG were digested with heparitinase and mixed with Mab S1 coupled to CNBr-activated Sepharose beads. S1-bound material was analysed by SDS/4–16% polyacrylamide-gradient-gel electrophoresis and autoradiography: non-reduced material (lane 1); blank lane (lane 2); molecular-mass markers (lane 3); reduced material (lane 4). (*b*) Alternatively, non-digested ^{125}I-labelled membrane proteoglycans were fractionated by immunoaffinity chromatography and then analysed by electrophoresis under reducing conditions and autoradiography, with or without prior heparitinase digestion: heparitinase digestion of membrane proteoglycans that were not binding to control immunobeads (lane 1) or not binding to Mab S1-substituted beads (lane 2); Mab S1-bound membrane proteoglycans analysed without (lane 3) and with heparitinase (lane 4) digestion.

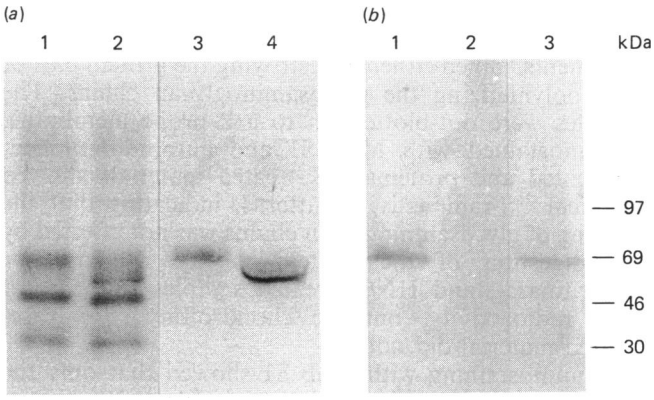

Fig. 5. Characterization of the Mab S1-reactive HSPG core protein by Western blotting

(*a*) Detergent-extracted, purified and ^{125}I-labelled membrane HSPG were digested with heparitinase (lanes 1 and 3) or with heparitinase and *N*-glycanase (lane 2 and 4). After SDS/polyacrylamide-gradient-gel electrophoresis, the core proteins were detected by autoradiography (lanes 1 and 2), or by electrotransfer to a Z-probe membrane and immunostaining with Mab S1 (lanes 3 and 4). (*b*) Cellular [^{35}S]sulphate-labelled materials extracted in 4 M-guanidinium chloride and eluted in the void volume (lane 1) or in the included volume (lane 2) of Sepharose CL-2B columns (see Fig. 4), and HSPG purified from the Sepharose CL-2B-excluded fraction by ion-exchange chromatography on Mono Q (lane 3), were ethanol-precipitated, heparitinase-digested, fractionated by SDS/polyacrylamide-gradient-gel electrophoresis, electroblotted on to Z-probe and immunostained with Mab S1.

Mono Q and by the same combination of autoradiography and immunostaining of dot-blotted column fractions. Label and epitope were nearly co-eluted in the fractions containing approx. 0.8 M-NaCl (results not shown). These fractions were pooled, incorporated into liposomes and resubmitted to gel-filtration chromatography on Sepharose CL-4B in the absence of detergent. Again label and S1-epitope were co-eluted. Part became excluded from the column, and hence appeared to be incorporated into liposomes (results not shown).

Alternatively, the human lung fibroblast monolayers were directly extracted in 4 M-guanidinium chloride in the absence of detergent. This procedure effectively solubilizes matrix-associated and cell-associated HSPG (Lories *et al.*, 1986). Under these circumstances the hydrophobic membrane-associated HSPG are extracted in micellar form, and behave as a detergent-susceptible aggregate that is excluded from Sepharose CL-2B, whereas the matrix-associated HSPG are included (Lories *et al.*, 1986). Under similar extraction and gel-filtration conditions, virtually all the materials that carried the S1 epitope were excluded from Sepharose CL-2B (Fig. 2*c*). Thus the S1 epitope appeared not to be part of the matrix HSPG ($K_{av.} \simeq 0.4$) or any other included materials. Approx. 20% of the total monolayer-associated ^{35}S label was co-eluted with the S1 epitope (Fig. 2*a*) and hydrophobic membrane HSPG represented nearly 80% of this excluded label. When these excluded materials were further fractionated by gel filtration and by ion-exchange chromatography in the presence of detergent, and by liposome incorporation as described above, the S1-reactive material was found to behave like the membrane HSPG present in this fraction (results not shown). This behaviour was similar to that of the S1-reactive material extracted with detergent (see above).

This co-purification suggested that Mab S1 was directed against membrane HSPG. To assess this possibility directly, purified hydrophobic membrane HSPG were treated with heparinase and heparitinase. Untreated and treated samples were analysed by SDS/agarose electrophoresis, followed by electrotransfer on to Z-probe membrane and immunostaining with S1 (Fig. 3*a*). Undigested material in the control lane ran as a smear in the high-molecular-mass region. In the heparinase-treated and the heparitinase-treated samples, however, all the material carrying the S1 epitope was shifted to a lower molecular mass. To verify whether this low-molecular-mass material was generated solely as the result of the action of heparinase, and not by contaminating proteinases, the HSPG were *N*-desulphated, a treatment that renders heparan sulphate resistant to heparinase digestion (Hovingh & Linker, 1970). Analysis by SDS/agarose-gel electrophoresis and immunostaining with Mab S1 (Fig. 3*b*) showed that upon *N*-desulphation the material carrying the S1 epitope indeed became resistant to heparinase treatment, since it remained in the high-molecular-mass region as did undigested control and desulphated samples.

To assess further whether the S1 epitope resides in the proteoglycan core protein moiety or in its glycosamino-

glycan chains, purified [^{35}S]sulphate-labelled hydrophobic membrane HSPG were subjected to different treatments, aimed either at destroying the protein part or at depolymerizing the glycosaminoglycan chains. The samples were dot-blotted on to a Z-probe membrane, immunostained with Mab S1 and autoradiographed. Untreated and proteinase K-treated materials showed identical ^{35}S-radioactivity patterns, indicating that the binding of glycosaminoglycan chains was not affected by the digestion of the protein moiety. Heparinase-, heparitinase-, and HNO_2-treated samples lost part of their radioactivity, but the chondroitin ABC lyase-treated material did not.

Immunostaining with Mab S1 showed that only the proteinase K-digested sample had lost the S1 epitope, suggesting that Mab S1 recognizes a part of the core protein or at least not the heparan sulphate chains. Experiments with thermolysin confirmed this proteinase-sensitivity. Incubation with thermolysin at 54 °C abolished the recognition by S1, whereas incubation at high temperature alone or with inactivated thermolysin had no effect on the recognition by S1. Finally, upon reduction with dithiothreitol and alkylation the material lost its S1 epitope, although binding of ^{125}I-labelled HSPG on to the Z-probe membrane was not affected (results not shown). Thus Mab S1 appeared specific for a determinant on the core protein of a heparan sulphate proteoglycan. This specificity and the effect of reduction were confirmed by immunostaining of Western blots of reduced and non-reduced SDS extracts of whole human lung fibroblast monolayers analyzed by SDS/agarose-gel electrophoresis. S1 only recognized a smear in the high-molecular-mass region of non-reduced material (results not shown).

Characterization of the S1-reactive core protein

When heparitinase-digested ^{125}I-labelled membrane HSPG were incubated with Mab S1 that had been immobilized on CNBr-activated Sepharose approx. 10% of the label was bound to the beads. After elution, SDS/polyacrylamide-gradient-gel electrophoresis and autoradiography, a single labelled 64 kDa band was detected in non-reduced samples (Fig. 4*a*). After reduction with 2-mercaptoethanol, the eluted band was slightly more retarded, suggesting an increase in apparent molecular mass, consequent to the rupture of (an) intrachain disulphide bond(s). Reduced ^{125}I-labelled HSPG, however, did not bind to the immuno-affinity matrix, consistent with the results described above. Analysis of ^{125}I-labelled HSPG incubated with immunobeads before heparitinase digestion and reduction yielded similar results (Fig. 4*b*). Only a single 64 kDa band was detected in the heparitinase digest of Mab S1-bound HSPG. In contrast, several distinct bands were present in the digests of membrane HSPG fractions that did not bind to control or to Mab S1 immunobeads. Thus the S1 epitope appeared to be unique to a 64 kDa HSPG core protein of human lung fibroblasts.

SDS/polyacrylamide-gel electrophoresis of heparitinase-digested membrane HSPG, followed by electrotransfer to Z-probe membranes and immunostaining with Mab S1, also revealed a single immunoreactive 64 kDa band (Fig. 5). Similar results were obtained with iodinated hydrophobic proteoglycans isolated from detergent extracts (Fig. 5*a*), and with metabolically labelled proteoglycans that were directly extracted in 4 M-guanidinium chloride and fractionated over Sepharose CL-2B (Fig. 5*b*, lane 1) and then over Mono Q (Fig. 5*b*), lane 3), which suggested that no significant fragmentation of the S1-reactive core protein occurred during the isolation and iodination procedures. Treatment of the heparitinase-digested proteoglycans with *N*-glycanase did not abolish the reactivity with the monoclonal antibody, but caused a small decrease (approx. 8000) in the apparent molecular mass of the immunoreactive materials (Fig. 5*a*). Interestingly, of the different core proteins, only some 64 kDa forms were clearly *N*-glycanase-susceptible (Fig. 5*a*). Thus the membrane HSPG of human lung fibroblasts that is recognized by the monoclonal antibody S1 appears composed of a monomeric ~ 64 kDa core protein that displays (an) internal sulphide bond(s) and carries one or more heparan sulphate chains and *N*-linked oligosaccharide chains. Probably this is only one of several distinct membrane core protein forms existing within human lung fibroblasts.

DISCUSSION

Immunization of Balb/c mice with the HSPG isolated from detergent extracts of human lung fibroblasts has yielded monoclonal antibody S1, which recognizes an epitope that appears to be specific for one of these membrane proteoglycans. The epitope appears to be part of a peptide, since its reactivity with the antibody is destroyed by proteinase treatment and by the reduction of disulphide bonds. It is unlikely that the association of the epitope with heparan sulphate is of non-covalent nature or is mediated through disulphide bonds. The former is suggested by the observation that the ability to detect the epitope as a sharp band after electrophoresis in the presence of SDS and immunoblotting is absolutely dependent upon prior digestion of the samples with heparitinase. The second is suggested by the observation that the radiolabelled core protein that can be purified by immuno-affinity chromatography yields only a single radioactive band, with and without disulphide-bond reduction (Fig. 4). Thus, in all likelihood, the epitope is part of the heparan sulphate proteoglycan core protein itself. Moreover, the epitope appears to be restricted to specific membrane proteoglycans. Other proteoglycans, including the matrix-associated HSPG, lack the epitope, as do all other Sepharose CL-2B-included or -excluded cellular proteins. Yet, one would expect some unsubstituted core protein to be present in cells. This result could mean that the heparitinase-resistant glycosaminoglycan chain linkage region is part of the epitope. Alternatively, the size of the pool of unsubstituted core proteins may be very small compared with that of the complete proteoglycan. Pulse–chase experiments with and without β-xylosides and limited proteolytic fragmentations might help to resolve this issue.

The heparitinase-digested proteoglycan that is recognized by the S1 antibody has a monomeric 64 kDa core protein. The occurrence of *N*-linked oligosaccharides also appears restricted to 64 kDa core proteins, but is not a determinant of the epitope. Besides the S1-reactive 64 kDa core protein, 125 kDa, 90 kDa, 48 kDa and 35 kDa monomeric core proteins (Figs. 4 and 5) and a 64 kDa dimeric core protein occur in detergent-extracted ^{125}I-labelled membrane HSPG (Lories *et al.*, 1987). The latter yields a heparan sulphate-free and a heparan

sulphate-carrying 35 kDa fragment upon disulphide-bond reduction. The absence of the S1 epitope from these other core proteins provides no direct information on the relatedness of the different forms. They may be unrelated to the S1 protein, explaining the absence of cross-reactivity. On the other hand, one possible relationship is that the different core proteins in the detergent extracts represent non-overlapping or only partially overlapping fragments of a larger intact molecule, and that this fragmentation occurs during the isolation procedure. Even under conditions that should minimize proteolysis (extraction and fractionation in 4 M guanidinium chloride, in the cold, in the presence of several proteinase inhibitors, and in the absence of radioiodination) the heparitinase-digested S1-reactive material had the size of a 64 kDa peptide, suggesting that the S1-reactive core protein occurs as such in the cells. The results provide no exclusion, however, on possible degradations that might yield the other core protein forms from the 64 kDa S1-reactive core protein and, *a fortiori*, from other precursor forms. Indeed, although the amount of S1-reactive 64 kDa protein appeared to be fairly stable throughout the isolation procedure and thereafter, the present data are not quantitative enough to exclude any further breakdown into unreactive species. For example, proteolytic cleavage of the 64 kDa core protein in the disulphide-bond-created structure that sustains the S1 epitope may destroy the epitope and yield the dimeric 64 kDa protein. On the other hand, S1-reactive core protein forms that were smaller than the 64 kDa protein were also never detected, which would imply that during degradation only those 64 kDa conversions would occur that also destroy the S1 epitope. This possibility appears rather unlikely. Taken together, the absence of any other S1-reactive proteoglycan, larger or smaller, than the 64 kDa core proteoglycan may be indicative that little or no core protein degradation occurs, whatever the isolation procedure employed. It ensues that the observed heterogeneity of the core proteins is likely to be genuine and existing within cells. The basis and functional significance of this heterogeneity remain to be established.

These investigations were supported by Grant 3.0030.81 from the Nationaal Fonds voor Geneeskundig Wetenschappelijk Onderzoek, Belgium, and by U.S. Public Health Service Research Grant HL-31750, awarded to G.D. V.L. is a Specialisatiebursaal of the Instituut tot aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw, Belgium. G.D. is an Onderzoeksleider of the Nationaal Fonds voor Wetenschappelijk Onderzoek, Belgium.

REFERENCES

Cheng, C.-F., Oosta, G. M., Bensadoun, A. & Rosenberg, R. D. (1981) J. Biol. Chem. **256**, 12893–12898

Cole, G. J., Schubert, D. & Glaser, L. (1985) J. Cell Biol. **100**, 1192–1199

Cöster, L., Malmström, A., Carlstedt, I. & Fransson, L.-Å. (1983) Biochem. J. **215**, 417–419

Cöster, L., Carlstedt, I., Kendall, S., Malmström, A., Schmidtchen, A. & Fransson, L.-Å. (1986) J. Biol. Chem. **261**, 12079–12088

David, G. & Van den Berghe, H. (1983) J. Biol. Chem. **258**, 7338–7344

David, G. & Van den Berghe, H. (1985) J. Biol. Chem. **260**, 11067–11074

Farell, D. H. & Cunningham, D. D. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 6858–6862

Fransson, L.-Å, Carlstedt, I., Cöster, L. & Malmström, A. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 5657–5661

Fritze, L. M. S., Reilly, C. F. & Rosenberg, R. D. (1985) J. Cell Biol. **100**, 1041–1049

Gallagher, J. T., Lyon, M. & Stewart, W. P. (1986) Biochem. J. **236**, 313–325

Gill, P. J., Silbert, C. K. & Silbert, J. E. (1986) Biochemistry **25**, 405–410

Höök, M., Kjellén, L., Johansson, S. & Robinson, J. (1984) Annu. Rev. Biochem. **53**, 847–869

Hovingh, P. & Linker, A. (1970) J. Biol. Chem. **245**, 6170–6175

Kawakami, H. & Terayama, H. (1981) Biochim. Biophys. Acta **646**, 161–168

Kjellén, L., Pettersson, I. & Höök, M. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 5371–5375

Koda, J. E., Rapraeger, A. & Bernfield, M. (1985) J. Biol. Chem. **260**, 8157–8162

Laemmli, U. K. (1970) Nature (London) **277**, 680–682

Laterra, J., Silbert, J. E. & Culp, L. A. (1983) J. Cell Biol. **96**, 112–123

Lories, V., David, G., Cassiman, J. J. & Van den Berghe, H. (1986) Eur. J. Biochem. **158**, 351–360

Lories, V., De Boeck, H., David, G., Cassiman, J. J. & Van den Berghe, H. (1987) J. Biol. Chem. **262**, 854–859

Marcum, J. A. & Rosenberg, R. D. (1984) Biochemistry **23**, 1730–1737

Marcum, J. A., Conway, E. M., Youssoufian, H. & Rosenberg, R. D. (1986) Exp. Cell Res. **166**, 253–258

Marynen, P., Van Leuven, F., Cassiman, J. J. & Van den Berghe, H. (1981) J. Immunol. **127**, 1782–1786

Norling, B., Glimelius, B. & Wasteson, Å. (1981) Biochem. Biophys. Res. Commun. **103**, 1265–1272

Rapraeger, A. C. & Bernfield, M. R. (1982) in Extracellular Matrix (Hawkes, S. & Way, J. L., eds.), pp. 265–269, Academic Press, New York

Rapraeger, A. C. & Bernfield, M. R. (1983) J. Biol. Chem. **258**, 3632–3636

Ratner, N., Bunge, R. P. & Glaser, L. (1985) J. Cell Biol. **101**, 744–754

Schubert, D. & La Corbière, M. (1985) J. Cell Biol. **100**, 56–63

Shimada, K., Gill, P. J., Silbert, J. E., Douglas, W. H. & Fanbury, B. L. (1981) J. Clin. Invest. **68**, 995–1002

Shively, J. E. & Conrad, H. E. (1976) Biochemistry **15**, 3932–3942

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Van Leuven, F., Marynen, P., Cassiman, J. J. & Van den Berghe, H. (1986) J. Immunol. Methods **90**, 125–130

Woods, A., Höök, M., Kjellén, L., Smith, C. G. & Rees, D. A. (1984) J. Cell Biol. **99**, 1743–1753

Woods, A., Couchman, J. R. & Höök, M. (1985) J. Biol. Chem. **260**, 10872–10879

Woods, A., Couchman, J. R., Johansson, S. & Höök, M. (1986) EMBO J. **5**, 665–670

Received 23 February 1987/15 May 1987; accepted 31 July 1987