

Membrane-associated Chondroitin Sulfate Proteoglycans of Human Lung Fibroblasts

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Abstract. Cultured human fetal lung fibroblasts produce some chondroitin sulfate proteoglycans that are extracted as an aggregate in chaotropic buffers containing 4 M guanidinium chloride. The aggregated proteoglycans are excluded from Sepharose CL4B and 2B, but become included, eluting with a K_{av} value of 0.53 from Sepharose CL4B, when Triton X-100 is included in the buffer. Conversely, some of the detergent-extractable chondroitin sulfate proteoglycans can be incorporated into liposomes, suggesting the existence of a hydrophobic membrane-intercalated chondroitin sulfate proteoglycan fraction. Purified preparations of hydrophobic chondroitin sulfate proteoglycans contain two major core protein forms of 90 and 52 kD. A

monoclonal antibody (F58-7D8) obtained from the fusion of myeloma cells with spleen cells of BALB/c mice that were immunized with hydrophobic proteoglycans recognized the 90- but not the 52-kD core protein. The epitope that is recognized by the antibody is exposed at the surface of cultured human lung fibroblasts and at the surface of several stromal cells *in vivo*, but also at the surface of Kupffer cells and of epidermal cells. The core proteins of these small membrane-associated chondroitin sulfate proteoglycans are probably distinct from those previously identified in human fibroblasts by biochemical, immunological, and molecular biological approaches.

CULTURED human fibroblasts produce several distinct chondroitin sulfate proteoglycans (CSPGs)¹ (5, 6, 37). These CSPGs differ in overall size, relative glucuronic acid/iduronic acid content, and distribution between culture compartments (7). Some have been characterized in considerable detail.

The very large CSPGs from the fibroblast matrix harbor large core proteins that interact with hyaluronic acid (22, 33), which is reminiscent of the properties of the large aggregating cartilage proteoglycans (17). The large CSPGs from fibroblasts and from cartilage can be distinguished immunohistochemically (22), but comparative peptide maps show also structural similarities (20). Recent cDNA sequence data imply that the carboxy-terminal ends of a large human fibroblast CSPG and of the large aggregating rat cartilage CSPG are strongly homologous, containing sequences with lectin-like and growth factor-like structural features (10, 25).

Cultured human fibroblasts also secrete some small iduronic acid-rich proteoglycans characterized by ~40-kD core proteins (2, 14). Immunological and peptide mapping data suggest that these small proteoglycans are related to small interstitial CSPGs with 50–40-kD core proteins that can be iso-

lated from a number of different tissues (20, 30). These small CSPGs accumulate in the extracellular matrix during culture on collagen supports (11) and *in vivo*, where they occur in close association with fibrous structures (39). *In vitro*, they bind to fibronectin (35) and to collagen (34) through their core proteins, affect collagen fibril formation (38), and modulate the adhesion of cells to collagen-fibronectin matrices (26). Recent cDNA and peptide sequence data suggest that these small CSPGs are synthesized by the human fibroblasts as preproteins, containing a signal peptide, a propeptide, and the mature core protein (24).

However, poly- (18) and monoclonal (1) antibody probes, recognizing epitopes in the residual chain stubs of chondroitinase-treated proteoglycans or recognizing the native glycosaminoglycan chains themselves, detect also some nonfibrillar chondroitin sulfate (CS) in close association with the outer aspect of the plasma membrane of the fibroblasts. Ligand-induced capping upon incubation of living cells with the antibodies (1) and a patchy surface distribution of immunoferritin complexes in electron microscopy (18) imply that this surface CS is free to move laterally along the plane of the membrane, and raise the possibility of integral membrane CSPG.

In the present report we provide direct evidence in support of this proposal, and demonstrate the occurrence of CSPGs at the cell surface of human lung fibroblasts by means of a monoclonal antibody probe which defines an epitope in the core protein moiety of these proteoglycans.

1. *Abbreviations used in this paper:* CS, chondroitin sulfate; CSPG(s), chondroitin sulfate proteoglycan(s); GdnHCl, guanidine hydrochloride; HSPG(s), heparan sulfate proteoglycan(s).

Materials and Methods

Cell Culture, Fractionation, and Extraction Procedures

Human lung fibroblasts were grown in Dulbecco's modified minimal essential medium (Gibco Europe, Gent, Belgium) containing 10% (vol/vol) newborn calf serum (27). Confluent cultures were labeled for 48 h with $5 \mu\text{Ci}$ ($1.8 \times 10^5 \text{ Bq}$) of carrier free $\text{H}_2^{35}\text{SO}_4$ (New England Nuclear, Boston, MA) per milliliter of culture medium (27). Labeled monolayers were rinsed with Dulbecco's PBS before fractionation and extraction. Whole monolayers were extracted in 4 M guanidine hydrochloride, 10 mM *N*-ethylmaleimide, 5 mM benzamidine, 50 mM sodium acetate pH 5.8 (GdnHCl buffer), supplemented with 2.5 mg/ml pepstatin A, 50 $\mu\text{g}/\text{ml}$ leupeptin, and 2 mM phenylmethylsulfonyl fluoride just before use (27). Plasma membrane fractions were prepared as described before (23, 27) and extracted in 4 M GdnHCl buffer supplemented as described above. Extracellular matrix fractions were prepared by sequential extraction of the monolayers with Triton X-100 buffer (see below), sodium deoxycholate buffer, hypotonic Tris-HCl buffer and hypertonic KCl buffer as described before (19, 21, 27), and extracted in 4 M GdnHCl buffer. The whole monolayer, plasma membrane, and matrix extracts were cleared by centrifugation (100,000 g, 30 min) before gel filtration. In every instance, the cleared 4 M GdnHCl extract contained 95% or more of the total ^{35}S -chondroitin sulfate in the fraction.

Gel Filtration and Ion-Exchange Chromatography

Gel filtration on (1 \times 100 cm) Sepharose CL2B and 4B (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) columns in 4 M GdnHCl buffer, or in 4 M GdnHCl buffer supplemented with 0.5% (wt/vol) Triton X-100, was performed at 4°C with a flow rate of 4 ml/h. Carrier heparin and chondroitin sulfate (10 $\mu\text{g}/\text{ml}$) and bovine serum albumin (50 $\mu\text{g}/\text{ml}$) were included in the 4 M GdnHCl buffer used for chromatography, except when the sample was prepared for radioiodination (28).

Ion-exchange chromatography on Mono Q (Pharmacia LKB Biotechnology Inc.) in urea buffer (0.5% Triton X-100, 6 M urea, 50 mM Tris-HCl pH 8.0) with a (0–1.2 M) linear NaCl gradient was as described before (8).

Glycosaminoglycan Identification

Aliquots of the eluted fractions were digested with protease K (Merck, Darmstadt, FRG) and ethanol precipitated. Ethanol-insoluble protease-resistant label which became ethanol soluble after chondroitinase ABC (Miles Laboratories Inc., Naperville, IL) digestion was defined as chondroitin sulfate (8). The remaining chondroitinase ABC-resistant label was identified as heparan sulfate previously (27). For digestions with chondroitinase AC (Miles Laboratories Inc.) the pH of the Tris-HCl buffer was adjusted to 7.0.

Purification of the CSPGs

Proteoglycans extracted from whole monolayers in Triton X-100 buffer (10 mg/ml Triton X-100, 150 mM NaCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 5 mM *N*-ethylmaleimide, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu\text{g}/\text{ml}$ of pepstatin A, pH 7.4), were concentrated by absorption on DEAE-Trisacryl M, eluted in 4 M GdnHCl buffer containing 0.5% Triton X-100, and fractionated over Sepharose CL4B in 4 M GdnHCl containing 0.5% Triton X-100. Fractions containing ^{35}S -chondroitin sulfate were pooled, dialyzed against urea buffer, and submitted to ion-exchange chromatography on Mono Q. The eluted ^{35}S -chondroitin sulfate was reabsorbed on Mono Q, rinsed with urea buffer containing 50 mM octylglucoside instead of Triton X-100, and eluted in 4 M GdnHCl buffer supplemented with 50 mM octylglucoside. After the addition of 5 mg/ml of phosphatidyl choline, the proteoglycan sample was dialyzed against 4 M GdnHCl. Resulting proteoglycan-liposome mixtures were fractionated over Sepharose CL4B in 4 M GdnHCl buffer, to separate hydrophobic liposome-associated proteoglycans from the nonhydrophobic proteoglycans and free glycosaminoglycan chains. In some experiments [^3H]phosphatidylcholine was included in the proteoglycan-phospholipid mixtures to mark the position of the liposomes (27). These eluted in the excluded volume of the Sepharose CL4B columns. Small CSPGs accumulating in the culture media of the cells (2, 14) were purified by gel filtration on Sepharose CL4B and ion-exchange chromatography on Mono Q as described above.

Characterization of the Core Protein Moieties

Purified hydrophobic CSPGs were made free of carrier and phospholipid by gel filtration on Sepharose CL4B in 4 M GdnHCl buffer containing 0.5% Triton X-100. Carrier-free CSPGs were ^{125}I iodinated by the chloramine T method as described before (28). ^{125}I -labeled proteoglycan was separated from free label by ion-exchange chromatography and gel filtration in the presence of detergent. Digestion of the ^{125}I -labeled proteoglycans with chondroitinase ABC, or with heparitinase (Miles Laboratories Inc.), or with both enzymes and electrophoresis on 4–16% polyacrylamide gradient gels or in 4% Nu-sieve agarose gels in the presence of SDS were as described before (8, 9, 28). Binding of the CSPGs and of their core proteins to fibrillar type I collagen was assessed as described previously (21).

Immunochemical Procedures

mAb F58-7D8 was obtained after immunizing BALB/c mice with partially purified hydrophobic proteoglycan fractions, using the standard procedures of immunization, cell fusion, hybrid selection, cloning, and subcloning described before (9). Affinity-purified polyclonal rabbit antibodies (14, 39) and a mixture of murine monoclonal antibodies (LN1, LN3, LN4; reference 35) specific for the core proteins of the small dermatan sulfate (iduronic acid-rich chondroitin sulfate) proteoglycans from human fibroblast secretions were kind gifts of Dr. Hans Kresse (University of Münster, Federal Republic of Germany). Dot-blotted eluate fractions and Western blots on Zeta-probe nylon membrane (Bio-Rad Laboratories, Richmond, CA) were incubated with 20 $\mu\text{g}/\text{ml}$ of mAb 7D8 diluted in PBS containing 0.5% casein, and stained using peroxidase-linked rabbit anti-mouse Ig, 3,3'-diaminobenzidine, and H_2O_2 (9, 21). The polyclonal and the monoclonal anti-dermatan sulfate proteoglycan antibodies were used at dilutions of 1:400 and 1:100, respectively. Coupling of mAb 7D8 to CNBr-activated Sepharose and immunopurification of the ^{125}I -labeled proteoglycans were as described before (28).

Histological Procedures

Immunoperoxidase Staining of Cells and Tissues for Light Microscopy. Cultured cells were fixed with 0.3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. mAb 7D8 was added at a concentration of 10–100 $\mu\text{g}/\text{ml}$ in PBS containing 1% BSA. Binding of mAb 7D8 was detected with rabbit anti-mouse peroxidase, diluted 1:50 in PBS-BSA, using 3,3'-diaminobenzidine (0.05%) and hydrogen peroxide (0.01%) as substrates. Tissues were frozen in liquid nitrogen and cryosections (6 μm thick) were incubated with mAb 7D8 (25 $\mu\text{g}/\text{ml}$) without prior fixation.

Immunoperoxidase and Gold Labeling for Transmission Electron Microscopy. Cryosections were immediately fixed for 20 min with 2% acrolein in phosphate buffer at 4°C, and incubated with mAb 7D8 (100 $\mu\text{g}/\text{ml}$). After the immunogold labeling (5-nm gold-tagged goat anti-mouse IgG, diluted 1:10, overnight) or the immunoperoxidase labeling (peroxidase-conjugated rabbit anti-mouse IgG, diluted 1:50, 1 h), a second fixation with 3% glutaraldehyde in sodium cacodylate preceded dehydration and embedding. For ultracytosections cultured cells were fixed *in situ* with acrolein and scraped. Sections (\sim 100 nm thick) were made as described before (36) and incubated with mAb 7D8 (100 $\mu\text{g}/\text{ml}$) for 1 h and with gold-coupled goat anti-mouse IgG.

Gold Labeling for Scanning Electron Microscopy. Cells were allowed to spread for 1.5 h on a fibronectin coat and fixed with 2% acrolein for 20 min at 4°C. After incubation with mAb 7D8 (0.5 $\mu\text{g}/\text{ml}$) and gold labeling with goat anti-mouse (diluted 1:40) a silver enhancement treatment was performed following the instructions of Janssen Pharmaceuticals, Beerse, Belgium. Cells were fixed again with glutaraldehyde (3%), dehydrated, critical point dried, and covered with gold in a sputter coater (Polaron Instruments Inc., Hatfield, PA) before viewing in a Philips PSEM 500. To test whether the binding of mAb 7D8 was specific, uncoated, CSPG-coated or heparan sulfate proteoglycan (HSPG)-coated beads were added during the incubation of the cells with the antibody. To prepare coated beads, the cellular CSPG and HSPG present in the detergent-extract of \sim 1 \times 10⁷ human lung fibroblasts were fractionated on a Sepharose CL4B column (as in Fig. 3 A). The CSPG and HSPG peaks were dialyzed against urea buffer and adsorbed on 200 μl packed DEAE-Trisacryl M beads. After extensive rinsing with 150 mM NaCl, 20 mM Tris-Cl, pH 7.4 to remove all traces of detergent, the beads were mixed with 0.5 μg mAb 7D8 in 1 ml 150 mM NaCl, 20 mM Tris-Cl, pH 7.4, and the mixture was added to the cells. Prior experiments had shown that mAb 7D8 did not bind to DEAE in this buffer. After a 1-h incubation the mixture of beads and mAb was removed, and the

monolayers were rinsed, further treated with second antibody, and processed as above.

Results

Integral Membrane CSPG of Human Lung Fibroblasts

When 4 M GdnHCl extracts of continuously $^{35}\text{SO}_4$ -labeled fibroblast monolayers were fractionated over Sepharose CL2B, three distinct ^{35}S -chondroitin sulfate-containing peaks were obtained: excluded materials, materials eluting with $K_{av} \sim 0.3$ and a peak eluting with $K_{av} \sim 0.8$ (Fig. 1 A). Further experiments indicated that the excluded fractions contained minor (only $\sim 7\%$ of the total ^{35}S -CS in the monolayer) but specific CSPGs with properties of integral membrane molecules. First, this Sepharose CL2B-excluded ^{35}S -CS became partially included when treated with 0.5% Triton X-100 and rechromatographed on Sepharose CL4B in the presence of detergent (Fig. 2 A). This 4B-included ^{35}S -CS eluted with $K_{av} = 0.53$, and was identified as proteoglycan by virtue of its susceptibility to protease K. The alkali borohydride-treated ^{35}S -CS chains from this proteoglycan eluted with $K_{av} = 0.69$ (estimated approximate $M_r = 25,000$, not shown). The Sepharose CL2B-included ^{35}S -CS peaks which eluted with $K_{av} = 0.3$ and with $K_{av} = 0.8$, in contrast, were not affected in their chromatographic behavior when treated with detergent (not shown). When rechromatographed

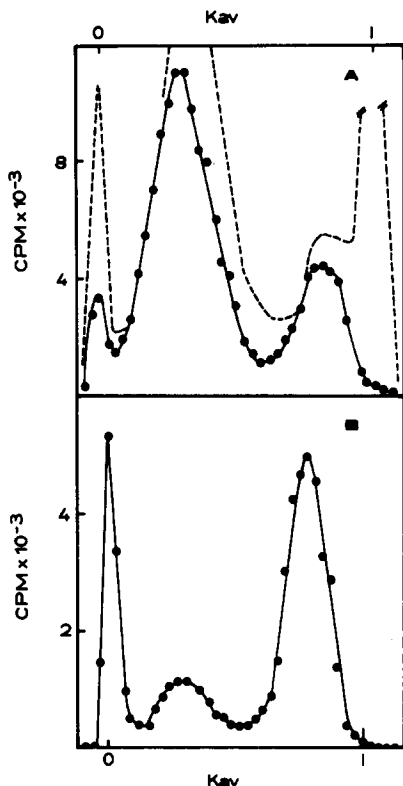


Figure 1. Fractionation of the culture extracts. Whole monolayers (A) and plasma membrane fractions (B) of cultured $^{35}\text{SO}_4$ -labeled human lung fibroblasts were extracted in 4 M GdnHCl and chromatographed on Sepharose CL2B in the absence of detergent. Samples were taken to determine the amount of total ^{35}S (---) and ^{35}S -CS (●) eluted in each fraction.

on Sepharose CL4B the former eluted in the void volume of the column (not shown, but as in Fig. 2 B).

Second, in comparison with whole monolayer extracts (Fig. 1 A), 4 M GdnHCl extracts of partially purified plasma membrane preparations (nearly 13-fold enriched in 5' nucleotidase, see reference 27), were enriched in Sepharose CL2B-excluded ^{35}S -CS (Fig. 1 B). The K_{av} 0.3 CSPGs, in contrast, were relatively depleted from the membranes, but were the predominant CSPG present in extracellular matrix extracts. With or without detergent, the latter were excluded from Sepharose CL4B (Fig. 2 B). After treatment with detergent most of the 2B-excluded membrane-associated ^{35}S -CS eluted with $K_{av} = 0.53$ from Sepharose CL4B (Fig. 2 B), a result similar to that obtained for Sepharose CL2B-excluded materials from whole monolayer extracts (Fig. 2 A).

Third, some 10% of the ^{35}S -CS which could be extracted from human lung fibroblast monolayers with nonionic detergent and in the absence of chaotrope (Triton X-100 buffer extracted $\sim 70\%$ of the amount of ^{35}S -CS extractable with

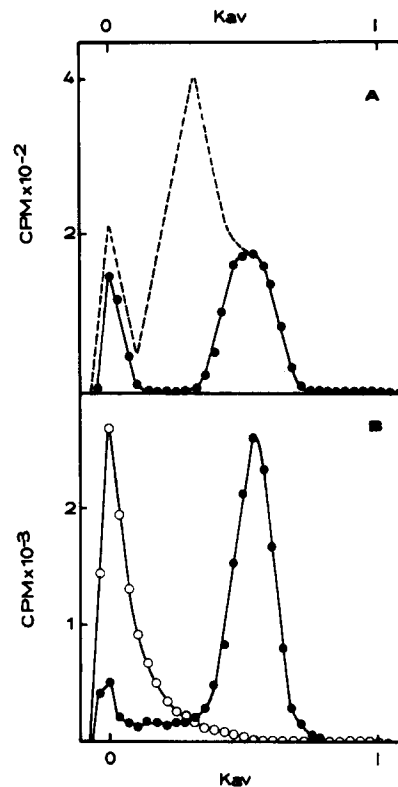


Figure 2. Detergent-susceptible CSPG complexes. Sepharose CL2B-excluded label originating from whole monolayer extracts (A) and from plasma membrane extracts (B) was treated with Triton X-100 and rechromatographed on Sepharose CL4B in the presence of the detergent. Samples were taken to determine the amount of ^{35}S -CS (●) eluted in each fraction. Indicating the amount of total ^{35}S (---) eluted in A reveals a CS-poor peak which corresponds to the hydrophobic heparan sulfate proteoglycan present in these cells (27). The elution of the large ^{35}S -CSPG present in extracellular matrix preparations of the fibroblasts (○) is given in B for comparison.

4 M GdnHCl buffer), could be incorporated into liposomes (Fig. 3, *A* and *B*). After treatment with detergent, those liposome-associated ^{35}S -CSPGs eluted with $K_{av} = 0.53$ from Sepharose CL4B (not shown). Similar results were obtained with the ^{35}S -CSPG, isolated from 4 M GdnHCl extracts, which were originally excluded from Sepharose CL2B (as in Fig. 1 *A*) but became 4B-included after detergent treatment (as in Fig. 2 *A*), except that the association of these "detergent-susceptible" CSPG with the liposomes was nearly quantitative (not shown). The ^{35}S -CSPG isolated from the detergent-insoluble matrix (tested on Sepharose CL2B columns, as in reference 27), and ^{35}S -CSPG isolated from conditioned culture medium, in contrast, did not associate with liposomes (not shown). Analysis of the chondroitinase susceptibility of the glycosaminoglycan chains indicated that a specific ^{35}S -CS fraction of the detergent extracts became liposome associated (Fig. 4). Chondroitinase AC-digestion of the ^{35}S -CS chains present in the non-interacting materials yielded virtually no Sephadex G25-included label (Fig. 4, *D-F*). In contrast, chondroitinase AC converted nearly half of the label of the liposome-associated

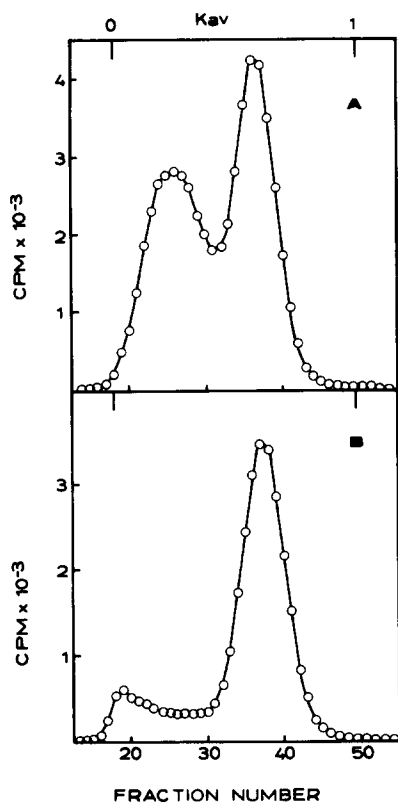


Figure 3. Liposome-interacting CSPG. Detergent extracts of $^{35}\text{SO}_4$ -labeled human lung fibroblast monolayers were fractionated on Sepharose CL4B in 4 M GdnHCl containing Triton X-100, yielding a heparan sulfate-rich peak ($K_{av} \sim 0.27$) and a chondroitin sulfate-rich peak ($K_{av} \sim 0.57$) (*A*). The chondroitin sulfate peak was further purified by ion-exchange on Mono Q. After exchanging Triton X-100 for octylglucoside, liposomes were formed by adding phosphatidylcholine and dialyzing the mixture against detergent-free 4 M GdnHCl buffer. The liposome-CSPG mixture was then rechromatographed on Sepharose CL4B in the absence of detergent (*B*). In some experiments [^3H]phosphatidylcholine was included to trace the elution of the liposomes.

^{35}S -CS chains to the size of disaccharides (Fig. 4, *A-C*), indicative for a higher glucuronic acid content in these lipophilic materials. Analysis of the ^{35}S -CS chains present in the detergent-susceptible peak of the 4 M GdnHCl extract, revealed a chondroitinase AC susceptibility similar to that of the CS chains present on the liposome-interacting materials of the detergent extract (not shown). Thus, human lung fibroblasts contain some distinct ^{35}S -CSPGs which are enriched in plasma membrane preparations, from where they can be extracted with chaotropes as (Sepharose CL2B-excluded) micellar aggregates, or be solubilized with detergent. This requirement for detergent to obtain complete solubilization and their ability to interact with liposomes would identify these proteoglycans as integral membrane components.

Characterization of the Integral Membrane CSPG Core Proteins

These putative integral membrane CSPGs were purified from Triton X-100 extracts of the fibroblast monolayers, by gel filtration on Sepharose CL4B in the presence of detergent (as in Fig. 3 *A*), ion-exchange chromatography of the ^{35}S -CS peak on Mono Q (not shown), and by liposome incorporation followed by chromatography on Sepharose CL4B in

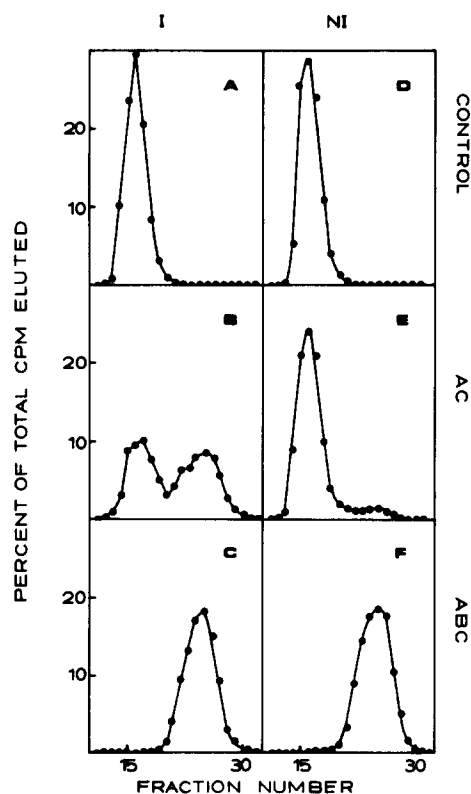


Figure 4. Chondroitinase susceptibility of the CSPG. Chondroitin-sulfate chains were isolated from liposome-interacting (*I*) ^{35}S -CSPG (excluded from Sepharose CL4B, as in Fig. 3 *B*) and from noninteracting (*NI*) materials (Sepharose CL4B included, as in Fig. 3 *B*) by digestion with protease K and treatment with nitrous acid to insure removal of any contaminating heparan sulfate (27). I-CS chains (*A-C*) and NI-CS chains (*D-F*) were chromatographed on Sephadex G25 in 100 mM Tris-HCl, pH 8.0 without further treatment (*A* and *B*), after treatment with chondroitinase AC (*B* and *E*), or after treatment with chondroitinase ABC (*C* and *F*).

the absence of detergent (as in Fig. 3 B). During these isolation procedures the epitope recognized by mAb 7D8 (obtained from the immunization of BALB/c mice with membrane proteoglycans of human fibroblasts) appeared to copurify with the lipophilic CSPGs. The mAb stained only dot spots derived from fractions of detergent-extracts eluting with $K_{av} \sim 0.5-0.6$ from Sepharose CL4B in the presence of detergent (Fig. 5 A); and from fractions that were excluded from Sepharose CL4B after liposome incorporation and chromatography of the materials in the absence of detergent (Fig. 5 B). Apparently, the mAb did not react with any heparan sulfate proteoglycan (Fig. 5 A) or with non-lipophilic cellular CSPG (Fig. 5 B). It also did not react with any matrix or secreted PG (not shown).

The purified lipophilic CSPGs were radioiodinated and further analyzed by SDS-polyacrylamide gradient gel electrophoresis and autoradiography (Fig. 6 A). Without further treatment the ^{125}I -labeled proteoglycans migrated as two broad bands with apparent molecular mass of 110,000–180,000 and of 60,000–85,000 kD. After chondroitinase ABC-treatment two major sharp bands of 90 and 52 kD were obtained, together with minor bands of 130, 119, and 46 kD. The migration of these bands was not affected by additional digestion of the CSPGs with heparitinase, indicating absence of heparan sulfate-chondroitin sulfate hybrid proteoglycans as found in other cell types (8). Omitting the reduction of disulfide bonds before the electrophoresis had no distinct effect on the migration of the major labeled bands (not shown, but compare with Fig. 7 B and Fig. 8 A). Analysis of the ^{125}I -CSPG which could be immunopurified by adsorption on Sepharose-linked mAb 7D8, in contrast, yielded a broad 110–180 kD smear without enzyme treatment, and a single sharp ~ 90 -kD band after chondroitinase ABC digestion (Fig. 6 B). Reduction had no effect on the migration of this material (not shown).

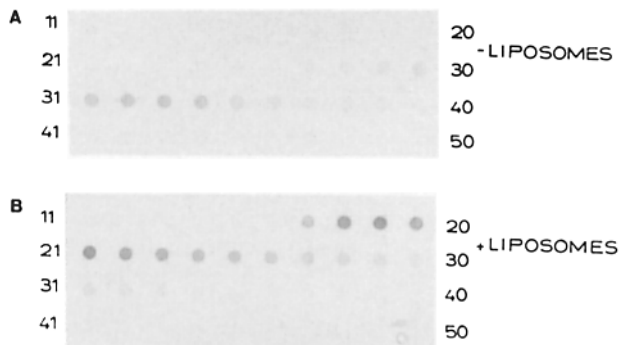


Figure 5. Monoclonal antibody 7D8 marks a lipophilic molecule. Detergent extracts of human lung fibroblasts were fractionated on Sepharose CL4B in 4 M GdnHCl buffer containing Triton X-100 as in Fig. 3 A. Aliquots of the fractions (11–50) were spotted on Zeta-probe membrane and stained with mAb 7D8 and peroxidase-conjugated rabbit anti-mouse immunoglobulins (A). The CSPG- and 7D8-positive fractions (28–45) were further purified, incorporated in liposomes, and rechromatographed on Sepharose CL4B in the absence of detergent as in Fig. 3 B. Aliquots of the eluate fractions (11–50) were spotted on Zeta probe membrane and stained with mAb 7D8 and second antibody (B).

The comparison of the immunostaining (Fig. 7 A) and of the autoradiography (Fig. 7 B) of Western blots of unreduced chondroitinase ABC-digested ^{125}I -labeled CSPG indicated that the 90-kD core was recognized by mAb 7D8. Reduction with β mercaptoethanol before electrophoresis abolished this recognition of the 90-kD band by mAb 7D8, without affecting the migration or the electrotransfer of the band (not shown), suggesting that this core protein had a single subunit structure, with one or more internal disulfide bonds.

In the absence of chondroitinase ABC digestion the immunoreactive and the labeled materials transferred poorly from the polyacrylamide gradient gels (compare lanes 1 and 2 in Fig. 7, A and B). Both the digested and the undigested materials transferred well, however, when the electrophoresis was performed in SDS-agarose gels. Both were intensely stained by mAb 7D8, and chondroitinase ABC digestion decreased the apparent molecular mass of the immunoreactive materials (Fig. 7 C). This result confirmed that the 7D8 epitope was part of the CSPG core protein and exposed in the native molecule.

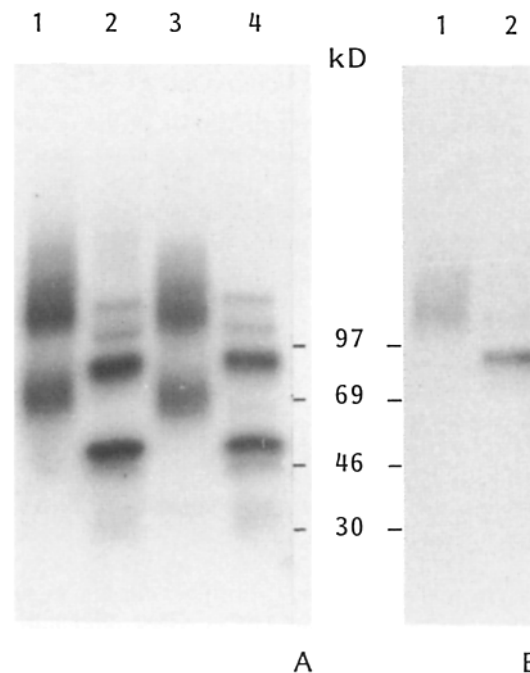


Figure 6. Heterogeneity of the lipophilic CSPG. (A) Purified liposome-interacting CSPGs were iodinated using Na^{125}I and chloramine T, and submitted to polyacrylamide gradient gel electrophoresis in the presence of SDS after reduction with β -mercaptoethanol and analyzed by autoradiography: nondigested CSPG (1); chondroitinase ABC-digested CSPG (2); heparitinase-digested CSPG (3); doubly heparitinase- and chondroitinase ABC-digested CSPG (4). (B) The ^{125}I -labeled lipophilic CSPGs were further fractionated by affinity chromatography on Sepharose-linked mAb 7D8. The bound materials were eluted in 4M GdnHCl buffer, ethanol precipitated, and analyzed by SDS polyacrylamide gradient gel electrophoresis and autoradiography before (1) and after (2) digestion with chondroitinase ABC.

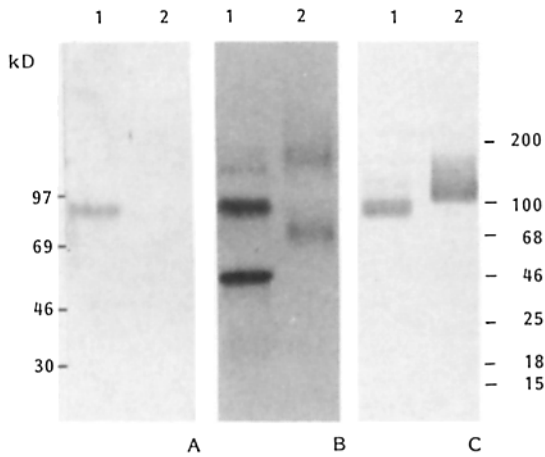


Figure 7. Monoclonal antibody 7D8 marks the unreduced 90-kD core protein. Purified ^{125}I -iodinated liposome-interacting CSPGs were treated with chondroitinase ABC (lane 1) or left untreated (lane 2) and submitted to polyacrylamide gradient gel electrophoresis in the presence of SDS, but without prior reduction of disulfide bonds. After electrotransfer to Zeta probe membrane, the membrane was stained with mAb 7D8 using an indirect immunoperoxidase technique (A) and submitted to autoradiography (B). Alternatively, the untreated (1) and the chondroitinase ABC-digested (2) liposome-interacting CSPGs were submitted to SDS-agarose gel electrophoresis, electroblotted on a Zeta probe membrane, and immunostained with mAb 7D8 (C).

Comparison of Lipophilic and Nonlipophilic CSPGs

The purified nonliposome-interacting CSPGs of the detergent extracts were similarly radioiodinated, and compared with the liposome-interacting CSPGs by SDS-polyacrylamide gradient gel electrophoresis and autoradiography (Fig. 8 A). In contrast to the lipophilic CSPGs which ran as two separate smears if undigested or as two distinct bands of 90 and 52 kD after chondroitinase ABC-digestion, the ^{125}I -labeled nonlipophilic CSPGs migrated as a single broad band with apparent molecular mass of 70,000–100,000 kD before chondroitinase ABC digestion and as a closely spaced doublet of 46–44-kD bands after chondroitinase ABC digestion. Some label associated with the nonlipophilic CSPGs resisted the digestion with chondroitinase or had a residual mobility similar to that of the bulk of the nonenzyme-treated proteoglycans, its nature remaining unclear. Whereas intact nonliposome-interacting CSPGs or the 46–44-kD core proteins of these proteoglycans bound to fibrillar collagen, none of the intact or chondroitinase ABC-digested lipophilic CSPG had collagen-binding properties (Fig. 8 B). Finally, dot-blot analysis (Fig. 9 A), using core protein-specific polyclonal and monoclonal antibody probes raised against the small iduronic acid-rich CSPGs of human skin fibroblasts (14, 35), indicated that the nonlipophilic CSPGs of the detergent extracts were immunologically related to the small CSPGs that were secreted in the culture medium by the cells. After digestion with chondroitinase ABC, both yielded a doublet of immunoreactive 46–44-kD core proteins on Western blots (Fig. 9 B). In contrast, none of these antibodies detected any of the lipophilic proteoglycans or core proteins (Fig. 9, A and B) and conversely, mAb 7D8 did not detect the nonlipophilic cellular or secreted CSPG (Fig. 9 A), under circumstances where each of these proteoglycans was readily

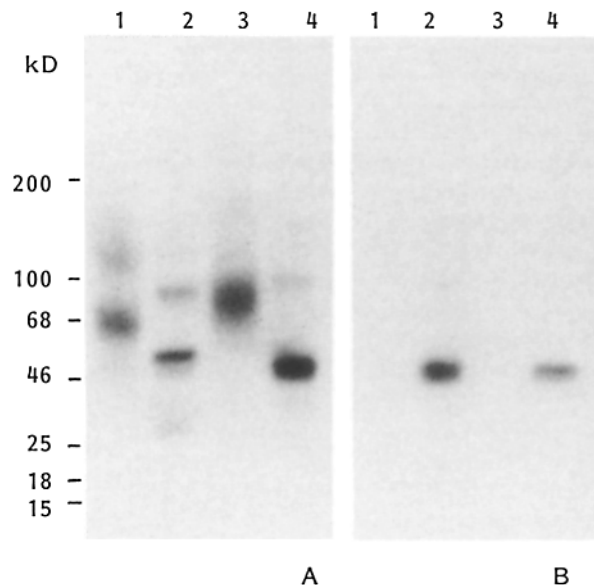


Figure 8. Lipophilic and nonlipophilic CSPGs contain structurally and functionally distinct core proteins. (A) Purified ^{125}I -iodinated lipophilic (1 and 2) and nonlipophilic (3 and 4) CSPGs were treated with chondroitinase ABC (2 and 4) or left untreated (1 and 3) and submitted to polyacrylamide gradient gel electrophoresis in SDS, without reduction of disulfide bonds, followed by autoradiography of the gel. (B) Lipophilic (1 and 3) and nonlipophilic (2 and 4) ^{125}I -labeled CSPGs were digested with chondroitinase ABC or left untreated and tested for binding to fibrillar type I collagen. Collagen-binding chondroitinase-treated CSPGs (1 and 2) and chondroitinase-digested collagen-binding CSPGs (3 and 4) were submitted to polyacrylamide gradient gel electrophoresis in SDS, followed by autoradiography of the gel.

detected by its corresponding immunoprobe. Thus, lipophilic and nonlipophilic small CSPGs of human lung fibroblasts likely represent distinct molecular species.

Cell Surface Localization and Tissue Distribution

The epitope recognized by mAb 7D8 showed a specific cellular and tissue distribution, both in vitro and in vivo. Indirect immunoperoxidase staining with mAb 7D8 of whole mounts of nonpermeabilized cultured human lung fibroblasts revealed a distinct and homogeneous staining of cell surfaces (Fig. 10 A). This homogeneous cell-surface distribution of the epitope was confirmed by scanning electron microscopy of silver-enhanced immunogold complexes. Except for the supranuclear area, the entire surface and the finest extensions of fibroblasts spreading on fibronectin coats were heavily labeled (Fig. 10 E). This heavy labeling of the cell surface was not observed on cells which had not been exposed to the mAb (Fig. 10 D), and was markedly inhibited by the addition of DEAE-bound CSPG (Fig. 10 G) during the incubation of the cells with the mAb, but not by the addition of DEAE-bound HSPG (Fig. 10 H) or by DEAE-beads only (Fig. 10 F). Thus, most of the gold deposits were due to the specific binding of the monoclonal antibody to the cell surface. Indirect immunogold staining of frozen sections, however, indicated that the distribution of the epitope was not limited to the surface of the cultured fibroblast (Fig. 10 C). The label also decorated internal cell structures, but it was not possible

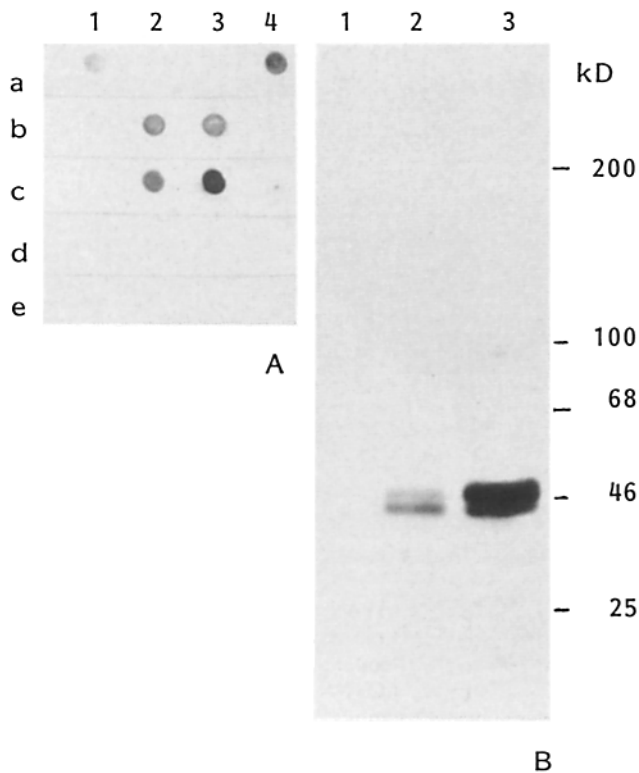


Figure 9. Lipophilic and nonlipophilic CSPGs contain immunologically distinct core proteins. (A) The lipophilic (1) and the nonlipophilic (2) CSPGs of the detergent extract, and the small CSPGs secreted in the culture medium (3) of one lung fibroblast monolayer, and amounts of lipophilic CSPGs from three times as many cells (4) were dot-spotted on a Zeta probe membrane, and stained with mAb 7D8 (a); with a polyclonal affinity-purified anti-dermatan sulfate proteoglycan antibody (b), with a mixture of three monoclonal anti-dermatan sulfate proteoglycan antibodies (c), or only with the peroxidase-conjugated rabbit anti-mouse antibodies (d), and the peroxidase-conjugated swine anti-rabbit antibodies (e) used in the direct immunoassay. (B) The lipophilic (1), and the nonlipophilic (2) CSPGs of the detergent extract and the small secreted CSPGs of human lung fibroblasts (3) were digested with chondroitinase ABC and submitted to SDS polyacrylamide gradient gel electrophoresis, electroblotted on Zeta probe membrane, and stained with the mixture of monoclonal anti-dermatan sulfate proteoglycan antibodies using an indirect immunoperoxidase assay.

to determine whether these were membrane limited. The mAb also stained the contours and surface structures of cultured human mammary epithelial HBI-100 cells (Fig. 10 B). Thus, the epitope is not restricted to cultured human fibroblasts.

Indirect immunoperoxidase staining and light microscopy of tissue sections indicated that, in situ, the 7D8 epitope occurred on connective tissue and some epithelial cells. In skin, reaction occurred in both the dermis and epidermis (Fig. 11 A). In the dermis the reaction was restricted to the cells. In the epidermis all cell layers, except the most superficial, were stained. In liver the reaction was confined to the portal spaces and to some cells (presumably Kupffer cells) localized in the sinusoids, but the hepatocytes were negative (Fig. 11 B). In kidney, the interstitial connective tissue was strongly positive, but the glomerular endo- and epi-

thelium, and the tubular epithelia were negative (Fig. 11 C). Immunoperoxidase and immunogold staining of skin sections followed by electron microscopy localized the epitope at the external surface of the epidermal cells (Fig. 11 D) and dermal fibroblasts (Fig. 11 E). Internal cell structures or matrix fibers were not decorated. In every instance the staining was shown to depend on the inclusion of mAb 7D8 in the first incubation. These data indicate that expression of the 7D8 epitope may show some restriction, occurring at the surface of human fibroblasts and of some (ectodermal) epithelial cells.

Discussion

Some of the CS residues produced by human lung fibroblasts are closely associated with the outer aspect of the plasma membrane (1, 18). The present results document that some CSPGs of cultured human lung fibroblasts indeed behave like integral membrane components, and that one form of these lipophilic CSPGs can be traced, in vitro during isolation procedures and in situ at the cell surface, by a murine monoclonal antibody which defines an epitope within the 90-kD core protein of this proteoglycan.

The cell surface exposure (Fig. 10) and the lipophilic properties (Fig. 5) of the CSPG which is marked by the 7D8 epitope, are two of the criteria which tentatively identify this proteoglycan as an "integral" membrane molecule. Unreported investigations have, in addition, indicated that the 7D8 epitope can be extracted as a micellar aggregate when whole fibroblasts or (placenta) plasma membrane fractions are exposed to 4 M GdnHCl buffer without detergent, and that it becomes included in Sepharose CL4B columns when this extract is treated with detergent. Possibly, this proteoglycan is only one of several integral membrane CSPGs present in fibroblasts, as, indeed, the final preparation of hydrophobic CSPGs contains multiple core protein bands (Fig. 6) of which only one could be shown to react with the 7D8 mAb (Figs. 6 and 7). The larger minor bands could, however, also represent glycosylation variants of the 90-kD core protein which are not sufficiently abundant for ready detection in Western blots, whereas the bands of lower relative molecular mass may represent core proteins which became truncated during isolation and have lost the epitope. If such were the case, it would mean that the 7D8 epitope is situated more distally on the core protein with respect to the hydrophobic membrane anchor, than the chondroitin sulfate chains. The issue of whether only one or several integral membrane CSPG species may occur in these cells needs further clarification.

In the presence of detergent, the overall size of the integral membrane CSPGs resembles that of the small iduronic acid-rich CSPGs which constitute major fibroblast secretion products (14). Based on cDNA sequencing data (24) these proteoglycans seem to lack the necessary intrinsic properties of hydrophobicity to become directly embedded in the membrane. They are, however, subject to continuous internalization through a process of receptor-mediated endocytosis which involves a lysine recognition marker on the proteoglycan core protein (15), so that these CSPGs may, at least temporarily, become peripherally membrane associated. It seems possible that some of the Sepharose-included CSPGs

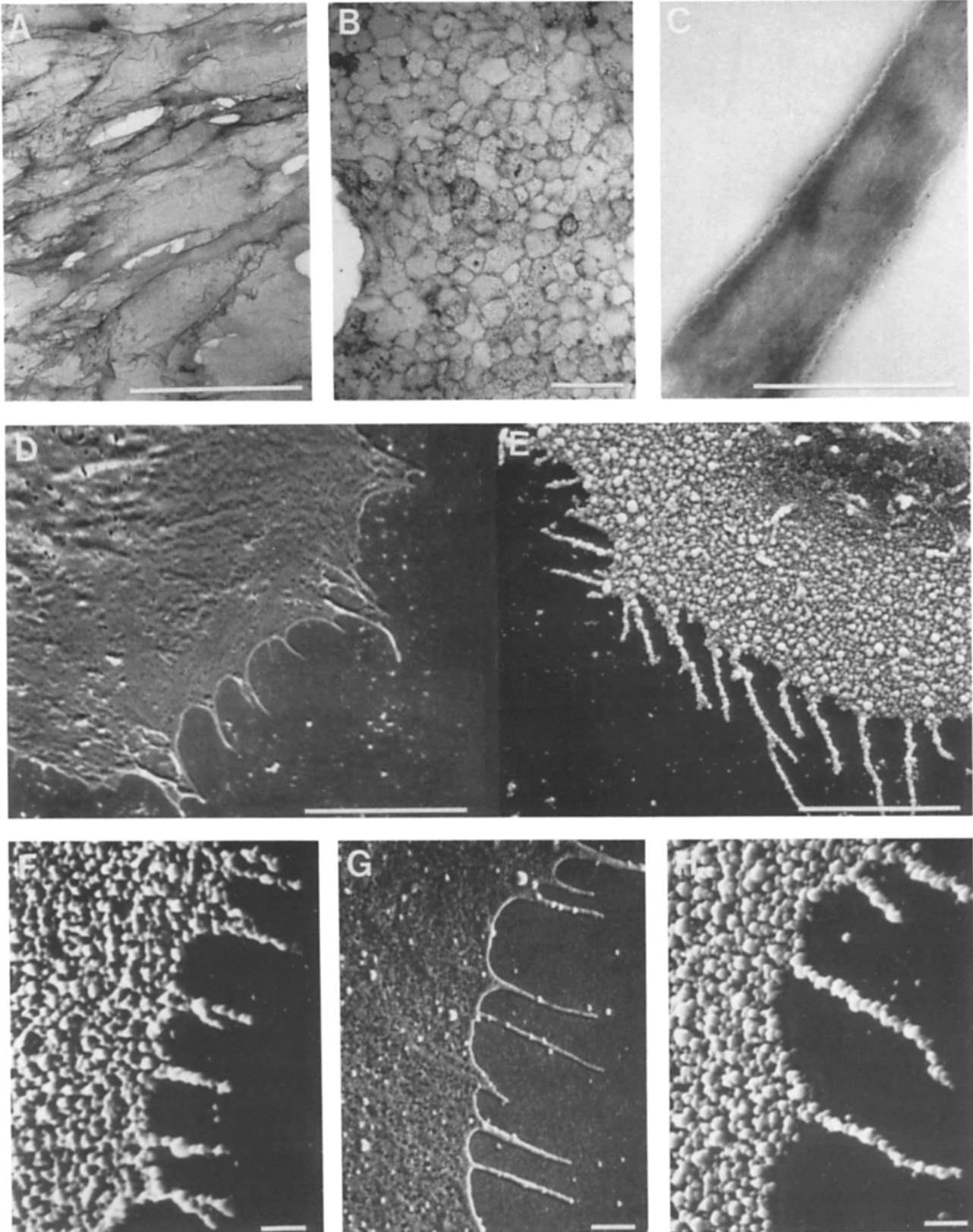


Figure 10. Monoclonal antibody 7D8 decorates the cell surface of cultured human fibroblasts. (A) Cultured human lung fibroblasts and, (B) HBL-100 cells were fixed, stained with mAb 7D8 using an indirect immunoperoxidase technique, and inspected in light microscopy. (C) Ultracryosections of cultured human fibroblasts were labeled with mAb 7D8 using an indirect immunogold technique, and analyzed in transmission electron microscopy. (D and E) Human lung fibroblasts were allowed to adhere and spread on a fibronectin coat for 90 min and fixed. Control cells (D) were labeled with gold-labeled goat anti-mouse Ig only, before silver enhancement. Others (E) were labeled with mAb 7D8 and gold-linked goat anti-mouse Ig followed by silver enhancement, and analyzed by scanning electron microscopy. Binding of mAb 7D8 to the cells was also assessed in the presence of competing DEAE-beads (F), of DEAE-bound CSPG (G), and of DEAE-bound HSPG (H). Bars: (A and B) 100 μ m; (D and E) 10 μ m; (C, F, G and H) 1 μ m.

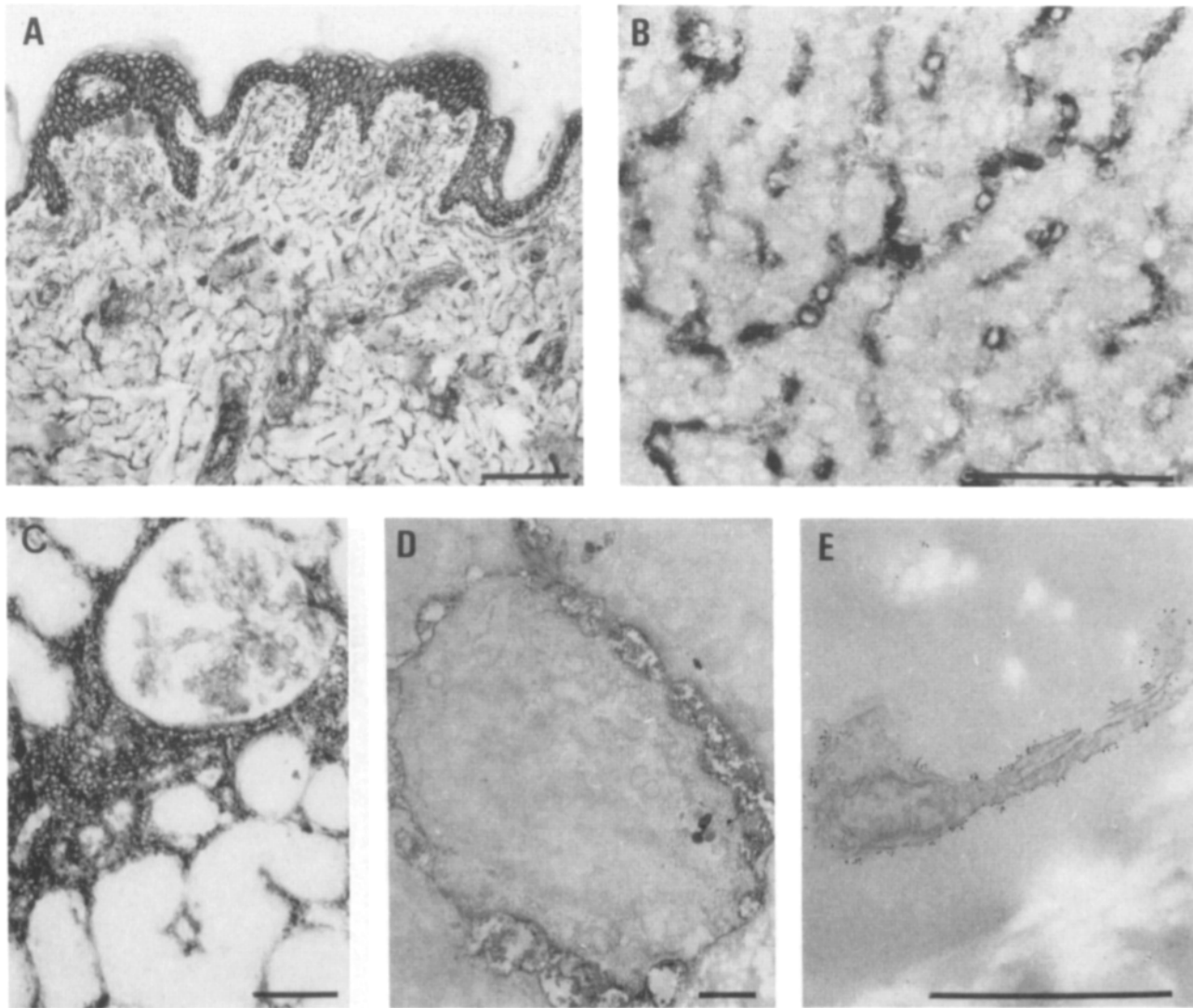


Figure 11. Tissue distribution of the 7D8 epitope. (A) Human skin, (B) liver, and (C) kidney cryosections were treated with mAb 7D8 and stained using an indirect immunoperoxidase technique and analyzed in light microscopy. (D) A human epidermal cell, stained with mAb 7D8 using an indirect immunoperoxidase technique, and (E) a human fibroblast, decorated with mAb 7D8 and gold-labeled goat anti-mouse Ig, viewed in transmission electron microscopy. Bar: (A, B, and C) 100 μm ; (D and E) 1 μm .

present in extracts of fibroblast membranes (Fig. 1 B) may represent such receptor-bound proteoglycans, but it is unlikely that, under the dissociative conditions used for chromatography, receptor-bound proteoglycans would contaminate the micellar aggregates (or liposomes) which remain excluded from the columns. Moreover, the dimension of at least the 7D8-positive core protein largely exceeds that known for the core proteins in these secreted CSPGs. After chondroitinase digestion the core moieties of the latter show some heterogeneity due to the variable substitution of a 38-kD protein with two or three N-linked oligosaccharides and two to four CS chains (14), with core sizes (44–46 kD) that are only half of that obtained for the 7D8-positive core, but close to that of the smaller 7D8-negative hydrophobic cores (see also Fig. 9 B). However, none of the hydrophobic cores reacts with antibodies raised against the core proteins of these secreted proteoglycans (Fig. 9, A and B). Finally, comparing the susceptibility of the CSPGs to chondroitinase

ABC and chondroitinase AC suggested that the small secreted forms are iduronic acid-rich (not shown), while the hydrophobic CSPGs are glucuronic acid-rich (Fig. 4). Thus, the integral membrane CSPGs and the small secreted CSPGs of human lung fibroblasts likely represent distinct molecular species. The nonlipophilic CSPGs of the detergent extracts, in contrast, display structural, functional, and immunological characteristics very reminiscent of those of the secreted CSPGs (Figs. 8 and 9).

Immunohistochemistry indicates that the expression of the 7D8 epitope is not restricted to human fibroblasts (Figs. 10 and 11). Cell surface CSPGs have, in fact, also been reported in other cell types, including cultured rat ovarian granulosa cells (40), human melanoma cells (3, 12), B lymphocytes (13, 32), and neurites (4). In some cells the cell surface CSPGs even appear to be hybrid proteoglycans and carry both heparan sulfate and chondroitin sulfate chains (8). Based on the apparent sizes of their core proteins, on their general

molecular characteristics, and on the apparently limited tissue distribution of some of them, all these CSPGs seem to represent different molecular species. Cell surface proteoglycan complexity appears further enhanced by the observation that at least some core proteins may also have alternative existences. The class II-associated CSPGs in lymphocytes indeed represent alternatively processed forms of the invariant chain proteins (13), whereas the precursor of the melanoma core protein may bypass the glycosaminoglycan assembly step and exist at the cell surface as a 250-kD glycoprotein (16). Positive histochemical findings do therefore not necessarily imply that the detected epitopes are expressed as part of proteoglycans. During the fractionation of cultured fibroblasts and HBL-100 cells, at least, and within the limits of the sensitivity of the blotting assays, all of the 7D8 epitope appeared proteoglycan associated (not shown), which would distinguish this CSPG from the category of "occasional" proteoglycans.

The functions of these cell surface CSPGs are not precisely known, and the apparent multiplicity of possible forms implies that these functions may be equally varied. Interfering with the substitution of the invariant chains with CS chains depresses the sensitization of target cells, consistent with a proposal that the proteoglycan form of the molecule may have a role in antigen processing or presentation (31). The membrane CSPG isolated from the electric organ, on the other hand, is thought to anchor the nerve terminal to the synaptic basement membrane (4), and the concentration of the melanoma proteoglycan on microspikes, microdomains of the cell surface involved in the initial interactions of cells with their neighbors or with the substratum, is also suggestive for a possible role in cell contact and adhesive phenomena (12). The subcellular distribution of the 7D8 epitope in spreading fibroblasts (Fig. 10) is clearly not as restricted as that of the melanoma proteoglycan, possibly reflecting alternative spreading mechanisms in these cells or alternative functions for this proteoglycan. Monoclonal antibody 7D8 did also not interfere with the adhesion or the spreading of the fibroblasts (not shown). The spreading of melanoma cells on endothelial matrix, in contrast, is inhibited by a mAb which reacts with the melanoma CSPG (3), but obviously the functional repercussions of mAb binding will very much depend on the function of the reactive epitopes. On the other hand, some cell surface CSPGs may participate in pericellular processes involving soluble ligands. The activation of heparin cofactor II by human fibroblasts and the concomitant inhibition of thrombin, for example, seems primarily due to the presence of some low relative molecular mass chondroitinase ABC-sensitive proteoglycans in the fibroblast monolayers (29). Since thrombin is mitogenic for fibroblasts, CSPGs of the cell surface with heparin cofactor activating effects could be involved in the modulation of tissue repair responses. Further investigations and the development of additional tools will be required, however, to clarify the function of the membrane CSPGs of human lung fibroblasts and to clarify their relationship to the membrane CSPGs of other cell types.

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