Multiple Distinct Membrane Heparan Sulfate Proteoglycans in Human Lung Fibroblasts*

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The abbreviations used are: HSPG, heparan sulfate proteoglycan; Mab(s), monoclonal antibody(ies); SDS, sodium dodecyl sulfonate; i.e., monomeric core proteins of 125, 90, 64, 48, and 35 kDa and a dimeric-linked dimeric core protein composed of ~35-kDa subunits. By immunizing BALB/c mice with liposome-incorporated HSPG, we have obtained a total of five anti-HSPG monoclonal antibodies (Mabs, i.e. Mabs S1, 1C7, 2E9, 6G12, and 10H4) with different specificities. Polyacrylamide gel electrophoresis of 1251-labeled membrane HSPG immunoprecipitated with these Mabs revealed that Mabs 1C7 and 2E9 bind only membrane HSPG which yield a 125-kDa core protein after heparitinase digestion, whereas Mab S1-bound HSPG yield a 64-kDa core protein, and Mabs 6G12 and 10H4 retain membrane HSPG with a 48-kDa core protein. Western blotting of the heparitinase-digested proteoglycans and immunostaining with the Mabs confirmed this pattern of reactivity. However, in this assay, Mabs 6G12 and 10H4 also detected a minor ~90-kDa core protein in addition to the 48-kDa core protein. Except perhaps for the 10H4 epitope, the epitopes recognized by these Mabs appear to be part of the peptide moieties as they resisted complete deglycosylation of the HSPG with trifluoromethanesulfonic acid. Since these data were inconsistent with a direct relationship between the major core proteins, the 48-, 64-, and 125-kDa core proteins were immunopurified and further compared 48-kDa core protein. The multiplicity of core proteins (35-240 kDa) (Oldberg et al., 1979) to 950,000 (IOZZO, 1984) with molecular weight estimates for the intact proteoglycans range from 75,060 (Oldberg et al., 1979) to 950,000 (Iozzo, 1984) with differences in the size of both the HS chains (14-50 kDa) and the core proteins (35-240 kDa) (Oldberg et al., 1979; Iozzo, 1984; Hiss et al., 1987; Yanagisita and Hascall, 1984). The extent and origin of this heterogeneity are not clear. Species and tissue specificities probably exist, but structural heterogeneity is also observed at the level of a single tissue (Bretscher, 1985; Cöster et al., 1986; Lories et al., 1987).

These cell surface HSPG have been postulated to play a regulatory role in cell adhesion (Cole et al., 1986), cell attachment and spreading (Johansson and Höök, 1984; Woods et al., 1986; Saunders and Bernfield, 1988), maintenance of cell shape (Woods et al., 1984; Rapraeger et al., 1986), growth control (Castellot et al., 1981; Ratner et al., 1985), anticoagulation (Marcus et al., 1986; Nader et al., 1987), and matrix assembly (Hedman et al., 1982; Koda et al., 1985). Possibly, the structural heterogeneity of the cell surface HSPG is a reflection of the functional diversity of these components.

In prior characterizations of the cell surface HSPG of human lung fibroblasts, we have identified four major (and one minor) monomeric core proteoglycans of 125, 90, 64, 48, and 35 kDa and a dimeric core protein composed of disulfide-bonded ~35-kDa subunits (Lories et al., 1987). These results implied that human lung fibroblasts may contain several membrane proteoglycans, but the possibility that the multiple protein forms were generated from a single species in situ by the cells or in vitro during the isolation of the HSPG remained.

In the present report, we identify the 48, the 64-, and the 125-kDa HSPG core proteins as structurally distinct molecules. Each of these core proteins carries unique epitopes which were identified by a panel of monoclonal antibodies. Moreover, protein degradation using Staphylococcus aureus protease V8, trypsin, and CNBr yielded distinct peptide maps for these three core proteins. Thus, the multiplicity of core proteins in human lung fibroblasts stems, at least in part, from the occurrence of several distinct membrane proteoglycan species.

MATERIALS AND METHODS

Cell Culture—Fetal human lung fibroblasts were cultured on plastic substrata in Dulbecco's modified Eagle's medium (GIBCO Europe) containing 10% (v/v) fetal calf serum (David and Van den Berghe, 1982). Confluent monolayers were labeled for 48 h in culture medium containing 1 μCi of carrier-free H235SO4/ml (Du Pont-New England Nuclear).

Isolation of the Hydrophobic Membrane HSPG—Rinsed 35SO42−-labeled human lung fibroblasts were extracted with Triton X-100 buffer (0.1% [v/v] Triton X-100, 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 60 mM 6-aminohexanoic acid, 10 mM EDTA, 5 mM N-ethylmaleimide, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A; 2 × 10 ml/150-cm2 culture flask). This

Cell surface-associated HSPG1 have been isolated from a number of different tissues and cells in culture. The characterization of these HSPG identifies a "family" of HSPG molecules with important structural diversity. Molecular weight estimates for the intact proteoglycans range from 75,060 (Oldberg et al., 1979) to 950,000 (Iozzo, 1984) with differences in the size of both the HS chains (14-50 kDa) and the core proteins (35-240 kDa) (Oldberg et al., 1979; Iozzo, 1984; Hiss et al., 1987; Yanagisita and Hascall, 1984). The extent and origin of this heterogeneity are not clear. Species and tissue specificities probably exist, but structural heterogeneity is also observed at the level of a single tissue (Bretscher, 1985; Cöster et al., 1986; Lories et al., 1987).

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buffer preferentially extracts the membrane-associated proteoglycans of cultured or conditioned human lung fibroblasts (Lories et al., 1986). The resulting extract was cleaved by trypsin (1 mg/ml) in 0.001 M Mops (pH 7.0) and concentrated by absorption on DEAE-Sepharose Fast Flow and Mono Q (Pharmacia). The hydrophobic membrane HSPG were isolated from these concentrated fractions by gel filtration on Sepharose CL-4B, by ion exchange chromatography on Mono Q, and by incorporation into liposomes as described previously (Lories et al., 1986). When chromatographed on Sepharose CL-4B columns in 4 M guanidinium chloride buffer (4 M guanidinium chloride, 100 mM 6-aminohexanoic acid, 10 mM EDTA, 10 mM N-ethylmaleimide, 5 mM benzamidine, 50 μg/ml bovine serum albumin, 10 μg/ml heparin, 10 μg/ml chondroitin sulfate, and 500 μM sodium azide (pH 5.8) containing 0.5% (v/v) Triton X-100, the Triton-extracted [35S]SO₄-labeled membrane proteoglycans elute with Kᵥ = 0.25 ± 0.3 (Lories et al., 1986). A distinct fraction of these HSPG has unique hydrophobic properties.

After the substitution of octyl glucoside for Triton X-100 followed by the addition of phosphatidylcholine and removal of the detergent by dialysis against detergent-free 4 M guanidinium chloride buffer, approximately one-half to two-thirds of the HSPG migrate as lipid-proteoglycan complexes when rechromatographed on the Sepharose columns in 4 M guanidinium chloride buffer without detergent (Lories et al., 1988). The affected proteoglycans coelute with the phosphatidylcholine, which is formed by the dialysis of the detergent at its original positions when treated with detergent. Moreover, this proteoglycan fraction becomes quantitatively reassociated with the lipids vesicles when retested for liposome incorporation. In contrast, the proteoglycans which do not change their chromatographic behavior upon removal of detergent and the addition of lipid are not affected by the addition of detergent and remain unaffected when retested for liposome incorporation. This indicates that the behavior of the proteoglycans in this assay is specific, reflecting unique hydrophobic properties (Lories et al., 1986).

Selection of Monoclonal Antibodies—BALB/c mice were injected with liposome- incorporated purified membrane HSPG according to the immunization scheme described previously (De Boeck et al., 1987). The culture supernatants of the hybridomas were screened by indirect immunofluorescence in microtiter wells using the immunization scheme described previously (De Boeck et al., 1987). Mab F69-3G10 was obtained by immunization of BALB/c mice with liposome-purified membrane HSPG according to the immunization scheme described previously (De Boeck et al., 1987). Mab S1 (De Boeck et al., 1987) and F69-3G10 were used for immunoaffinity chromatography-Mabs 1C7, 2E9, 6G12, 10H4, and S1 were purified from ascites fluid and immobilized on CNBr- activated Sepharose (3 μg of Mab/ml of beads). Radiolabeled HSPG in phosphate-buffered saline containing 100 μg/ml bovine serum albumin, 50 μg/ml heparin, and 0.5% (v/v) Triton X-100 were incubated with the Sepharose Immunobeads (overnight, 4°C). After the incubation, the beads were rinsed with 3 volumes of incubation buffer, with 3 volumes of incubation buffer supplemented with 1 M NaCl, and with 3 volumes of phosphate-buffered saline containing 0.5% Triton X-100. The materials were eluted with 0.1 M guanidinium chloride, 50 mM Tris-Cl, pH 8.0, 0.5% (v/v) Triton X-100 or were recovered by boiling the beads in the presence of 2% SDS, 50 mM Tris-Cl, pH 8.0.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed on gels (4-18% T, 10% C; or 6-26% T, 25% C) gels using the buffer system of Laemmli (1970). The samples were boiled for 5 min in the presence of 2% (v/v) SDS. Samples run under reducing conditions were supplemented with 2% (v/v) β-mercaptoethanol. The gels were prepared for autoradiography as previously (Lories et al., 1987). For fluorography, the gels were soaked in 1 volume of 20% (v/v) Tricine (20%, w/v) and 2.5% dimethyl sulfoxide for 3 h. The gel was left overnight in methanol/ H2O (1:1) and then dried under vacuum. Kodak XAR-5 film was used for autoradiography and fluororadiography at -70°C. For immunostaining, the electrophoresed materials were electrotransferred to E-probe membrane (20 V, 4-5 h) (De Boeck et al., 1987).

Radio labeling of the Core Proteins—Purified membrane HSPG were made carrier free and [35S]-labeled using the chioramine-T procedure or the Bolton-Hunter reagent as described previously (Lories et al., 1987). Because the iodotyrosine linkage is acid-labile, the HSPGs were labeled with [35S]sulfate (10,000 Ci/mmol) and [35S]methionine N-hydroxysuccinimidyl ester, Amersham International), following the same procedure as for the Bolton-Hunter reagent, when the core proteins were to be analyzed after deglycosylation with trifluoromethanesulfonic acid.

The labeled HSPG fractions were resubmitted to ion exchange chromatography on Mono Q gel and gel filtration in Sepharose CL-4B to check the purity of the labeled material (Lories et al., 1987).

Heparan Sulfate Digestion—After the addition of 50 mM 6-aminohexanoic acid, 0.1 M HCl, 100 mM NaCl, and 100 μM heparinase, the purified membrane HSPG were digested with (5–30 μIU/ml) heparin sulfate lyase (EC 4.2.2.8) (2 h, 37°C) in 50 mM Tris-Cl, pH 7.0, 100 mM NaCl, 1 mM CaCl2, as described before (Lories et al., 1987).

Deglycosylation with Trifluoromethanesulfonic Acid—Purified membrane HSPG, labeled in their core protein moiety with [35S] sulfur labeling reagent, were precipitated from a 0.3 M NaCl solution with 0.1% (v/v) cetylpyridinium chloride. The pellet was washed with ice-cold water and dried under N2. For deglycosylation of the [35S] HSPG, the pellet was dissolved in 100 μl of trifluoromethanesulfonic acid (30% v/v) kept under nitrogen for 4 h at 4°C. The reaction mixture was neutralized with pyridine/H2O (1:1) and extracted twice with diethyl ether (Edge et al., 1987). The aqueous phase containing the deglycosylated HSPG was supplemented with 0.5% (v/v) Triton X-100 and dialyzed extensively against phosphate-buffered saline before immunofluorescence chromatography or gel electrophoresis.

Reduction and Alkylation of Disulfide Bonds—Samples in 4 M guanidinium chloride, 50 mM Tris-Cl, pH 8.0, were reduced by dithiothreitol and alkylated with iodoacetic acid as has been described elsewhere (Lories et al., 1987).

Trypsin Proteolysis Digestion—Immunopurified HSPG were reduced and alkylated, treated or not treated with heparitinase as described above, supplied with 0.5% SDS (w/v) and 500 μg/ml bovine serum albumin, and boiled for 5 min. S. aureus protease V8 (Pierce Chemical Co.) was added (100 μg/ml), and the samples were left at room temperature for 10 min before electrophoresis in a 6-26% polyacrylamide gradient gel.

CNBr Degradation—Immunopurified HSPG were reduced and alkylated, treated or not treated with heparitinase, and precipitated with 3 volumes of 95% ethanol (containing 1% sodium acetate). The pelletted materials were dissolved in 70% formic acid containing 50 mg/ml CNBr. The samples were flushed with N2 and left in the dark for 18 h at room temperature. Three volumes of NH4HCO3 (100 mM) were added, and the mixture was evaporated under a stream of N2. The addition of NH4HCO3 and subsequent evaporation were repeated twice. The final pellets were dissolved in 2% SDS, 50 mM Tris-Cl, pH 6.5, boiled for 5 min, and applied on a 6-26% polyacrylamide gradient gel.

Alternatively, heparitinase-digested [35S]-HSPG were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions. The gel containing the deglycosylated core proteins was excised from this "first dimension" gel and was incubated with 75 mg/ml CNBr in 70% formic acid for 6 h (or overnight) at room temperature. The gel slice was then placed on top of a "second dimension" (6-26% gradient) gel for electrophoresis and autoradiography.

RESULTS

Core Protein Heterogeneity—Previous experiments have indicated that a sequence of separation steps which involves selective solubilization with detergent, gel filtration, and ion exchange chromatography in the presence of detergent and chaotropes, and incorporation into liposomes followed by gel filtration, in the absence of detergent yields a fraction of purified hydrophobic heparan sulfate proteoglycans (Lories et al., 1987). Indeed, after radioiodination of this fraction, all incorporated [125I]-labeled proteoglycans by polyacrylamide gradient gel electrophoresis after denaturation with SDS confirms this behavior, revealing a broad labeled smear of high Mr, in the absence
of heparitinase digestion but discrete labeled bands with apparent $M_r$ values of 125,000, 90,000, 64,000, 48,000, and 35,000 after heparitinase digestion (Lories et al., 1987). Reduction of the proteoglycans with dithiothreitol reveals additional heterogeneity, causing the release of a peptide with an $M_r$ of 35,000 from the non-heparitinase-digested proteoglycans. In the experiments described below, we provide evidence which supports this hypothesis.

Heparitinase Digestion Yields Multiple Hydrophobic Core Proteins—As reported previously (Lories et al., 1987), heparitinase digestion of the $^{125}$I-labeled hydrophobic HSPG of human lung fibroblasts yielded multiple bands in SDS-polyacrylamide electrophoresis (Fig. 1, lane 1). To investigate the significance of this heterogeneity, the heparitinase-digested HSPG was resubmitted to liposome incorporation and gel filtration in 4 M guanidinium chloride in the absence of detergent. After liposome incorporation, most of the $^{125}$I-labeled material eluted from Sepharose CL-4B with a $K_{av}$ of $-0.3$ (Fig. 2). In contrast, when the sample was supplemented with detergent and chromatographed on a similar column in the presence of detergent, a single peak was eluted with a $K_{av}$ value of $-0.5$ (Fig. 2). Thus, most of the labeled heparitinase-resistant proteoglycan core proteins had retained the hydrophobic properties of the parent proteoglycan molecules. Analysis of the hydrophobic cores by SDS-polyacrylamide gel electrophoresis and autoradiography after reincorporation into liposomes and gel filtration (Fig. 1, lane 2) identified the same kinds of bands as found for the starting materials (Fig. 1, lane 1). However, the relative density of the 35-kDa band was markedly decreased, indicating that most of these core proteins did not have a hydrophobic domain. On the other hand, after Western blotting of the heparitinase-digested hydrophobic HSPG and incubation with Mab 69-3G10, bands at 35, 48, 64, 90, and 125 kDa could be stained (Fig. 1, lane 3). This Mab, obtained after immunization of mice with heparitinase-digested HSPG, was found to react with HSPG and with (some fragments of) isolated heparan sulfate chains of several different sources (cells, matrix, culture medium) provided these were digested with heparitinase. Moreover, the reaction of Mab 3G10 with the heparitinase-digested HSPG was specifically inhibited by the presence of heparitinase-digested heparan sulfate but not by heparitinase or heparan sulfate added separately nor by chondroitin ABC-lyase-digested chondroitin sulfate chains. Thus, Mab 3G10 likely identifies (a) heparitinase digestion product(s).

These results imply that if the fragmentation of a single proteoglycan would be responsible for the occurrence of different core proteins, the major 125-, 64-, and 48-kDa fragments must be partially overlapping and at least share the sequences spanning the distance from the hydrophobic domain to at least part of the heparan sulfate-carrying domain.

Immunologically Distinct HSPG Core Proteins—We have previously reported the isolation of Mab S1 following the immunization of BALB/c mice with purified membrane HSPG (De Boeck et al., 1987). We demonstrated that the epitope recognized by Mab S1 copurified with the membrane HSPG of human lung fibroblasts and that it was part of an HSPG core protein (De Boeck et al., 1987). Following a similar immunization scheme and by the same criteria for selection of the hybridomas, we have now obtained four more anti-HSPG Mab-secreting hybridomas (i.e. 1C7, 2E9, 6G12, and 10H4). As for Mab S1, the materials which carried the epitopes that were recognized by these Mabs copurified with the HSPG fractions from human lung fibroblasts and were reduced in size upon treatment with heparitinase (results not shown).

In the present study, we used these Mabs to investigate the possible immunological relationships between the different membrane HSPG core proteins. Initial experiments with S1 had shown that this Mab recognized only HSPG which contained a monomeric core protein of 64 kDa, irrespective of whether the HSPG were treated with heparitinase before or

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2 G. David, unpublished results.
after immunoprecipitation (Lories et al., 1987). When Mabs 1C7, 2E9, 6G12, and 10H4 were immobilized on CNBr-activated Sepharose and incubated with [35S]labeled heparitinase-digested membrane HSPG, each Mab bound ∼10–15% of the labeled HSPG. Similar results were obtained in different experiments and with lower HSPG-antibody proportions. The bound materials were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Autoradiography revealed that Mabs 1C7 and 2E9 had bound a 125-kDa core protein, while Mabs 6G12 and 10H4 bound a 48-kDa protein (results are not shown but are as in Fig. 5, lanes 2, 5, 8, and 11). The starting heparitinase-digested membrane HSPG fraction, in contrast, contained protein bands of 125, 90, 64, 48, and 35 kDa (Fig. 3, lane 10) as described before (Lories et al., 1987). In other experiments, the immobilized Mabs were incubated with nondigested membrane HSPG. After elution of the bound materials, half of each sample was treated with heparitinase and applied on SDS-polyacrylamide (4-16%) gradient gel electrophoresis and autoradiography. Digestion of the bound materials, half of each sample was treated with heparitinase and applied on SDS-polyacrylamide (4-16%) gradient gels before Western blotting. Lane 1 was immunostained with Mab 1C7, lane 2 with Mab 2E9, lane 3 with Mab 6G12, lane 4 with Mab 10H4, and lane 5 with Mab S1.

Thus, the “smaller” membrane HSPG (i.e., those with the 48- and the 64-kDa core proteins) carry epitopes which reside in their core protein and which are absent from the “larger”...
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**FIG. 5.** Effect of deglycosylation on the immunoreactivity of the membrane HSPG. Non-heparitinase-digested (lanes 1, 4, 7, 10, and 13), heparitinase-digested (lanes 2, 5, 8, 11, and 14), and trifluoromethanesulfonic acid-treated (lanes 3, 6, 9, 12, and 15) 35S-membrane HSPG were applied on Sepharose-linked Mabs 1C7 (lanes 1–3), 2E9 (lanes 4–6), 6G12 (lanes 7–9), 10H4 (lanes 10–12), and S1 (lanes 13–15). Bound materials were released by boiling the beads in 2% (w/v) SDS and applied on an SDS-polyacrylamide (4–18% gradient) gel before fluorography.

**FIG. 6.** S. aureus protease V8 peptide mapping of the membrane HSPG. “48-,” “64-,” and “125-kDa” HSPG were isolated by immunoprecipitation of 125I-HSPG on Sepharose-linked Mabs 6G12 (lanes 1, 4, 7, and 10), S1 (lanes 2, 5, 8, and 11), and 2E9 (lanes 3, 6, 9, and 12), respectively. The bound 125I-HSPG were eluted in guanidinium chloride buffer, reduced and alkylated, and ethanol-precipitated (75%, v/v). The 125I-HSPG were applied on an SDS-polyacrylamide (6–26% gradient) gel either after heparitinase treatment alone (lanes 1–3), after S. aureus protease V8 digestion (lanes 4–6), or after combined heparitinase and protease V8 digestion (lanes 7–9). Lanes 10–12: same as lanes 4–6 but after prolonged exposure of the gel.

(i.e. those with 125-kDa core proteins) HSPG. Therefore, a direct precursor-product relationship between these different HSPG is not suggested.

Peptide Maps of the Major Membrane HSPG Core Proteins—To investigate further the structural relationship among the different membrane HSPG core proteins, we compared the peptide maps of the three major membrane HSPG forms. The “125-,” “64-,” and “48-kDa” 125I-HSPG forms were isolated by immunoprecipitation using Mabs 2E9, S1, and 6G12. These HSPG fractions were reduced and alkylated and digested with S. aureus protease V8 or with trypsin or cleaved with CNBr. Autoradiography of the electrophoretically separated peptides showed distinct patterns for the “48-,” the “64-,” and the “125-kDa” HSPG.

Without prior heparitinase treatment, all the protease V8 digests of the HSPG samples contained labeled materials that barely entered the gels. In addition to these M, materials, protease V8 treatment of the non-heparitinase-digested “48-kDa” HSPG yielded (a) ~14-kDa fragment(s) (Fig. 6, lane 4). After prolonged exposure of the gel, a 24-kDa fragment was also visualized (Fig. 6, lane 10). The “64-kDa” HSPG, on the other hand, yielded three major fragments of 36, 16, and ~10 kDa and two minor fragments of 42 and 28 kDa (Fig. 6, lanes 5 and 11). The “125-kDa” HSPG, in contrast, was more susceptible to the protease than the “64-kDa” HSPG and yielded a smear of peptides ranging from ~12 to 19 kDa. When the HSPG were digested with heparitinase before treatment with protease V8, label was no longer retained in the high M, region of the gels. In addition, an extra band at 20 kDa resulted from the 64-kDa core protein, but no other marked differences followed from the heparitinase treatment for the “48-kDa” and the “125-kDa” HSPG. These data allow two conclusions. First, the 64-kDa core cannot be a fragment of the 125-kDa core since it yields larger V8-resistant peptides than the 125-kDa core. Second, in the “48-” and “125-kDa” forms of the proteoglycans, the HS chains are clustered on a small V8-resistant domain of the core proteins, or the HS-carrying domains of these proteoglycans are largely V8-susceptible but efficiently protected from degradation by the enzyme when the chains are still present since no extra bands are observed in the corresponding heparitinase-digested samples.

Exhaustive digestion of the HSPG with trypsin yielded fragments of 32, 26, and 15 kDa for the “48-kDa” HSPG (Fig. 7, lane 4), whereas two major fragments of ~15 and 12 kDa were obtained for the “125-kDa” HSPG (Fig. 7, lane 6). The “64-kDa” HSPG, in comparison, was more susceptible to trypsin, yielding faint bands at ~15 and ~8 kDa (Fig. 7, lane 5), and therefore does not seem to contain the 48-kDa core. The digestions of the heparitinase-treated samples were less extensive (due to the presence of competing trypsin inhibitors in the heparitinase buffer) and generated a distinct pattern for each of the HSPG (Fig. 7, lanes 7–9).

CNBr degradation of the three membrane HSPG also resulted in clearly distinct peptide patterns. CNBr liberated an ~13-kDa fragment from non-heparitinase-digested “48-kDa” HSPG (Fig. 8, lane 4); 13-, 18-, 26-, 33-, 38-, and 52-kDa fragments from the “64-kDa” HSPG (Fig. 8, lane 5); and some large 80-, 100-, and 112-kDa fragments from the “125-kDa”
Finally, to relate these major core proteins to those which could not be purified by immunoaffinity chromatography, the electrophoretically separated core proteins were submitted to *in situ* CNBr mapping (Fig. 9). The lanes derived from the minor ~90-kDa core protein and from the 48-kDa core protein showed partial similarity, consistent with a possible relationship, as already suggested by the immunostaining results. Partial similarity was also observed between the lanes derived from the 64- and the 35-kDa core protein (Fig. 9). In conclusion, immunochemical data and peptide mapping suggest that the hydrophobic membrane proteoglycans of human lung fibroblasts may belong to at least three different groups: one yielding the 48/90-kDa core proteins; another, the 64/35-kDa core proteins; and finally, a third yielding the 125-kDa core protein.

**DISCUSSION**

Fetal human lung fibroblasts synthesize HSPG which are associated with the cell membrane through a hydrophobic moiety (Lories *et al.*, 1986). After heparitinase treatment, 125-, 90-, 64-, 48-, and 35-kDa monomeric core proteins and a dimeric core protein composed of disulfide bond-linked 35-kDa subunits have been isolated from these HSPG (Lories *et al.*, 1987). Here, we present data that indicate that the monomeric 125-, 64-, and 48-kDa core proteins are structurally distinct molecules.

In a previous report, we identified a monoclonal antibody (Mab S1) which is directed toward an epitope unique to the 64-kDa core protein (De Boeck *et al.*, 1987). We have now obtained four more anti-HSPG Mabs (*i.e.*, 1C7, 2E9, 6G12, and 10H4). In immunoaffinity chromatography, each of these antibodies recognizes only one of the major HSPG core proteins. Mabs 1C7 and 2E9 bind the HSPG with a 125-kDa core protein, whereas Mabs 6G12 and 10H4 bind HSPG with a 48-kDa core protein (Fig. 3). Only a single band is obtained whether the HSPG are treated with heparitinase before or after the immunoprecipitation, indicating that a possible proteolytic activity occurring during the heparitinase digestion is not responsible for the generation of multiple core protein forms.

The epitopes recognized by Mabs 1C7, 2E9, 6G12, and S1 are part of the peptide moieties of the HSPG as they resist deglycosylation with trifluoromethanesulfonic acid. The 10H4 epitope, in contrast, is trifluoromethanesulfonic acid-sensitive. It may, therefore, include a glycosidic component and is thus distinct from the 6G12 epitope. Also, the two 125-kDa-specific Mabs recognize distinct epitopes as they react with different proteolytic fragments of the core protein (results not shown). Thus, these data suggest that the "smaller" membrane HSPG forms carry epitopes (in their core protein moiety) that are not present or detected in the "larger" HSPG molecules. Therefore, the "48-kDa" and the "64-kDa" membrane HSPG forms do not seem to be fragments of the "125-kDa" HSPG resulting from some kind of proteolytic processing or from fragmentation during the isolation of the membrane HSPG.

However, the results obtained in immunoaffinity chromatography are not in complete agreement with the Western blot results. In the latter assay, both Mabs 6G12 and 10H4 detected an ~90-kDa core protein in addition to the 48-kDa core protein. Possibly, conformational restraints exist in the soluble ~90-kDa core proteins which interfere with Mab binding, whereas these restraints may be alleviated in nylon membrane-bound 90-kDa cores and may not exist in either the soluble or the membrane-bound 48-kDa core proteins. On the other hand, the ~90-kDa species represent only a minor

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**Fig. 8. CNBr peptide mapping of the membrane HSPG.** "48," "64," and "125-kDa" HSPG were immunopurified on Sepharose-linked Mabs 6G12 (lanes 1, 4, and 7), S1 (lanes 2, 5, and 8) and 2E9 (lanes 3, 6, and 9), respectively. The bound 125I-HSPG were eluted in guanidinium chloride buffer, reduced and alkylated, and ethanol-precipitated (75%, v/v). The 125I-HSPG were applied on an SDS-polyacrylamide gel under reducing conditions. The gel was sliced, and the lane containing the separated materials that did not enter the gels. Thus, high M proteins do not enter the gels. In addition to these fragments, all lanes contained high M, materials that did not enter the gels. CNBr degradation preceded by heparitinase digestion of the hydrophobic membrane proteoglycans of human lung fibroblasts may belong to at least three different groups: one yielding the 48/90-kDa core proteins; another, the 64/35-kDa core proteins; and finally, a third yielding the 125-kDa core protein.

**Fig. 9. CNBr peptide mapping of membrane HSPG in gel slices.** Heparitinase-digested 125I-membrane HSPG were electrophoresed on a 4–16% gradient SDS-polyacrylamide gel under reducing conditions. The gel was sliced, and the lane containing the separated core proteins was incubated with CNBr and then layered on top of a second 6–26% gradient SDS-polyacrylamide gel. The CNBr-generated peptides were separated by overnight electrophoresis. The *arrows* indicate the positions of the 125I-HSPG core proteins in the gel slice of the first dimension gel.
fraction of the labeled cores (see Fig. 3, lane 10). Assuming similar binding affinities, immunopurification is expected to isolate the 48- and ~90-kDa core proteins in relative proportions in which they actually occur, rendering the ~90-kDa core protein more difficult to detect than the 48-kDa core protein. In contrast, blotting of electrophoretically separated core proteins on membranes with a limiting binding capacity would relatively be in favor for the detection of the less abundant forms. Whatever the cause, this result indicates that the 48- and the ~90-kDa cores have structural features in common. Moreover, this similarity pertains to two distinct epitopes as indicated by the results of the trifluoromethanesulfonic acid treatment. Although not contradictory to the conclusions concerning the relationship between the 48-, 64-, and 125-kDa core proteins, the Western blot results indicate that the immunoaffinity results should be interpreted with caution.

The preliminary conclusion that the 48-, 64-, and 125-kDa core proteins are not fragments from each other is, however, also supported by the results obtained from the peptide-mapping data. The 48- and 64-kDa core proteins contain V8-resistant fragments which are larger than the V8 peptides generated from the 125-kDa core proteins, and the 48-kDa core protein, in turn, is more trypsin-resistant than the 64-kDa core protein. Also, CNBr degradation yields distinct peptide patterns for the three HSPG core proteins. Interestingly, the heparan sulfate-carrying domains of the different HSPG core proteins behave differently during the peptide mapping. The 64-kDa core protein contains a 20-kDa V8-resistant heparan sulfate-bearing domain not present e.g. in the 125-kDa core protein. On the other hand, CNBr releases 52- and 112-kDa heparitinase-resistant fragments from the 64- and the 125-kDa HSPG core proteins, respectively, indicating that their heparan sulfate chains are clustered on an ~10-kDa terminal CNBr fragment. For the 48-kDa core protein, in contrast, one can only conclude from the CNBr experiment that a fragment of at least 13 kDa separates the heparan sulfate chains from one of the termini of the core. As a whole, the absence of immunological cross-reactivity and of similarities in the peptide patterns does not support a model which proposes that these three core proteins would be partially overlapping fragments of a single proteoglycan which would at least share the hydrophobic domain and part of the heparan sulfate-carrying region (Fig. 1).

The second dimension electrophoresis of in situ CNBr-degraded HSPG core proteins, however, revealed a possible relation between a minor 90-kDa and the 48-kDa core proteins and between the 64- and 35-kDa core proteins. For the ~90- and 48-kDa core proteins, this confirms the results obtained by immunostaining of Western blots where both core proteins react with both Mabs 6G12 and 10F4. A relationship between the 64- and some 35-kDa core proteins may easily be explained. Before the in situ CNBr degradation, the HSPG were electrophoretically separated under reducing conditions so that in the second dimension electrophoresis, the lane containing the 35-kDa materials consisted of both the monomeric 35-kDa core protein and the 35-kDa subunits of the disulfide-bonded dimeric core protein. This latter HSPG might be generated by partial degradation of the monomeric 64-kDa core protein at a site located between the attachment points of an intrachain disulfide bond. Such intrachain disulfide bonds do occur since reduction of the 64-kDa core protein abolishes the reactivity with Mab S1 and slightly retards the migration of the core protein during electrophoresis (De Boeck et al., 1987). This partial degradation of the monomeric 64-kDa core protein would also destroy the S1 epitope since the dimeric core and the 35-kDa subunits do not react with Mab S1 (De Boeck et al., 1987). The possibility that some of the 35-kDa core proteins are degradation products of larger core proteins seems also to be illustrated by the reincorporation of isolated heparitinase-treated membrane HSPG into liposomes. Obviously, most of the 35-kDa core proteins were not retained in the liposome-incorporated HSPG fraction (Fig. 1, lane 2). Thus, fetal human lung fibroblasts synthesize at least three distinct membrane-associated HSPG types: one with 48/90-kDa core proteins; one with 35/64-kDa core proteins; and a third with a 125-kDa core protein.

When observing this remarkable complexity of the membrane-associated heparan sulfate proteoglycans, concern may be raised regarding the covalent nature of the heparan sulfate-protein association in these samples. Several considerations, however, argue against the occurrence of heparan sulfate-protein complexes as the source of the multiple labeled bands. First, the proteoglycans have been purified and analyzed under highly dissociative and denaturing conditions. Nonspecific ionic interactions and specific protein-carbohydrate interactions (e.g. like those occurring between heparin and antithrombin) may reasonably be assumed to be disrupted under these circumstances. Yet, the iodinated molecules behave as authentic proteoglycans during chromatography, and no discrete bands can be observed upon electrophoresis in SDS-containing gels unless the proteoglycans are heparitinase-digested. Second, the addition of tracer [35S]heparan sulfate, prepared by protease digestion of the proteoglycan samples, to the initial extracts showed no evidence for complex formation during the isolation procedure (not shown). Third, complete N-desulfation of the [35S]-proteoglycans does not alter their apparent size (Lories et al., 1987). Yet, this treatment dramatically reduces the net negative charge of the component heparan sulfate chains and is expected to affect ionic and specific complex formation. Fourth, the buffers used for chromatography during the purification of the samples contain an excess of carrier heparin which will compete with the endogenous chains for heparan sulfate-binding proteins. Heparin-protein complexes which might contaminate the final proteoglycan samples would be sensitive to heparinase but not to the heparitinase which is used for the analysis of the proteoglycans. Thus, it seems reasonable to conclude that the labeled bands which are observed after heparitinase digestion of the preparations represent authentic core proteins.

Multiple hydrophobic HSPG forms have also been isolated from human embryonic skin fibroblasts (Coster et al., 1986). Postconfluent skin fibroblasts synthesize hydrophobic HSPG which have a core that is composed of two disulfide-linked 90-kDa proteins and which is able to bind transferrin (Franson et al., 1984). No evidence could be obtained for a HSPG with similar size, subunit composition, and transferrin-binding properties in the cells studied here. Proliferating skin fibroblasts, however, also synthesize a detergent-extractable HSPG with a hydrophobic core protein of 50 kDa (Coster et al., 1986). This type of HSPG does not bind to transferrin. Having a similar size, this latter membrane HSPG might be related to the 48-kDa core protein described in the present report. However, the 50-kDa skin fibroblast core protein partly decomposes in 24- and 33-kDa peptides after reduction of disulfide bonds, a feature which the 48-kDa lung fibroblast core protein lacks but which is rather reminiscent of the dimeric 64-kDa lung fibroblast HSPG core protein which is composed of two disulfide-linked (~35-kDa) subunits (Lories et al., 1987). The discrepancy in M, estimates might be explained by the difference of techniques used: SDS-polyacrylamide gradient gel electrophoresis in our investigations (Lor-
ies et al., 1987) versus gel filtration by others (Coster et al., 1986). In these studies on human skin fibroblasts, the expression of the HSPG was found to be determined by the state of confluency of the cells, as e.g. only postconfluent cells synthesized the transferrin-binding core protein (Coster et al., 1986). In the present study, we only used human lung fibroblasts which were grown to confluency, but in some experiments, differences in the relative amounts of the minor and major core proteins were also noticed (compare Fig. 1, lane 1 with Fig. 3, lane 10). Together, these observations may indicate that the expression of cell surface HSPG indeed fluctuates and that it is possibly regulated.

In conclusion, fetal human lung fibroblasts synthesize at least three types of membrane-associated HSPG with structurally distinct core proteins of 125, 48/90, and 35/64 kDa. It is tempting to speculate that each of these HSPG might be predestined to fulfill one of the multiple functions that have been ascribed to the membrane-associated HSPG. Whether this multiplicity of membrane HSPG core proteins is restricted to fibroblasts or is a generally occurring phenomenon, however, remains also to be elucidated.

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