Identification of the Precursor Protein to Basement Membrane Heparan Sulfate Proteoglycans*

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The precursor protein of a basement membrane specific heparan sulfate proteoglycan has been identified as a 400,000 M₉ polypeptide. Antibodies against large and small forms of this proteoglycan, isolated from a basement membrane (Engelbreth-Holm-Swarm, EHS) tumor, immunoprecipitated the same 400,000 protein from pulse-labeled EHS cells. The proteoglycan precursor protein was not recognized by antibodies against other basement membrane components or by antibodies to the cartilage proteoglycan. Furthermore, heparan sulfate proteoglycan purified from the EHS tumor blocked the immunoprecipitation of the precursor protein. Pulse-chase studies with [³⁵S]methionine showed the precursor protein was converted to a proteoglycan. Pulse-chase studies with [³⁵S]SO₄ showed the large, low density proteoglycan appeared first and was degraded to a smaller, high density proteoglycan. We propose that the precursor protein is used after very little or no modification in the assembly of a large, low density heparan sulfate proteoglycan and that a portion of the population of these macromolecules are subsequently degraded to a smaller form.

Proteoglycans are a class of glycoproteins that are highly glycosylated and sulfated. These molecules are widely distributed in the body and are prominent as components of extracellular matrices. Various studies indicate that proteoglycans are tissue specific and are distinguished from one another by their carbohydrate chains as well as by their core proteins. For example, antibodies to proteoglycans isolated from cartilage localize, respectively, to those tissues and show that portions of their protein core are similar.

In recent studies, we have isolated a large (M₉ 300,000) and a small heparan sulfate proteoglycan (M₉ 350,000) from the EHS tumor (21), which is often used as a source of basement membrane components. Antibodies raised against each proteoglycan react with the extracellular matrix in the EHS tumor and with native basement membranes in various tissues suggesting that they contain similar macromolecules. Competitive immunoassay experiments showed that the large and small proteoglycan have common antigenic determinants suggesting that portions of their protein core are similar.

The results indicate that a 400,000 protein is converted to a large, low density proteoglycan which can then be degraded to a smaller, high density proteoglycan. The 400,000 precursor protein is immunologically distinct from other large connective tissue proteins such as laminin and the precursor protein to the cartilage proteoglycan.

MATERIALS AND METHODS

Large and small heparan sulfate proteoglycans were isolated from the EHS tumor as described (21). Briefly, sequential extraction of the tissue resulted in an initial separation of the large and small proteoglycans. The tissue was first extracted in a solution of 0.15 M NaCl, 0.05 M Tris-HCl, pH 6.8, 0.1 M 6-aminohexanoic acid, 0.04 M EDTA, 8 M N-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride. This extraction yielded mostly the small proteoglycan. The insoluble residue was then extracted in the saline solution described above but containing 7 M urea. This extraction yielded most of the large proteoglycan. Proteoglycans were further purified by column chromatography on DEAE-Sephacel. The material that bound was recovered and centrifuged in gradients of CsCl. Finally, the proteoglycan-enriched fractions from the CsCl gradients were chromatographed on a column of Sepharose CL-4B.

Antibodies against large and small heparan sulfate proteoglycans were prepared in rabbits by subdermal injection of 0.5 ml of saline/Freund's complete adjuvant emulsion containing 500 µg of the purified proteoglycan. The animals were boosted 4 and 6 weeks later with 0.5 ml of saline/Freund's incomplete adjuvant containing 300 µg of proteoglycan. The titer of antisera to the proteoglycan was measured by the enzyme-linked immunosorbent assay (22). Chondroitin sulfate proteoglycan was isolated from a rat chondrosarcoma using standard procedures (23) and antibodies against this proteoglycan were prepared in rabbits, as described above, and also obtained from Drs. Nancy Schwartz (University of Chicago, Chicago, IL) and Srinivasan Chandrasekhar (National Institutes of Health, Bethesda, MD). Antibodies against laminin were obtained from Dr. Hynda Kleinman (National Institutes of Health, Bethesda, MD).

Culture of EHS Tumor Cells—Initially, we pulse-labeled minced EHS tumor tissue with [³⁵S]methionine in culture and prepared

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1 The abbreviations used are: EHS, Engelbreth-Holm-Swarm; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodeyl sulfate; CHAPS, 3-[3-Cholamidopropyl]dimethylammonio]-1-propane sulfonate.
extracts of the tissue. However, antibodies to the proteoglycan failed to precipitate any of the labeled proteins. Since it was possible that the cultured tissue contained an excess of matrix-associated proteoglycan, we established conditions for isolating viable, matrix-free cells from the EHS tumor.

Freshly harvested EHS tumor, maintained in mice by serial passage as previously described (24), was pressed through a stainless steel sieve with 0.75-mm mesh openings. The tissue was placed in Ca"+/- Mg"+ free Hank's solution containing 30 mM Hepes buffer, pH 7.4, dispensed with repetitive pipetting, and then pelleted by low speed centrifugation. The pellet was resuspended in fresh Ca"+/- Mg"+ free Hank's solution containing 2.4% Dispase (Boehringer Mannheim) and incubated at 37°C for 45 min with gentle rocking. At that time, an equal volume of NCTC 109 containing 10% fetal calf serum was added and the cells were collected by centrifugation. The cells were resuspended in fresh culture medium (NCTC 109 with 10% fetal calf serum) and then passed through a 75-micron mesh Nitex screen to remove undissociated tissue. Cells that passed through the filter were collected by centrifugation, resuspended in fresh medium (NCTC 109 with 10% fetal calf serum) and plated at 4.0 x 10^6 cells/35-mm dish. Cells were radiolabeled after 18 h in culture.

Chondrocyte cell cultures were prepared from the Swarm rat chondrocyte line (19) according to the procedure of Kimura et al. (25).

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RESULTS

Cells were isolated from the EHS tumor after digesting the extracellular matrix with Dispase and plated at high cell density. The cells attached to tissue culture plastic (Fig. 1), but did not flatten, even after several days in culture. The incorporation of [35S]sulfate into proteoglycan was monitored by autoradiography shows that the isotope was incorporated with 0.5 g of guanidine HCl/ml. The unincorporated radioactivity was removed by acid digestion followed by chromatography on Sephadex G-50 (4). Aliquots, mixed with an equal amount of 70% ethanol, were allowed to swell in NCS (Amersham) according to the manufacturer's instructions and the radioactivity was measured using a liquid scintillation counter. Molecular weight of proteins under study were determined by comparison to high molecular weight standards obtained from Miles Laboratories or was purified from flavobacterium according to the procedure of Linker (28) as modified by Klein, et al. (29). Heparan sulfate side chains were removed from the low density heparan sulfate proteoglycan by digestion with heparitinase (1 unit of enzyme/100 g of proteoglycan) in 20 mM Tris-HCl, pH 7.4, containing 1 mCi CaCl_2, 37°C for 60 min. The action of the enzyme was stopped by heating at 100°C for 3 min.

\[ ^{35}S \text{SO}_4, \text{Radiolabeling} - \text{Cells were radiolabeled in 1.0 ml of medium containing 250 or 1000 } \mu \text{Ci of } ^{35}S \text{SO}_4, \text{(Amersham) for 20 min. The medium was removed at the end of the labeling period and the cell layer re-suspended with 0.5 g of guanidine HCl/ml. The unincorporated radioactivity was removed by acid digestion followed by chromatography on Sephadex G-25 (Pharmacia) using a solvent containing 0.5% CHAPS, 4 mM guanidine HCl, 0.05 mM sodium acetate, 0.01 mM EDTA, 0.005 mM benzamidene-HCl, and 0.1 mM 6-aminohexanoic acid, pH 6.5. The media plus rinse was adjusted to contain 4 mM guanidine HCl by the addition of 0.5 g of guanidine HCl/ml. The unincorporated radioactivity was removed by acid digestion followed by chromatography on Sephadex G-50 (4).} \]

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Enzyme and Chemical Degradation—Heparitinase was purchased from Miles Laboratories or was purified from flavobacterium according to the procedure of Linker (28) as modified by Klein, et al. (29). Heparan sulfate side chains were removed from the low density heparan sulfate proteoglycan by digestion with heparitinase (1 unit of enzyme/100 g of proteoglycan) in 20 mM Tris-HCl, pH 7.4, containing 1 mCi CaCl_2, 37°C for 60 min. The action of the enzyme was stopped by heating at 100°C for 3 min.

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preparation from the EHS tumor immunoprecipitated a single antibody against the large heparan sulfate proteoglycan cells/35-cm² culture dish and photographed after 24 h. Magnification × 200.

FIG. 1. EHS cells in culture. EHS cells were plated at 4 × 10⁶ cells/35-cm² culture dish and photographed after 24 h. Magnification × 200.

These results show that antibodies to both the large and small forms recognized the same protein, which indicates that both forms share common antigenic determinants. Antibodies against other basement membrane components, such as laminin (Fig. 2, lane C), type IV collagen, and entactin (not shown) did not recognize this 400,000 Mr protein. Antibodies against a large cartilage-specific chondroitin sulfate proteoglycan were also tested because this proteoglycan is also an extracellular matrix molecule and its precursor core protein is of similar (Mr = 370,000) size (2). These antibodies did not precipitate any proteins produced by EHS cell cultures (Fig. 2, lane F). This antibody, however, did precipitate a 370,000 molecular weight protein from extracts of [³⁵S]methionine-labeled cultured chondrosarcoma cells (not shown).

We next confirmed that the 400,000 protein precipitated by antibodies to the basement membrane proteoglycan is the precursor to the core protein of the basement membrane proteoglycan. In this and subsequent experiments, we used antibodies directed against the large heparan sulfate proteoglycan because it was shown to have a larger core protein than the small proteoglycan (21) and we assumed that these antibodies recognize a greater number of antigenic determinants on the precursor core protein (21). The precipitation of the precursor core protein was blocked in a dose-dependent manner by the addition of increasing amounts of heparitinase-treated proteoglycan (Fig. 3, lanes C2-7). Addition of heparitinase or heparitinase and bovine serum albumin (Fig. 3, lanes D and E) did not interfere with the immunoprecipitation of the precursor protein from the EHS cells, indicating that it was the added proteoglycan core protein that blocked the

Fig. 2. Fluorograph of [³⁵S]methionine-labeled proteins extracted from pulse-labeled EHS cells. EHS cells were labeled with [³⁵S]methionine for 10 min, the cells were then lysed, and aliquots were pre-adsorbed with protein A-Sepharose and preimmune serum. Then the proteins in the lysate were immunoprecipitated with antibodies to proteoglycan and laminin. Immunoprecipitates were solubilized in electrophoresis sample buffer containing dithiothreitol and electrophoresed on a 4–15% gradient (SDS) polyacrylamide gel and a fluorograph of the gel was prepared. Lane A, whole cell lysate; lane B, material precipitated by preimmune serum; lane C, material precipitated by antibodies against laminin; lane D, material precipitated by antibodies against the larger, low density heparan sulfate proteoglycan; lane E, material precipitated by antibodies against the smaller, high density proteoglycan; lane F, material precipitated by antibodies against a chondroitin sulfate proteoglycan from the Swarm rat chondrosarcoma.
Presence of heparitinase alone blocked in a dose-dependent manner by basement membrane antiserum to immunoprecipitate the proteoglycan precursor pro-

heparitinase for 60 min, and the reaction stopped by boiling for 3 min. The protein core was then added, in increasing amounts, to antiserum against the large heparan sulfate proteoglycan before using the antiserum to immunoprecipitate the proteoglycan precursor protein from aliquots of the cell lysate. Immunoprecipitates were solubi-

lized in sample buffer containing dithiothreitol and electrophoresed on a 4–15% gradient (SDS) polyacrylamide gel. A fluorograph of that gel is shown here. Lane A, aliquot of the cell lysate; lane B, laminin, immunoprecipitated from the cell lysate; lanes C1–C7, immunopre-

cipitation of the cell lysate with antibodies against the large proteo-

glycan. Antiserum was pre-absorbed with 0, 0.1, 1.0, 5.0, 10.0, 50.0, and 100.0 μg of whole proteoglycan that was treated with heparitinase. Lanes D and E, immunoprecipitation of the precursor protein in the presence of heparitinase alone (D) or heparitinase plus 100 μg of bovine serum albumin (E).

Immunoprecipitation of the precursor protein.

Biiosynthesis of the proteoglycan was examined in EHS cell cultures pulse-labeled (10 min) with [35S]methionine and switched to fresh, unlabeled medium for up to 4 h. Cell ex-

tacts and culture medium obtained from cultures harvested at timed intervals during the chase period were immunopre-

cipitated with antibodies to the basement membrane proteo-

glycan. Fluorographs of the electrophoresed samples show the 400,000 precursor protein present in extracts of the cell layer throughout the chase period (Fig. 4, lanes A–E). Only a trace amount of this protein was found in the culture medium (Fig. 4, lanes F–J) and this may be derived from the rupture of cells during the chase period. After 60 min chase, however, a very large molecule was precipitated from the medium (Fig. 4, see brackets, lanes F–J) and was identified as the basement membrane proteoglycan. It was recognized by the antibody and migrated in a similar position (21) as the intact proteo-

glycan.

The biosynthesis of laminin was also examined for compar-

ison. Cell layer extracts and culture media from the pulse-

chase experiment described above were immunoprecipitated with antibodies to laminin. Laminin (A and B chains) was present in cell layer extracts throughout the chase period (Fig. 5, lanes A–E). Occasionally, as in this experiment, the 400,000 protein co-precipitated with laminin in several of the cell layer extracts (Fig. 5, lanes B, C, and D). This may be due to the interactions thought to occur between these molecules (31, 32). Laminin, like the proteoglycan, appeared in the culture medium 1 h after the pulse (Fig. 5, lanes F–J). Laminin secreted into the medium electrophoresed somewhat slower and as a more diffuse band than laminin precipitated from the cell layer. This is probably due to glycosylation of the secreted molecule. The detection of the intact proteoglycan and glycosylated laminin only in the medium suggests that most of the completed macromolecules are secreted into the media under these culture conditions.

The labeling kinetics of the 400,000 protein, proteoglycan, and laminin were further examined by excising the bands of the polyacrylamide gels corresponding to these molecules and counting them to quantitate the amount of incorporated radioactivity (Fig. 6). The amount of radioactivity in the 400,000 protein in the cell layer increased in the first 30 min of the chase period and then declined with an approximate half-life of 3.5 h (Fig. 6A). Radioactivity in the intact proteoglycan first appeared at 30 min in the medium and continued to increase during the chase period (Fig. 6B). These labeling kinetics indicate that the 400,000 protein detected in the cell layer is secreted into the media as a proteoglycan. The radio-

activity in laminin showed similar kinetics except that the A and B chains appeared to be synthesized and secreted at different rates. In the cell layer, the radioactivity in the A chains was maximal at 30 min and declined sharply thereafter, whereas, the radioactivity in the B chains was maximal at 2 h and declined more slowly than the A chain (Fig. 6C). Radio-

activity in the A chain appeared more rapidly in the media than the B chains (Fig. 6A).
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FIG. 5. Immunoprecipitation of laminin during pulse-chase of EHS cells. Aliquots of pulse-labeled EHS cell lysates and culture medium, prepared as described in the legend to Fig. 4, were immunoprecipitated with antiserum against laminin. Immunoprecipitates were electrophoresed on a 4–10% gradient (SDS) polyacrylamide gel under reducing conditions and developed for fluorography. Lanes A–E are immunoprecipitates of the cell layer at 0, 30, 60, 120, and 240 min after the pulse. Lanes F–J are immunoprecipitates of the culture medium at the same time points.

The biosynthesis of low and high density proteoglycans was studied in EHS cell cultures pulse-labeled (20 min) with "SO4 and switched to fresh, unlabeled medium for up to 3 h. Cell extracts and culture medium obtained from cultures harvested at timed intervals during the chase period were subjected to CsCl density gradient centrifugation to separate proteoglycans according to density. Most of the incorporated "SO4 initially appeared (time 0) in low density material (top two-fifths of the gradient) extracted from the cell layer (Fig. 7). The proportion of radioactivity in this fraction declined during the chase period and increased in the high density fraction (bottom two-fifths of the gradient). There were also increases during the chase period in the proportion of radioactivity in both the low and high density fractions derived from the media (Fig. 7). Even after 3 h of chase there still was a substantial proportion of radioactivity remaining in the cell layer indicating it was not all secreted to the media as was the glycosylated products of the ["S]methionine pulse-chase. This difference may be due to the absence of serum in the media for the ["S]methionine pulse-chase which may effect secretion. The top two-fifths and bottom two-fifths of the gradient from selected time points of the cell layer were chromatographed of Sepharose CL-4B (Fig. 8). The top of the gradient from the time 0 contained a major peak of radioactivity at Kav of 0.15–0.30 and the bottom of the gradient a major peak at Kav 0.4–0.6. These correspond to the large, low and small high density proteoglycans isolated from the tumor (21). The proteoglycans isolated from these cell culture experiments are slightly smaller than their counterparts isolated from the tumor tissue. The size of the heparan sulfate glycosaminoglycan from cell culture proteoglycans are, however, only ~30,000 M, (Fig. 9) which is half the size of the heparan sulfate glycosaminoglycan isolated from the tumor tissue. The cell culture conditions must reduce the length of the side chains which in turn reduces the overall size of the proteoglycan. The proportion of radioactivity in smaller high density proteoglycans (Kav 0.6–0.9) increased during the chase period

FIG. 6. The precursor protein (M, = 400,000) is converted into a proteoglycan. The amount of radioactivity in the 400,000 protein, proteoglycan, and laminin immunoprecipitated from the pulse-chase experiment shown in Figs. 4 and 5, was measured. Bands of the gel, corresponding to each molecule, were cut out and the radioactivity was determined in a liquid scintillation counter. A and C, cell lysate; B and D, media.

CsCl DENSITY GRADIENT CENTRIFUGATION

FIG. 7. "SO4 pulse-chase. EHS cells in culture were labeled for 20 min with "SO4 and the medium changed to medium without isotope for up to 3 h. The cell layer and media were harvested at timed intervals, the incorporated radioactivity extracted, and separated into low and density material by CsCl density gradient centrifugation. The per cent of the total counts in each time period (cell layer plus media) are presented for the top two-fifths and bottom two-fifths of the gradient.
The results of these studies show a 400,000 $M_r$ protein to be the precursor to the basement membrane-specific heparan sulfate proteoglycan. This 400,000 precursor protein was distinct from other large extracellular matrix proteins such as the A chain of laminin (30), and the precursor protein of a chondroitin sulfate proteoglycan (2).

Two lines of evidence indicate that the 400,000 protein is the precursor to the basement membrane proteoglycan. First, the basement membrane proteoglycan core protein, produced by heparitinase treatment of the proteoglycan, blocked the immunoprecipitation of the 400,000 protein by competing for available antibody. Second, a pulse-chase experiment showed that the 400,000 protein was present in greatest amount in early time points of cell layer extracts and, as it decreased in amount during the chase, there was a corresponding increase in high molecular weight proteoglycan immunoprecipitated from the culture medium. However, it cannot be ruled out that small amounts of the precursor protein, seen in the culture media, are secreted as a glycoprotein. The sequential addition of heparan sulfate side chains to the precursor protein could not be detected during the pulse-chase experiment. The addition of one complete heparan sulfate side chain to a precursor protein should have been easily resolved from the 400,000 precursor protein on acrylamide gels. This absence of intermediates suggests that the addition of heparan sulfate side chains may occur very quickly or even simultaneously.

The half-life of this proteoglycan precursor protein was estimated to be 3.5 h, suggesting that there may be a large intracellular pool of precursor protein. A sizeable pool of proteoglycan precursor protein also exists in the Swarm rat chondrosarcoma that produces a cartilage-specific chondroitin sulfate proteoglycan (2) and in M21 human melanoma cells that produce an immunologically unrelated chondroitin sulfate proteoglycan (26). However, the depletion of the precursor protein varies widely with a half-life of about 90 min in the chondrosarcoma to several hours in both the EHS tumor and the M21 melanoma cell line.

The labeling kinetics of the 400,000 precursor protein were
**BIOSYNTHESIS OF THE BASEMENT MEMBRANE PROTEOGLYCAN**

![Diagram of Biosynthesis Scheme]

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**Fig. 10.** A schematic representation of the synthesis of the heparan sulfate proteoglycan in basement membranes. Synthesis of a 400,000 M<sub>d</sub> protein represents the initial step for the production of the heparan sulfate proteoglycan in basement membranes. This precursor protein acts as a substrate for the addition of heparan sulfate glycosaminoglycans to become a large, low density proteoglycan. The 400,000 precursor protein is used, with little modification, as the protein core of the large, low density proteoglycan. A portion of this population is converted to the small, high density proteoglycan.

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also compared with the labeling kinetics for laminin. In contrast to that found for the biosynthesis of the proteoglycan, the fully glycosylated laminin found in the media was only slightly larger than the incompletely glycosylated form found in the cell layer. However, both the proteoglycan, and laminin were detected in the culture medium after 60 min and in increasing amounts thereafter. A cell transit time of 60 min for both the heparan sulfate proteoglycan and laminin produced by EHS cells is in good agreement with previous transit studies of the same or similar proteins. For example, the transit time for the precursor proteins of chondroitin sulfate proteoglycans produced by the Swarm rat chondrosarcoma and M21 melanoma cells were approximately 60 min (2, 26). Likewise laminin produced by parietal endodermal cells is secreted 60 min after pulse-labeling (33).

The A and B chains of laminin were found to be synthesized and secreted at different rates. The two B chains of F9 cells have been shown to be synthesized at different rates and it has been suggested that the excess B chain is not assembled into the secreted molecules (34). The two B chains produced by the EHS cells did not completely resolve on the acrylamide gel for us to make a similar comparison but careful examination of lanes A and E on Fig. 5 indicate the upper B chain is retained more on the cell layer than the lower B chain.

Previously, we showed that the core protein of the large basement membrane proteoglycan is 350,000–400,000 M<sub>d</sub> after heparitinase digestion (21). The smaller proteoglycan had a heterodisperse core protein of 95,000–130,000 M<sub>d</sub> (21). In this study, antibodies to large and small forms identified the same precursor of the basement membrane proteoglycan as M<sub>d</sub> = 400,000. These observations indicate that the precursor protein is probably used directly with little or no proteolytic processing for the synthesis of the large, low density proteoglycan. It is possible that part of the population of precursor proteins are degraded to 95,000–130,000 and used as such for the synthesis of the smaller, high density proteoglycan. However, pulse-chase studies with <sup>35</sup>S04 show the large, low density proteoglycan to be synthesized first and a portion of this population is converted to the smaller, high density proteoglycan. The conversion occurs quickly, within 1–2 h after the synthesis of the low density proteoglycan. This change in density and size would involve removal of a section of the protein core from the large proteoglycan that contains little or no heparan sulfate side chains. This biosynthetic scheme is illustrated in Fig. 11. A similar conversion of large heparan sulfate proteoglycan to a smaller one has been described for granulosa cells (35, 36).

Heparan sulfate proteoglycans appear to be ubiquitous be-

cause they are produced by a variety of cells including fibroblasts (37–39), hepatocytes (40), epithelial cells (6–8, 13–15), endothelial cells (9, 13–15), muscle cells (41, 42), granulosa cells (35, 36), and many transformed cells (43–45). These proteoglycans have been characterized almost exclusively by their carbohydrate side chains and comparatively little attention has been devoted to their core protein, which would establish their real identity. However, monoclonal antibodies to a heparan sulfate proteoglycan from amphibian muscle have been used to show its core protein to be 350,000–400,000 (41); a value similar to that found here and in the accompanying study (21). Our antibodies to the basement membrane proteoglycan have been shown to stain basement membranes of a variety of cell types including epithelial cells, endothelial cells, and muscle cells (4, 21). Furthermore, they have been used to show the 400,000 precursor protein appears during the development of embryonic limb muscle (46). Consequently, proteoglycans with a ~400,000 core protein and fragments of this proteoglycan may constitute a class of proteoglycans found in all basement membranes. It has been recently shown that antibodies to a liver heparan sulfate proteoglycan stain cell surfaces and not basement membranes (47). The core protein of this proteoglycan is likely derived from a different gene product than the basement membrane proteoglycan.

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