Perlecan and Basement Membrane-Chondroitin Sulfate Proteoglycan (Bamacan) Are Two Basement Membrane Chondroitin/Dermatan Sulfate Proteoglycans in the Engelbreth-Holm-Swarm Tumor Matrix*

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The presence of proteoglycans bearing galactosaminoglycan chains has been reported, but none has been identified previously in the matrix of the Engelbreth-Holm-Swarm tumor, which is a source of several basement membrane components. This tumor matrix contains perlecan, a large, low buoyant density heparan sulfate proteoglycan, widespread in many basement membranes and connective tissues. We now identify two distinct proteoglycan species from this tumor source, which are substituted with galactosaminoglycans and which show basement membrane localization by immunohistochemistry. One species is perlecan but, in addition to being present as a heparan sulfate proteoglycan, it is also present as a hybrid molecule, with dermatan sulfate chains. A major population of perlecan apparently lacks heparan sulfate chains totally, and some of this is substituted with chondroitin sulfate. The second species is immunologically related to basement membrane-chondroitin sulfate proteoglycan (BM-CSPG) and bears chondroitin sulfate chains. No BM-CSPG was detectable which was substituted with heparan sulfate chains. A combination of immunological and molecular approaches, including cDNA cloning, showed that perlecan and BM-CSPG are distinct in core protein structure. Both are, however, basement membrane components, although there are tissue-specific differences in their distribution.

The most well-characterized basement membrane proteoglycan is perlecan, first isolated from the murine Engelbreth-Holm-Swarm (EHS) tumor (1). This complex molecule is probably the most abundant basement membrane proteoglycan, whose properties include interaction with other basement membrane components to form the matrix, participation in selective filtration, e.g. in the kidney glomerulus, and interactions with cell surface integrin receptors (2–4). In addition, perlecan may both sequester heparin-binding growth factors, such as fibroblast growth factor 2, and potentiate their interaction with high affinity receptors (5). The entire sequences of murine and human perlecan core proteins are now known and represent one of the largest and most complex extracellular matrix protein structures identified (6–8). The core protein of M, ~ 396,000–467,000 consists of five domains; the small N-terminal domain is the only one unique in structure and potentially bears 1–3 heparan sulfate chains. Domain I1 has homology to the low density lipoprotein receptor, while domain II has homology to laminin 1 short arms. Domain IV consists of 14–21 I-strings most closely resembling those of neural cell adhesion (N-CAM), and is potentially subject to alternate splicing at the mRNA level (2), while domain V contains neuraminic repeats and is homologous to the C-terminal domains of agrin, and, to a lesser extent, the C-terminal globular region of laminin a chains (2–4). Perlecan is widely distributed in basement membranes and some other extracellular matrices, as detected by monoclonal and polyclonal antibodies, including some which are domain-specific (2–4, 9, 10).

The presence of other, distinct basement membrane proteoglycans is clear. Agrin is now recognized as a heparan sulfate proteoglycan (HSPG), and other HSPG core proteins may yet be characterized (11). In addition, we have identified a chondroitin sulfate proteoglycan (CSPG), distributed in most, but not all, adult rat basement membranes (BM-CSPG; Refs. 12 and 13). It is immunologically unrelated to perlecan, is apparently regulated in embryonic development, and has been implicated in pathogenic changes in basement membranes accompanying several unrelated diseases (14–16). All this may point to a role for BM-CSPG in basement membrane stability.

The EHS tumor, while containing perlecan as its major proteoglycan component, has not been investigated for its galactosaminoglycan-bearing proteoglycans. Perlecan itself probably represents <10% of the basement membrane components present in the tumor matrix. Chondroitin sulfate has been detected as a minor proportion of the glycosaminoglycan, representing from <5% to 19% of the tumor matrix glycosaminoglycan content in various preparations (17–19). The core proteins bearing galactosaminoglycan have not been determined. One high density, small proteoglycan bearing both chondroitin and heparan sulfate chains has been identified (21), but is a poor antigen, and so its relationship, if any, to known species such as syndecans (2) or a fragment of perlecan is unclear. In this work, we utilize immunological and molecular techniques to characterize two chondroitin/dermatan proteoglycans (CS/
EHS Tumor Chondroitin Proteoglycans

DSPGs of the EHS tumor. One is perlecan itself, which is present as both a heparan sulfate-substituted species and as a hybrid heparan/dermatan sulfate proteoglycan. The second is BM-CSPG, present as a CSPG and confirmed to be distinct from other proteoglycans.

MATERIALS AND METHODS

Extraction and Purification of Proteoglycans from Engelbreth-Holm-Swarm (EHS) Tumor—The extraction and purification steps were performed by a modification of the procedure described by Hassell et al. (19). All procedures were performed at 4°C in the presence of protease inhibitors (0.1 M 6-aminohexanoic acid, 0.005 M EDTA, 0.005 M N-ethylmaleimide, 0.002 M phenylmethylsulfonyl fluoride) unless stated otherwise. The EHS tumor (400 g) was minced with a scalpel. The minced tissue was washed with 5 volumes (w/v) of 0.15 M NaCl, 0.05 M Tris-HCl, pH 6.8, with vigorous stirring for 1 h. The mixture was centrifuged at 10,000 × g for 20 min, and the pellet was extracted with 10 volumes (w/v) of 4 M guanidinium HCl, 0.05 M Tris-HCl, pH 6.8, for 24 h with vigorous stirring. The extract was concentrated 10-fold using an Amicon ultrafiltration device with a YM100 membrane filter. The concentration was dialyzed into 6 M urea, 0.05 M Tris-HCl, pH 6.8, followed by centrifugation at 15,000 × g for 30 min to remove any insoluble material.

The concentrate was chromatographed on a column of DEAE-Sepharose (40 cm; Pharmacia Biotech Inc.) equilibrated with 6 M urea, 0.05 M Tris-HCl, pH 6.8. After sample loading, the column was washed with 5 bed volumes of the equilibration buffer. Subsequently, the column was eluted with 3 bed volumes each of 0.3 M NaCl and 2 M NaCl in 6 M urea, 0.05 M Tris-HCl, pH 6.8. The 2 M NaCl eluate was dialyzed against 6 M urea, 0.05 M Tris-HCl, pH 6.8, and chromatographed on a DEAE-Sepharose column (10 × 40 cm) equilibrated with 6 M urea, 0.05 M Tris-HCl, pH 6.8. The column was washed with 5–7 bed volumes of the equilibration buffer followed by elution with a linear gradient of 0.1 M NaCl to 2 M NaCl in 6 M urea, 0.05 M Tris-HCl, pH 6.8. Approximately 3-ml fractions were collected and analyzed for uronic acid (22). Fractions containing uronic acid were pooled, dialyzed against distilled H2O (with protease inhibitors), and lyophilized.

The lyophilized material was resolubilized (5 mg/ml) in 4 M guanidinium HCl, 0.05 M Tris-HCl, pH 7.4 (with protease inhibitors) and chromatographed on a column of Sepharose CL-4B (2.5 × 100 cm; Pharmacia). Approximately 5-ml fractions were collected and analyzed for uronic acid. Fractions containing uronic acid were appropriately pooled, dialyzed against distilled H2O, lyophilized, and stored desiccated at 4°C for further analysis. The column was calibrated with a rooster comb hyaluronic for the V0 and [1^4]proline for the Vt.

Immunological Procedures—The primary antibodies used in these studies were as follows. A rabbit polyclonal antiserum raised against chondroitin ABC-treated bovine nasal cartilage aggrecan was used to detect the total complement of CS/DSG core proteins (R36). The antibody specifically for aggrecan core protein epitopes, but, in addition, binds unsaturated uronic acid residues arising from chondroitinase treatment. Therefore, it can recognize the core proteins of all CS/DSGs, but only after enzyme treatment. This antibody has been characterized previously (23). Five rat monoclonal antibodies against murine perlecan core protein were also used in Western blotting. All were IgG1, subisotype, and their specificity has been demonstrated (9, 24). The domain specificity of some of these antibodies has now been mapped (9). A further mouse monoclonal antibody (1B48) against rat perlecan core protein was also used. Two rabbit polyclonal antibodies recognizing pericranin core protein were used, one prepared previously in the laboratory (25), the other a gift from Dr. J. R. Hassell (University of Pittsburgh). In the latter case, a rabbit polyclonal antibody was raised against polypeptide 2 CS/DSGs (R63). Approximately 3 mg of pool 2 proteoglycan (see below) was solubilized in 2 ml of 4 M guanidinium HCl in 0.1 M sodium acetate, pH 7.0, and dialyzed against 0.1 M sodium acetate, pH 7.0, containing 0.1 M calcium acetate (heparinase buffer). The proteoglycans were treated twice with 10 milliunits of heparinase III (EC 4.2.2.8) in the presence of 10% of ovalbumin. The digests from R63, which had been covalently cross-linked, using a published procedure (26).

The column flow-through, including a column volume of PBS wash, was collected, and 100 μl of R63 antiserum was added before incubation on a rotator for 90 min at 37°C. Three mg of Protein A-Sepharose (Pharmacia) was preincubated with 10% fetal bovine serum in PBS containing 0.02% sodium azide to block nonspecific binding sites, washed copiously by centrifugation and resuspension in PBS, then mixed with the proteoglycan/antibody preparation for 45 min at 37°C. The immunoprecipitates were washed repeatedly with PBS, Immunohistochemistry—Monoclonal and polyclonal antibodies were used in immunohistochemistry on frozen and paraffin-embedded human and rat tissue sections as previously (12, 13). Hybridoma supernatants were used either undiluted or 1:4 in PBS, ascites fluids were diluted 1:50, and polyclonal antibodies were used at 1:30–1:100 dilution in PBS. Secondary antibodies (Cappel Laboratories, Organon Teknika Corp., Durham, NC) were affinity-purified, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or goat anti-rabbit IgG. In some cases, particularly for polyclonal primary antibodies, an F(ab) fragment of goat anti-rabbit IgG was used. All hybridomas were constructed with the use of preimmune sera, including that from rabbit R63, and omission of primary antibody. All controls gave background staining, in contrast to the test antibodies.

Western Blotting—Proteoglycan samples (5 μg) were subjected to enzyme digestions in 15 μl of heparinase buffer, containing 0.5–1 milliunit of heparinase III (heparitinase, EC 4.2.2.8), and/or 1–2 milliunits of protease-free chondroitin ABC (chondroitin ABC lyase, EC 4.2.2.4) or 1–2 milliunits of chondroitin ACII (chondroitin AC lyase, EC 4.2.2.5) overnight at 37°C. These enzymes were from Sekagaku America Inc., Rockville, MD. In some cases, 0.5 milliunit of heparinase II was also added (Sigma). To each sample was added 12 μl of sample buffer containing 20% glycerol, before dialysis against 0.1 M sodium acetate, pH 7.0, for 3 min. Samples were resolved on 3–15% SDS-PAGE and transferred to nitrocellulose as described previously (9, 12). Membranes were blocked for 30 min with 0.5–1% dried milk dissolved in PBS for monoclonal antibody studies, or Tris-buffered saline (PBS, 0.5 mM sodium chloride in 50 mM Tris-HCl, pH 7.5) for polyclonal antibody studies, both containing 0.02% sodium azide. All hybridoma supernatants were diluted 1:8 in PBS containing 0.1% dried milk, 0.02% bovine serum albumin, and 0.1% Tween 20. Polyclonal antibodies were diluted 1:200–1:2000 in PBS containing the same additives. Secondary antibodies were alkaline phosphatase conjugates of goat anti-mouse IgG or goat anti-rabbit IgG, diluted 1:3000 in the same buffers as the primary antibodies. Secondary antibodies and color development chemicals were from Bio-Rad Laboratories Inc., Richmond, CA.

Slot Blot Analysis of Proteoglycans Recognized by R63 Polyclonal Antibody—Total proteoglycans from 900 ml of L2 rat yolk sac cell culture supernatants were prepared under nondenaturing conditions. The supernatant was clarified by centrifugation and passed over a 20-ml column of DEAE-Sepharose (Pharmacia) at 9 ml/h. The column was washed sequentially with PBS, 50 mM sodium acetate buffer, pH 4.0, containing 0.15 M NaCl and PBS. Proteoglycans were eluted with PBS containing NaCl made up to 1.5 M. In each case, 0.2 ml PMSF, 10 ml EDTA, and 1 ml benzamidine HCl were included. The second and third wash steps also included 0.1% Tween 20. Two ml fractions were collected and monitored spectrophotometrically at 260 nm for protein absorbance. Five volumes of 1 M sodium acetate were added to each to facilitate proteoglycan preparation and incubated overnight at −20°C. Samples of washed and dried precipitate were weighed for total proteoglycan content. Of this material, 3.1 mg were solubilized in 4 ml of PBS, whose NaCl content was adjusted to 0.3 M. This was passed over a column of Protein A-Sepharose (Pharmacia) to which saturating amounts of polyclonal antibody were bound in PBS containing 0.02% sodium azide. The column was washed twice with 5 ml of PBS plus 1 ml benzamidine HCl and ethanol. A post-bleed from the same rabbit before primary injection was included in all cases. The immunoprecipitates were washed repeatedly with PBS, Immunohistochemistry on frozen and paraffin-embedded human and rat tissue sections as previously (12, 13). Hybridoma supernatants were used either undiluted or 1:4 in PBS.
precipitated proteoglycans were eluted with two 0.5-ml aliquots of 100 mM glycine-HCl, pH 2.8. An equal volume of 0.2 M NaCl, 50 mM sodium acetate, pH 5.5, was added, and the pH was adjusted, if necessary, to 4.0. Aliquots of 200 μl were applied to DEAE-membrane (NA45; Schleicher & Schuell, Keene, NH) assembled in a Bio-Rad blot apparatus, for 2 h at room temperature. Under these buffer conditions, the transfemoral proteoglycans, but not the majority of the R63, bound to the membrane.

The membrane was washed with 0.2 M NaCl, 50 mM sodium acetate, pH 4.0, then blocked with 0.5% dried milk, 1% normal goat serum in PBS containing 0.05% Tween 20 (PBST). After brief washing in PBST, duplicate wells were incubated with 1:1000 dilutions in PBST of pre-immune serum, immune R63 (negative control, respectively), or 1:30 dilutions of SA3 mouse monoclonal antibody against BM-CSPG (12), an irrelevant monoclonal antibody AYB of the same IgG subclass, and 11B4 monoclonal antibody against rat perlecan core protein. Additional wells received no primary antibody. After a 1-h incubation at 37°C, the membrane was washed extensively with PBST, followed by 1:2000 dilutions in PBST of alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG. Additional washings in PBST after incubation for 1 h at 37°C with secondary antibodies was followed by color development, according to the manufacturer’s protocol (Bio-Rad). The blots were photographedinmediately. The entire experiment was repeated three times, with identical results.

cDNA Library Screening and Fusion Protein Production—A cDNA expression library prepared from L2 rat yolk sac carcinoma cells (Stratagene) was screened with R63 polyclonal antiserum raised against basement membrane CS/DSPGs. The library was prepared in the Uni-Zap™ XR vector, and 10⁵ plaques were grown in 150-mm plates in LB agar medium at 32°C for 3.5 h. The plates were then covered with nitrocellulose filters (Schleicher & Schuell) presoaked in 1 mM isopropanol. Filters were incubated for 1 h at 37°C with secondary antibodies was followed by color development, according to the manufacturer’s protocol (Bio-Rad). The blots were photographed immediately. The entire experiment was repeated three times, with identical results.

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RESULTS

EHS Tumor-derived Chondroitin/ Dermatan Sulfate Proteoglycans—Three pools of glycosaminoglycan-containing molecules from EHS tumor matrix were separated on the basis of hydrodynamic size. The largest (P1) and intermediate-sized (P2) contained CS/DSPGs (Fig. 1). The smallest-sized (P3) pool contained very little detectable core protein and probably consisted of large quantities of free glycosaminoglycans and small proteoglycan fragments. The P3 pool was not considered further. Samples from P1 were subjected to digestion with chondroitinase ABC ± heparinases II and III, followed by SDS-PAGE and Western blotting with an antisem recognizing chondroitin or dermatan sulfate stubs remaining after enzyme treatment (R36). The antibody could therefore recognize all proteoglycans bearing galactosaminoglycan chains. A single, or more commonly a closely spaced doublet, of polypeptides of approximately M_r ~ 400,000 were resolved, together with a second species with greater electrophoretic mobility, approximately M_r ~ 200,000 (Fig. 2). The former required both chondroitinase ABC and heparitinase enzyme treatments to be detectable as a distinct core protein, indicating that most was a hybrid proteoglycan. The latter, smaller core protein was present after chondroitinase ABC treatment alone, showing that it was a CS/DSPG. Material from P2 yielded essentially similar results.

Since the larger species had a core protein of approximate M_r ~ 400,000, an obvious candidate molecule was perlecan. Therefore, five core protein-specific monoclonal antibodies recognizing murine perlecan were used separately in Western blotting of P1 and P2 material following treatment with chondroitinase ABC and/or heparitinase III. These all recognized polypeptides of an identical high molecular mass, the largest of which was of M_r ~ 400,000 (Fig. 3), confirming that the high molecular weight core protein was perlecan. The perlecan core protein could be resolved after heparitinase III treatment alone and was therefore also present as a proteoglycan species bearing only heparan sulfate chains (lane 2, Fig. 3, A, B, and C). Perlecan in the form of an HSPG has been reported previously (1, 17–19). These five antibodies recognized at least four different epitopes, and three have been mapped to epitopes within domains III, IV, or V of the core protein (9). Two different polyclonal antibodies were also tested, one raised against perlecan, the other against an HSPG prepared from the PYS-2 murine endodermal cell line. Both gave results virtually identical with the five monoclonal antibodies (not shown). In addition, three other features emerged. First, in the P2 material, the monoclonal antibodies were able to detect small amounts of M_r ~ 400,000 polypeptide in samples not enzyme-treated (lane 1, Fig. 3B) or treated with chondroitinase ABC alone (lane 4, Fig. 3B). This shows that some perlecan can be purified, which either lacks or has extremely small glycosaminoglycan chains, in addition to some which contains only chondroitin/dermatan sulfate chains. Second, after heparitinase III treatment, at least two perlecan core proteins were resolved as a closely spaced doublet, whereas only a single core was detected after chondroitinase ABC treatment. The latter corresponded to the largest form seen after heparitinase III treatment (lanes 2, Fig. 3). The species with higher mobility was not, however, a protein contaminant from the heparitinase III, since no polypeptides were detected by any antibody in lanes where heparitinase III alone had been loaded (lanes 5, Fig. 3). Third, no perlecan antibody, either monoclonal or polyclonal, recognized the CS/DSPG with a core of M_r ~ 200,000.

Production of Antiserum against EHS Tumor CS/DSPGs—To characterize the EHS tumor CS/DSPGs more fully, 
a polyclonal antiserum was prepared against P2 proteoglycans which had been digested exhaustively with heparinase III and repurified by anion exchange chromatography. This rabbit antibody (R63) was then used in Western blotting of P1 and P2 proteoglycans. It detected both the perlecan core proteins of Mr ~ 400,000 after heparinase III ± chondroitinase ABC treatments, as well as the CS/DSPG with a core protein of Mr ~ 200,000 (Fig. 4). The latter was visible as a discrete core protein after chondroitinase ABC treatment, but not heparinase III alone. In indirect immunofluorescence microscopy of rodent and human skin and other organs, the antiserum gave characteristic linear staining patterns, expected for basement membrane components. Basement membranes underlying epithelia, vascular endothelia, smooth and skeletal muscle, as well as fat cells and peripheral nerve fibers were all positive (Fig. 5).

Similar staining patterns were seen with monoclonal antibodies against perlecan or BM-CSPG. In particular, it was noted that R63 gave very weak or no staining of adult rat glomerular basement membrane, a characteristic pattern noted previously for BM-CSPG (12, 13). Perlecan is present in this basement membrane, however (9, 10, 24, 27, 28). Evidence from this study and cDNA expression library screening (described below) indicated that reactivity of R63 to perlecan was weak. The staining pattern with R63 indicated, therefore, that both of the two CS/DSPGs from the EHS tumor have basement membrane distributions. Neither of these two components had a staining pattern consistent with a cell surface distribution.

Identification of Two Distinct Polypeptides from a cDNA Library—A second source of basement membrane proteoglycans was examined, the rat yolk sac carcinoma cell line L2. In addition to synthesizing a large amount of proteoglycans in tissue culture, a rat source was appropriate for some of our immunological probes. Furthermore, a commercially available cDNA expression library was available for these cells. The polyclonal antiserum R63 also detected a similar spectrum of core proteins in a total proteoglycan preparation from rat yolk sac L2 cell supernatants (Fig. 6A). In this case, the core protein of the CS/DSPG had a slightly higher mobility, with an Mr ~ 150,000. An additional smaller core protein species was also labeled (Mr ~ 100,000), often strongly, possibly representing a degradation product. These were detectable after chondroitinase ABC treatments of L2 proteoglycans (lanes 3 and 4, Fig. 6A). The presence of perlecan was confirmed by both a monoclonal (Fig. 6B) and polyclonal antibody (not shown) against perlecan core protein. This is consistent with the previous identification of perlecan as a product of this cell line (20). Total CS/DSPG core proteins were also detected by Western blotting.
was consistent with perlecan, having a $M_r \approx 400,000$, and identical with that recognized by 11B4 monoclonal antibody against rat perlecan core protein (cf. Fig. 6B). For perlecan core protein resolution and detection by R36, both heparinase III and chondroitinase ABC treatments were required, indicative of a hybrid proteoglycan. No perlecan bearing galactosaminoglycan chains only was detected in L2 preparations. The other two core proteins were $M_r \approx 150,000$ and 100,000, identical with those detected by R63, and representing the basement membrane CS/DSPG. As before, only chondroitinase ABC treatment was required to resolve the latter two core proteins.

The antisera R63 was used to screen an expression cDNA library from L2 cell mRNA in the Uni-ZAP vector. Of 11 clones detected from preliminary screening and subsequent rounds of clonal selection, two different polypeptides were identified by DNA sequencing. Two overlapping clones of 2.2 kb yielded portions of domain IV of perlecan core protein, as judged by very high homology to the previously reported murine and human sequences. These were only weakly detected by the R63 serum. The remaining nine clones, all overlapping, gave a predicted amino acid sequence not related to any lodged with EMBL or GenBank. In total these latter clones encompassed 3.4 kb, including an open reading frame of 2.9 kb and 0.5 kb of 3'-noncoding sequence. This sequence will be reported elsewhere. The two bacterial clones expressing a portion of rat perlecan core protein (5a and 11a), and two of the nine putatively expressing BM-CSPG (4a and 15a) were grown in liquid culture in the presence of isopropyl-1-thio-β-D-galactopyranoside to induce fusion protein expression. Bacterial lysates were probed by Western blotting with R63 or EY#S, a polyclonal anti-murine perlecan serum (Fig. 7). While clone 5a and 11a fusion proteins could be detected with anti-perlecan sera, as expected, clone 4a and 15a fusion proteins could not. This was consistent with these latter proteins being a portion of a distinct gene product. Clone 4a and 15a fusion proteins were strongly detected by R63, however, while 5a and 11a perlecan clones were only weakly detected. As predicted from immunological studies, therefore, at least two distinct basement mem-

**Fig. 5.** Indirect immunofluorescence microscopy of human skin (a and b) and rat kidney cortex (c) stained with R63 antibody against EHS tumor CS/DSPGs. Basement membranes of the dermal-epidermal junction (J) and papillary dermal capillaries (arrow, a), sweat glands and ducts (S) and their associated vasculature (V in b), and kidney tubules (T), Bowman’s capsule (P), and glomerular mesangium (M in c) are stained. Glomerular capillary loop basement membranes are virtually unstained. Bar = 100 μm.

**Fig. 6.** L2 cell culture medium proteoglycans immunoblotted with R63 against EHS tumor CS/DSPGs (A), 11B4 monoclonal antibody against rat perlecan core protein (B), and R36 against chondroitin/dermatan sulfate chain stubs remaining attached to core proteins after chondroitinase ABC treatment (C). In each case, lanes 1 contain untreated proteoglycans, in lanes 2 the proteoglycans are heparinase III-treated, in lanes 3 the samples are heparinase III- and chondroitinase ABC-treated, and in lanes 4 chondroitinase-treated only. The BM-CSPG core proteins are labeled with arrowheads, and the positions of molecular mass standards, in kilodaltons, are shown by arrows.
brane proteoglycan core proteins are present within the EHS tumor matrix.

The CSPG Is Immunologically Related to BM-CSPG—The CSPG unrelated to perlecan has characteristics similar to the previously described BM-CSPG, first isolated from embryonic rat Reichert's membranes (12, 29). To confirm its identity, we immunoprecipitated proteoglycans from rat L2 cell supernatants with the antibody R63. The products were dot-blotted onto DEAE-membrane and probed with monoclonal antibodies against BM-CSPG or perlecan. As shown in Fig. 8, both proteoglycans could be detected, while irrelevant mouse monoclonal antibodies did not react. It therefore appears that L2 cells, as well as the EHS tumor matrix, are sources of BM-CSPG and perlecan.

Nature of the Galactosaminoglycans in EHS Tumor CS/DS PGs—In order to identify which galactosaminoglycan type is present on perlecan and BM-CSPG, we utilized the differing specificities of chondroitinase ABC and ACII enzymes. The former will cleave most forms of chondroitin and dermanatan sulfates while the latter is unable to cleave adjacent to iduronic acid residues of dermanatan sulfate. Since hybrid perlecan is much more abundant than forms bearing only chondroitin/dermanatan sulfate chains, samples of P2 proteoglycans were treated with chondroitinase ABC or ACII in conjunction with heparinase III and resolved on SDS-PAGE, followed by Western blotting with R63 or R36. Clear and discrete perlecan core proteins were visible after chondroitinase ABC or ACII, indicating that at least some of the hybrid perlecan contains no dermanatan sulfate and bears chondroitin and heparan sulfates. BM-CSPG core protein was also resolved after either chondroitinase ABC or ACII enzyme, showing that some or all was substituted with chondroitin sulfate chains (Fig. 9). In this case, as above, heparinase III was not required additionally to yield discrete core protein.

DISCUSSION

Many basement membrane components have been purified and characterized from the EHS tumor matrix, and, in some cases, their first isolation from this source has been the catalyst for new directions of research. Perlecan is such a case and remains the best characterized basement membrane proteoglycan. It is now clear, however, that not only is the extracellular matrix from the tumor very complex, containing a number of macromolecules and growth factors (1, 17–19, 30, 31), but the spectrum of basement membrane macromolecules described from this source is only a subset of those found in mammalian basement membranes in vivo. For example, the laminin family has expanded from the prototypical heterotrimer, first isolated from the EHS tumor, and it is now appreciated that at least 10 distinct chains can be identified from a variety of sources (32).

While perlecan is the most abundant proteoglycan in the EHS tumor matrix, other smaller proteoglycans have previously been described. A small high density HSPG has been purified, apparently bearing shorter glycosaminoglycan chains than the lower buoyant density perlecan. This may be a fragment of perlecan, but it has also been suggested that it has only a weak immunological relationship to the larger, low density species (33). It seems quite likely that while perlecan may be cleaved to generate smaller proteoglycans, there are additional unrelated HSPGs, with distinct core proteins. One small, high density hybrid proteoglycan has been described which bears both chondroitin and heparan sulfate chains (21), and it has been postulated to be related to cell surface syndecan, possibly syndecan 1 (2). However, direct immunological data are currently lacking, preventing a more confident identification of this molecule. Previous analyses indicate that up to 20% of the tumor matrix glycosaminoglycans are chondroitin or dermanatan sulfates, but no core proteins have been associated with these chains (17–19).

This report identifies two distinct proteoglycans as galactosaminoglycan-bearing. The first is perlecan itself, which, in addition to being substituted with heparan sulfate alone, was also present as a hybrid, with additional chondroitin sulfate chains. Indeed, small amounts of perlecan bearing galactosaminoglycan in the absence of heparan sulfate could also be
detected, as well as very small amounts bearing no, or very small, glycosaminoglycans. This data are consistent with other observations. First, a cell line derived from the EHS tumor (34) was shown to synthesize perlecan as a hybrid molecule, and a similar form with heparan and dermatan sulfate chains was purified from human placenta (35) and bovine cartilage (36). This indicates that perlecan can occur as a hybrid in vivo and, therefore, is one member of the overall basement membrane CS/DSPGs. One report also shows that perlecan can be found in vivo as a CS/DSPG, with no heparan sulfate chains (37). However, it has not been reported previously that perlecan may exist in the EHS tumor matrix in these various forms. The impact of alternate glycosylation of the perlecan core protein is not understood, but, in the light of the potential importance of heparan sulfate in its interactions with integrins and growth factors (5, 38), it is possible that substitution with dermatan sulfate chains in place of, or in addition to, heparan sulfate may impact the biological activity of perlecan. Further, perlecan forms bearing heparan sulfate yield two core proteins, unlike the single species resolved after chondroitinase ABC treatment. The reasons for this are unclear, but it probably does not result from proteolytic activity in the heparinase III enzyme. Not only were protease inhibitors present, but perlecan from other sources (L2 cells, human keratinocytes, or murine PYS-2 or PFHR-9 cells) can be resolved as single core proteins after identical enzyme treatments. It is possible that endogenous proteolytic activity in the tumor matrix is responsible, but why heparan sulfate, but not glycosaminoglycan-substituted forms, should be more susceptible is unclear, although potentially interesting. It is currently unknown where in the core protein perlecan is substituted with chondroitin sulfate and in which tissues this occurs. Indeed, the site of heparan sulfate chain substitution is inferred from the SGD sequences in domain I, but has yet to be confirmed directly (2).

The other proteoglycan identified was immunologically related to BM-CSPG and probably is identical with this for the following reasons. First, the glycosaminoglycan and core protein characteristics are very similar, and, since R63 stained only basement membranes, the proteoglycan is basement membrane-restricted in its distribution. Staining of adult rat kidney sections showed marked depletion in the glomerular capillary basement membrane, entirely consistent with our previous reports (12, 13) for BM-CSPG. Immunopurification of L2 cell proteoglycans by R63, followed by slot-blotting with previously characterized monoclonal antibodies, confirmed the presence of both BM-CSPG and perlecan reactivity in the antiserum raised against EHS tumor matrix CS/DSPGs. Screening of an expression cDNA library with the R63 antibody also led to the identification of two distinct proteins. One was perlecan, on the basis of high homology of the (rat) sequence to mouse and human perlecan domain IV. The other was unique and is currently being completed. However, preliminary data indicate an mRNA of approximately 4.2 kb, consistent with a protein smaller than perlecan (12–14 kb), and a sequence with total lack of identity or homology with perlecan or other basement membrane components. The sequence indicates a five domain structure in a head-rod-tail configuration, with large domains predicted to form coiled-coil structures (39, 40). There is no structural similarity with perlecan or agrin, therefore, nor with any currently reported chondroitin/dermatan sulfate proteoglycan. Since at least two distinct proteoglycans in basement membranes bear galactosaminoglycans, it is appropriate to name these precisely to avoid confusion. As we have reported in preliminary communications, BM-CSPG will be called bamancan (basement membrane-associated chondroitin sulfate proteoglycan).

The presence of BM-CSPG in the EHS tumor matrix has not been noted before, but is consistent with other features of this matrix. We did not detect the presence of a small hybrid proteoglycan that was noted previously (21), even though we used antibodies capable of detecting all CS/DSPGs (R36) by virtue of its reactivity to the unsaturated terminal uronic acid residues created by chondroitinase treatments. Nor did we detect small HSPGs in these proteoglycan preparations, but lack of appropriate immunological reagents may explain this, assuming that the core proteins are unrelated to perlecan. One such HSPG with a core protein of $M_r \sim 40,000$ has been reported from liver tissue (41), for example. Therefore, while we have determined the presence of two distinct proteoglycans in the EHS tumor matrix, BM-CSPG being a minor component consistent with earlier findings, we do not rule out the possibility of further basement membrane proteoglycans in this matrix. It would not be surprising, given the large number of type IV collagen chains and laminin isoforms now recognized, that multiple species of proteoglycan are also present in tissue basement membranes, perhaps regulated in developmental and/or tissue specific patterns. Our information on BM-CSPG indicates that it is developmentally regulated, but also affected in diseases such as diabetes and polycystic kidney disease (14, 15). We have also gained indications that basement membrane chondroitin sulfate may be substantially missing in the dermal-dermal junction of patients with dystrophic forms of epidermolysis bullosa (42). Perlecan core protein was present in an apparently normal pattern, but since this may be a hybrid proteoglycan, the possibility arises that its glycosylation may be affected, in addition to any effects on BM-CSPG, in disease states.

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Perlecan and Basement Membrane-Chondroitin Sulfate Proteoglycan (Bamacan) Are Two Basement Membrane Chondroitin/Dermatan Sulfate Proteoglycans in the Engelbreth-Holm-Swarm Tumor Matrix

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