Basement Membrane Proteoglycan in Various Tissues: Characterization Using Monoclonal Antibodies to the Engelbreth-Holm-Swarm Mouse Tumor Low Density Heparan Sulfate Proteoglycan

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Abstract. The Engelbreth-Holm-Swarm mouse tumor has been found to produce at least two molecular species of heparan sulfate proteoglycan, a low density one (LD) and a high density one, which differ not only in core proteins but also in glycosaminoglycan structures (Kato, M., Y. Koike, Y. Ito, S. Suzuki, and K. Kimata. 1987. *J. Biol. Chem.* 262:7180–7188). With aim at investigating their distribution and possible functions in tissues, monoclonal antibodies were produced. Hybridomas obtained by fusion of NS-1 mouse myeloma cells with spleen cells from the rat immunized with a mixture of these proteoglycans were selected by their ability to react with the antigen. Two of them secreted monoclonal antibodies (IgG2a), designated HK-84 and HK-102, that recognize specifically the core protein moiety of LD.

Immunofluorescent staining of various tissues (skeletal muscle, cardiac muscle, lung, brain, and kidney) with these monoclonal antibodies has demonstrated that the antigen molecules were present in all basement membranes of these tissues. SDS-PAGE of heparitinase-treated proteoglycan fractions prepared from these tissues and subsequent immunoblotting using these monoclonal antibodies have confirmed that the antigen molecule was LD, and further suggested that there was a tissue-specific variation in the core molecular size.

Based on these results, we propose that LD may be an essential component in all basement membranes.

HEPARAN sulfate proteoglycans have been classified into at least two families, cell surface type and basement membrane type, which are different not only in their distributions but also in their functions (Stow et al., 1985*a, b*; Jalkanen et al., 1985).

Antibodies to basement membrane heparan sulfate proteoglycan were first raised against the one from the Engelbreth-Holm-Swarm (EHS)[1] mouse tumor, designated BM-1 (Hassell et al., 1980). Immunohistological studies using these antibodies have shown that most if not all basement membranes contain determinants of the antibodies (Hassell et al., 1980; Kimata et al., 1985; Thesleff et al., 1981).

However, heparan sulfate proteoglycan in the EHS tumor was then found to be separated into two forms, low density form (LD) and high density form (HD) (Dziadek et al., 1985; Fujiwara et al., 1984; Hassell et al., 1985), and we have recently demonstrated that LD and most of HD are different molecules, not only in their core molecular species but also in their glycosaminoglycan chain structures (Kato et al., 1987). LD is a large proteoglycan containing heparan sulfate chains attached to a single core molecule (M_r = 450,000), whereas HD is a mixture of small proteoglycans with four different size core molecules (M_r = 34,000, 29,000, 27,000, and 21,000), most if not all of which bear both heparan sulfate and chondroitin sulfate.

Therefore, the wide reactivity of anti–BM-1 antibody with basement membranes of various tissues might be explained by assuming that anti–BM-1 antibody can recognize various molecules immunologically related to both LD and HD. Antibodies that were subsequently raised against purified LD or HD, however, crossreacted each other (Dziadek et al., 1985; Hassell et al., 1985) and LD and HD appeared to have common antigenic determinants although shared determinants differed in an affinity to the antibodies between LD and HD (Dziadek et al., 1985). This motivated us to produce a mAb specific for either LD or HD, to use as a reliable tool to investigate their tissue distributions and functions.

In this paper we first describe the establishment of two mAbs against LD. By using them, we have examined the distribution in various tissues and characterized the core molecules of LD isolated from those tissues. We show here that these mAbs stained all basement membranes in all tissues

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1. *Abbreviations used in this paper*: EHS, Engelbreth-Holm-Swarm; HD, high density species of heparan sulfate proteoglycan; LD, low density species of heparan sulfate proteoglycan.

tested (skeletal muscle, cardiac muscle, lung, brain, and kidney) and detected core molecules identical in size to LD.

Materials and Methods

Materials

DEAE-Sephacel, Sepharose CL-4B, and low molecular mass protein standards for SDS-PAGE were purchased from Pharmacia, Tokyo Japan; phenylmethylsulfonyl fluoride, DNase I (type IV; protease free), V8-protease (protease type XVII), BSA, and myosin were from Sigma Chemical Co., St. Louis, MO; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was from Dojindo Laboratories, Kumamoto Japan; polystyrene 96-well plates were from Linbro, Flow Laboratories, Hamden, CT; peroxidase-conjugated rabbit anti-rat Ig(G+M) immunoglobulins were from DAKOPATTS, Copenhagen, Denmark; peroxidase-conjugated protein A was from E. Y. Laboratories, Inc., San Mateo, CA; FITC-conjugated rabbit anti-rat Ig(G+M) immunoglobulins were from Zymed Laboratories, Inc., San Francisco, CA; FITC-conjugated goat anti-rabbit IgG immunoglobulins were from Cappel Laboratories, Inc., Cochranville, PA; mAb typing kit for rat immunoglobulins was from Miles Laboratories Inc., Naperville, IL.

Laminin and type IV collagen were prepared from the EHS mouse tumor according to the method of Kleinman et al. (Kleinman et al., 1982). Anti-type IV collagen serum was obtained from a rabbit immunized with the mouse EHS tumor type IV collagen as described previously (Kimata et al., 1985). As the serum had a slight cross-reactivity with laminin, the serum was passed through a laminin-conjugated Sepharose 4B column. The preparation thus obtained had no reactivity with other basement membrane components, laminin, heparan sulfate proteoglycan, and other types of collagen as tested by ELISA and immunoblotting (not shown). Affinity-purified anti-BM-1 polyclonal antibodies (Hassell et al., 1980) were a gift from Drs. J. R. Hassell, H.-J. Barrach, and G. R. Martin of the National Institutes of Health, Bethesda, MD. Heparitinase[2] was a generous gift from M. Mizutani of Seikagaku Kogyo, Co., Tokyo Japan.

Preparation of Heparan Sulfate Proteoglycans from the EHS Tumor

The preparation was described previously (Hassell et al., 1985; Kato et al., 1987). Briefly, freshly harvested tumor tissue was extracted with 4 M guanidine-HCl, 50 mM Tris-HCl, pH 7.4, 1% CHAPS (wt/vol), protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 10 mM EDTA), and the extract was fractionated by CsCl density gradient at starting density of $\rho_0 = 1.35$ g/ml. The bottom fractions ($\rho > 1.35$ g/ml) were pooled and applied to DEAE–Sephacel column in 7 M urea, 50 mM Tris-HCl, pH 7.4, 0.5% (vol/vol) Triton X-100, protease inhibitors. Bound materials were eluted with a linear gradient of 0–0.75 M NaCl in the same solution. Hexuronate-positive fractions (eluted with 0.3–0.6 M NaCl) were pooled and subjected to Sepharose CL-4B column chromatography in 4 M guanidine-HCl, 50 mM Tris-HCl, pH 7.4, 1% CHAPS (wt/vol), protease inhibitors. The fractions ($K_{av} = 0$–0.5) were pooled and further fractionated into two forms, LD and HD, by CsCl density gradient ($\rho_0 = 1.50$ g/ml) in the same solution.

Preparation of the Immunogen for Monoclonal Antibodies

An aliquot of the heparan sulfate proteoglycan fraction after DEAE-Sephacel column chromatography (a mixture of LD and HD) was reduced with 10 mM dithiothreitol at 50°C for 2 h in 4 M guanidine-HCl, 100 mM Tris-HCl, pH 8.0. After addition of solid iodoacetamide to give a final concentration of 30 mM, the sample was allowed to stand at room temperature for 3 h in the dark. The reduced heparan sulfate proteoglycan fraction thus obtained and the unreduced one were mixed and desalted by ethanol precipitation (Kimata et al., 1974), and the precipitate was dissolved in PBS.

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2. One unit of heparitinase was defined as the amount of the enzyme required to release 1 μmol of unsaturated oligosaccharides/min.

Immunization and Production of Monoclonal Antibodies

A mixture of unreduced and reduced heparan sulfate proteoglycan fractions (each contained 200 μg heparan sulfate proteoglycan as protein) in 250 μl of PBS was mixed with the equal volume of Freund's complete adjuvant and intraperitoneally injected into a 5-wk-old Wister rat. The rat received booster injections with Freund's incomplete adjuvand at 2-wk intervals. 1 wk after each injection, blood samples were taken to test reactivity with heparan sulfate proteoglycan by ELISA (see below). The rat with the fifth injection gave a high titer (>50,000) and 3 d after the sixth injection, the spleen was harvested.

For hybridoma production, a single cell suspension of rat spleen cells (1.5 × 10⁸ cells) was mixed with NS-1 mouse myeloma cells at a lymphocyte/myeloma ratio of 10:1 and treated with polyethylene glycol 4,000. The cells were plated in 96-well plates at a density of 1.5×10^5 cells/well in RPMI medium containing 10% (vol/vol) FCS and hypoxantine-aminopterin-thymidine. Hybridoma supernatants were screened by ELISA (see below). Hybridomas that showed a positive reaction were cloned and then subcloned by limiting dilution.

Screening and Characterization of mAbs

ELISA was done as described by Rennard et al. (Rennard et al., 1981) with a slight modification. Briefly, antigen solution containing 0.1 μg (as protein) of either heparan sulfate proteoglycan, or laminin, or type IV collagen, in 100 μl Voller's buffer was added to each well of a 96-well polystyrene plate and the plate was incubated overnight at 4°C. The solution was replaced with 100 μl PBS/1% (vol/vol) BSA and incubated for 1 h at 37°C to block the nonspecific binding of the antibody solution, and the wells were rinsed with 200 μl PBS, 0.05% (vol/vol) Tween 20 (PBS/Tween) three times. In some cases, each well was treated with 0.1 mU heparitinase in 100 μl 100 mM Tris-HCl, pH 7.2, 5 mM calcium acetate, protease inhibitors (Kato et al., 1985) for 1 h at 37°C before blocking. After the addition of 100 μl hybridoma medium (pH was adjusted to 7.4 with Tris-HCl buffer), the plate was incubated for 2 h at room temperature. After rinsing the wells with PBS/Tween three times, 100 μl peroxidase-conjugated anti-rat Ig(G+M) serum (final dilution 1:500 in PBS/Tween) was added to each well and the plate was incubated for 1 h at room temperature. The wells were rinsed four times with 200 μl of PBS/Tween. Peroxidase binding was measured by developing color using 0.01% (wt/vol) o-phenylenediamine containing 0.03% (vol/vol) hydrogen peroxide. For positive control, diluted (1:1,000 in PBS/Tween) rat anti-heparan sulfate proteoglycan antiserum obtained from the immunized rat was used. Nonspecific color reaction was minimized under these conditions (not shown).

SDS-PAGE and Immunoblotting

LD samples (1 μg as protein each) were treated with 1 mU heparitinase each for 1 h at 37°C (Kato et al., 1985). The digests were lyophilized, redissolved in 40 μl of 2% (wt/vol) SDS, 2% (wt/vol) glycerol, 0.002% (wt/vol) bromophenol blue with or without 5% (vol/vol) mercaptoethanol, and incubated at 37°C for 30 min. The samples were subjected to SDS-PAGE under the unreduced condition using a 5% separation gel (Laemmli, 1970). After the electrophoresis, proteins were transferred to a nitrocellulose membrane according to the method of Towbin et al. (Towbin et al., 1979). The membrane was soaked with 0.15 M NaCl/50 mM Tris-HCl, pH 7.4 (TBS), 1% (wt/vol) BSA for 1 h at 37°C, and incubated with hybridoma medium for 2 h at room temperature. After rinsing the membrane three times with TBS, 0.02% (vol/vol) Tween 20 (TBS/Tween), the membrane was incubated with peroxidase-conjugated anti-rat Ig(G+M) (final dilution, 1:500 in TBS/Tween) for 1 h at room temperature. The membrane was rinsed with TBS/Tween four times and the binding peroxidase was visualized by developing color using 0.05% (wt/vol) 4-chloro-1-naphthol containing 0.03% (vol/vol) hydrogen peroxide in TBS. For detection of proteins, the SDS-gel running at the same time was stained with Coomassie Blue R-250.

Detection of V8 Protease Fragments of LD with mAbs

LD samples (4 μg as protein each) were incubated with V8 protease (proteoglycan/protease = 100:1 dry weight ratio) in 50 mM Tris-HCl, pH 7.4, for 5 h at 37°C. The digests were subjected to SDS-PAGE using a 5–15% gradient gel, and followed by immunoblotting as described above. For detection of proteins, the SDS-gel running at the same time was stained with

Coomassie Blue R-250. Myosin heavy chain (200,000 M_r), phosphorylase b (94,000 M_r), albumin (67,000 M_r), ovalbumin (43,000 M_r), carbonic anhydrase (30,000 M_r), trypsin inhibitor (20,000 M_r), α-lactalbumin (14,000 M_r) were used for determination of molecular masses of immunoreactive bands.

Immunofluorescent Staining

Cryostat sections (6 μm thick) of tissues from 4-wk-old C57 black mice were prepared without fixation. They were incubated with the hybridoma medium or diluted anti-mouse type IV collagen antibodies (1:30 in PBS), and processed for immunofluorescent staining as described previously (Kimata et al., 1985). For positive controls, EHS tumor tissue sections were stained as above. For negative controls, RPMI, 10% (vol/vol) FCS or diluted (1:30 in PBS) nonimmunized rat serum were replaced with the hybridoma medium.

Immunodetection of Core Molecules of Heparan Sulfate Proteoglycans Isolated from Various Tissues

1 g of various tissues from 4-wk-old C57 black mice (skeletal muscle, cardiac muscle, lung, whole brain [including cerebrum, mesencephalon, and cerebellum], and kidney), were homogenized with a glass homogenizer in 7.5 ml of 7 M urea, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% (vol/vol) Triton X-100, protease inhibitors, and extracted twice with the same solution for 12 h at 4°C. The extracts were applied to DEAE-Sephacel columns (1.5 × 3 cm), and bound materials were eluted with two-fold column volume of 1 M NaCl in the same solution. After ethanol precipitation, samples were treated with 200 μg DNase I in 2 ml of 10 mM Tris-HCl, pH 7.0, 10 mM KCl, 3 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride at 37°C for 15 min. After ethanol precipitation to remove DNA fragments, samples (2 nmol hexuronate each) were digested with 10 mU of heparitinase for 1 h at 37°C, and subjected to SDS-PAGE using a 5–15% gradient gel and subsequent immunoblotting as described above. For positive control, the EHS tumor tissue (1 g) was extracted and processed the same way. Affinity-purified anti-BM-1 polyclonal antibodies (1:2,000 in TBS/Tween) were also used as a probe for basement membrane heparan sulfate proteoglycans, and detected with peroxidase-conjugated protein A (1:1,000 in TBS/Tween). Molecular masses of immunoreactive bands were determined as described above.

Preparation of Glomeruli and Analysis of the Heparan Sulfate Proteoglycans

Glomeruli were isolated by the graded sieving technique (Kobayashi et al., 1983). Briefly, 40 kidneys from 20 of 4-wk-old C57 black mice were used as a starting material. The preparation contained at least 70% pure glomeruli under the observation by phase contrast microscopy (not shown).

Urea/Triton X-100 extract of the glomerulus preparation was applied to DEAE-Sephacel column (0.8 × 1.5 cm) as described above. Bound materials were eluted and used for SDS-PAGE and subsequent immunoblotting.

Results

Production and Characterization of mAbs

A rat was immunized with a mixture of LD and HD, half of which had been subjected to reduction of disulfide bonds with dithiothreitol. The antiserum obtained from the rat reacted with both LD and HD although the reactivity with HD was fairly low (Fig. 1 C). Hybridization of the spleen cells and mouse myeloma was performed as described in Materials and Methods. Hybridoma supernatants were tested for the reactivity by ELISA with the heparan sulfate proteoglycan preparation used as immunogen. 23 hybridomas showed a positive reaction and cloned two times by limiting dilution.

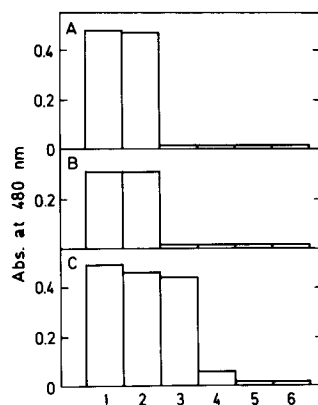Two of the cloned hybridomas, designated HK-84 (IgG2a) and HK-102 (IgG2a), recognized LD and heparitinase-

Figure 1. Specificities of HK-84 (A), HK-102 (B), or rat anti–heparan sulfate proteoglycan antiserum (C). Reactivities with LD (column 1), heparitinase-digested LD (column 2), reduced LD (column 3), HD (column 4), laminin (column 5), and type IV collagen (column 6) were examined by ELISA. Note that both monoclonal antibodies had no reactivity with reduced LD and HD although polyclonal antibodies had reactivity with both. Absorbance at 480 nm of a blank well was 0.02.

treated LD (the core molecules of LD), but not reduced LD, HD, laminin, and type IV collagen (Fig. 1). It should be noted here that both monoclonal antibodies did not show any reactivity with HD. Both monoclonal antibodies could only react with antigens from mice as far as we tested (not shown).

The reactivities of HK-84 and HK-102 with the core molecules of LD were further examined using the immunoblotting technique. The heparitinase-treated LD was subjected to SDS-PAGE without reduction and subsequent immunoblotting. Both HK-84 and HK-102 stained the major two bands of 450,000 and 360,000 M_r (Fig. 2, lanes 4 and 6). The staining patterns were the same as the one with Coomassie Blue of the core molecules of LD (Fig. 2, lane 2) (Ledbetter et al., 1987; Kato et al., 1987). The results clearly indicate that mAbs of HK-84 and HK-102 recognize the core molecule of LD. When the reduced core molecule of LD was subjected to SDS-PAGE and subsequent immunoblotting, neither HK-84 nor HK-102 stained the blotting membrane (not shown). The complete loss of the reactivity with the mAb suggested that their epitopes may be on the core protein moiety of LD.[3]

We further assessed the epitope structures on the core molecules of LD. LD was digested with V8 protease for 5 h at 37°C. The digests were subjected to SDS-PAGE without heparitinase digestion and subsequent immunoblotting with HK-84 and HK-102. Coomassie Blue staining showed that the digestion produced major peptides of 92,000, 80,000, 46,000, 44,000, 29,000, 28,000, 15,000, and 13,000 M_r plus numerous minor peptides varying in size (Fig. 3, lane 1). Both HK-84 and HK-102 showed the reactivity with the fragments of 150,000, 120,000, 92,000, 80,000, and 64,000 M_r released from LD (Fig. 3, lanes 2 and 3). Since the glycos-

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3. This conclusion, however, should be considered with certain reservations, because it is still possible that the epitopes were conformation-dependent oligosaccharide–protein interactions in which disulfide bonds were involved. Both epitopes appeared to be resistant to endoglycosidase F digestion under unreduced conditions (data not shown). However, the conditions used here were not yet confirmatory for the complete loss of the oligosaccharide.
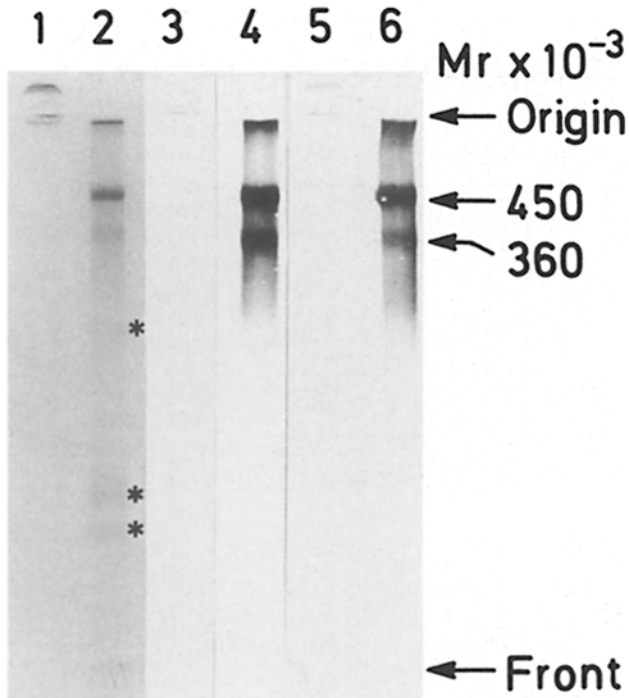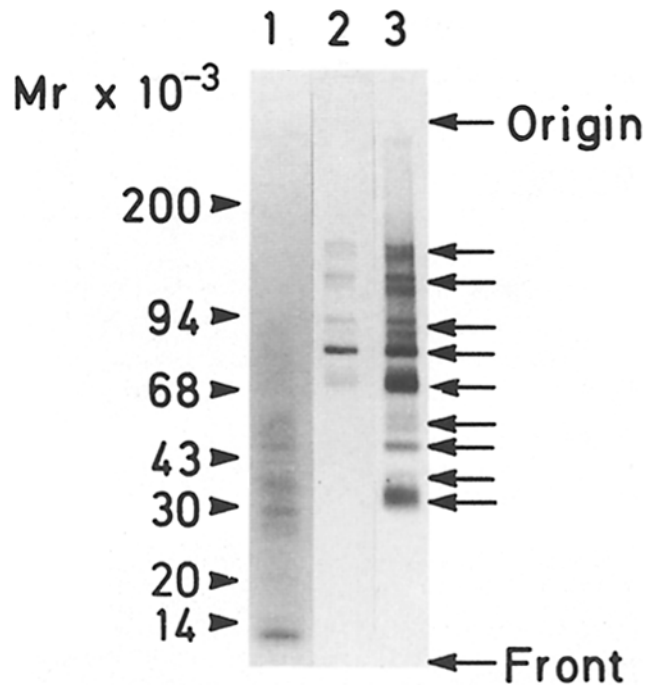
Figure 2. SDS-PAGE of LD (lanes *1* and *2*) and its immunoblotting with HK-84 (lanes *3* and *4*) or HK-102 (lanes *5* and *6*) before (lanes *1, 3,* and *5*) and after (lanes *2, 4,* and *6*) heparitinase digestion. Lanes *1* and *2*, stained with Coomassie Blue. Arrows indicate the positions of immunoreactive bands. Bands marked by asterisks in lane *2* were derived from a heparitinase preparation.

Figure 3. SDS-PAGE of V8-digested LD (lane *1*) and its immunoblotting with HK-84 (lane *2*) or HK-102 (lane *3*). Lane *1*, stained with Coomassie Blue. Arrows indicate the positions of immunoreactive fragments from LD; from the top, 150,000, 120,000, 92,000, 80,000, 64,000, 52,000, 46,000, 39,000, and 33,000 M_r. Arrowheads indicate the positions of standard proteins.

aminoglycan-bound fragments derived from LD by the digestion could hardly penetrate into the gel (Kato et al., 1987),[4] these antibody-reactive fragments would be derived from glycosaminoglycan-free core protein domains of LD. HK-84 could not react with the lower molecular mass fragments smaller than these (see the staining pattern with Coomassie Blue, Fig. 3, lane *1*). However, HK-102 yet recognized four additional smaller fragments of 52,000, 46,000, 39,000, and 33,000 M_r (Fig. 3, lane *3*). The difference in the reactivity suggests that the epitope of HK-84 may have been on the peptides released from the 64,000-M_r fragments when it was converted into the smaller ones.

Immunofluorescent Staining of Various Tissues with mAbs

HK-102 was first used to examine the distribution of LD in various tissues from mouse. The extracellular matrix of the EHS tumor tissue from which the immunogen was prepared was stained intensely (Fig. 4 *A*). In normal mouse tissues, basement membranes surrounding skeletal muscle fibers (endomysium) and ones surrounding each cardiac muscle cells were stained (Fig. 4, *B* and *C*, respectively). Alveolar and bronchial basement membranes in lung were also stained (Fig. 4 *D*). Blood vessel endothelial basement mem-

branes in all tissues were intensely stained. Especially in brain, they were stained with characteristic features (Fig. 4 *E*) and no significant difference was observed between cerebrum and cerebellum (not shown). In kidney, all basement membranes of Bowman's capsule, glomerulus, and renal tubules, as far as could be detected, were stained strongly (Fig. 5 *A*). When HK-84 was used to stain these tissues, the completely same patterns were observed (not shown). These tissues were also stained with anti–mouse type IV collagen antibodies. The patterns were again completely the same as those with HK-102 (Fig. 5 *B*; data on the tissues other than kidney were not shown). Since type IV collagen has been well characterized as a constitutive component for all basement membranes (Timpl and Martin, 1982), these results suggest that the antigen of HK-84 or HK-102 is present in all basement membranes of all tissues.

Immunodetection of Heparan Sulfate Proteoglycan Core Molecules Isolated from Various Tissues

We characterized molecules reactive with HK-84 and HK-102 in the tissues in which basement membranes were stained with these monoclonal antibodies with expectation that the reactive molecules were the core molecule of LD. Direct application of the tissue extracts to SDS-PAGE disturbed the subsequent immunoblotting analysis, due to the large amounts of proteinous contaminants. Since the antigen of HK-84 and HK-102 is a negatively charged molecule, we have taken advantage of this nature. Proteoglycan fractions were prepared from various tissues by DEAE–Sephacel

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4. This was confirmed by the complete retention of the Alcian blue–positive fragments at the origin of the gel, which is probably due to the fact that the heparan sulfate chains of LD are extremely large in size (average 60,000 M_r) (unpublished observations).
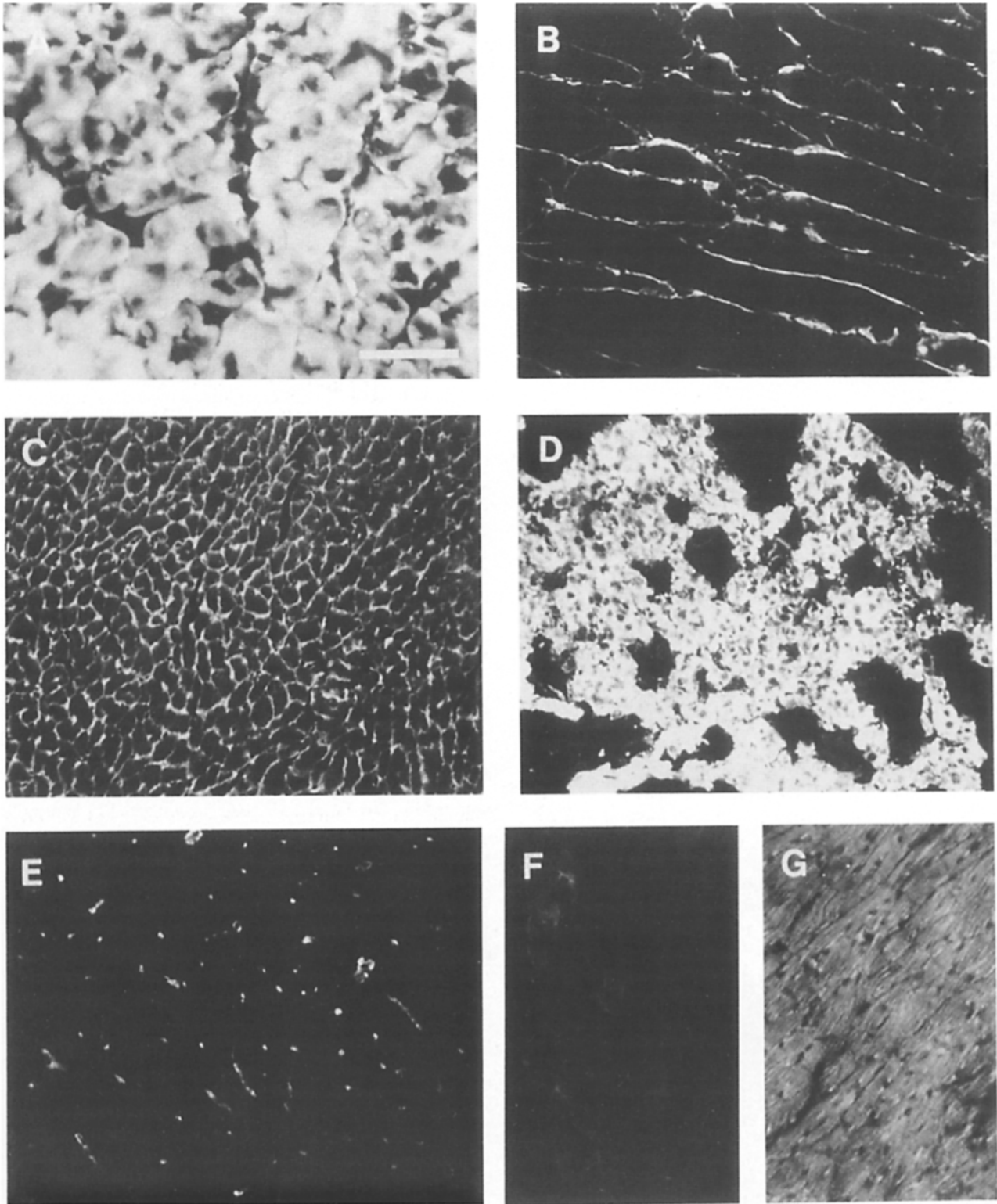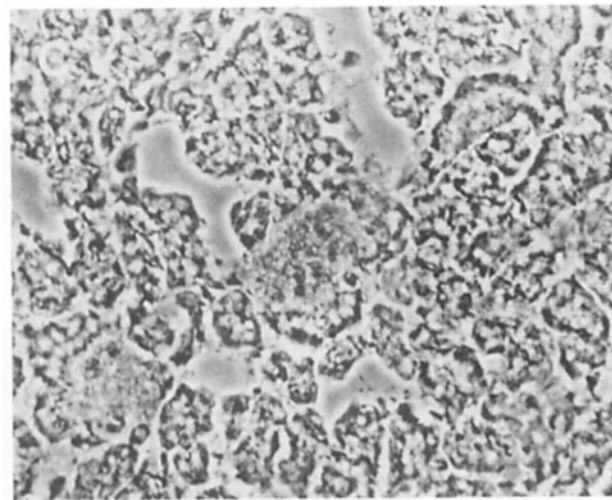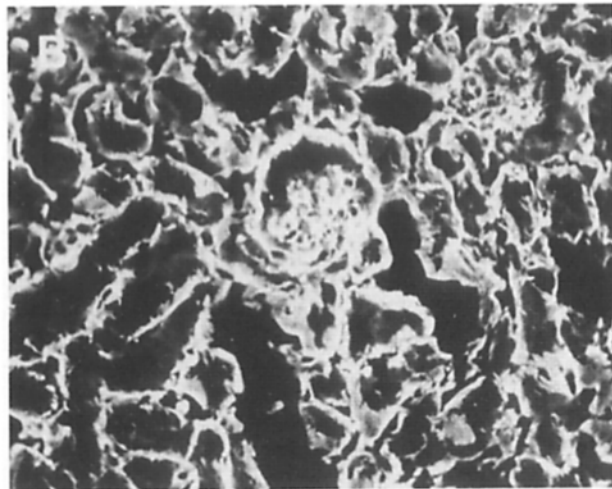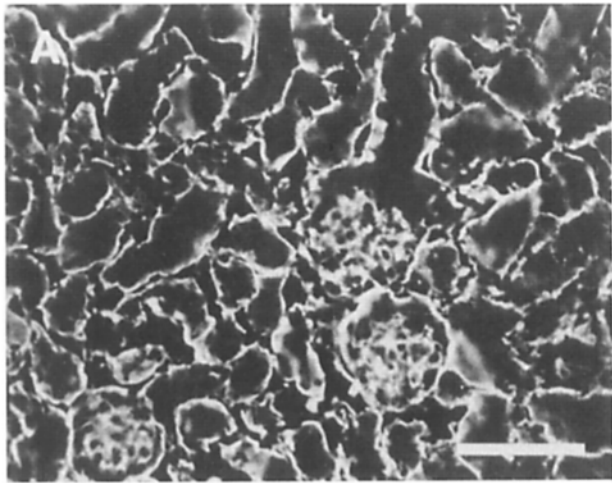
Figure 4. Immunofluorescent staining of various mouse tissues with HK-102. (*A*) The EHS tumor; (*B*) skeletal muscle; (*C*) cardiac muscle; (*D*) lung; (*E*) brain (cerebellum). Skeletal and cardiac muscle were stained with nonimmunized rat serum (*G* and *H*, respectively). Nonspecific fluorescence was observed inside the cells but not on the basement membranes in the latter sample. Bar, 100 μm.

column chromatography as described in Materials and Methods. The fractions were then treated with or without heparitinase and subjected to SDS-PAGE and subsequent immunoblotting. During preparing proteoglycans, care was taken to minimize degradation of core molecules as described in Materials and Methods.

In the proteoglycan preparations from the EHS tumor tissue 450,000- and 360,000-M_r core molecules of LD were

Figure 5. Immunofluorescent staining of mouse kidney with HK-102 (*A*) and anti–type IV collagen polyclonal antibodies (*B*). (*C*) Phase contrast micrograph of *B*. Note that HK-102 could react with all basement membranes that were recognized by anti–type IV collagen antibodies. Bar, 100 μm.

detected (Fig. 6, lane *1*), but an additional minor double band of 280,000 M_r was also detected. Since the fractions were prepared without CsCl density gradient centrifugation in the present study, the appearance of the minor doublet bands after heparitinase digestion was probably due to the difference in the preparation and revealed the presence of the LD which had a higher density than the typical LD in the EHS tumor tissue.[5]

In all samples without heparitinase digestion, smear and broad bands near the gel origin ($M_r > 450,000$) were detected (Fig. 6, lanes *1–7*) with weakly stained bands of 450,000 M_r, suggesting that some have less heparan sulfate chains and some are almost heparan sulfate free. When treated with heparitinase, large core molecules of 450,000 M_r, the same size as ones of LD, were commonly detected as major bands in all tissue samples (Fig. 6, lanes *2–7*). However, as observed with the EHS tumor sample, there were additional bands with tissue-specific variations. In skeletal muscle and cardiac muscle, a double band of ∿360,000 M_r was detected (Fig. 6, lanes *2* and *3*). In lung, there was a single 360,000-M_r band (Fig. 6, lane *4*). In kidney, two double bands of 360,000 and 280,000 M_r were detected (Fig. 6, lane *6*). In the glomerulus preparation, a double band of 360,000 M_r and a single band of 280,000 M_r were detected (Fig. 6, lane *7*). It is noteworthy that any core molecules bands smaller than 280,000 M_r could not be detected in this preparation. In addition, even when anti–BM-1 antibodies were used for the staining, we also obtained essentially the same immunoblotting pattern as those with the monoclonal antibodies (Fig. 7).

Together with these results, heparan sulfate proteoglycans that are identical with LD are present in all basement membranes in all tissues examined.

Discussion

Our mAbs, HK-84 and HK-102 recognized LD and molecules derived from LD, but did not have any reactivity with HD (Figs. 1 and 3).

We have recently shown that some degradation products of LD were recovered in the HD preparation (Kato et al., 1987). The observations that polyclonal antibodies to purified HD showed the reactivity with LD (Dziadek et al., 1985; Hassell et al., 1985) might be in part explained by this contamination. If so, one may speculate that the contaminants in the HD preparation must be derived from the glycosaminoglycan-bound regions of LD in view of no reactivity of the monoclonal antibodies with HD together with the epitope locations on the gycosaminoglycan-unbound regions of core molecule of LD as discussed below. This possibility is also supported by the previous observation that the glycosaminoglycan-bound derivatives from LD by V8 protease digestion had a density as high as HD (Kato et al., 1987).

According to the assumption by Ledbetter et al. (1987)
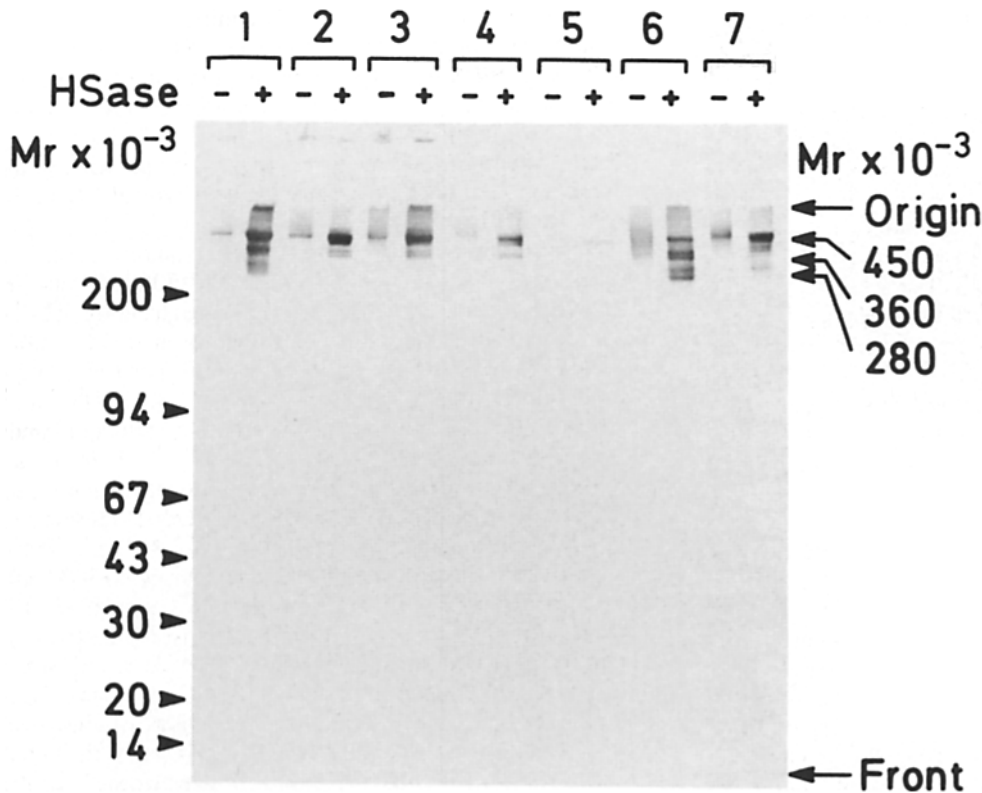
Figure 6. Immunoblotting of proteoglycan fractions from various mouse tissues with HK 102. Samples from the EHS tumor (lane *1*), skeletal muscle (lane *2*), cardiac muscle (lane *3*), lung (lane *4*), brain (lane *5*), kidney (lane *6*), and glomerulus preparation (lane *7*) were subjected to SDS–gel electrophoresis before (−) and after (+) heparitinase digestion and subsequently to immunoblotting. Arrows indicate the positions of immunoreactive bands. Arrowheads indicate the positions of standard proteins. The completely identical pattern was obtained when HK-84 was used for the immunostaining (see Fig. 7).
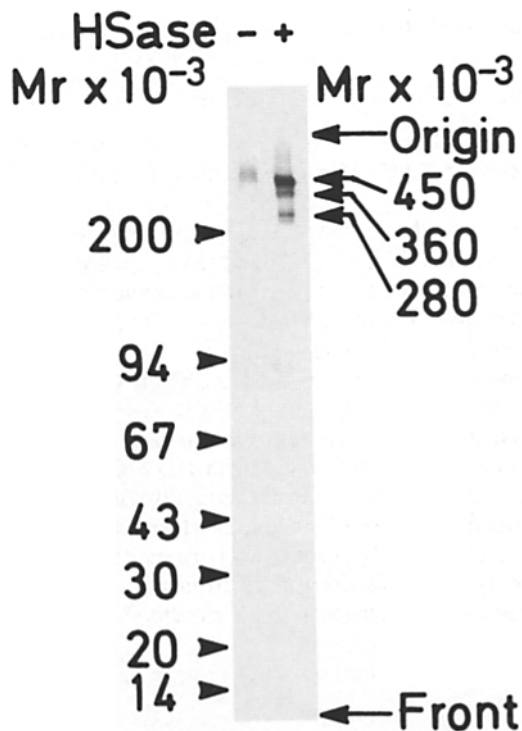
Figure 7. Immunoblotting of proteoglycan fractions from mouse glomeruli with anti–BM-1 polyclonal antibodies. Samples were subjected to SDS-PAGE before (−) and after (+) heparitinase digestion and then to immunoblotting. Arrows indicate the positions of immunoreactive bands. Arrowheads indicate the positions of standard proteins.

based on the kinetic study on the V8 protease digestion of LD, the 200,000-M_r peptide that lacks heparan sulfate chains is located at one end of the core protein of LD and the two 46,000- and 44,000-M_r peptides stable to the further digestion (probably by holding the structures with disulfide bonds) are contained within this polypeptide. Although strict comparison is difficult between their assumption and our findings in terms of the mobilities of the V8 protease digestion products on SDS-PAGE because of the differences in used experimental conditions, their 46,000-M_r peptide appeared to correspond to our HK-102–positive 46,000-M_r fragment. If so, the epitope of HK-102 may be on this fragment, the one of HK-84 may be on the peptide close to this fragment, and the loss of the epitopes by reduction may be related to the disulfide-bonded holding structure of these fragments.

Both HK-84 and HK-102 showed positive immunofluorescent staining to all basement membranes in tissues of skeletal muscle, cardiac muscle, lung, brain, and kidney (Figs. 4 and 5). Moreover, by the immunoblotting technique using HK-84 and HK-102, we observed in all proteoglycan fractions from these tissues the common existence of core molecules of heparan sulfate proteoglycan which were identical in size with the ones of LD, although there were tissue-specific variations in the relative amounts of core molecules of smaller size (Fig. 6). The result suggests that the positive fluorescent staining of the basement membranes in the tissues tested is due to the existence of LD, and therefore, this heparan sulfate proteoglycan may be commonly present in basement membranes.

Heparan sulfate proteoglycans that have a 450,000-M_r core molecule have been also found in amphibian muscle

(Anderson and Fambrough, 1983), L2 rat yolk sack tumor (Wewer et al., 1985), bovine corneal endothelial cells (Lander et al., 1985), and mouse Reichert's membranes (Paulsson et al., 1985). Moreover, in immunoblottings using HK-84, HK-102, or anti–BM-1 polyclonal antibodies, we recently observed the occurrence of LD in various cultured cells tested, mouse mammary epithelial cells, mouse mammary mesenchymal cells, mouse and bovine endothelial cells, mouse dermal fibroblasts, baby hamster kidney (BHK) cells, and rat smooth muscle cells (Kato, M., T. Okado, S. Suzuki, and K. Kamata, manuscript in preparation). Therefore, LD might be an essential component not only in tissues but also in various types of cultured cells.

Many mechanisms for the observed tissue-specific variations in size of the core molecules of LD could be operative. Ledbetter et al. (1985) observed that a precursor core molecule found within the EHS tumor cells was of a single polypeptide of 400,000 M_r. Therefore, the heterogeneity might be due to tissue-specific posttranslational modification of the same precursor protein and might represent some functional differences in the various basement membranes.

Heparan sulfate proteoglycan of murine glomerular basement membranes has been characterized previously (Edge and Spiro, 1987; Kanwer et al., 1984; Kobayashi et al., 1983; Stow et al., 1985a). According to their reports, the intact size of the proteoglycan ranges from 130,000 to 200,000 M_r and its core molecule from 18,000 to 143,000 M_r. We observed the existence of LD having core molecules of 450,000, 360,000, and 280,000 M_r not only in immunofluorescent staining of kidney (Fig. 5) but also in immunoblotting of the glomerular proteoglycan fraction with both HK-84 and HK-102 (Fig. 6). Since even anti–BM-1 polyclonal antibodies which were raised against the mixture of LD and HD (Hassell et al., 1980, 1985) did not stain any core molecules <280,000 M_r (Fig. 7), the results suggest the following alternative possibility. In glomeruli, there may be other types of basement membrane heparan sulfate proteoglycans that are not immunologically related to either LD or HD. Alternatively, proteolysis that could occur during the extensive purification procedure used to isolate glomerular basement membrane proteoglycans might have caused the differences in the sizes of the core molecules and the loss of antigenicity. Additional experiments are needed to establish the relationship between glomerular and EHS tumor heparan sulfate proteoglycans.

References

Anderson, M. J., and D. M. Fambrough. 1983. Aggregates of acetylcholine receptors are associated with plaques of a basal lamina heparan sulfate proteoglycan on the surface of skeletal muscle fibers. J. Cell Biol. 97:1396–1411.

Dziadek, M., S. Fujiwara, M. Paulsson, and R. Timpl. 1985. Immunological characterization of basement membrane types of heparan sulfate proteoglycan. EMBO (Eur. Mol. Biol. Organ.) J. 4:905–912.

Edge, A. S. B., and R. G. Spiro. 1987. Selective deglycosylation of the heparan sulfate proteoglycan of bovine glomerular basement membrane and identification of the core protein. J. Biol. Chem. 262:6893–6898.

Fujiwara, S., H. Wiedemann, R. Timpl, A. Lustig, and J. Engel. 1984. Structure and interactions of heparan sulfate proteoglycans from a mouse tumor basement membrane. Eur. J. Biochem. 143:145–157.

Hassell, J. R., W. C. Leyshon, S. R. Ledbetter, B. Tyree, S. Suzuki, M. Kato, K. Kimata, and H. K. Kleinman. 1985. Isolation of two forms of basement membrane proteoglycans. J. Biol. Chem. 260:8098–8105.

Hassell, J. R., P. G. Robey, H.-J. Barrach, J. Wilczek, S. I. Rennard, and G. R. Martin. 1980. Isolation of a heparan sulfate-containing proteoglycan from basement membrane. Proc. Natl. Acad. Sci. USA. 77:4494–4498.

Jalkanen, M., H. Nguyen, A. Rapraeger, N. Kurn, and M. Bernfield. 1985. Heparan sulfate proteoglycans from mouse mammary epithelial cells: localization on the cell surface with monoclonal antibody. J. Cell Biol. 101:976–984.

Kanwer, Y. S., A. Veis, J. H. Kimura, and M. L. Jakubowski. 1984. Characterization of heparan sulfate-proteoglycan of glomerular basement membrane. Proc. Natl. Acad. Sci. USA. 81:762–776.

Kato, M., Y. Koike, Y. Ito, S. Suzuki, and K. Kimata. 1987. Multiple forms of heparan sulfate proteoglycans in the Engelbreth-Holm-Swarm tumor: the occurrence of high density forms bearing both heparan sulfate and chondroitin sulfate side chains. J. Biol. Chem. 262:7180–7188.

Kato, M., Y. Oike, S. Suzuki, and K. Kimata. 1985. Selective removal of heparan sulfate chains from proteoheparan sulfate with a commercial heparitinase. Anal. Biochem. 148:479–784.

Kimata, K., M. Okayama, A. Oohira, and S. Suzuki. 1974. Heterogeneity of proteochondroitin sulfates produced by chondrocytes at different stages of cytodifferentiation. J. Biol. Chem. 249:1646–1653.

Kimata, K., T. Sakakura, Y. Inaguma, M. Kato, and Y. Nishizuka. 1985. Participation of two different mesenchymes in the developing mouse mammary gland: synthesis of basement membrane components by fat pad precursor cells. J. Embryol. Exp. Morph. 89:243–257.

Kleinman, H. K., M. L. McGarvey, L. A. Liotta, P. G. Robey, K. Tryggvason, and G. R. Martin. 1982. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS tumor. Biochemistry. 21:6188–6193.

Kobayashi, S., K. Oguri, K. Kobayashi, and M. Okayama. 1983. Isolation and characterization of proteoheparan sulfate synthesized in vitro by rat glomeruli. J. Biol. Chem. 258:12051–12057.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.

Lander, A. D., D. Fujii, and L. D. Reichardt. 1985. Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules. J. Cell Biol. 101:898–913.

Ledbetter, S. R., L. Fisher, and J. R. Hassell. 1987. Domain structure of the basement membrane heparan sulfate proteoglycan. Biochemistry. 26:988–995.

Ledbetter, S. R., B. Tyree, J. R. Hassell, and E. A. Horigan. 1985. Identification of precursor protein to basement membrane heparan sulfate proteoglycans. J. Biol. Chem. 260:8106–8113.

Paulsson, M., M. Dziadek, C. Suchanek, W. Hunttner, and R. Timpl. 1985. Nature of sulfated macromolecules in Reichert's membrane. Biochem. J. 231:571–579.

Rennard, S. I., K. Kimata, B. Dusemund, H.-J. Barrach, J. Wilczek, J. H. Kimura, and V. C. Hascall. 1981. An enzyme-linked immunoassay for the cartilage proteoglycan. Arch. Biochem. Biophys. 207:399–406.

Stow, J. L., H. Sawada, and M. G. Farquhar. 1985a. Basement membrane heparan sulfate proteoglycans are concentrated in the laminae rare and in podocytes of the rat renal glomerulus. Proc. Natl. Acad. Sci. USA. 82:3296–3300.

Stow, J. L., L. Kjellen, E. Unger, M. Hook, and M. G. Farquhar. 1985b. Heparan sulfate proteoglycans are concentrated on the sinusoidal plasmalemmal domain and in intracellular organelles of hepatocytes. J. Cell Biol. 100:975–980.

Thesleff, I., H.-J. Barrach, J. M. Foidart, A. Vaheri, R. M. Pratt, and G. R. Martin. 1981. Changes in the distribution of type IV collagen, laminin, proteoglycan, and fibronectin during mouse tooth development. Dev. Biol. 81:182–192.

Timpl, R., and G. R. Martin. 1982. Components of basement membranes. In Immunochemistry of Extracellular Matrix. Vol. 2. M. Furthmayar, editor. CRC Press, Inc., Boca Raton, Florida. 119–150.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350–4354.

Wewer, U. M., R. Albechtsen, and J. R. Hassell. 1985. Heparan sulfate proteoglycans made by different basement-membrane-producing tumors have immunological and structural similarities. Differentiation. 30:61–67.