Collagen XVIII Is a Basement Membrane Heparan Sulfate Proteoglycan*

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The present study shows that collagen XVIII is, next to perlecan and agrin, the third basal lamina heparan sulfate proteoglycan (HSPG) and the first collagen/proteoglycan with heparan sulfate side chains. By using monoclonal antibodies to an unidentified HSPG in chick, 14 cDNA clones were isolated from a chick yolk sac library. All clones had a common nucleotide sequence that was homologous to the mRNA sequences of mouse and human collagen XVIII. The deduced amino acid sequence of the chick fragment shows an 83% overall homology with the human and mouse collagen XVIII. Similar to the human and mouse homologue, the chick collagen XVIII mRNA has a size of 4.5 kilobase pairs. In Western blots, collagen XVIII appeared as a smear with a molecular mass of 300 kDa. After treatment with heparitinase, the protein was reduced in molecular mass by 120 kDa to a protein core of 180 kDa. Collagen XVIII has typical features of a collagen, such as its existence, under non-denaturing conditions, as a non-covalently linked oligomer, and a sensitivity of the core protein to collagenase digestion. It also has characteristics of an HSPG, such as long heparitinase-sensitive carbohydrate chains and a highly negative net charge. Collagen XVIII is abundant in basal laminae of the retina, epidermis, pia, cardiac and striated muscle, kidney, blood vessels, and lung. In situ hybridization showed that the main expression of collagen XVIII HSPG in the chick embryo is in the kidney and the peripheral nervous system. As a substrate, collagen XVIII moderately promoted the adhesion of Schwann cells but had no such activity on peripheral nervous system neurons and axons.

Heparan sulfate proteoglycans (HSPGs)1 are members of a family of cell-surface proteins with long carbohydrate chains of repeating disaccharide units (1–3). They exist as integral membrane proteins (4) or as secreted extracellular matrix proteins. HSPGs have a variety of biological functions, such as serving as molecular sieves for the ultrafiltration of blood in the kidney (5), sequestering growth factors (6–8), lining blood vessels (9), and a highly negative net charge. Collagen XVIII is a member of the collagen family with heparan sulfate side chains. Collagen XVIII was identified several years ago by screening cDNA libraries with probes for collagen-like proteins (20). Here, we present evidence that collagen XVIII is the first member of the collagen family with heparan sulfate side chains. Collagen XVIII was also shown to be associated with collagen protein cores (21). We present evidence that collagen XVIII is indeed a proteoglycan (21, 24). However, attempts to show that collagen XVIII is a chondroitin sulfate proteoglycan were unsuccessful, as the digestion with chondroitinase did not lead to a shift in the molecular weight of the protein (25). The present data demonstrate that collagen XVIII is indeed a proteoglycan with almost half of its molecular weight in heparan sulfate side chains. Furthermore, collagen XVIII is a constituent of almost all embryonic and adult basal laminae. Based on the presence of several consensus sequences for glycosaminoglycan attachment, it was suggested that collagen XVIII could be a proteoglycan (21, 24). However, attempts to show that collagen XVIII is a chondroitin sulfate proteoglycan were unsuccessful, as the digestion with chondroitinase did not lead to a shift in the molecular weight of the protein (25). The present data demonstrate that collagen XVIII is indeed a proteoglycan with almost half of its molecular weight in heparan sulfate side chains. Furthermore, collagen XVIII is a constituent of almost all embryonic and adult basal laminae.

**EXPERIMENTAL PROCEDURES**

Antibodies—The two monoclonal antibodies (mAb 6C4 and 1B11) against a heparitinase-sensitive proteoglycan from chick embryonic basal lamina were obtained from hybridoma clones of X63Ag8.653 myeloma cells (29) fused with splenocytes of a mouse immunized with embryonic day (E) 10 chick retinal basal lamina. Cross-reactivity studies (30) and the isolation of cDNAs with identical nucleotide sequences with both antibodies (this study) show that the 1B11 and 6C4 antibodies recognize the same protein. Monoclonal antibodies to agrin (clone 6D2; see Ref. 31), collagen IV (clones IA8, II1B2, ID2, see Ref. 32), kindly provided by Drs. Fitch and Linsenmayer (Tufts University, Bos-
ton), NCAM (clone 9H2; see Ref. 33), and tenascin (clone M1, see Ref. 34, Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore) were mouse IgGs. Rabbit antisera to Engelbreth-Holm-Swarm mouse tumor perlecan (10, 35) were a generous gift from Dr. M. Vigny (Institute National de la Sante, Paris) and Dr. J. Hassel (Shriners Hospital for Crippled Children, Tampa Bay, FL). A mouse monoclonal antibody (clone 33-2, see Ref. 36) to a chicken heparan sulfate proteoglycan was obtained from the Developmental Studies Hybridoma Bank (John Hopkins University, Baltimore). The tissue distribution and the molecular weight of the antigen strongly suggest that the 33-2 antibody recognizes chick perlecan.

Molecular Cloning of Chick Collagen XVIII and Northern Blots—The 6C4 and 1B11 hybridoma supernatant was used to screen each 600,000 and 300,000 recombinants from an E5 chick yolk sac lambda-ZAP II cDNA expression library (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Eleven positive phage clones from the 6C4 screen and 3 clones from the 1B11 screen were plaque-purified, and Bluescript II SK(+) phagemids containing the inserts were rescued by in vivo excision. The plasmids were purified using the Wizard plasmid purification kit (Promega, Madison, WI) and sequenced on both strands at the University of Pittsburgh sequencing facility by using T3, T7, and internal sequencing primers and the Dye Terminator Cycle Sequencing Ready Reaction Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. 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FIG. 1. a, diagram comparing the domain structure of human collagen α1(XVIII) and the chick α1(XVIII) fragment. Triple helical (COL) regions are indicated by wide rectangular areas; non-triple helical (NC) domains are indicated by thin rectangular areas. The most homologous regions in the two chains are indicated by dark-stippled boxes. Vertical lines indicate potential O-linked glycan attachment sites. b, alignment of the C-terminal part of the human collagen XVIII (24) with the partial chick collagen XVIII sequence. The COL 9 and 10 as well as the NC 10 and 11 domains are indicated. The beginning of the endostatin sequence in the NC 11 domain is also indicated. The chick and human sequences show homologies throughout the entire sequence. Note the almost 100% homology in the COL 9 and 10, and in the N-terminal part of the endostatin sequence of the NC 11 domain.

FIG. 2. Northern blots showing the size and abundance of chick collagen XVIII mRNA in kidney (lane 1), liver (lane 2), brain (lane 3), and heart (lane 4). A strong band of 4.5 kb was detected in samples of kidney and heart, whereas faint bands were detected in liver and brain. The blot with lanes 1–4 was probed with a digoxigenin-labeled RNA. Another blot, probed with a radiolabeled DNA probe (lanes 5 and 6), showed a strong band of 4.5 kb in a sample from cultured astrocytes and a faint 4.5-kb band in an mRNA sample of brain.
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Mannheim). A digoxigenin-labeled RNA ladder (Boehringer Mannheim) was used to determine the size of the collagen XVIII mRNA. Another set of Northern blots, containing mRNA samples of E9 chick brain, gut, heart, liver, and cultured astrocytes (38), was probed with the radiolabeled insert from the p10d plasmid. The probe was synthesized using random primers and [α-32P]dATP. Membranes were hybridized for 20 h with radioactive probe and washed with high stringency.

**Gel Electrophoresis and Western Blotting—Amnion, kidneys, and meninges were dissected from E10 chick embryos and homogenized in calcium- and magnesium-free Hank's solution (CMF), 50 μg/ml DNase I (Sigma). The homogenates were spun at 3000 rpm for 5 min, and the pellets were re-extracted in 8 M urea in CMF and centrifuged again at 3000 rpm. The supernatants from the urea extracts were used as the samples. To characterize further the biochemical nature of the protein, samples were treated with 0.5 units of heparitinase (Seikagaku, Rockville, MA), 0.5 units of chondroitinase ABC (Sigma), or 5 units/ml collagenase (Sigma type VII) for 1 h at 37 °C in PBS, 2% bovine serum albumin, pH 7.4. Intact tissues were treated first with the enzymes for 1 h and washed in CMF twice. Following homogenization in CMF and centrifugation, the pellets were extracted in 8 M urea, spun again, and the supernatants from the urea extracts were used as samples. Vitreous bodies were collected from E10 chick eyes, centrifuged at 15,000 rpm, and the supernatant was used, undigested or digested, as samples. Vitreous body supernatant and tissue extracts were mixed with SDS sample buffer/β-mercaptoethanol and were loaded either boiled or non-boiled onto the gels. The proteins were separated by 3.6–14% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon P membrane filters (Millipore, Bedford, MA). After blocking with 5% skim milk in CMF, the blots were incubated with the 6C4 mAb to collagen XVIII, and only with a 1.5 M salt wash (lane 12), the bulk of collagen XVIII was eluted from the beads.

**Histology—** The trunks of E3 to E16 chick embryos and the dissected spinal cord of a 5-day-old chick (P5) were fixed in 4% paraformaldehyde in 0.1 M potassium phosphate buffer, pH 7.4, for 1 h. After washing in CMF and cryoprotecting with 30% sucrose for 4 h, the specimens were embedded in Tissue-Tek (Miles, Elkhart, IN) and sectioned with a cryostat at 25 μm. Muscle samples from post-hatched (P) 5 chicken were sectioned unfixed. Sections were mounted on Superfrost slides (Fisher) and incubated with hybridoma supernatants for 1 h. After 3 rinses, the sections were incubated with 1:500 Cy3-labeled goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h. Following 2 final rinses, the specimens were examined with an epifluorescence microscope (Zeiss, Thornwood, NY). Basal lamina whole mounts from E6 chick retinas were prepared as described (39). The whole mounts were rinsed several times with 2% Triton X-100 to remove all cellular components and stained with the 6C4 mAb to collagen XVIII or the 9H2 mAb to NCAM (see above).

**In Situ Hybridization—** The p10d plasmid was linearized, and a digoxigenin-labeled antisense and sense cRNA probe were synthesized from the template using an RNA polymerase labeling kit (Boehringer Mannheim). The in situ hybridization procedure of tissue sections follows the procedure described previously (40). Adjacent sections were
also labeled with probes to agrin and collagen XII. The isolation of the agrin plasmid has been described previously (13), and the plasmid to collagen XII was obtained by antibody screening of a E5 chick yolk sac cDNA library with a polyclonal antisera to chondroitin sulfate proteoglycans from chick embryos. The identity of the collagen XII clone was confirmed by matching the DNA sequence with the mRNA sequence of collagen XII (bp 5070–5604) in the data bank using the Blast search.2

Collagen XVIII Isolation—Collagen XVIII was isolated from E10 chicken vitreous bodies by ion exchange chromatography followed by immunofinity purification using the 6C4 mAb coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, see Ref. 30). The vitreous bodies from approximately 400 eyes were collected and spun at 15,000 rpm for 20 min. The supernatant (100 ml) was run over a 3-ml column of Q-Sepharose (Amersham Pharmacia Biotech). The column was washed with 50 mM Tris, pH 7.4, and eluted with a salt gradient from 0 to 2 M NaCl in 50 mM Tris, pH 7.4. Fractions containing collagen XVIII immunoreactivity were collected, dialyzed against CMF, and run over an affinity column containing 6C4 mAb conjugated to Sepharose. After extensive washing with CMF, 0.5% Tween, and CMF alone, collagen XVIII was eluted with 0.1 M diethylamine buffer, pH 11.5. Fractions were tested by a dot assay on nitrocellulose membrane filters, and collagen XVIII-containing fractions were concentrated using Centricon filters (Millipore). The concentration of the proteoglycan was estimated both by the dye-binding method of Minamide and Bamburg (41) and by gel electrophoresis. Gels were stained with Coomassie and a modified Alcian blue/silver staining procedure for the detection of proteoglycans (42). The concentration of the samples was also estimated by comparing the staining density of the proteoglycan band with that of known concentrations of laminin and fibronectin (Life Technologies, Inc.) in adjacent lanes of the gel. Each purification yielded between 10 and 50 μg of proteoglycan.

Cell Adhesion Assays—As substrates for cell adhesion and neurite outgrowth assays, collagen XVIII (50 μg/ml), merosin (Life Technologies, Inc., 50 μg/ml), and ovalbumin (Sigma, 50 μg/ml) were spotted onto nitrocellulose-coated dishes (43). The dishes were rinsed 3 times in CMF, 2% ovalbumin, and 60 μl of a cell suspension from trypsin-dissociated E12 chick dorsal root ganglia in Dulbecco’s modified Eagle’s medium; 2% ovalbumin was added to the substrates. After 6 h, the cultures were rinsed 3 times with Dulbecco’s modified Eagle’s medium, 2% ovalbumin, and finally fixed in 4% paraformaldehyde for 1 h. The number of cells per microscopic field were counted at 3 different areas of every culture dish. For each substrate, the assays were run in triplicate. To account for variations between different test runs, the average number of cells on the ovalbumin control substrate was set at 100%. The average number of cells attached on the other substrates was expressed as multiples of the ovalbumin control. The identity of the cells as Schwann cells was done by staining cultures with a mAb to P0 (clone 1E8, kindly provided by Dr. E. Frank, University of Pittsburgh, see Ref. 44), a protein specific for Schwann cells. According to the immunohistochemistry, over 90% of the cells in the ovalbumin and collagen XVIII-coated dishes were Schwann cells.

RESULTS

Cloning of Partial cDNA Sequence of Chick XVIII Collagen—Two monoclonal antibodies to a putatively new basal lamina HSPG were used to screen an E5 chick yolk sac cDNA expression library. The screen resulted in the isolation of 14 cDNA clones ranging in size from 2 to 2.5 kb. All 14 clones overlapped in their sequence. A Blast search revealed that the common sequence was highly homologous to the 3’ third of human and mouse collagen XVIII mRNA. The 2.5- kb p10d cDNA clone was the longest and most complete cDNA clone, as it contained at its 3’ end an AATAAA termination signal followed by a poly(A) tail (Gene Bank™ accession number AF083440). The p10d clone also contained 1.2 kb of 3’-untranslated region and 1.2 kb of a coding sequence that was homologous to the 3’ third of the coding region of human and mouse collagen XVIII. Translation of the coding sequence showed a 387-amino acid long peptide that started at its N-terminal end with a typical collagen sequence that was followed by a short, non-collagenous stretch. After yet another short collagen sequence, the peptide ended at its C-terminal part with a long, non-collagenous domain (Fig. 1). The two chick collagen domains were over 90% homologous with the 9th and 10th collagen domain (Col9 and Col10) of the human and mouse collagen XVIII. Both non-collagenous domains (NC10 and NC11) were between 60 and 90% identical to the human and mouse collagen XVIII counterparts. The most homologous sequence in the NC11 domain corresponded to the N-terminal part of the 18-kDa endostatin peptide (Fig. 1). The overall amino acid sequence identity of the chick and human collagen XVIII was 65%, and the overall homology was 83%. The identity and homology between the chick and human amino acid sequences are slightly lower than the identity and homology between the human and mouse collagen XVIII amino acid sequences, which are 79 and 95%, respectively. The chick sequence showed one serine-glycine consensus sequence as a potential GAG attachment site, which was conserved in the human and mouse homologue.

FIG. 5. Fluorescent micrograph of a cross-sections through the retina (R) shows the presence of collagen XVIII in the retinal basal lamina (arrowhead), pigment epithelial basal lamina (small arrow), and in the periorcular mesenchyme (a). In a cell-free retinal basal lamina whole mount preparation (b), collagen XVIII was abundant in the extracellular matrix of the basal lamina. The basal lamina of the contralateral eye was prepared the same way and stained with an antibody to NCAM, which stains the plasma membranes of retinal cells. There was no NCAM immunoreactivity detectable in the basal lamina preparation, showing the absence of retinal cell in these whole mounts (c). The edge of the basal lamina is indicated by arrowheads. Bar, 100 μm.

2 W. Halfter, unpublished observations.
was in all tissues 4.5 kb (Fig. 2, lanes 1–4). The Northern blots were repeated but using a radiolabeled DNA probe. The blots showed a single band of 4.5 kb in all samples that was faint in samples of brain (Fig. 2, lane 6), gut, and liver (not shown) but prominent in samples from cultured astrocytes (Fig. 2, lane 5). Based on the overall sequence homology, we assumed that the chick HSPG is the chick homologue of collagen XVIII, and we decided to tentatively refer to the unidentified chick HSPG as collagen XVIII in further studies.

Biochemical Characteristics of Collagen XVIII—In Western blots, intact chick collagen XVIII from chick vitreous body appeared as a smear with an apparent molecular mass of 300 kDa (Fig. 3, lane 1, and Fig. 4, lane 4). Digestion with heparitinase led to a reduction of the size of the protein by 120 kDa to a core protein of 180 kDa (Fig. 3, lane 2, and Fig. 4, lane 5). Treatment with chondroitinase had no effect on the size or banding pattern of the molecule (Fig. 3, lane 4, and Fig. 4, lane 6), confirming previous experiments with mouse collagen XVIII (25). When the samples were treated with collagenase, the protein was no longer detectable in Western blots (Fig. 3, lane 3, and Fig. 4, lane 7), demonstrating a collagenase-sensitive core protein. To check the collagenase and heparitinase preparations for potential protease contamination, the same samples that were used to detect collagen XVIII were also probed with an antibody to tenasin. Tenasin, a large 220-kDa glycoprotein, was degraded neither by collagenase (Fig. 3, lane 6) nor heparitinase (Fig. 3, lane 7). The specificity of collagenase for only collagenous proteins was confirmed in an independent study by digesting chick vitreous body and meninges with the enzyme (45, 46); all collagenous components from vitreous body and meninges, such as collagen I, II, IV, and IX, were no longer detectable in Western blots, whereas the abundance and the banding pattern of non-collagenous vitreous body and meningeal proteins, such as laminin, agrin, nidogen, tenasin, fibronectin, and axonin I, were unchanged.

Collagen XVIII was also detectable in urea extracts of meninges (Fig. 3, lanes 8 and 9), amnion (Fig. 3, lanes 10–12), and kidney (Fig. 3, lanes 13 and 14). In contrast to agrin, collagen XVIII was not detectable in PBS extract (Fig. 3, lane 11) and only solubilized in the presence of high molar urea. In all tissue samples tested, heparitinase treatment led to a major reduction in size, and in all tissue samples tested the protein core had a molecular mass of 180 kDa (Fig. 3, lanes 2, 8, 9, 12, and 14). Without heparitinase treatment, only in meningeal tissue a minor 180-kDa band was detectable (Fig. 3, lane 8), indicating that collagen XVIII exists almost exclusively in the glycosylated version as an HSPG.

When unboiled sample was loaded onto the gel, collagen XVIII appeared in Western blots with a molecular mass between 700 and 1000 kDa (Fig. 4, lane 1), most likely representing a trimer of the 300-kDa collagen XVIII monomers. The oligomerization was dependent on interactions between the core protein monomers and was independent of the heparan sulfate side chains, as heparitinase treatment of the samples did not lead to the dissociation of the complex but rather showed only the predicted drop in molecular weight that reflected the loss of the heparan sulfate side chains (Fig. 4, lane 2). Chondroitinase treatment did not affect the size and banding pattern of the collagen XVIII oligomer (Fig. 4, lane 3).

A hallmark of proteoglycans is their negative net charge and a strong binding to anion exchange beads, such as Q-Sepharose. Incubating vitreous body supernatant with Q-Sepharose showed that the collagen XVIII that is present in vitreous body (Fig. 4, lane 8) quantitatively bound to the Q-Sepharose beads (Fig. 4, lane 9). Washing the beads with buffer containing increasing salt concentrations showed that at 0.5 and 1 M NaCl,
only minor quantities of the protein were eluted, whereas the bulk of collagen XVIII was eluted at 1.5 M salt (Fig. 4, lanes 10–12). With a continuous salt gradient, collagen XVIII eluted from the Q-Sepharose beads between 0.8 and 1.4 M NaCl (not shown), at a similar ionic strength as agrin and collagen IX, both of which are proteoglycans and present in vitreous body (13, 31). Collagen II, which is also present in vitreous body, but is not a proteoglycan, was eluted at salt concentrations less that 0.5 M NaCl (not shown).

Distribution of Collagen XVIII—Immunocytochemistry showed that collagen XVIII was abundant in almost all basal laminae, like the classical basal lamina proteins collagen IV, laminin, agrin, and perlecan. In the eye, collagen XVIII was present in the inner limiting membrane of the retina (Fig. 5a), the pigment epithelium (Fig. 5a), and the lens capsule (not shown). The strong staining of cell-free whole mount preparations of chick retinal basal lamina (Fig. 5a) demonstrated that collagen XVIII is part of the extracellular basal lamina matrix, rather than associated with retinal cells. Collagen XVIII was also detected in the basal laminae of the epidermis, skeletal muscle, kidney tubules and glomeruli, lung tissue, the cardiac muscle (not shown), blood vessels (Fig. 7a), the pial basal lamina (Figs. 6a and 7a), and, especially prominent, in ganglia and nerves of the peripheral nervous system (Figs. 6a and 7a). In the post-hatched chick, collagen XVIII was prominent in basal laminae of skeletal muscle fibers, in Schwann cell basal laminae of peripheral ganglia and nerves (not shown), and along blood vessels (Fig. 7b). In the vascular system, collagen XVIII immunoreactivity appeared slightly later in development compared with that of collagen IV (compare Fig. 7, a and c); however, in post-hatched chick spinal cord, the distribution of collagen XVIII and IV in the spinal cord vascular system was indistinguishable (compare Figs. 7, b and d). Despite similarities (Figs. 6–8), the distribution of collagen XVIII was not entirely identical with that of perlecan, agrin, and collagen IV. The distinction was most obvious in the gut (Fig. 8), where agrin, perlecan, and collagen IV decorated the inner and outer smooth muscle layers, whereas collagen XVIII was absent from these areas.

Distribution of Collagen XVIII mRNA—In situ hybridization showed that collagen XVIII mRNA was abundant in the developing heart (not shown), kidney, and the peripheral nervous system (Fig. 9, a and c). mRNA was also detected in the meninges surrounding the spinal cord. Except for the strong labeling of the roof plate of the spinal cord (Fig. 9a, large arrow), small amounts of collagen XVIII mRNA were detected along the ventricular lining in the central nervous system, reflecting a minor expression of collagen XVIII by the radial glia cells. The scarcity of collagen XVIII mRNA in the central nervous system contrasts to the abundance of agrin mRNA, which is particularly prominent in radial glia cells and motoneurons of the spinal cord (Fig. 9d, 47). Agrin mRNA, on the other hand, was much less abundant in peripheral nerves or muscle compared with collagen XVIII mRNA. Collagen XII, which is a part-time collagen/chondroitin sulfate proteoglycan, was detectable only as a minor streak dorsal to the neural tube and...
Collagen XVIII as a Substrate for Schwann Cell Attachment—Collagen XVIII was isolated from vitreous body and used as a substrate in vitro for cell adhesion and neurite outgrowth assays. The collagen XVIII substrate promoted the attachment of Schwann cells more effectively than ovalbumin as the control. Between 3 and 8 cells per microscopic field (mean 5.2 ± 1) were counted on the ovalbumin substrates, whereas the number of Schwann cells on collagen XVIII substrates was between 30 and 70 (mean 49 ± 7) per microscopic field. Averaged for all experiments, the number of Schwann cells on collagen XVIII was 9.1-fold greater than on ovalbumin. Not only was the number of attached cells on collagen XVIII substrates greater, but the Schwann cells were also wider spread on the collagen XVIII substrate than on the ovalbumin control (Fig. 10). Compared with merosin, which served as a positive control, however, collagen XVIII was less effective in the promotion of Schwann cell attachment (between 500 and 530 cells/microscopic field; mean 515 ± 180), showing that cell adhesion promotive activity of collagen XVIII is not very strong. Furthermore, collagen XVIII substrates did not promote the attachment of neurons and did not promote neurite outgrowth from dorsal root ganglia explants, whereas control explants on merosin substrates showed numerous neurons in the single cell cultures and abundant neurite outgrowth during the same time of incubation (not shown).

DISCUSSION

Identity of the Chick Collagen XVIII—Screening of a chick yolk sac cDNA library with antibodies to a previously unknown HSPG led to the isolation of 14 cDNA clones with a common DNA and amino acid sequence that were both homologous to the 3’ third and the N-terminal part of human and mouse collagen XVIII. The deduced chick amino acid sequence comprised two collagen domains and two non-collagenous domains. The collagen sequences, corresponding to the 9th and 10th human and mouse collagen XVIII domain, are close to 100% conserved compared with mouse and human collagen XVIII, whereas the non-collagenous domains are less homologous to the human and mouse sequence. The average 83% homology between the chick sequence with the human collagen XVIII throughout the entire sequence indicated to us that the HSPG is chick collagen XVIII. Further support that the unknown chick HSPG is collagen XVIII is the size of the mRNA that is with 4.5 kb very similar to the size of the collagen XVIII mRNA of mouse and human (21, 23, 24). The most convincing evidence that the unknown HSPG is indeed a collagen is derived from biochemical studies. Western blots showed that the protein core of the HSPG is sensitive to collagenase digestion and exists under non-denaturing conditions as a non-covalently linked oligomer, most likely a trimer, which is typical for all collagens. The oligomerization of the core protein does not require the presence of the heparan sulfate side chains but rather occurs by the interaction of the core protein monomers. The protein also showed typical features of HSPGs as follows: the protein bound strongly to anion exchange beads and dissociated from the beads only at high salt concentration, similar to agrin and collagen IX, both proteoglycans. Furthermore, the most important identification for proteoglycans is the presence of long carbohydrate chains that are sensitive to proteoglycan-specific glycosidases. As shown in Figs. 3 and 4, the chick HSPG/collagen carries glycosaminoglycans side chains that were sensitive to heparitinase. After digestion with heparitinase, but not with chondroitinase, the molecular mass was...
reduced by 120 kDa, which implies that these carbohydrate side chains are several thousand kDa in size. That collagen XVIII is an HSPG is consistent with previous assumptions, since sequencing data of the human and mouse collagen XVIII revealed the existence of several SG consensus sequences as potential sites for GAG attachment (21, 23, 24). Our data demonstrate that the SG consensus sequences are indeed used to link heparan sulfate side chains to the protein core. With exception of a minor quantity in the meninges, the 180-kDa core protein of collagen XVIII was not detected in Western blots without heparitinase treatment, indicating that collagen XVIII almost always carries its heparan sulfate side chains and is constitutively an HSPG. The HSPG nature of collagen XVIII applies to all three possible splice variants, since the mAbs used in this study recognize an antigenic site of the unspliced and conserved C-terminal part of chick collagen XVIII, and based on this fact, our Western blots detected all possible variants of collagen XVIII. In summary, our sequencing and biochemical data combined demonstrate the existence of an HSPG that has the typical features of a collagen. According to

Fig. 9. In situ hybridization showing the distribution of collagen XVIII (a and c), collagen XII (b), and agrin mRNA (d) in cross-sections of the trunk of E5 (a and b) and E7 (c and d) chick embryos. At both stages of development, collagen XVIII was prominent in the kidney (K), gonads (G), the meninges (arrowhead in c), dorsal root ganglia (D), and peripheral nerves (N). Collagen XII, which served as a control, was detected as a narrow stripe dorsal to the spinal cord (b). Agrin was abundant in spinal cord and the dorsal root ganglia (d), showing a strikingly different distribution as collagen XVIII. Bar, 200 μm.

Fig. 10. Cell adhesion of dissociated cells from E10 dorsal root ganglia to substrates of collagen XVIII (a) and ovalbumin (b). Phase contrast micrographs show that more cells attached to the collagen XVIII (a) than on ovalbumin (b) substrate, and the cells are wider spread on collagen XVIII than on ovalbumin. Bar, 25 μm.
the amino acid sequence homology, this HSPG/collagen chimera is chick collagen XVIII.

Distribution and Possible Function of the HSPG—Immunocytochemistry showed that collagen XVIII is an extracellular matrix protein and a constituent of basal laminae. Collagen XVIII was found in the basal laminae of the retina, epidermis, pia, heart and skeletal muscle, kidney, lung, and endothelial cells. With the exception of smooth muscle fibers in the gastrointestinal tracts, the distribution of collagen XVIII was similar to that of perlecan, agrin, collagen IV, and laminin, the classical basal constituents. Collagen XVIII is present in the basal laminae of Schwann cells and peripheral ganglia and nerve, collagen XVIII is abundant in kidney and the peripheral nervous system. In chick. In addition to blood vessels, however, collagen XVIII is present in basal laminae of blood vessels of the embryonic and adult course “Molecular Cloning of Neural Genes” for introducing one of us (W. H.) to the molecular cloning techniques.

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