Lack of Collagen XVIII Long Isoforms Affects Kidney Podocytes, whereas the Short Form Is Needed in the Proximal Tubular Basement Membrane*

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Collagen XVIII is characterized by three variant N termini, an interrupted collagenous domain, and a C-terminal antiangiogenic domain known as endostatin. We studied here the roles of this collagen type and its variant isoforms in the mouse kidney. Collagen XVIII appeared to be in a polarized orientation in the tubular basement membranes (BMs), the endostatin domain embedded in the BM, and the N terminus residing at the BM-fibrillar matrix interface. In the case of the glomerular BM (GBM), collagen XVIII was expressed in different isoforms depending on the side of the GBM. The orientation appeared polarized here, too, both the endothelial promoter 1-derived short variant of collagen XVIII and the epithelial promoter 2-derived longer variants having their C-terminal endostatin domains embedded in the BM and the N termini at the respective BM-cell interfaces. In addition to loosening of the proximal tubular BM structure, the Col18a1+/− mice showed effacement of the glomerular podocyte foot processes, and microindentation studies showed changes in the mechanical properties of the glomeruli, the Col18a1−/− glomeruli being ~30% softer than the wild-type. Analysis of promoter-specific knockouts (Col18a1P1/P1 and Col18a1P2/P2) indicated that tubular BM loosening is due to a lack of the shortest isoform, whereas the glomerular podocyte effacement was due to a lack of the longer isoforms. We suggest that lack of collagen XVIII may also have disparate effects on kidney function in man, but considering the mild physiological findings in the mutant mice, such effects may manifest themselves only late in life or require other compounding molecular changes.

Basement membranes (BMs)2 are sheet-like, highly specialized extracellular matrix (ECM) structures lining most tissues. In addition to their cell adherent and supporting functions, they have other important biological functions both during development and in the mature state. Although the central BM scaffold consists of networks composed of collagen IV, laminins, and nidogens, all BMs also contain several other molecules, including perlecan, agrin, fibulin, fibronectin, and the proteoglycan collagens XV and XVIII. In addition to variations in molecular composition, the fine structure of BMs varies from tissue to tissue (reviewed in Refs. 1 and 2). Many cells adhere to BMs by binding to specific receptors. This adhesion is primarily mediated by a subfamily of integrin receptors composed of α and β heterodimers that bind extracellular proteins, including laminins, collagen IV, perlecan, and other BM components (reviewed in Ref. 2). In the epidermal BM of the skin, where extra strength is needed for dermal-epidermal adhesion, specific molecules such as collagens XVII and VII link cells to the BM and also the BM to the adjacent fibrillar ECM (reviewed in Refs. 3 and 4). The function of the outer zone of the BM is less well understood, although its important role in connecting the cells to the matrix is clear. Among the ubiquitous BM components, collagen XVIII is the only one that has been shown to localize specifically at the fibrillar matrix interface (5, 6), which suggests a role in anchoring the BM to other matrix proteins.

Collagen XVIII is found in most BM structures throughout the body (7–9). It occurs in three N-terminal isoforms, the “short form” being derived from transcription of promoter 1, and the long and middle variants being derived from promoter 2 and alternative splicing of exon 3 sequences (8, 10, 11). The properties of the variant N-terminal domains are largely unknown, but the C-terminal endostatin domain, present in all isoforms, can inhibit tumor growth in injectable models (12). Mutations in this collagen lead to Knobloch syndrome in humans, characterized by a severe eye phenotype and occipital encephalocele (13, 14), and knock-out (Col18a1−/−) mice likewise suffer from multiple eye defects (6, 15–17). Moreover, ultrastructural studies of Col18a1−/− mice have identified broadened BM structures in the choroid plexus, kidney proximal tubule, skin epidermis, and heart atrioventricular regions, suggesting a structural role for collagen XVIII in some BMs (18).

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2 The abbreviations used are: BM, basement membrane; GBM, glomerular BM; HPF-FS, high pressure freezing and freeze substitution; ECM, extracellular matrix.
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4 The abbreviations used are: BM, basement membrane; GBM, glomerular BM; HPF-FS, high pressure freezing and freeze substitution; ECM, extracellular matrix.
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The kidney provides an interesting repertoire of BMs ranging from those with the permeabilities required for ion transport in distal tubules to those capable of absorbing larger molecules such as glucose and amino acids in the proximal tubules. The glomerular BM (GBM) plays a critical role in the kidney filtration barrier, and its structural anomalies are known to lead to hereditary kidney diseases such as Alport syndrome (reviewed in Ref. 19). To further our understanding of the role of collagen XVIII in the kidney, we performed more detailed ultrastructural studies of Col18a1−/− kidneys using high pressure freezing of tissue samples combined with the classical freeze substitution (HPF-FS) method. HPF avoids artifacts induced by chemical fixatives, and in combination with FS and embedding in plastic significantly improves the preservation of the tissue morphology, thus retaining the in vivo state (reviewed in Ref. 20). We draw attention here to previously unnoticed ultrastructural defects in the kidneys of mice lacking collagen XVIII. We have generated promoter–specific knock-outs of collagen XVIII by inactivation of either exon 1 (promoter 1-specific knockout Col18a1P1/P1) or exon 3 (promoter 2-specific knockout Col18a1P2/P2), and we now set out with the help of these promoter-specific collagen XVIII knockouts (Col18a1P1/P1 mice lacking expression of the shortest N-terminal variant and Col18a1P2/P2 mice lacking expression of the middle and long variants) to identify specific expression patterns of the promorter-specific isoforms and assign distinct functions to them. We also address here the effect of collagen XVIII on the mechanical properties of the glomeruli.

EXPERIMENTAL PROCEDURES

Mouse Lines—Heterozygous collagen XVIII total knock-out (Col18a1−/−) mice (15) were back-crossed with the C57BL/6J mouse strain (The Jackson Laboratory) for >15 generations to produce an inbred C57BL/6J Col18a1−/− mouse line. This strain was then crossed once with the C57BL/6OlaHsd (Harlan) mouse strain, and the resulting line was maintained by means of heterozygous Col18a1+/− matings. Wild-type littermates were used as controls in all the experimental analyses. The mouse genotypes were confirmed by polymerase chain reaction as described previously (17).

The Col18a1 gene encodes three variant polypeptides. Promoter 1 directs synthesis of the short variant in conjunction with exons 1 and 2, whereas promoter 2 directs synthesis of the two longer variants in conjunction with exon 3 (21). Promoter-specific knockouts of collagen XVIII were generated by inactivation of either exon 1 (promoter 1-specific knock-out Col18a1P1/P1) or exon 3 (promoter 2-specific knock-out Col18a1P2/P2), as described and back-crossed with the C57BL/6OlaHsd (Harlan) mouse strain for seven generations. The mouse genotypes were confirmed by polymerase chain reaction as described.3

Electron Microscopy

Chemical Fixation for Epon Embedding—The mice were sacrificed by carbon dioxide and tissue samples from 4–7-month-old control, and mutant mice were fixed in a 1% glutaraldehyde, 4% formaldehyde mixture in 0.1 M phosphate buffer. They were postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon LX 112 (Ladd Research Industries, Williston, VT). Thin sections were cut with a Leica Ultracut UCT ultramicrotome and stained with uranyl acetate and lead citrate.

HPF-FS—Specimens for HPF were taken with a Leica punch tool (Leica Microsystems, Vienna, Austria) or biopsy needle (Gallini, S.R.L. Medical Products and Services, Modena, Italy) from the kidneys of 4–7-month-old control and mutant mice after carbon dioxide sacrifice. The specimens were first placed in cell culture media and then transferred to flat specimen carriers (Leica) and cryofixed in a Leica EM Pact-1 high pressure freezer using 1-hexadecene (Sigma Aldrich) as a freezing medium. They were freeze-substituted for Epon embedding in a Leica Automated Freeze Substitution device using the method described by Walther and Ziegler (22). The specimens were infiltrated in 1.7% osmium tetroxide and 0.1% uranyl acetate in acetone containing 1% water for 10 h at −90 °C and warmed to 0 °C in 18 h. They were then moved from the specimen carriers and infiltrated into Epon LX 112 at room temperature and polymerized at 60 °C for 48 h.

Immuneelectron Microscopy

Cryoimmunoelectron Microscopy—Fresh tissue from 4–7-month-old control and mutant mice were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h. Small pieces of tissue were immersed in 2.3 M sucrose and frozen in liquid nitrogen. Thin cryosections were cut with a Leica EM UC6 ultramicrotome.

HPF Specimens Combined with Immunoelectron Microscopy of Frozen Sections—The method described by van Donselaar et al. (23) was employed. Kidney biopsies were taken from 4–7-month-old wild-type and mutant mice and placed in cell culture medium containing 5% minimum Eagle’s medium and 5% FBS. The specimens were immediately immersed in 1-hexadecene (Sigma-Aldrich), transferred to flat specimen carriers (Leica), and frozen in the HPF apparatus (EM Pact-1).

Freeze Substitution—The cryoimmobilized specimens were further transferred to a freeze substitution apparatus (AFS, Leica), where they were kept first at a temperature of −90 °C for 48 h in acetone containing 1% distilled H2O. The temperature was then raised to −60 °C in 15 h, and the specimens were kept at this temperature for 8 h. Next, the temperature was raised again to −30 °C within 15 h, and the specimens were kept at this temperature for 8 h. The substitution solution was then changed to acetone containing 0.5% glutaraldehyde, 0.1% uranylacetate, and 1% water, and the specimens were kept at −30 °C for a further 8 h. Next, they were transferred to a solution containing 0.5% glutaraldehyde, acetone, and 1% distilled H2O for 2 h. After washings in acetone, the specimens were kept on ice for 1 h.

Rehydration—Rehydration was performed on ice, steps of 95, 90, 80, and 70% acetone in distilled H2O containing 0.5% glutaraldehyde, each for 10 min, then 50 and 30% acetone in PHEM buffer (60 mM PIPES, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl2, 3 H. Elamaa, M. Aikio, L. Seppinen, S. Mäkelä, R. Sormunen, M. Muiu, I. Erikson, M. Jauhainen, L. Kjellen, M. J. Savolainen, R. Soininen, C. C. Shoulders, and T. Pihlajaniemi, unpublished results.
pH 6.9) containing 0.5% glutaraldehyde, and twice in 0.5% glutaraldehyde in PHEM, and finally twice in PHEM.

After these rehydration steps, the specimens were removed from the specimen carriers at room temperature and incubated in 12% gelatin in PHEM buffer for 10 min at 37 °C and transferred onto ice for 15 min. Gelatin blocks were infiltrated into 2.3 M sucrose in PHEM buffer and kept rotating at 4 °C overnight. Thin cryosections were cut as described above.

**Immunolabeling**—The same immunolabeling protocol was used for both the HPF-cryosections and conventional cryosections. The sections were first incubated in 5% BSA and 0.1% gelatin in PBS. Antibodies and gold conjugates were diluted in 0.1% BSA-C (Aurion, Wageningen, Netherlands) in PBS. All washings were performed in 0.1% BSA-C in PBS.

The sections were incubated with antibodies to α1(IV) (H11 against human and mouse α1 chain NC1 domain), α3(IV) (H31 against human and mouse α3 chain NC1 domain) and α5(IV) (M54 against human and mouse α5 chain NC1 domain) chains of collagen IV (24) (each diluted 1:50), polyclonal collagen IV antibody (5 μg/ml, rabbit anti-mouse collagen IV, Chemicon), collagen XVIII polyclonal antibodies recognizing the N-terminal part of all three collagen XVIII variants (anti-all rabbit antibody against collagen XVIII, 5 μg/ml) or only the two longer variants (anti-long, 10 μg/ml)4 or the C-terminal endostatin part (anti-endostatin, 4.9 μg/ml) of collagen XVIII (25) or perlecans (monoclonal anti-heparan sulfate proteoglycan antibody, Seikagaku Corp., 5 μg/ml) for 60 min. After washings, sections for perlecans and α1(IV), α3(IV), and α5(IV) stainings were exposed to rabbit anti-rat IgG (The Jackson Laboratory) for 30 min, followed by protein A-gold complex (size, 10 nm; Molecular Probes) for 30 min, made per Slot and Geuze (26). The controls were prepared by carrying out the labeling procedure without the primary antibody. The cryosections were embedded in methylcellulose. All of the specimens were examined in a Philips CM100 transmission electron microscope, and the images were captured with a Morada CCD camera (Olympus Soft Imaging Solutions GMBH, Munster Germany).

**Polyethyleneimine Staining for Anionic Sites of GBM**—To stain the anionic sites of the GBM, kidney specimens from 2-month-old male mice were treated as described by Schurer et al. (27) with some modifications. The samples were incubated in 0.5% polyethyleneimine in 0.1 M NaHPO4 (pH 7.2) on ice for 20 min. Polyethyleneimine blocks were infiltrated into 2.3 M sucrose in PHEM buffer for 10 min at 37 °C and transferred onto ice for 15 min. Gelatin blocks were infiltrated into 2.3 M sucrose in PHEM buffer and kept rotating at 4 °C overnight. Thin cryosections were cut as described above.

**Isolation of Mouse Kidney Glomeruli**—Glomeruli from wild-type and Col18a1<sup>−/−</sup> mice were isolated using a modified sieving technique. The kidneys were removed from the sacrificed mice, cleaned of fat, and decapsulated at 4 °C in PBS. Each kidney was minced to a fine paste with a razor blade at 4 °C in Dulbecco’s PBS. The paste was then applied to the first of three stacked sieves (180 μm, 90 μm and 45 μm, W.S. Tyler Co., Cleveland, OH), pressed gently through the mesh, and washed with Dulbecco’s PBS. The material on the bottom of the sieves was washed into 45-μm sieves and finally processed for Epon embedding as described earlier.

**RESULTS**

**Improved Preservation of Mouse Kidney Ultrastructure Using HPF-FS**—We compared the conventional and HPF-FS sample preparation methods to determine which approach would lead to best results when analyzing the roles of collagen XVIII in the BMs of mouse kidneys. First, the quality of freezing in HPF-FS was evaluated by observing the nuclear morphology. Those blocks with clear nuclear matrix distortion were omitted. The conventionally fixed samples of both distal and proximal tubular BMs (Fig. 1, A and E) showed a clearly detectable lamina lucida, which was not seen in the HPF-FS samples (Fig. 1, B and F), where the BM is seen as a homogenous network structure.
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The kidney cortex areas of wild-type mice and all three mutant mouse lines were prepared and processed by the HPF-FS technique, and the isoforms deleted in the promoter-specific knock-out mouse lines are illustrated in Fig. 2A. The N-terminal antibody shows consistently staining at the BM-fibrillar ECM interface in the proximal tubular BM (Fig. 2B), whereas the immunosignal of the C-terminal antibody (Fig. 2C) is consistently located in the lamina densa. Similarly, the N-terminal antibody in the GBM shows staining on the edges of the BM, localized to both the podocyte and endothelial cell surfaces in the HPF-FS sample shows a well preserved glycocalyx on both (Fig. 1, L and N), whereas this is scarcely visible in the conventionally fixed sample (Fig. 1, K and M).

Collagen XVIII Has a Polarized Orientation in Kidney BMs—It has been suggested that collagen XVIII has a polarized orientation in the epidermal BM and Bruch’s membrane BMs of the eye, the C-terminal part being localized within the lamina densa and the N-terminal part facing toward the fibrillar ECM (5, 6). To test whether a similar polarization also exists in the kidney BMs, kidney samples from wild-type mice were high pressure frozen, freeze-substituted, and rehydrated to form cryosections as described in the “Experimental Procedures” and stained with antibodies against the N-terminal part of collagen XVIII (anti-endostatin antibody) or against the C-terminal endostatin domain of collagen XVIII (anti-endostatin antibody). The three isoforms of collagen XVIII, the binding sites of the collagen XVIII antibodies, and the isoforms deleted in the collagen XVIII knockout mouse lines are illustrated in Fig. 2A.

The N-terminal antibody shows consistently staining at the BM-fibrillar ECM interface in the proximal tubular BM (Fig. 2B), whereas the immunosignal of the C-terminal antibody (Fig. 2C) is consistently located in the lamina densa. Similarly, the N-terminal antibody in the GBM shows staining on the edges of the BM, localized to both the podocyte and endothelial cell surfaces (Fig. 2D), whereas the immunosignal of the C-terminal antibody is localized within the GBM (Fig. 2E). Our antibody recognizes both the endostatin domain of the full-length collagen XVIII as well as the proteolytically cleaved form of endostatin, and thus we cannot totally exclude the possibility that some of the staining represents the cleaved form of endostatin. On the other hand, previous work with other tissues suggests that the endostatin staining is largely lost in the BMs after proteolytic processing (6). Based on the results of the immunolocalization studies we therefore suggest that collagen XVIII has a polarized orientation in kidney BMs, the C-terminal part lying within the lamina densa and the N-terminal part facing toward the fibrillar ECM.

Short Collagen XVIII Isoform Is Needed for Proximal Tubule Integrity—The mice lacking all isoforms of collagen XVIII (Col18a1−/−) and the Col18a1P1/P1 and Col18a1P2/P2 promoter-specific knock-out mice, lacking the short isoform or the middle and long isoforms, respectively (Fig. 2A), were all viable. The kidney cortex areas of wild-type mice and all three mutant mouse lines were prepared and processed by the HPF-FS technique, and the isoforms deleted in the promoter-specific knock-out mouse lines are illustrated in Fig. 2A.

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FIGURE 1. Comparison of conventionally fixed and HPF-FS wild-type mouse kidney samples. The conventionally fixed distal (A) and proximal (G) BMs show a clearly detectable lamina lucida (asterisk), whereas this is missing from the corresponding HPF-FS samples (B and F), where the BM in seen as a homogenous network structure slightly broader in appearance (arrows) than conventionally fixed samples. Higher magnification of the HPF-FS samples shows the broader, looser network of the proximal tubular BM (H) by comparison with the distal tubular BM (D). The structural difference is not as visible in the corresponding conventionally fixed samples (C and G). The GBM of the HPF-FS sample (J) is also broader in appearance than the conventionally fixed sample (I) and the podocyte foot processes (p) are rounder in shape. The glycocalyx is well preserved on both the podocyte (p) and endothelial cell (e) surfaces (arrow head) in the HPF-FS sample (L), whereas it is almost completely missing from the conventionally fixed sample (K). Higher magnification of K shows only remnants of the glycocalyx (arrowhead) on the conventionally fixed podocytes (M), whereas the podocytes of the HPF-FS sample show a well preserved glycocalyx (N, showing a higher magnification of L). e, endothelium; dt, epithelial cell of the distal tubulus; pt, epithelial cell of the proximal tubulus; p, podocyte cell.
method as described in the “Experimental Procedures.” As previously reported for conventionally fixed EM samples (18), the proximal tubular BM of the \textit{Col18a1}/H11002/H11002 mice (Fig. 3C) was broader than that of the wild-type (Fig. 3A), and a similar broadening of the proximal tubular BM was also seen in the \textit{Col18a1P1/P1} sample lacking only the short isoform of collagen XVIII (Fig. 3E), whereas the lack of only the two longer variants did not lead to such broadening (Fig. 3G). In contrast, neither promoter-specific (Fig. 3, F and H) nor total knock-out (Fig. 3D) of collagen XVIII led to alterations in the distal tubular BM structure.

\textbf{Tubular BMs in Kidney Contain Short Collagen XVIII Isoform}—To test whether the knock-out mouse findings correlate with expression of the collagen XVIII isoforms in the tubular BMs, HPF-FS samples of the promoter-specific knockouts were stained with antibodies against all three collagen XVIII variants or against only the two longer variants. In addition, the wild-type and total collagen XVIII knock-out samples were compared in terms of the localization and intensity of immunostaining with antibodies against perlecan and collagen IV, the latter antibody recognizing several of the \(\alpha(IV)\) chains.

Both the proximal tubular BM (Fig. 4A) and the distal tubular BM (data not shown) of the wild-type sample showed a strong immunosignal with the antibody recognizing all three collagen XVIII isoforms, and a similar staining pattern was also detected in the sections from a \textit{Col18a1P2/P2} mouse lacking only the two
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FIGURE 4. Immunostaining of mouse kidney proximal tubule samples from HPF-FS-rehydrated cryosections of wild-type (A, E, and G) and total collagen XVIII knock-out (Col18a1−/−) mice (F and H) or promoter-specific knock-out mice lacking either the two longer variants (Col18a1P2/P2) (B) or only the short isoform (Col18a1P1/P1) (C and D) with antibodies against all three collagen XVIII variants (Col18 anti-all) or against only the two longer variants (Col18 anti-long), perlecan, or collagen IV polyclonal antibody. The proximal tubular BM of the wild-type sample (A) shows a strong immunosignal (arrows) with the antibody recognizing all the collagen XVIII isoforms, and a similar staining pattern is detectable in the mice lacking only the two longer variants (B). By contrast, the tubular immunosignal is lost in the mice lacking only the short isoform of collagen XVIII both with the antibody against all three variants (C) and with the antibody against only the two longer variants (D). Immunostaining of wild-type samples with antibodies against perlecan (E) and collagen IV (G) shows evenly distributed gold particles in the proximal tubular BM with both antibodies, whereas no changes in the staining intensity or localization were detected in the total collagen XVIII knock-out samples (F and H).

longer variants using the antibody against all three isoforms (Fig. 4B), whereas no gold particles could be detected with the antibody against the two longer variants (data not shown). However, the proximal tubular signal was lost when the Col18a1P1/P1 mouse that lacked only the short isoform of collagen XVIII was studied with the antibody against all three variants (Fig. 4C) and that against only the two longer variants (Fig. 4D). A similar loss of the tubular BM signal was also detected in the distal tubules of the promoter 1-specific knock-out mouse (data not shown). Thus the immuno-EM studies identified the promoter 1-derived protein as the sole isoform in the tubular BMs, which is in perfect agreement with the Col18a1P1/P1 knock-out mouse data.

Staining with antibodies against the classical BM components perlecan and collagen IV was evenly distributed in both the proximal (Fig. 4, E and G) and distal (data not shown) tubular BMs, and similar staining performed on the Col18a1−/− mouse kidney samples with both antibodies showed no difference in either staining intensity or localization relative to the wild-type samples (Fig. 4, F and H).

Lack of Collagen XVIII Promoter 2 Isoforms Leads to Podocyte Effacement and Softening of Kidney Glomeruli—Analysis of HPF-FS-prepared specimens of the kidney GBM revealed no differences between the Col18a1−/−, Col18a1P1/P1, and Col18a1P2/P2 mice (Fig. 5, B–D) and their controls (Fig. 5A), with the exception that the Col18a1−/− mice lacking all three collagen XVIII isoforms (Fig. 5B) and the Col18a1P2/P2 mice lacking the two longer variants (Fig. 5D) showed podocyte foot process effacement, with a flat, elongated cell shape, whereas the podocyte structures of both the wild-type control mice (Fig. 5A) and the Col18a1P1/P1 mice lacking only the short isoform (Fig. 5C) were unchanged.

Because the charge of the GBM can affect podocyte growth and development, we determined whether loss of collagen XVIII affects the GBM charge by visualizing the anionic sites of the GBM in kidney samples from wild-type (Fig. 5E) and Col18a1P2/P2 (Fig. 5F) mice by staining with the cationic probe polyethyleneimine, as described in the “Experimental Procedures.” No differences in polyethyleneimine staining were found among the GBMs from these different mice when analyzed for number, size, or intensity of staining.

To determine whether loss of collagen XVIII affects the mechanical properties of the glomeruli, we measured the elasticity of whole glomeruli using microindentation, in which individual glomeruli were compressed ∼15 µm against a probe capable of measuring nanonewton forces. Force-indentation curves for four wild-type and five Col18a1−/− glomeruli are shown in Fig. 5G. The force-indentation relations of the wild-type glomeruli are remarkably similar, strongly suggesting that glomerular stiffness is a tightly controlled characteristic. In contrast, the glomeruli from the Col18a1−/− mice were on average significantly softer and more heterogeneous. One of the five Col18a1−/− glomeruli was similar to the wild-type but slightly softer, whereas the other four knock-out glomeruli were significantly softer and similar to each other. Effacement could be detected in ∼60% of the podocytes in both the Col18a1−/− and the Col18a1P2/P2 mouse lines, probably accounting for the near-normal mechanical properties of one of the glomeruli examined. Finite element analysis of a uniform spherical model predicts that force-indentation relations will be linear under our conditions and shows that the elastic modulus is proportional to their slope. Comparison of the average slopes of the wild-type and Col18a1−/− glomeruli suggests that Col18a1−/− glomeruli are ∼30% softer than the wild-type. The deviation of the data for Col18a1−/− glomeruli from a straight line also suggests that there are structural changes.

5 Q. Wen, unpublished results.
altering their responses to small stresses. Given the generally normal appearance of the glomeruli in light and phase-contrast microscopy, the reduction in glomerular stiffness probably represents a general property of Col18a1−/− glomeruli and is not limited to overtly diseased capillaries or glomeruli.

Promoter 2 Isoforms of Collagen XVIII Are Expressed Specifically on Podocyte Side of GBM—As reported previously (18), the antibody against all three isoforms of collagen XVIII gave a clear staining on both the podocyte and endothelial sides of the GBM of the wild-type sample (Fig. 6A). However, the staining

FIGURE 5. Shown is a comparison of the glomerular ultrastructure of HPF-FS samples of kidneys from wild-type mice (A), total collagen XVIII knock-out (Col18a1−/−) mice (B), and collagen XVIII promoter-specific knock-out (Col18a1P1/P1 and Col18a1P2/P2) mice (C and D). The sample lacking all three collagen XVIII isoforms (B) and that lacking only the two longer variants (D) show podocyte foot process (p) effacement, whereas the podocyte structures of both the wild-type sample (A) and that lacking only the short isoform (C) are unchanged. Polyethyleneimine staining (arrows) of the anionic sites did not show any differences within the GBM between the wild-type (E) and promoter 2-specific knock-out mice (F). Force indentation measurements performed on four separate wild-type glomeruli (open circles) and five separate Col18a1−/− glomeruli (filled circles) (G) show softening of the total knock-out glomeruli. e, endothelium.

FIGURE 6. Immunostaining of kidney glomerular samples from HPF-FS-rehydrated cryosections of wild-type mice (A, E, G, and I), total collagen XVIII knock-out (Col18a1−/−) mice (F, H, and J), or collagen XVIII promoter-specific knock-out mice lacking either the two longer variants (Col18a1P2/P2) (B) or only the short isoform (Col18a1P1/P1) (C and D) with antibodies against all three collagen XVIII variants (Col18 anti-all) or against only the two longer variants (Col18 anti-long), perlecan, or different α chains of collagen IV. The antibody against all three isoforms of collagen XVIII gives clear staining (arrows) on both the podocyte (p) and endothelial (e) sides of the GBM of the wild-type sample (A). The immunosignal is preserved on the endothelial side of the GBM but lost on the podocyte side in the mice lacking only the two longer isoforms of collagen XVIII (B), whereas it is preserved on the podocyte side of the GBM in the samples lacking only the short isoform of collagen XVIII and is lost on the endothelial side both with the antibody against all three variants (C) and with the antibody against only the two longer variants (D). Immunostaining of both wild-type (E) and total collagen XVIII knock-out (F) samples with an antibody against perlecan gives a clear immunosignal on both the podocyte and endothelial sides of the GBM. The immunosignal of the collagen IV antibody recognizing the α1(IV) chain is preferentially located only on the endothelial side of both the wild-type (G) and total knock-out samples (H), whereas the antibody recognizing the α5(IV) chain is staining the podocyte side of the GBM in both the wild-type (I) and total knock-out samples (J).
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on the podocyte side was lost in the samples from the Col18a1<sup>P2/P2</sup> mice that lacked the two longer variants when using the antibody against all three variants (Fig. 6B), whereas the immunosignal on the endothelial side persisted. In contrast, the signal was seen on the podocyte side but almost lost on the endothelial side of the GBM in the Col18a1<sup>P2/P2</sup> mice lacking only the short isoform of collagen XVIII both with the antibody against all three variants (Fig. 6C) and with the antibody against only the two longer variants (Fig. 6D).

Staining of wild-type samples with an antibody against perlecan showed localization of gold particles on both the podocyte and endothelial sides of the GBM (Fig. 6E), whereas the staining pattern of collagen IV depended on the α(IV) chain the antibody was recognizing. The antibody against the α1(IV) chain gave a clear immunosignal only on the endothelial side (Fig. 6G), whereas no gold particles could be detected on the podocyte side. In contrast, the antibodies against α3(IV) and α5(IV) chains were staining preferentially the podocyte side of the GBM (data not shown and Fig. 6I, respectively). Thus, our findings are in support of previous results showing the localization of different collagen IV α chains in the GBM (29). Similar stainings performed on the Col18a1<sup>−/−</sup> mouse kidney samples with both perlecan and collagen IV antibodies showed no difference in either staining intensity or localization relative to the wild-type (Fig. 6, F, H, and J).

DISCUSSION

Given that messenger RNAs corresponding to all three collagen XVIII isoforms are expressed in the kidney (8, 10, 30), we show here that the shortest isoform is virtually the sole collagen XVIII variant in the tubular BMs, whereas the longer variants are also present in the GBM. Ultrastructural analysis of samples preserved with minimal extraction artifacts revealed an abnormally loose, network-like BM in the proximal tubules of Col18a1<sup>−/−</sup> mouse kidneys, a change that could be attributed to a specific lack of the short isoform of collagen XVIII. In keeping with the lack of immunosignals for the other two isoforms in the tubular BMs, the lack of the middle and longest isoforms had no effect on the tubular BM ultrastructure. Immunostaining with the classical BM components perlecan and collagen IV showed no differences in either staining intensity or localization between the wild-type and the collagen XVIII isoforms are expressed in the kidney (8, 10, 30), we suggest here that the shortest isoform is virtually the sole collagen XVIII variant in the tubular BMs, whereas the longer variants are also present in the GBM. Ultrastructural analysis of samples preserved with minimal extraction artifacts revealed an abnormally loose, network-like BM in the proximal tubules of Col18a1<sup>−/−</sup> mouse kidneys, a change that could be attributed to a specific lack of the short isoform of collagen XVIII. In keeping with the lack of immunosignals for the other two isoforms in the tubular BMs, the lack of the middle and longest isoforms had no effect on the tubular BM ultrastructure. Immunostaining with the classical BM components perlecan and collagen IV showed no differences in either staining intensity or localization between the wild-type and the collagen XVIII mutants, suggesting a true loosening of the BM scaffold rather than thickening due to abnormal accumulation of other BM components in these mice.

BMs from different regions of the nephron were distinct in their ultrastructure, the distal tubular BM being more compact than the proximal tubular BM. This difference probably reflects molecular and structural heterogeneity, enabling them to perform specialized functions, the proximal tubules being able to absorb larger substances (such as glucose, amino acids, and proteins) from the glomerular filtrate than the distal tubules, which transport mostly ions. Although the short isoform of collagen XVIII is normally present at similar levels in both the proximal and distal tubular BMs, its absence affects the fine structure, and hence possibly the function, only of the former.

Studies of the epidermal BM in skin and Bruch’s membrane in the eye suggest that collagen XVIII is the only one of the common BM components that is localized specifically at the fibrillar matrix interface (5, 6), and they also point to a polarized orientation of collagen XVIII molecules, with the C-terminal endostatin domain embedded in the lamina densa and the N-terminal domain residing at the interface between the BM and the fibrillar matrix. Recombinantly produced endostatin has been shown to bind strongly to certain known BM components, namely laminin-1, perlecan, heparin, nidogen, and fibulin-1 (31), whereas little is known about the binding activities of the N termini. Studies of the longest isoform, containing the frizzled domain, suggest that this part of the molecule can be proteolytically released, and when released from the parent molecule can affect Wnt signaling (10, 30).

We also provide further information here on the molecular localization of collagen XVIII and suggest that it has a similar polarized orientation in the kidney tubular BM. Interestingly, we also found that the endothelial side of the GBM contains the promoter 1-derived short variant of collagen XVIII, whereas the podocyte side contains only the promoter 2-derived longer variants of collagen XVIII. In addition to the specific locations of the variant collagen XVIII isoforms at the different sides of the GBM, the molecules appear to reside in a polarized manner also in the GBM, both the endothelial and epithelial isoforms having their C-terminal endostatin domains embedded in the BM and the N termini at the BM-cell interface in the case of the short form at the BM-endothelial cell junction and in the case of the middle and long forms at the BM-podocyte junction. It should be noted that it was not possible for us to distinguish with the methods available whether one or both of the promoter 2 isoforms of collagen XVIII, the middle or the long (frizzled) variant, is localized on the podocyte side of the GBM. Previous studies at the mRNA level nevertheless indicate that both the middle form and the longest form (containing a frizzled domain) are present in the kidney (8, 10).

The GBM is formed during kidney development by the fusion of two separate layers, the glomerular endothelial BM and the podocyte epithelial BM (32, 33), but recent evidence suggests that the epithelial and endothelial sides of the GBM differ in their molecular composition, at least in the localization of collagen IV isoforms (reviewed in Ref. 34). The mature GBM is known to consist of both α1α2α3 and α2α4α5 subtypes of collagen IV, the former produced by the endothelium and the latter by the podocytes (29). This view is supported by our stainings with the different α chain-specific antibodies of collagen IV, the α1(IV) antibody giving a clear immunosignal only on the endothelial side of the GBM, whereas the α3(IV) and α5(IV) antibodies are staining the podocyte side of the GBM. Similarly to the collagen IV isoforms, we show here that collagen XVIII is expressed in different isoforms on each side of the GBM. However, no clear difference within the GBM ultrastructure was detected in either of the promoter-specific knock-out mouse lines, nor in the Col18a1<sup>−/−</sup> mice lacking all the isoforms. Instead, we found that the total knock-out and promoter 2-specific knock-out mice were affected by podocyte foot process effacement, whereas the podocyte structure of the promoter 1-specific knock-out was normal. Thus, loss of the two longer collagen XVIII variants evidently leads to abnormalities in
podocyte structure, whereas loss of the short variant has no effect on the podocytes.

Podocytes are highly differentiated epithelial cells connected to each other by specific adherens junctions, the slit diaphragms. The interdigitating foot processes of the podocytes are attached to the GBM (reviewed in Ref. 35), and podocyte injury and loss have been observed in various glomerular diseases leading to kidney failure (reviewed in Ref. 36). A common final pathway in the response of the podocytes to injury is a change in shape called effacement, during which the foot processes widen and shorten, leading to a flat, elongated cell shape with a reduced number of filtration slits. Correct interaction of the podocytes with the ECM is crucial for maintaining the order of the foot process architecture (reviewed in Ref. 37). The podocytes are connected to the underlining GBM through two major cell adhesion complexes: α/β-dystroglycans and the α3β1 integrin (38, 39), both of which are capable of binding several common BM components (40, 41). Alterations in the binding properties of either one of these adhesion complexes have been shown to lead to glomerular defects characterized by podocyte foot process effacement (38, 39, 42–44).

Thus, it is possible that the podocyte effacement in the Col18a1−/− and Col18a1P2/P2 kidneys may have been due to defects in the interaction between the podocytes and the GBM. Endostatin, the C-terminal domain of collagen XVIII, has been shown to bind α3β1- and α3β4-integrins (45–47) and could be required for podocyte-GBM interaction. However, considering the polarized orientation of collagen XVIII molecules in the GBM, the endostatin domain appears rather to be employed for interactions with molecules within the BM. We therefore favor the possibility that the N termini of the middle and long isoforms interact with the podocytes. In light of the multidomain nature of the N termini, several possibilities exist. Both the middle and long variants contain a 247-amino acid residue domain of unknown function (11), and so far, there is no information on its binding activities. The N termini of all three variants contain a thrombospondin 1 homology domain (48), which may possess biological activities. Thrombospondin 1 itself is known to bind many growth factors, proteases, cell surface receptors, and ECM proteins mediating specific cellular responses (reviewed in Ref. 49). Last but not least, the frizzled module of the longest isoform has been shown to be capable of binding Wnt3a in vitro and of suppressing the stabilization of β-catenin in tumor cell lines (30). Interestingly, Wnt-1 up-regulation and active β-catenin have been observed in podocytes in cases of human proteinuric kidney disease with podocyte dysfunction (50). It is thus also possible that the podocyte effacement seen in the Col18a1−/− and Col18a1P2/P2 kidneys could have been due to a lack of the inhibitory effects of the frizzled module of collagen XVIII on Wnt signaling, leading to anomalous activation of the pathway.

Regardless of whether the function of collagen XVIII is to cross-link other BM components or to interact with cell receptors, loss of this collagen could alter the mechanical properties of the BM. If this is the case, the podocyte pathology could be a response to an abnormal mechanical environment. The mechanical environment is known to have dramatic effects on cell behavior in podocytes (51) and in other situations (52). At this time, we cannot separate the mechanical characteristics of the whole glomerulus from those of the GBM, but the Col18a1−/− glomeruli are clearly softer than normal and to a degree that is physiologically significant (28, 53)

The softening of the glomeruli in Col18a1−/− mice could have contributed to podocyte effacement and organ dysfunction. A number of recent findings show that changes in the elastic modulus of cells or matrix can have direct effects on cell structure and function, leading to the hypothesis that modulation of the factors that control the elastic modulus of organs and tissues both in the cytoskeleton and in the ECM not only help us identify disease mechanisms but also have potential therapeutic applications (52, 54).

Despite the ultrastructural changes found in the podocytes and proximal tubular BMs, Col18a1−/− mice have a normal lifespan without any obvious signs of kidney malfunction. During our previous studies, however, the serum creatinine levels of the Col18a1−/− mice were shown to be elevated (10.11 + 1.59 umol/liter versus 8.42 + 2.07 mm in controls) (18). Creatinine is a muscular waste product that is filtered out of the serum by both glomerular filtration and proximal tubular secretion (10–20% of urine creatinine). Thus, in the light of the ultrastructural findings, the elevated creatinine levels of the Col18a1−/− mice could reflect minor malfunction of the glomeruli, proximal tubules, or both.

In a recent study, collagen XVIII expression was shown to be elevated in the Bowman’s capsule and the GBM in a mouse model of anti-GBM glomerulonephritis, and the Col18a1−/− mice developed more severe glomerular and tubulointerstitial injury with altered matrix remodelling, enhanced inflammatory response and endothelial cell damage, than wild-type mice (55). Interestingly, treatment of the knock-out mice with endostatin did not affect the progression of anti-GBM disease, suggesting that the whole collagen XVIII molecule is needed to preserve the integrity of the ECM and capillaries in the kidney and to protect against progressive glomerulonephritis. It is conceivable that a lack of collagen XVIII may also have disparate effects on kidney function in man, but considering the mild physiological findings in the mutant mice, such effects may manifest themselves only late in life or require other compounding molecular changes.

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