Matrix Pathobiology

Disruption of Glomerular Basement Membrane Charge through Podocyte-Specific Mutation of Agrin Does Not Alter Glomerular Permselectivity

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Glomerular charge selectivity has been attributed to anionic heparan sulfate proteoglycans (HSPGs) in the glomerular basement membrane (GBM). Agrin is the predominant GBM-HSPG, but evidence that it contributes to the charge barrier is lacking, because newborn agrin-deficient mice die from neuromuscular defects. To study agrin in adult kidney, a new conditional allele was used to generate podocyte-specific knockouts. Mutants were viable and displayed no renal histopathology up to 9 months of age. Perlecan, a HSPG normally confined to the mesangium in mature glomeruli, did not appear in the mutant GBM, which lacked heparan sulfate. Moreover, GBM agrin was found to be derived primarily from podocytes. Polyethyleneimine labeling of fetal kidneys revealed anionic sites along both laminae rarae of the GBM that became most prominent along the subepithelial aspect at maturity; labeling was greatly reduced along the subepithelial aspect in agrin-deficient and conditional knockout mice. Despite this severe charge disruption, the glomerular filtration barrier was not compromised, even when challenged with bovine serum albumin overload. We conclude that agrin is not required for establishment or maintenance of GBM architecture. Although agrin contributes significantly to the anionic charge to the GBM, both it and its

charge are not needed for glomerular permselectivity. This calls into question whether charge selectivity is a feature of the GBM. (*Am J Pathol* 2007, 171:139–152; DOI: 10.2353/ajpath.2007.061116)

The glomerular capillary wall is thought to function as both a size- and charge-selective barrier. The concept of charge selectivity emerged from a series of now classic studies examining the clearance of tracers differing in charge.^{1–3} The permeability of anionic tracers was lower than their neutral counterparts, whereas that of cationic forms was enhanced. It was concluded that an intrinsic or “fixed” negative charge in the capillary wall poses an electrostatic barrier to anionic plasma proteins, such as albumin. These types of studies have been challenged on several fronts, particularly on the basis of deformation, degradation, or selective uptake of the differentially charged tracers. Whether charge selectivity exists and is important for glomerular function is a subject of intense debate.⁴ Nevertheless, the concept remains a cornerstone of renal physiology.

Anionic sites can be detected based on their affinity for cationic probes and have been found in association with each layer of the capillary wall. The anionic glycocalyx of podocytes and endothelial cells that is formed largely by podocalyxin⁵ may contribute to the barrier. Podocalyxin serves a critical role in dictating podocyte foot process architecture, presumably through charge-related repulsive effects.⁶ However, the glomerular basement membrane (GBM) is generally considered to be of primary importance. GBM charge is imparted by sulfated glycos-

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aminoglycan (GAG) side chains of proteoglycans and to a lesser extent by carboxyl and sialyl groups of glycoproteins. GBM anionic sites are distributed in a quasi-regular pattern along both laminae rarae but are most prominent along the subepithelial aspect.^{7,8} These were identified as heparan sulfate proteoglycans (HSPGs) based on their susceptibility to enzymatic digestion.⁹ Other sulfated GAGs are not present in the GBM in significant amounts.

Charge barrier dysfunction is considered an important factor in the pathogenesis of glomerular disease.^{10–13} This may be brought about by decreased expression or undersulfation of GBM-HSPGs.^{14–16} In animal models, enzymatic removal of glomerular heparan sulfate (HS) or charge neutralization results in proteinuria and promotes the permeability of anionic tracers.⁴ However, a recent study has challenged the notion that GBM-HS is important for glomerular permselectivity.¹⁷

Three genetically distinct basement membrane (BM)-HSPGs are recognized: perlecan, collagen XVIII, and agrin. Perlecan and collagen XVIII are both found in the glomerulus but are localized primarily to the mesangial matrix and Bowman's capsule and are only prominent in the GBM during development.^{15,18} Mice lacking the attachment sites for HS on perlecan have normal glomerular ultrastructure and no renal disease but show increased susceptibility to protein-overload proteinuria.^{19,20} Collagen XVIII mutants have mild mesangial expansion and only slightly elevated serum creatinine levels compared with controls.²¹

Agrin has been identified as the predominant GBM-HSPG in all species studied, prompting speculation that it may be a critical determinant of the charge barrier.^{22,23} It is characterized by an ~2000-residue core protein of ~220 kd that carries at least two GAG chains, bringing its mass to ~400 kd. Agrin is generally classified as an HSPG, but it can carry both heparan and chondroitin sulfate (CS) GAGs.^{24,25} Sites for GAG attachment have been mapped experimentally in chick agrin to one site located between the seventh and eighth follistatin-like domains that carries exclusively HS and to a second in the serine/threonine-rich region that carries predominantly CS.²⁵

Alternative promoters give rise to two isoforms of agrin that are either BM or cell associated, with the latter being specific to neurons.²⁶ BM-associated agrin binds to the laminin γ 1 chain via a globular domain (NtA) at its N terminus and to dystroglycan and integrin receptors through its C terminus.^{27–29} By virtue of these interactions, agrin is thought to be an integral part of the molecular complex linking podocytes to the GBM.³⁰

Agrin-deficient mice are grossly normal but die at birth with severe neuromuscular defects.³¹ The objective of this study was to assess the role of agrin in glomerular development and function through the study of agrin mutant mice. To overcome the perinatal lethal phenotype, we generated conditional knockouts in which *Agrn* was ablated in podocytes. Here, we demonstrate that agrin contributes significantly to GBM anionic charge but is not needed for glomerular function.

Materials and Methods

Agrin Mutant Mice

Kidneys were studied from agrin-deficient mice homozygous for a knockout allele (*Agrn*^{tm4Jrs}; denoted *Agrn*^{del}) in which genomic sequence from within exon 6 to intron 33 (numbering according Rupp et al³²) is replaced by a loxP-flanked PGK-*neo* cassette³¹ or a gene trap allele (*Agrn*^{Gt(p1.8TM)192Wcs}; denoted *Agrn* ^{β -geo}) that disrupts *Agrn* 3' of the last NtA-encoding exon.²⁶ A conditional allele harboring loxP sites within introns 6 and 33 (*Agrn*^{tm1Rwb}; denoted *Agrn*^{fl}) was generated through "recombineering" in EL350 cells,³³ followed by electroporation of the construct into R1 embryonic stem cells. Embryonic stem cell clones were injected into C57BL/6J blastocysts to generate germline chimeras that were bred to obtain heterozygous and homozygous mice. In some cases, these were crossed to *FLPe* transgenic mice³⁴ to eliminate the frt-flanked *neo* selectable marker.

Conditional knockouts were generated by crossing to mice that express Cre recombinase from the human *NPf2* promoter (2.5P-Cre³⁵) or the murine *Pax3* promoter (P3pro-Cre³⁶). Genotyping was performed by polymerase chain reaction (PCR) using primers for the following alleles: *Agrn*^{fl}, 5'-AGCCCGGAAACTCTGGATTCC-3' (exon 33) and 5'-CAAAGTGGTTGCTCTGCAGCG-3' (exon 34); *Agrn*^{fl Δ neo}, 5'-CGGACACACATATGCTAGTGA-3' (exon 6) and 5'-ACTGTCCAGCTGAGCACACAGC-3' (exon 7); *Agrn*^{del}, 5'-TGCCAAGTTCTAATTCCATCAGAAGCTGAC-3' (*neo*) and 5'-GGGCTAACACCAACAACAATGCAACAAAGG-3' (intron 33), or 5'-CAGTGAAGAATGGGAAAGCTG-3' (exon 5) and the exon 34 primer for *Agrn*^{del Δ neo}; *Agrn* ^{β -geo}, 5'-GGATTGGTGGCGACGAC-TCC-3' and 5'-AATGGGCGAGGTAGCCGGATCAAGCG-3'; *Cre*, 5'-CGGTCTGATGCAACGAGTGATGAG-3' and 5'-ACGAACCTGGTCAAATCAGTGCG-3'; and *FLP*, 5'-GTGGATCGATCCTACCCCTTGCG-3' and 5'-GGTCCA-ACTGCAGCCCAAGCTTCC-3'. Mice were studied on a mixed 129 $\times$ C57BL/6J genetic background. Animal experiments were approved by the Washington University Animal Studies Committee.

Analysis of Mutant mRNA Transcripts

Total RNA extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH) was used to synthesize cDNA with the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA). PCR reactions contained 2 μ l of cDNA template, 125 μ mol/L dNTPs, 2.5 U of *Taq* polymerase (Bioline, Randolph, MA) in the supplied buffer, 0.2 μ mol/L each of a forward primer in exon 5 or 6 (as above), and a reverse primer in exon 34 (as above), exon 35 (5'-GCCCACCTGAAGGGAACC-3'), or exon 36 (5'-CACAAAACCCGTGCCATAG-3'). Amplicons were cloned in pCR2.1-TOPO (Invitrogen) and sequenced.

Expression of Recombinant Agrin

A cDNA (GenBank accession no. BP758133) encoding mouse agrin in pBC SK⁺ (Stratagene, La Jolla, CA) was

Table 1. Antibodies Used for Immunohistochemistry

Antibody	Dilution	Species	Supplier
Agrin 4,8 (C terminus)	300	Rabbit anti-human	Regeneron Pharmaceuticals (Tarrytown, NY)
Agrin LG (C terminus)	25,000	Rabbit anti-mouse	T. Sasaki (Max Planck Institute, Martinsried, Germany)
Agrin (C terminus)	300	Hamster anti-rat	P. Noakes (University of Queensland, Brisbane, Australia)*
Agrin (N terminus; mAb MI-91)	300	Hamster anti-rat	J. Berden (Radboud University, Nijmegen, The Netherlands)
Agrin (N terminus; GR-14)	800	Sheep anti-rat	J. Berden (Radboud University)
Perlecan (mAb 1948)	300	Rat anti-mouse	Chemicon, Temecula, CA
HS (mAb JM-403)	100	Mouse anti-rat	J.H. Berden (Radboud University)
HS (mAb NAH46)	100	Mouse anti- <i>E. coli</i>	Seikagaku, Tokyo, Japan
CS (mAb CS-56)	200	Mouse anti-chicken	Sigma, St. Louis, MO
Integrin $\alpha 3$	200	Rabbit anti-chicken	M. DiPersio (Albany Medical College, Albany, NY)
α -Dystroglycan (mAb IIH6)	100	Mouse anti-rabbit	K. Campbell (University of Iowa, Iowa City, IA)
Laminin $\beta 2$	2000	Rabbit anti-mouse	T. Sasaki (Max Planck Institute)
Nidogen (mAb 1946)	1000	Rat anti-mouse	Chemicon

The following secondary antibodies were used for detection: Cy3-conjugated goat anti-rabbit IgG (1:1500), Cy3- or Cy2-conjugated goat anti-hamster IgG (1:1500 or 1:200, respectively), Cy3-conjugated goat anti-rat IgG (1:1500), fluorescein isothiocyanate-conjugated goat anti-mouse IgM (1:100; Jackson ImmunoResearch, West Grove, PA); Alexa Fluor 488-conjugated donkey anti-sheep IgG or goat anti-mouse IgM (1:200; Invitrogen); and Alexa 488 Fluor-conjugated goat anti-mouse IgG₁ (1:200; Molecular Probes).

*Hamster antiserum raised against rat agrin fusion proteins.⁶³

provided by Dr. Susumu Seino (Kobe University, Kobe, Japan). A 3632-bp *KpnI-EcoRI* fragment of this clone that begins with 56 bp of the 5'-untranslated region and terminates within exon 18 was subcloned into pcDNA3.1/*myc*-His (Invitrogen). The resulting construct, agrin₁₋₁₁₇₁, encodes an 1171-amino acid (128 kd) epitope-tagged fragment that includes both GAG attachment sites identified in the chick sequence. A truncated construct was generated that recapitulates the form of agrin expressed from the *Agrn*^{fl} allele after Cre recombination. A cDNA of 1825 bp was generated by PCR using agrin₁₋₁₁₇₁ as template, 0.2 μ mol/L of the primers 5'-CCAAGCTTCGC-CATGGTCCGCCCGCGGC-3' and 5'-CAGAATTCTG-GCAGTGTCCGGCTGAGGCC-3' mismatched (underlined) to introduce *HindIII* and *EcoRI* adapters, 200 μ mol/L dNTPs, and 5 U of *Herculase Taq* polymerase (Stratagene) in the supplied buffer. The PCR product was cloned into the *HindIII* and *EcoRI* sites of pcDNA3.1/*myc*-His. The resulting construct, agrin₁₋₆₀₂, comprises all coding sequence up to the 3' end of exon 6 and encodes a 602-amino acid (70 kd) epitope-tagged fragment of agrin.

Cell Culture, Transfection, and Western Blotting

COS-7 and 293 cells maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum were transiently transfected with agrin₁₋₁₁₇₁, agrin₁₋₆₀₂, or the empty vector using Lipofectamine (Invitrogen). After culturing in serum-free Opti-MEM (Invitrogen), conditioned medium was recovered and cleared by centrifugation (300 $\times$ g for 15 minutes at 4°C). Cells lysates were prepared in 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) containing Complete protease inhibitor (Roche, Indianapolis, IN) and cleared by centrifugation (16,000 $\times$ g for 15 minutes at 4°C). Samples were subjected to reducing SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were blocked with 3% bovine serum albumin (BSA) in 10 mmol/L Tris-HCl, pH 8.0, 150

mmol/L NaCl, and 0.1% Tween 20 and incubated with mouse anti-human Myc antibody 9E10 (Calbiochem, San Diego, CA) diluted 1:2000 or rat anti-mouse perlecan antibody (Chemicon, Temecula, CA) diluted 1:1000. After washing in Tris-buffered saline/Tween 20, blots were probed with biotinylated anti-mouse or anti-rat antibodies diluted 1:1000, then incubated in ABC-horseradish peroxidase reagent (Vector Laboratories, Burlingame, CA), and detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL). For deglycosylation, cell lysates or media (buffered with the addition of NaOAc, pH 7.0, to 50 mmol/L and CaCl₂ to 2 mmol/L) were incubated with PNGase F (0.1 U/ml; Prozyme, San Leandro, CA), heparinase III (2 U/ml; Sigma, St. Louis, MO), or chondroitinase ABC (0.4 U/ml; Seikagaku, Tokyo, Japan) overnight at 37°C.

Histology and Immunostaining

Formalin-fixed, paraffin-embedded kidney sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff reagent. Kidneys were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and frozen in 2-methylbutane cooled in a dry-ice/ethanol bath. Unfixed cryosections were blocked with 1.5% goat serum in phosphate-buffered saline and stained with the antibodies indicated in Table 1. Phosphate-buffered saline was substituted with 20 mmol/L Tris-HCl, pH 7.4, and 100 mmol/L NaCl for detection of α -dystroglycan. For HS staining, sections were fixed in acetone for 10 minutes at 4°C and blocked with 10% goat serum and 1% BSA in phosphate-buffered saline using the Mouse-on-Mouse kit (Vector Laboratories). Neuromuscular junctions were labeled with rhodamine-conjugated α -bungarotoxin (Molecular Probes, Eugene, OR) diluted 1:200.

Electron Microscopy and Polyethyleneimine Labeling

Minced cortex was immersion-fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L cacodylate

buffer, pH 7.2, postfixed in 1% OsO₄, and then dehydrated and embedded in Polybed (Polysciences, Warrington, PA). Sections were counterstained with uranyl acetate/lead citrate and examined with a CX-100 electron microscope (JEOL, Tokyo, Japan). For polyethyleneimine (PEI) labeling, samples were incubated 30 minutes in 0.5% PEI (1.8 kD; Sigma) in 0.9% NaCl, pH 7.3. After washing in 0.1 mol/L cacodylate, specimens were fixed in the same buffer containing 2.5% glutaraldehyde and 2% phosphotungstic acid, pH 7.3, and then postfixed and embedded as above. Glomerular capillary loops were photographed at magnification $\times 14,000$ using a blinded experimental design, and the number of PEI aggregates per micrometer along each aspect of the GBM was counted. Analysis of E17.5-P0 *Agrn*^{del/del} ($n = 4$) and control littermates ($n = 4$) was based on 40 micrographs representing ≥ 22 glomeruli for each group. For podocyte-specific knockouts ($n = 7$) and controls ($n = 8$) 7 weeks to ~ 11 months of age, ≥ 97 micrographs representing 38 glomeruli for each group were used.

Clinical Chemistry, Ficoll Clearance Studies, and Protein-Overload Proteinuria

Urine samples were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. Urinary protein and creatinine concentrations were measured using Biuret and Jaffé reactions, respectively, on a Cobas Mira Plus analyzer (Roche). Enzyme-linked immunosorbent assays were used to quantify albumin (Exocell, Philadelphia, PA) and total IgG (Alpha Diagnostics, San Antonio, TX). To assess glomerular charge selectivity, mice were given a bolus of fluorescein isothiocyanate-labeled carboxymethyl Ficoll-70 (250 μ g/g body weight; TdB Consultancy, Uppsala, Sweden) in 0.9% NaCl by tail vein injection and then housed 24 hours in metabolic cages (Hatteras Instruments, Cary, NC). The concentration of the tracer in urine was determined by diluting samples and standards in 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, and measuring the fluorescence with a QuantaMaster fluorimeter (λ_{ex} 488 nm, λ_{em} 529 nm; Photon Technology International, Lawrenceville, NJ). The fraction of the dose excreted over 24 hours was calculated for comparison. To induce overload proteinuria, mice were given daily intraperitoneal injections of endotoxin-free BSA (15 mg/g body weight; Sigma) in 0.9% NaCl for 5 days. Urine was collected at 24-hour intervals before injection.

Statistical Analyses

Minitab v13.1 statistical software (State College, PA) was used to analyze PEI and urinalysis data using two-sample *t*-tests, and a general linear model for analysis of variance was applied to analyze the protein-overload data. Differences were considered significant at $P < 0.05$.

Figure 1. Structure of mouse agrin and properties of the mutant *Agrn* alleles. The N-terminal domain of agrin (NtA) that binds laminin $\gamma 1$ is followed by follistatin-like (F), laminin EGF-like (L), serine/threonine-rich (ST), sperm protein/enterokinase/agrin (SEA), and EGF-like (E) domains. Laminin globular (G) domains at the C terminus bind integrin and dystroglycan receptors. Two putative attachment sites for GAGs are shown. **A:** A conditional allele (*Agrn*^{fl}) was generated by introducing loxP sites within introns 6 and 33. Cre-mediated excision deletes most coding exons (light gray, numbered according to Rupp et al³²) but leaves flanking exons (dark gray) and the untranslated regions (black) intact. **B:** In a knockout allele (*Agrn*^{del}), sequence from within exon 6 through intron 33 is replaced by a loxP-flanked *neo* cassette. Transcripts derived from the *Agrn*^{del} and *Agrn*^{fl} alleles encode truncated proteins that lack the C terminus due to frameshifts. **C:** A gene trap allele (*Agrn* ^{β -geo}) intercepts the *Agrn* gene 3' of the last NtA-encoding exon. The positions of primers used for genotyping are indicated (arrows).

Results

Generation of a Conditional *Agrn* Allele

Agrin is essential for neuromuscular synaptogenesis and has been extensively studied in this context. However, the early lethality of agrin-deficient mice poses a barrier to studies that might define important roles for agrin during postnatal life. We therefore generated a conditional allele (*Agrn*^{fl}; Figure 1A) by introducing loxP sites within introns 6 and 33 of the mouse *Agrn* gene. *Agrn*^{fl/fl} mice were phenotypically normal, indicating agrin was properly expressed from the “floxed” allele. Excision of the frt-flanked *neo* cassette (*Agrn*^{fl Δ neo}) had no effect; alleles containing or lacking this insert were used interchangeably and are hereafter referred to as *Agrn*^{fl}.

The experiments herein also made use of previously described "null" alleles. The first was a targeted knockout ($Agrn^{del}$; Figure 1B) in which genomic sequence from within exon 6 to intron 33 was replaced by a loxP-flanked PGK-*neo* cassette.³¹ Deletion of the *neo* insert ($Agrn^{del\Delta neo}$) by crossing to β -actin-Cre mice³⁷ had no phenotypic effect, and both forms of this allele are hereafter referred to as $Agrn^{del}$, unless otherwise noted. Finally, we used a gene trap allele ($Agrn^{\beta-geo}$; Figure 1C) that disrupts the *Agrn* gene 3' of the NtA-encoding exons.²⁶

Agrn Knockout Mice Synthesize N-Terminal Truncated Forms of Agrin

Each mutant allele retains the capacity to encode variants of agrin with biological activity. $Agrn^{\beta-geo/\beta-geo}$ mutants, for example, lack BM-associated agrin but express normal levels of the neuron-specific isoform.²⁶ The $Agrn^{del}$ and recombined $Agrn^{fl}$ alleles represent extensive and nearly identical genomic deletions, but both could theoretically encode truncated forms of agrin, because they retain multiple intact exons. Importantly, the splicing of mutant transcripts from either exon 5 or 6 to exon 36 would result in an in-frame mRNA encoding a form of "miniagrin" that could be biologically active.³⁸ Reverse transcriptase-PCR using primers in exons 6 and 36 yielded a single product from podocyte-specific knockout kidney ($Agrn^{fl/fl};2.5P-Cre$; see below) that was not amplified from liver of the same mice or from wild-type kidney. Sequencing revealed splicing occurred from exons 6 to 34, thereby introducing a frameshift that disrupts the C terminus of any protein derived from a recombined $Agrn^{fl}$ allele (Figure 1A). No products were amplified from fetal $Agrn^{del/del}$ kidney using a forward primer in exon 5 and reverse primers in exons 34, 35, or 36 due to the intervening *neo* cassette, but the same reactions using RNA from $Agrn^{del\Delta neo/+}$ kidney yielded amplicons spliced from exons 5 to 34, which also introduces a frameshift (Figure 1B).

Agrin is present in the GBM of early capillary loop stage glomeruli in fetal human kidney.³⁹ Here, normal fetal mouse kidney was stained with a panel of three different antisera raised against the C terminus of agrin. Each gave the same pattern, labeling the BM and vascular cleft of S-shaped nephrons and pre-capillary loop-stage glomeruli (Figure 2A). At later stages of glomerular development, there was prominent staining of the GBM (Figure 2B). The BMs of collecting ducts, peritubular capillaries, and vascular smooth muscle cells were positive with all antisera. $Agrn^{del/del}$ and $Agrn^{\beta-geo/\beta-geo}$ kidneys failed to stain with C-terminal antibodies (Figure 2, C and D, respectively), confirming that they are agrin specific.

C-terminal agrin antibodies stain rat and human GBM intensely, whereas most tubular BMs are negative or only weakly labeled.^{22,23,40} In contrast, N-terminal antibodies label glomerular, tubular, and vascular smooth muscle BMs uniformly.⁴⁰ The reason for this discrepancy is unknown, but it might reflect alternative splicing or process-

ing of agrin. The N-terminal monoclonal antibody (mAb) MI-91 labeled most BMs in normal fetal kidney (Figure 2, E and F). Unexpectedly, MI-91 also stained $Agrn^{del/del}$ mutant kidney in a manner indistinguishable from controls (Figure 2G), but its failure to label $Agrn^{\beta-geo/\beta-geo}$ kidney (Figure 2H) proves its specificity for agrin. These findings were confirmed using a polyclonal N-terminal antibody, GR-14 (results not shown). Thus, although the $Agrn^{del}$ allele is functionally null, it encodes an N-terminal fragment of agrin that is properly localized to BMs. Based on the structure of the mutant alleles, the epitopes recognized by MI-91 and GR-14 must be located within the first five follistatin-like domains.

Podocytes Are Responsible for Deposition of Agrin in Mature GBM

Transgenic mice expressing Cre from the *Pax3* promoter (P3pro-Cre) were used to mutate *Agrn* broadly in developing kidney. P3pro-Cre is expressed by cells of the metanephric mesenchyme and targets all of their descendants, including glomerular and tubular epithelial cells.⁴¹ A potentially complicating issue is that the transgene is also expressed at other sites throughout the embryo.³⁶ $Agrn^{fl/fl};P3pro-Cre$ mutants phenocopied agrin-deficient mice; mutants were stillborn, and their diaphragms lacked neuromuscular junctions (results not shown). This suggests that Cre was active in motoneurons, and it establishes that the $Agrn^{fl}$ allele is functionally null upon recombination. Nevertheless, these mutants proved useful for investigating the cellular origin of agrin in the GBM. In late fetal $Agrn^{fl/fl};P3pro-Cre$ kidneys labeled with C-terminal agrin antibodies, the BM of S-shaped nephrons was negative, and the GBM of mature glomeruli was very weakly positive. Ureteric bud and collecting duct BMs were positive, because P3pro-Cre is not expressed in ureteric bud derivatives. The small amount of agrin detected in mutant GBM is probably endothelial-derived, because the BM of earlier avascular S-shaped nephrons was already negative. These findings suggest that glomerular endothelial cells, at least in late fetal kidney, are not a significant source of GBM agrin.

Transgenic mice that express Cre under the human *NPHS2* promoter (2.5P-Cre) were used to specifically target podocytes. In this system, recombination occurs when Cre is expressed by immature podocytes in early capillary loop stage glomeruli.³⁵ Mice lacking podocyte-derived agrin displayed no overt phenotype. Mutants were fertile, lived until at least 1 year of age, and had body weights not significantly different from sex-matched control littermates. The GBM of normal adults was intensely labeled with C-terminal agrin antibodies (Figure 2, I and J). As in fetal mice, all BMs in normal adult kidney were labeled with the N-terminal mAb MI-91 (Figure 2, M and N). The GBM of $Agrn^{del/fl};2.5P-Cre$ mutants analyzed between 1 and 3 weeks of age showed a marked reduction in staining with C-terminal antibodies. By 3 weeks, labeling of mutant GBM was segmental, with only short stretches of some capillary

Figure 2. Podocytes are responsible for deposition of agrin in the GBM, and mutants synthesize N-terminal truncated forms of the protein. C-terminal agrin antibodies label the BM of pre-capillary loop-stage glomeruli (**A**) and mature GBM (**B**) in normal fetal kidney. BMs of collecting ducts, peritubular capillaries, and vascular smooth muscle cells are also stained. All BMs in *Agrn*^{del/del} (**C**) and *Agrn* ^{β -geo/ β -geo} (**D**) mutants are negative. N-terminal antibodies label tubular, vascular, and glomerular BMs in normal fetal mice (**E** and **F**) and *Agrn*^{del/del} mutants (**G**), whereas *Agrn* ^{β -geo/ β -geo} mutants are negative (**H**). C-terminal antibodies give the same staining pattern in normal adult kidney (**I** and **J**) noted in fetal mice but fail to label the GBM of adult podocyte-specific knockouts (**K** and **L**), indicating endothelial cells are not a significant source of agrin in mature GBMs. The N terminus of agrin is detected in most BMs of normal adults (**M** and **N**), and there is comparable labeling in conditional mutants (**O** and **P**). Thus, the *Agrn*^{del} and *Agrn*^{fl} alleles encode N-terminal truncated forms of agrin that localize to BMs. Scale bar: 150 μ m in **I**, **K**, **M**, and **O**; 100 μ m in **F**–**H**; 50 μ m all other panels.

loops weakly positive. The GBM of conditional mutants beyond 3 weeks of age failed to stain with C-terminal antibodies (Figure 2, K and L), implicating podocytes as the source of agrin in mature GBM. However, GBM agrin levels were clearly influenced by the mutant genotype. In *Agrn*^{del/fl};2.5P-Cre and *Agrn* ^{β -geo/fl};2.5P-Cre mice ~2 to 9.5 months of age, the GBM was either negative ($n = 23$) or showed weak segmental staining ($n = 4$). GBM labeling was more prevalent in *Agrn*^{fl/fl};2.5P-Cre mutants, as noted in 8 of 11 adult mice examined. We hypothesize that this reflects inefficient recombination of both *Agrn*^{fl} alleles in some podocytes, resulting in early deposition of agrin in the GBM that persists because of slow turnover. To avoid this confounding issue, the studies herein used only conditional mutants that were confirmed to lack GBM agrin by immunostaining. All BMs in conditionally mutant kidneys were labeled with the N-terminal mAb MI-91 (Figure 2, O and

P), irrespective of genotype, because the recombined *Agrn*^{fl} allele, like the *Agrn*^{del} allele, encodes an N-terminal fragment of agrin.

Truncated Forms of Agrin Expressed by Mutants Are Not Glycanated

Mouse agrin contains many potential sites for GAG attachment. Although the truncated mutant proteins lack the sites identified in other species, they retain others that could carry GAG chains *in vivo* (Figure 3A). To investigate this, *in vitro* studies were performed using an epitope-tagged construct, agrin_{1–602}, that recapitulates the form synthesized from the recombined *Agrn*^{fl} allele. This cDNA includes all of the coding sequence of the *Agrn*^{del} allele. A longer construct, agrin_{1–1171}, that includes both GAG attachment sites identified in chick served as a control.

Figure 3. Truncated forms of agrin synthesized in mutants do not carry GAG chains. **A:** The *Agrin*^{fl} and *Agrin*^{del} alleles encode fragments of agrin with potential GAG attachment sites. COS-7 cells were transfected with a Myc-tagged construct (Agrin₁₋₆₀₂) that represents the protein derived from a Cre-recombined *Agrin*^{fl} allele to assess whether these sites (arrows) carry GAG chains. Agrin₁₋₁₁₇₁ includes known attachment sites and served as a control. **B:** Western blotting of medium and cell lysates for the Myc epitope detected bands of the expected size in samples from Agrin₁₋₁₁₇₁ and Agrin₁₋₆₀₂-transfected cells whereas mock-transfected controls were negative. **C:** Agrin₁₋₁₁₇₁ was detected in the medium as a smear that shifted to a more discrete band on chondroitinase (cABC) but not heparinase (Hep) digestion, indicating that it carries predominantly CS-GAGs. Agrin₁₋₆₀₂ was insensitive to digestion with cABC and Hep (not shown), indicating that it lacks GAG chains. **D:** Blotting of medium for perlecan revealed a high-molecular weight species that shifted to a discrete band consistent with the size of the perlecan core protein after Hep digestion, demonstrating that COS-7 cells possess the biosynthetic pathways necessary for synthesis of HSPGs.

COS-7 cells transfected with agrin₁₋₁₁₇₁ or agrin₁₋₆₀₂ showed positive cytoplasmic staining for the Myc epitope and with mAb MI-91 (results not shown). Western blotting for Myc revealed a band of 142 kD in agrin₁₋₁₁₇₁-transfected cell lysates but not in mock-transfected controls (Figure 3B). This size is slightly greater than that predicted from the deduced amino acid sequence (128 kD), a discrepancy explained in part by *N*-linked glycosylation, because PNGase F digestion shifted this band to 134 kD (results not shown). In the medium, agrin₁₋₁₁₇₁ was detected as a smear typical of proteoglycans that ranged from ~190 to ~250 kD. Chondroitinase digestion shifted this species to a

more discrete band of 172 kD, indicating that agrin₁₋₁₁₇₁ carries predominantly CS-GAGs (Figure 3C). A diffuse smear remained that may be explained by incomplete digestion or by the presence of chondroitinase-insensitive GAGs. This protein was also *N*-glycosylated, because PNGase F digestion further reduced its size to 163 kD. Heparinase digestion had little effect on secreted agrin₁₋₁₁₇₁, indicating that it does not carry HS-GAG (Figure 3C). As a control, blotting for perlecan revealed a high-molecular weight species in undigested samples of media that shifted to a discrete band of ~400 kD after heparinase digestion, consistent with the size of the perlecan core

protein (Figure 3D). This proves that COS-7 cells possess the necessary pathways for biosynthesis of HSPGs and confirms that heparinase digestion was efficient.

Recombinant agrin₁₋₆₀₂ was detected as a band of 79 kD in cell lysates (Figure 3B). PNGase F digestion shifted the band to 74 kD, closer to its predicted mass of 70 kD (not shown). The protein was secreted to the medium, where it was detected as a band of 86 kD (Figure 3B). The fact that it was detected as a discrete species suggests that agrin₁₋₆₀₂ does not carry GAGs; consistent with this, it was insensitive to digestion with chondroitinase (Figure 3C) and heparinase (results not shown). The same findings were made using transfected 293 cells.

Loss of GBM-HS in Podocyte-Specific Knockouts

Perlecan was detected in all BMs of normal fetal kidney and colocalized with agrin in the vascular cleft of S-shaped nephrons and the nascent GBM of early capillary loop stage glomeruli. GBM labeling (Figure 4A) diminished as glomeruli matured, and perlecan became prominent in the mesangium. The same staining pattern was noted in *Agrn*^{del/del} (Figure 4B) and *Agrn* ^{β -geo/ β -geo} mutants (results not shown). In adult glomeruli, only the mesangial matrix and Bowman's capsule BM were significantly labeled (Figure 4C). In podocyte-specific knockouts up to ~7 months of age, glomerular staining for perlecan was properly localized to the mesangium (Figure 4D).

Because perlecan is not aberrantly deposited in mutant GBM and the truncated forms of agrin are unlikely to carry GAGs, there should be a reduction in the amount of HS and/or CS present in this site. This was assessed first by staining with mAb JM-403, which recognizes *N*-unsubstituted glucosamine units in native HS.^{42,43} In normal adults, this antibody labeled the GBM intensely (Figure 4E). In adult conditional mutants, staining of the GBM was either reduced ($n = 1$) or absent ($n = 7$), consistent with a loss of GBM-HS (Figure 4F). This finding was supported by staining with mAb NAH46 directed against *N*-acetylated domains in precursor forms of HS (results not shown). Dual-labeling with mAb MI-91 revealed glomeruli present in both control and mutant tissue sections (Figure 4, G and H, respectively). CS-GAGs were detected using mAb CS-56.⁴⁴ In normal adults, it diffusely stained the interstitium, Bowman's capsule, and tubular BMs but did not label the GBM. The staining pattern in adult conditional knockouts was identical, indicating that no CSPG carrying the CS-56 epitope is aberrantly deposited in mutant GBM (results not shown).

Distribution of Other Renal BM Components and Their Receptors in Agrin Mutants

Laminin in mature GBM is a heterotrimer consisting of the $\alpha 5$, $\beta 2$, and $\gamma 1$ chains. An antibody to the $\beta 2$ chain

Figure 4. Agrin mutants lose GBM-HS in the absence of compensation by perlecan. Perlecan is detected in all BMs of normal fetal kidney (A) and in agrin-deficient mice (B; E18.5 *Agrn*^{del/del} shown). In podocyte-specific knockouts up to ~7 months of age (D), staining for perlecan is properly localized to the mesangium and indistinguishable from controls (C). Antibody JM-403 recognizing native HS labels the GBM in normal adults (E) but not conditional mutants (F). Dual-labeling with mAb MI-91 to the N terminus of agrin is shown to localize glomeruli in control and mutant sections (G and H, respectively). Scale bar: 50 μ m in A–D; 75 μ m in E–H.

labeled the GBM and vascular smooth muscle in normal fetal mice (Figure 5A). In *Agrn* ^{β -geo/ β -geo} mutants, laminin $\beta 2$ was detected in these sites in a linear BM-like distribution but also in a punctate pattern consistent with an intracellular localization (Figure 5B). Punctate labeling was observed for all other laminin isoforms evaluated in *Agrn* ^{β -geo/ β -geo} mutants ($\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 1$, and $\gamma 1$) but not in *Agrn*^{del/del} mice, where the staining pattern for each was indistinguishable from controls (results not shown). The *Agrn* ^{β -geo} allele encodes a chimeric protein consisting of the agrin NtA domain fused to β -geo. This is expected to be membrane-bound in the ER in a configuration that exposes the NtA to the lumen where it would be free to interact with its only known binding partner, laminin $\gamma 1$; we hypothesize that this leads to “co-trapping” of laminin trimers containing $\gamma 1$, as previously shown.⁴⁵ In contrast, nidogen-1 and the type IV collagen chains of mature GBM ($\alpha 3$, $\alpha 4$, and $\alpha 5$) were detected in the normal pattern and at normal levels in agrin-deficient mice (Figure 5, C and D; results not shown). All of these BM components

Figure 5. Expression of BM components and their receptors in mutant kidney. The laminin $\beta 2$ chain is detected in normal fetal GBM (**A**), whereas $Agrn^{\beta\text{-geo}/\beta\text{-geo}}$ mutants (**B**) show both BM and punctate labeling, suggesting $\beta 2$ -containing trimers were co-trapped by the NtA/ β -geo fusion protein. Nidogen is detected in all BMs of normal fetal mice (**C**), and labeling is comparable in $Agrn^{\beta\text{-geo}/\beta\text{-geo}}$ mutants (**D**). Glomerular staining for integrin $\alpha 3$ (**E**) and dystroglycan (**G**) in normal fetal mice shows a localization similar to that observed in $Agrn^{\text{del}/\text{del}}$ glomeruli (**F** and **H**, respectively). Scale bar = 50 μm .

were expressed normally in adult conditional knockout kidney.

Agrin binds to integrins and dystroglycan via globular domains at its C terminus, which are missing in mutants. Therefore, we assayed expression and localization of these receptors. Integrin $\alpha 3$ was detected in normal fetal glomeruli in a podocyte distribution, and there was comparable labeling of $Agrn^{\text{del}/\text{del}}$ mutants (Figure 5, E and F, respectively). Staining was also normal in adult conditional mutants (results not shown). Dystroglycan is expressed by all epithelial cells in normal fetal kidney, including podocytes (Figure 5G). There was comparable labeling in $Agrn^{\text{del}/\text{del}}$ mutants

(Figure 5H) and in 3-week-old conditional knockouts (results not shown), so agrin is not necessary for proper dystroglycan localization.

Agrin Mutants Show No Significant Renal Histological or Ultrastructural Abnormalities

Compared with normal controls (Figure 6, A and B), no pathological changes were noted in $Agrn^{\text{del}/\text{del}}$ (Figure 6, D and E) or $Agrn^{\beta\text{-geo}/\beta\text{-geo}}$ (not shown) kidneys. Glomerular ultrastructure in $Agrn^{\text{del}/\text{del}}$ (Figure 6F) and $Agrn^{\beta\text{-geo}/\beta\text{-geo}}$ mutants (not shown) was also indistin-

Figure 6. Agrin mutants show no significant renal histological or ultrastructural abnormalities. Shown are representative H&E sections from E18.5 control (A and B) and *Agrin*^{del/del} mutant kidneys (D and E). Glomerular ultrastructure in E18 *Agrin*^{del/del} mutants (F) was indistinguishable from controls (C). Incomplete fusion of the GBM evident in the mutant is normal at late stages of glomerulogenesis. At 7 weeks of age, glomeruli of conditional mutants (J and K) display no histological or ultrastructural defects compared with controls (G and H). Mutant kidneys were normal by light microscopy up to ~8 months of age, the latest time point examined. By EM, subepithelial irregularities of the GBM (ie, “bumps”) were noted in mutants beyond 4 months of age (L) that were more prominent than in controls (I). Scale bars: 500 μ m in A and D; 50 μ m in B, E, G, and J; 1 μ m in all other panels.

guishable from controls (Figure 6C); thus, agrin is not required for nephrogenesis. Conditional mutants (Figure 6J) showed no histopathology compared with controls

(Figure 6G) until at least 8 months of age. Likewise, glomerular ultrastructure in conditional mutants was indistinguishable from controls up to ~4 months of age (Figure 6, K and H, respectively). GBM irregularities, characterized by focal thickening and epithelial protrusions of GBM material, were noted in mutants beyond 4 months (Figure 6L). Although these changes were occasionally observed in control mice, they were more prominent in mutants, especially when comparisons were made between littermates (Figure 6I). Morphometric analyses were not performed and may be confounded by the mixed genetic background of these mice. In any event, agrin is dispensable for the structural organization and integrity of the glomerular capillary wall.

Agrin Mutants Have a Severe GBM Charge Defect

Anionic sites were visualized by EM after labeling with the cationic probe PEI. In mature glomeruli of normal fetal mice, there was punctate labeling of the GBM concentrated in a regular pattern along both the subepithelial and subendothelial laminae rarae (Figure 7, A and C). This staining pattern presumably reflects concentration of the probe at sites with the greatest negative charge density. In littermate *Agrin*^{del/del} mutants, subendothelial labeling was comparable with controls, but there was a marked reduction in the number of anionic sites underlying podocytes (Figure 7, B and D). Quantitation of PEI aggregates per micrometer of GBM length (Figure 7E) revealed a significant reduction in subepithelial anionic sites compared with controls (5.41 ± 0.24 versus 13.95 ± 0.33 ; $P < 0.001$). There was also a small reduction in subendothelial anionic sites in mutants (11.25 ± 0.33 versus 12.67 ± 0.36 ; $P = 0.005$). Labeling of mesangial matrix, Bowman’s capsule, and tubular BMs was

Figure 7. Agrin mutants have a severe GBM charge defect. Anionic sites in the glomerular capillary wall were detected with the cationic probe PEI. In normal fetal mice (A, E17.5; C, E19), there is punctate labeling of the GBM distributed in a regular pattern along both laminae rarae. Subendothelial labeling was comparable in littermate *Agrin*^{del/del} mutants, but subepithelial labeling was markedly decreased (B and D). Quantitative analysis revealed a significant reduction in the number of anionic sites in mutants (E). In normal adults, subendothelial labeling is diminished, but discrete subepithelial labeling remains (F, 4 months; G, 11 months). In adult conditional mutants, PEI labeling is markedly diminished (H, 4 months; I, 9 months), and quantitative analysis revealed a significant reduction in subepithelial anionic sites (J). Scale bars: 200 nm in C and D; 500 nm in all other panels.

Figure 8. The glomerular filtration barrier is not compromised in conditional agrin knockout mice. **A:** Urinary protein-to-creatinine ratios of adult conditional knockouts and controls were not significantly different ($P = 0.34$). Mutants do not show elevated urinary excretion of albumin (**B**) or immunoglobulin (**C**) compared with controls ($P = 0.99$ and 0.70 , respectively). **D:** Mice were administered daily injections of BSA to test the integrity of the glomerular filtration barrier in the setting of a challenge. There was no significant difference in urinary protein-to-creatinine ratios between conditional knockout and control mice over time ($P = 0.99$). **E:** Renal excretion of the anionic tracer carboxymethyl Ficoll-70 was equivalent in conditional mutants and controls ($P = 0.83$), supporting the lack of a contribution to the charge barrier by the GBM. Values are mean $\pm$ SEM.

comparable in all mice and provided an internal control for probe penetration.

Subepithelial labeling remained prominent in normal adult GBM, but subendothelial anionic sites were diminished compared with fetal mice (Figure 7, F and G). PEI labeling was reduced throughout the GBM in adult conditional mutants (Figure 7, H and I). Quantitative analysis revealed subepithelial anionic sites per micrometer of normal adult GBM numbered 15.39 ± 0.27 (Figure 7J). Subepithelial labeling was significantly reduced in adult conditional mutants (8.00 ± 0.26 ; $P < 0.001$), whereas subendothelial anionic sites were equivalent in number to controls (9.29 ± 0.21 versus 8.99 ± 0.19 ; $P = 0.29$). In addition, many subepithelial anionic sites were less intensely labeled in the mutant than in the control, a qualitative difference that probably reflects reduced charge density in the GBM. The charge defects in mutants show that podocyte-derived agrin contributes significantly to the fixed negative charge of adult GBM.

The Glomerular Filtration Barrier Is Not Compromised in Agrin Mutants

Agrin-deficient mice die shortly after birth and fail to fill their bladders, precluding urinalysis as a means of assessing renal function. To test this indirectly, amniotic fluid from E16.5 to E18.5 embryos was analyzed by SDS-PAGE. Albumin was detected as a prominent ~ 70 -kd band present at equivalent levels in samples from mutant and control mice (results not shown).

By SDS-PAGE and determination of protein-, albumin-, and IgG-to-creatinine ratios, conditional mutants displayed no evidence of significantly elevated urinary pro-

tein levels up to ~ 10 months of age (Figure 8, A–C; results not shown). Mutant and control urines were also indistinguishable when analyzed by nondenaturing PAGE to assess differences relating to protein charge (results not shown). In mutants, tubular resorption of filtered proteins may be sufficient to compensate for a loss of glomerular permselectivity. To test the glomerular barrier in the setting of an experimental challenge, mice were injected with BSA for 5 consecutive days to induce protein-overload proteinuria. Mutant and control mice responded with a marked increase in urinary protein/creatinine ratios, but at no time point were these values significantly different between groups (Figure 8D). Finally, glomerular charge selectivity was assessed by measuring the clearance of carboxymethyl Ficoll-70. The amount of this inert and highly anionic tracer recovered in urine directly reflects its glomerular permeability and would be expected to differ between mutant and controls only if the intrinsic negative charge of the GBM serves as a physiologically relevant barrier. The fraction of the administered dose excreted over 24 hours by conditional mutants was not significantly different from controls (Figure 8E).

Discussion

This work addresses the theory that the intrinsic negative charge of the GBM, thought to be imparted largely by HSPGs, contributes to glomerular permselectivity. We showed that removal of agrin from the GBM leads to the loss of HS and anionic charge in this site, but this has no discernable effect on glomerular filtration. By virtue of its binding to laminin in the BM and dystroglycan and integrin receptors that are expressed by podocytes, agrin has been ascribed important structural and signaling roles in the glomerulus. The kidneys of conditional mutants showed no abnormalities by routine histology and only minor changes of GBM ultrastructure. Therefore, agrin is not required for initial assembly of the glomerular capillary wall or for maintenance of its structural integrity. Our findings challenge the theory that cell-matrix interactions involving agrin serve as an important link between podocytes and the GBM.

Much of our studies involved defining the nature of the truncated agrin proteins produced by the *Agrin^{del}* and recombined *Agrin^{fl}* alleles. Both mutant proteins are expected to be composed of the NtA domain and roughly one-half the complement of follistatin-like domains; the interaction between the NtA and the laminin $\gamma 1$ chain²⁷ is apparently sufficient for incorporation of the truncated proteins into BMs. It is a formal possibility that the mutant proteins retain unknown functions despite their lack of GAGs and the C terminus that binds cellular receptors. To resolve this may ultimately require the generation of a conditional allele that deletes the entire coding region.

Agrin is present in most renal BMs as revealed by N-terminal antibodies, but it is not required in these sites for nephrogenesis. Kidney development is also normal in mice lacking collagen XVIII²¹ and perlecan-HS.¹⁹ In contrast, HS itself is critical for this process, because defi-

ciency of certain enzymes involved in HS biosynthesis causes renal agenesis.^{46,47} Presumably, this dramatic phenotype reflects the fact that all forms of HS linked to both BM- and cell-associated proteoglycans (eg, syndecans and glypicans) are disrupted.

The GBM is assembled from the same basic repertoire of components found in other BMs, but it contains specific isoforms essential for its role as a filtration barrier. Mature GBM isoforms of laminin ($\alpha 5\beta 2\gamma 1$) and type IV collagen ($\alpha 3\alpha 4\alpha 5$) were detected in mutants, indicating that the well-defined developmental transitions involving these proteins^{48,49} proceed normally in the absence of agrin. Perlecan was not deposited ectopically to compensate for the loss of agrin, so we expected the amount of HS in mutant GBM to be disrupted. This was assessed by immunostaining using antibodies JM-403 and NAH46 that recognize specific HS structures in the GBM. The former has been used to demonstrate the loss of GBM-HS in human and experimental kidney disease, including human membranous nephritis, lupus nephritis, minimal change disease, and diabetic nephropathy^{14,39,50} and rat adriamycin nephropathy and Heymann nephritis.^{51,52} Although these studies support the theory that reductions in GBM-HS contribute directly to loss of barrier function, labeling with mAb JM-403 was recently reported to be normal in diabetic humans and rats with microalbuminuria, and it was concluded that loss of GBM-HS may be a secondary event that occurs in advanced disease.⁵³ Here, immunostaining with both antibodies revealed that adult conditional mutants lack GBM-HS. This confirms that mouse agrin exists as a HSPG *in vivo* but questions the importance of GBM-HS for kidney function.

Anionic sites in the laminae rarae of the GBM were described decades ago and attributed to HSPGs based on their susceptibility to GAG-degrading enzymes, but the specific forms that impart charge to the GBM have never been formally identified. Here, we show that the majority of anionic sites in the lamina rara externa of fetal and adult GBM represent agrin. GBM charge was not disrupted in adult perlecan-HS mutants,²⁰ which in light of the present study can be explained by the presence of agrin. Agrin mutants showed a ~50% reduction in the number of subepithelial anionic sites, and on a qualitative basis, those that remained appeared less intensely stained by PEI. This charge defect is, in both respects, as profound as that typically attributed to be a causative factor in human and experimental kidney disease; reported alterations range from a 63% reduction in human congenital nephrotic syndrome,⁵⁴ to 57% in membranous nephritis, to 52 to 20% in focal segmental glomerulosclerosis, to 21% in minimal change disease,^{55,56} and to 28 to 18% in rat models of diabetic nephropathy and puromycin nephrosis.^{57,58}

The key finding of this work is that agrin mutant mice have normal renal function despite a severe disruption of GBM charge. To test the glomerular barrier in the setting of an experimental challenge, we used a model of protein-overload proteinuria. This choice was based on an earlier study of perlecan-HS-deficient mice that used this technique to reveal a role for perlecan in glomerular

filtration.²⁰ No such defect was found in agrin mutants under similar experimental conditions. These findings are difficult to reconcile, especially considering the restriction of perlecan to the mesangium in adults. We also performed a clearance study using the anionic tracer carboxymethyl Ficoll to test glomerular charge selectivity. Clearance rates were nearly identical in mutant and control mice, suggesting that if a charge barrier exists, it is probably associated with cellular constituents of the capillary wall.

Although agrin is not needed for glomerular filtration, it may play important roles in other aspects of renal pathophysiology. By virtue of their charge, HSPGs are thought to influence the deposition and localization of circulating immune complexes and promote planting of cationic antigens that can elicit *in situ* formation of immune deposits.^{16,59–61} Conditional agrin knockout mice may provide a useful model to investigate the impact of GBM charge on these processes.

Beyond excluding a role for agrin in glomerular filtration, our findings challenge the notion that the GBM serves as a charge barrier and should force a reevaluation of this concept. In doing so, they direct attention to other sites, particularly the anionic glycocalyx of podocytes and glomerular endothelial cells. Although a role for the former in maintaining the elaborate architecture of podocytes has been defined, attention is only now becoming focused on the endothelial glycocalyx proper as a component of the filtration barrier.⁶² Dissecting these factors will hopefully advance our understanding of the pathogenesis of human kidney disease and lead to new approaches for its treatment.

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References

1. Chang RL, Deen WM, Robertson CR, Brenner BM: Permselectivity of the glomerular capillary wall: III. Restricted transport of polyanions. *Kidney Int* 1975, 8:212–218
2. Bohrer MP, Baylis C, Humes HD, Glasscock RJ, Robertson CR, Brenner BM: Permselectivity of the glomerular capillary wall: facilitated filtration of circulating polycations. *J Clin Invest* 1978, 61:72–78
3. Rennke HG, Patel Y, Venkatachalam MA: Glomerular filtration of proteins: clearance of anionic, neutral and cationic horseradish peroxidase in the rat. *Kidney Int* 1978, 13:278–288
4. Comper WD, Glasgow EF: Charge selectivity in kidney ultrafiltration. *Kidney Int* 1995, 47:1242–1251
5. Kerjaschki D, Sharkey DJ, Farquhar MG: Identification and characterization of podocalyxin: the major sialoprotein of the renal glomerular epithelial cell. *J Cell Biol* 1984, 98:1591–1596
6. Doyonnas R, Kershaw DB, Duhme C, Merckens H, Chelliah S, Graf T,

- McNagny KM: Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin. *J Exp Med* 2001, 194:13–27
7. Caulfield JP, Farquhar MG: Distribution of anionic sites in glomerular basement membranes: their possible role in filtration and attachment. *Proc Natl Acad Sci* 1976, 73:1646–1650
8. Kanwar YS, Farquhar MG: Anionic sites in the glomerular basement membrane: in vivo and in vitro localization to the laminae rarae by cationic probes. *J Cell Biol* 1979, 81:137–153
9. Kanwar YS, Linker A, Farquhar MG: Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *J Cell Biol* 1980, 86:688–693
10. Carrie BJ, Salyer WR, Myers BD: Minimal change nephropathy: an electrochemical disorder of the glomerular membrane. *Am J Med* 1981, 70:262–268
11. Bridges CR, Myers BD, Brenner BM, Deen WM: Glomerular charge alterations in human minimal change nephropathy. *Kidney Int* 1982, 22:677–684
12. Myers BD, Okarma TB, Friedman S, Bridges C, Ross J, Asseff S, Deen WM: Mechanisms of proteinuria in human glomerulonephritis. *J Clin Invest* 1982, 70:732–746
13. Guasch A, Deen WM, Myers BD: Charge selectivity of the glomerular filtration barrier in healthy and nephrotic humans. *J Clin Invest* 1993, 92:2274–2282
14. van den Born J, van den Heuvel LP, Bakker MA, Veerkamp JH, Assmann KJ, Weening JJ, Berden JH: Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular disease. *Kidney Int* 1993, 43:454–463
15. Groffen AJ, Veerkamp JH, Monnens LA, van den Heuvel LP: Recent insights into the structure and functions of heparan sulfate proteoglycans in the human glomerular basement membrane. *Nephrol Dial Transplant* 1999, 14:2119–2129
16. Raats CJ, van den Born J, Berden JH: Glomerular heparan sulfate alterations: mechanisms and relevance for proteinuria. *Kidney Int* 2000, 57:385–400
17. Wijnhoven TJ, Lensen JF, Wismans RG, Lamrani M, Monnens LA, Wevers AL, Rops AL, van der Vlag J, Berden JH, van den Heuvel LP, van Kuppevelt TH: In vivo degradation of heparan sulfates in the glomerular basement membrane does not result in proteinuria. *J Am Soc Nephrol* 2007, 18:823–832
18. Saarela J, Rhen M, Oikarinen A, Autio-Harmainen H, Pihlajaniemi T: The short and long forms of type XVIII collagen show clear tissue specificities in their expression and location in basement membrane zones in humans. *Am J Pathol* 1998, 153:611–626
19. Rossi M, Morita H, Sormunen R, Airenne S, Kreivi M, Wang L, Fukai N, Olsen BR, Tryggvason K, Soininen R: Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. *EMBO J* 2003, 22:236–245
20. Morita H, Yoshimura A, Inui K, Ideura T, Watanabe H, Wang L, Soininen R, Tryggvason K: Heparan sulfate of perlecan is involved in glomerular filtration. *J Am Soc Nephrol* 2005, 16:1703–1710
21. Utriainen A, Sormunen R, Kettunen M, Carvalhaes LS, Sajanti E, Eklund L, Kaappinen R, Kitten GT, Pihlajaniemi T: Structurally altered basement membranes and hydrocephalus in a type XVIII collagen deficient mouse line. *Hum Mol Genet* 2004, 13:2089–2099
22. Groffen AJ, Ruegg MA, Dijkman H, van de Velden TJ, Buskens CA, van den Born J, Assmann KJ, Monnens LA, Veerkamp JH, van den Heuvel LP: Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. *J Histochem Cytochem* 1998, 46:19–27
23. Groffen AJ, Buskens CA, van Kuppevelt TH, Veerkamp JH, Monnens LA, van den Heuvel LP: Primary structure and high expression of human agrin in basement membranes of adult lung and kidney. *Eur J Biochem* 1998, 254:123–128
24. Tsen G, Halfter W, Kroger S, Cole GJ: Agrin is a heparan sulfate proteoglycan. *J Biol Chem* 1995, 270:3392–3399
25. Winzen U, Cole GJ, Halfter W: Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine-glycine clusters. *J Biol Chem* 2003, 278:30106–30114
26. Burgess RW, Skarnes WC, Sanes JR: Agrin isoforms with distinct amino termini: differential expression, localization, and function. *J Cell Biol* 2000, 151:41–52
27. Kammerer RA, Schulthess T, Landwehr R, Schumacher B, Lustig A, Yurchenco PD, Ruegg MA, Engel J, Denzer AJ: Interaction of agrin with laminin requires a coiled-coil conformation of the agrin binding site within the laminin γ 1 chain. *EMBO J* 1999, 18:6762–6770
28. Gesemann M, Brancaccio A, Schumacher B, Ruegg MA: Agrin is a high-affinity binding protein of dystroglycan in non-muscle tissue. *J Biol Chem* 1998, 273:600–605
29. Martin PT, Sanes JJ: Integrins mediate adhesion to agrin and modulate agrin signaling. *Development* 1997, 124:3909–3917
30. Raats CJ, van Den Born J, Bakker MA, Oppers-Walgreen B, Pisa BJ, Dijkman HB, Assmann KJ, Berden JH: Expression of agrin, dystroglycan and utrophin in normal renal tissue and in experimental glomerulopathies. *Am J Pathol* 2000, 156:1749–1765
31. Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF: Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* 2001, 410:1057–1064
32. Rupp F, Ozcelik T, Linial M, Peterson K, Francke U, Scheller R: Structure and chromosomal localization of the mammalian agrin gene. *J Neurosci* 1992, 12:3535–3544
33. Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG: A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 2001, 73:56–65
34. Rodríguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF, Dymecki SM: High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat Genet* 2000, 25:139–140
35. Moeller MJ, Sanden SK, Soofi A, Wiggins RC, Holzman LB: Podocyte-specific expression of Cre recombinase in transgenic mice. *Genesis* 2003, 35:39–42
36. Li J, Chen F, Epstein JA: Neural crest expression of Cre recombinase directed by the proximal Pax3 promoter in transgenic mice. *Genesis* 2000, 26:162–164
37. Lewandoski M, Meyers EN, Martin GR: Analysis of fgf8 gene function in vertebrate development. *Cold Spring Harb Symp Quant Biol* 1997, 62:159–168
38. Moll J, Barzaghi P, Lin P, Bezakova G, Lochmuller H, Engvall E, Muller U, Ruegg MA: An agrin minigene rescues dystrophic symptoms in a mouse model for congenital muscular dystrophy. *Nature* 2001, 413:302–307
39. Yard BA, Kahlert S, Engelleiter R, Resche S, Waldherr R, Groffen AJ, van den Heuvel LP, van den Born J, Berden JH, Kroger S, Hafner M, van den Woude FJ: Decreased glomerular expression of agrin in diabetic nephropathy and podocytes, cultured in high glucose medium. *Exp Nephrol* 2001, 9:214–222
40. Raats CJ, Bakker MA, Hoch W, Tamboer WP, Groffen AJ, van den Heuvel LP, Berden JH, van den Born J: Differential expression of agrin in renal basement membranes as revealed by domain-specific antibodies. *J Biol Chem* 1998, 273:17832–17838
41. Chang CP, McDill BW, Neilson JR, Joist HE, Epstein JA, Crabtree GR, Chen F: Calcineurin is required in urinary tract mesenchyme for the development of the pyeloureteral peristaltic machinery. *J Clin Invest* 2004, 113:1051–1058
42. van den Born J, van den Heuvel L, Bakker MA, Veerkamp JH, Assmann KJ, Berden JH: A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats. *Kidney Int* 1992, 41:115–123
43. van den Born J, Gunnarsson K, Bakker MA, Kjellen L, Kusche-Gullberg M, Maccarana M, Berden JH, Lindahl U: Presence of N-unsubstituted glucosamine units in native heparan sulfate revealed by a monoclonal antibody. *J Biol Chem* 1995, 270:31303–31309
44. Avnur Z, Geiger B: Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. *Cell* 1984, 38:811–822
45. Yin Y, Kikkawa Y, Mudd JL, Skarnes WC, Sanes JR, Miner JH: Expression of laminin chains by central neurons: analysis with gene and protein trapping techniques. *Genesis* 2003, 36:114–127
46. Bullock SL, Fletcher JM, Beddington RS, Wilson VA: Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev* 1998, 12:1894–1906
47. Li JP, Gong F, Hagner-McWhirter A, Forsberg E, Abrik M, Kiselevsky R, Zhang X, Lindahl U: Targeted disruption of a murine glucuronyl C5-epimerase gene results in heparan sulfate lacking

- L-iduronic acid and in neonatal lethality. *J Biol Chem* 2003, 278:28363–28366
48. Miner JH, Patton BL, Lentz SI, Gilbert DJ, Snider WD, Jenkins NA, Copeland NG, Sanes JR: The laminin α chains: expression, developmental transitions, and chromosomal locations of $\alpha 1$ – $\alpha 5$, identification of heterotrimeric laminins 8–11, and cloning of a novel $\alpha 3$ isoform. *J Cell Biol* 1997, 137:685–701
49. Miner JH, Sanes JR: Collagen IV $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains in rodent basal laminae: sequence, distribution, association with laminins, and developmental switches. *J Cell Biol* 1994, 127:879–891
50. Tamsma JT, van den Born J, Bruijn JA, Assmann KJ, Weening JJ, Berden JH, Wieslander J, Schrama E, Hermans J, Veerkamp JH, Lemkes HH, van der Woude FJ: Expression of glomerular extracellular matrix components in human diabetic nephropathy: decrease of heparan sulphate in the glomerular basement membrane. *Diabetologia* 1994, 37:313–320
51. Raats CJ, Luca ME, Bakker MA, van Der Wal A, Heeringa P, van Goor H, van Den Born J, De Heer E, Berden JH: Reduction in glomerular heparan sulfate correlates with complement deposition and albuminuria in active Heymann nephritis. *J Am Soc Nephrol* 1999, 10:1689–1699
52. Wapstra FH, Navis GJ, van Goor J, van den Born J, Berden JH, de Jong PF, de Zeeuw D: ACE inhibition preserves heparan sulfate proteoglycans in the glomerular basement membrane of rats with established adriamycin nephropathy. *Exp Nephrol* 2001, 9:21–27
53. van den Born J, Pisa B, Bakker MA, Celie JW, Straatman C, Thomas S, Viberti GC, Kjellen L, Berden JH: No change in glomerular heparan sulfate structure in early human and experimental diabetic nephropathy. *J Biol Chem* 2006, 281:29606–29613
54. Vernier RL, Klein DJ, Sisson S, Mahan JD, Oegema TR, Brown DM: Heparan sulfate-rich anionic sites in the human glomerular basement membrane. *N Engl J Med* 1983, 309:1001–1009
55. Kitano Y, Yoshikawa N, Nakamura H: Glomerular anionic sites in minimal change nephrotic syndrome and focal segmental glomerulosclerosis. *Clin Nephrol* 1993, 40:199–204
56. Ramjee G, Coovadia HM, Adhikari M: Direct and indirect tests of pore size and charge selectivity in nephrotic syndrome. *J Lab Clin Med* 1996, 127:195–199
57. Isogai S, Mogami K, Ouchi H, Yoshino G: An impairment of barrier size and charge selectivity of glomerular basement membrane in streptozotocin-induced diabetes and prevention by pharmacological therapy. *Med Electron Microsc* 2000, 33:123–129
58. Mahan JD, Sisson-Ross S, Vernier RL: Glomerular basement membrane anionic charge site changes early in aminonucleoside nephrosis. *Am J Pathol* 1986, 125:393–401
59. Gallo GR, Caulin-Glaser T, Lamm ME: Charge of circulating immune complexes as a factor in glomerular basement membrane localization in mice. *J Clin Invest* 1981, 67:1305–1313
60. Kanwar YS, Caulin-Glaser T, Gallo GR, Lamm ME: Interaction of immune complexes with glomerular heparan sulfate-proteoglycans. *Kidney Int* 1986, 30:842–851
61. Madaio MP, Adler S, Groggel GC, Couser WG, Salant DJ: Charge selective properties of the glomerular capillary wall influence antibody binding in rat membranous nephropathy. *Clin Immunol Immunopathol* 1986, 39:131–138
62. Jeansson M, Haraldsson B: Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. *Am J Physiol* 2006, 290:F111–F116
63. Campanelli JT, Hoch W, Rupp F, Kreiner T, Scheller RH: Agrin mediates cell contact-induced acetylcholine receptor clustering. *Cell* 1991, 67:909–916