Synthesis of Anticoagulantly Active Heparan Sulfate Proteoglycans by Glomerular Epithelial Cells Involves Multiple 3-O-Sulfotransferase Isoforms and a Limiting Precursor Pool*

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Endothelial and other select cell types synthesize a subpopulation of heparan sulfate (HS) proteoglycans (HSPGs), anticoagulant HSPGs (aHSPGs) that bear aHS-HS chains with the cognate 3-O-sulfated pentasaccharide motif that can bind and activate antithrombin (AT). Endothelial cells regulate aHSPG production by limiting levels of HS 3-O-sulfotransferase-1 (3-OST-1), which modifies a non-limiting pool of aHS-precursors. By probing kidney cryosections with 125I-AT and fluorescently tagged AT we found that the glomerular basement membrane contains aHSPGs, with the staining pattern implicating synthesis by glomerular epithelial cells (GECs). Indeed, cultured GECs synthesized aHS with high AT affinity that was comparable with the endothelial product. Disaccharide analyses of human GEC (hGEC) HS in conjunction with transcript sections with 125I-AT and fluorescently tagged AT we found that the disaccharide pattern implicating synthesis by glomerular epithelial cells (GECs). Indeed, cultured GECs synthesized aHS with high AT affinity that was comparable with the endothelial product. Disaccharide analyses of human GEC (hGEC) HS in conjunction with transcript analyses revealed that hGECs express predominantly 3-OST-1 and 3-OST-3A, aHS production has not been previously examined in cells expressing multiple 3-OST isoforms. This unusual situation appears to involve novel mechanisms to regulate aHS production, as HS structural analyses suggest hGECs exhibit excess levels of 3-OST-1 and an extremely limiting pool of aHS-precursor. A limiting aHS-precursor pool may serve to minimize aHS synthesis by non-3-OST-1 isoforms. Indeed, we show that high in vitro levels of 3-OST-3A can efficiently generate aHS. Non-3-OST-1 isoforms can generate aHS in vivo, as the probing of kidney sections from 3-OST-1-deficient mice revealed GEC synthesis of aHS-precursors. Surprisingly, Hs3st1−/− kidney only expresses 3-OST isoforms having a low specificity for aHS synthesis. Thus, our analyses reveal a cell type that expresses multiple 3-OST isoforms and produces minimal amounts of aHS-precursor. In part, this mechanism should prevent aHS overproduction by non-3-OST-1 isoforms. Such a role may be essential, as 3-OST isoforms that have a low specificity for aHS synthesis can generate substantial levels of aHSPGs in vivo.

The majority of mammalian cell types express heparan sulfate proteoglycans (HSPGs), which are comprised of a protein core to which is attached linear chains of heparan sulfate (HS). These pericellular components regulate a myriad of biologic processes (1–6). Tremendous functional diversity stems from the structural complexity of the HS component. The HS chains have a repeated disaccharide unit of N-acetylgalactosamine(1→4)glucuronic/iduronic acid(1→4) (GlcNAc → GlcA/IdoA) that is partially decorated with N- and O-sulfate groups. The specific arrangement of these substituents, in large part, gives rise to distinct binding motifs that activate an array of important biologic effector molecules. HS motifs arise through the remodeling of the copolymer backbone by a relativley ordered series of reactions involving an epimerase and four families of sulfotransferases (reviewed in Refs. 1, 3, 7, and 8). 3-O-Sulfation of glucosamine residues is the rarest modification, yet is accomplished by the largest family of HS sulfotransferases, comprised of seven distinct 3-O-sulfotransferase (3-OST) isoforms (9–13). Modification by these isoforms can complete the synthesis of distinct ligand binding motifs, and individual isoforms exhibit distinct sequence specificities. 3-OST-1 preferentially generates a pentasaccharide motif that binds and activates antithrombin (AT) (9). HS with this motif is referred to as anticoagulant HS (aHS). 3-OST-3-type isoforms prefer to generate HS-binding sites for the glycoprotein gD of herpes simplex virus-1 (14, 15). HS with this motif can mediate cellular entry of this virus (16). An intermediate specificity occurs for 3-OST-5, which is equally efficient at generating both motifs (11). Thus, the biologic activity of HSPGs can, in part, be regulated by isoform-specific 3-O-sulfation.

Glomerular filtration is a process of molecular exclusion whereby the glomerular basement membrane (GBM) functions as an ultrafiltration barrier that prevents high molecular weight proteins from entering the urinary space. The selective permeability of the GBM is imparted by HSPGs, which are secreted by the three major cell types of the glomerulus: glomerular epithelial cells (GECs), mesangial cells, and endothelial cells (17–20). In part, perselectivity is thought to involve the extreme negative charge of the HS chains. However, GBM HS also exhibits very

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2 The abbreviations used are: HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; aHS, heparan sulfate; HSPG, anticoagulant heparan sulfate proteoglycan; AT, antithrombin; HSPG, anticoagulantly inactive heparan sulfate proteoglycan; 3-OST, HS 3-O-sulfotransferase; Hs3st1, 3-OST-1 deficient mice; Hs3st3A−/−, wild-type mice; GAG, glycosaminoglycan; AnMan, 2,5-anhydromannitol; RT, reverse transcriptase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; PAPS, adenosine 3’-phosphate,5’-phosphosulfate; HPLC, high pressure liquid chromatography.
unknown 3-O-sulfated residues (21–23). It is unknown whether these residues contribute to permeselectivity or other properties of the GBM. In particular, the GBM is exposed to clotting factors and other plasma proteins that pass through the highly permeable glomerular endothelium. Thus, the glomerulus constitutes a physiological functional unit in which the extravascular environment is exposed to clotting factors, but where clotting does not occur. Given that 3-O-sulfated residues are an essential component of anticoagulant HSPGs (aHSPGs), it is conceivable that certain glomerular cell types might synthesize aHSPGs. However, this possibility has not been directly evaluated.

It is well established that endothelial cells produce aHSPGs, which bear heparin-like HS chains containing the cognate pentasaccharide motif that binds and activates AT (24–27). However, aHSPGs can also be synthesized by a variety of cells of fibroblastic or epithelial origin (28–31). Given that aHSPGs can be generated extracellularly, it is conceivable that GECs might also synthesize aHSPGs.

The endothelial cell mechanisms that regulate aHSPG production have been well characterized. Endothelial cells produce only a minor subpopulation of aHS chains, containing the AT-binding motif (26). The remaining HS chains lack this motif and are anticoagulantly inactive (iHS). The core proteins of the active and inactive forms were found to be structurally similar, which suggests that synthesis of aHS is not regulated by the polypeptide chains to which the side chains are covalently linked (32). Moreover, multiple core proteins bear both aHS and iHS (33). Overexpression of the core protein syndecan-4 in fibroblastic and endothelial cells increased the amount of aHS but did not increase the amount of aHS from these mice are able to grow when cultured under permissive conditions, at 33 °C in the presence of interferon-γ. By contrast if they are grown at 37 °C in the absence of interferon-γ, cell division does not occur and they resume a differentiated phenotype. GECs were identified as mentioned above. Under nonpermissive conditions, these mGECs acquired positive staining with markers found in vivo in fully differentiated podocytes. We found positive staining with anti-Glepp1 and anti-podocalyxin antibodies; whereas, staining was absent in cells grown under permissive conditions.

### EXPERIMENTAL PROCEDURES

**Mesangial Cell Culture**—Mesangial cells were prepared from male Sprague-Dawley rat kidneys. Procedures for the separation, identification, and culture of the cells were as previously described (36). We employed subcultures of the 5th to 12th passages of mesangial cells grown at 37 °C in a humidified 5% CO₂ atmosphere.

**Glomerular Epithelial Cell Culture**—Experiments were done with rat primary GECs (rGECs), human primary GECs (hGECs), or with an immortalized murine GEC line (mGECs) derived by us from H-2Kb–primary GECs (rGECs), human primary GECs (hGECs), or with an immortalized murine GEC line (mGECs) purchased from Charles River Co. (United Kingdom) (37). rGECs were a gift from Prof. David Salant (Boston University). rGECs were cultured in Dulbecco's modified Eagle's medium and Ham's F10 culture media (Invitrogen) supplemented with 5% Nu serum (BD Biosciences), 0.5 ng/ml insulin (Sigma), 25 ng/ml prostaglandin E (Sigma), and 5 μg/ml transferrin (Sigma). They were incubated in nitrogen-coated Petri culture dishes at 37 °C in 5% CO₂.

For hGEC cultures, renal tissue was obtained from normal cortex regions of kidneys removed from patients who had unilateral nephrectomy for malignant neoplasms or urologic malformations. The cortex was separated from the medulla, homogenized, and filtered through 80- and 140-mesh sieves. The glomeruli were incubated with collagenase type 1, 300 units/ml at 37 °C. After 30 min, the digestion was filtered through a 500-mesh sieve, GECs were recovered in culture media (RPMI 1640 (Invitrogen) supplemented with 10 mM HEPES, 20% fetal calf serum, 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin) and incubated in gelatin-coated culture flasks at 37 °C, 5% CO₂. GECs were identified by their typical morphology on light microscopy, positive staining with cytokeratin and vimentin, and negative staining for factor VIII (38). Subcultures of the 3rd and 4th passages were used.

Primary cultures of GECs are difficult to achieve because GECs are terminally differentiated; whereas, culture of GECs under standard conditions leads to de-differentiation. For this reason, we developed an immortalized mGEC line from H-2Kb-tsA58 transgenic mice (immortomice), purchased from Charles River Co. (37). GECs from these mice are able to grow when cultured under permissive conditions, at 33 °C in the presence of interferon-γ. By contrast if they are grown at 37 °C in the absence of interferon-γ, cell division does not occur and they resume a differentiated phenotype. GECs were identified as mentioned above.

Under nonpermissive conditions, these mGECs acquired positive staining with markers found in vivo in fully differentiated podocytes. We found positive staining with anti-Glepp1 and anti-podocalyxin antibodies; whereas, staining was absent in cells grown under permissive conditions.

**Metabolic Labeling of Cells**—Confluent monolayers of cells were incubated in labeling medium containing Na₂[³⁵S]SO₄ (0.108 Ci/ml) and supplemented with 0.5% fetal calf serum for mesangial cells or 5% fetal calf serum (or 5% plasma) for GECs. The medium was collected, and the cells were labeled for an additional 24 h with fresh labeling medium. The media was then centrifuged to be free of floating cells, boiled at 100 °C for 5 min, filtered through a 0.45-μm filter, and kept frozen at −20 °C. The pooled media was referred thereafter as conditioned media. At the end of the 48 h, the cells were washed twice with phosphate-buffered saline, and cell layer-associated glycosaminoglycans (GAGs) were released by treatment with 0.05% trypsin, 0.53 mM EDTA (Invitrogen) for 10 min at 37 °C. The action of trypsin was stopped with 1 mg/ml soybean trypsin inhibitor (Sigma). After centrifugation the supernatant was collected and boiled at 100 °C for 5 min, filtered through a 0.45-μm filter and kept frozen at −20 °C. The cell layer-associated fraction was referred thereafter as trypsinate.

**Purification of ³⁵S-HS**—The HS chains of the conditioned media and trypsinate were purified in parallel. Samples were incubated first with 0.1 mg/ml protease (Sigma) overnight at 37 °C and then with 0.16 mg/ml papain (Sigma), and its cofactors (10 mM NaCN, 1 mM cysteine-HCl, 0.5 mM EDTA) for 5 h at 37 °C. The chondroitin sulfate chains were degraded with chondroitinase ABC (Sigma), 0.1 units/ml, for 3 h at 37 °C and then 0.05 units/ml overnight at 37 °C. ³⁵S-HS chains were isolated by ion exchange chromatography on DEAE-Sephadex (Amer sham Biosciences). The labeled samples were loaded in phosphate-buffered saline, pH 7.2; the gel was washed with 10 column volumes of the same buffer followed by 2 column volumes of 50 mM sodium acetate, pH 5. Then ³⁵S-HS was eluted with phosphate-buffered saline with 1 M...
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NaCl, pH 7.2. The 35S-HS was cleaved from peptides by β-elimination, and proteins were removed by phenol extraction. 35S-HS was dialyzed against 10 mM NH4HCO3 and evaporated to dryness in a SpeedVac concentrator (35). The purity of the preparation was confirmed by the absence of degradation with chondroitinase ABC (Sigma) and complete digestion with flavobacterium heparitinase (Seikagaku, Japan) (31).

Isolation of aHS by Affinity Chromatography—aHS was isolated from anticoagulant iHS by AT affinity on concanavalin A-Sepharose. To form aHS-AT complexes, 35S-HS was incubated with 2.5 μM AT in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, containing 10 μM dextran sulfate, 0.002% Triton X-100, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2, for 1 h at room temperature. aHS-AT complexes were bound to concanavalin A by mixing samples with a suspension of concanavalin A-Sepharose 4B (Sigma) equilibrated in the same buffer and incubating for 4 h by gentle agitation at room temperature. iHS was removed by washing the matrix. aHS was eluted from the gel by dissociation of the aHS-AT complexes in buffer containing 1 M NaCl. AT was removed by phenol extraction and NaCl by dialysis. The HS fractions were concentrated by ethanol precipitation. aHS and iHS content were quantified by scintillation counting.

Affinity Coelectrophoresis—At neutral pH the electrophoretic mobility of HS is higher than that of AT and the binding of AT to HS retards its migration (39). Electrophoresis was performed on a 1% agarose gel (Sea-Plaque-agarose, Bioconcept) in 0.005% CHAPS (Sigma), 50 mM formic acid, 0.002% Triton X-100, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2, for 1 h at room temperature. aHS-AT complexes were equilibrated in the same buffer and incubating for 4 h by gentle agitation at room temperature, iHS was removed by washing the matrix. aHS was eluted from the gel by dissociation of the aHS-AT complexes in buffer containing 1 M NaCl. AT was removed by phenol extraction and NaCl by dialysis. The HS fractions were concentrated by ethanol precipitation. aHS and iHS content were quantified by scintillation counting.

Molecular Size Determination—The hydrodynamic size distribution of 35S-HS was analyzed by gel filtration on a Superdex 200 (Amersham Biosciences) column using a fast protein liquid chromatography system. The column was run in 50 mM Tris, 150 mM NaCl, pH 7.4, and loaded with 100,000 cpm of 35S-HS. GAGs were quantified by scintillation counting. The V0 marker was dextran blue 2000 (M, 2,000,000) and the V1 marker was 35S-SO4.

Alternatively the Mr was determined by electrophoresis of 35S-HS on polyacrylamide gel (7.5–15%) gels, without SDS, in 0.25 M Tris, pH 7.4. The column was run in 50 mM Tris, 150 mM NaCl, pH 7.4, and loaded with 100,000 cpm of 35S-HS. GAGs were quantified by scintillation counting. The V0 marker was dextran blue 2000 (M, 2,000,000) and the V1 marker was 35S-SO4. GAGs were quantified by scintillation counting.

Alternatively the Mr was determined by electrophoresis of 35S-HS on polyacrylamide gel (7.5–15%) gels, without SDS, in 0.25 M Tris buffer containing 0.1 M NaCl to limit the GAG hydration volume. Calibrated GAG molecular weight standards were heparin (Mr = 16,400), chondroitin sulfate (Mr = 21,600), and heparan sulfate (Mr = 15,500). 1 μg of unlabeled GAGs or 50,000 cpm of 35S-HS chains were loaded per lane. Migration profiles of 35S-HS were recorded with a phosphorimager analyzer (PerkinElmer Life Sciences). Migration profiles of standards were determined after staining the gel with azure A and AgNO3. Modal Rg of GAGs and 35S-HS were measured by densitometry scanning of the stained gel or phosphorimager analysis of 35S counts. 35S-HS Mr was determined by extrapolation from the regression curve of the standard GAGs Rg and the log of molecular weights (31, 40).

Disaccharide Analysis—GEC aHS and iHS were degraded to disaccharides by deacetylation through hydroxynolysis followed by high pH nitrous acid and then low pH nitrous acid treatments as described previously. The distribution of sulfated species was analyzed by reverse ion pairing HPLC on a C18 column (0.46 × 24 cm, Vydac); 0.5-ml fractions were collected, and radioactivity was quantified in a scintillation coun-

ter. Similar to the procedure of Guo and Conrad (41), the samples were eluted at 0.5 ml/min with 1 mM tetrabutylammonium phosphate, pH 3.6, containing acetonitrile at 4.8 (45 min), 9 (15 min), and 11.7%. The identification of each disaccharide was confirmed by co-chromatography on ion pairing reverse phase HPLC with 3H-labeled disaccharide standards, as described in prior publications (34, 41, 42).

Measurement of aHS Precursor Pool Size—The aHS-precursor pool size was measured by the ability of 3-OST-1 or 3-OST-3, to convert iHS into aHS in vitro. Metabolically labeled 35S-HS purified from cultured hGECs was incubated with purified recombinantly expressed 3-OST-1 or 3-OST-3, in the presence of the sulfate donor PAPS, as described (9, 43). aHS was subsequently quantified by AT affinity chromatography on concanavalin A-Sepharose. As control, we also evaluated iHS purified from the L-cell clone, L-33+ (43).

aHSPG Localization by Autoradiographic and Fluorescence Microscopy—aHSPGs were localized by 125I-AT binding on kidney cryosections followed by microscopic autoradiography, as described (44). Kidneys were obtained from adult male Sprague-Dawley rats and 5-μm cryosections were cut from OCT-embedded tissues. Kidney serial cryosections were preincubated in phosphate-buffered saline, incubated with 20 μl of 125I-AT (15,000 cpm/μl), coveredslipped, and kept in humidified chambers for 1 h at 4°C. Slides were washed five times and fixed in absolute ethanol, immersed in autoradiographic emulsion, and revealed in Dektol. The specificity of 125I-AT binding to kidney cells was verified by using heparin (100 μg/ml) as a competitor. Alternatively, cryosections were incubated with either 100 millimolars/ml chondroitinase ABC (Sigma) or 20 millimolars/ml heparitinase (Seikagaku, Japan) for 1 h at 37°C prior to incubation with 125I-AT. Similar analyses were conducted on kidney sections from 3-OST-1-deficient mice (Hs3st1−/−), which we have previously described (45).

aHSPGs were also localized by fluorescent labeling using AT-Alexa 488 or AT-Alexa 647 conjugates. AT labeled with Alexa Fluor 488 or 647 succinimidyl esters (Molecular Probes), respectively. The generation of fluorescent AT forms is more fully described elsewhere. In brief, AT (Cutter) was initially repurified over heparin-Sepharose, with washing at 250 mM NaCl and elution at 1 M NaCl. The AT flow-through fraction, which lacks heparin affinity, was used as a negative control for nonspecific binding. Both AT preparations were labeled according to the manufacturer's instructions. After coupling, the AT-Alexa conjugates were repurified on heparin-Sepharose to obtain high affinity AT-Alexa 488 or 647, which lacked heparin affinity. Labeling conditions yielded ~1.5 fluorophores per molecule of AT. Compared with non-labeled AT, the conjugates retained 80–90% of heparin-induced anti-Xa activity by the Coamatic AT assay (Chromogenix Instruments). Fluorescent AT was incubated on cryosections at a concentration of 200 nm for 1 h and excess unbound ligand was eliminated by washing. Specificity verification of the AT-Alexa conjugates included coincubation with a synthetic aHS cognate pentasaccharide (100 μg/ml ~ 58 μm) (Atrixtra, Organon Synoﬁ-Synthelabo LLC).

Histological staining and autoradiographs were photographed using a Coolpix 990 digital camera (Nikon) attached to a Nikon Optiphot microscope. Fluorescently labeled sections were photographically detected using a Axiovert 200 epifluorescence microscope (Zeiss) and a digital CCD camera directed by the Openlab software. The pictures were mounted using the Photoshop 5.5 software.

Confocal Deconvolution Microscopy—Mice were given a lethal injection of avertin, then blood was removed by perfusing phosphate-buff-

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Images were obtained with a Zeiss LSM 510 Meta Laser Scanning Confocal Microscope System with a 63× Apochromat oil objective (NA 1.4). Z-stacks spanned >12 μm to capture all detectable axial emissions and were comprised of 1.1-μm optical sections offset every 0.53 μm. Each channel was collected separately using non-saturating conditions with the minimum possible laser power and a pinhole approaching 1.0 Airy units. Stacks were deconvolved with AutoDeblur version 9.3 (AutoQuant Imaging, Inc.) using default conditions.

3-OST mRNA Expression in GECs—Northern blot analysis for 3-OST-1, 3-OST-3, and 3-OST-6, which was initially identified in a characterization of chromosome 16 by the Human Genome Project and referred to as 3-OST-6 (12). The results were expressed as 3-OST mRNA copies per 400,000 copies of actin mRNA, corresponding approximately to 10 ng of total RNA and to about 1000 cells.
**RESULTS**

**Localization of aHSPGs within the Kidney**—To identify sites in the rat kidney that produce aHSPGs, we initially employed an established autoradiographic microscopy approach in which cryosections were probed with \(^{125}\text{I}}\)AT. The kidney gave a strong positive signal for aHSPGs, as compared with other tissues such as muscle or testis (not shown). In particular, abundant AT-binding sites occurred in the rat kidney cortex. Fig. 1 shows specific binding sites for AT in the glomeruli, peritubular basement membrane, peritubular capillaries, and blood vessels. The label was localized diffusely on the glomeruli, on the basolateral side of the tubular epithelial cells, and in vessel walls (Fig. 1A). The \(^{125}\text{I}}\)-AT selectively reveals aHS, as tissue binding of \(^{125}\text{I}}\)-AT was abolished by competition with heparin (Fig. 1B), and as \(^{125}\text{I}}\)-AT binding was prevented when the kidney sections were preincubated with heparitinase (Fig. 1D), but not with chondroitinase ABC (Fig. 1D). Thus, aHSPGs are abundant in the rat kidney and are localized to defined structures, including the glomerulus.

We sought to assess which glomerular cell type may be generating aHSPGs; however, visualization with \(^{125}\text{I}}\)-AT lacks sufficient resolution for this purpose. To obtain high resolution localization of aHSPGs, we generated fluorescently labeled AT forms (AT–Alexa 488 and AT–Alexa 647). Similar to \(^{125}\text{I}}\)-AT, AT–Alexa 488 revealed glomeruli, blood vessels, and the basolateral side of tubular epithelial cells (Fig. 1G). The signal was competed out with a synthetic pentasaccharide that encompasses the AT-binding motif of heparin/aHS (Fig. 1H), indicating that AT–Alexa 488 staining occurs through the sequence-specific heparin-binding site of the AT. Binding was specific, as dextran sulfate (a non-specific polyanion) di not interfere with AT–Alexa 488 binding (Fig. 1I). Signal was also reduced by coinoculation with excess unlabeled AT (Fig. 1J), indicating that modified and native AT bind the same target. Furthermore, no fluorescence was detected when sections were probed with an AT form that lacks affinity for heparin (Fig. 1L) and AT–Alexa 488 binding was prevented by predigestion of sections with heparitinase, but not chondroitinase ABC (data not shown). Combined, these controls indicate that fluorescently labeled AT, similar to the well-established \(^{125}\text{I}}\)-AT probe, allows for specific visualization of aHSPGs.

The glomerulus contains epithelial, endothelial, and mesangial cells. At high magnification the fluorescent AT revealed that aHSPGs closely follow the GBM and adjacent cells in the vascular and urinary space (Fig. 1K). This pattern of labeling is inconsistent with the glomerular location of mesangial cells. Moreover, the observed labeling was wider than that which occurred in peritubular capillaries, which implicates production by at least glomerular epithelial cells. Thus, the high resolution fluorescence microscopy suggests that glomerular epithelial cells might synthesize aHSPGs.

**Glomerular Epithelial Cells Synthesize aHSPGs**—To address whether glomerular cells can indeed synthesize aHSPGs, we evaluated primary GECs from human and rat (hGECs and rGECs, respectively), as well as an immortalized line that we generated from mouse (mGECs). Rat primary mesangial cells were used as control. Cells were cultured with [\(^{35}\text{S}}\])SO\(_4\), then \(^{35}\text{S}}\)-aHS was purified from the cell surface (cellular fraction) or the culture medium (secreted fraction), as described under “Experimental Procedures.” For mGECs and rGECs these fractions were pooled as material was limiting. AT affinity chromatography was employed to isolate aHS and iHS. All three types of GECs produced substantial levels of aHS (TABLE TWO); whereas, \(^{35}\text{S}}\)-aHS recovery for human and mouse GECs demonstrated that the cell surface and secreted HSPGs contained an equivalent fraction of aHS (~1%, TABLE TWO).

The ability of purified aHS versus iHS to form specific complexes with AT was assessed by affinity coelectrophoresis (Fig. 2). \(^{35}\text{S}}\)-HS samples were resolved through an agarose gel containing various amounts of AT. The presence of AT retarded the migration of aHS but not iHS, indicating that AT selectively forms complexes with GEC aHS (Fig. 2). All HS from rat mesangial cells showed unretarded migration, confirming the absence of aHS (not shown). Fig. 2 presents data for rGEC samples but comparable results were also obtained for human and mouse GEC (not shown). For all three species, the migration of aHS was ~50% retarded at 30 nM AT and maximally retarded at 500 nM AT, which is consistent with the known \(K_d\) of endothelial cell aHS for AT (~50 nM) (27, 31). Moreover, aHS behaved homogeneously in the pres-
Table Two

| Cell-type          | aHS | h  |
|--------------------|-----|----|
| Mouse GECs         |     |    |
| Pooled fractions   | 4.55 ± 0.39 | 5   |
| Human GECs         |     |    |
| Cellular fraction  | 1.12 ± 0.25 | 5   |
| Secreted fraction  | 1.10 ± 0.23 | 5   |
| Rat GECs           |     |    |
| Pooled fractions   | 19.4 ± 9.8 | 3   |
| Rat mesangial cells|     |    |
| Cellular fraction  | 0.1 | 2   |
| Secreted fraction  | 0.1 | 2   |

* a The assay background is ~0.1%.

3B shows a representative phosphorimager analysis of the migration profile of hGEC-secreted 35S-aHS and 35S-iHS. hGEC aHS and iHS have a similar size distribution and appear broadly smeared reflecting a high molecular weight polydispersity, concordant with the broad peak observed by gel filtration in Fig. 2A. We have determined that the model M$_r$ of hGEC secreted aHS and iHS is ~30,000 (Fig. 3B). Comparable M$_r$ values were obtained for aHS and iHS prepared from hGEC cell surface HS (not shown). Thus, GEC synthesis of aHS is independent of HS chain size, as has been shown for other cell types.

**Disaccharide Composition of hGEC iHS and aHS**—hGEC synthesized iHS and aHS were subjected to chemical degradation, and their disaccharide compositions were analyzed by reverse phase ion pairing HPLC (Table Three). Cell surface and secreted iHS were analyzed independently and generated very comparable results, confirming the reproducibility of this method (data not shown). hGEC aHS and iHS yielded three 3-O-sulfated disaccharides, IdoA2S-AnMan3S, GlcA-AnMan3S, and GlcA-AnMan3S6S, which were readily resolved (Fig. 4). The IdoA2S-AnMan3S residue is known to derive from 3-OST-3 isoforms, and shows similar levels in aHS and iHS samples. This product is not derived from HS of other cell types that generate aHS (31, 34, 47), indicating that aHS synthesis in hGEC may exhibit unique properties. The GlcA-AnMan3S and GlcA-AnMan3S6S residues typically originate from critical disaccharides in the AT-binding sites of aHS and are the main products of 3-OST-1 activity (9, 43). However, aHS and iHS samples produced almost equal amounts of these residues. This similarity is extremely unusual as aHS, compared with iHS, typically shows >10-fold enrichment for these products (31, 34, 47). Such high levels of these residues from iHS are perplexing, as it suggests that hGECs may have a high activity of 3-OST-1 (and/or other 3-OST isoforms). However, high 3-OST-1 activity is typically associated with high levels of aHS synthesis (35), but hGECs produce only a modest level of aHS (Table Two). These paradoxical results might be reconciled if hGECs produce a very limited amount of aHS-precursor chains.

**Determination of aHS-precursor Pool Within iHS**—We have previously shown that iHS contains a subpopulation of HS chains that functions as a substrate for aHS synthesis (the aHS-precursor) (35). Modification by 3-OST-1 completes the formation of functional AT-binding sites and thereby converts aHS-precursor chains into functional aHS.
The size of the aHS-precursor pool can be determined by exhaustively modifying iHS with 3-OST-1 and then measuring the level of generated aHS (35). We have employed a similar procedure to measure the aHS-precursor pool size of hGECs. Given that hGECs produce a 3-OST-3-type product, IdoA2S-AnMan3S, we additionally evaluated the potential of 3-OST-3A to generate aHS. Thus, we used recombinantly expressed 3-OST-1 or 3-OST-3A to exhaustively modify hGEC 35S-iHS and then measured the resulting aHS by AT affinity chromatography (Table Four). 3-OST-1 modification of hGEC iHS produced a low level of aHS (~1%). Modification of hGEC iHS by 3-OST-3A also produced aHS, but at slightly lower levels corresponding to about half of what was generated by 3-OST-1. In contrast, we also evaluated iHS from L-33 cells, which are known to have high aHS-precursor levels (35). 3-OST-1 converted 16% of the L-33 iHS into aHS; whereas, 3-OST-3A converted only ~17% of iHS into aHS. Combined, these data demonstrate that the aHS-precursor pool is very limited in hGECs. In addition, they also suggest that the nature of the aHS-precursor in hGECs is much more susceptible to conversion by 3-OST-3A than is the aHS-precursor from L-33 cells. Thus in hGECs, it is possible that 3-OST-3-type isoforms might contribute to aHS production.

**Identification of the Major 3-OST Isoforms in hGECs**—The above disaccharide analyses suggest that hGECs should express 3-OST-1 and 3-OST-3A, and/or 3-OST-3B. Indeed, by Northern blot analysis of hGEC poly(A) RNA we have detected positive signals for all three isoforms (data not shown). To obtain a more comprehensive analysis, we measured the expression of all seven 3-OST isoforms by real time RT-PCR. This quantitative analysis revealed that hGECs predominantly express 3-OST-1 and 3-OST-3A (Fig. 5). Minor levels of 3-OST-2 and 3-OST-3B are also present. Such low expression probably contributes very little to the total 3-O-sulfated residues, as hGEC disaccharides lacked detectable levels of an additional known product of 3-OST-2, GlcA2S-AnMan3S. Thus, it is likely that hGEC 3-O-sulfated residues are predominantly derived from 3-OST-1 and 3-OST-3A. Moreover, expression of just these two isoforms would be sufficient to account for all 3-O-sulfated disaccharides detected in hGEC HS.

**Glomeruli of Hs3st11−/− Mice Generate aHSPGs**—The above data demonstrate that hGECs predominantly express both 3-OST-1 and 3-OST-3A. Moreover in hGECs, 3-OST-3A might contribute to aHS production. To evaluate whether aHS might be generated by non-3-OST-1 isoforms in hGECs, we examined the localization of aHSPGs in kidney cryosections of wild-type (Hs3st11+/+) and 3-OST-1-deficient mice (Hs3st11−/−) (Fig. 6). In wild-type mice, the localization of aHSPGs in the kidney is similar to that observed in the rat (Fig. 6A) with strong labeling of the glomeruli, tubules, and blood vessels. In 3-OST-1 null mice, AT binding is reduced to background levels in the kidney tubules and in the vasculature. In contrast, the labeling seen on the glomeruli persists (Fig. 6B). To confirm that 125I-AT binding to kidney glomeruli in Hs3st11−/− mice is specific for aHS, sections were pretreated with heparitinase (Fig. 6C). In wild-type mice, the localization of aHSPGs in the kidney is similar to that observed in the rat (Fig. 6A) with strong labeling of the glomeruli, tubules, and blood vessels. In 3-OST-1 null mice, AT binding is reduced to background levels in the kidney tubules and in the vasculature. In contrast, the labeling seen on the glomeruli persists (Fig. 6B). To confirm that 125I-AT binding to kidney glomeruli in Hs3st11−/− mice is specific for aHS, sections were pretreated with GAG lyases prior to the incubation with 125I-AT. Chondroitinase ABC did not modify the labeling (Fig. 6C), but the labeling was abolished for sections treated with heparitinase (Fig. 6D). These data demonstrate that even in the absence of 3-OST-1, significant amounts of aHSPGs are synthesized in the kidney glomerulus.

The glomerular cell type responsible aHSPG production in Hs3st11−/− glomeruli was assessed by imaging aHSPGs at high resolution with fluorophore-tagged AT (AT-Alexa 647) via confocal deconvolution microscopy. Similar to AT-Alexa 488, AT-Alexa 647 staining is
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**FIGURE 6. Autoradiographic localization of aHSPGs in kidneys of Hs3st1+/+ and Hs3st1−/− mice.** Cryosections of mouse kidney isolated from Hs3st1+/+ (A) and Hs3st1−/− (B–D) mice were labeled with 125I-AT. A, kidney cryosections incubated with 125I-AT alone; C, section preincubated with chondroitinase ABC prior to the labeling with 125I-AT; D, section preincubated with heparitinase prior to the labeling with 125I-AT. Phase-contrast microscopy of sections B and C confirmed labeling is on glomeruli (not shown).

Hs3st1+/+ kidneys predominantly expressed the two major isoforms found in hGECs, 3-OST-1 and 3-OST-3, as well as 3-OST-3b and 3-OST-6. In Hs3st1−/− kidneys, transcript levels were indistinguishable between genotypes, except for the anticipated absence of 3-OST-1 (Fig. 8). Thus, the only isoforms that can potentially account for aHSPG production by Hs3st1−/− GECs are 3-OST-3, 3-OST-3b, and 3-OST-6. We acknowledge that GECs are a minor kidney cell population, so it is presently unclear whether all three of these isoforms are expressed in mouse GECs. The minor contribution of GEC RNA to the total kidney pool also prevents us from determining whether any of these isoforms are up-regulated in Hs3st1−/− GECs. Despite these limitations, this analysis indicates candidates for potentially producing aHSPGs in Hs3st1−/− GECs. 3-OST-3, 3-OST-3b, and 3-OST-6 are particularly strong candidates as they are also expressed in hGECs.

**DISCUSSION**

Universal Features of aHSPG Synthesis by GECs—We believe this is the first report describing GEC synthesis of aHSPGs and its very unique features. The probing of rat kidney cryosections with 125I-AT revealed that aHSPGs are not restricted to blood vessels but also occur in the glomerulus and on the basolateral surface of the tubular epithelium. These data are consistent with immunohistochemical studies that found endogenous antithrombin localizes to aHSPGs of capillaries and the basement membrane of tubular epithelium (48, 49). However, endogenous AT was only detected on the endothelial surface of the GBM (49). In contrast, our high resolution imaging of the glomerulus using fluorescent AT clearly revealed that aHSPGs are localized throughout the entire width of the GBM. Presumably the GEC side of the GBM exhibits a very low accumulation of endogenous AT, which may have precluded detection by the prior studies. Regardless, our in vitro data demonstrate that primary and immortalized GECs can synthesize aHSPGs; whereas, rat mesangial cells do not. Thus, the aHSPGs of the GBM must normally derive from both endothelial cells and epithelial cells.

GEC biosynthesis of aHSPGs shares some mechanistic features with other cell types and these commonalities reveal fundamental aspects of aHSPG production. First, it has been established that aHSPG synthesis is governed by the expression pattern of the HS biosynthetic enzymes, with no absolute specificity for a particular core protein (32, 34). In this regard, aHSPG localization in the kidney does not completely overlap with the known expression sites of various core proteins. Both perlecan and agrin are major HSPGs of the GBM. Perlecan occurs on the endothelial side of the GBM but not the epithelial side, so cannot account for all aHSPGs of the GBM (50). Similarly, agrin occurs on both sides of the GBM but does not occur in the tubular basement membrane so it cannot fully explain all renal sites of aHSPGs (51). Second, aHSPG production is controlled by a "late" biosynthetic modification and so is relatively independent of chain size (52). Our analyses show that GEC aHS has a modal M, of ~30,000. This value falls within the published data; aHS isolated from endothelial cells, ovarian granulosa cells, or fibroblastic L-cells has a modal M, ranging from 13,000 to 79,000 (31, 32, 52–54). Moreover, hGEC-generated aHS and iHS exhibited comparable chain size, which has also been found for aHS and iHS isolated from other cell types (34, 36, 59). Thus, similar to these cell types, GEC synthesis of aHS is independent of HS chain size. Third, for all three GEC lines, affinity coelectrophoresis showed that the dissociation constant of aHS for AT is in the order of ~50 nM, the dissociation constant of AT for aHS generated by endothelial cells, granulosa cells, and L-cells (27, 31, 55). This similarity suggests that the AT-binding site in GEC aHS is similar...
documented. In orange-yellow (surrounded by GECs (nearly abolished in endothelial basement membrane that is not adjacent to GECs (anti-podocalyxin antibodies indirectly labeled with Alexa Fluor 568 (CD31, blue). False colored confocal images were acquired by laser scanning microscopy and processed with deconvolution software to generate optical sections of 0.53 μm, as described under “Experimental Procedures.” For each genotype, the outside column shows an enlarged region from the inside column, as indicated (Merge, thin lines). GECs are identified by their extensive podocalyxin staining and a GEC nucleus is indicated (Merge, G). CD31 staining reveals endothelial cells and some representatives are indicated (Merge, E). Occasional endothelial cells also exhibit a weak, thin podocalyxin signal that colocalizes with CD31 (Merge, magenta). Such endothelial expression of podocalyxin is well documented. In Hs3st1+/− mice, aHSPGs are apparent in the GBM between GECs and endothelial cells (●), and also occurs in the basement membrane of endothelial cells not surrounded by GECs (●). In Hs3st1−/− mice, aHSPGs are still evident in the GBM (●) and in GEC matrix that is not immediately adjacent to endothelial cells (●); however, aHSPGs are nearly abolished in endothelial basement membrane that is not adjacent to GECs (KO, ●). Both genotypes show considerable colocalization of aHSPGs with GEC podocalyxin (Merge, orange-yellow). The bar indicates 2 μm.

to the well characterized pentasaccharide motif that has been structurally determined for heparin and endothelial cell aHS (56–58).

Unusual Features of aHSPG Synthesis by GECs—Despite these fundamental similarities, GEC synthesis of aHS also exhibits very unique features. Disaccharide analyses of HS from hGECs not only exhibited the expected products of 3-OST-1 (GlcA-AnMan3S±6S) but also showed the additional 3-O-sulfated disaccharide, IdoA2S-AnMan3S. This unusual disaccharide was identified by the early work of Edge and Spiro (21) as being a characteristic product of HS from the GBM. These studies did not detect GlcA-AnMan3S±6S; however, their disaccharide analyses were conducted by thin layer chromatography systems that did not detect GlcA-AnMan3S±6S from endothelial cell HS (22). The presence of multiple 3-O-sulfated residues raised the possibility that hGECs express multiple 3-OST isofoms. IdoA2S-AnMan3S was initially identified as the predominant product derived from 3-OST-3-type isofoms, but it can also result from the remaining non-3-OST-1 isofoms (14, 59–61). We found that hGECs predominantly express 3-OST-1 and 3-OST-3a transcripts; so the derived enzymes are likely the major sources of GlcA-AnMan3S±6S and IdoA2S-AnMan3S, respectively. The extremely low levels of 3-OST-2 and 3-OST-3b in hGECs likely provide only a minor contribution to total IdoA2S-AnMan3S levels. Other aHSPG producing cells are only known to express 3-OST-1 (9, 31). Thus, GECs represent the first example of an aHSPG producing cell type that expresses multiple 3-OST isofoms.

This scenario shows additional unusual biosynthetic features. In particular, hGECs appear to exhibit excess levels of 3-OST-1 activity with a low capacity to generate aHS. Excess 3-OST-1 activity is indicated by two factors. First, hGEC iHS yielded exceptionally high amounts of GlcA-AnMan3S±6S, which were comparable with aHS levels. When 3-OST-1 activity is limiting, levels of these 3-O-sulfated residues from iHS are typically >10-fold lower than that from aHS (31, 32, 35, 52). Conversely, a transition in 3-OST-1 levels from being limiting to being in excess was accompanied by a 50-fold rise in GlcA-AnMan3S±6S derived from iHS, as found during differentiation of F9 cells (42). Second, exhaustive modification of hGEC iHS with 3-OST-1 revealed an extremely small pool of residual aHS-precursor, amounting to 1% of total HS, indicating consumption of the pool by excess 3-OST-1. When

5 R. Lawrence, T. Yabe, S. HajMohammadi, J. M. Rhodes, M. McNeely, J. Liu, E. D. Lamperti, P. A. Toselli, P. G. Spear, R. D. Rosenberg, and N. W. Shworak, submitted for publication.
cellular 3-OST-1 activity is limiting, the aHS-precursor pool typically represents 10–30% of total HS chains (35, 42). Conversely, minimal pools have been observed in HS from differentiated F9 cells (42) and in heparin (62, 63), which both are produced under conditions of 3-OST-1 excess. Thus, minimal aHS-precursor pools have previously been found only in conjunction with high aHS production (20–30% of total HS/heparin). In contrast, the total potential aHS production of hGECs is a surprisingly low 2% of total HS (actual aHS production of 1% + aHS-precursor of 1%). Such a low synthesis of aHS-precursor has not been previously documented for any cell type. Thus, hGECs likely exhibit a unique and extreme limitation of an early biosynthetic step required for the formation of the aHS-precursor.

We speculate that limiting the production of the aHS-precursor may well be a key feature of cells that express multiple 3-OST isoforms at high levels, in part, because enzymatic sequence specificity is not absolute. For example, our previous in vitro analyses of 3-OST-3A revealed that this isoform can generate aHS, but at 270-fold lower efficiency than 3-OST-1 (64). However, such analyses are conducted under enzyme limiting conditions. Specificity can also be evaluated under conditions of enzyme excess, such as are used to measure the aHS-precursor pool size. From our exhaustive modification of L-33 HS (TABLE FOUR), 3-OST-3A was only ~10-fold less efficient than 3-OST-1 at generating aHS. Specificity is additionally influenced by the precise structure of the HS substrate. Indeed for exhaustive modification of hGEC iHS, 3-OST-3A was only 2-fold less efficient than 3-OST-1. Thus, 3-OST isoforms that exhibit an extremely low specificity for aHS production under limiting enzyme levels can produce substantial aHS levels when high enzyme levels occur and when a preferential substrate is present. So, when multiple 3-OST isoforms are co-expressed at high levels, it may not be possible to control aHS synthesis simply by regulating 3-OST-1 levels. In this context, limiting the production of aHS-precursor may be the preferred means to prevent overproduction of aHS.

It is likely that in vivo conditions are suitable for GECs to produce aHS from 3-OST-3A, or a comparable isoform. First, the above analysis shows hGECs produce a preferential substrate for aHS production by 3-OST-3A. Second, 3-OST-3A levels in hGECs are likely high, as hGEC total HS has a high content of IdoA2S-AnMan3S. This component accounted for 1% of sulfated residues; whereas, less than 0.1% of residues are 3-O-sulfated when 3-OST activity is limiting (31, 32, 52). The in vivo situation is even more extreme, as GBM HS directly isolated from kidney yields massive levels of IdoA2S-AnMan3S (accounting for 14% of total sulfated residues) (21). Thus, GECs in vivo must express extremely high levels of 3-OST-3A and/or 3-OST-3-like isoforms.

To evaluate the potential for in vivo production of aHS in the absence of 3-OST-1, we examined the localization of aHSPGs in kidneys of 3-OST-1-deficient mice (Hs3st1+/−). We have previously shown that aHS levels in kidney are reduced by >95% in Hs3st1+/−, as compared with Hs3st1+/+ mice (45). Consistent with this reduction, Hs3st1+/− kidney sections were largely devoid of 125I-AT binding, with a complete loss of peritubular and vascular labeling. In contrast, glomerular aHSPGs remained, as confirmed by GAG lyase digestions. Thus, substantial amounts of aHSPGs are synthesized in the kidney glomerulus in the absence of 3-OST-1. Confoital deconvolution microscopy indicated that GECs are the principal source of aHSPG in the Hs3st1+/− glomerulus. Conversely, the observed low contribution by endothelial cells is expected, as prior studies have indicated that these cells exclusively express the 3-OST-1 isoform (9).

It has previously been proposed that 3-OST-5 may be the source of aHSPGs in Hs3st1+/− mice (45), as this enzyme produces aHS far more efficiently than the remaining non-3-OST-1 isoforms (11). Despite this anticipation, 3-OST-5 is clearly not responsible for glomerular aHSPGs, as real time RT-PCR shows this isoform is completely absent in Hs3st1+/− and Hs3st1+/+ kidneys. This result is somewhat surprising, because the remaining isoforms are, at best, much less efficient at generating aHS. However, such characterizations were conducted under enzyme limiting conditions, which may not always be representative of in vivo conditions. Transcript analyses additionally rule out 3-OST-2 and 3-OST-4, which are also absent in the mouse kidney and are known to be preferentially expressed in neuronal tissues (10). In contrast, 3-OST-3, 3-OST-3B, and 3-OST-6 are expressed in both Hs3st1+/+ and Hs3st1−/− kidneys. Of these, 3-OST-3A might be the principal source of aHSPGs of Hs3st1+/− GECs, given that 3-OST-3A is overwhelmingly the major non-3-OST-1 isoform of hGECs, and given that 3-OST-3A can produce aHS. 3-OST-3B should exhibit a comparable aHS production capacity, as the 3-OST-3 isoforms exhibit virtually identical sulfotransferase domains, which is the region that defines enzymatic sequence specificity (10, 64). Moreover, in vitro analysis indicates that 3-OST-3A and 3-OST-3B generate identical HS modifications (14). Because hGECs show a low expression of 3-OST-3A, this isoform might provide a minor contribution to aHSPG production by Hs3st1+/− GECs. Conversely, 3-OST-6 is the least likely candidate as this isoform does not appear to generate aHS (61) and is not expressed in hGECs. However, all three isoforms remain candidates as their expression and properties within Hs3st1−/− GECs are unknown.

It is unclear whether synthesis of aHSPGs by Hs3st1−/− GECs results from a compensatory up-regulation of a 3-OST isoyme(s) or simply reflects aHSPG synthesis from a pre-existing 3-OST isoform(s). The latter possibility appears more likely, because levels of the non-3-OST-1 isoforms were comparable between Hs3st1+/− and Hs3st1+/+ kidneys. However, the glomerulus represents an extremely small component of the total kidney mass, so this analysis might be insensitive to changes that selectively occur in GECs. Conclusive resolution of this issue will require direct analysis of mGECs. Unfortunately, this analysis was not possible as our immortalized mGEC line failed to maintain expression of non-3-OST-1 isoforms.6 Regardless, our studies not only describe
unusual features of aHSPG synthesis by GECs, but also provide the first description of the in vivo biosynthesis of aHSPGs independent of 3-OST-1. Surprisingly this synthesis also occurs independent of 3-OST-5, which has been considered as the most likely candidate for aHSPG production in Hs3st1+/− mice. Instead our data implicate 3-OST isoforms that have a low specificity for aHS production. Thus, a low biochemical specificity does not preclude such isoforms from generating substantial amounts of aHSPGs in vivo.

Possible Roles of 3-O-Sulfated HS within the Glomerulus—Our data demonstrate that the blood-urea interface in the kidney is coated with aHSPGs. Although the role of aHSPGs in glomerular pathophysiology is unknown, at least three potential functions seem feasible. First, aHSPGs may serve to activate AT and thereby regulate the local balance between potentially harmful effectors, such as fibroblast growth factors. Among these, fibroblast growth factor-2 can induce focal and segmental glomerulonephritis via proinflammatory signaling through protease-activated receptor-1 (65). Thrombin can also stimulate mesangial cell expression, Warren Kett for generating AT-Alexa488/647, and Ulrike Brandt for cryosectioning.

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