Heparan Sulfate Chains With Antimitogenic Properties Arise From Mesangial Cell-Surface Proteoglycans

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Heparan sulfate (HS) chains accumulate in both the medium and the cell layer of mesangial cell cultures. When given in fresh medium to quiescent cultures at naturally occurring concentrations, they suppress entry into the cell cycle and progression to DNA synthesis. We have attempted to identify the proteoglycan (PG) source of the antimitogenic HS chains from mesangial cell layers ($HS_{(c)}$) and medium ($HS_{(m)}$). When cells were labeled for 16 hours with [^{35}S]sulfate, 25% of the label was found in intracellular HS chains and 5% in extracellular HSPGs. Cell-surface HSPGs accounted for the remaining 70% of the label associated with cell-layer HS and were released by either trypsin or 2% Triton X-100. About 20% of this cell-surface fraction was released by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), and probably represents glypican-like PG; glypican mRNA was present in the cells. The remainder of this fraction could be incorporated into liposomes, indicating the presence of hydrophobic transmembrane regions suggestive of syndecans. Upon purification and deglycosylation, an antiserum to rat liver HSPGs that reacts primarily with syndecan-2 showed a strong signal corresponding to this protein and three weaker bands that may represent additional syndecans. mRNAs for syndecan-1, -2, and -4 were present in the cultures. Syndecan-1 and -2 mRNAs were increased 30 minutes after stimulation of quiescent rat mesangial cells (RMCs) with serum. Heparin, $HS_{(c)}$, and $HS_{(m)}$ all prevented this increase. Syndecan-4 mRNA was not affected by serum, heparin, or HS. In pulse-chase experiments, the amount of ^{35}S appearing in the cellular protein-free HS fraction was accounted for almost entirely by cell-surface PGs, as matrix-associated label was a minor contribution at the end of the pulse-labeling. The appearance of [^{35}S]HS in cell extracts was unaffected by phospholipase C treatment, indicating that turnover of the newly labeled syndecan fraction is the source of the antimitogenic HS chains.

HEPARAN SULFATES (HSs) subserve diverse functions in cell growth and regulation by virtue of their ability to bind with various growth factors, proteases and protease inhibitors, enzymes, adhesion molecules, and matrix components.[1-4] These extensive binding interactions are facilitated by the high negative charge of the heparan chain and the additional electrostatic contacts contributed by numerous sites of sulfation. Current information indicates that the addition and location of sulfate are regulated during development and in concert with cellular phenotypic variation.[5] David,[4] noting that the functional polymorphism of the HS chain depends on the cell type and stage of differentiation,[6] has suggested that the cell controls glycosaminoglycan (GAG) chain synthesis independently of information contained in the core protein.

HS proteoglycans (PGs) are localized both in the extracellular matrix and on the cell surface.[2] The basement membrane component, perlecan, typifies the former class.[7] The best-characterized of the cell-surface PGs include (1) those that are anchored in the plasma membrane by glycosyl phosphatidylinositol (GPI) linkages and (2) those with membrane-spanning regions and short cytoplasmic tails. These have been termed, respectively, GRIPS (glypican-related integral membrane proteoglycans) and SLIPS (syndecan-like intercalated proteoglycans) after their respective prototypes, glypican[8] and syndecan-1.[9] Known glypicans include (1) glypican, synthesized by many different cell types, (2) cerebroglycan, apparently confined to the central nervous system,[10] (3) OCI-5, or glypican-3, expressed in the colon during development,[11,12] and (4) the recently characterized K-glypican, expressed at high levels in the mouse kidney and developing brain.[13] Four syndecans, each a distinct gene product, are also known in mammalian cells[6]: syndecan-1 (syndecan),[9] syndecan-2 (fibroglycan),[14] syndecan-3 (N-syndecan),[15,16] and syndecan-4 (ryudocan/amphiglycan).[17,18] They are distributed widely but have a tissue-specific pattern of expression.[6,19] Most cell types generate more than one member of the syndecan family. Syndecan-1 is expressed predominantly in epithelial cells and syndecan-2 in tissues rich in endothelial cells. Syndecan-4 is found in most tissues while syndecan-3 is found primarily in neural tissues in early development.[6,19,20]

Mesangial cells are smooth muscle–like cells found normally in a quiescent state.[21] However, increased mesangial cell proliferation is a common feature of progressive renal disease triggered by a variety of immune, physical, toxic, or unknown stimuli.[22,23] We reported that HS chains isolated from rat mesangial cell (RMC) cultures, as well as exogenous heparin at concentrations as low as 100 ng/mL, inhibit the mitogenic response of RMCs to serum.[24,25] Analogous to the known effects of heparin on vascular smooth muscle cells,[26,27] heparin and RMC-derived HS inhibit serum-dependent and phorbol ester–stimulated induction of c-*fos* mRNA[25] and activation of mitogen-activated protein kinase.[28] These antimitogenic properties of the protein-free HS chains are not shared by intact RMC PGs.[24] Because HS is synthesized on intact core proteins, we concluded that the antimitogenic species arise as metabolic products of mesangial cell PGs.[24,25] The present study was undertaken to determine the source of the antimitogenic HS chains in RMC cultures.

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MATERIALS AND METHODS

Cell Culture

RMCs were cultured from the glomeruli of 100-g male Wistar rats using the procedure of Simonson and Dunn,[29] and were characterized by morphology and positive staining for desmin and smooth muscle actin[30] as described previously.[24] They were used between passages five and 15, when they contract in response to angiotensin II and endothelin and exhibit growth suppression in the presence of heparin (1 μg/mL). Cultures were maintained in RPMI 1640 containing 10% fetal bovine serum with penicillin and streptomycin and passaged by trypsinization.[24] All culture media and reagents were obtained from GIBCO-BRL (Burlington, Ontario, Canada). In some experiments, cells were rendered quiescent by transferring subconfluent cultures to 0.4% serum for 48 hours, followed by stimulation to proliferate by treatment with 5% NuSerum (Collaborative Medical Products, Bedford, MA) as described previously.[25]

Labeling and Extraction of PGs

Confluent RMC cultures were washed twice with Ham's F-12 medium, which has an inorganic sulfate concentration of 6 μmol/L. PGs/GAGs were labeled by addition of 0.1 mCi $H_2{}^{35}SO_4$ (carrier-free; ICN, Mississauga, Ontario, Canada) to 3 mL of the same medium. At the end of the desired labeling period, the medium was removed and lyophilized. Cell layers were washed twice with Hanks balanced salt solution and extracted with 4-mol/L guanidine · HCl/50-mmol/L sodium acetate/1% (vol/vol) Triton X-100, pH 5.8, containing protease inhibitors, as previously described.[24] Greater than 98% of ^{35}S-labeled macromolecules were extracted, as determined after solubilizing the remaining pelleted extract and culture plate washings in 0.1 mol/L NaOH. Chondroitin/dermatan sulfates were removed from the samples by treatment with chondroitinase ABC (Sigma, St Louis, MO). Partially purified desalted samples following DEAE-Sephacel chromatography (described later) were dissolved in 50 μL 50-mmol/L Tris/50-mmol/L sodium acetate, pH 7.5, containing the same protease inhibitors as the extraction solution for digestion with 0.04 U enzyme overnight at 37°C.

In some experiments, cell layer PGs were selectively extracted with trypsin (Sigma) or phosphatidylinositol-specific phospholipase C ([PI-PLC] Boehringer-Mannheim Canada, Laval, Quebec). Trypsin (0.5 mL of a 100-μg/mL solution) was added to serum-free medium, and the cultures were incubated for 3 minutes at 37°C. After each enzyme digestion, the incubation medium was removed for analysis and the cell layers were extracted overnight at 4°C with 1 mL 4-mol/L guanidine · HCl buffer containing protease inhibitors and 1% (vol/vol) Triton X-100. PI-PLC treatment was performed by adding 0.5 mL of the enzyme (0.2 U/mL) to fresh medium and incubating for 30 minutes at 37°C. PGs were also released by incubation with heparin (1 mg/mL; Sigma) or 2% Triton X-100 for 15 minutes on ice.

Chromatography

Cell-layer extracts were eluted from Sephadex G-50 (Pharmacia, Montreal, Quebec, Canada) columns (30 × 0.9 cm) with 7-mol/L urea/50-mmol/L sodium acetate/0.15-mol/L NaCl, pH 5.9 (DEAE buffer), and the void-volume fractions were pooled. Lyophilized media were dissolved in DEAE buffer and eluted with the same buffer from Sephadex G-50 poured into disposable plastic 10-mL pipettes. The columns were discarded after collection of the void-volume fractions. PGs from Sephadex G-50 chromatography were loaded onto DEAE-Sephacel (Pharmacia) columns (1-mL bed volume in 3-mL plastic syringes) equilibrated with DEAE buffer. The columns were washed with 10 column vol starting buffer, and a 40-mL linear gradient from 0.15 to 1.15 mol/L NaCl in DEAE buffer was used to elute 0.5-mL fractions. Nonspecific binding was minimized by pretreating the DEAE-Sephacel columns with 3 mL DEAE buffer containing 1 mg bovine serum albumin (BSA), 1 mg chondroitin sulfate A, and 1 mg heparin (all from Sigma). Sample recovery was 85% to 90% of the applied radioactivity.

Size fractionation was performed on a Pharmacia Superose 6 fast protein liquid chromatography (FPLC) column. Samples reconstituted in 250 μL 7-mol/L urea buffer were injected into the column and eluted in DEAE buffer containing 0.2% (vol/vol) Triton X-100. The elution buffer was delivered by Pharmacia Liquid Chromatography Controller model LCC-500 at 0.1 mL/min, and 0.5-mL fractions were collected. Sephacryl S-300 analytical-scale chromatography was performed on a 40 × 0.7-cm column in the same buffer. Recovery from both columns was greater than 90% of the applied radioactivity.

Purification of Syndecan Core Proteins

Cell-surface HSPGs were purified from confluent RMC cultures using the procedure of Lories et al.[31] Confluent RMC monolayers (156 petri dishes, 75 cm^2) were rinsed twice with 3 mL cold phosphate-buffered saline and then extracted on ice with 3 mL/plate Triton X-100 buffer (10 mmol/L Na_2HPO_4 and 2 mmol/L KH_2PO_4, pH 7.5, containing 2% vol/vol Triton X-100, 150 mmol/L NaCl, 50 mmol/L 6-aminohexanoic acid, 10 mmol/L EDTA, 5 mmol/L *N*-ethylmaleimide, 5 mmol/L benzamidine, 1 mmol/L PMSF, and 1 μg/mL pepstatin A). ^{35}S-labeled PGs from four petri dishes of confluent RMCs were used as a tracer for purification. The suspension was cleared by centrifugation (10,000 × *g* for 60 minutes at 4°C). PGs in this crude extract (about 500 mL) were bound to 5 mL DEAE-Sephacel and eluted with 35 mL urea buffer containing 0.8 mol/L NaCl. This eluate was concentrated further to 10 mL and the NaCl concentration was decreased to 0.2 mol/L by ultrafiltration against a PM30 membrane (Amicon, Beverly, MA). The sample was applied to a Mono-Q anion-exchange FPLC column (Pharmacia) in urea buffer with 0.2 mol/L NaCl, and the bulk of the bound proteins were eluted with a 0 to 0.6-mol/L linear NaCl gradient in urea buffer (total 40 mL) containing 0.5% (vol/vol) Triton X-100. The retained PGs were then eluted with guanidine · HCl buffer (4 mol/L guanidine · HCl, 100 mmol/L 6-aminohexanoic acid, 10 mmol/L EDTA, 10 mmol/L *N*-ethylmaleimide, 5 mmol/L benzamidine · Hcl, 50 μg/mL BSA, 10 μg/mL heparin, 10 μg/mL chondroitin sulfate A, and 50 mmol/L Na acetate, pH 5.8) containing 0.5% (vol/vol) Triton X-100. Hydrophobic cell-associated HSPGs were further purified by gel filtration, ion exchange on Mono-Q, and incorporation of the HSPGs into liposomes.

One milliliter of phosphatidylcholine (10 mg/mL) in chloroform was dried using a rotary evaporator. Pooled fractions (2 mL) from Mono-Q were added and mixed by vortexing. Liposomes were formed by dialyzing the PG-lipid mixture at a 12 to 14-kd cutoff against 100 mL detergent-free 4 mol/L guanidine · HCl buffer with four changes during 4 days at 4°C. About 2.4 mL dialyzate was centrifuged at 16,000 × *g* (10 minutes at 4°C), and the PG-lipid mixture was loaded onto a Sepharose CL-4B column (1.5 × 100 cm) preequilibrated in 4 mol/L guanidine · HCl buffer. ^{35}S-containing fractions with a K_d less than 0.1 were pooled, and Triton X-100 was added to 0.5% by volume to dissolve the liposomes and free the labeled PGs.

Characterization of Core Proteins

PGs recovered from the liposome preparation (170 μL from a total 350-μL ultrafiltrate) were diluted to 3 mL with urea buffer and loaded onto 0.2 mL DEAE-Sephacel in Tris/glucoside buffer (10 mmol/L octylglucoside and 100 mmol/L Tris · HCl, pH 7.5). The DEAE-beads were suspended in iodination solution (20 μL $Na^{125}I$, 300 μL Tris/glucoside buffer and an Iodo-Bead; Pierce, Rockford, IL; preincubated at room temperature for 2 minutes) for 15 minutes. ^{125}I-PGs were then

isolated by ion-exchange chromatography on Mono-Q and gel filtration on Sepharose CL-4B.

Some samples of ^{125}I-PGs were treated with chondroitinase ABC as before. Others were digested with heparin lyase III (Sigma) at 5 U/mL in 20 mmol/L Tris · HCl, pH 7.5, containing 4 mmol/L $CaCl_2$, 50 mmol/L NaCl, and 0.1 mg/mL BSA in the presence of protease inhibitors (50 mmol/L 6-aminohexanoic acid, 1 mmol/L PMSF, 2.5 μg/mL pepstatin A, and 20 μg/mL leupeptin). Digestion was performed at 25°C overnight. Proteinase K (Boehringer-Mannheim) digestion was performed following digestion with heparin lyase III. The sample still contained in heparin lyase buffer was made to 0.5% (wt/vol) in SDS and boiled for 5 minutes.[31] Digestion of the denatured samples was performed at 60°C for 40 minutes at a concentration of 70 μg proteinase K/mL. Deglycosylation with trifluoromethane sulfonic acid ([TFMS] Sigma) was performed according to the protocol of Sojar and Bahl.[32] Briefly, desalted ^{125}I-PG was mixed with 40 μg aprotinin and then lyophilized overnight. TFMS (20 μL) was added to the dried samples on ice under N_2, incubated for 2 hours, cooled in dry-ice/acetone, neutralized by gradual addition of 150 μL precooled (−20°C) 60% (vol/vol) pyridine in water, and kept on ice for a further 30 minutes.

Electrophoresis of ^{125}I-labeled samples was performed on gradient polyacrylamide gels (4% to 12%) using the buffer system of Laemmli.[33] Gels were fixed and dried for autoradiography.

Western Blotting

The iodinated, fully deglycosylated core proteins could not be detected with rat syndecan antiserum, so an alternative purification scheme was used based on the study by Lyon and Gallagher.[34] Briefly, after extraction of cell homogenates and chromatography on DEAE-Sephacel, PGs were digested with chondroitinase ABC and desalted on Sephadex G-50. Following extensive dialysis against 4-mol/L guanidinium chloride/0.2% (wt/vol) CHAPS/20-mmol/L sodium phosphate, pH 7.3, samples were precipitated with 9 vol 95% ethanol at −20°C with carrier heparin and digested with heparitinase (Seikagaku America, Falmouth, MA). After electrophoresis on polyacrylamide (5% stacking gel and 8% running gel) in Laemmli buffer, pH 7.2, the proteins were transferred (40 V for 90 minutes) to 0.45 μm nitrocellulose. Western blotting was performed with a 1:250 dilution of rat liver syndecan antiserum, generously provided by Dr Malcolm Lyon,[35] after blocking in 5% BSA. The secondary antibody was goat anti–rabbit immunoglobulin G (1:500), and detection was made with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, England).

Preparation of Syndecan cDNA Fragments

DNA fragments of syndecans-1, -2, and -4 were prepared by polymerase chain reaction (PCR) to use as probes for Northern blotting. Total RNA from confluent RMC cultures was used to amplify syndecan core-protein sequences by reverse transcriptase–PCR. Total RNA was treated with deoxyribonuclease I (amplification-grade; GIBCO-BRL), and RMC cDNAs were synthesized from total RNA with reverse transcriptase using a GeneAmp RNA PCR kit (Perkin-Elmer, Branchburg, NJ). Oligonucleotide primer sequences were based on published sequences of cDNA for syndecan-1,[36] syndecan-2,[35] and syndecan-4[17] and were as follows: syndecan-1, sense 5′ (+259) GCCACCCTCACCTCTATCCTGC (+280) and antisense 5′ (+563) GTGCCTCCATCCTCCACACTTG (+542); syndecan-2, sense 5′ (+326)TGCTCCCGAAGTGGAAACCA (+345) and antisense 5′ (+461) TCCGTCCGCTTGAACAGATTG (+441); and syndecan-4, sense 5′ (+5) CGCCTGTCTGCCTGTTTGC (+23) and antisense 5′ (+161) CCAGCGTCTTCATCGTCCG (+143).

Amplification resulted in a single ethidium bromide–staining band for each set of syndecan primers (data not shown). Syndecan-1 primers produced a band of about 300 base pairs. A sequence of 305 base pairs is expected from the known sequence and choice of primers.[36] Likewise, the syndecan-2 and -4 primers produced bands of the expected sizes: syndecan-2, 180 base pairs (expected, 186 base pairs[35]); and syndecan-4, 160 base pairs (expected, 157 base pairs[17]). The identity of the products was confirmed by sequencing.

Northern Blotting

Total RNA was isolated from RMCs grown on 75-cm^2 culture plates by lysis in guanidinium thiocyanate and extraction with phenol/chloroform using a Promega RNAgents total RNA isolation kit (Fisher Scientific, Fairlawn, NJ). Equal amounts of RNA were denatured by the method of Gong[37] and fractionated on a 1.2% agarose gel containing 2.2 mol/L formaldehyde, 1 mmol/L EDTA, 5 mmol/L sodium acetate, and 20 mmol/L 3-(*N*-morpholino)propanesulfonic acid, pH 7.0. Fractionated RNA was visualized by UV transillumination, transferred by overnight capillary blotting to Hybond-N nylon membranes (Amersham, Oakville, Canada) in 20× SSC according to the method of Southern,[38] and cross-linked to the membrane by UV irradiation (600 μW/cm^2 at 250 nm for 3 minutes). To remove formaldehyde and small molecules produced from UV cross-linking, the membrane was washed with 2× SSC, air-dried, and baked at 80°C under vacuum for 2 hours. cDNA probes were labeled with [α-^{32}P]dCTP by the random-primer method using a random-primer DNA labeling kit (Boehringer-Mannheim). Membranes were prehybridized for 15 minutes at 65°C in Quik Hyb hybridization solution (Stratagene, La Jolla, CA). Hybridization was then performed at 65°C for 1 hour in Quik Hyb solution containing 500 μg/mL heat-denatured salmon sperm DNA and ^{32}P-labeled cDNA probes. mRNA levels were quantified by densitometry of Northern blot signals using a gel-documentation system (Diamed Lab Supplies, Mississauga, Ontario, Canada). A fragment of rat glypican cDNA, designated 4EX,[39] was obtained from Dr Arthur Lander, Massachusetts Institute of Technology (Boston, MA). Rat OCI-5/glypican 3 cDNA was a gift from Dr Jorge Filmus, Sunnybrook Health Sciences Center (Toronto, Ontario, Canada).[40]

RESULTS

Localization of Cell-Layer HSPG

We showed previously that a proportion of mesangial cell–associated HS binds to C_{18} hydrophobic columns, elutes in the void volume of a Superose 6 gel filtration column, and represents HSPG. The remainder fails to bind to C_{18}, fractionates on Superose 6, and contains no detectable protein, ie, it represents HS chains free of significant core protein.[24] In prior studies,[24,25] we designated the free HS chains from the cell layer and medium as HS1 and HS2, respectively. Here, we refer to them as $HS_{(c)}$ and $HS_{(m)}$ to avoid confusion with recently named HS genes. To localize cell layer–associated HS chains, which we designate $HS_{(c)}$, confluent RMCs were labeled for 16 hours with $^{35}SO_4$ and then extracted with either heparin, trypsin, or 4 mol/L guanidine · HCl. After partial purification on DEAE-Sephacel and degradation with chondroitinase ABC, the resulting labeled HS species (consisting of HSPG and HS chains) were subjected to gel filtration (Fig 1). Only about 2% of the labeled HS species were heparin-displaceable. Most of the label was recovered in the void volume after trypsin treatment. In contrast, $HS_{(c)}$—recovered at a K_d value centered on 0.78—was only released by 4 mol/L guanidine · HCl extraction and accounted for 25% of the total label. Thus, during 16 hours of labeling, most newly synthesized cell-associated HSPGs are exposed on the cell surface or in an extracellular site, where they are accessible to trypsin. A significant proportion is

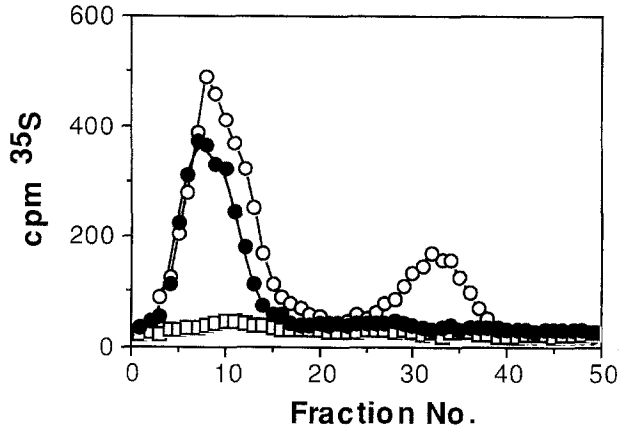

Fig 1. Selective release of cell-associated HS species from RMC cultures. Confluent RMC cultures were labeled with $^{35}SO_4^{2-}$ for 16 hours and then extracted with heparin 1 mg/mL (□), trypsin 100 μg/mL (●), or guanidine · HCl extraction solution (○). HSPGs recovered from DEAE-Sephacel after digestion with chondroitinase ABC were subjected to Superose 6 FPLC in urea buffer, and the ^{35}S elution profiles (0.5 mL/fraction) are plotted. Total cpm applied were proportional to the original amounts of the extracts. Void volume, fraction 8; total volume, fraction 39.

metabolized to HS chains that accumulate in a trypsin-inaccessible compartment, presumably within the cell.

Extraction of labeled cells with 2% Triton X-100 released 70% of the ^{35}S-HS/HSPG, and of this, 90% could be incorporated into liposomes, indicating the presence of membrane-associated regions. When PI-PLC treatment preceded trypsinization, 15% of cell layer–associated ^{35}S was released by the phospholipase and a further 60% was released by trypsin (not shown). Together, these results indicate that membrane-intercalated (syndecan-like) HSPGs account for most of the newly labeled HSPGs, with GPI-linked HSPGs representing a significant but minor fraction. In summary, these data indicate that after 16 hours, ^{35}S is distributed approximately as follows: 25% in intracellular HS chains, 15% in GPI-linked membrane HSPGs, 55% in other cell-surface HSPGs, and 5% in other extracellular HSPGs. The latter 5% is consistent with the amount of matrix [^{35}S]-HSPG species remaining on the plate when cells are removed by gentle agitation in Ca^{2+}-free medium with EGTA (not shown). These values are reproducible to within 10% when the same purification steps are repeated with cells at the same degree of confluence, ie, freshly confluent cultures. The relative amount of $HS_{(m)}$ and $HS_{(c)}$ varies with the age of the culture as $HS_{(m)}$ accumulates in conditioned medium.

$HS_{(c)}$ and $HS_{(m)}$ Do Not Arise From GPI-Linked HSPGs

Confluent RMC cultures were pulse-labeled with $^{35}SO_4$ for 1 hour, and the labeled PGs were extracted, digested with chondroitinase ABC, and chromatographed on Sephacryl S-300. At the beginning of the chase period (t = 0 hours), greater than 95% of the label eluted in the void volume (Fig 2), indicating that it was associated with intact HSPGs. With time, this peak diminished and a peak appeared in the lower-mass region, representing $HS_{(c)}$. This $HS_{(c)}$ peak was also diminished by 6 hours, but when the lysosomotropic reagent, chloroquine, was included in the chase medium, $HS_{(c)}$ accumulated (Fig 2). These results indicate a precursor-product relationship between HSPGs and $HS_{(c)}$, and further support an intracellular (lysosomal or prelysosomal) location for $HS_{(c)}$.

Because cell-surface HSPGs account for most of the incorporated label at the end of the pulse-labeling period, we treated the cells with PI-PLC at this time to determine whether syndecans and (or) GPI-linked PGs yield $HS_{(c)}$. Treatment of the pulse-labeled cells with PI-PLC caused a diminution of the void-volume fraction but did not affect the rate of appearance or quantity of ^{35}S in $HS_{(c)}$ during the chase period (Fig 3). We conclude that $HS_{(c)}$ is not derived from GPI-linked PGs on the cell surface.

Similar pulse-chase studies were undertaken with $HS_{(m)}$. In contrast to the short pulse used to label HSPG and then observe the relatively rapid appearance of free labeled HS arising from the higher–molecular weight material in the chase period (Figs 2 and 3), cells were now labeled for 16 hours to steady state.[24] This also allows labeling of free HS chains and observance of the release of different pools into the medium. Regardless of whether cells were treated with PI-PLC at the beginning of the chase period, the rate of appearance of [^{35}S]-HS(PG) in the medium was the same (Fig 4). As in our previous studies,[24] this ^{35}S was nearly equally divided between HSPG and $HS_{(m)}$ (not shown). Treatment of the cell layers with PI-PLC after increasing chase times showed the expected decrease in the amount of ^{35}S released (Fig 4), which nevertheless did not account for the

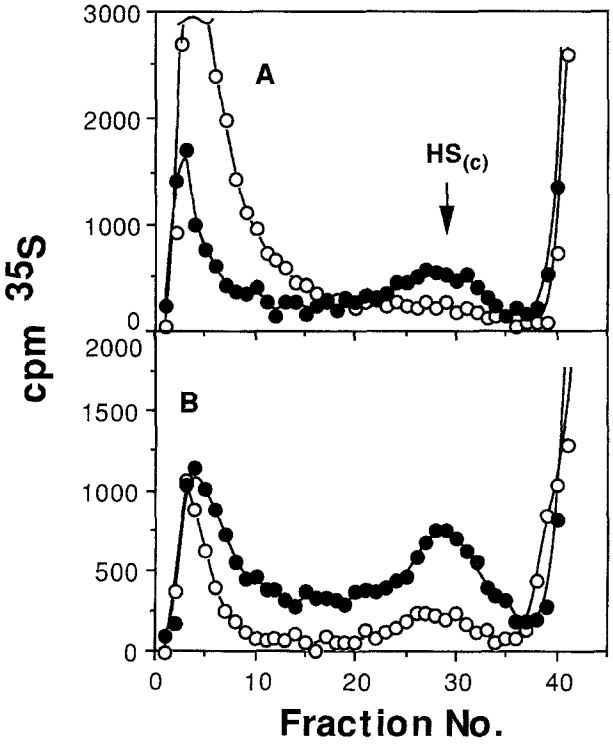

Fig 2. Turnover of RMC HS species. Cells were pulse-labeled with $^{35}SO_4^{2-}$ for 1 hour and then returned to label-free medium. Total PG was extracted from the cell layer with 4 mol/L guanidine · HCl after 0, 3, or 6 hours and chromatographed on Sephacryl S-300 after digestion with chondroitinase ABC. Arrow indicates the position of $HS_{(c)}$. (A) ○, t = 0; ●, t = 3 hours. (B) t = 6 hours without (○) or with (●) chloroquine 100 μg/mL.

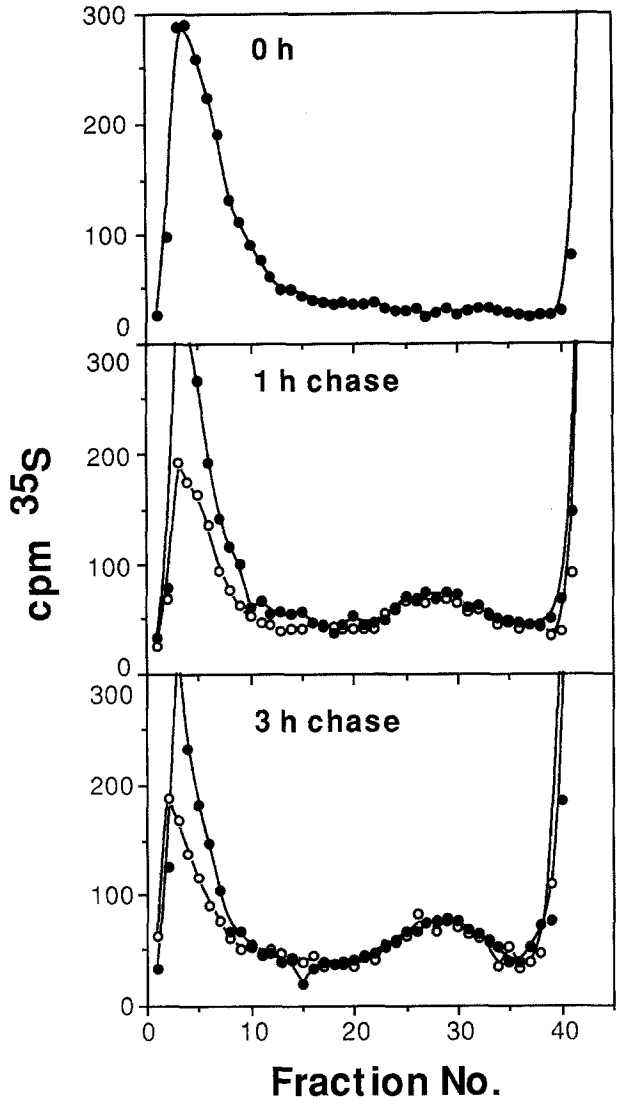

Fig 3. Degradation of cell-surface HSPGs. Confluent RMC cultures were pulse-labeled with $^{35}SO_4^{2-}$ for 30 minutes and ^{35}S-HSPGs were chromatographed on Sephacryl S-300 as in Fig 2 at $t = 0$ or after chase periods of 1 and 3 hours in the presence (○) or absence (●) of PI-PLC 0.2 U/mL.

^{35}S appearing in the medium. For example, between 0 and 8 hours after labeling, about 10^4 cpm ^{35}S have been lost that cannot be accounted for in the medium (Fig 4). Therefore, GPI-linked HSPG does not produce either $HS_{(c)}$ or $HS_{(m)}$.

Northern Blotting of Syndecan and Glypican mRNA

Because these results indicated the presence of both PI-PLC–sensitive and –insensitive cell-surface HSPG, we tested for the presence of mRNAs for the major syndecans and glypicans. Syndecan-1 cDNA hybridized with two bands of 3.2 and 2.6 kb on a Northern blot of RMC RNA samples (Fig 5), consistent with the two sizes of syndecan-1 mRNA detected previously,[9,36] in addition to a variable and unexpected band of about 4 kb. The syndecan-2 cDNA fragment detected three bands at 3.4, 2.4, and 1.2 kb, again consistent with the typical pattern of expression of this syndecan.[36,41] Hybridization of syndecan-4 cDNA to the same blot revealed a single band of 2.6 kb, consistent with the size of syndecan-4 mRNA isolated from rat endothelial cells.[42] Therefore, RMCs in culture synthesize mRNA for each of the three syndecans investigated. The transcripts of syndecan-1 and syndecan-2 are increased by a 30-minute treatment of quiescent cells with serum. This increase is prevented by heparin, $HS_{(c)}$, or $HS_{(m)}$, all at 1 μg/mL (Fig 5), consistent with the ability of these molecules to prevent other responses to serum such as cell-cycle entry and c-*fos* induction.[25] Syndecan-4 mRNA is insensitive to the presence of serum, heparin, or HS.

To determine the nature of the GPI-linked HSPGs in RMCs, we used a cDNA of glypican isolated from the rat central nervous system to determine by Northern blotting whether glypican mRNA is expressed in RMCs. The probe detected a single message of 3.8 kb (Fig 6), in agreement with the size of glypican mRNA from the adult rat.[39] The transcript was most abundant in subconfluent cells and diminished as the cells reached confluence. When the same filter was probed with cDNA for the developmentally regulated GPI-linked HSPG, OCI-5/glypican 3, no message was detected (not shown), suggesting that the GPI-linked HSPG in RMCs is glypican.

Purification of Putative RMC Syndecan Core Proteins

Triton X-100 extracts of RMCs were eluted from DEAE-Sephacel and concentrated on a Mono-Q ion-exchange column prior to Sepharose CL-4B chromatography in the presence of detergent (Fig 7A). The fractions containing HSPGs were pooled and rechromatographed on Mono-Q, and the indicated fractions (Fig 7B) were pooled, incorporated into liposomes, and chromatographed on Sepharose CL-4B in the absence of detergent (Fig 7C). Liposome-associated material eluted in the void volume of the column. To remove the lipid and the carrier material (heparin, CS, and BSA) from the purified hydrophobic HSPGs, liposome-associated HSPGs were solubilized with

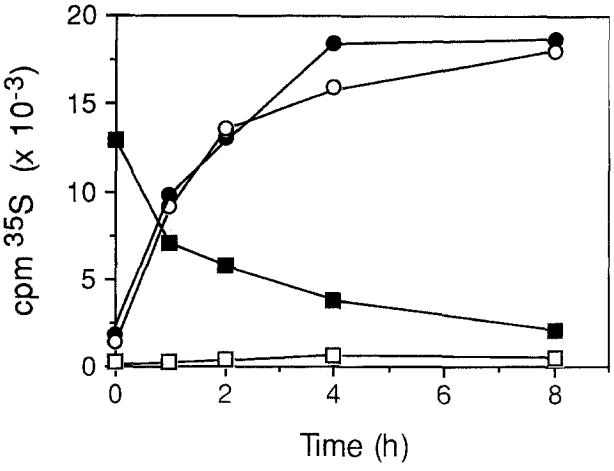

Fig 4. Release of pulse-labeled HS(PG) into the medium. The origin of HS(PG) shed into the medium was determined by a pulse-chase protocol. RMCs were labeled for 16 hours with [^{35}S]sulfate and then transferred at time 0 to label-free medium after treatment with PI-PLC (○, □) to remove GPI-linked PGs or no treatment (●, ■). After each indicated chase period, ^{35}S-labeled HS(PG) released into the medium spontaneously (●, ○) or by treatment with PI-PLC (■, □) was determined by liquid scintillation counting.

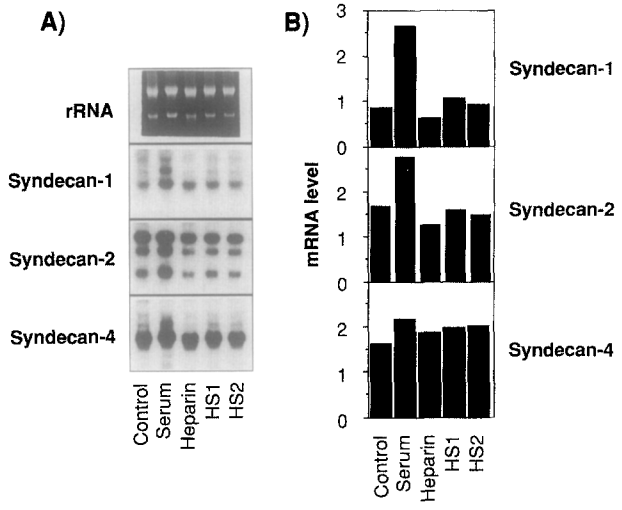

Fig 5. Northern blots of total RMC RNA probed with cDNAs to syndecans. (A) Autoradiograms after probing with cDNAs for syndecan-1, -2, and -4. Top panel shows the ethidium bromide–stained agarose gel, with the 28S rRNA signal used to correct for loading. RNA was isolated from quiescent cells (control, lane 1) or cells stimulated with 5% NuSerum for 30 minutes (lanes 2-5). In lanes 3-5, heparin, $HS_{(c)}$ (HS1), or $HS_{(m)}$ (HS2) were added to NuSerum at a concentration of 1 μg/mL. $HS_{(c)}$ and $HS_{(m)}$ are free HS chains isolated from the cell layer and conditioned medium, respectively, of RMC cultures, as described previously.[24] (B) Quantitation of band intensities by densitometry. mRNA levels are corrected for the 28S signal from the ethidium bromide gel. For syndecan-1 and -2, the intensities of the 2 and 3 prominent bands, respectively, were added. These observations were repeated at least 4 times with serum and heparin, with consistent results. Because of limited amounts of material, the results with HS chains are from a single experiment.

0.5% (vol/vol) Triton X-100 and rechromatographed on Sepharose CL-4B in carrier-free 4 mol/L guanidine · HCl buffer containing 0.5% Triton X-100 (Fig 7C). This purified preparation contained about 4.6% of the total ^{35}S activity originally extracted by detergent from the RMCs and was resistant to chondroitinase ABC but susceptible to heparan lyase III treatment, indicating that it contained HSPGs (not shown). Based on our previous inventory of RMC PGs,[24] 9% of the total cell-layer ^{35}S-labeled PGs/GAGs were HSPGs. Seventy percent of the HSPGs can be extracted with Triton X-100, so about 73% (ie, 4.6/[0.7 × 9]) of the total cell-layer HSPG was recovered.

These HSPGs isolated from liposomes were labeled with $Na^{125}I$ for further characterization. On electrophoresis under reducing conditions, undigested ^{125}I-HSPG migrated as a smear in the high–molecular mass region (>200 kd; Fig 8). Chondroitinase ABC treatment did not affect the SDS-PAGE profile of labeled material, while heparin lyase III treatment removed label from the high-M_r region, indicating that the high–molecular mass molecules are HSPGs. Inclusion of proteinase inhibitors during heparin lyase III treatment produced the same SDS-PAGE profile of labeled samples as treatment with heparin lyase III alone, whereas proteinase K treatment fully degraded the labeled material, supporting the conclusion that the ^{125}I label was exclusively on the protein cores. Chemical deglycosylation with TFMS yielded three major protein bands with apparent M_r values of 61, 46, and 35 kd. Two bands at about 40 and 50 kd in untreated samples (Fig 8) were not observed on nonreducing SDS-PAGE (not shown), suggesting that they are disulfide-bonded to high–molecular mass components under these conditions. Their identity has not been pursued further.

Western Blotting of Syndecans

The iodinated, TFMS-deglycosylated proteins were not reactive with syndecan antiserum, so we alternatively prepared mesangial cell HSPGs by DEAE-Sephacel chromatography followed by digestion with chondroitinase and heparitinase. An antiserum to rat syndecan-2 detected a prominent band at 56 kd with three fainter bands in the range of 70 to 80 kd (Fig 9). This corresponds to published electrophoretic patterns of rat liver syndecans with this same immune serum, where the lower band is also reactive with a monoclonal antibody to syndecan-2 and the higher bands are detected with antiserum to syndecan ectodomains.[35] The higher apparent molecular weight of these species compared with TFMS-treated samples probably reflects incomplete deglycosylation.

DISCUSSION

In previous studies, we identified three HSPG fractions associated with the mesangial cell layer, which we designated HSPG1, HSPG2, and HSPG3.[24] HSPG3 is a minor component of unknown identity. Because HSPG1 is a large PG resistant to Triton X-100 extraction[24] and remains behind when cells are detached by treatment with EGTA (not shown), it is likely located in the extracellular matrix. (In fact, mRNA for the extracellular matrix HSPG, perlecan, is readily detected in these cells by Northern blotting.[43]) The major fraction, HSPG2, consists of cell-surface HSPGs with both GPI-linked and membrane-intercalated members. However, although both syn-

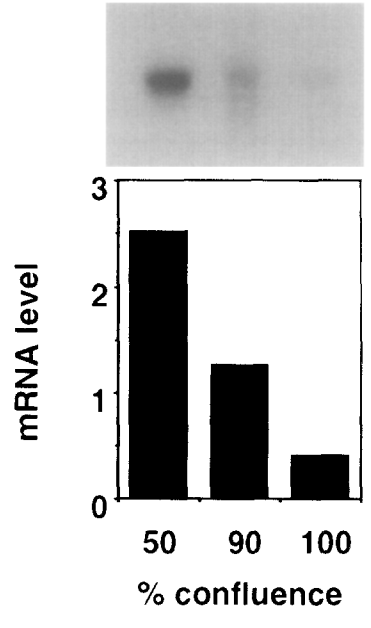

Fig 6. Northern blot of total RMC RNA probed with cDNA to glypican. Cells were growing in serum-replete culture medium at the stage of confluence indicated at the time of harvest. The degree of confluence was estimated visually. Bars show the intensity of the glypican signal relative to that of 18S rRNA as determined by densitometry, in arbitrary units.

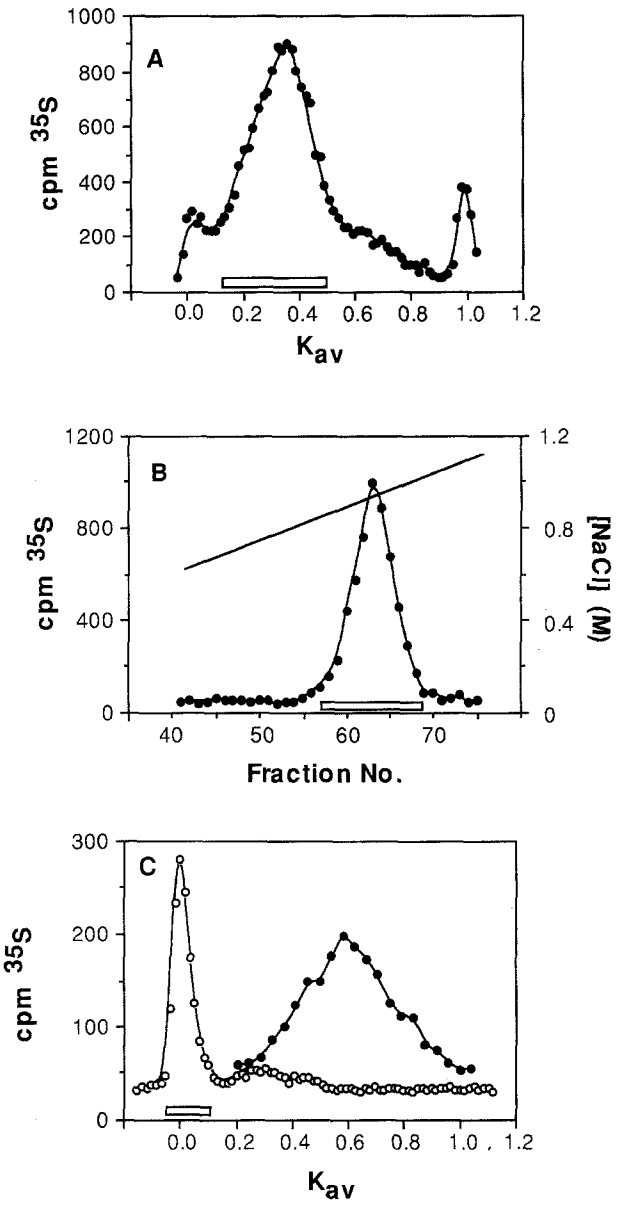

Fig 7. Purification of RMC membrane-intercalated HSPGs. Metabolically ^{35}S-labeled RMC cultures were extracted with Triton X-100 in the presence of protease inhibitors and concentrated. (A) Sepharose CL-4B in 4 mol/L guanidine · HCl/Triton X-100 buffer. Fractions from ^{35}S elution profiles were collected and pooled as indicated by the open bar. (B) Mono-Q ion-exchange chromatography of pooled fractions from Sepharose CL-4B. Radioactivity was eluted with a NaCl gradient as indicated by the solid line. Fractions were pooled as indicated by the open bar for incorporation into liposomes. (C) Sepharose CL-4B chromatography in 4 mol/L guanidine · HCl buffer of material incorporated into liposomes (○) and pooled as indicated by the open bar. Pooled material was rechromatographed after release from liposomes with 0.5% Triton X-100 (●).

decans and glypican appear to contribute to the HSPG2 fraction, glypican does not contribute to either the intracellular or extracellular pools of free HS chains. This is consistent with the observation by Yanagishita[44] that in ovarian granulosa cell cultures, membrane-intercalated PGs are internalized and give rise to identifiable HS fragments, whereas GPI-linked HSPGs are degraded rapidly without generating such fragments. More rapid degradation of GPI-linked HSPGs as compared with other cell-surface HSPGs has also been reported in an osteosarcoma cell line.[45] This appears to be a general phenomenon, accounting for the lack of contribution from PI-PLC–sensitive ^{35}S to either $HS_{(c)}$ or $HS_{(m)}$ in the present study.

Several lines of evidence indicate that one or more syndecans are the source of $HS_{(c)}$ and $HS_{(m)}$. These include the location of the parent HSPG on the cell surface, its ability to incorporate into liposomes, its resistance to PI-PLC, the presence of mRNAs for the three syndecans, and the presence of three core proteins in liposome preparations that migrate with the known apparent M_r values of the same three syndecans.[6] Syndecan-2 is prominent at the protein level as detected by Western blotting.

It has been postulated that syndecans may participate in regulating a variety of cellular functions by serving, in general, as coreceptors for a broad range of bioactive ligands.[19] Syndecans also bind a variety of extracellular matrix molecules and

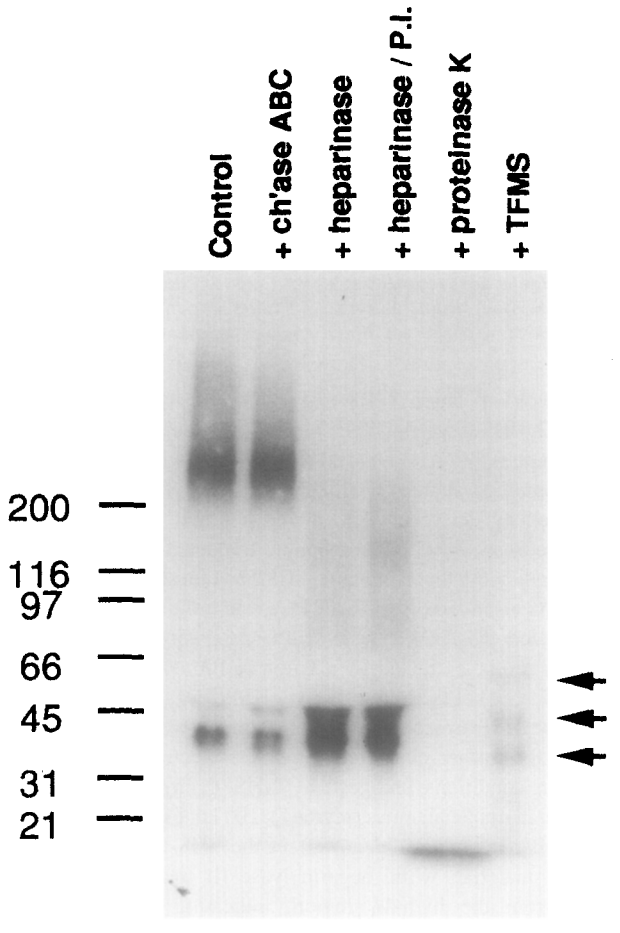

Fig 8. Electrophoresis of ^{125}I-labeled RMC membrane-intercalated HSPGs. Membrane HSPGs released from liposomes as in Fig 6C were iodinated and subjected to SDS-PAGE (4%-12% gradient gel) under reducing conditions. An autoradiogram is shown. Samples were run without further treatment (control) or after digestion with chondroitinase ABC (ch'ase ABC), heparinase with or without protease inhibitors (P.I.), or proteinase K. Samples were also subjected to total deglycosylation with TFMS. Arrows indicate the position of 3 protein bands. The position of migration of molecular mass marker proteins is indicated in kd at left.

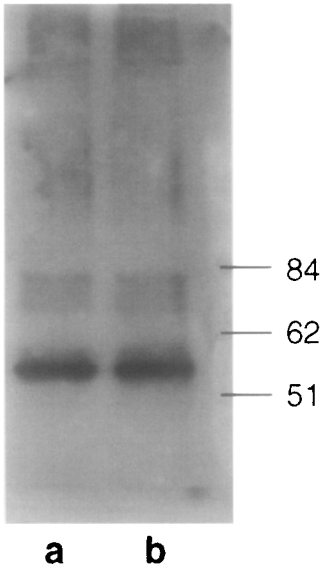

Fig 9. Western blot of RMC syndecans. Core proteins were prepared and deglycosylated with chondroitinase ABC and heparitinase. The blot was probed with an antiserum to rat liver HSPG and visualized by enhanced chemiluminescence detection. The 2 lanes show samples from 2 independent preparations. The omission of primary antibody or use of preimmune serum did not produce any detectable signal. The position of molecular weight markers is shown to the right, with mass in kd.

therefore may act as receptors for cell adhesion and spreading.[46-48] Each of the known interactions of syndecans with growth factors or matrix components occur through recognition of the ligand by GAG chains; the core protein does not play a role in binding.[20] GAGs may also affect cellular events independently of their matrix-binding and growth factor–coreceptor properties. When quiescent RMCs are stimulated with serum, they respond with activation of mitogen-activated protein kinase as early as 1 minute later and induction of c-*fos* shortly thereafter. The addition of heparin to the serum inhibits these early responses.[28] These effects do not appear to be mediated through growth factor or growth factor receptor interactions, because they are also exerted when Ca^{2+} ionophores or phorbol ester are used to induce c-*fos* under serum-free (and therefore growth factor–free) conditions[28] and when serum stimulation is performed with serum from which heparin-binding factors have been removed by heparin-affinity chromatography.[24,25] Like heparin, $HS_{(c)}$ and $HS_{(m)}$, but not intact HSPG, suppress the progression of RMCs to mitosis,[25] and at similar concentrations, both block c-*fos* induction in response to serum stimulation to the same extent.[28] Therefore, metabolism of cell-surface HSPG represents another potential point of regulation of GAG-related function.

Because cell-surface–derived HS can influence cell proliferation, the possibility of autoregulation arises. The turnover of newly labeled cell-surface HSPG is sufficiently rapid that peak quantities of $HS_{(c)}$ are found about 3 hours later in pulse-chase experiments. Previously, we showed that mesangial cells in culture have about 0.5 μg/mL $HS_{(m)}$ in the medium at confluence, and comparable amounts of $HS_{(c)}$ can be recovered from the cells.[24] This is in the range of biological activity, with antimitogenic properties of heparin being observed down to 100 ng/mL.[25] Therefore, the released HS chains may be positioned to influence cell proliferation and regulate their own production. Syndecan-1 and -2 mRNAs both increase after quiescent RMCs are stimulated with serum, and heparin suppresses this increase. This suggests that syndecan-1 and -2 expression is linked to proliferation in RMCs. Significantly, $HS_{(c)}$ and $HS_{(m)}$ also block the increased mRNA levels of syndecan-1 and -2 that follow serum stimulation. Syndecan-4 mRNA is influenced little by serum or heparin treatment, consistent with the distinct biological properties and roles for the various syndecans.

Interestingly, overexpression of syndecan-1 in epithelial cells increased serum-dependent growth,[49] perhaps by enhancing the response to growth factors in serum. In contrast, the extracellular domain of syndecan-1 suppressed the growth of several tumor cell lines, and this required both HS chains and the protein core.[50,51] The present study, together with our earlier study,[24,25] shows that an antimitogenic signal is derived from cell-surface HSPGs by liberation and partial degradation of the HS chains. HSPG2, a mixture of cell-surface PGs, does not inhibit RMC mitogenesis, and therefore the metabolic release of $HS_{(c)}$ and $HS_{(m)}$ from the core protein(s) is necessary to unmask the antimitogenic activity of the GAG chains.[24] This introduces an additional potential level of regulation on the putative autocrine function of the syndecans: in mesangial cells, intact cell-surface HSPGs may serve as growth stimuli by acting as growth factor coreceptors, but their turnover reverses this role by generating antimitogenic signals.

It should also be noted that the antimitogenic properties of $HS_{(c)}$ and $HS_{(m)}$ were demonstrated by partially purifying them and adding them to fresh RMC cultures.[24] $HS_{(c)}$ sequestered in a prelysosomal or lysosomal compartment is unlikely to be positioned to exhibit this activity in vivo, and the balance between $HS_{(m)}$ and $HS_{(c)}$ may determine whether antimitogenic activity is exhibited or masked. The relationship between these two pools is unknown. It has been shown that PGs can be metabolized by release of the GAG chain at the cell surface,[52,53] or they may be endocytosed by a pathway that has been studied for cell-surface HSPG in ovarian granulosa cells.[54] The rate of internalization approximates first-order kinetics with a half-life of 3 to 8 hours.[54-56] Cell-surface PGs are internalized as membrane-intercalated species rather than by a receptor-mediated process. In the presence of chloroquine, hydrophobic PGs accumulated inside the cell, suggesting that their core proteins are not degraded and that they are still probably intercalated into the membrane of the endosome.[56,57] The PGs then undergo stepwise degradation in prelysosomal compartments.[54,58] First, their protein cores are degraded with a half-life of about 30 minutes to release peptide-linked HS chains of about 30 kd, which are then acted upon by an endoglycosidase that produces HS fragments of about 10 kd, similar in size to $HS_{(c)}$ in our studies. These two steps are tightly coordinated.[59] The 10-kd HS fragments are further digested by an endoglycosidase at acidic pH in a separate compartment to fragments of about 5 kd that are rapidly and completely degraded to free sugars and sulfate in the lysosome. It is also possible that HSPG is hydrolyzed on the cell surface or in the pericellular matrix by endoglycosidase activity and then endocytosed.[60,61] A number of different cell types secrete endoglycosidases that degrade HS

to low–molecular weight products,[62-65] and a HS-degrading endoglycosidase has been found in the rat liver plasma membrane.[66]

In summary, RMCs express mRNA for syndecan-1, -2, and -4 and glypican, and express at least syndecan-2 at the protein level. They accumulate HS chains in the medium and cell layer that are derived from the cell-surface PG pool. These HS chains have been identified as arising from syndecans by their resistance to PI-PLC, and they have previously been shown to have antimitogenic activity when added to fresh RMC cultures. They also block the increase in syndecan-1 and -2 mRNAs resulting from serum stimulation of quiescent cells. Therefore, cell-surface HSPGs may potentially act as growth-promoting coreceptors that become growth-inhibitory upon metabolism. The balance between intracellular and extracellular metabolites may determine the extent to which the inhibitory activity is expressed.

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