Heparan Sulfate Chains from Glypican and Syndecans Bind the Hep II Domain of Fibronectin Similarly Despite Minor Structural Differences*

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Sarka Tumova‡, Anne Woods, and John R. Couchman§

From the Department of Cell Biology and Cell Adhesion and the Matrix Research Center, University of Alabama, Birmingham, Alabama 35294

Numerous functions of heparan sulfate proteoglycans are mediated through interactions between their heparan sulfate glycosaminoglycan chains and extracellular ligands. Ligand binding specificity for some molecules, including many growth factors, is determined by complex heparan sulfate fine structure, where highly sulfated, iduronate-rich domains alternate with N-acetylated domains. Syndecan-4, a cell surface heparan sulfate proteoglycan, has a distinct role in cell adhesion, suggesting its chains may differ from those of other cell surface proteoglycans. To determine whether the specific role of syndecan-4 correlates with a distinct heparan sulfate structure, we have analyzed heparan sulfate chains from the different surface proteoglycans of a single fibroblast strain and compared their ability to bind the Hep II domain of fibronectin, a ligand known to promote focal adhesion formation through syndecan-4. Despite distinct molecular masses of glypican and syndecan glycosaminoglycans and minor differences in disaccharide composition and sulfation pattern, the overall proportion and distribution of sulfated regions and the affinity for the Hep II domain were similar. Therefore, adhesion regulation requires core protein determinants of syndecan-4.

Heparan sulfate proteoglycans (HSPGs)† are ubiquitous cell surface and extracellular matrix molecules involved in cell adhesion, migration, proliferation, and differentiation (1–5). They consist of a protein core and usually three covalently attached glycosaminoglycan chains (6) composed of alternating glucosamine and hexuronic residues with various degrees of sulfation and epimerization (7, 8). Heparan sulfate (HS) chains have a characteristic sulfation pattern responsible for their ligand binding specificity and their subsequent biological functions (9). Although the HS chains mediate interactions with various extracellular ligands (4), core proteins of many cell surface proteoglycans can interact with cytoplasmic molecules (10), including cytoskeletal components and signaling molecules, thus linking extracellular and intracellular events. Major cell surface proteoglycans are members of the syndecan and glypican family (1, 4, 5, 11). Syndecans 1–4 are integral membrane proteoglycans with highly conserved transmembrane domains and two constant regions in the cytoplasmic domain, whereas glypicans, which are tethered to the membrane via glycosylphosphatidylinositol (GPI) anchor, lack the cytoplasmic connection. Expression of HSPGs is cell type-specific (12), and different cells can decorate a specific core protein with HS chains of distinct fine structure and ligand binding properties (13, 14). Physiological processes occurring during development, tumorigenesis, or in aging can also be accompanied by HS with specific structural features (15–17). At present, the overall sulfation pattern is thought to be determined by the cell type or physiological state rather than the core protein, although it has not been clearly established whether different proteoglycans from the same cell can carry HS chains with distinct structure and functions.

Cell adhesion is one of the processes requiring the presence of proteoglycans on the cell surface (1, 2, 4). Focal adhesions in many cell types, including rat embryo fibroblasts (REFs), growing on various substrates contain syndecan-4 (2, 18), and fibroblast adhesion on a fibronectin matrix requires both interaction with HS chains and signal transduction involving the core protein (19–22). Specific residues in the variable region of syndecan-4 cytoplasmic domain were shown to participate in intracellular signaling by interacting with phosphatidylinositol 4,5-bisphosphate and protein kinase C (20, 23, 24), but little information is available on the regulation of extracellular events. Under normal circumstances, interaction of HS chains with extracellular ligands may aid in clustering proteoglycans that appears to be critical for focal adhesion formation (25, 26), although the need for HS chains can be bypassed and syndecan-4 oligomerization achieved by overexpressing unglycanated core protein (27). In REFs, HS chains are present on multiple cell surface proteoglycans, but only syndecan-4 is localized in focal adhesion contacts, without apparent interference from other HSPG species. To explore the possibility that syndecan-4 HS chains have specific structural features that would support the unique ability of syndecan-4 to interact with extracellular matrix ligands in focal adhesion contacts, we examined whether REFs produce proteoglycans with distinct heparan sulfate chains based on the protein core. We isolated separate populations of radioactively labeled GPI-anchored proteoglycans (glypican-1) and integral membrane proteoglycans (syndecan-1, -2, and -4). In addition, syndecan-4 was im-

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‡ Current address: Dept. of Bioscience, University of Helsinki, P. O. Box 56, Helsinki 00014, Finland.
§ To whom correspondence should be addressed: Dept. of Cell Biology, UAB, 1670 University Blvd. VH 201A, Birmingham, AL 35294. Tel.: 205-934-2626; Fax: 205-975-9956; E-mail: jrcouchman@cellbio.bhs.uab.edu.
† The abbreviations are: HSPG, heparan sulfate (HS) proteoglycan; CHAPS, 3-[{3-cholamidopropyl}dimethylammonio]-1-propanesulfonic acid; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-dependent phospholipase C; REF, rat embryo fibroblast; SAX, strong anion exchange; GlcNAc, N-acetylated glucosamine; GlcNAc6-O-S, 6-sulfated N-acetylated glucosamine; GlcNS, N-sulfated glucosamine; GlcNS6-O-S, N-sulfated-6-O-sulfated glucosamine; ΔUA, unsaturated hexuronic acid; ΔUA2(2-O-S), unsaturated 2-O-sulfated hexuronic acid; MOPS, 3-(N-morpholino)-2-hydroxypropanesulfonic acid.
mucinoprecipitated from REFs using a specific monoclonal antibody. The size of their respective HS chains was compared by gel filtration HPLC. Fine structure of the chains was examined by enzymatic and chemical treatment, and their ligand binding ability was determined by affinity coelectrophoresis.

MATERIALS AND METHODS

Cell Culture—Rat embryo fibroblasts were cultured as described previously (28).

Proteoglycan Detection—Cells monolayers with or without prior PI-PLC treatment were washed and scraped in phosphate-buffered saline, then transferred into 0.1 M sodium chloride, 1 mM sodium citrate, 0.05 mM HEPES-acetate, pH 7.0. Samples (50 μl of cell suspension from a 1×75 cm² flask) were incubated for 30 min at 37 °C with 2.5 μlilumins of heparinase III (heparinase 1, EC 4.2.2.7, Seikagaku America, Fal-

mouth, MA) to remove HS chains. Phospholipase treatment resulted in cell fragility, so protease inhibitors 10 μM N-ethylmaleimide, 1 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM benzami-
dine hydrochloride were retained during the harvest and heparinase III incubation. The presence or absence of protease inhibitors resulted in no major differences in samples not exposed to PI-PLC. Samples corre-
sponding to equal cell numbers were electrophoresed on a 3−15% gra-
dient polyacrylamide gel and transferred to a nitrocellulose membrane. Coomassie blue-stained bands were detected using a specific monoclonal antibody (Seikagaku America), which specifically recog-
nizes an epitope created by the heparinase III treatment, including a terminal unsaturated uronic acid residue (29).

Isolation of GPI-Linked and Integral Proteoglycans—Nearly conflu-
ent REFs were labeled overnight with 50 μCi/mL [35S]H2SO4 (NEN Life Science Products) or [3H]glucosamine (American Radiolabeled Chemi-
cals, St. Louis, MO) in the absence of serum. Cell monolayers were washed with phosphate-buffered saline and incubated for 1 h with serum-free medium containing 0.1 million units of PI-PLC (Roche Molec-
ular Biochemicals, Molecular Probes, Inc., Eugene, OR) at 37 °C to release GPI-linked proteoglycans (30). Cells were then washed with Tris-buffered saline, 0.5 mM EDTA, and remaining cell surface proteoglycans were eluted by a 10-min incubation with 70 μg/mL chymotryp-
sin (Sigma) at 4 °C (31). GPI-linked and integral radioactively labeled proteoglycans were purified by anion exchange chromatography and lyophilized.

Purification of HS Chains—Free glycosaminoglycan chains were ob-
tained by alkaline elimination and purified as described previously (32). Briefly, proteoglycan samples were incubated for 16 h at 4 °C with 0.5 mM NaOH, 1 mM NaBH₄, which destroys the core proteins. After neutral-
ization with 10 M acetic acid, the glycosaminoglycans were reprecipi-
tated by alkaline elimination and purified as described previously (32).

Affinity Coelectrophoresis—The affinity of the interaction between free HS chains and their ligands was determined by affinity coelectrophoresis (39). Labeled HS samples were applied into horizontal slots in a 1% agarose gel (FMC BioProducts, Rockland, ME) and electrophore-
sed through precast 15-mm lanes containing different concentrations of the recombinant Hep II domain of fibronectin (isolated by affinity chromatography on nickel-agarose and heparin-Sepharose as described previously (34)). The gels were prepared as indicated by Lee and Lander (39) using 1% agarose in 50 mM MOPS, 125 mM sodium acetate, pH 7.0, and 0.5% CHAPS. Electrophoresis was performed for 120 min at 50 V (constant voltage) using 50 mM MOPS, 125 mM sodium acetate, pH 7.0, as a running buffer. Gels were dried under vacuum and subjected to autoradiography. Reaction coefficient R derived from relative mobilities of [3H]HS in the presence and absence of the ligand was used to calculate dissociation constants (39).

RESULTS

Isolation of Syndecan-4—REFs were labeled at seeding (1:2 split) with 10 μCi of [35S]H2SO4 in 10 ml of basal medium Eagle (ICN, Costa Mesa, CA) containing 20 μM NaSO₄ as a chaperone with 4 μCi of [3H]glucosamine in a minimum-Eagle’s medium with 5% dialyzed se-
rum. Cells were allowed to attach and spread overnight. The next day, monolayers were washed with warm phosphate-buffered saline, scraped into 50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 1% Triton, and incubated for 60 min on ice. Syndecan-4 was immunoprecipitated from the lysate as described previously (33). Briefly, precleared lysate was incubated overnight at 4 °C with monoclonal anti-syndecan-4 an-
tibody 150.9 and then sequentially with rabbit anti-mouse IgG and protein A-Sepharose beads for 1 h each. After washing the beads ex-
tensively with the lysis buffer, syndecan-4 was eluted by boiling in 4 M guanidinium hydrochloride buffer. The lysate was subjected to several rounds of immunoprecipitation to maximize the yield.

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ization with 10 mM acetic acid, the glycosaminoglycans were reprecipi-
tated with ethanol, dried, and incubated with chondroitin-ABC lyase (Seikagaku America) for 16 h at 37 °C to degrade chondroitin sulfate. Heparan sulfate chains were separated from chondroitin sulfate disaccharides by anion exchange chromatography on DEAE-Sephacel col-

ums (Amersham Pharmacia Biotech), desalted on a PD-10 gel filtration column (Amersham Pharmacia Biotech, Inc.), and lyophilized.

Gel Filtration—The size of [3H]HS chains was examined by gel filtration chromatography on TSK 4000 (35). The TSK 4000 HPLC column (TosoHaas, Montgomeryville, PA) was equilibrated and run in 0.1 mM KH₂PO₄ buffer, pH 6.0, 0.5 mM NaCl, 0.2% Tween at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were assayed for radioactivity. Similar results were obtained when the column was equilibrated in 4 mM guanidine hydrochloride, 50 mM Tris, 0.5% Triton X-100, pH 6.0, to prevent self-association of glycosaminoglycans.

A Superdex peptide fast protein liquid chromatography column (Am-

ersham Pharmacia Biotech) was used for separation of short oligosac-
charides (36). It was equilibrated in 0.5 mM pyridine acetate, pH 5.0, and HS chains were eluted from the column at a flow rate of 0.5 ml/min. The column was calibrated with oligosaccharides generated by low pH nitrous acid treatment from [3H]glucosamine-labeled glycos-
aminoglycans.

Enzymatic Digestion of HS Chains—[3H]HS chains were mixed with unlabeled heparin or HS (0.02 mg/ml final) and incubated for 6 h at 37 °C with 1.2 million units of heparinase I (heparinase EC 4.2.2.7, Sei-

kagaku America) or heparinase III. The reaction buffer was 1 mM calcium acetate, 40 mM sodium acetate, pH 7.0, for heparinase I or 0.1 mM calcium acetate, pH 7.0, 0.1% Tween for hepari-

nase III. To ensure the reaction went to completion, another 1.2-milli-

lagram of the enzyme was added after 6 h, and the mixture was incubated overnight. Heparinase I degradation products were analyzed by gel filtration on a TSK 4000 column, whereas the sizes of heparinase III degradation products were examined on a Superdex peptide column.

Nitrous Acid Treatment—[3H]HS chains were incubated with low pH nitrous acid as described by Shively and Conrad (37). This treatment specifically cleaves the glycosaminoglycan at N-sulfated glucosamine residues. The degradation products were resolved by gel filtration on a Superdex column.

Strong Anion Exchange (SAX) HPLC—[3H]HS chains were digested by three additions of 1.25 million units of heparinase I and heparinase III (EC 4.2.2.7, Seikagaku America) for 16 h at 37 °C with 0.5 mM MOPS, 125 mM sodium acetate, pH 7.0, containing 1 mg/ml BSA and 0.1 mg/ml heparin. This treatment degraded all HS chains mostly to disaccharides (60−90%), which were then isolated by gel filtration on a Superdex column, lyophilized, and separated by SAX chromatography. Samples were applied to a SAX Partisil column (0.5 × 25 mm, Whatman, Chiston, NJ) equilibrated in 28 mM KH₂PO₄, and eluted by a step gradient with increasing concentra-
tions of KFPO₄. Fractions of 1 ml were collected and assayed for radioactivity. The identity of HSdisaccharides was determined by comparison with heparin disaccharide standards (Sigma).

Affinity Coelectrophoresis—The affinity of the interaction between free HS chains and their ligands was determined by affinity coelectrophoresis (39). Labeled HS samples were applied into horizontal slots in a 1% agarose gel (FMC BioProducts, Rockland, ME) and electrophore-
sed through precast 15-mm lanes containing different concentrations of the recombinant Hep II domain of fibronectin (isolated by affinity chromatography on nickel-agarose and heparin-Sepharose as described previously (34)). The gels were prepared as indicated by Lee and Lander (39) using 1% agarose in 50 mM MOPS, 125 mM sodium acetate, pH 7.0, and 0.5% CHAPS. Electrophoresis was performed for 120 min at 50 V (constant voltage) using 50 mM MOPS, 125 mM sodium acetate, pH 7.0, as a running buffer. Gels were dried under vacuum and subjected to autoradiography. Reaction coefficient R derived from relative mobilities of [3H]HS in the presence and absence of the ligand was used to calculate dissociation constants (39).

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kDa. Reproducible differences were observed between HS chains of different origin. Glypican-de-TSK 4000 HPLC column (Fig. 2). Reproducible differences were analyzed by gel filtration chromatography on a treatment as well as HS chains from immunoprecipitated syn-
derived glycosaminoglycans representing syndecan-2, glycican-1, and synde-
can-1, respectively. A control sample from cells without heparinase treatment (lane 3) shows two nonspecific polypeptides at 75 and 120 kDa.

remaining cell surface [35S]HSPGs were released by chymotrypsin digestion. Under the conditions used in the labelling, at least 50% of cell surface [35S]HS chains originated from glypican. Based on the proteoglycan identification in the Fig. 1, the pool derived from integral proteoglycans contains mostly syndecan-2 and syndecan-4 as well as syndecan-1 and will be henceforth referred to as the syndecan population. In addition, immunoprecipitation with monoclonal antibody 150.9 was used to specifically isolate radioactively labeled synde-
can-4 proteoglycan.

Fibroblast Glypican and Syndecans Carry HS Chains of Diff- erent Length but Similar Net Charge—Sizes of [3H]HS chains from glypican and syndecan populations obtained by enzymatic treatment as well as HS chains from immunoprecipitated synde-
can-4 were analyzed by gel filtration chromatography on a TSK 4000 HPLC column (Fig. 2). Reproducible differences were observed between HS chains of different origin. Glypican-de-
duced glycosaminoglycans (Kav – 0.57) were shorter than HS from both the total syndecan population (Kav – 0.43) and syndecan-4 (Kav – 0.41) (Table I), indicating that HS chains of specific length can be attached to different core proteins in REFs. This may not be entirely unexpected, as matrix HSPGs were shown previously to contain longer HS chains than those of the cell surface HSPG population (42), suggesting the cellular synthetic machinery is able to distinguish between different core proteins. Glypican HS chains in our study we were slightly bigger than the largest standard, indicating the mass was close to 60 kDa, whereas the syndecan glycosaminoglycans far exceeded the calibration standards, making it impossible to accurately determine their molecular mass. Despite their differ-
ent sizes, glypican and syndecan HS chains displayed comparable net charge, since similar salt concentrations (0.63 and 0.66 M NaCl, respectively) were required to elute them from a 1-mL column of DEAE-Sephaecel (Table I).

Syndecan and Glypican HS Chains Have Comparable Fine Structure—Heparan sulfate chains have a characteristic structural organization, which is critical for ligand binding abilities (7–9). Heparan sulfate polymers consist of continuously N-sulfated S-domains rich in 2-O-sulfated iduronate interspersed with N-acetylated domains containing predominantly glucur-
onate residues and low sulfate levels. Mixed sequences with alternating N-sulfated and N-acetylated glucosamine are found at the borders. To examine the sulfation pattern and distribution of S-domains, radioactively labeled HS chains were subjected to chemical and enzymatic digestion with de-
defined specificity, and the resulting oligosaccharides were ana-
yzed by gel filtration (Fig. 3). Since using the [35S]H2SO4 label can obscure the results for shorter oligosaccharides that may lack sulfated residues, fine structure analysis was performed using HS labeled with [3H]glucosamine, which incorporates uniformly along the chain.

Low pH nitrous acid treatment cleaves HS specifically at N-sulfated glucosamine, and thus, sizes of generated fragments indicate the length of sequences separating N-sulfated resi-
dues. Consequently, nitrous acid-generated oligosaccharides cor-
respond to contiguous N-sulfated sequences, tetrasaccharides indicate mixed N-sulfated/N-acetylated sequences, and longer oligosaccharides originate from sequences with a low level of N-sulfation. Therefore, by comparing the oligosaccharide com-
position of nitrous acid digests from different HS species, one can analyze the differences in the distribution of N-sulfated domains. Nitrous acid treatment produced comparable profiles for glypican, total syndecan, and syndecan-4-derived HS chains (Fig. 3A). Overall the level of N-sulfation was similar for all chains (approximately 30%, Table I), which is lower than that previously reported for skin fibroblast HS (40–50%) (42). How-
ever, a reproducible difference was observed in the level of contiguous N-sulfation. In glypican HS chains, approximately 32% N-sulfated disaccharides were present in contiguous se-
quencies, whereas in total syndecan and syndecan-4 HS popu-
lations contiguous sequences accounted for only 21 and 23% N-sulfation, respectively (Table I). This suggests that the distri-
bution, rather than level, of N-sulfation may be slightly different between the HSPGs.

Heparinase I and III are bacterial enzymes that cleave HS with defined specificity. Heparinase III acts on low sulfated, N-acetylated regions, cleaving predominantly the sequence GlcNAc-glucuronic acid, but it can also tolerate N-sulfation and 6-O-sulfation of the glucosamine residue, so it degrades the major part of a typical HS chain to disaccharides, whereas it leaves S-domains intact (43, 44). Therefore, analysis of the degradation products can be used to estimate the size of S-
domains. When heparinase III-generated oligosaccharides from all three HS species were resolved on a Superdex column, the profiles were nearly identical. More than 50% of the material was degraded to disaccharides, whereas a small amount of tetrasaccharides and 10–12 residue oligosaccharides remained uncleaved, representing the resistant sequences within S-domains (Fig. 3B). Heparinase I, on the other hand, cleaves within S-domains, at the GlcNS(6-O-S)-2-
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small amounts of disaccharides were present at the $V_p$, suggesting very few 2-O-sulfated iduronate residues are present in neighboring disaccharides. This finding is also consistent with the relatively high susceptibility to heparinase III degradation.

**HS Chains from Different Proteoglycans Have Comparable Disaccharide Composition**—SAX chromatography can separate disaccharides based on the number as well as position of the sulfate groups and, thus, allows comparison of the relative proportions of O- and/or N-sulfated disaccharides in each of the HS species. Therefore, radioactively labeled HS chains from the three pools were digested with a mixture of heparinase I and III. Resulting disaccharides were isolated by gel filtration on a Superdex column and subjected to SAX chromatography on a Partisil column, which was calibrated with mono- and disulfated standards (Fig. 4). Because of low labeling efficiency of $[^3H]N\text{glucosamine}$ in syndecan-4 immunoprecipitations, $[^35S]N\text{HS}$ was used in these studies. Consequently, only sulfated disaccharides were detected, and therefore this method could not be used for the absolute determination of disaccharide content. However, it allowed for comparison between different HS species. Very little difference was seen in the sulfated disaccharide composition (Fig. 4 and Table II), especially for di- and trisulfated disaccharides. In comparison to glypicans and syndecan-4 HS, the total syndecan population displayed an increased amount of ΔUA(2-O-S)-GlcNAc, which was compensated for by a relative decrease in ΔUA(2-O-S)-GlcNS. Limited resolution as well as some specimen variation in the region corresponding to monosulfated disaccharides made a precise estimation of the disaccharide content difficult; however, syndecan-4 apparently contained a higher amount of ΔUA-GlcNS than the other proteoglycans. These findings suggest that minor differences may exist in the disaccharide composition of HS from different cell surface proteoglycans.

**Glypican and Syndecan HS Chains Bind the Hep II Domain of Fibronectin with Similar Affinity**—The structural studies suggested that both level and distribution of the sulfation and epimerization on HS chains from glypicans and syndecans in REFs are comparable, especially regarding the S-domains, which would predict that they will bind most ligands with similar affinity. However, since minor structural variations could still remain undetected by conventional methods, the ultimate test of function is a ligand binding assay. In particular, we were interested in HS binding to the Hep II domain of fibronectin because of its involvement in focal adhesion formation. Affinity coelectrophoresis was used to determine the affinity of isolated HS chains for the recombinant Hep II domain (39). Radioactively labeled HS chains were electrophoresed through lanes in agarose gel containing different concentrations of the protein ligand, and dissociation constants were determined from relative retardation of the glycosaminoglycan in the presence of the fibronectin domain (Fig. 5). HS chains derived from glypicans and total syndecan population bind Hep II with nearly identical affinity ($K_d$ of 67 and 66 nM, respectively), and the apparent dissociation constant for the interaction between Hep II and syndecan-4 HS (63 nM) is within the same range (Table I), indicating that all HS chains have comparable ability to bind the Hep II domain of fibronectin. When intact ectodomains of glypicans and syndecan HSPGs were assayed for the interaction with Hep II, the dissociation constant decreased to ~30 nM for both populations (data not shown), indicating that the presence of the core proteins does not affect significantly Hep II binding, although clustering HS chains may slightly improve their ability to interact with this particular ligand.

**DISCUSSION**

In agreement with previous findings for fibroblastic cells (40–42), rat embryo fibroblasts in our study contained significant amounts of syndecan-2 and -4 and a GPI-linked proteoglycan, most likely glypican-1. In addition, a proteoglycan with a protein core of apparent mass of 90 kDa was detected, possibly representing syndecan-1. Treatment with PI-PLC was used to separate two populations of HSPGs. The enzyme specifically liberated glypican-1, whereas the resistant integral proteogly-
cans, consisting mainly of syndecan-1, -2, and -4, were subsequently recovered by proteolytic release. Heparan sulfate glycosaminoglycans derived from these pools as well as chains from immunoprecipitated syndecan-4 share the typical HS structural organization (Fig. 6), consisting of S-domains rich in iduronate and N- and O-sulfation interspersed with N-acetylated sequences that are rich in glucuronate and contain low sulfation level. Susceptibility of these chains to the specific bacterial enzymes heparinase I and III indicated that all syndecan and glypican HS contain over 50% of extended (10–15 kDa), predominantly N-acetylated regions with low sulfation levels. Low pH treatment with nitrous acid determined that in all HS chains, approximately 30% of glucosamine residues are N-sulfated, which is less than that shown previously for skin fibroblasts (40–50%) (42). It is possible that culture conditions or the state of differentiation may affect the level of modification. The distribution of N-sulfated disaccharides along the HS chains was also similar, with the exception of contiguous N-sulfated sequences, where small but reproducible differences were observed between HS species derived from syndecans (23% of N-sulfated disaccharides for the whole population and 21% for syndecan-4) and glypican (32% N-sulfated disaccharides). This would indicate that glypican HS has either more or longer contiguous N-sulfated sequences. The latter possibility appears to be more likely, since S-domains, which are typically contained within the contiguously N-sulfated region, are distributed similarly along syndecan and glypican HS. Comparative analysis of sulfated disaccharides derived from the HS by enzymatic treatment did not reveal any dramatic changes in the specific residue content, except for a relatively higher content of ΔUA-GlcNS disaccharide in syndecan-4 and an altered proportion of ΔUA(2-O-S)-GlcNAc and ΔUA(2-O-S)-GlcNS in the total syndecan population. It is not clear at present whether these changes have functional implications.

The most pronounced difference between HS from syndecan and glypican species was their size. Glypican HS chains were significantly shorter than HS isolated from syndecan-4 or total syndecan population, all with molecular masses greater than 60 kDa. Even with equal affinity for ligands, this difference could have an effect on HS biological functions. For example, the length of HS extending from the protein core could affect the accessibility for HS binding molecules, making the longer syndecan HS chains more easily recognized. This would be augmented by the arrangement of chains on the core protein; although glypican-1 HS chains are located close to the plasma membrane, most of syndecan glycosaminoglycan attachment sites are positioned at the N terminus, relatively far from the cell surface. Thus, the topological array of glypican and syndecan HS may be distinct. Also, since the average distribution pattern of the sulfation and epimerization appears to be similar for all HS, it implies that longer HS will contain more S-domains per chain (Fig. 6), thus increasing their capacity to interact with ligands. We speculate that the difference in HS size may result from a distinct transport mechanism of the core
proteins during the synthetic pathway. As the newly synthesized core protein is transported through the Golgi system, HS are synthesized on a serine residue within the glycosaminoglycan attachment sequence and are concurrently modified by sulfation and epimerization (6). Chain length rather than the extent of modification may be affected by the time the proteoglycan spends in the Golgi compartment. Our result would be an indication that glypicans are shuttled through the glycosylation pathway faster than syndecans. This may not be entirely unexpected, since the GPI anchor provides proteins with many characteristic transport/localization features, including specific targeting on the cell surface (45, 46) or alternative degradation pathway (47). Another explanation for the different HS sizes could be differential degradation and recycling of proteoglycans on the cell surface. A minor fraction of glypican-1 in human skin fibroblasts was shown previously to undergo partial degradation and reglycanation before being recycled back to the cell surface (48). Unlike glypican HS chains in our study, the reglycanated chains were significantly longer than the normal HS population. However, acylation of the GPI anchor occurred simultaneously with the glycanation process, rendering it resistant to PLC treatment. A similar glypican recycling process reported in skin fibroblasts only concerned a small subpopulation and also produced overmodified HS, which we did not detect, so it is unlikely that any contaminating extended glypican HS in the syndecan pool was responsible for the size differences observed in our study. Also, Western blotting of proteoglycan species from rat embryo fibroblasts confirmed that the majority of glypican was removed from the cell surface by the PI-PLC treatment.

Comparable fine structure of HS chains from syndecans and glypicans suggested that many of their ligand binding properties are likely to be the same. Indeed, all HS species displayed similar affinity for the Hep II domain of fibronectin, with the dissociation constants in the range of 60–70 nM. These dissociation constants are similar to the dissociation constants reported for heparin and intact or fragmented fibronectin (49–52). Dif-

**Fibroblast Heparan Sulfate Structure**

**TABLE II**

Relative content of sulfated disaccharides in [35S]HS from different proteoglycan pools determined by SAX anion exchange

| Disaccharides                  | Syndecan-4 | Glypican |
|-------------------------------|------------|---------|
| 1 ΔUA-GlcNS                   | 26.1 ± 7.0 | 34.8 ± 3.9 | 28.1 ± 5.4 |
| 2 ΔUA-GlcNAc(6-O-S)           | 17.1 ± 7.2 | 14.8 ± 0.3 | 13.5 ± 5.4 |
| 3 ΔUA(2-O-S)-GlcNAc           | 8.5 ± 0.5  | 4.4 ± 0.9  | 4.6 ± 1.5  |
| 4 ΔUA-GlcNS(6-O-S)            | 25.4 ± 4.2 | 21.9 ± 3.2 | 21.3 ± 3.9 |
| 5 ΔUA(2-O-S)-GlcNS            | 6.5 ± 1.3  | 10.2 ± 1.2 | 14.5 ± 2.6 |
| 6 ΔUA(2-O-S)-GlcNS(6-O-S)     | 13.9 ± 0.1 | 11.3 ± 1.5 | 14.5 ± 3.6 |

**FIG. 5.** The affinity of different HS populations for the Hep II domain of fibronectin. The ability of [35S]HS from glypican (open circles), syndecan-4 (closed triangles), and total syndecan population (closed circles) to bind Hep II was determined by affinity coelectrophoresis. The retardation coefficient R was calculated from relative mobilities of [35S]HS on agarose gel in the presence and absence of various ligand concentrations and represents the fraction of bound ligand. The dissociation constants (K_d) were determined by fitting the experimental data in the equation $R = R^*([\text{Hep II}]K_d + [\text{Hep II}])$, where $R^*$ is the maximum retardation coefficient at saturating ligand concentration and are reported in Table I. The best fit for glypican (dashed line), syndecan-4 (full line), and total syndecan HS (dotted line) are shown.

**FIG. 4.** SAX chromatography of sulfated HS disaccharides. [35S]HS from glypican (A), total syndecan population (B), and syndecan-4 (C) was exhaustively digested with a mixture of heparinase I and III, and the resulting disaccharides were purified on a Superdex column and subjected to SAX chromatography. The SAX Partisil column was equilibrated in 28 mM KH2PO4, and the disaccharides were eluted by a step gradient of 150 mM KH2PO4 and 400 mM KH2PO4, as indicated by the arrows. The disaccharide peak positions were correlated with elution of heparin disaccharide standards: 1. [UA-GlcNS], 2. [UA-GlcNAc(6-O-S)]; 3. [UA(2-O-S)-GlcNAc]; 4. [UA-GlcNS(6-O-S)]; 5. [UA(2-O-S)-GlcNS]; and 6. [UA(2-O-S)-GlcNS(6-O-S)].
Different methods used for the analysis and conditions of the measurement such as pH and ionic strength (49) are likely to contribute to the variations.

The Hep II domain has been implicated in the interaction between fibronectin and syndecan-4 (19, 21, 34) that, accompanied by integrin engagement, leads to focal adhesion formation (25, 26). One possible explanation for the exclusive role that syndecan-4 plays in this process would be a specific ability of its glycosaminoglycan chains to interact with fibronectin. However, our study shows that all cell surface HS PGs in rat embryo fibroblasts carry HS chains of comparable structure and fibronectin binding abilities. The extracellular interactions that define syndecan-4 participation in focal adhesion formation are likely to be determined by other factors. These could involve a specific location on the cell surface determined by other parts of the protein core or alternative extracellular interactions within the syndecan-4 extracellular domain. The region of core protein between the plasma membrane and glycosaminoglycan attachment sites has been shown previously to involve a specific location on the cell surface determined by other parts of the protein core or alternative extracellular interactions within the syndecan-4 extracellular domain. The region of core protein between the plasma membrane and glycosaminoglycan attachment sites has been shown previously to involve a specific location on the cell surface determined by other parts of the protein core or alternative extracellular interactions within the syndecan-4 extracellular domain. The region of core protein between the plasma membrane and glycosaminoglycan attachment sites has been shown previously to involve a specific location on the cell surface determined by other parts of the protein core or alternative extracellular interactions within the syndecan-4 extracellular domain.
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Sarka Tumova, Anne Woods and John R. Couchman

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