Modulations of Glypican-1 Heparan Sulfate Structure by Inhibition of Endogenous Polyamine Synthesis

Mapping of Spermine-Binding Sites and Heparanase, Heparin Lyase, and Nitric Oxide/Nitrite Cleavage Sites*

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Cell surface heparan sulfate proteoglycans facilitate uptake of growth-promoting polyamines (Belting, M., Persson, S., and Fransson, L.-Å. (1999) Biochem. J. 338, 317–323; Belting, M., Borsig, L., Fuster, M. M., Brown, J. R., Persson, L., Fransson, L.-Å., and Esko, J. D. (2001) Proc. Natl. Acad. Sci. U. S. A., in press). Here, we have analyzed the effect of polyamine deprivation on the structure and polyamine affinity of the heparan sulfate chains in various glypican-1 glycoforms synthesized by a transformed cell line (ECV 304). Heparan sulfate chains of glypican-1 were either cleaved with heparanase at sites embracing the highly modified regions or with nitrite at N-unsubstituted glucosamine residues. The products were separated and further degraded by heparin lyase to identify sulfated iduronic acid. Polyamine affinity was assessed by chromatography on agarose substituted with the polyamine spermine. In heparan sulfate made by cells with undisturbed endogenous polyanhydride synthesis, free amino groups were restricted to the unmodified, unsulfated segments, especially near the core protein. Spermine high affinity binding sites in derivatives were located to the modified and highly sulfated segments that were released by heparanase. In cells with up-regulated polyanhydride uptake, heparan sulfate contained an increased number of clustered N-unsubstituted glucosamines and sulfated iduronic acid residues. This resulted in a greater number of NO/nitrite-sensitive cleavage sites near the potential spermine-binding sites. Endogenous degradation by heparanase and NO-derived nitrite in polyanhydride-deprived cells generated a separate pool of heparan sulfate oligosaccharides with an exceptionally high affinity for spermine. Spermine uptake in polyanhydride-deprived cells was reduced when NO/nitrite-generated degradation of heparan sulfate was inhibited. The results suggest a functional interplay between glypican recycling, NO/nitrite-generated heparan sulfate degradation, and polyanhydride uptake.

Proteoglycans (PGs)† are glycosaminoglycan-substituted proteins that can be found in the extracellular matrix or at the cell surface. Glypican constitutes a family of cell surface-bound PGs where the protein is covalently connected at the C terminus to membrane lipids via a so-called glycosylphosphatidylinositol anchor. The central part of the protein consists of a cysteine-rich globular domain that contains information that ensures a high level of heparan sulfate (HS) glycosaminoglycan substitution at three sites located close to the C terminus (1). So far six different human glypicans with the same overall design have been molecularly cloned (for review, see Ref. 2). Biosynthesis of the HS glycan chains proceeds in a stepwise manner. Serine residues in sequences like DDGSGSGSD (glypican-1) are first substituted with xylose and then the common glycosaminoglycan-to-protein linkage region GlcUA-Gal-Gal-xylose is formed. HS assembly is initiated by a unique 3-O-GlcNac-transferase that adds the first GlcNac (3). By the alternating addition of GlcUA and GlcNac, catalyzed by HS-copolymerases (4), the extended, linear heparan backbone is formed. A unique step in HS biosynthesis is the regional exchange of N-acetyl for sulfate on glucosamine (4) catalyzed by various isoforms of N-deacetylase/sulfotransferase (NDST). NDST-1, -2, and -4 have an NDST ratio below 1, whereas NDST-3 has a 10-fold higher deacetylase activity (5). Therefore, expression and participation of this isoform during HS biosynthesis would yield a significant proportion of N-unsubstituted glucosamine (GlcNH₂O³). Free amino groups could also be generated by N-desulfation catalyzed by sulfamidases.

After the formation of N-sulfate (-NSO₃) groups, further modifications of the HS chain take place, including epimerization of GlcUA to iduronic acid (IdoUA) and O-sulfations at various positions, yielding a characteristic pattern of alternating modified and highly modified segments separated by transition regions. A hypothetical HS sequence is shown in Scheme 1, and the overall structural pattern is depicted in Scheme 2A, top.

We have previously studied the nature of recycling glypicans glycosaminoglycans in fibroblasts and ECV 304 cells (for review, see Ref. 6). In the latter cells, a brefeldin A (BFA)-arrested, large size glypican-1 glycoform with long HS chains containing multiple GlcNH₂O³ residues is degraded by heparanase during a chase, generating HS oligosaccharides and a glypican-1 glycoform.

DFMO, α-difluoromethylornithine; GlcNac, N-acetylgalactosamine; GlcNH₂O³, N-unsubstituted glucosamine; GlcNR, glucosamine with unspecified N substituent; GlcNOSO₃, N-sulfamidoglucosamine; GlcUA, α-glucuronic acid; HexUA, unspecified hexuronic acid; Δ1HexUA, 4,5-unsaturated hexuronic acid; HS, heparan sulfate; IdoUA, 1-iduronic acid; ST, sulfotransferase; NDST, N-deacetylase/sulfotransferase; OSO₃, sulfate; OST, O-sulfotransferase; CHO, Chinese hamster ovary.

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1 The abbreviations used are: PG, proteoglycan; BFA, brefeldin A;
with truncated HS chains (7). This glycoform can serve as a precursor for the reformation of a PG with full size HS chains. Re-synthesis of HS on the stubs is prevented by nitrite deprivation and restored when an NO donor is supplied (7). We have proposed that heparanase degradation proceeds until the GlcNH₃ residues are near the nonreducing end of the stubs, then NO-derived nitrite cleaves at these residues, providing fresh acceptor sites. More recent studies have shown that the GlcNH₃ residues are indeed concentrated to sites near the reducing side of heparanase cleavage sites in the transition region between unmodified and modified chain segments near the core protein (Ref. 8; see also Scheme 2, top).

Polyamines (putrescine, spermidine, and spermine) are essential for growth and differentiation of all cells, and they bind electrostatically to polyanions like nucleic acids. The intracellular polyamine levels are tightly regulated by synthesis, degradation, and transport (for review, see Ref. 9). Inhibition of endogenous synthesis, e.g. by inhibition of ornithine decarboxylase with α-Difluoromethylornithine (DFMO) results in increased polyamine uptake from the environment. The partly disappointing results with DFMO in anti-cancer trials may be explained by compensatory retrieval of extracellular polyamines by tumor cells (9). Inhibition of both polyamine synthesis and uptake could therefore be a useful anticancer strategy. However, the nature of a polyamine transport system in mammalian cells still remains elusive. Studies from this laboratory show that HS binds the polyamine spermine with an affinity that is 10 times greater than that of DNA (10). We have also obtained direct evidence for an involvement of HS in the uptake of polyamines by cultured fibroblasts (11). Removal of cell surface HS or inhibition of PG synthesis or sulfation reduces spermine uptake. Upon depletion of the intracellular polyamine pool, cells respond by synthesizing increased amounts of HSG forms that have higher spermine affinity. Mutant CHO cells deficient in PG synthesis have a reduced polyamine uptake and fail to proliferate and form colonies in the presence of the ornithine decarboxylase inhibitor despite the presence of exogenous spermine. Transfection with cDNA for the missing enzyme restores polyamine uptake.²

It is thus conceivable that the HS chains of recycling glypic-an could carry polyamines into cells when endogenous synthesis is inhibited by DFMO. After degradation of the HS chains, polyamines bound to HS oligosaccharides would be separated from the recycling truncated PG. To explore whether polyamine deprivation induces changes in HS structure and function that would facilitate polyamine uptake, we have made a comparative study of glypic-an HS from ECV 304 cells with undisturbed polyamine synthesis and from cells treated with DFMO. The PG and its HS chains were examined for spermine affinity as well as for content and location of sulfated hexuronic acids (HexUA) and heparanase and nitrite cleavage sites. We provide evidence that polyamine deprivation induces structural changes that favor increased formation of HS oligosaccharides with higher spermine affinity and that inhibition of HS degradation reduces spermine uptake.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cells, culture media, sera, antiserum to glypic-an-1, BFA, α-DFMO, radioactive precursors, enzymes, prepacked columns, a spermine-substituted HiTrap column, Centriplus tubes, and other media or chemicals were obtained from sources listed previously (7, 8, 11).

**Cell Treatments and Radiolabeling—**ECV cells were maintained as described (7, 8, 11) and preincubated with the appropriate medium before radiolabeling. Pretreatments with 5 mM DFMO and 1 μM spermine were carried out as described (11). Radiolabeling was carried out with 20 μCi/ml [6-3H]glucosamine and 50 μCi/ml [35S]sulfate in sulfate-poor medium (7, 8). Radiolabeling in the absence of BFA was generally carried out with media that had been preincubated with n-6-3H]glucosamine alone to achieve labeling of the HS stubs on the small, resident precursor PG (7). Trypsin digestions were performed as described (8).

**Extraction and Isolation of PG and PG Products—**Cells were extracted with radiolabeled precipitation buffer followed by immunoaffinity purification on glypican-1-glycoprotein using anti-glypican antiserum as described previously (7). Cells were also extracted with Triton X-100 and PG, and PG-derived material were recovered either by passage over DEAE-cellulose or by desalting on PD-10 (7, 8). Separation into PG and the various degradation products was performed by gel-permeation chromatography on Superose 6 or Superdex peptide, and further purification of PG material was achieved by ion exchange chromatography on MonoQ (gradient elution) and sometimes by hydrophobic interaction chromatography on octyl-Sepharose (7, 8). Affinity chromatography on spermine-substituted agarose has been described elsewhere (11).

**Degradation Procedures—**Purified radiolabeled PG was incubated with 75-cm² cultures of CHO-K1 cells in 6 ml of medium. Degradation products were recovered from the cell layer after extraction with Triton X-100. HS chains and chain stubs were released from the core protein by treatment with alkaline borohydride (7, 8). Enzymatic digestions of HS were performed with HS or heparin lyase and deaminative cleavage by treatment with alkaline borohydride (7, 8). Enzymatic digestions of HS were performed with HS or heparin lyase and deaminative cleavage at GlcNH₃⁺ with HNO₂ at pH 3.9 as previously described (7, 8). Unlabeled nonreducing terminal hexuronic acid was removed with mercury (II) acetate (12). Radioactivity measurements, buffer changes, concentrations, and recovery procedures as well as carriers were the same as described previously (7, 8).

**Spermine Uptake Measurements—**ECV cells were grown in regular medium with or without 5 mM DFMO for 24 h. The rate of uptake of different concentrations of [14C]spermine was measured as described (11).

**RESULTS**

**General Strategy—**A flow chart for the structural analysis of glypic-an-1 HS is shown in Scheme 2. We first examined HS chains of large (A) and small (B) glypic-an-1 glycoforms as well as HS oligosaccharides from ECV cells with undisturbed polyamine synthesis and then corresponding HS material from ECV cells that were treated with DFMO to up-regulate polyamine import. HS chains and oligosaccharides were analyzed for the content and location of GlcNH₃⁺ residues, sulfated IdoUA, and heparanase cleavage sites in relation to the unmodified and modified regions and for high affinity spermine-binding sites (C). Finally, we explored whether inhibition of HS degradation could affect polyamine uptake.

**Mapping of GlcNH₃⁺ Residues in HS of the Large Glypic-an-1 Glycoform from Cells with Undisturbed Polyamine Synthesis—**HS chains from the ³Hglucosamine- and [³⁵S]sulfate-labeled large size glypic-an PG of BFA-treated ECV cells were cleaved with nitrite at pH 3.9 at GlcNH₃⁺ residues (Scheme 1, site 4), and the various products (Scheme 2) were separated by chromatography on Superdex peptide (Fig. 1A). As expected, most

² M. Belting, L.-Å. Fransson, and J. D. Esko, unpublished observations.
SCHEME 2. Flow chart of the strategy for analyzing HS structure and spermine affinity. ECV cells not treated or treated with DFMO to deplete endogenous polyamine pools were used in all cases (A–C). In A, large size glypican-1 precursor was isolated from cells that were also exposed to BFA, and in B, smaller-size glypican-1 and HS oligosaccharides obtained from cells not treated with BFA were separately isolated. In C, selected fractions were examined for spermine affinity. As shown under A, HS chains consist of a backbone (GlcUA-GlcNAc, solid line) that is modified by exchange of N-acetyl for N-sulfate by epimerization of GlcUA to IdoUA and by various O-sulfations (boxes) either extensively (filled boxes) or partially (open boxes). Possible positions for GlcNH$_3$/H$_{11001}$ and sulfated IdoUA (S) are indicated. Previous studies indicated clustering of GlcNH$_3$/H$_{11001}$ to regions near the core protein attachment (Ser) and clustering of sulfated IdoUA to the highly modified domains (8). Heparanase cleavage sites are located at the reducing (filled arrow) or nonreducing side (open arrow) of highly modified regions (20). The glypican preparations were either treated with alkali to release HS chains or directly with heparanase (see II and III) to generate HS fragments and core protein with remaining HS stubs. Intact, alkali-released HS chains were exposed to HNO$_2$ at pH 3.9 to identify the location of GlcNH$_3$/H$_{11001}$ residues. Fragments obtained were separated and digested with heparin (Hep) lyase to identify sulfated HexUA and by HS lyase to cleave the unmodified backbone. Alternatively, HS chains were directly cleaved by HS lyase to liberate the modified sections and those containing GlcNH$_3$/H$_{11001}$. These products were also separated and subsequently treated with either HNO$_2$ at pH 3.9 or heparin lyase. Cleavage of HS in intact glypican by heparanase yields both long and short fragments (II and III, respectively). The former pool includes stubs attached to the core protein via Ser. The core protein has a C-terminal, hydrophobic glycosylphosphatidylinositol-anchor (filled circle with two rods). The latter property was used to separate the degradation products, and HS stubs were released from the core protein by alkali treatment. Heparanase-released HS fragments (from II and III) were further degraded by heparin lyase or HNO$_2$ at pH 3.9.
of the fragments were large and appeared in the excluded volume (8). The presence of clustered GlcNH₃⁺ residues would yield small fragments (Scheme 2A). The results showed that no disaccharides were generated, indicating that consecutive GlcNH₃⁺-containing repeats were very rare or nonexisting. The tetra- and hexasaccharide pools in A were treated with either heparin lyase (B and D, respectively) or HS lyase (C and E, respectively) and rechromatographed. Alkali-released HS chains from the same PG were directly degraded by HS lyase and chromatographed in (F). The decasaccharide (I0) and tetrasaccharide (4) pools in (F) were treated with either heparin lyase (G and I, respectively) or nitrite at pH 3.9 (H and J, respectively) and rechromatographed. O, ³H; □, ³⁵S; V₀, void volume; Vₜ, total volume.

Fig. 1. Localization of nitrite, heparin lyase, and HS lyase cleavage sites in high molecular weight HS chains. Gel permeation chromatography is shown on Superdex peptides of [³H]glucosamine and [⁵⁵S]sulfate-labeled HS chains from large size glypican PG after deaminative cleavage by nitrite at GlcNH₃⁺ (A). Numbers 2, 4, 6 etc. refer to di-, tetra-, hexasaccharides, and so on. The hexasaccharide (6) and tetrasaccharide (4) pools in A were treated with either heparin lyase (B and D, respectively) or HS lyase (C and E, respectively) and rechromatographed. Alkali-released HS chains from the same PG were directly degraded by HS lyase and chromatographed in (F). The decasaccharide (I0) and tetrasaccharide (4) pools in (F) were treated with either heparin lyase (G and I, respectively) or nitrite at pH 3.9 (H and J, respectively) and rechromatographed.

Polyamine-dependent Modulation of Heparan Sulfate Structure
Mapping of GlcNH₃⁺ Residues in HS Lyase-resistant Segments of HS Chains—HS chains from the [³H]glucosamine- and [³⁵S]sulfate-labeled glypicin PG of BFA-treated cells were directly treated with HS lyase (Scheme 2A). Highly modified regions and repeats containing GlcNH₃⁺ were expected to be spared. The HS lyase degradation products were separated on Superdex peptide into oligosaccharides ranging from di- to decasaccharides or even larger (Fig. 1F). The disaccharides (2 in Fig. 1F), which are the major products, emanate from the extended unmodified stretches of the HS backbone (Scheme 2A). Each oligosaccharide pool was treated with heparin lyase to identify GlcNSO₃-HexUA(2-OSO₃) bonds or with nitrite to identify GlcNH₃⁺-HexUA bonds. The decasaccharides (10 in Fig. 1F) were partly degraded by heparin lyase to sulfate-enriched smaller saccharides (2–8 in Fig. 1G), indicating the presence of heparin-type repeats. Very little if any GlcNH₃⁺-containing repeats were detected (Fig. 1H). Thus, highly modified regions contain few if any GlcNH₃⁺ residues (see Scheme 2A, top). The tetrasaccharides (4 in Fig. 1F), derived from the sequence GlcNR-GlcUA-GlcNR-HexUA-GlcNR-GlcUA, were insensitive to heparin lyase (Fig. 1I) but sensitive to nitrous acid at pH 3.9, indicating the presence of GlcNH₃⁺ residues (see 2 in Fig. 1J). Hence, the saccharide (HexUA-GlcNH₃⁺-HexUA-GlcNR, where HexUA probably is GlcUA, may be derived from stretches of the unmodified backbone containing a solitary GlcNH₃⁺ residue (see Scheme 2A, top). The octasaccharide (8 in Fig. 1F) had the same reaction patterns as the decasaccharide, and the hexasaccharide (6 in Fig. 1F) resembled the tetrasaccharide (data not shown).

Mapping of Heparanase Cleavage Sites in HS of the Large Glypicin Glycoform—Heparanase is expected to cleave HS at certain glucuronidic linkages (Scheme 1, site 2; see also Ref. 19) located on either side of the highly modified regions (Scheme 2A, for review, see Ref. 20). However, CHO cell-derived heparanase may not require the 2-O-sulfate group on the HexUA (20). Moreover, the site closest to the core protein (Scheme 2A) may not always be cleaved (8). Because the unmodified stretches generally are longer than the more modified ones, two types of fragments should be obtained (Scheme 2A, bottom half), i.e. longer ones (pool II), comprising the unmodified and less modified stretches and including the stubs still attached to the core protein as well as shorter ones (pool III) carrying the highly modified regions. Previous studies show (8) that ECV cells express a surface-located, suramin-inhibited heparanase that degrades exogenously supplied, mature glypicin PG into the above-mentioned products. Because we subsequently found that many CHO cell lines express more potent heparanase activity, these cells were used in most of the following experiments. To obtain sufficient amounts of material for further analysis, several batches of glypicin PG were repeatedly incubated with the CHO cells, and the products were recovered and separated on Superose 6. One such example is shown in Fig. 2. [³⁵S]Sulfate-labeled glypicin PG obtained from BFA-treated ECV cells and purified via both gel-permeation and ion-exchange chromatography (8) was incubated with heparanase-containing CHO-K1 cells, and the products were separated by gel permeation chromatography (Fig. 2A). Pool I consisted of undegraded and partially degraded PG. Pool II, which should consist of long HS-chain fragments and glypicin core protein with truncated HS chains (see Scheme 2A, pool II), was passed over octyl-Sepharose. The unbound, free HS-chain fragments were rechromatographed in B, pooled (see bar), treated with nitrite at pH 3.9, and rechromatographed again (C). [³⁵S]; Vₒ, void volume; Vₜ, total volume.

**Fig. 2.** Degradation by heparanase of high molecular weight HS chains in glypicin PG. Gel permeation chromatography is shown on Superose 6 of [³⁵S]sulfate-labeled glypicin PG from BFA-treated ECV 304 cells (see Fig. 1) after incubation with heparanase of CHO-K1 cells (A). Pool I consists of undegraded and partially degraded PG. Pool II, which consists of long HS-chain fragments and glypicin core protein with truncated HS chains (see Scheme 3), was passed over octyl-Sepharose. The unbound, free HS-chain fragments were rechromatographed in B, pooled (see bar), treated with nitrite at pH 3.9, and rechromatographed again (C). [³⁵S]; Vₒ, void volume; Vₜ, total volume.

### Polyamine-dependent Modulation of Heparan Sulfate Structure

Mapping of GlcNH₃⁺ Residues and Heparin-type Repeats in Heparanase-generated Fragments Comprising Unmodified Regions—Because heparanase cleaves a glucuronidic bond, subsequent cleavage of a glucosaminidic bond by nitrous acid or heparin lyase would yield odd-numbered saccharide fragments (see Scheme 1 if sites 2 and 4 were cleaved). To assess whether we could distinguish between odd- and even-numbered saccharides, HS-lyase digests of [³H]glucosamine and [³⁵S]sulfate-labeled HS chains, comprising a series of even-numbered saccharides, were chromatographed on Superdex peptide either
directly (Fig. 3A) or after subsequent removal of nonreducing terminal \( \Delta \text{HexUA} \) residues to obtain odd-numbered saccharides (Fig. 3B). The results showed that it might be possible to distinguish between these saccharide series, at least when they appear separately.

The long HS-chain fragments released by heparanase treatment of \([3H]\text{glucosamine} \text{ and } [35S]\text{sulfate-labeled} \) large size glypican PG (see Scheme 2A, pool II, and obtained as in Fig. 2, A–C) were treated with nitrite at pH 3.9 and chromatographed on Superdex peptide (Fig. 3C) to demonstrate that no small odd-numbered terminal saccharide fragments had been released. Hence GlcNH\(_3\) residues and heparanase cleavage sites were not located close to one another in the peripheral parts of HS chains. To check whether IdoUA(2-OSO\(_3\))/GlcUA(2-OSO\(_3\)) residues were located close to the nitrite cleavage sites, the nitrite-treated material was subsequently digested with heparin lyase and rechromatographed (Fig. 3D). Again, there was no release of small saccharides. We can thus conclude that IdoUA(2-OSO\(_3\))/GlcUA(2-OSO\(_3\)) residues were rare in these segments of the HS chain (Scheme 2A).

**Mapping of GlcNH\(_3\) Residues in HS Segments near the Core Protein**—The glypican core protein with HS stubs remaining after heparanase treatment of the large size PG (see Scheme 2A) was recovered from pool II (as in Fig. 2A) by adsorption to octyl-Sepharose. This material, which was alkali-sensitive (data not shown), was excluded from Superdex peptide (Fig. 3E). To identify GlcNH\(_3\) residues, it was treated with nitrite and rechromatographed (Fig. 3F). A series of saccharide fragments, probably mostly small odd-numbered ones but also larger even-numbered ones, were obtained. A monosaccharide that might be present could only be derived from a sequence (GlcUA)-GlcNH\(_3\)-HexUA(-OSO\(_3\))-, provided that the heparanase can cleave adjacent to a GlcNH\(_3\) residue (19). A trisaccharide could be derived from a sequence (GlcUA)-GlcNRC-HexUA(-OSO\(_3\))-GlcNH\(_3\)-. However, few of the small fragments obtained (1–5 in Fig. 3F) appeared to be sulfated. Therefore, this arrangement was probably very rare. Consecutive GlcNH\(_3\) residues that were not detected in preceding experiments (Fig. 1A) should have yielded disaccharides. The smallest fragment obtained from the core protein-attached HS stubs may thus be a monosaccharide (1 in Fig. 3F). Tetra-, hexa-, and octasaccharides that were obtained (4, 6, and 8 in Fig. 3F) should be derived from sequences containing clustered GlcNH\(_3\) residues that are known (8) to be concentrated to the region near the core protein (see Scheme 2A). The radiosulfate-labeled material eluting in the excluded volume (\( \psi_1 \) in Fig. 3F) may correspond to long stubs still attached to the core protein (see Scheme 2A, pool II) but devoid of GlcNH\(_3\) residues and connected to a highly modified region (from pool III in Scheme 2A) because a heparanase site was lacking or not cleaved. Alternatively or in addition there could be fragments obtained by cleavage of widely spaced heparanase and nitrite cleavage sites (Scheme 1, sites 2 and 4) with the latter located close to the core protein.

**Mapping the Distance between the Core Protein and the First GlcNH\(_3\) Residue**—To determine the distance between the core protein and the first GlcNH\(_3\) residue, \([3H]\text{glucosamine-} \text{ and } [35S]\text{sulfate-labeled} \) glypican PG from BFA-treated cells and comprising the highly modified regions were recovered from pool III (see Scheme 2A) as in Fig. 2A. To check whether this pool included some small saccharides, it was also chromatographed on Superdex peptide (Fig. 3H). Some trisaccharide was obtained (3 in Fig. 3H), indicating that cleavage of a nitrite-sensitive site near the heparanase site could take place when exogenous PG is incubated with cells. However, these sites appear to be rare.

Most of the heparanase-generated fragments that contained the highly modified regions (Scheme 2A, pool III) were excluded from Superdex peptide (Fig. 3H). This material was pooled (see bar), digested with heparin lyase, and rechromatographed (Fig. 3I). Small sulfated saccharides were formed, indicating the presence of IdoUA(2-OSO\(_3\))/GlcUA(2-OSO\(_3\)) residues. Some of them appeared to be odd-numbered (1 and 3 in Fig. 3I). A monosaccharide could only be derived from the nonreducing end of an heparanase-generated oligosaccharide because cleavage of the glucuronic (bold face) linkage in -GlcNR-HexUA-GlcNR-GlcUA-GlcNR-HexUA(-OSO\(_3\))-GlcNR- would yield GlcNR-HexUA(-OSO\(_3\))-GlcNR- terminating fragments. If the HexUA was IdoUA, R in the nonreducing terminal GlcNR would have to be N-sulfated (5), and then heparin lyase would release the monosaccharide GlcNSO\(_3\). The results were inconclusive (Fig. 3I), and it appears more likely that most of the heparin lyase-released saccharides were derived from the highly modified regions in the center of the heparanase-released fragments (Scheme 2A, pool III). Treatment with nitrite did not cleave the oligosaccharide backbone (Fig. 3J), confirming that GlcNH\(_3\) residues are absent from the highly modified regions.

**Localization of High Affinity Spermine-binding Sites in HS of the Large Glypican Glycoform**—Spermine binding was assessed by affinity chromatography on spermine-substituted agarose eluted with a linear guanidinium chloride gradient (Fig. 4). BFA-arrested, \([3H]\text{glucosamine-} \text{ and } [35S]\text{sulfate-labeled} \) glypican PG precursor with long, GlcNH\(_3\)-containing HS chains (Scheme 2A and Ref. 8), eluted as a uniform peak centered at an ionic strength of ~0.8 m (Fig. 4A). The degradation products obtained after heparanase treatment of this PG (Scheme 2A), i.e. long HS-chain fragments (from pool II), core protein with HS stubs (from pool II) and smaller HS fragments (pool III) were chromatographed separately on spermine-agarose (Fig. 4, B–D). Most of the long and free HS-chain fragments comprising the unmodified regions appeared at a somewhat lower ionic strength of ~0.7 m (Fig. 4B). The core protein with HS stubs showed relatively low affinity and eluted as a broad distribution between 0.2 and 0.7 m (Fig. 4C). A major population of the smaller HS fragments comprising the modified regions eluted at the same ionic strength as...
FIG. 3. Localization of heparin lyase and nitrite cleavage sites in heparanase degradation products from high molecular weight glypican PG. Gel permeation chromatography on Superdex peptide of [\(^3\)H]glucosamine- and [\(^35\)S]sulfate-labeled HS chains of glypican PG from BFA-treated ECV 304 cells after treatment with HS lyase (A) and HS lyase followed by mercury (II) acetate (B). The recovery of small saccharides after treatment with mercury (II) acetate was suboptimal. [\(^3\)H]Glucosamine- and [\(^35\)S]sulfate-labeled glypican PG from BFA-treated ECV 304 cells was subjected to cleavage by heparanase of CHO-K1 cells followed by separation on Superose 6 into large and small cleavage products (as in Fig. 2A, pools II and III, respectively). Free HS-chain fragments and core protein with HS stubs were separated by passage of pool II over octyl-Sepharose (see Scheme 2A). Free chain fragments were treated with nitrite at pH 3.9 (as in Fig. 2C) and rechromatographed on the Superdex peptide column (C). The same material was both treated with nitrite at pH 3.9 and digested with heparin lyase and rechromatographed (D). Core protein with HS stubs (see Scheme 2) was chromatographed both before (E) and after treatment with nitrite at pH 3.9 (F). [\(^3\)H]Glucosamine- and [\(^35\)S]sulfate-labeled glypican PG from BFA-treated ECV 304 cells was directly subjected to cleavage by nitrite, passed over octyl-Sepharose to recover core protein with HS stubs, and treated with alkali to release these stubs, which were then chromatographed (G). HS oligosaccharides (as in pool III in Fig. 2A) were chromatographed directly (H), after digestion with heparin lyase (I), and after treatment with nitrite at pH 3.9 (J). In the latter case, only \(^3\)H was recorded. Numbers 2, 4, 6 etc. refer to di-, tetra-, hexa saccharide and so on; 1, 3, 5 etc. refer to mono-, tri-, pentasaccharide and so on. \(V_0\), void volume; \(V_t\), total volume.
the long fragments, indicating that the smaller fragments have the highest relative spermine affinity (Fig. 4D). As shown previously (10), there is also a nonspecific correlation between HS chain size and spermine affinity. The high affinity HS fragments displayed reduced affinity upon digestion with heparin lyase (Fig. 4E), indicating that IdUA(2-OSO₃)/GlcUA(2-OSO₃) residues contribute to the high affinity as shown previously (10).

**Localization of High Affinity Spermine-binding Sites in HS of the Small Glypican Glycoform**—The small size, immunoisolated glypican-1 glycoform present in untreated ECV cells appeared to contain three populations of spermine binding material (Fig. 4F). The most retarded major component eluted at an ionic strength of 0.9 M, i.e. somewhat later than the large glypican PG from BFA-treated cells (Fig. 4A). Although the large glycoform carries HS chains with high affinity spermine-binding sites (see Fig. 4D), there is also a greater number of GlcNH₃⁺ residues. The presence of positive charges is expected to reduce overall spermine affinity.

**Effects of Polyamine Deprivation on the Structure of HS in the Small Glypican Glycoform**—ECV cells were made dependant on an exogenous supply of spermine by treatment with DFMO during growth (11). When the cultures had reached confluency after growth in spermine-containing medium, cells were incubated with [³H]glucosamine and [³⁵S]sulfate for 24 h in the continued presence of DFMO with or without exogenous spermine. The small glypican-1 glycoform was immunoisolated from both cultures, and the HS chains were released by alkali and analyzed for size and GlcNH₃⁺ content (Scheme 2B). As shown in Fig. 5A, the HS chains of glypican-1 from cells radiolabeled during spermine uptake comprised both large and smaller size chains, whereas those obtained in the absence of spermine were mainly of smaller size (Fig. 5E). Furthermore, HS chains of glypican-1 from cells radiolabeled during spermine uptake were partly degraded by nitrite to a series of fragments (Fig. 5B) including sulfated oligosaccharides (V₃), indicating the presence of multiple sites containing GlcNH₃⁺ residues close to the sulfated, highly modified regions. In contrast, HS chains derived from glypican radiolabeled in the absence of concomitant spermine uptake comprised both large and smaller size chains, whereas those obtained in the absence of spermine were mainly of smaller size (Fig. 5E). Although this may reflect greater sulfation, it is also possible that the

**Fig. 4.** Affinity chromatography on spermine-agarose of various glypican PG glycoforms and its degradation products produced in untreated or DFMO-treated cells. [³H]Glucosamine and [³⁵S]sulfate-labeled large size glypican PG obtained by gel and ion exchange chromatography from BFA-treated ECV 304 cells was subjected to cleavage by heparanase of CHO-K1 cells followed by separation on Superose 6 into large and small cleavage products (as in Fig. 2A, pools II and III, respectively). Free HS-chain fragments and core protein with HS stubs were separated by passage of pool II over octyl-Sepharose (see Scheme 2A). The graphs (A–E) show intact large size glypican PG precursor (A) and heparanase degradation products (B–E) of this PG, i.e. long, free HS-chain fragments from pool II (B), glypican core protein with truncated HS chains from pool II (C), and short HS-chain fragments from pool III directly (D) and after further digestion with heparin lyase (E). The graphs (F–H) show [³H]glucosamine- and [³⁵S]sulfate-labeled immunoisolated, small-size glypican PG obtained from untreated ECV 304 cells (F) and from DFMO-treated cells after radiolabeling in the presence (G) or absence of spermine uptake (H). The graphs (I–J) show [³H]glucosamine- and [³⁵S]sulfate-labeled large size glypican PG obtained from BFA- and DFMO-treated ECV 304 cells by gel and ion exchange chromatography after radiolabeling in the presence (I) or absence of spermine uptake (J). The graphs (K–M) show results obtained with endogenously formed intracellular HS oligosaccharides isolated from Triton X-100 extracts by desalting on PD-10, chromatography on Superdex peptide to remove small saccharides, and recovery of the excluded material (fractions 18–28) followed by chromatography on Superose 6 to remove PG material. The pooled oligosaccharides (fractions 39–51) were from untreated (K) and from DFMO-treated cells after radiolabeling in the presence (L) or absence of spermine uptake (M). The column was eluted with a linear guanidinium chloride gradient (see the dotted line in the top panels). ○, [³H]; □, [³⁵S].
presence of cationic spermine affects uptake of sulfate and glucosamine.

Effects of Polyamine Deprivation on the Structure of HS in the Large Glypican Glycoform—The HS chains of the BFA-arrested, large glypican PG precursor produced in cells with up-regulated polyamine uptake were also examined for free amino groups. When the cultures had reached confluence under the same regimen as above, cells were incubated with $[^3H]$glucosamine and $[^35S]$sulfate in the presence of both DFMO and BFA with or without exogenous spermine. Glypican PG was isolated from both cultures, and the HS chains were released by alkali and analyzed for size and GlcNH$_3^+$ content. As shown in Fig. 5, C–D and G–H, respectively, the HS chains were similar in size and extensively degraded by nitrite in both cases. The number of GlcNH$_3^+$ residues clearly exceeded that seen in HS chains of BFA-arrested glypican from ECV cells with undisturbed endogenous polyamine synthesis (see Ref. 8 and Fig. 4).

To investigate possible effects of intracellular polyamine deprivation on the sulfation of HS, we measured the content of sulfated IdoUA residues by degradations with heparin lyase. Mature HS chains derived from the large BFA-arrested PG precursor isolated from ECV cells with undisturbed endogenous polyamine synthesis were digested with heparin lyase and chromatographed on Superose 6 (Fig. 6A). One pool of larger, lower sulfated fragments and another pool of smaller, higher sulfated fragments were obtained. The latter were examined for GlcNH$_3^+$ residues by treatment with nitrite at pH

**FIG. 5.** Effects of DFMO treatment on the content of N-unsubstituted glucosamine in HS. $[^3H]$Glucosamine- and $[^35S]$sulfate-labeled immunoisolated, small-size glypican PG (A and E) and radiolabeled, large size BFA-arrested glypican PG (C and G) isolated by gel and ion exchange chromatography were obtained from DFMO-treated cells after radiolabeling in the presence (A–D) or absence of spermine uptake (E–H). HS chains were released from the respective PG by alkali and chromatographed on Superose 6 before (A, C, E, and G) and after subsequent deaminative cleavage (B, D, F, and H). O, $[^3H]$; ■, $[^35S]$; V, void volume; V, total volume.
3.9 followed by gel permeation chromatography on Superose 6 (Fig. 6B). No degradation could be observed. Furthermore, there was no release of small saccharides from the termini of the fragments, as indicated by chromatography on Superdex peptide (data not shown). Hence, there was little clustering of IdoUA(2-OSO₃)/GlcUA(2-OSO₃) and GlcNH₃/H₁₁₀₀₁ residues in accordance with the results presented above (Fig. 1H and 3J).

The results obtained with HS chains of corresponding PG from DFMO-treated cells obtained after radiolabeling in the presence or absence of spermine are shown in Fig. 6, C and D, respectively. Both HS preparations were sensitive to degradation by heparin lyase and afforded, as in the preceding case, one pool of larger fragments and one pool of smaller ones. The yields of the latter fragments (see the bars in Fig. 6, A, C, and D) were 1.5-fold greater in the case of material from cells treated with DFMO alone are shown in G and H, respectively. 

The results obtained with HS chains of corresponding PG from DFMO-treated cells obtained after radiolabeling in the presence or absence of spermine are shown in Fig. 6, C and D, respectively. Both HS preparations were sensitive to degradation by heparin lyase and afforded, as in the preceding case, one pool of larger fragments and one pool of smaller ones. The yields of the latter fragments (see the bars in Fig. 6, A, C, and D) were 1.5-fold greater in the case of material from cells treated with DFMO alone. Material from cells treated with DFMO and spermine were chromatographed before (E) and after deaminative cleavage (F), and the corresponding results for material from cells treated with DFMO alone are shown in G and H, respectively.

Effect of Polyamine Deprivation on the Affinity of Heparan Sulfate for Spermine (Scheme 2C)—We examined the effects of polyamine deprivation on the spermine affinity of the various glypican-1 glycoforms and HS-degradation products by affinity chromatography (see Fig. 4). The small size GlcNH₃-rich glypican-1 glycoform obtained from DFMO-treated cells radiolabeled in the presence of spermine contained more components with lower affinity for spermine (Fig. 4G) compared with material from cells with ongoing endogenous polyamine synthesis (Fig. 4F). The extreme clustering of IdoUA(2-OSO₃)/GlcUA(2-OSO₃) and GlcNH₃ residues in HS of this glypican glycoform from DFMO-treated cells (see Figs. 5B and 6C) may have inhibited spermine binding. Accordingly, GlcNH₃-poor glypican-1, produced by DFMO-treated cells in the absence of spermine uptake, comprised a major high affinity population (Fig. 4H) of similar affinity (0.8–0.9 M) to that of glypican from unperturbed cells (Fig. 4F).

We also examined the effect of DFMO treatment on the spermine affinity of the large size, BFA-arrested glypican. The
PG precursor produced in cells with up-regulated polyamine transport had a greater affinity for spermine (peak elution positions 0.9–1.0 M in Fig. 4, I and J) than the PG from cells with undisturbed endogenous polyamine synthesis (0.8 M, Fig. 4A). It should be pointed out that the two PG forms from DFMO-treated cells contained more free amino groups, i.e. more positive charges, than PG from untreated cells that would counteract the binding to spermine-agarose. Hence, they should harbor sites with increased spermine affinity to compensate for their lower net negative charge. Accordingly, upon ion exchange chromatography on MonoQ the BFA-arrested PG from cells with endogenous polyamine synthesis bound more strongly than the corresponding PG from DFMO-treated cells (data not shown). This is in agreement with their net negative charges and indicates that spermine binding can be specific and not only a nonspecific electrostatic attraction.

We finally examined the endogenously generated HS oligosaccharides that are degradation products of the large size glypican PG precursor (7) from cells with undisturbed polyamine synthesis and from cells treated with DFMO to inhibit polyamine synthesis and increase polyamine uptake. HS oligosaccharides formed in cells with endogenous polyamine synthesis showed relatively low spermine affinity and eluted in three pools at 0.4, 0.5, and 0.7 M (Fig. 4K). In the oligosaccharide pool obtained from DFMO-treated cells, a separate component with high spermine affinity (elution position, 0.7 M) was markedly increased (Fig. 4, L and M).

Effect of Nitrite Deprivation on Spermine Uptake—As shown in Fig. 5D, up-regulation of spermine uptake by DFMO treatment resulted in an increased content of GlcNH₃ in the HS chains of the large size glypican PG precursor. The HS degradation products (oligosaccharides) generated endogenously from this glycome during glypican recycling (7) were also examined for the presence of GlcNH₃ residues by treatment with nitrite and chromatography on Superdex peptide. There was no indication of cleavage in any of the samples (data not shown), indicating that both heparanase- and nitrite-generated cleavage was taking place during their endogenous formation. It is possible that NO/nitrite-generated cleavage of HS is required for spermine uptake. We therefore examined whether spermine uptake was affected by nitrite deprivation (Fig. 7). ECV cells displayed Michaelis-Menten polyamine uptake kinetics with a $K_m$ value of $\sim$0.5 mM, i.e. in the same range as other cultured cell lines (11). DFMO treatment resulted in a 1.5-fold increase in $V_{max}$ for spermine uptake but no change in $K_m$, as indicated by a Lineweaver-Burke plot (data not shown), suggesting an increased number of spermine-binding sites or transporters. Uptake was reduced to control level either by scavenging nitrite with sulfamate or by preventing NO release from nitrosothiols by the Cu(I)-selective chelator neocuproine. By treatment with the selective endothelial NO synthase inhibitor N-nitroarginine, uptake of spermine was reduced even further, presumably because the ultimate source of nitrite (NO released from Arg) was depleted.

DISCUSSION

Cell surface HSPG are selective regulators of ligand-receptor encounters. Thereby they can regulate the signaling of growth factors and morphogens during growth and developmental patterning (for reviews, see Refs. 2, 21, and 22). HSPG are also involved in the uptake and internalization of small basic molecules such as polyamines (11) and basic peptides such as fibroblast growth factor (23) and human immunodeficiency virus-1 Tat (24) as well as of virus particles (25, 26). The glycosylphosphatidylinositol-anchored HSPG glypicans may localize to caveolae, membrane domains involved in specific forms of internalization (27). However, the mechanism for transfer of HS-
Polyamines are bound electrostatically to the HS chains of glypican and are carried into the cell when glypican is internalized, possibly via caveolae. As heparanase degrades HS, polyamines are released, probably still bound to the HS fragments. Glypican with the residual HS stubs return to the Golgi where HS chains are resynthesized provided that the GlcNH$_3^+$ units are cleaved-off via deaminative cleavage catalyzed by endogenously formed NO-derived nitrite. Polyamines exit from caveolae, endosomes, or possibly caveosomes (27) by some unknown mechanism. Small HS fragments generated by the combined action of heparanase and nitrite on the structurally altered HS in polyamine-deprived cells could support membrane penetration of polyamines.

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