Syndecan-1 and -4 Synthesized Simultaneously by Mouse Mammary Gland Epithelial Cells Bear Heparan Sulfate Chains That Are Apparently Structurally Indistinguishable*

Received for publication, September 20, 2002, and in revised form, February 4, 2003
Published, JBC Papers in Press, February 5, 2003, DOI 10.1074/jbc.M209658200

Masahiro Zako‡, Jianying Dong‡, Olga Goldberger‡, Merton Bernfield‡, John T. Gallagher¶, and Jon A. Deakin¶
From the ‡Division of Newborn Medicine, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02215 and the ¶Department of Medical Oncology, Cancer Research UK and University of Manchester, Christie Hospital NHS Trust, Manchester M20 4BX, United Kingdom

Many of the biological functions attributed to cell surface heparan sulfate (HS) proteoglycans, including the Syndecan family, are elicited through the interaction of their HS chains with soluble extracellular molecules. Tightly controlled, cell-specific sulfation and epimerization of HS precursors endows these chains with highly sulfated, iduronic acid-rich regions, which are major determinants of cytokine and matrix-protein binding and which are interspersed by N-acetylated, poorly sulfated regions. Until this study, there have been no comprehensive structural comparisons made on HS chains decorating simultaneously expressed, but different, syndecan core proteins. In this paper we demonstrate that the HS chains on affinity-purified syndecan-1 and -4 from murine mammary gland cells are essentially identical by a number of parameters. Size determination, disaccharide analyses, enzymatic and chemical scission methods, and affinity co-electrophoresis all failed to reveal any significant differences in fine structure, domain organization, or ligand-binding properties of these HS species. These findings lead us to suggest that the imposition of the fine structure onto HS occurs independently of the core protein to which it is attached and that these core proteins, in addition to the HS chains, may play a pivotal role in the various biological functions ascribed to these macromolecules.

Heparan sulfate proteoglycans (HSPGs)† are critically involved in a wide variety of biological phenomena including organogenesis, angiogenesis, regulation of blood coagulation, growth factor/cytokine action, cell adhesion, lipid metabolism, and wound healing (1–4). All these activities are elicited primarily through the heparan sulfate (HS) chains at the cell surface. The high degree of structural diversity observed in these HS chains is believed to impart the specificity of function of these macromolecules. Such diversity of structure is imposed by tightly regulated patterns of sulfation and epimerization upon the basic polysaccharide backbone during synthesis within the Golgi (2, 5). Highly regulated HS synthesis permits HSPGs to accomplish their distinct biological roles, such as basic FGF binding and activation, which are mediated through specific sequences within the chains (6) and which can be markedly modulated by the physiological degradation of HS in vivo (7). Recently, HSPGs have been shown to regulate signaling by many diverse growth factors, including FGFs, members of the TGF-β superfamily, Wnt, heparin-binding-EGF, and hepatocyte growth factor through HS (reviewed in Refs. 4, 8, and 9). Thus, the HS chains are believed to be the principal determinants of the binding and regulatory activity of HSPGs on the external surface of the plasma membrane.

Syndecans are a major family of four kinds of transmembrane HSPGs (4, 10–13). The mammalian syndecans are similar in primary sequence in the cytoplasmic and membrane-spanning regions, but the extracellular domains (ectodomains) are largely non-homologous. According to the chromosomal localization, exon organization, and sequence relationships with Drosophila syndecan, the syndecan gene is thought to have arisen by gene duplication and divergent evolution from a single ancestral gene and that syndecan-1 and -3 and syndecan-2 and -4 comprise separate subfamilies (14). These members of the syndecan family are expressed in distinct cell-, tissue-, and developmental stage-specific patterns (15, 16). It suggests that each syndecan family member may have distinct functions (17), although some shared activities of, for example syndecan-1 and -4, have been observed (4, 11, 18, 19). Thus far, evidence from knock-out mice indicates that no critical step in development depends on a specific syndecan gene.

Syndecan-1 and -4 promote intercellular adhesion following their translocation into human B lymphoid cells, suggesting that both of them are important mediators of this event (20). Both syndecan-1 and -4 are induced during wound repair, but their cellular localization is different (21), suggesting that although both are involved in the wound healing process their functions may be dissimilar. Kinnunen et al. describe a unique role for syndecan-3 in the binding and activity of heparin-binding growth-associated molecule at E11 in the rat rhomboencephalon during neurite outgrowth (22), while syndecan-4 has a distinctive role in the generation and maintenance of focal adhesion complexes (23, 24) and in signal transmission during dendritic process formation (25). The ectodomains of all synde-
cans may be shed intact by proteolytic cleavage of the core protein in a site adjacent to the plasma membrane. Proteolytic activity causes the release of syndecan-1 and -4 ectodomains in acute human dermal wound fluids (26) where they modify the protease/antiprotease balance (27). Recently, it was shown that the shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive cell surface metalloproteinase (28). Although it is well documented that these molecules may display sometimes similar and sometimes very different biological activities, it is unclear whether it is the protein core component or the GAG chains of these proteoglycans that are the major determinants of differences in biological activity between syndecan family members. Comparative structural and functional analyses of the HS chains on syndecan-1 and -4 have not been undertaken to date. In the present study we describe such analyses on HS chains that were prepared simultaneously from the same cell type, namely NMuMG (normal murine mammary gland epithelium), and show that HS of syndecan-1 and -4 have highly similar, fine structural profiles and ligand-binding activities. These data provide further insights into the regulation of HS biosynthesis within a particular cell population. The findings indicate that heparan sulfate structure, although highly dependent upon cell type, appears to display very little variability within the syndecan population of an individual cell type (in this instance NMuMG), an observation that may have notable implications in the understanding of the biological roles of glycosaminoglycan chains.

**EXPERIMENTAL PROCEDURES**

**Materials**—Na₂³⁵SO₄ (carrier-free, -specific activity, 1200–1400 Ci/mmol) was obtained from PerkinElmer Life Sciences. Heparinase I (Flavobacterium heparinum, heparanase, heparin lyase, EC 4.2.2.7), heparinase II (F. heparinum; no EC number assigned), and heparinase III/heparitinase (F. heparinum; heparin-sulfate lyase, EC 4.2.2.8) were purchased from Gakushu Enzymes (Aberdeen, UK). Chondroitin ABC lyase (Proteus vulgaris; EC 4.2.2.4) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Sodium chloride (HiPerSolv grade) for HPLC and all other analytical grade reagents were supplied from BDH-Merck Ltd. (Lutterworth, Leicestershire, UK). Ultra-pure water (resistance 18 megohms) for all HPLC analysis was dispensed from a Milli-Q water system (Millipore, Milford, MA). Bio-Gel P-6 (fine grade) was from Bio-Rad (Hemel Hempstead, Hertfordshire, UK). ProPac PA-1 analytical columns were purchased from Dionex (Camberley, Surrey, UK). Sepharose CL-6B was obtained from Pharmacia Biotech (Upsala, Sweden). Low-melting point agarose (SeaPlaque) and Gelbond were from FMC Corp. Bioproducts (Rockland, ME). Polyvinylidene difluoride membranes (Millipore, UK). Bio-Gel P-6 column (1 × 106 cm, eluted at 3 ml/min in 0.1 mM ammonium hydrogen carbonate) or on a Sepharose CL-6B column (as described above), collecting 1 ml fractions. Peaks of radioactivity were determined by liquid scintillation counting. Additional digests, containing 13,000 cpm each of syndecan-1 and syndecan-4, were undertaken in which the resulting oligosaccharides were then analyzed as described above, except for non-specific staining omitted the primary antibody. No staining was observed in control samples. Preparation of Cell Surface Syndecan-1 and -4—To prepare the extracellular domains of syndecan-1 and -4, the conditioned media of NMuMG were used (15). After anion exchange chromatography, the samples were applied to density gradient centrifugation, and the fractions whose specific gravity were more than 1.35 g/ml were collected. Syndecan-1 and -4 were purified by using 281–2 and KY/8.2 affinity columns, respectively. To purify the labeled syndecan-1 and -4, mildly treated trypsin was also used (15). For the following HS structural and affinity co-electrophoresis analyses, the labeled samples were digested with chondroitin ABC lyase before the affinity chromatography.

**Size Determination of the HS Chains of Syndecan-1 and -4—**Proteoglycans were dissolved in 500 μl of distilled water containing 0.1% (w/v) CHAPS (Sigma) and then subjected to eliminative cleavage and reduction by adjusting to 100 mM NaOH/1 mM NaBH₄ for 24 h at 37 °C. After neutralizing samples by dropwise addition of 1 M HCl, free HS chains were recovered by separation on a Sepharose CL-6B column (1 × 97 cm) eluting in phosphate-buffered saline at a flow-rate of 10 ml/h and collecting 1-ml fractions. Radioactivity in each fraction was determined by liquid scintillation counting. The void volume (V₀, fraction 25) and total volume (V₅, fraction 63) of the column were established using Dextran Blue and sodium dichromate, respectively.

**Disaccharide Compositional Analysis of HS Chains—**Aliquots of 7000 cpm (32S) each of syndecan-1 and -4 HS chains were adjusted to 50 mM sodium acetate/0.5 mM calcium acetate, pH 7.0, before addition of 20 μM each of heparinase I, II and III in a total volume of 100 μl or less. Samples were incubated for 4 h at 37 °C followed by a second addition of the same amount of enzymes and incubation for a further 24 h at 37 °C. Each digest was diluted to 1 ml with distilled water adjusted to pH 3.5 by addition of HCl before application to a ProPac PA-1 strong-anion exchange (SAX) column (4.6 mm × 25 cm, Dionex UK) attached to a gradient HPLC pump (Dionex, Sunnyvale, CA). Disaccharides were eluted in a gradient of 0–1.0 M NaCl, pH 3.5, over 45 min at a flow rate of 1 ml/min, collecting 0.5-ml fractions (31). Radioactivity in each fraction was determined by liquid scintillation counting. Disaccharides were identified by comparison with unlabeled disaccharide standards of known composition that were monitored on-line UV absorption at λ₂₃₂ nm. Recoveries of radio-labeled sample were typically between 85–90%. Very little radioactivity was detected after elution of the most sulfated disaccharide, indicating almost complete depolymerization of GAG chains. Analysis of Heparan I, Heparan III, and Nitrous Acid-derived Oligosaccharides—Aliquots of 70000 cpm (32S) each of syndecan-1 or -4 in distilled water containing 0.1% CHAPS were adjusted to 50 mM sodium acetate/0.5 mM calcium acetate, pH 7.0 before addition of 40 μM each of either heparinase I or heparinase III (heparitinase) and incubation at 25 °C for 16 h. Additionally, similar aliquots were subjected to low pH nitrous acid cleavage (32) for 20 min, followed by neutralization by dropwise addition of 2 mM sodium carbonate. The resulting oligosaccharide digestion products from the above treatments were then resolved by size exclusion chromatography on a Bio-Gel P-6 column (1 × 106 cm, eluted at 3 ml/min in 0.1 mM ammonium hydrogen carbonate) or on a Sepharose CL-6B column (as described above), collecting 1 ml fractions. Peaks of radioactivity were determined by liquid scintillation counting. Additional digests, containing 13,000 cpm each of syndecan-1 and syndecan-4, were undertaken in which the resulting oligosaccharides were then analyzed on a ProPac PA-1 SAX-HPLC column eluted with a gradient of 0–1.2 M NaCl, pH 3.5, over 90 min at a flow-rate of 1 ml/min (Dionex, Sunnyvale, CA), monitoring UV absorption at λ₂₃₂ nm. Recoveries for the above were typically above 85% of added material.

**Affinity Co-electrophoresis (ACE) Analysis**—NMuMG cells were labeled with radio-sulfate, and the [³⁵S]sulfate-labeled syndecan-1 and -4 ectodomains from conditioned medium was purified as described above. HS chains from syndecan-1 and -4 were released by incubation with 0.5 M NaOH/1 M NaCl at 4 °C for 24 h (15). Binding of each iodinated syndecan-1 and -4 (12,500 cpm) was electrophoresed through these lanes. The migration on syndecan-1 and -4 was detected on a Phosphorimage (Molecular Dynamics, Sunnyvale, CA). The pixel intensities were integrated and used to determine the migration distance of the major peak.
Simultaneously Expressed Syndecan-1 and -4 on the NMuMG Cells—Histochemical analysis was performed on the cultured NMuMG cells to investigate the simultaneous expressions of syndecan-1 and -4. For this purpose, we used 281–2 monoclonal and M5E-4 polyclonal antibodies to detect syndecan-1 and -4, respectively (Fig. 1). Syndecan-1 was expressed at the cell surfaces of NMuMG cells uniformly as shown previously (34, 35), while the focal expression of syndecan-4 was seen at the extracellular matrix regions as previously shown by Woods and Couchman (23). Syndecan-1 expressed rather strongly along the cell-cell interaction regions, whereas the syndecan-4 was expressed faintly. No signals were detected on the cells stained by the second antibody alone (data not shown).

RESULTS

Simultaneously Expressed Syndecan-1 and -4 on the NMuMG Cells—Histochemical analysis was performed on the cultured NMuMG cells to investigate the simultaneous expressions of syndecan-1 and -4. For this purpose, we used 281–2 monoclonal and M5E-4 polyclonal antibodies to detect syndecan-1 and -4, respectively (Fig. 1). Syndecan-1 was expressed at the cell surfaces of NMuMG cells uniformly as shown previously (34, 35), while the focal expression of syndecan-4 was seen at the extracellular matrix regions as previously shown by Woods and Couchman (23). Syndecan-1 expressed rather strongly along the cell-cell interaction regions, whereas the syndecan-4 was expressed faintly. No signals were detected on the cells stained by the second antibody alone (data not shown).

We also performed the immunohistochemical staining of syndecan-4 on the NMuMG cells using KY/8.2 monoclonal antibody that we used for the purification of syndecan-4 for the following analyses. KY/8.2 monoclonal antibody also showed the focal expression of syndecan-4. These results indicate that syndecan-1 and -4 are co-expressed in NMuMG, albeit with spatially different distributions. Therefore, metabolically labeled syndecan-1 and -4 were purified from these cells for HS structural analyses.

Alkali/Borohydride Eliminative Cleavage of HS on Syndecan-1 and -4—NMuMG cells that were taken at 50% confluence were metabolically radiolabeled for 48 h with $^{35}$S-orthosulfate. Radiolabeled syndecan-1 and -4 were purified from the radiolabeled conditioned medium and cells as described under “Experimental Procedures.” HS chains from syndecan-1 and -4 were produced by alkali borohydride eliminative cleavage from the respective proteoglycans and then applied to Sepharose CL-6B column to compare the relative sizes of the HS chains. There is no major size difference on the HS chain sizes of syndecan-1 and -4.

Low pH Nitrous Acid Scission of HS on Syndecan-1 and -4—The HS chains from both syndecans were individually subjected to low pH nitrous acid degradation, which results in specific scission at N-sulfated disaccharides with the concomitant loss of the N-sulfate groups (32). In these $^{35}$S-labeled HS preparations, wherever N-sulfated disaccharides are situated consecutively within the chain, nitrous acid digestion will result in the liberation of disaccharides and free $^{35}$SU.

When N-sulfated disaccharides are interspersed by disaccharides containing N-acetylated glucosamine residues (which are resistant to attack by nitrous acid) fragments larger than disaccharides will be generated, but with a $^{35}$S-label these will be visible only if they contain additional O-sulfates. The size of resistant oligosaccharides will be a reflection of the number of consecutive N-acetylated disaccharides possessed by that oligosaccharide. Fig. 3 indicates that there is a close association between N- and O-sulfation, which is a characteristic feature of HS. Approximately 89 and 90% of the radiolabel from syndecan-1 and -4, respectively, is represented in the above peak. The remainder of the sulfates, i.e. O-sulfates are located in alternating sequences, represented by peaks of radioactivity corresponding to tetrasaccharides, and containing 11 and 10% of the total incorporated $^{35}$SU for syndecan-1 and -4, respectively. Larger, sulfated oligosaccharides are not observed, demonstrating that in both HS species, there is a strong linkage between N- and O-sulfates, such that O-sulfate does not appear to occur more than one disaccharide away from an N-sulfate.
Heparinase III Scission—Heparinase III (heparitinase) can cleave only at unsulfated hexuronic acid (HexUA) (glucuronic acid (GlcUA) or iduronic acid (IdoUA)) residues, especially if there are relatively few SO₄ groups on the adjacent residues. Heparinase III largely cleaves HS at hexosaminidic linkages to glucuronic acid residues (GlcN(NS or NAc, +6S)-GlcUA) (37–39), although the enzyme also shows activity against some glucosaminidic linkages to iduronic acid (40) but with reduced efficiency. The samples of digested syndecan-1 and -4 were subjected to heparinase III digestion and then applied to a Bio-Gel P-6 chromatography to assess the distribution and ratios of the resulting oligosaccharides. Fig. 4 illustrates the profiles of heparinase III-resistant fragments derived from both syndecan-1 and -4 heparan sulfates. This approach revealed no significant structural differences between the two HS species under investigation, with both displaying a similar pattern of resistant fragments ranging in size from disaccharide (dp2) to around tetradecasaccharide (dp14) in very similar proportions (Table I). Minor variations in the proportional spread of radiolabel among the resistant fragments of this digest may reflect incomplete enzymic scission at a small proportion of its potential cleavage sites. The small peaks of radioactivity between fractions 84 and 85 arose due to partial resolution of the disaccharides into lower and higher sulfated constituents. The contents of 2-O-sulfation, 6-0-sulfation and N-sulfation in the labeled syndecan-1 and -4 samples are ~54, 46–51, and 89% of the total sulfated disaccharide population, respectively (see Table II). Regions of the chains resistant to heparitinase scission represent the higher sulfated domains (S-domains) of HS.

The oligosaccharides generated by this enzyme were also subjected to SAX-HPLC (Fig. 5) and similarly displayed only minor differences between the two species under investigation. Both the pattern and proportion of resulting oligosaccharides were similar for both syndecans, indicating a comparable distribution of glucuronate residues within the two HS species. The small differences between profiles may be due to incomplete cleavage of a minority of the potential susceptible linkages between the chains.

Heparinase I Scission—To gain further information on the structure of HS chains, the enzyme heparinase I was used. This enzyme cleaves HS essentially where GlcNS(±6S)-IdoUA(2S) residues occur (37, 38), although the enzyme is active against glucuronate-2-O-sulfate (41), a rare constituent of HS (42, 43). This enzyme generated very similar amounts of di-, tetra-, hexa-, and octasaccharide in both syndecan-1 and -4 HS (Fig. 6). Furthermore, the resistant sulfated fragments resulting from heparinase I digestion were the same average size ($K_w$ of...
0.73 (7 kDa), which corresponds to an average size of around 14−16 disaccharides, and had the same size distribution when the total digest was analyzed by Sepharose CL-6B size exclusion chromatography (Fig. 7).

**Total Disaccharide Composition—**$^{35}$S-radiolabeled HS chains from both syndecan-1 and -4 were completely degraded to disaccharides by the combined actions of heparinases I, II, and III. The disaccharides were separated by gradient SAX-HPLC, and the results are shown graphically (Fig. 8) and numerically (Table II). Disaccharides were identified by comparison of their elution positions relative to those of known, unlabelled disaccharide standards. It was not possible to determine the levels of the non-sulfated disaccharide, $\Delta$UA-GlcNAc as the material was metabolically radiolabeled with $^{35}$S alone. The major $^{35}$S-radiolabeled disaccharides in both labeled syndecan-1 and -4 were $\Delta$UA(2S)-GlcNS(6S) and $\Delta$UA(2S)-GlcNAc(6S). The $\Delta$UA(2S)-GlcNAc(6S) was not detected in syndecan-1 and -4. The data indicate no major differences between the two types of HS chain at the gross level of analysis. The overall levels of both N- and 2-O-sulfation (89.3/53.9% and 88.9/54.0% for syndecan-1 and -4, respectively) of the two HS types are in very good agreement with each other as are the total sulfation levels (189.4% and 193.6% for syndecan-1 and -4, respectively). Similarly, the estimated levels of N-sulfation for both syndecans by this enzymatic technique are in very close agreement with those established by the chemical method employing low pH nitrous acid (see above). A relatively small difference in 6-O-sulfation of 4.5% was noted, though this is possibly within experimental variation for this system using this quantity of radiolabeled material. These findings are compatible with the same elution profiles of syndecan-1 and -4 treated with nitrous acid, heparinase I, II, and III (Figs. 3−8).

Summarizing the above, the following can be concluded about the two HS species investigated in this study: 1) the chain lengths, the size distribution of chains, and overall di-

### Table II

| Disaccharide                        | Syndecan-1 | Syndecan-4 |
|------------------------------------|------------|------------|
| $\Delta$UA-GlcNAc                  | ND         | ND         |
| $\Delta$UA-GlcNAc(6S)              | 8.5        | 8.9        |
| $\Delta$UA-GlcNS                  | 28.7       | 27.4       |
| $\Delta$UA-GlcNS(6S)              | 8.9        | 9.7        |
| $\Delta$UA(2S)-GlcNAc             | 2.2        | 2.2        |
| $\Delta$UA(2S)-GlcNAc(6S)         | ND         | ND         |
| $\Delta$UA(2S)-GlcNS              | 22.9       | 19.7       |
| $\Delta$UA(2S)-GlcNS(6S)          | 28.8       | 32.1       |

**Values represent the proportion of the total disaccharides produced by combined enzymic digestion, and depolymerization was greater than 95% complete. ND, not detected.**

Fig. 5. **Strong anion-exchange HPLC analysis of the oligosaccharides of syndecan-1 and -4 after depolymerization with heparinase III.** Aliquots of syndecan-1 and -4 HS chains were subjected to heparinase III (heparitinase) digestion, and the resulting total oligosaccharides were completely degraded by combined heparinase I, II, and III digestion, and the resulting disaccharides were analyzed by HPLC anion-exchange chromatography as described under "Experimental Procedures." Values represent the proportion of the total disaccharides produced by combined enzymic digestion, and depolymerization was greater than 95% complete. ND, not detected.

Fig. 6. **Bio-Gel P-6 column chromatography of HS chains on syndecan-1 and -4 after depolymerization with heparinase I.** Aliquots of each of the syndecan chains were subjected to heparinase I digestion and then applied to a Bio-Gel P-6 column to assess the distribution and ratios of the resulting oligosaccharides. The syndecan-1 and -4 HS gave very similar yields of low molecular weight oligosaccharides. The number associated with each peak corresponds to the degree of polymerization of oligosaccharides (e.g. dp 2, disaccharides). $V_o$ and $V_t$ were determined using hemoglobin and sodium dichromate, respectively.
saccharide compositions are almost indistinguishable; 2) the distribution of N-sulfated residues within the chains are apparently the same; 3) the proportion and distribution of IdoUA(2S) and GlcUA residues within the chains are almost identical. It can only be concluded that the two different syndecans (syndecan-1 and -4) produced by the same cell, although having very different core proteins, are decorated by substantially similar HS chains.

**Syndecan-1 and -4 Show Same Specificity in Binding to Type I Collagen and FGF-2**

To examine whether these two proteoglycans, bearing essentially the same HS, bind type I collagen and FGF-2 in a similar manner, metabolically labeled ectodomains or alkali-released HS chains were purified from the culture medium of NMuMG cells and subjected to ACE (33). Type I collagen and FGF-2 are both known to bind syndecan-1 (15).

The ACE profiles with type I collagen and both syndecan-1 and -4 ectodomains showed similar binding activities (Figs. 9 and 10). Based on the median mobilities, the apparent \( K_d \) values for the interaction of syndecan-1 and -4 with type I collagen were 38 nM in both instances, confirming previously determined values (15). Intact syndecans bind type I collagen with a slightly higher affinity than the isolated HS chains, presumably because of the multivalent nature of the proteoglycan or possibly due to a small but significant contribution of the protein core to the binding. The apparent \( K_d \) values for the binding of isolated HS chains of both syndecans to type I collagen were 175 nM.

The ACE profiles utilizing FGF-2 again revealed apparently identical affinities for both syndecan-1 and -4 (Fig. 10). The calculated \( K_d \) values for intact syndecan-1 and -4 were both 28 nM, similar to values shown previously (15). Similar ACE profiles were obtained with the isolated HS chains, and once more these \( K_d \) values were almost indistinguishable (56 and 52 nM for syndecan-1 and -4, respectively).

The agreement in the binding values for type I collagen and FGF-2 with syndecan-1 and -4 support the hypothesis that, when prepared simultaneously from NMuMG cells, these proteoglycans possess HS chains that have almost identical structures. These data also indicate that the binding of both of these proteoglycans to these protein ligands is mediated primarily through their GAG chains with only a minor contribution being made from their protein core components.

**DISCUSSION**

This paper describes detailed analyses of the structures and ligand-binding properties of two related but functionally different members of the syndecan family, syndecan-1 and -4, derived from the surfaces of identical cell types. Though related, these two proteoglycans and the other two members of the syndecan family display dissimilar topological distribution and histotypic organization, and although some of their biological activities overlap (for example they are capable of activating bFGF in experimental systems), there are nonetheless some important functional differences between them (see the Introduction).

Studies to date have indicated that the fine structures of the HS chains borne on syndecan-1 can vary considerably in their
molecular fine structures in an apparently cell type-specific manner (15, 44), but prior to this study it was unclear whether different members of this proteoglycan family, synthesized in the same cells, are modified with structurally similar HS chains or with uniquely different ones. The present study demonstrates that the HS moieties of syndecan-1 and -4, synthesized simultaneously in NMuMG cells are, indeed, almost indistinguishable at the gross and fine structural levels and with respect to protein ligand binding.

The molecular mass of syndecan-1 may differ according to cell type (NMuMG cell, NIH/3T3, BALB/3T3) due primarily to differences in the length of its constituent HS chains as opposed to alterations in the gross domain organization of the HS (15). We now reveal that syndecan-1 and -4 derived from the same NMuMG cells bear HS chains of very similar molecular size (both ~40 kilodaltons as determined by CL-6B size exclusion chromatography). Furthermore, the overall molecular organization of the polysaccharides and their sulfation patterns are remarkably similar as determined by comparison of the frequency and disposition of N-sulfated glucosamine residues (Fig. 3 enzymic depolymerization profiles and Table I), disaccharide compositions and O-sulfate ratios (Figs. 4–7 and Table I).

The syndecan core proteins share highly conserved transmembrane and cytoplasmic domains, with the exception of the V-regions (10) and, as illustrated by this study, essentially identical HS chains; therefore the question arises as to how these proteoglycans exhibit their biologically different functions. The V-regions of syndecans vary in both length and amino acid sequence, such that, for instance, syndecan-4 through binding and activation of protein kinase-C and phosphatidylinositol-4,5-diphosphate participates in the formation of focal adhesions, whereas that of syndecan-2 being a substrate for a different array of kinases does not (reviewed in Ref. 4). Another possibility derives from their structurally divergent extracellular domains (ectodomains). Sequence variability is greatest in these domains, with the exception of the glycosami-
noglycan attachment sites proximal to the N terminus, which are relatively well conserved. The calculated $M_r$ values for the core proteins of syndecan-1 and -4, for example, are quite different at 30.6 and 19.5 kDa, respectively. The syndecan-4 ectodomain core protein has a high affinity binding site for an unknown ligand on the surfaces of several human and mouse cell types, whereas the syndecan-1 core protein ectodomain shows only weak binding to the surface of Swiss 3T3 cells (45). The syndecan-4 interaction with cell surfaces shows specificity in that its ectodomain, but not that of any other proteoglycan, can block this binding (46). There is also a potential contribution of chondroitin sulfate (CS) chains to consider; however, there is limited information about the role of CS chains on syndecans-1 and -4, but by analogy with other proteoglycans they could modify the protein binding characteristics of these macromolecules (47, 48).

Core protein synthesis is cell type-specific and developmentally regulated (reviewed in Ref. 9). Contained within the sequences of these proteins is the information needed to instruct cells to initiate GAG chain synthesis upon them (either HS or CS/DS), but increasing evidence suggests that their role stops there and that subsequent postpolymeric modification is dictated by the Golgi apparatus, utilizing a particular repertoire of biosynthetic enzymes possessed by that cell. This was illustrated by studies on the matrix proteoglycan perlecain, in which three proteoglycans from three different cellular sources were investigated. Essentially the same protein core was shown to be HS chains that were not only structurally dissimilar but also varied in their ability to modulate the biological activities of FGF-1 and FGF-2 (49). Similarly, during glial cell progenitor differentiation down to astrocytic or oligodendritic lineages, it was observed that a marked change from the expression of heparin to heparan sulfate occurred upon the surface of these cells, although no concomitant alteration in the core protein expression pattern was detectable (50). Additionally, two unrelated HSPGs extracted from fibroblasts, namely syndecan-4 (membrane intercalated) and glypican-1 (GPI anchored), were demonstrated to possess HS chains with no major domain or fine structural differences between them and almost inseparable affinities for the Hep-II domain of the matrix component fibronectin (51). Thus, it would appear that HS structure, per se does not appear to be dictated by the core protein. Instead the available evidence supports the hypothesis that the synthesis of HS chains and core proteins of individual HSPGs are independently regulated in any particular cell type and possibly tailored for the desired role(s) of that cell. Interestingly, various cloned and non-cloned vascular endothelial cells, which form part of the non-thermogenetic surfaces of blood vessels, were all shown to synthesize similar, albeit low, levels of anti-coagulantly active heparan sulfate, whereas in the same study smooth muscle cells did not (52).

The reproducibly high levels of structural similarity observed between the HS chains of both syndecan-1 and -4 may suggest that not only the cell type but also the cell status could affect the HS structures of the cell surface of HSPGs. The composition of HS derived from the culture medium, the cell surface, the intracellular, and nuclear pools of a rat hepatoma cell line are clearly different (43, 53). Furthermore the amount of heparan sulfate as well as its composition in these pools changes when growing cells reach confluence (43). At the time of these publications we had no information on the structure of HSPG core proteins. A similar scenario was illustrated convincingly by Brickman et al. (54), who isolated two separate pools of HS from neuroepithelial cells derived from embryonic day 10 mice; one from cells in log-phase growth, which greatly potentiated the activity of FGF-2, and a second from contact-inhib-ited cells, which preferentially activated FGF-1. In the present study, we used two different in vitro assay systems to evaluate the ligand-binding activities of syndecan-1 and -4. Although these limited assays may not reflect all biological activities of HS chains on syndecans, it does suggest that these two HSPGs may display the same activities in vivo. These results, however, do mirror those outlined above in which HS derived simultaneously from fibroblast syndecan-4 or glypican-1 displayed very similar affinities for the Hep-II domain of fibronectin. Similarly, in a study into the production of proteoglycans in L cells by Shworak et al. (55), the investigators were unable to identify any major structural differences between HS chains (in terms of sulfation/epimerization) produced in cultures overexpressing transfected syndecan-4 (Ryu- docan) and those of control cultures. Additionally, the proportion of antithrombin III binding sites (which are uniquely specific for high affinity binding to antithrombin III) in both of these HS species were remarkably constant. The above article supports findings that the production of anticoagulant HS does not require a unique protein core (56).

One can only speculate as to the benefits of expressing cell surface proteoglycans that all possess the same potential to bind a vast array of ligands. In normal, undamaged tissues, these proteoglycans may not necessarily encounter all of these ligands, but after perturbation of the tissue, for instance during wounding, previously unseen soluble effectors may need to be sequestered via HS chains to initiate the correct repair process. Many of these soluble effectors are, indeed, heparin-binding molecules (reviewed in Ref. 4).

In conclusion, the findings from the present study indicate that the HS chains on syndecan-1 and -4 derived from the same NMuMG cells display minimal detectable fine structural or domain structural differences. The implication is that the HS chains on HSPGs synthesized simultaneously by other cell types may also show this characteristic. This apparent identity in structure leads one to speculate that protein core-independent sulfation and epimerization of HS chains during biosynthesis in a particular cell type may be determined not only by the repertoire of HS synthetic enzymes possessed by that cell but also by other factors, such as its growth status or developmental role. Precisely how these factors dictate HS structure merits further investigation.

Acknowledgment—We thank Mie Abe for excellent technical assistance.

REFERENCES

1. David, G. (1993) FASEB J. 7, 1023–1030
2. Salmivirta, M., Lihdahl, K., and Lindahl, U. (1996) FASEB J. 10, 1270–1279
3. Rosenzweig, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) J. Clin. Invest. 99, 2062–2070
4. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Linecum, J., and Zako, M. (1999) Annu. Rev. Biochem. 68, 729–777
5. Gallagher, J. T., and Walker, A. (1985) Biochem. J. 220, 665–674
6. Turnbull, J. E., Farnig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. (1992) J. Biol. Chem. 267, 10337–10341
7. Kato, M., Wang, H., Kainulainen, V., Fitzgerald, M. L., Ledbetter, S., Ornitz, D. B., and Bernfield, M. (1998) Nat. Med. 4, 691–697
8. Eko, J. D., and Selleck, B. S., (2002) Annu. Rev. Biochem. 71, 435–471
9. Rapraeger, A. C. (2001) Cell Dev. Biol. 12, 107–116
10. Bernfield, M., Kohenesi, R., Kato, M., Hinkel, M. T., Spring, J., Gallo, R. L., and Lose, E. J. (1992) Annu. Rev. Cell Biol. 8, 365–393
11. Carey, D. J. (1997) Biochem. J. 327, 1–16
12. Fitzgerald, M. L., and Bernfield, M. (1999) in Syndecans Guidebook to the Extracellular Matrix, Anchor, and Adhesion Proteins (Kreis, T., Yale, R. D., eds) pp. 306–311, Oxford University Press, Oxford, UK
13. Zimmermann, P., and David, G. (1989) FASEB J. 3, 891–890
14. Bernfield, M., Hinkel, M. T., and Gallo, R. L. (1996) Deo. Suppl., 205–212
15. Kato, M., Wang, H., Bernfield, M., Gallagher, J. T., and Turnbull, J. E. (1994) J. Biol. Chem. 269, 18881–18889
16. Lindahl, B., Eriksson, L., and Lindahl, U. (1995) Biochem. J. 306, 177–184
17. Kim, C. W., Goldberger, O. A., Gallo, R. L., and Bernfield, M. (1994) Mol. Biol. Cell 5, 797–805
18. Woods, A., Oh, E. S., and Couchman, J. R. (1998) Matrix Biol. 17, 477–483
19. Woods, A., and Couchman, J. R. (1998) Trends Cell Biol. 8, 189–192
20. Stanley, M. J., Liebersbach, B. F., Liu, W., Anhalt, D. J., and Sanderson, R. D.
Identical Heparan Sulfate Chains on Syndecan-1 and -4

21. Gallo, R., Kim, C., Kokkori, R., Adzick, N. S., and Bernfield, M. (1996) J. Invest. Dermatol. 107, 676–683
22. Kinnunen, A., Kinnunen, T., Kakonen, M., Nelo, R., Panula, P., and Rauvala, H. (1998) Eur. J. Neurosci. 10, 635–648
23. Woods, A., and Couchman, J. R. (1994) Mol. Biol. Cell 5, 183–192
24. Woods, A., and Couchman, J. R. (2001) Curr. Opin. Cell Biol. 13, 578–583
25. Yamashita, Y., Oritani, K., Miyoshi, E. K., Wall, R., Bernfield, M., and Kincade, P. W. (1999) J. Immunol. 162, 5940–5948
26. Subramanian, S. V., Fitzgerald, M. L., and Bernfield, M. (1997) J. Biol. Chem. 272, 14713–14720
27. Kainulainen, V., Wang, H., Schick, C., and Bernfield, M. (1998) J. Biol. Chem. 273, 11563–11569
28. Fitzgerald, M. L., Wang, Z., Park, P. W., Murphy, G., and Bernfield, M. (2000) J. Cell Biol. 148, 811–824
29. Jalkanen, M., Nguyen, H., Rapraeger, A., Kurn, N., and Bernfield, M. (1985) J. Cell Biol. 101, 976–984
30. David, G., Van der Schueren, B., and Bernfield, M. (1981) J. Natl. Cancer Inst. 67, 719–728
31. Lyon, M., Deakin, J. A., and Gallagher, J. T. (1994) J. Biol. Chem. 269, 11208–11215
32. Shively, J. E., and Conrad, H. E. (1976) Biochemistry 15, 3932–3942
33. Lee, M. K., and Lander, A. D. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2768–2772
34. Rapraeger, A., Jalkanen, M., and Bernfield, M. (1986) J. Cell Biol. 103, 2683–2698
35. Hayashi, K., Hayashi, M., Jalkanen, M., Firestone, J., Trelodat, R. L., and Bernfield, M. (1987) J. Histochem. Cytochem. 35, 1079–1088
36. Wasteson, A. (1971) J. Chromatogr. 59, 87–97
37. Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D., and Gallagher, J. T. (1990) Biochemistry 29, 2611–2617
38. Lohse, D. L., and Linhardt, R. J. (1992) J. Biol. Chem. 267, 24347–24355
39. Yamada, S., Sakamoto, K., Tsuda, H., Yoshida, K., Sugahara, K., Khoo, K. H., Morris, H. R., and Dell, A. (1994) Glycobiology 4, 69–78
40. Sugahara, K., Tohno-oka, R., Yamada, S., Khoo, K. H., Morris, H. R., and Dell, A. (1994) Glycobiology 4, 535–544
41. Yamada, S., Murakami, T., Tsuda, H., Yoshida, K., and Sugahara, K. (1995) J. Biol. Chem. 270, 8696–8705
42. Maceyama, M., Saka, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) J. Biol. Chem. 271, 17804–17810
43. Fedarko, N. S., and Conrad, H. E. (1986) J. Cell Biol. 102, 587–599
44. Sanderson, R. D., Turnbull, J. E., Gallagher, J. T., and Lander, A. D. (1994) J. Biol. Chem. 269, 13160–13166
45. McFall, A. J., and Rapraeger A. C. (1997) J. Biol. Chem. 272, 12901–12904
46. McFall, A. J., and Rapraeger A. C. (1998) J. Biol. Chem. 273, 28270–28276
47. Yamagata, M., Suzuki, S., Akiyama, S. K., Yamada, K. M., and Kimata, K. (1989) J. Biol. Chem. 264, 8012–8018
48. Ueno, M., Yamada, S., Zako, M., Bernfield, M., and Sugahara, K. (2001) J. Biol. Chem. 276, 29134–29140
49. Knox, S., Merry, C., Stringer, S., Melrose, J., and Whitelock, J. (2002) J. Biol. Chem. 277, 14657–14665
50. Stringer, S. E., Mayer-Koriech, M., Kalyani, A., Rao, M., and Gallagher, J. T. (1999) J. Biol. Chem. 274, 25452–25460
51. Tumova, S., Woods, A., and Couchman, J. R. (2000) J. Biol. Chem. 275, 9410–9417
52. Marcum, J. A., Ata, D. H., Fritze, L. M. S., Nawroth, P., Stern, D., and Rosenberg, R. D. (1986) J. Biol. Chem. 16, 7507–7517
53. Bienkowski, M. J., and Conrad, H. E. (1984) J. Biol. Chem. 259, 12889–12896
54. Brickman, Y. G., Nurcombe, V., Ford, M. D., Gallagher, J. T., Bartlett, P. F., and Turnbull, J. E. (1998) Glycobiology 8, 463–471
55. Shworak, N. W., Shirakawa, M., Colliec-Jouault, S., Liu, J., Mulligan, R. C., Birinyi, L. K., and Rosenberg, R. D. (1994) J. Biol. Chem. 269, 24941–24952
56. Mertens, G., Cassiman, J. J., Van den Berghe, H., Vermuyen, J., and David, G. (1992) J. Biol. Chem. 269, 2270–2276
Syndecan-1 and -4 Synthesized Simultaneously by Mouse Mammary Gland Epithelial Cells Bear Heparan Sulfate Chains That Are Apparently Structurally Indistinguishable
Masahiro Zako, Jianying Dong, Olga Goldberger, Merton Bernfield, John T. Gallagher and Jon A. Deakin

J. Biol. Chem. 2003, 278:13561-13569.
doi: 10.1074/jbc.M209658200 originally published online February 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M209658200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 29 of which can be accessed free at http://www.jbc.org/content/278/15/13561.full.html#ref-list-1