Sulphated and undersulphated heparan sulphate proteoglycans in a Chinese hamster ovary cell mutant defective in *N*-sulphotransferase

Karen J. BAME,*‡ Lijuan ZHANG,* Guido DAVID† and Jeffrey D. ESKO*

*Department of Biochemistry and Molecular Genetics, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A. and †Center for Human Genetics, University of Leuven, B-3000 Leuven, Belgium

The Chinese hamster ovary cell mutant, *pgsE*-606, synthesizes undersulphated heparan sulphate glycosaminoglycans because of a deficiency in *N*-sulphotransferase activity [Bame and Esko (1989) *J. Biol. Chem.* **264**, 8059–8065]. We compared the heparan sulphate proteoglycans synthesized by mutant and wild-type cells to determine what effect the undersulphation defect had on proteoglycan structure. The majority of heparan sulphate proteoglycans synthesized by *pgsE*-606 were undersulphated, but the mutant also synthesized a population of proteoglycans that were sulphated to the same extent as wild-type molecules. Anion-exchange analysis of the glycosaminoglycans in each proteoglycan population showed that they were all modified in the same way. The length of the glycosaminoglycans in each proteoglycan

population were similar, suggesting that *N*-sulphation does not affect chain polymerization. To examine whether the sulphation state of the attached heparan sulphate glycosaminoglycans was dependent on the protein core, we purified syndecan-1 from mutant and wild-type cells using antibodies against the core protein. As with the unfractionated heparan sulphate proteoglycans, *pgsE*-606 synthesized both undersulphated and sulphated syndecan-1. Each pool contained either undersulphated or sulphated glycosaminoglycan chains respectively. Thus the modification of all heparan sulphate chains on a core protein occurs on a proteoglycan-wide basis (i.e. to the same extent).

INTRODUCTION

Studies of Chinese hamster ovary (CHO) cell mutants defective in heparan sulphate biosynthesis [1–6] have suggested that the synthesis and modification of heparan sulphate and heparin are similar [7]. The glycosaminoglycan chains assemble on core proteins by the sequential addition of *N*-acetylglucosamine and glucuronic acid residues, and the newly polymerized chain is modified by a series of interdependent enzyme reactions which include (1) *N*-deacetylation and *N*-sulphation of *N*-acetylglucosamine residues, (2) conversion of glucuronic acid residues into iduronic acid by epimerization at C-5 and (3) *O*-sulphation at C-2 of iduronic acid residues and C-6 of glucosamine residues. In addition, minor *O*-sulphation reactions can occur at C-2 or C-3 of glucuronic acid and C-3 of glucosamine residues. As the epimerization and *O*-sulphation reactions require *N*-sulphate groups, overall modification of the glycosaminoglycan chain depends on the initial *N*-deacetylation and *N*-sulphation reactions [1,3,7]. These reactions are tightly regulated [3], and recent experiments with the *N*-sulphotransferase gene cloned from rat liver indicate that the *N*-deacetylase and *N*-sulphotransferase activities exist on the same protein [8].

Lidholt and Lindahl [9] have proposed a model for heparin biosynthesis in which *N*-deacetylation and *N*-sulphation occur in concert with chain polymerization, and may, in fact, promote chain elongation. Concurrent polymerization and *N*-sulphation may explain the relatively even distribution of *N*-sulphated glucosamine residues along the heparin chain [7]; however, heparan sulphate is not as extensively modified as heparin [10], and structural analysis of heparan sulphate chains from fibroblasts and endothelial cells shows that modified residues are

clustered on the glycosaminoglycan [11,12]. These findings indicate that *N*-deacetylation and *N*-sulphation occur in blocks during heparan sulphate biosynthesis [13], and suggests that the modification of heparan sulphate and heparin may be different.

Different species of cell-associated heparan sulphate proteoglycans have been identified on the basis of their association with the plasma membrane [14–16], the size of the protein core [14–20], or the number, size or sulphation state of the heparan sulphate chains attached to the core protein [17–19,21]. For the most part, all the heparan sulphate chains attached to the same core protein are modified to the same extent [18,19,22]. However, there are examples of proteoglycans that have differentially modified glycosaminoglycans on the same core protein. These differences can be subtle, as in the case of the high-affinity heparin proteoglycans found in rat skin, where only one-third of the glycosaminoglycan chains contain the pentasaccharide sequence that binds to antithrombin III [21]. On the other hand, these differences can be extreme, as in the case of the heparan sulphate proteoglycans synthesized by human colon carcinoma cells which have both fully modified and completely unmodified heparan sulphate glycosaminoglycan chains on the same core protein [23].

In this report, we analyse the heparan sulphate proteoglycans in a mutant CHO cell line defective in *N*-sulphotransferase (*pgsE*-606) to determine how the decrease in enzyme activity affects the size and modification of the glycosaminoglycan chains. Our studies show that chain elongation is not altered by reduced sulphation. Moreover, they demonstrate that two populations of proteoglycans exist, one that contains undersulphated chains and one that contains chains sulphated to the same extent as found in wild-type cells.

Abbreviations used: CHO, Chinese hamster ovary; PAPS, adenosine 3'-phosphate 5'-phosphosulphate.

‡ To whom correspondence should be addressed. Present address: Division of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO 64110-2499, U.S.A.

EXPERIMENTAL

Cell culture

CHO cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61; Rockville, MD, U.S.A.). Mutant *pgsE*-606 was identified by $^{35}\text{SO}_4$ -colony autoradiography as described previously [24], and was shown to be defective in the enzyme heparan sulphate *N*-sulphotransferase [1]. Cells were maintained in Ham's F12 medium (Mediatech, Washington, DC, U.S.A.), supplemented with 7.5% (v/v) fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 100 $\mu\text{g}/\text{ml}$ streptomycin sulphate and 100 units/ml penicillin G. Sulphate-free low-glucose (1 mM) medium was used for all radioactive labelling experiments, and was prepared as described previously [24]. Cells were grown at $37 \pm 0.2^\circ\text{C}$ in an atmosphere of 5% CO_2 in air and 100% relative humidity. They were subcultured every 3–4 days with 0.125% trypsin, and after 15–20 passages, fresh cells were revived from frozen stocks stored in liquid nitrogen. To isolate [^3H]heparan sulphate proteoglycans, cell monolayers were cultured in F12 medium for 2 days, then equilibrated in low-sulphate low-glucose medium overnight. The cultures were labelled with 50–100 $\mu\text{Ci}/\text{ml}$ D-[6- ^3H]glucosamine (40 Ci/mmol) for 1–4 h, the media were removed and the cells washed three times with cold PBS (0.137 M NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4 , 6.48 mM Na_2HPO_4) before the cell-associated proteoglycans were extracted from the monolayers.

Purification of heparan sulphate proteoglycans

Cells were solubilized for 5 h at 4°C in a solution containing 4 M guanidinium chloride, 50 mM sodium acetate (pH 5.0), 10 mM EDTA, 0.2% Zwittergent 3–12, 10 mM *N*-ethylmaleimide, 1 mM phenylmethanesulphonyl fluoride, 1 $\mu\text{g}/\text{ml}$ leupeptin and 1.5 $\mu\text{g}/\text{ml}$ pepstatin A. All remaining purification steps were performed at 4°C in the presence of protease inhibitors. The extracts were dialysed (Spectrapor; molecular-mass cut-off 6000–8000 Da) against 50 mM sodium acetate buffer (pH 5.0) containing 4 M urea, 0.1 M NaCl and 10 mM EDTA to remove the guanidinium salts. After the addition of CHAPS (0.5% final concn.), the urea extracts were applied to 0.5 ml DEAE-Sephacel columns [1], equilibrated in the dialysis buffer; the columns were washed with 15 ml of 4 M urea buffer containing 0.1 M NaCl, and the [^3H]proteoglycans were eluted with 2.5 ml of buffer containing 1.0 M NaCl. The isolated proteoglycans were desalted by chromatography on a PD10 column equilibrated in ethanol/water (1:9, v/v) (Pharmacia LKB Biotechnology), and separated into heparan sulphate proteoglycans and chondroitin sulphate proteoglycan species by anion-exchange h.p.l.c. [1]. The heparan sulphate proteoglycans were pooled, diluted with a solution of 10 mM KH_2PO_4 (pH 6.0) containing 0.2% Zwittergent 3–12, and repurified over DEAE-Sephacel columns [1]. The purified [^3H]heparan sulphate species were desalted over a PD10 column, lyophilized, resuspended in 0.1 M KH_2PO_4 (pH 6.0) containing 0.5 M NaCl and 0.2% Zwittergent 3–12 and chromatographed on a TSK 4000 gel-filtration column (7.5 mm $\times$ 300 mm) (Pharmacia LKB Biotechnology) to separate intact proteoglycans from partially degraded glycosaminoglycan chains [25]. The column was run at a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected. V_0 and V_i values for the column were determined by the elution position of bovine nasal cartilage proteoglycan and glucuronic acid respectively. Proteoglycans eluted at or near the V_0 were purified for further analysis.

Purification of syndecan

Cells were labelled with [6- ^3H]glucosamine and solubilized over-

night at 4°C in a Triton X-100 buffer (1% Triton X-100, 150 mM NaCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) containing the above protease inhibitors plus 5 mM benzamidine, 10 mM EDTA and 50 mM aminohexanoic acid. The extract was clarified by centrifugation (10000 g; 60 min; Sorvall SS-34), and the pellet was re-extracted with 5 ml of Triton X-100 buffer for 1 h at room temperature. The supernatants were combined, and solid urea was added to a final concentration of 6 M. After the addition of 1 mg of bovine tracheal chondroitin sulphate-A as carrier, the samples were applied to 0.5 ml DEAE-Sephacel columns [1] equilibrated in a solution containing 6 M urea, 0.2 M NaCl, 0.5% Triton X-100 and 25 mM Tris/HCl (pH 7.0). The columns were washed with 15 ml of buffer, and [^3H]proteoglycans were eluted with the same buffer containing 1.0 M NaCl. The isolated [^3H]proteoglycans were desalted over a PD10 column, lyophilized, resuspended in PBS containing protease inhibitors, and incubated with chondroitinase ABC to degrade the chondroitin sulphate chains on core proteins. The remaining [^3H]heparan sulphate-bearing proteoglycans were reisolated by anion-exchange chromatography, desalted over a PD10 column, and applied to a 0.25 ml affinity column of anti-syndecan monoclonal antibodies, 2E9, coupled to Sepharose [20,26]. The column was washed with 8 ml of PBS, followed by 2 ml of PBS containing 0.5 M NaCl. Syndecan was eluted from the resin with 5 ml of 4 M guanidinium chloride in 50 mM sodium acetate (pH 5.8) containing protease inhibitors. Salt was removed from the purified [^3H]syndecan by PD10 chromatography before further analysis.

Characterization of proteoglycans and glycosaminoglycans

Purified heparan sulphate proteoglycans or syndecan were resuspended in 0.5 M NaBH_4 in 0.4 M NaOH, and incubated at 4°C for 24 h [27] to release the glycosaminoglycan chains from the protein core. Base treatment was stopped by adding 5 μl portions of 5 M acetic acid until bubble formation ceased. Proteoglycans and the released glycosaminoglycan chains were analysed by anion-exchange h.p.l.c. [1] and gel-filtration h.p.l.c., as described above.

RESULTS

PgsE-606 synthesizes both undersulphated and sulphated heparan sulphate proteoglycans

Previous studies have shown that a mutant CHO cell line, *pgsE*-606, synthesizes highly acetylated undersulphated heparan sulphate chains compared with wild-type cells, because of a 3- to 4-fold decrease in the activity of the modification enzyme *N*-sulphotransferase [1–3]. The decrease in *N*-sulphotransferase activity does not affect the synthesis of [^3H]heparan sulphate in *pgsE*-606 [1], but the $^{35}\text{SO}_4$ incorporated into the mutant glycosaminoglycans is only 30–40% of that found in wild-type chains [1], causing the ^{35}S , ^3H -labelled heparan sulphate chains from *pgsE*-606 cells to be eluted from anion-exchange resins earlier than wild-type molecules [1]. Structural analysis of the heparan sulphate glycosaminoglycans synthesized by the mutant shows that the *N*-sulphotransferase defect causes an increase in the spacing between *N*-sulphated glucosamine residues [1], and a decrease in the amount of iduronic acid residues and 2-*O*- and 6-*O*-sulphate groups on the chain [2].

To examine how decreased *N*-sulphotransferase activity affected proteoglycan structure, wild-type and *pgsE*-606 cells were labelled with [6- ^3H]glucosamine for 1, 2 or 4 h, and the proteoglycans were isolated and analysed by anion-exchange h.p.l.c. (Figure 1). The ^3H -labelled material was eluted from the

Figure 1 Anion-exchange h.p.l.c. of [^3H]proteoglycans from wild-type and *pgsE-606* cells

Wild-type (a) and *PgsE-606* (b) cells were labelled with [$6\text{-}^3\text{H}$]glucosamine for 1 (●, ○), 2 (▲, △) or 4 (■, □) h, and the isolated proteoglycans were analysed by anion-exchange h.p.l.c. using a linear salt gradient from 0.05 to 0.7 M NaCl (-----). Fractions 1–15 and 40–50 contain negatively charged glycoproteins and chondroitin sulphate proteoglycans respectively. Fractions indicated by the solid bars were pooled as undersulphated heparan sulphate (pool I) and 'normally' sulphated heparan sulphate (pool II).

anion-exchange resin in three regions (Figure 1). Neuraminidase degraded the material eluted in fractions 1–15, suggesting that it consisted of acidic glycoproteins, and the ^3H -labelled species eluted in fractions 40–50 represent chondroitin sulphate proteoglycans and free chondroitin sulphate chains, identified on the basis of susceptibility to chondroitinase ABC treatment [25]. The majority of wild-type [^3H]heparan sulphate proteoglycans were eluted as a broad peak centred at 0.45 M NaCl (Figure 1a, peak II), which is the elution position of the free glycosaminoglycan chains [1]. A small peak of material was eluted at lower salt concentration (0.3 M NaCl; peak I), at the same position at which undersulphated chains from *pgsE-606* cells are eluted [1]. As we have previously shown that the majority of *pgsE-606* heparan sulphate chains have two to three times fewer sulphate groups than wild-type chains [1,2], these [^3H]proteoglycans are presumably undersulphated species. The ratio of undersulphated (I) and sulphated (II) peaks in wild-type cells did not change with incubation time: peak II represented 75% of wild-type [^3H]heparan sulphate proteoglycans at 1, 2 and 4 h.

[^3H]Heparan sulphate proteoglycans synthesized by *pgsE-606*

Figure 2 Size analysis of purified wild-type and *pgsE-606* heparan sulphate proteoglycans and glycosaminoglycans

^3H -labelled heparan sulphate proteoglycans free glycosaminoglycan chains were analysed by TSK 4000 gel chromatography (see the Experimental section). (a) Wild-type pool II; (b) *pgsE-606* pool I; (c) *pgsE-606* pool II. ○, □, △, Intact proteoglycans; ●, ■, ▲, glycosaminoglycans liberated from the proteoglycans.

cells were eluted very differently (Figure 1b). One population was eluted around 0.35 M NaCl (peak I), and the remainder were eluted in the same position as wild-type heparan sulphate proteoglycan (0.45 M NaCl; Peak II). Unlike the wild-type proteoglycans, the percentage of undersulphated (peak I) material made by the mutant increased from 60% at 1 h to 80% at 2 and 4 h, so that by 2 h the second peak had been obscured by the material in peak I. On the basis of the proposed biosynthetic pathway for heparan sulphate, it is unlikely that a highly sulphated proteoglycan would mature into an undersulphated

Figure 3 Anion-exchange h.p.l.c. of wild-type and *pgsE-606* heparan sulphate proteoglycan and glycosaminoglycan pools

Purified ^3H -labelled heparan sulphate proteoglycans and liberated glycosaminoglycans (see the Experimental section) were analysed by anion-exchange h.p.l.c. using a linear salt gradient from 0.05 to 0.7 M NaCl (---). (a) Wild-type pool II; (b) *pgsE-606* pool I; (c) *pgsE-606* pool II. ○, □, △, Intact proteoglycans; ●, ■, ▲, liberated glycosaminoglycan chains.

form. Thus the data suggest that the mutant synthesizes two different populations of proteoglycans that vary in degree of sulphation.

The glycosaminoglycans on the protein cores are modified to the same extent

To determine whether the chains on each population of proteoglycans were homogeneous, wild-type and *pgsE-606* [^3H]heparan sulphate proteoglycans from the 4 h time points were pooled into 'undersulphated' (pool I) and 'sulphated' (pool II) species, and purified by gel filtration (results not shown). This step is necessary because free glycosaminoglycans derived from proteoglycan degradation are present in the pooled populations [25]. Pool I from wild-type cells contained no proteoglycan species, and thus was not analysed further. (This wild-type pool-I material is eluted from the TSK 4000 column as three peaks with K_{av} values

of 0.33, 0.60 and 0.77. The material eluted with a K_{av} of 0.77 is probably heparanase-cleaved heparan sulphate glycosaminoglycans derived from the catabolism of pool-II proteoglycans, as the shorter chains are eluted from the anion-exchange resin before chains or intact proteoglycans [25]. We have not yet characterized the other two peaks of ^3H -labelled material. *PgsE-606* may also contain these substances, but they are masked by the undersulphated proteoglycan when analysed by anion-exchange chromatography; however, as gel filtration is a prerequisite for purifying the proteoglycans, we have presumably lost this material.)

The size of the purified proteoglycans from the wild-type and *pgsE-606* pools were examined by gel-filtration h.p.l.c. (Figure 2). The [^3H]proteoglycans from all three pools were eluted close to the void volume of the TSK 4000 column. Treating the samples with alkali under reducing conditions released free glycosaminoglycan chains which were eluted in a more included position (Figure 2). Thus each pool consisted of intact proteoglycans. The size of the [^3H]heparan sulphate chains from the mutant were as large and more homogeneous than those from the wild-type. They did not vary according to the degree of sulphation as the liberated glycosaminoglycans from the undersulphated (pool I) and sulphated (pool II) proteoglycans were eluted from the column in the same position (Figures 2b and 2c). Base treatment of all three proteoglycan pools also released ^3H -labelled species that were eluted at or near the V_i of the column (Figure 2). These presumably represent other O-linked oligosaccharides attached to the protein core.

Intact proteoglycan and base-released chains were analysed by anion-exchange h.p.l.c. to determine whether the glycosaminoglycans on each proteoglycan population were sulphated homogeneously (Figure 3). Only a single peak of base-released ^3H -labelled chains is observed for each of the proteoglycan species, indicating that all of the heparan sulphate chains in the different proteoglycan populations were modified to the same extent (Figure 3). A slight shift to a lower salt concentration was observed with the base-released [^3H]glycosaminoglycans, possibly as a result of differences in the way free chains and intact proteoglycans permeate the anion-exchange gel.

Two populations of syndecan-1 are present in *pgsE-606*

It is possible that the differences observed in proteoglycan sulphation in the mutant were core-protein dependent. To examine whether the core protein plays a role in the sulphation state of the chains attached to it, we purified a specific heparan sulphate proteoglycan, syndecan-1, from wild-type and *pgsE-606* cells. Syndecan-1, a transmembrane proteoglycan, was first described in mouse mammary epithelial cells [28], and has both heparan sulphate and chondroitin sulphate chains covalently bound to the core protein. Antibodies to syndecan-1 from human lung fibroblasts cross-react with a proteoglycan population in CHO cells that represents approx. $20 \pm 5\%$ of the steady-state [^3H]proteoglycans in either wild-type or *pgsE-606* cells. About $15 \pm 5\%$ of the [^3H]glycosaminoglycans on both wild-type and mutant syndecan-1 are sensitive to chondroitinase ABC (results not shown), indicating that the CHO proteoglycan contains both heparan sulphate and chondroitin sulphate glycosaminoglycans. Thus the defect in N-sulphation does not affect the expression of syndecan-1 in the mutant, nor does it alter the proportion of heparan sulphate and chondroitin sulphate chains attached to the syndecan-1 core protein.

Because the more highly sulphated chondroitin sulphate chains might affect the elution position of syndecan-1, the proteoglycans were treated with chondroitinase ABC before syndecan-1 was

Figure 4 Anion-exchange h.p.l.c. of [^3H]syndecan-1 from wild-type and *pgsE-606* cells

Wild-type (a) and *pgsE-606* (b) cells were incubated with [^3H]glucosamine for 4 h, and the [^3H]syndecan-1 synthesized by each cell type isolated by using an anti-syndecan-1 antibody column (see the Experimental section) and analysed by anion-exchange h.p.l.c. using a linear salt gradient from 0.05 to 0.7 M NaCl (.....). Fractions indicated by the bars were pooled as undersulphated (I) and sulphated (II) syndecan-1. The proteoglycans were treated with chondroitinase ABC before syndecan-1 was analysed (see the Experimental section), so the elution position of the molecule is due only to the sulphation of the heparan sulphate glycosaminoglycans on the core protein.

purified (see the Experimental section). Thus the differences observed between wild-type and *pgsE-606* syndecan-1 are due solely to the sulphation state of the heparan sulphate chains. Wild-type [^3H]syndecan-1 was eluted from an anion-exchange h.p.l.c. column as a symmetrical peak around 0.48 M NaCl (Figure 4a). This salt concentration is slightly higher than that required to elute the unfractionated heparan sulphate proteoglycans (Figure 1a compared with Figure 4a), indicating that syndecan-1 behaves as a distinct population of the wild-type molecules. Most of the *pgsE-606* [^3H]syndecan-1 is undersulphated (Figure 4b, pool I); however, as observed with the unfractionated heparan sulphate proteoglycans synthesized by *pgsE-606*, a proportion of the mutant [^3H]syndecan-1 requires a higher salt concentration to be eluted from the resin (Figure 4b, pool II). These results indicate that syndecan-1 exists in both undersulphated and sulphated forms in *pgsE-606* cells.

As with the unfractionated heparan sulphate proteoglycans, wild-type and *pgsE-606* syndecan-1 was eluted from the TSK 4000 gel-filtration column near the void volume (Figure 5), and

Figure 5 Size analysis of purified wild-type and *pgsE-606* syndecan-1, and heparan sulphate glycosaminoglycans released from syndecan-1

Purified [^3H]syndecan-1 (devoid of chondroitin sulphate chains) and liberated heparan sulphate glycosaminoglycans from wild-type (a) or *pgsE-606* (b) cells (see the Experimental section) were analysed by TSK 4000 gel chromatography as described in Figure 2. $\circ$, Wild-type syndecan-1; $\bullet$, wild-type glycosaminoglycans; $\square$, *pgsE-606* syndecan-1; $\blacksquare$, *pgsE-606* pool-I glycosaminoglycans; $\blacktriangle$, *pgsE-606* pool-II glycosaminoglycans.

base treatment released [^3H]heparan sulphate chains which were more included (Figure 5). Again, the sulphation state of [^3H]syndecan does not affect the size of the heparan sulphate chains attached to it, as all three syndecan-1 populations have glycosaminoglycans of similar size (Figure 5). Mature syndecan-1 core protein has a molecular mass of 31 kDa and five possible glycosaminoglycan-attachment sites [5]. Although we do not know the exact number of heparan sulphate chains found on CHO syndecan-1, the difference in the elution position of intact syndecan and base-released chains on the TSK 4000 gel-filtration column (Figure 5) suggests that there are at least two heparan sulphate glycosaminoglycan chains on each core protein. As the undersulphated and sulphated syndecan synthesized by *pgsE-606* behave similarly on the column, it appears that the N-sulphation defect does not affect the number of glycosaminoglycan chains on the core protein.

Figure 6 Anion-exchange h.p.l.c. of wild-type and *pgsE*-606 heparan sulphate glycosaminoglycans released from syndecan-1 core proteins

Purified ${}^3\text{H}$ -labelled heparan sulphate released from wild-type (a) or *pgsE*-606 (b) syndecan-1 core proteins (see the Experimental section) were analysed by anion-exchange h.p.l.c. using a linear salt gradient from 0.05 to 0.7 M NaCl (---). ●, Wild-type glycosaminoglycans; ■, *pgsE*-606 pool-I glycosaminoglycans; ▲, *pgsE*-606 pool-II glycosaminoglycans.

The [${}^3\text{H}$]heparan sulphate released from each syndecan population was analysed by anion-exchange h.p.l.c. to determine whether the chains on each core protein were homogeneously sulphated (Figure 6). Both wild-type and *pgsE*-606 pool-I ${}^3\text{H}$ -labelled chains were eluted as single peaks near the same salt concentration as the parent syndecan-1 (Figure 6), indicating that all the heparan sulphate on each core protein was modified to the same extent. As seen with the unfractionated heparan sulphate proteoglycans, the base-released glycosaminoglycans were eluted at a slightly lower salt concentration than the intact proteoglycan (compare Figures 4 and 6). The [${}^3\text{H}$]heparan sulphate released from *pgsE*-606 pool-II syndecan-1 required a high salt concentration to be eluted from the anion-exchange resin (Figure 6b; filled triangles). Some heterogeneity of the material may exist as it was eluted in several subpeaks. Nevertheless, the data suggest that *pgsE*-606 cells synthesize both undersulphated and sulphated forms of syndecan-1.

DISCUSSION

Our previous studies analysing the *N*-sulphotransferase-deficient mutant *pgsE*-606 suggested that the level of *N*-sulphotransferase

activity was the determining factor for the degree of chain modification [1–3]. However, evidence presented in this report suggests that other factors, in addition to *N*-sulphotransferase activity, control the modification state of individual heparan sulphate glycosaminoglycans. Whereas the majority of *pgsE*-606 proteoglycans have undersulphated heparan sulphate chains, some of the newly synthesized mutant proteoglycans have glycosaminoglycans that are modified to the same extent as wild-type CHO-K1 heparan sulphate (Figures 1 and 3). The overall degree of chain modification does not appear to be regulated by the core protein, as CHO cell syndecan-1 also exists in undersulphated and sulphated forms (Figures 4 and 6).

With the possible exception of the highly sulphated mutant syndecan-1, all the heparan sulphate chains on the same core protein are modified to the same extent in *pgsE*-606 (Figures 3 and 6), indicating that, however the glycosaminoglycans are modified, every chain on a core protein is treated the same way. This is different from the situation in the human colon carcinoma cell line where both fully sulphated chains and non-sulphated chains were found on the same protein core [23]. The colon carcinoma cells overexpress a heparan sulphate proteoglycan core protein, and differences in the capacities of the chain-polymerizing and chain-modifying machinery are thought to explain the presence of non-sulphated glycosaminoglycans [23]. *PgsE*-606 cells are somewhat analogous to these cells, in that the decrease in *N*-sulphotransferase activity reduces the capacity for *N*-sulphation in the mutant. However, our results indicate that the level of active *N*-deacetylase/*N*-sulphotransferase enzyme in *pgsE*-606 is sufficient to modify all the chains to some extent, and some of the chains to a full extent.

Gallagher et al. [13] have proposed two models to explain the formation of modified domains on the heparan sulphate chain. One model, based on the studies of heparin biosynthesis, proposes that *N*-sulphation is required at discrete stages of chain elongation in order for polymerization to proceed [13]. Regions of *N*-sulphated groups would be formed during chain synthesis, and spacing would be dependent on polymerization requirements. Further modification would occur at these domains, as the subsequent epimerization and *O*-sulphation reactions require the presence of *N*-sulphated glucosamine groups [7]. Our present studies do not support this model, as *N*-sulphation does not appear to drive elongation of the heparan sulphate chain. If sulphation were required to promote polymerization, we would expect the undersulphated glycosaminoglycans synthesized by *pgsE*-606 to be shorter than the fully sulphated chains, yet both types of glycosaminoglycan are the same size (Figures 2 and 5). The observation that the sizes of the newly synthesized heparan sulphate chains are unaffected when wild-type cells are incubated in the presence of 5 mM chlorate (K. J. Bame, unpublished work) provides further evidence that *N*-sulphation is not required for chain polymerization. Chlorate inhibits the formation of the sulphate donor adenosine 3'-phosphate 5'-phosphosulphate (PAPS) by acting as a non-competitive inhibitor of the adenosine 5'-phosphosulphate sulphurylase. When CHO cells are cultured with chlorate the heparan sulphate chains become dramatically undersulphated [3].

Our results support the second model proposed by Gallagher et al. [13], in which the *N*-deacetylase/*N*-sulphotransferase enzyme recognizes a feature of the secondary structure of the heparan sulphate chain, and modifies the glycosaminoglycan at this region. This feature will occur periodically along the glycosaminoglycan, resulting in a definite spacing of *N*-sulphated domains. These *N*-sulphated sequences will be further modified by epimerization and *O*-sulphation reactions. In *pgsE*-606 cells the chains apparently assume an appropriate secondary structure,

allowing the modification reactions to initiate. If this 'structural modification' model is correct, we would predict that the frequency, but not the size, of the modified domains are altered on the heparan sulphate synthesized by *pgsE*-606. Previous studies comparing the oligosaccharides generated by low-pH HNO₂ treatment of wild-type and *pgsE*-606 heparan sulphate support this idea [1]. The distance between N-sulphated residues is increased in the mutant chains, but the distribution of O-sulphate residues among the depolymerization products is similar for both wild-type and *pgsE*-606 heparan sulphates [1].

The presence of undersulphated to fully sulphated heparan sulphate glycosaminoglycans synthesized by *pgsE*-606 (Figures 3b and 3c) suggests that formation of the first N-sulphated domain promotes the formation of subsequent domains. Thus, once an N-sulphated domain is formed on the chain, the glycosaminoglycan is more likely to be further N-sulphated. This 'positive feedback' mechanism is more easily seen in *pgsE*-606 because of the deficiency in N-sulphotransferase activity. In addition to promoting subsequent N-sulphation on the same heparan sulphate chain, the formation of modified domains drives sulphation of all the chains on the proteoglycan, as the heparan sulphate chains attached to a core protein are modified to similar extents (Figure 3). The formation of the structural domain recognized by the N-deacetylase/N-sulphotransferase enzyme does not appear to be dependent on the type of protein core, as the syndecan-1 synthesized in *pgsE*-606 is sulphated in a similar manner to the total heparan sulphate proteoglycan population in the mutant (Figures 1 and 4).

One possible mechanism for promotion of further N-sulphation could be to increase the affinity of the newly sulphated glycosaminoglycan for the N-deacetylase/N-sulphotransferase enzyme. This appears to be the case in heparin biosynthesis, as studies with the N-deacetylase from mouse mastocytoma cells showed that the K_m of the substrate for the enzyme was decreased if PAPS was included in the assay [3]. However, the substrate K_m for either wild-type CHO or *pgsE*-606 N-deacetylase was the same whether or not PAPS was present [3], suggesting that the activity of the CHO enzyme is not affected by N-sulphation of the substrate.

Another plausible explanation for undersulphated and sulphated populations of proteoglycans may relate to the content of the modifying enzymes between cells, or the compartmentalization of the enzymes within cells. We do not know conclusively that the content of N-sulphotransferase is homogeneous over the *pgsE*-606 cells on one plate; however, as the mutant was isolated from a single cell that exhibited reduced incorporation of ³⁵SO₄ [1], and because the fresh cells are revived from frozen stocks after 15–20 passages, it is unlikely that there are cells in the mutant population that have wild-type levels of the modification enzyme. However, it may be that the level of N-sulphotransferase is not homogeneous within a cell. Some proteoglycan cores may pass through a compartment containing a high concentration of

enzymes, which results in extensive modification of the chains. Others may move through compartments where the concentrations of modifying enzymes are reduced, resulting in poorly sulphated molecules. Mutant *pgsE*-606 contains less N-sulphotransferase which might accentuate the differences in these compartments. With purified N-sulphotransferase available, it should be possible to examine whether the enzyme is heterogeneously distributed across the mutant cell population, or across the Golgi stacks, where N-sulphation, O-sulphation and epimerization are thought to occur [7,10].

This work was supported in part by grant GM33063 from the N.I.H. to J.D.E. and an American Cancer Society postdoctoral fellowship, PF-3051, awarded to K.J.B.

REFERENCES

- Bame, K. J. and Esko, J. D. (1989) *J. Biol. Chem.* **264**, 8059–8065
- Bame, K. J., Lidholt, K., Lindahl, U. and Esko, J. D. (1991) *J. Biol. Chem.* **266**, 10287–10293
- Bame, K. J., Rethy, R. V. and Esko, J. D. (1991) *J. Biol. Chem.* **266**, 12461–12468
- Esko, J. D., Stewart, T. E. and Taylor, W. H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3197–3200
- Esko, J. D., Weinke, J. L., Taylor, W. H., Ekborg, G., Roden, L., Ananthramiah, G. and Gawish, A. (1987) *J. Biol. Chem.* **262**, 12189–12195
- Lidholt, K., Weinke, J. L., Kiser, C. S., Lugemwa, F. N., Bame, K. J., Cheifetz, S., Massague, J., Lindahl, U. and Esko, J. D. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2267–2271
- Lindahl, U. (1989) in *Heparin, Chemical and Biological Properties: Clinical Applications* (Lane, D. A. and Lindahl, U., eds.), pp. 159–189, Edward Arnold, London
- Wei, Z., Swiedler, S. J., Ishihara, M., Orellana, A. and Hirschberg, C. B. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3885–3888
- Lidholt, K. and Lindahl, U. (1992) *Biochem. J.* **287**, 21–29
- Gallagher, J. T. and Walker, A. (1985) *Biochem. J.* **230**, 665–674
- Lindblom, A., Bengtsson-Olivercrona, G. and Fransson, L. Å. (1991) *Biochem. J.* **279**, 821–829
- Turnbull, J. E. and Gallagher, J. T. (1991) *Biochem. J.* **273**, 553–559
- Gallagher, J. T., Turnbull, J. E. and Lyon, M. (1992) *Int. J. Biochem.* **24**, 553–560
- David, G. (1991) *Biochem. Soc. Trans.* **19**, 816–820
- Gallagher, J. T. (1989) *Curr. Opin. Cell Biol.* **1**, 1201–1218
- Yanagishita, M. and Hascall, V. C. (1992) *J. Biol. Chem.* **267**, 9451–9454
- David, G. and Van Den Berghe, (1989) *Eur. J. Biochem.* **178**, 609–617
- Hampson, I. N., Kumar, S. and Gallagher, J. T. (1984) *Biochim. Biophys. Acta* **801**, 306–313
- Kinsella, M. G. and Wight, T. N. (1988) *Biochemistry* **27**, 2136–2144
- Lories, V., Cassiman, J.-J., Van Den Berghe, H. and David, G. (1989) *J. Biol. Chem.* **264**, 7009–7016
- Horner, A. (1987) *Biochem. J.* **244**, 693–698
- Schmidtchen, A. and Fransson, L.-Å. (1992) *Eur. J. Biochem.* **208**, 537–546
- Iozzo, R. V. (1989) *J. Biol. Chem.* **264**, 2690–2699
- Esko, J. D., Elgavish, A., Prasthofer, T., Taylor, W. H. and Weinke, J. L. (1986) *J. Biol. Chem.* **261**, 15725–15733
- Bame, K. J. (1993) *J. Biol. Chem.* **268**, 19956–19964
- Lories, V., Cassiman, J.-J., Van Den Berghe, H. and David, G. (1992) *J. Biol. Chem.* **267**, 1116–1122
- Bienkowski, M. J. and Conrad, H. E. (1984) *J. Biol. Chem.* **259**, 12989–12996
- Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. and Lose, E. J. (1992) *Annu. Rev. Cell Biol.* **8**, 365–393