Basement-membrane heparan sulphate with high affinity for antithrombin synthesized by normal and transformed mouse mammary epithelial cells

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Basement-membrane proteoglycans, biosynthetically labelled with [³⁵S]sulphate, were isolated from normal and transformed mouse mammary epithelial cells. Proteoglycans synthesized by normal cells contained mainly heparan sulphate and, in addition, small amounts of chondroitin sulphate chains, whereas transformed cells synthesized a relatively higher proportion of chondroitin sulphate. Polysaccharide chains from transformed cells were of lower average M_r and of lower anionic charge density compared with chains isolated from the untransformed counterparts, confirming results reported previously [David & Van den Berghe (1983) *J. Biol. Chem.* **258**, 7338–7344]. A large proportion of the chains isolated from normal cells bound with high affinity to immobilized antithrombin, and the presence of 3-*O*-sulphated glucosamine residues, previously identified as unique markers for the antithrombin-binding region of heparin [Lindahl, Bäckström, Thunberg & Leder (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6551–6555], could be demonstrated. A significantly lower proportion of the chains derived from transformed cells bound with high affinity to antithrombin, and a corresponding decrease in the amount of incorporated 3-*O*-sulphate was observed.

INTRODUCTION

Heparin and heparan sulphate are structurally related sulphated polysaccharides belonging to the glycosaminoglycan family. Both are synthesized as proteoglycans, heparin proteoglycans by connective-tissue-type mast cells and heparan sulphate proteoglycans by a variety of cell types (Rodén, 1980; Gallagher *et al.*, 1986). The glycosaminoglycan chains of both heparin and heparan sulphate are composed of hexuronic acid (D-glucuronic acid or L-iduronic acid) and D-glucosamine units in alternating sequence. The glucosamine residues can be either *N*-sulphated or *N*-acetylated, rarely *N*-unsubstituted, and, in addition, both the hexuronic acid and the glucosamine residues carry *O*-sulphate groups at various positions (Lindahl & Höök, 1978; Rodén, 1980; Bienkowski & Conrad, 1985; Gallagher *et al.*, 1986; Lindahl & Kjellén, 1987). Heparin and heparan sulphate are distinguished by quantitative rather than qualitative differences, heparan sulphate generally having a lower overall sulphate content, less iduronic acid and more *N*-acetyl groups than heparin (Gallagher & Walker, 1985).

The blood anticoagulant activity of heparin is mediated by a specific pentasaccharide sequence (Lindahl *et al.*, 1984; Atha *et al.*, 1985), which contains, as a unique component, a 3-*O*-sulphated glucosamine residue (Lindahl *et al.*, 1980). Heparin molecules possessing this structure bind with high affinity to antithrombin, thereby accelerating the reaction between the proteinase inhibitor

and serine proteinases of the coagulation cascade (Björk & Lindahl, 1982). The presence of an antithrombin-binding region, possibly similar to that of heparin, has been demonstrated also in heparan sulphate (Marcum & Rosenberg, 1985; Lane *et al.*, 1986; Marcum *et al.*, 1986).

Basement membranes contain, in addition to a variety of proteins, appreciable amounts of chondroitin sulphate proteoglycans and, in particular, heparan sulphate proteoglycans. Structural studies of the proteoglycans isolated from the Engelbreth-Holm-Swarm tumour (Fujiwara *et al.*, 1984; Hassell *et al.*, 1985) and from Reichert's membrane (Paulsson *et al.*, 1985) have demonstrated the presence of two forms of heparan sulphate proteoglycans in basement membranes, a protein-rich low-buoyant-density type and a protein-poor high-buoyant-density type.

The functions of the basement-membrane proteoglycans remain largely unknown. At certain sites they appear to contribute a fixed negative charge, which is important for the filtration properties of the basement membrane (Kanwar *et al.*, 1980). Other observations, supported by direct studies *in vitro* on the mutual interactions of isolated basement-membrane components (Fujiwara *et al.*, 1984), imply a possible role for the proteoglycans in the processes that lead to the assembly of a basement membrane. Mouse mammary-gland epithelial (MuMG) cells grown *in vitro*, for example, respond to the presence of collagen by producing a continuous basement membrane (David *et al.*, 1981).

Abbreviations used: MuMG cells, murine mammary-gland cells; BM, basement membrane; HexA, unspecified hexuronic acid; IdA, L-iduronic acid; GlcA, D-glucuronic acid; GlcNAc, 2-deoxy-2-acetoamido-D-glucose (*N*-acetyl-D-glucosamine); aMan_n, 2,5-anhydro-D-mannitol formed by reduction of terminal 2,5-anhydro-D-mannose residues with NaBH₄; -OSO₃, *O*-sulphate, ester sulphate group; the locations of *O*-sulphate groups are indicated in parentheses.

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Collagen appears to promote the assembly of basal lamina by binding basal extracellular proteoglycan (Koda & Bernfield, 1984) and decreasing the rate at which heparan sulphate is degraded (David & Bernfield, 1981).

In numerous reports a change in glycosaminoglycan synthesis upon neoplastic transformation has been described (Underhill & Keller, 1975; Winterbourne & Mora, 1978, 1981; Keller *et al.*, 1980; Fransson *et al.*, 1982; Underhill & Toole, 1982; Shanley *et al.*, 1983). When analysed for transformation-associated changes, spontaneously transformed MuMG cells that produce invasive tumours *in vivo* (David *et al.*, 1981) were found to produce basement-membrane heparan sulphate of lower M_r and of lower anionic charge density than the untransformed parental counterpart (David & Van den Berghe, 1983). In contrast with the parental cell line, the transformed MuMG cells respond poorly to collagen, and show weak basal lamina formation, apparently owing to an impaired ability to decrease the degradation of heparan sulphate (David & Bernfield, 1982).

In the present study we have investigated the structure and antithrombin-binding properties of basement-membrane heparan sulphate derived from normal and transformed MuMG cells.

MATERIALS

Unlabelled standard pig mucosal heparin and ^3H -labelled heparin, chondroitin sulphate and hyaluronan were as described previously (Höök *et al.*, 1982). Reference ^3H -labelled HexA-aMan_R disaccharides with *O*-sulphate groups at various positions were obtained by HNO_2 (pH 1.5)– NaB^3H_4 treatment of unlabelled heparin followed by anion-exchange h.p.l.c. (Thunberg *et al.*, 1982). The tetrasaccharide standards, IdA-GlcNAc(6-OSO₃)-GlcA-[1- ^3H]aMan_R(3-OSO₃) and IdA-GlcNAc(6-OSO₃)-GlcA-[1- ^3H]aMan_R(3,6-di-OSO₃), were prepared as described previously (Thunberg *et al.*, 1982).

Sephacrose CL-6B, Sepharose CL-4B and Sephadex G-25 (superfine grade) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), DEAE-cellulose (DE 52) was from Whatman (Maidstone, Kent, U.K.) and chondroitin ABC lyase was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Antithrombin covalently attached to Sepharose 4B was prepared as described previously (Höök *et al.*, 1976).

METHODS

Collagen gels (3 ml per 100 mm-diameter Falcon tissue-culture dish) were prepared as described previously (David & Van den Berghe, 1983). The 'normal' MuMG cells and their transformed derivatives were plated on collagen gels and grown to confluency for 3 days in special formula medium as described previously (David & Van den Berghe, 1985). Labelling, which was sustained for 48 h, was initiated after 3 days, by replacing the medium used for plating by 20 ml of fresh medium containing 100 μCi of carrier-free $\text{H}_2^{35}\text{SO}_4$ (New England Nuclear, Boston, MA, U.S.A.)/ml.

After labelling, the cells and collagen gels were harvested as described previously (David & Van den Berghe, 1983). Labelling media were discarded. Cells, released from the collagen gels by incubation at 37 °C in

0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4, containing 0.02% EDTA, were extracted in buffer A containing 4 M-guanidinium chloride, 50 mM-sodium acetate, 100 mM-6-aminohexanoic acid, 10 mM-EDTA, 5 mM-benzamidine, 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride and 1 μg of pepstatin A/ml at pH 5.8. Gels, essentially free of cells but still retaining the basal lamina produced *in vitro* were extracted in buffer A, supplemented with 0.5% (w/v) Triton X-100.

After incubation at 4 °C for 24 h, the extracts were cleared by centrifugation (30 000 *g* for 30 min) and fractionated at 4 °C by gel filtration on Sepharose CL-4B in buffer A with or without 0.5% Triton X-100. The columns (1 cm $\times$ 100 cm) were run at 4 °C with a flow rate of 3 ml/h. Cell extracts were chromatographed in the absence of detergent, to separate hydrophobic and non-hydrophobic proteoglycan fractions (Rapraeger & Bernfield, 1985). Further purification of the ^{35}S -labelled proteoglycans was achieved by anion-exchange chromatography on an HR5/5/Mono Q column (Pharmacia) in 6 M-urea/50 mM-Tris/HCl buffer, pH 8.0, containing 0.5% Triton X-100, with a linear NaCl gradient (0–1.2 M; 15 mM/ml) delivered by a Pharmacia fast protein liquid chromatography system at a flow rate of 0.5 ml/min. Determination of the amount of heparan [^{35}S]sulphate and chondroitin [^{35}S]sulphate in each eluted fraction was as described previously (David & Van den Berghe, 1985).

Free glycosaminoglycan chains were prepared by β -elimination of the various proteoglycans with 0.5 M-NaOH for approx. 18 h (4 °C) followed by neutralization with 4 M-HCl and dialysis against water. Digestion with chondroitin ABC lyase was performed in accordance with Kolset *et al.* (1983). HNO_2 degradation (pH 1.5) of glycosaminoglycans followed by NaBH_4 reduction of the products was carried out with a slight modification of the method described previously (Thunberg *et al.*, 1982). HNO_2 (200 μl ; pH 1.5) was added to the samples and after 10 min at room temperature the reaction was interrupted by addition of 75 μl of 1 M- Na_2CO_3 containing 13 mg of NaBH_4 /ml. After reduction of the deamination products for approx. 18 h at room temperature the pH was lowered to approx. 4 with acetic acid and finally the samples were neutralized with 4 M-NaOH. Uronic acid was detected by the carbazole reaction (Bitter & Muir, 1962).

Gel chromatography was performed on columns of Sephadex G-25 (1 cm $\times$ 190 cm, eluted with 0.2 M- NH_4HCO_3 at approx. 6 ml/h) or Sepharose CL-6B (1 cm $\times$ 90 cm, eluted with 0.15 M-NaCl/0.1% SDS/50 mM-Tris/HCl buffer, pH 8.0, at approx. 3 ml/h). Anion-exchange chromatography was carried out on columns (3 ml) of DEAE-cellulose, eluted at approx. 6 ml/h with linear salt gradients extending from 0.05 M- to 1.5 M-LiCl in 0.05 M-sodium acetate buffer, pH 4.0. Affinity chromatography on antithrombin-Sepharose was performed as previously described (Thunberg *et al.*, 1982), with columns (3 ml) eluted with linear salt gradients.

Anion-exchange h.p.l.c. of ^{35}S -labelled di- and tetrasaccharides was performed on a Partisil-10 SAX (Whatman, Clifton, NJ, U.S.A.) column eluted stepwise with KH_2PO_4 buffers (Bienkowski & Conrad, 1985) at 1 ml/min. Radioactivity eluted from the column was detected with a Flo-One HS radioactive-flow detector

Fig. 1. Gel chromatography on Sepharose CL-4B of intact basement-membrane proteoglycans

Biosynthetically ^{35}S -labelled BM proteoglycans from cultures of (a) normal and (b) transformed MuMG cells were extracted and chromatographed at 4°C in buffer containing 4 M-guanidinium chloride and Triton X-100. Collected fractions were analysed for their total [^{35}S]sulphate (—), heparan [^{35}S]sulphate (●) and chondroitin [^{35}S]sulphate (○). The fractions were combined in two separate pools as indicated by bars 1 and 2 at the top of the Figure.

(Radiomatic Instruments, Tampa, FL, U.S.A.), and was continuously registered on a strip-chart double-channel recorder. As scintillation medium was used Flow-Scint III (Radiomatic), which was pumped through the instrument at a rate of 5 ml/min. Peaks of ^{35}S radioactivity were identified by comparison of their retention times with those of ^3H -labelled di- and tetra-saccharide internal standards.

RESULTS

Characterization of intact proteoglycans

The ^{35}S -labelled proteoglycans that accumulated in the basement membranes of cells cultured on collagen gels were extracted and subjected to gel filtration on Sepharose CL-4B in the presence of Triton X-100.

Fig. 2. Gel chromatography on Sepharose CL-6B of BM glycosaminoglycans isolated from normal (○) and transformed (●) MuMG cells

Biosynthetically ^{35}S -labelled glycosaminoglycan chains were obtained by alkaline elimination of the corresponding BM1 proteoglycans. Samples (approx. 10000 c.p.m.) were applied to columns (1 cm $\times$ 90 cm) of Sepharose CL-6B equilibrated with 0.15 M-NaCl/0.1% SDS/50 mM-Tris/HCl buffer, pH 8.0. Fractions (approx. 1 ml) were collected and analysed for radioactivity.

Basement-membrane (BM) proteoglycans originating from both normal and transformed MuMG cell cultures were eluted as two incompletely separated peaks near $K_{av} = 0.2$ and $K_{av} = 0.35-0.40$ (Fig. 1). In contrast with the material obtained from normal cells, a significant proportion of label in proteoglycans derived from transformed cells was in chondroitin sulphate, mostly in the fractions appearing near $K_{av} = 0.2$.

The eluted fractions were combined in two pools, as indicated (further designated as BM1 and BM2 proteoglycans). The relative amounts of label present in pools 1 and 2 varied slightly in different experiments. Characteristically, however, the matrix of transformed cells contains less heparan [^{35}S]sulphate, and a smaller proportion of this heparan [^{35}S]sulphate is present in pool 1. In addition, a larger proportion of the matrix-associated label is accumulated in chondroitin sulphate as compared with the normal cells (David & Bernfield, 1981; David & Van den Berghe, 1983; Fig. 1).

^{35}S -labelled proteoglycans were also extracted from cells that were released from the collagen gels during the monolayer dissociation procedure, and chromatographed on Sepharose CL-4B in the absence of detergent. Extracts from both cell types contained an excluded proteoglycan fraction, enriched in hydrophobic proteoglycans, and an included fraction ($K_{av} = 0.33-0.36$), enriched in non-hydrophobic proteoglycans (results not shown), consistent with previous results (David & Bernfield, 1981; Rapraeger & Bernfield, 1985).

Characterization of glycosaminoglycan chains

Molecular size. Free ^{35}S -labelled glycosaminoglycan chains, prepared by alkaline elimination of intact BM proteoglycans, were subjected to gel filtration on Sepharose CL-6B. Chains isolated from BM1 and BM2 proteoglycans of normal MuMG cells were eluted as essentially single peaks with K_{av} values of 0.34 and 0.37

Table 1. Properties of ^{35}S -labelled glycosaminoglycan chains isolated from MuMG cells

Glycosaminoglycan chains were released from the corresponding proteoglycans by alkaline elimination.

Source	K_{av} of peak from Sepharose CL-6B	Chondroitin sulphate content (%)	N-/O-Sulphate ratio*	Proportion bound with high affinity to antithrombin (%)	3-O-Sulphate (% of total sulphate)*
BM1 proteoglycans from normal cells	0.34	5	58:42	29	0.9
BM2 proteoglycans from normal cells	0.37	5	55:45	27	1.2
BM1 proteoglycans from transformed cells	0.42 (0.58)†	30	58:42	7	0.3
BM2 proteoglycans from transformed cells	0.46	5	53:47	14	0.5
Hydrophobic cellular proteoglycans from normal cells‡	—	—	—	22	—
Non-hydrophobic cellular proteoglycans from normal cells	—	—	—	23	—
Hydrophobic cellular proteoglycans from transformed cells	—	—	—	6	—
Non-hydrophobic cellular proteoglycans from transformed cells	—	—	—	6	—

* Refers to the heparan sulphate portion, corrected for the amount of chondroitin sulphate in each sample.

† Two peaks were observed on gel filtration. The K_{av} = 0.58 peak was degraded by chondroitin ABC lyase.

‡ The cellular proteoglycans were pure heparan sulphate proteoglycans since they were digested with chondroitin ABC lyase before affinity chromatography on antithrombin-Sepharose.

Fig. 3. Anion-exchange chromatography of BM glycosaminoglycans isolated from normal (○) and transformed (●) MuMG cells

^{35}S -labelled glycosaminoglycan chains were released from BM1 proteoglycans by alkaline elimination. Samples (approx. 10000 c.p.m.) were mixed with ^3H -labelled standards of hyaluronan (Hy), chondroitin sulphate (CS) and heparin (Hep) (approx. 50000 c.p.m. each) and applied to columns (3 ml) of DEAE-cellulose, which were eluted with linear salt gradients (see the Methods section). Effluent fractions (approx. 3 ml) were collected and analysed for ^{35}S radioactivity (○ and ●) and ^3H radioactivity (—). ———, Conc. of LiCl.

respectively (Fig. 2 and Table 1). The chains derived from the BM1 proteoglycan of transformed cells were divided into two peaks, one with K_{av} = 0.42 and a second peak with K_{av} = 0.58 (Fig. 2 and Table 1). Chondroitin ABC lyase digestion of this material revealed that the smaller chains, corresponding to the K_{av} = 0.58

peak, were completely degraded, whereas the larger chains were resistant to the enzyme treatment (results not shown). Polysaccharide chains released from the BM2 proteoglycans of transformed cells emerged as a symmetrical peak with K_{av} = 0.46 on Sepharose CL-6B chromatography (Table 1).

Fig. 4. Affinity chromatography on immobilized antithrombin of BM glycosaminoglycans isolated from normal (*a* and *b*) and transformed (*c* and *d*) MuMG cells

Metabolically ^{35}S -labelled glycosaminoglycan chains (approx. 20000 c.p.m.), obtained by alkaline elimination of BM1 (*a* and *c*) and BM2 (*b* and *d*) proteoglycans, were mixed with 1 mg of unlabelled standard pig mucosal heparin and applied to columns (3 ml) of antithrombin-Sepharose, which were eluted with linear salt gradients (Thunberg *et al.*, 1982). Effluent fractions (2–3 ml) were collected and analysed for ^{35}S radioactivity ($\circ$) and for hexuronic acid (—). Arrows indicate the beginning of the salt gradients. The elution positions of standard heparin with high affinity (HA) and low affinity (LA) for antithrombin are indicated.

Anionic properties. ^{35}S -labelled free polysaccharide chains derived from the various BM proteoglycans were subjected to anion-exchange chromatography on

Fig. 5. Gel chromatography of products obtained by deaminative cleavage of BM glycosaminoglycans isolated from normal MuMG cells

^{35}S -labelled polysaccharide chains, released by alkaline elimination from BM1 proteoglycans, were treated with HNO_2 (pH 1.5)– NaBH_4 and applied to a column (1 cm $\times$ 190 cm) of Sephadex G-25 equilibrated with 0.2 M- NH_4HCO_3 . Effluent fractions corresponding to disaccharides (including free inorganic sulphate) ($\text{Di} + ^{35}\text{SO}_4^{2-}$), tetrasaccharides (Tetra) and larger oligosaccharides ($\geq$ Hexa) were combined into separate pools (indicated by horizontal bars) and freeze-dried.

DEAE-cellulose along with ^3H -labelled standards of hyaluronan, chondroitin sulphate and heparin. Chains from both normal and transformed MuMG cells yielded single symmetrical peaks. Chains from normal cells appeared to have a slightly higher anionic charge density, as they were eluted somewhat later in the gradient than chains isolated from transformed cells (Fig. 3, results shown for chains derived from BM1 proteoglycans).

Binding to antithrombin. ^{35}S -labelled free polysaccharide chains, prepared by alkali treatment of intact proteoglycans, were mixed with unlabelled standard pig mucosal heparin and subjected to affinity chromatography on columns of antithrombin-Sepharose. The standard heparin divided into two distinct peaks, with high affinity and low affinity for the proteinase inhibitor respectively (— in Fig. 4), a pattern characteristic for commercial heparin preparations (Björk & Lindahl, 1982). Also, a substantial proportion (27–29%) of the ^{35}S -labelled chains obtained from BM proteoglycans produced by normal MuMG cells was bound with high affinity to antithrombin, the peak being eluted slightly earlier in the salt gradient than standard high-affinity heparin (Figs. 4*a* and 4*b* and Table 1). In contrast, a significantly lower proportion (7–14%) of chains isolated from BM proteoglycans derived from transformed cells bound with high affinity to antithrombin (Figs. 4*c* and 4*d* and Table 1).

In an additional experiment, the antithrombin-binding properties of ^{35}S -labelled chains isolated from cellular heparan sulphate proteoglycans (both hydrophobic and non-hydrophobic proteoglycans) of normal and transformed MuMG cells were investigated. Affinity chromatography on antithrombin-Sepharose showed that

Fig. 6. Anion-exchange h.p.l.c. of disaccharides (a) and tetrasaccharides (b) obtained by deaminative cleavage of biosynthetically ^{35}S -labelled BM1 proteoglycans isolated from normal MuMG cells

Di- and tetra-saccharides were recovered (Fig. 5) and analysed on a Partisil-10 SAX column. Monosulphated disaccharides were separated in 0.025 M- KH_2PO_4 , disulphated disaccharides and free inorganic sulphate in 0.16 M- KH_2PO_4 , monosulphated tetrasaccharides in 0.16 M- KH_2PO_4 , disulphated tetrasaccharides in 0.24 M- KH_2PO_4 and trisulphated tetrasaccharides in 0.35 M- KH_2PO_4 . Arrows indicate the retention times of reference ^3H -labelled di- and tetra-saccharides: 1, tentatively identified as $\text{GlcA}(2\text{-OSO}_3)\text{-aMan}_R$ (see Bienkowski & Conrad, 1985); 2, $\text{GlcA-aMan}_R(6\text{-OSO}_3)$; 3, $\text{IdA-aMan}_R(6\text{-OSO}_3)$; 4, $\text{IdA}(2\text{-OSO}_3)\text{-aMan}_R$; 5, $\text{IdA}(2\text{-OSO}_3)\text{-aMan}_R(6\text{-OSO}_3)$; 6, $\text{IdA-GlcNAc}(6\text{-OSO}_3)\text{-GlcA-aMan}_R(3\text{-OSO}_3)$ and 7, $\text{IdA-GlcNAc}(6\text{-OSO}_3)\text{-GlcA-aMan}_R(3,6\text{-di-OSO}_3)$. -----, Conc. of KH_2PO_4 .

22–23 % of the chains obtained from normal cells compared with only about 6 % of those derived from the transformed counterparts bound to the immobilized proteinase inhibitor with high affinity (Table 1).

Compositional analysis

HNO_2 depolymerization. HNO_2 depolymerization (pH 1.5) and NaBH_4 reduction of heparin-related saccharides results in conversion of *N*-sulphated glucosamine residues into the corresponding 2,5-anhydromannitol derivative with cleavage of the glucosaminidic bond and release of free inorganic sulphate. Consequently disaccharides obtained in this way will have the general structure HexA-aMan_R with *O*-sulphate groups at various positions. As *N*-acetylated glucosamine residues are resistant to HNO_2 degradation, tetrasaccharides with the general structure $\text{HexA-GlcNAc-GlcA-aMan}_R$ are formed whenever an *N*-acetylglucosamine residue is surrounded by two *N*-sulphated glucosamine units. Hexa- and larger oligosaccharides are formed in regions of the polysaccharide that contain two or more consecutive *N*-acetylated

disaccharide units (Shively & Conrad, 1976; Thunberg *et al.*, 1982).

Biosynthetically ^{35}S -labelled glycosaminoglycan chains, obtained by alkaline elimination of various BM proteoglycans, were subjected to deaminative cleavage with HNO_2 (pH 1.5) followed by reduction with NaBH_4 . Gel chromatography on Sephadex G-25 of the products yielded essentially similar patterns for the polysaccharides isolated from BM1 and BM2 proteoglycans of normal and transformed MuMG cells when corrected for the amount of galactosaminoglycan in each sample. [Chondroitin sulphate was eluted in the void volume of the column together with large heparan sulphate fragments. By subjecting this material to chondroitin ABC lyase digestion followed by repeated Sephadex G-25 chromatography, the overall percentage of chondroitin sulphate versus heparan sulphate could be calculated for the various proteoglycan fractions (Table 1).] A major peak, corresponding to 75–80 % of the label, emerged as disaccharides (including free inorganic ^{35}S sulphate), but also significant amounts of tetrasaccharides (11–13 %) and larger oligosaccharides (10–12 %) were observed (Fig. 5, results shown for BM1 proteoglycans derived from normal cells).

The *O*-sulphated disaccharides were separated from free inorganic ^{35}S sulphate by anion-exchange h.p.l.c. (Fig. 6a). The percentage of inorganic ^{35}S sulphate in each disaccharide fraction (corresponding to *N*-sulphate groups of the intact polysaccharide chains) combined with information about the size distribution of *O*-sulphated oligosaccharides formed on HNO_2 depolymerization (Fig. 5) was used to calculate the *N*-/*O*-sulphate ratio of the various BM heparan sulphate samples. The values obtained showed that the *N*-/*O*-sulphate ratios of all heparan sulphates were very similar (53–58 % *N*-sulphate), and no differences between heparan sulphate from transformed and normal cells could be demonstrated (Table 1).

H.p.l.c. analysis. ^{35}S -labelled di- and tetra-saccharides recovered from the various BM proteoglycans (see Fig. 5) were analysed by anion-exchange h.p.l.c. The identities of ^{35}S radioactivity peaks were ascertained by comparison of their retention times with those of ^3H -labelled standard oligosaccharides. Analysis of disaccharide fractions showed that the mono-*O*-sulphated disaccharide $\text{IdA}(2\text{-OSO}_3)\text{-aMan}_R$ was the most abundant component of all BM heparan sulphate fractions (Fig. 6a and Table 2). The disulphated disaccharide $\text{IdA}(2\text{-OSO}_3)\text{-aMan}_R(6\text{-OSO}_3)$ was present in lesser amounts and, in addition, smaller peaks corresponding to the monosulphated disaccharides $\text{GlcA-aMan}_R(6\text{-OSO}_3)$ and $\text{IdA-aMan}_R(6\text{-OSO}_3)$ were detected (Fig. 6a and Table 2). An interesting finding is the appearance of an additional peak (peak no. 1 in Fig. 6a) emerging ahead of standard $\text{GlcA-aMan}_R(6\text{-OSO}_3)$ on h.p.l.c. analysis of disaccharide fractions. A disaccharide with identical elution position in a similar h.p.l.c. system was previously identified as $\text{GlcA}(2\text{-OSO}_3)\text{-aMan}_R$ by Bienkowski & Conrad (1985).

The tetrasaccharide fractions contained largely monosulphated species but also significant amounts of di- and tri-sulphated tetrasaccharides (Fig. 6b and Table 2). The two 3-*O*-sulphated tetrasaccharides $\text{IdA-GlcNAc}(6\text{-OSO}_3)\text{-GlcA-aMan}_R(3\text{-OSO}_3)$ and $\text{IdA-GlcNAc}(6\text{-OSO}_3)\text{-GlcA-aMan}_R(3,6\text{-di-OSO}_3)$, previously used as markers for the antithrombin-binding region of heparin

Table 2. Composition of [³⁵S]sulphated di- and tetra-saccharides obtained by HNO₂ depolymerization of [³⁵S]sulphate-labelled heparan sulphate proteoglycans isolated from basement membranes produced by MuMG cells

The composition was determined by anion-exchange h.p.l.c. as described in the Methods section. Molar compositions of total *O*-sulphated saccharides were calculated with regard to the number of *O*-[³⁵S]sulphate groups in each component. Abbreviation: N.D., none detected.

Source	Composition of disaccharides (mol/100 mol)				
	GlcA(2-OSO ₃)-aMan _R *	GlcA-aMan _R (6-OSO ₃)	IdA-aMan _R (6-OSO ₃)	IdA(2-OSO ₃)-aMan _R	IdA(2-OSO ₃)-aMan _R (6-OSO ₃)
BM1 proteoglycans from normal cells	6.4	9.6	5.5	49	29
BM2 proteoglycans from normal cells	2.4	8.5	3.6	51	35
BM1 proteoglycans from transformed cells	N.D.	4.6	1.2	67	28
BM2 proteoglycans from transformed cells	9.5	2.3	7.9	56	24

Source	Composition of tetrasaccharides (mol/100 mol)				
	Mono-sulphated tetra-saccharides	IdA-GlcNAc(6-OSO ₃)-GlcA-aMan _R (3-OSO ₃)	Other disulphated tetra-saccharides	IdA-GlcNAc(6-OSO ₃)-GlcA-aMan _R (3,6-di-OSO ₃)	Other-trisulphated tetra-saccharides
BM1 proteoglycans from normal cells	83	7.3	2.8	N.D.	6.7
BM2 proteoglycans from normal cells	76	6.1	6.0	1.7	9.7
BM1 proteoglycans from transformed cells	85	1.0	6.6	1.1	6.1
BM2 proteoglycans from transformed cells	83	4.1	5.5	N.D.	7.2

* Tentative identification (see the Results section).

(Thunberg *et al.*, 1982), were identified among the deamination products of the various BM proteoglycans (Fig. 6*b* and Table 2). However, the contents of these tetrasaccharides were higher in fractions obtained from normal cells than those relating to transformed cells (Table 2). From the proportion of 3-*O*-sulphated anhydromannitol units in the tetrasaccharide fractions and the overall tetrasaccharide contents of the labelled products formed on deamination of the proteoglycan, the percentage of 3-*O*-[³⁵S]sulphate groups out of the total incorporated [³⁵S]sulphate was calculated. Assuming that no 3-*O*-sulphate groups were present in monosulphated tetrasaccharides or in oligosaccharides larger than tetrasaccharides, it was estimated that 0.9% and 1.2% of the sulphate groups incorporated into BM1 and BM2 proteoglycans of normal cells and 0.3% and 0.5% of the sulphate groups in BM1 and BM2 proteoglycans of transformed cells respectively were 3-*O*-substituents (Table 1). Transformation of the MuMG cells thus appears to result in a 2–4-fold decrease in the incorporation of 3-*O*-sulphate groups into the BM heparan sulphate and a corresponding decrease in the number of heparan sulphate molecules with high affinity for antithrombin (see above).

DISCUSSION

It has been shown that heparan sulphates from various sources possess anticoagulant activity (Marcum &

Rosenberg, 1985; Lane *et al.*, 1986; Marcum *et al.*, 1986). Moreover, the presence of the unique 3-*O*-sulphated glucosamine units, previously identified as markers for the antithrombin-binding region of heparin (Lindahl *et al.*, 1980), was demonstrated in these heparan sulphates. It has thus been suggested that heparan sulphate with anticoagulant activity located at the surface of vascular endothelial cells serves to render the vessel wall non-thrombogenic (Marcum & Rosenberg, 1985; Marcum *et al.*, 1986). However, in these heparan sulphate preparations only a modest proportion of the polysaccharide chains showed high affinity for antithrombin. In contrast, we have recently shown that approx. 80% of the heparan sulphate isolated from Reichert's membrane, an extra-embryonic basement membrane produced by rodent embryos, binds to antithrombin–Sepharose with high affinity (Pejler *et al.*, 1987*a*). A correspondingly large proportion of 3-*O*-sulphated glucosamine units could also be demonstrated in this material.

In the present work we show that basement-membrane heparan sulphate isolated from another source, the mouse mammary-gland epithelial cells, also contains unusually large amounts of molecules with high affinity for antithrombin (Fig. 4). The presence of the unique 3-*O*-sulphate group is also demonstrated (Fig. 6*b*). These findings, along with our previous findings (Pejler *et al.*, 1987*a*), suggest that the presence of heparan sulphate with high affinity for antithrombin may be characteristic for basement-membrane heparan sulphate, and it will be

important to corroborate this proposal by examining heparan sulphate derived from other basement membrane sources.

Transformation of the mammary-gland epithelial cells apparently results in a decreased biosynthesis of molecules with high affinity for antithrombin (Fig. 4 and Table 1) and a corresponding decrease in the amount of 3-*O*-sulphated glucosamine residues (Table 1). A similar difference between normal and transformed cells was observed in our previous study of basement-membrane heparan sulphate isolated from Reichert's membrane (produced by normal cells) and from the Engelbreth-Holm-Swarm tumour tissue (Pejler *et al.*, 1987a). Heparan sulphate derived from the tumour tissue contained decreased amounts of molecules with high affinity for antithrombin compared with the polysaccharide from Reichert's membrane; however, the basement-membrane-producing cells were of different origin, and the difference in antithrombin binding could just be an expression of tissue specificity rather than neoplastic transformation. The normal and transformed cells used in the present study were of identical origin, and it therefore seems justified to ascribe the structural and functional differences between the heparan sulphates to the neoplastic transformation.

It may be recalled that the proteoglycans of pool 1 and pool 2 are very similar in their chromatographic behaviour (Fig. 1) and in their buoyant densities (results not shown), to the BM proteoglycans isolated from the Engelbreth-Holm-Swarm mouse tumour, originally designated BM1 and BM2 proteoglycans by Hassell *et al.* (1980) and later referred to as low-density and high-density proteoglycans (Fujiwara *et al.*, 1984; Ledbetter *et al.*, 1985). Moreover, at least some of the BM proteoglycans produced by the normal and the transformed MuMG cells carry epitopes that are recognized by a specific antiserum raised against the BM proteoglycans isolated from the mouse tumour (G. David, B. Nusgens, B. Van der Schueren, D. Van Cauwenbergh, H. Van den Berghe & C. M. Lapiere, unpublished work), further suggesting that the proteoglycans studied in the different systems are related.

The biological function of anticoagulant-active heparan sulphate present in basement membranes is unclear. It is possible that prevention of fibrin deposition at sites of vascular injury or in filtration processes might be an important role for heparan sulphate in at least some types of basement membranes. On the other hand, the involvement of extravascular coagulation in certain inflammatory processes (Geczy, 1983) and the presence of heparin in animals lacking a blood coagulation system (Jordan & Marcum, 1986; Pejler *et al.*, 1987b) suggest that the function of the antithrombin-binding saccharide sequence is not necessarily restricted to regulation of haemostasis.

Biosynthesis of the antithrombin-binding sequence requires a highly organized series of specific polymer modification reactions (Lindahl & Kjellén, 1987). Apparently, cell transformation may affect the regulation of this modification process, in an as yet poorly understood manner, such that the formation of antithrombin-binding regions is impaired.

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REFERENCES

- Atha, D. H., Lormeau, J.-C., Petitou, M., Rosenberg, R. D. & Choay, J. (1985) *Biochemistry* **24**, 6723-6729
- Bienkowski, M. J. & Conrad, H. E. (1985) *J. Biol. Chem.* **260**, 356-365
- Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330-334
- Björk, I. & Lindahl, U. (1982) *Mol. Cell. Biochem.* **48**, 161-182
- David, G. & Bernfield, M. (1981) *J. Cell Biol.* **91**, 281-286
- David, G. & Bernfield, M. (1982) *J. Cell. Physiol.* **110**, 56-62
- David, G. & Van den Berghe, H. (1983) *J. Biol. Chem.* **258**, 7338-7344
- David, G. & Van den Berghe, H. (1985) *J. Biol. Chem.* **260**, 11067-11074
- David, G., Van der Schueren, B. & Bernfield, M. (1981) *J. Natl. Cancer Inst.* **67**, 719-728
- Fransson, L.-Å., Havsmark, B. & Chiarugi, V. (1982) *Biochem. J.* **201**, 233-240
- Fujiwara, S., Widemann, H., Timpl, R., Lustig, A. & Engel, J. (1984) *Eur. J. Biochem.* **143**, 145-157
- Gallagher, J. T. & Walker, A. (1985) *Biochem. J.* **230**, 665-674
- Gallagher, J. T., Lyon, M. & Steward, W. P. (1986) *Biochem. J.* **236**, 313-325
- Geczy, C. L. (1983) *Lymphokines* **8**, 201-247
- Hassell, J. R., Gehron Robey, P., Barrach, H. J., Wilczek, J., Rennard, S. I. & Martin, G. R. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4494-4498
- Hassell, J. R., Leyshon, W. C., Ledbetter, S. R., Tyree, B., Suzuki, S., Kato, M., Kimata, K. & Kleinman, H. K. (1985) *J. Biol. Chem.* **260**, 8098-8105
- Höök, M., Björk, I., Hopwood, J. & Lindahl, U. (1976) *FEBS Lett.* **66**, 90-93
- Höök, M., Riesenfeld, J. & Lindahl, U. (1982) *Anal. Biochem.* **119**, 236-245
- Jordan, R. E. & Marcum, J. A. (1986) *Arch. Biochem. Biophys.* **248**, 690-695
- Kanwar, Y. S., Linke, A. & Farquhar, M. G. (1980) *J. Cell Biol.* **86**, 688-693
- Keller, K. L., Keller, J. M. & Moy, J. N. (1980) *Biochemistry* **19**, 2529-2536
- Koda, J. E. & Bernfield, M. (1984) *J. Biol. Chem.* **259**, 11763-11770
- Kolset, S. O., Kjellén, L., Seljelid, R. & Lindahl, U. (1983) *Biochem. J.* **210**, 661-667
- Lane, D. A., Pejler, G., Flynn, A. M., Thompson, E. A. & Lindahl, U. (1986) *J. Biol. Chem.* **261**, 3980-3986
- Ledbetter, S. R., Tyree, B., Hassell, J. R. & Horgan, E. A. (1985) *J. Biol. Chem.* **260**, 8106-8113
- Lindahl, U. & Höök, M. (1978) *Annu. Rev. Biochem.* **47**, 385-417
- Lindahl, U. & Kjellén, L. (1987) in *The Biology of the Extracellular Matrix: Proteoglycans* (Wight, T. N. & Mecham, R., eds.), pp. 59-104, Academic Press, New York
- Lindahl, U., Bäckström, G., Thunberg, L. & Leder, I. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6551-6555
- Lindahl, U., Thunberg, L., Bäckström, G., Riesenfeld, J., Nordling, K. & Björk, I. (1984) *J. Biol. Chem.* **259**, 12368-12376
- Marcum, J. A. & Rosenberg, R. D. (1985) *Biochem. Biophys. Res. Commun.* **126**, 365-372
- Marcum, J. A., Atha, D. H., Fritze, L. M. S., Nawroth, P., Stern, D. & Rosenberg, R. D. (1986) *J. Biol. Chem.* **261**, 7507-7517

- Paulsson, M., Dziadek, M., Suchanek, C., Huttner, W. B. & Timpl, R. (1985) *Biochem. J.* **231**, 571–579
- Pejler, G., Bäckström, G., Lindahl, U., Paulsson, M., Dziadek, M., Fujiwara, S. & Timpl, R. (1987*a*) *J. Biol. Chem.* **262**, 5036–5043
- Pejler, G., Danielsson, Å., Björk, I., Lindahl, U., Nader, H. B. & Dietrich, C. P. (1987*b*) *J. Biol. Chem.* **262**, 11413–11421
- Rapraeger, A. & Bernfield, M. (1985) *J. Biol. Chem.* **260**, 4103–4109
- Rodén, L. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 267–371, Plenum Press, New York
- Shanley, D. J., Cossu, G., Boettiger, D., Holtzer, H. & Pacifici, M. (1983) *J. Biol. Chem.* **258**, 810–816
- Shively, J. E. & Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
- Thunberg, L., Bäckström, G. & Lindahl, U. (1982) *Carbohydr. Res.* **100**, 393–410
- Underhill, C. B. & Keller, J. M. (1975) *Biochem. Biophys. Res. Commun.* **63**, 448–454
- Underhill, C. B. & Toole, B. P. (1982) *J. Cell. Physiol.* **110**, 123–128
- Winterbourne, D. J. & Mora, P. T. (1978) *J. Biol. Chem.* **253**, 5109–5120
- Winterbourne, D. J. & Mora, P. T. (1981) *J. Biol. Chem.* **256**, 4310–4320

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