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Structure of a heparan sulphate oligosaccharide that binds to basic fibroblast growth factor

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Binding of basic fibroblast growth factor (bFGF) to the extracellular matrix of cultured bovine aorta smooth muscle cells is likely to be mediated via heparan sulphate, since not only exogenous addition of heparan sulphate to the culture medium but also pretreatment of the cells with heparitinase (but not chondroitinase ABC) resulted in loss of binding. Comparison of the affinity of bFGF to various glycosaminoglycan-conjugated gels showed a direct and specific binding of bFGF to heparan sulphate. Heparan sulphate also bound to a bFGF affinity gel. However, the proportion of heparan sulphate bound varied depending on the source of the HS (more than 90% and 45% with pig aorta heparan sulphate and mouse EHS tumour heparan sulphate respectively). The bound heparan sulphate had the ability to protect bFGF from proteolytic digestion, but the unbound heparan sulphate did not. The results suggest the presence in the bound heparan sulphate of a specific structure involved in binding. Limited digestion with heparitinase I of porcine aorta heparan sulphate yielded 13% oligosaccharides bound to the gel, of which the smallest were octasaccharides. Analysis of a hexadecasaccharide fraction which was obtained at the highest yield among the bound oligosaccharides was performed by h.p.l.c. of the deamination products obtained with nitrous acid and the unsaturated disaccharide products formed by heparitinase digestion. Comparison of the disaccharide unit compositions exhibited a marked difference in $IdoA(2SO_4)GlcNSO_3$ and $IdoA(2SO_4)GlcNSO_3(6SO_4)$ units between the bound and unbound hexadecasaccharides. The amounts measured were 3 mol and 1 mol per mol of the former and 0.4 mol and 0.6 mol per mol of the latter. It is likely that the binding of bFGF to heparan sulphate may require the domain structure of the heparan sulphate to be composed of clustering $IdoA(2SO_4)$-$GlcNSO_3$ units.

INTRODUCTION

A large body of evidence indicates that many types of proteoglycans are involved in the regulation of cell growth. For example, the cell-surface heparan sulphate proteoglycan isolated from post-confluent cultures of aorta smooth muscle cells exhibits a potent inhibition of cell growth [1]. The heparan sulphate isolated from the surfaces of rat hepatocytes, when added to culture medium, inhibits the growth of hepatocytes where heparan sulphate enriched with $GlcA(2SO_4)GlcNSO_3(6SO_4)$ units appeared in the nucleus [2,3]. Epidermal growth factor-dependent proliferation of aorta smooth muscle cells was also regulated by heparan sulphate, perhaps owing to the interaction with thrombospondin [4].

Basic fibroblast growth factor (bFGF) is a potent mitogen that stimulates proliferation and differentiation of various types of mesenchymal and neuroectodermal cells, thereby mediating various biological processes such as wound repairing, angiogenesis and neurite outgrowth [5,6]. bFGF binds strongly to heparin, and this property has greatly facilitated the purification and characterization of bFGF [7]. It has also been shown that bFGF may bind to heparan sulphate proteoglycans in the extracellular matrix (ECM) formed by cultured cells such as vascular and capillary endothelial cells [8–10], and to heparan sulphate proteoglycans in their plasma membranes [11]. Immunohistochemical studies have revealed bFGF deposition in the basement membranes and ECM of a variety of tissues of which heparan sulphate proteoglycans are the major constituents [12–14]. Thus bFGF is likely to be stored and stabilized by binding to the heparan sulphate proteoglycans in the ECM and basement membranes. More recently, Yayon *et al.* [15] and Rapraeger *et al.* [16] have suggested that the binding of bFGF to its high-affinity receptor requires its binding to heparan sulphate or heparin. The three-dimensional structure of bFGF induced by heparan sulphate binding has been suggested to be important for the interaction between bFGF and the receptor [17].

In the present study we have isolated heparan sulphate oligosaccharides capable of binding to bFGF, and analysed products of these oligosaccharides produced by deamination cleavage with nitrous acid and digestion with heparinase and heparitinases. The results suggest that the binding may be dependent upon specific sugar sequences and sulphation patterns in heparan sulphate. We have also investigated the effect of binding to heparan sulphate on the degradation of bFGF.

Abbreviations used: bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; IdoA, iduronic acid; EHS, Engelbreth–Holm–Swarm; ECM, extracellular matrix; AMan, 2,5-anhydro-D-mannose (when a subscript R follows this abbreviation, this refers to the corresponding alditol formed by reduction of the compound with $NaBH_4$); ΔDi-OS, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-glucose; ΔDi-6S, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-6-*O*-sulpho-D-glucose; ΔDi-NS, 2-deoxy-2-sulphamino-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-glucose; ΔDi-(N,6)diS, 2-deoxy-2-sulphamino-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-6-*O*-sulpho-D-glucose; ΔDi-(N,U)diS, 2-deoxy-2-sulphamino-(4-deoxy-2-*O*-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-glucose; ΔDi-(N,6,U)triS, 2-deoxy-2-sulphamino-(4-deoxy-2-*O*-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid)-6-*O*-sulpho-D-glucose; GAG, glycosaminoglycan; DMEM, Dulbecco's modified Eagle's medium; PBS(−), phosphate-buffered saline (0.1 M-sodium phosphate/1.37 M-NaCl/2.7 mM-KCl, pH 7.2); PBS(+), PBS containing Ca^{2+} and Mg^{2+}.

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EXPERIMENTAL

Materials

^{125}I-labelled bFGF [bovine recombinant, 297 TBq (8000 Ci)/mmol], bFGF (bovine recombinant) and [^{3}H]$NaBH_4$ [455 GBq (12.3 Ci)/mmol in 0.1 M-NaOH] were purchased from Amersham Japan, Tokyo, Japan. Heparan sulphates from pig aorta and EHS tumour, chondroitin 6-sulphate from shark cartilage, dermatan sulphate from pig skin, chondroitin sulphate E from squid cartilage, chondroitin 4-sulphate from whale cartilage and hyaluronic acid were obtained from Seikagaku Corporation, Tokyo, Japan. Porcine intestinal mucosa heparin and trypsin (type III) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Heparitinases I, II and IV, heparinase and chondroitinase ABC were from Seikagaku. Ready-safe scintillant was from Beckman, Palo Alto, CA, U.S.A. X-ray film (AIF RX) was from Fuji Photo Film Co., Tokyo, Japan. Rainbow protein M_r markers were from Amersham Japan, Tokyo, Japan. Epoxy-activated Sepharose 6B, CNBr-activated Sepharose 4B, Sepharose 6B and Sephadex G-50 were from Pharmacia, Uppsala, Sweden. [^{3}H]Tetrasaccharide and [^{3}H]octasaccharide prepared from testicular hyaluronidase digests of chondroitin [18] were gifts from Dr. O. Habuchi, Aichi Kyoiku University.

Cell culture

Smooth muscle cells were obtained by culturing explants of bovine aorta as described by Ross [19]. Cells were subcultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), penicillin and streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. SMC in the sixth passage were used for the experiments.

Preparation of ECM from cultured bovine aorta smooth muscle cells and ^{125}I-bFGF binding to the ECM

Bovine aorta smooth muscle cells (2×10^4) were seeded on to 16 mm wells and grown to post-confluency. The cell layer was washed with phosphate-buffered saline [PBS(−)] and treated with 0.5% (w/v) Triton X-100 in PBS(−) at 37 °C for 30 min with shaking. The residual cell layer was washed with PBS(−) four times. ECM thus prepared was incubated with ^{125}I-bFGF for 60 min at room temperature with gentle shaking. After four washes with PBS(−), the radioactivity of ^{125}I-bFGF bound to ECM was counted in a γ-radiation counter.

Preparation of glycosaminoglycan (GAG)-conjugated Sepharose gels

GAG–Sepharose gels were prepared by the reported method with a minor modification [20]. 3-Amino-2-hydroxypropyl-derivatized Sepharose gels were prepared from epoxy-activated Sepharose 6B gels according to the method recommended by the manufacturer. Portions of 3 g of amino–Sepharose gels thus obtained were suspended in 3 ml of 0.2 M-phosphate buffer, pH 7.2, containing 90 mg of GAG and 9 mg of $NaBH_3CN$. The suspension was kept at room temperature for 48 h with gentle shaking. The gels were washed several times with PBS(−). The amount of immobilized GAG was about 1.8–3.7 mg/ml of gel.

Preparation of bFGF-conjugated Sepharose gels

bFGF (200 μg) was coupled to 0.5 ml of CNBr-activated Sepharose 4B gels according to the method recommended by the manufacturer. *N*-Acetylated heparan sulphate (200 μg) was added to 0.5 ml of bFGF solution (400 μg/ml) before the coupling reaction to protect the heparan sulphate-binding sites in bFGF. A total of 120 μg of bFGF was immobilized to the gel.

Binding assays of ^{125}I-bFGF to immobilized GAG

GAG–Sepharose gels were suspended in 20 mg of BSA/ml in PBS containing Ca^{2+} and Mg^{2+} [PBS(+)], and then shaken for 1 h at room temperature in order to block non-specific binding sites. The gels were washed extensively with PBS(+), suspended in PBS(+) containing 0.02% NaN_3 to give a 25% (w/v) suspension, and stored at 4 °C until use. The binding reaction was performed in 100 μl of solution containing 3.75% (w/v) GAG–Sepharose, 2×10^4 c.p.m. of ^{125}I-bFGF, 0.1% (w/v) BSA and 0.02% Tween 20 in PBS(+). After a 1 h incubation at room temperature with gentle agitation, the mixtures were diluted with 3 vol. of PBS(+)/0.02% Tween 20 (solution A) and immediately centrifuged (640 *g*, 3 min) in a microcentrifuge tube with a membrane filter (UFC30HV00; Millipore, Bedford, MA, U.S.A.). The gel on the membrane was washed thoroughly with solution A and the radioactivity bound to the gel was determined in a γ-radiation counter. Non-specific binding was determined using Sepharose 6B in place of GAG–Sepharose.

bFGF affinity chromatography of heparan sulphates and their constituent oligosaccharides

About 100 μg of ^{3}H-labelled or unlabelled heparan sulphate dissolved in 300 μl of PBS(+) containing 200 μg of chondroitin 6-sulphate/ml (solution B) was applied to a syringe column of bFGF–Sepharose (0.5 ml) equilibrated with solution B at 4 °C, and the column was shaken gently for 2 h. The column was then washed with 10 ml of solution B and eluted with a linear gradient from 0 to 3 M-NaCl in solution B at 5 ml/h. ^{3}H-labelled or unlabelled oligosaccharides were subjected to affinity chromatography as described above, except that a stepwise elution method was used. The eluates containing chondroitin 6-sulphate were desalted using a Fast desalting column (Pharmacia) and then digested with chondroitinase ABC [21]. After digestion, heparan sulphate was recovered by ethanol precipitation, and for separation of oligosaccharides the digests were directly subjected to gel filtration.

Preparation of ^{3}H-labelled heparan sulphates and their constituent oligosaccharides

Heparan sulphate (1 mg) was dissolved in 200 μl of 0.1 M-Tris/HCl, pH 8.8, and reduced with 0.5 mCi of [^{3}H]$NaBH_4$ (specific radioactivity 1.72 Ci/mmol) in 35 μl of 0.1 M-NaOH at room temperature for 3 h. The solution was adjusted to pH 4 with acetic acid to destroy excess [^{3}H]$NaBH_4$ and then to pH 7 with NaOH. ^{3}H-labelled heparan sulphate was precipitated twice with 75% (v/v) ethanol and desalted using a Fast desalting column. The specific radioactivity of ^{3}H-labelled heparan sulphate was approx. 9×10^4 d.p.m./μg. Pig aorta heparan sulphate (5 mg) was partially digested with 50 munits of heparitinase I in 500 μl of 0.05 M-Tris/HCl (pH 7.2)/1 mM-$CaCl_2$/50 μg of BSA. After incubation for 60 min at 37 °C, the mixture was placed in a boiling-water bath for 2 min. Aliquots (50 μl) of the digest were lyophilized and dissolved in [^{3}H]$NaBH_4$ in 0.1 M-NaOH as described above. The reaction was carried out at 0 °C for 2 h. ^{3}H-labelled oligosaccharides were desalted using a Fast desalting column (final specific radioactivity 1.9×10^4 d.p.m./μg).

Compositional analyses of heparan sulphates and their constituent oligosaccharides

Samples of 1–20 μg of heparan sulphates or oligosaccharides were digested with a mixture of 8 munits of heparitinase I, 3 munits of heparitinase II and 8 munits of heparinase in 20 μl of 50 mM-Tris/HCl (pH 7.2)/1 mM-$CaCl_2$/2 μg of BSA at 37 °C for

2 h. As far as we could ascertain, heparan sulphate preparations used in this study were depolymerized to disaccharides with 96–98% yields under the above digestion conditions. The yields were determined by comparing the relative proportions of the hexuronic acid content corresponding to disaccharides recovered by gel permeation with those in the starting preparations. The unsaturated disaccharide products were analysed by h.p.l.c. using a polyamine-bound silica PAMN column (YMC). The elution was performed by a linear gradient from 40 mM- to 550 mM-KH_2PO_4, and a subsequent elution with 550 mM-KH_2PO_4 at a flow rate of 1.5 ml/min at 40 °C. The elution was monitored by u.v. absorption at 232 nm. Each peak was identified by comparison of its retention time with those of standard unsaturated disaccharides, as described previously [22].

Degradation of the saccharides with nitrous acid at pH 1.5 and reduction of the products with [^{3}H]$NaBH_4$ were carried out as described by Shively & Conrad [23]. The products were subjected to gel chromatography using an HW40S column (98 cm × 1.6 cm) (Toso Co., Tokyo, Japan) which had been equilibrated with 0.5 M-NH_4HCO_3. The fractions corresponding to labelled disaccharides were collected and analysed by h.p.l.c. on a Partisil-10 SAX column (Whatman, Clifton, NJ, U.S.A.) as described by Bienkowski & Conrad [24]. The elution was monitored by measuring the radioactivity in a liquid scintillation counter.

Determination of oligosaccharide size

For determination of molecular size, oligosaccharide samples were chromatographed on a Sephadex G-50 column (120 cm × 1.2 cm) that had been calibrated with various M_r standards. The standards used were [^{3}H]tetrasaccharide (M_r 758) and [^{3}H]octasaccharide (M_r 1516) prepared from the [^{3}H]$NaBH_4$ reduction of chondroitin-derived octa- and tetra-saccharides, and ΔDi-6S (M_r 475) prepared from chondroitinase ABC digests of chondroitin 6-sulphate. M_r values of heparan sulphate oligosaccharides were calculated based on the assumption that the mean M_r of the repeating disaccharide units of heparan sulphate is 416.

Column chromatography

Anion-exchange chromatography of oligosaccharides was performed on a Mono Q column (Pharmacia) at a flow rate of 1 ml/min with a linear gradient from 0 to 2 M-NaCl in 0.05 M-Tris/HCl, pH 7.2. Desalting was performed by passing through a Fast desalting column (Pharmacia) equilibrated with distilled water.

Trypsin digestion of ^{125}I-bFGF in the presence of various GAGs

The reaction mixture contained 2.7×10^4 d.p.m. of ^{125}I-bFGF and 25 μg of BSA in 45 μl of solution C [25 mM-Tris/HCl (pH 7.2)/0.15 M-NaCl/0.4 mM-$MgCl_2$/0.9 mM-$CaCl_2$]. GAG (0–10 μg) was added to the above reaction mixture. After shaking at 37 °C for 5 min, 5 μl of 0.1% (w/v) trypsin was added and the mixtures were incubated at 37 °C for 1 or 3 h. Aliquots of the digests were analysed by SDS/PAGE in a 20% gel [25]. The gels were dried and exposed at −80 °C to X-ray film for 2 days. To the remaining digests were added 100 μg of BSA and 10% (final concentration) cold trichloroacetic acid. The precipitates were centrifuged at 12100 *g* for 30 min at 4 °C. The radioactivity in the supernatants was measured to determine the extent of degradation of ^{125}I-bFGF.

The activity of trypsin was determined by measuring the increase in absorbance at 410 nm using Bz-DL-Arg-*p*-nitroanilide as a substrate.

RESULTS

Binding of bFGF to the ECM of cultured aorta smooth muscle cells

^{125}I-bFGF was added to the ECM of cultured aorta smooth muscle cells as described in the Experimental section. After four washes with PBS(−), significant radioactivity was retained in the ECM (Table 1). The ECM components that could bind bFGF were then analysed by examining the effects of exogenously added GAGs on bFGF binding to the ECM (Table 1, expt. A). Heparin and heparan sulphate inhibited the binding, but none of chondroitin 6-sulphate, dermatan sulphate and chondroitin 4,6-sulphate (chondroitin sulphate E) showed such an effect. Pretreatment of the ECM with various GAG lyases had different effects on binding (Table 1, expt. B). Digestion with either heparitinase I or heparinase resulted in a decrease in the ability of ECM to bind ^{125}I-bFGF, but chondroitinase ABC digestion had no effect. The results suggest that heparan sulphate proteoglycans or heparan sulphate-associated molecules in the ECM participate in the binding of bFGF.

To test the possibility that bFGF binds directly to heparan sulphate chains, various species of GAG were conjugated to

Table 1. Binding of ^{125}I-bFGF to the ECM of cultured aorta smooth muscle cells

ECM was prepared from cultured bovine aorta smooth muscle cells as described in the Experimental section and incubated with ^{125}I-bFGF at room temperature for 60 min. In expt. A, ^{125}I-bFGF was incubated with the ECM preparation in the absence or the presence of 100 μg of each GAG/ml. In expt. B, ^{125}I-bFGF was incubated with ECM which had been treated without or with each enzyme at 37 °C for 1 h. Values represent means ± S.D. of triplicate binding.

GAGs or enzymes	$10^{-2} \times$ ^{125}I-bFGF bound (c.p.m.)
Expt. A	
None	17.0 ± 0.7
Heparin	7.0 ± 1.5
Heparan sulphate (pig aorta)	9.0 ± 0.5
Heparan sulphate (EHS tumour)	8.5 ± 0.7
Chondroitin 6-sulphate	17.3 ± 0.7
Dermatan sulphate	20.3 ± 2.5
Chondroitin sulphate E	21.5 ± 2.3
Expt. B	
None	24.8 ± 2.8
Heparitinase I (6 munits)	13.0 ± 0.3
Heparinase (5 munits)	18.5 ± 0.5
Chondroitinase ABC (80 munits)	27.0 ± 2.4

Table 2. Binding of ^{125}I-bFGF to GAG–Sepharose gels

^{125}I-bFGF (2×10^4 c.p.m.) was added to GAG–Sepharose gels conjugated with 4 μg of each GAG. ^{125}I-bFGF bound to the gel was measured as described in the Experimental section. ^{125}I-bFGF bound to Sepharose 6B gels was due to non-specific binding. Values represent means ± S.D. of triplicate binding.

GAG	$10^{-3} \times$ ^{125}I-bFGF bound (c.p.m.)
Heparin	8.50 ± 0.71
Chondroitin 4-sulphate	0.89 ± 0.13
Hyaluronic acid	0.89 ± 0.15
Dermatan sulphate	1.48 ± 0.18
Heparan sulphate (pig aorta)	7.90 ± 0.42
None (Sepharose 6B)	0.59 ± 0.14

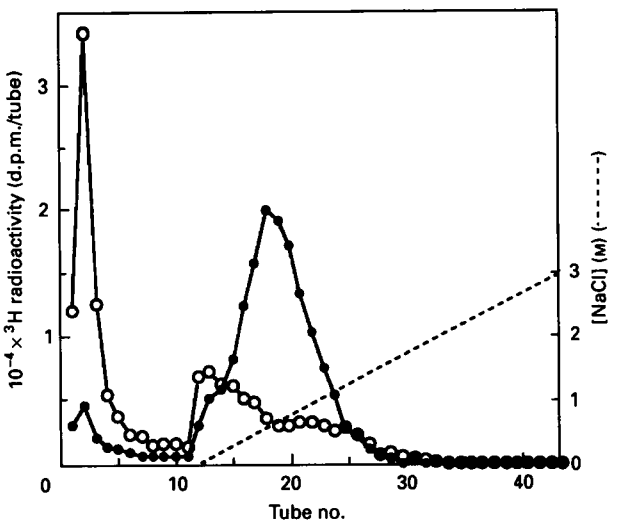

Fig. 1. bFGF affinity fractionation of [^{3}H]heparan sulphates

[^{3}H]Heparan sulphates were prepared by labelling the reducing end with [^{3}H]$NaBH_4$ as described in the Experimental section. A total of 1.5×10^5 d.p.m. of [^{3}H]heparan sulphate from pig aorta (●) or from EHS tumour (○) was dissolved in 300 μl of PBS(+) containing 200 μg of chondroitin 6-sulphate/ml (solution B) and applied to a bFGF–Sepharose column (0.5 ml) equilibrated with solution B. After standing at 4 °C for 2 h with gentle shaking, the column was washed with 10 ml of solution B, and then eluted with a 0–3 M-NaCl gradient in PBS(+) at 4 ml/h. Fractions (1 ml/tube) were collected and analysed for radioactivity. Fractions 1–5 and 12–27 from EHS tumour heparan sulphate were pooled, desalted, and concentrated (referred to as unbound HS and bound HS respectively) for further analyses.

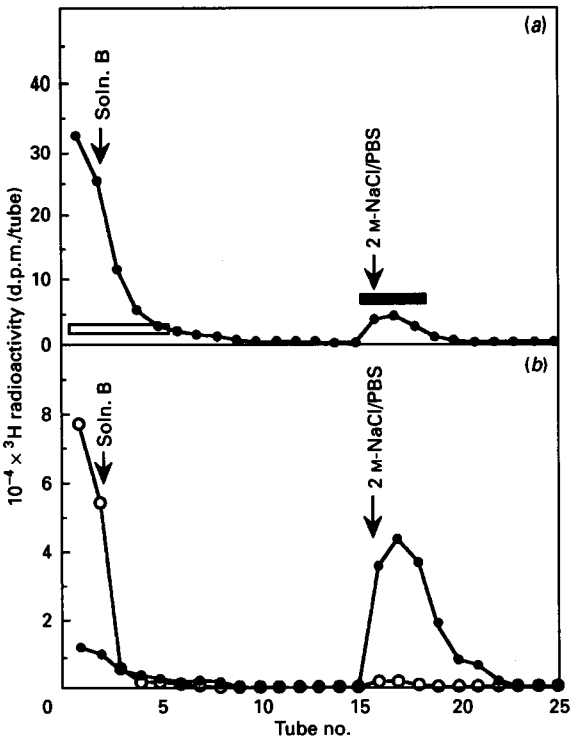

Fig. 2. bFGF affinity fractionation of [^{3}H]heparan sulphate oligosaccharides

Pig aorta heparan sulphate (5 mg) was subjected to partial digestion with 50 munits of heparitinase I at 37 °C for 1 h, and aliquots of the digests were then reduced with [^{3}H]$NaBH_4$ as described in the Experimental section. (*a*) [^{3}H]Heparan sulphate oligosaccharides thus obtained (9×10^5 d.p.m.) were applied to a bFGF–Sepharose column as described in the legend to Fig. 1. The column was washed with 7.5 ml of solution B, and then eluted with 5 ml of 2 M-NaCl in PBS(+) at 3 ml/h. Fractions (0.5 ml/tube) were collected and analysed for radioactivity (●). The open and solid horizontal bars indicate the unbound and bound fractions respectively, which were pooled, desalted and digested with chondroitinase ABC for re-chromatography. (*b*) Re-chromatography of the unbound fraction (○) and the bound fraction (●) indicated in (*a*). The resulting unbound and bound fractions were desalted using a Fast desalting column and digested with chondroitinase ABC to remove chondroitin 6-sulphate (referred to as unbound oligo and bound oligo respectively) for further analyses. The same column was used throughout the experiments.

Sepharose gels and their affinity interaction with ^{125}I-bFGF was examined (Table 2). The radioactivity was specifically bound to the heparan sulphate- or heparin-conjugated gels, but not to gels conjugated with hyaluronic acid, chondroitin 4-sulphate or dermatan sulphate. Since the net negative charge contributed by sulphate of heparan sulphate (0.42/disaccharide) is apparently lower than that of chondroitin 4- or 6-sulphate (1.0/disaccharide) or chondroitin sulphate E (1.7/disaccharide), the results in Tables 1 and 2 indicate that the binding of bFGF to heparan sulphate is not simply due to an electrostatic interaction.

Separation of heparan sulphate species with different affinities for bFGF

When ^{3}H-labelled heparan sulphate from pig aorta was applied to a column of bFGF-conjugated Sepharose equilibrated with PBS(+) containing 200 μg of chondroitin 6-sulphate/ml (solution B; chondroitin 6-sulphate was included to prevent non-specific binding), more than 90% of the starting radioactivity was retained on the column. Subsequent elution with a linear salt gradient from 0 to 3 M-NaCl in PBS(+) recovered most of the bound heparan sulphate from the column, with a peak at 0.6 M-NaCl (Fig. 1). In contrast, about 54% of ^{3}H-labelled EHS tumour heparan sulphate appeared in the washings with solution

Table 3. Unsaturated disaccharide compositions of bound HS, unbound HS and starting EHS tumour heparan sulphate preparations

The samples were digested with a mixture of heparitinases I and II and heparinase. The products were identified by h.p.l.c. on a polyamine-bound silica PAMN column and quantified at an absorbance of 232 nm as described in the Experimental section. Unidentified disaccharide represented less than 2% of the total.

	Composition (% of total)					
Fraction	ΔDi-OS	ΔDi-NS	ΔDi-6S	ΔDi-(N,6)diS	ΔDi-(N,U)diS	ΔDi-(N,6,U)triS
Unfractionated	33.6	59.4	0.4	2.6	2.3	0.7
Bound	34.1	57.4	0.6	2.9	2.7	0.8
Unbound	32.0	64.1	0.3	2.4	0.3	0.5

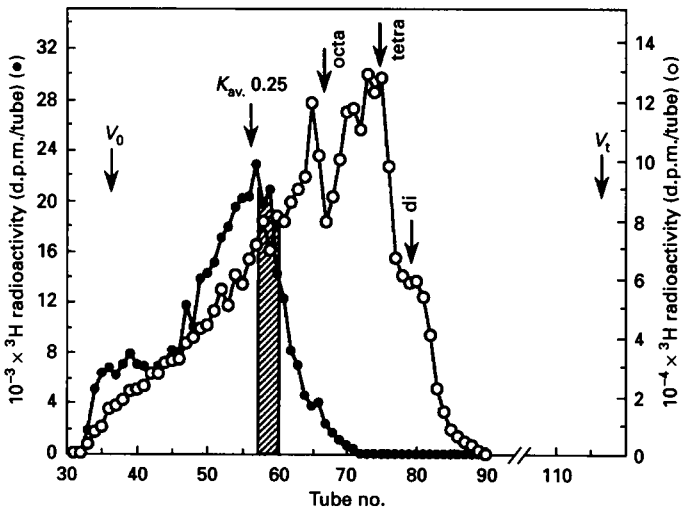

Fig. 3. Sephadex G-50 chromatography of [^{3}H]heparan sulphate oligosaccharides

Unbound oligo (3×10^6 d.p.m.; ○) and bound oligo (3.5×10^5 d.p.m.; ●) were subjected to gel chromatography, and fractions (1.4 ml/tube) were collected. The shaded bar indicates the fractions which were pooled for further analysis. V_0, void volume; V_t, total volume; the elution positions of the M_r markers Δdi-6S, [^{3}H]chondroitin tetrasaccharide and [^{3}H]chondroitin octasaccharide are indicated by the arrows labelled di, tetra and octa respectively.

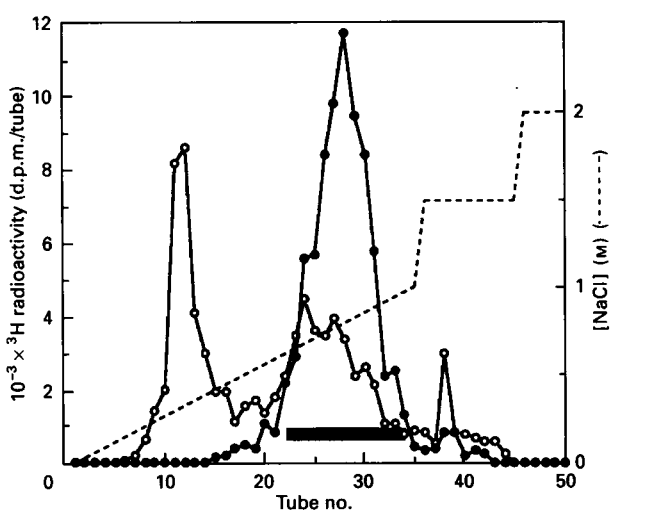

Fig. 4. Mono Q anion-exchange chromatography of [^{3}H]heparan sulphate hexadecasaccharide fractions

The hexasaccharide fractions from bound oligo (●) and those from unbound oligo (○) (8.4×10^4 d.p.m.; shaded area of Fig. 3) were desalted, concentrated and applied to a Mono Q column. The column was eluted with the indicated NaCl gradient in 0.05 M-Tris/HCl, pH 7.2, and fractions of 1 ml were collected. The fractions shown by the solid horizontal bar were pooled and desalted for further analysis; the pooled fractions from unbound oligo and bound oligo are referred to as U16-H and B16-H respectively.

B and the remainder was eluted with a broader peak from the beginning of the gradient. Re-chromatography of both bound fractions showed that essentially all of the radioactivity was again retained in the column. Conversely, none of the unbound fractions gave any radioactivity retained on the column (results not shown). The results suggest that there are at least two different types of heparan sulphates with respect to their affinity for bFGF [i.e. the bound (high-affinity) and unbound (low-affinity) species] in both aorta and EHS tumour samples. Hereafter, the unbound and bound fractions from the column of EHS tumour heparan sulphate are referred to as 'unbound HS' and 'bound HS' respectively.

Characterization of bound and unbound HS

Bound HS and unbound HS from EHS tumour heparan sulphate were digested with a mixture of heparitinases I and II and heparinase (heparitinase III), and the products were then subjected to h.p.l.c. on a polyamine silica column eluted with a linear gradient of KH_2PO_4. The disaccharide compositions were estimated from the percentage yield of each unsaturated disaccharide product compared with the total (Table 3). The results indicate that, although there was no significant difference between bound HS and unbound HS in the degree of sulphation (mol of sulphate/mol of disaccharide), the content of ΔDi-(N,U)diS was notably higher in bound HS than in unbound HS (2.7% compared with 0.3%). Comparative studies with pig aorta heparan sulphates were not carried out, because most of them bound to the bFGF affinity gel.

Binding of oligosaccharides to bFGF–Sepharose

If a unique structural unit is involved in the binding of bFGF, the smaller-sized bound heparan sulphate should have the higher relative content of such a unit. ^{3}H-labelled heparan sulphate oligosaccharides were prepared by limited digestion of pig aorta heparan sulphate with heparitinase I; this enzyme attacks preferentially glucosaminidic linkages to non-sulphated hexuronic acid residues. The products were radiolabelled by reduction with [^{3}H]$NaBH_4$ and applied to a bFGF-conjugated Sepharose gel column equilibrated with solution B. After washing with solution B, the column was eluted with 2 M-NaCl in PBS(+) as described above (Fig. 2*a*). About 13% of the applied radioactivity was bound to the bFGF column. When the bound and unbound oligosaccharide fractions were re-chromatographed on the same bFGF–Sepharose column, the unbound fraction did not yield any bound radioactivity, and most radioactivity from the bound fraction was reproducibly retained (Fig. 2*b*). These oligosaccharide fractions will be referred to subsequently as unbound oligo and bound oligo respectively.

Characterization of bound oligo and unbound oligo

Bound oligo and unbound oligo were subjected to gel chromatography on Sephadex G-50 (Fig. 3). Bound oligo was eluted between V_0 and the position of octasaccharides, with a peak at $K_{av.} = 0.25$, corresponding to octadeca- or hexadeca-saccharides (M_r 3700–4000). In contrast, unbound oligo showed a broad distribution with a peak around the position of hexasaccharides. It should be noted that, although the distribution of unbound oligo overlapped with that of bound oligo, oligosaccharides smaller in size than hexasaccharides were only detected in unbound oligo. The results suggest that octasaccharides are the minimal size required for the binding of bFGF, and that a factor other than chain length is also involved in bFGF binding.

[^{3}H]Hexadecasaccharides from bound oligo and unbound oligo were separately pooled (fractions 58–60; shaded bar in Fig. 3), since oligosaccharides of this size formed the majority in bound oligo. Both fractions were subjected to further fractionation by ion-exchange chromatography on a Mono Q column (Fig. 4). Most of the bound hexadecasaccharides were eluted in a single peak at 0.7 M-NaCl (fractions 22–34; termed B16-H in Fig. 4). On the other hand, the unbound hexadecasaccharides were eluted broadly with at least two major peaks at 0.3 M- and 0.7 M-

Table 4. Unsaturated disaccharide compositions of fractions B16-H and U16-H

B16-H and U16-H were prepared as shown in Fig. 4. The analysis was performed as described in Table 3. Unidentified disaccharides represented less than 5% of the total.

Sample	Composition (% of total)					
	ΔDi-OS	ΔDi-NS	ΔDi-6S	ΔDi-(N,6)diS	ΔDi-(N,U)diS	ΔDi-(N,6,U)triS
B16-H (bound)	27.6	14.5	6.8	3.4	35.7	11.2
U16-H (unbound)	39.4	25.1	13.5	5.0	5.5	7.3
Pig aorta HS (starting material)	63.9	18.5	6.0	2.3	4.4	3.8

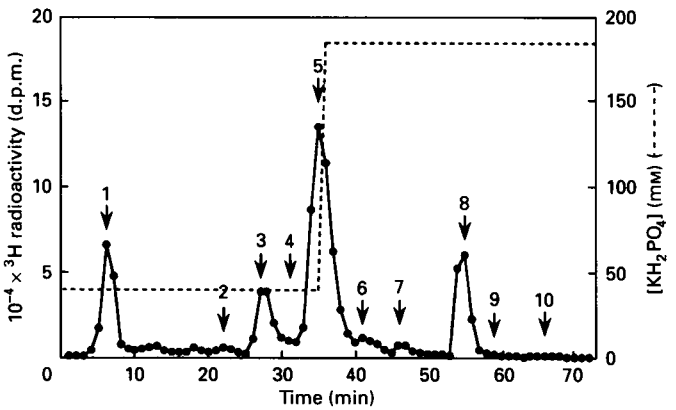

Fig. 5. H.p.l.c. of ^3H-labelled disaccharides produced after deaminative cleavage of the bound hexadecasaccharide

An unlabelled sample of B16-H was prepared as in Fig. 4 and treated with nitrous acid at pH 1.5. The products were labelled by reduction with $[^3H]NaBH_4$ and then subjected to gel chromatography on a HW40S column (not shown). An aliquot of the disaccharide fraction was applied to a Partisil-10 SAX column as described in the Experimental section. The column was eluted by stepwise elutions with increasing concentrations of KH_2PO_4 (broken line). Fractions of 1 ml were collected and analysed for radioactivity. The percentage yield of each disaccharide fraction is given below. Elution positions of the disaccharide references are indicated by arrows: 1, HexUA-AMan$_R$ (14%); 2, GlcA(2SO$_4$)AMan$_R$ (2%); 3, GlcA-AMan$_R$(6SO$_4$) (12%); 4, IdoA-AMan$_R$(6SO$_4$) (2%); 5, IdoA(2SO$_4$)AMan$_R$ (47%); 6, GlcA-AMan$_R$(3SO$_4$) (3%); 7, GlcA(2SO$_4$)AMan$_R$(6SO$_4$) (2%); 8, IdoA(2SO$_4$)AMan$_R$(6SO$_4$) (14%); 9, GlcA-AMan$_R$(3,6-di-SO$_4$) (not detected); 10, IdoA-AMan$_R$(3,6-di-SO$_4$) (not detected).

Table 5. Disaccharide composition per mol of bound hexadecasaccharide (B16-H)

The approximate molar ratios of the disaccharides per mol of B16-H were estimated from the data in Table 4 and Fig. 5.

Disaccharide	Content (mol/mol of B16-H)
IdoA(2SO$_4$)GlcNSO$_3$	3
IdoA(2SO$_4$)GlcNSO$_3$(6SO$_4$)	1
GlcAGlcNAc	2
GlcAGlcNAc(6SO$_4$)	0.65
GlcAGlcNSO$_3$	1
GlcAGlcNSO$_3$(6SO$_4$)	0.35
Unidentified	$\leqslant$ 0.02

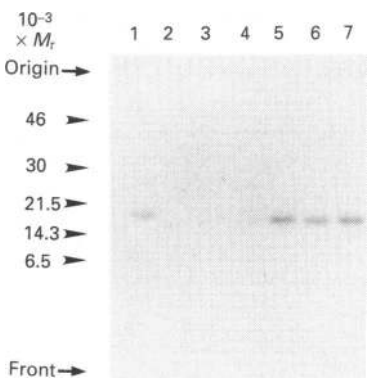

Fig. 6. SDS/PAGE of ^{125}I-bFGF digested with trypsin with or without GAGs

^{125}I-bFGF was digested with trypsin at 37 °C for 1 h in the absence of GAG (lane 2) or in the presence of 1 μg of chondroitin 6-sulphate (lane 3), 1 μg of unbound HS fraction (lane 4) 1 μg of bound HS fraction (lane 5), 0.1 μg of heparin (lane 6) or 1 μg of unfractionated heparan sulphate (lane 7). The sample was also incubated at 37 °C for 1 h without trypsin (lane 1). Aliquots of the digests were subjected to SDS/PAGE (20% gel). After drying, the gel was exposed to an X-ray film. The positions of standard proteins are indicated by the arrowheads.

NaCl. About 30% of the starting unbound hexadecasaccharide was recovered in the 0.7 M-NaCl fraction (fractions 22–34, termed U16-H in Fig. 4). The results show that the difference in bFGF affinity between B16-H and U16-H cannot be accounted for by a difference in the net negative charge alone.

B16-H and U16-H were exhaustively digested with a mixture of heparitinase I, heparitinase II and heparinase. The digested products were subjected to compositional analyses by h.p.l.c. on a polyamine silica column with a linear gradient elution of KH_2PO_4. Comparison of the disaccharide compositions of B16-H and U16-H revealed a marked difference (Table 4): 47% of the disaccharides obtained from the former were 2-*O*-sulphated hexuronic acid-containing disaccharide units [ΔDi-(N,U)diS plus ΔDi-(N,6,U)triS], whereas only 14% were 2-*O*-sulphated in the latter. When the dodecasaccharide fraction from bound oligo (cf. Fig. 3) was subjected to compositional analysis as above, about 50% of the disaccharides were 2-*O*-sulphated (results not shown). No further analysis has been done with this fraction, owing to the small amount available.

To identify the hexuronic acid residues participating in bFGF binding, B16-H was treated with nitrous acid at pH 1.5 and then reduced with $[^3H]NaBH_4$ according to the method of Shively & Conrad [23]. The labelled deamination products thus obtained were subjected to gel chromatography as described in the

Experimental section. Of the total labelled saccharides, 58% were recovered in the disaccharide fraction, 21% were in the tetrasaccharide fraction, and the remainder were in the hexasaccharide or larger oligosaccharide fractions (results not shown). The disaccharide fraction was then subjected to h.p.l.c. on a SAX column with stepwise elution with increasing concentrations of KH_2PO_4 (Fig. 5). More than 90% of the major disaccharide products [$HexUA(2SO_4)AMan_R$ and $HexUA(2SO_4)AMan_R(6SO_4)$] were of iduronic acid (IdoA)-type (compare peak 2 with peak 5 and peak 7 with peak 8 in Fig. 5). In contrast, most of the minor disaccharide fractions [$HexUAAMan_R(6SO_4)$ etc.] were of the GlcA type (Fig. 5, compare peak 3 with peak 4). The non-reducing end of B16-H should be non-sulphated unsaturated hexuronic acid, considering both the substrate specificity and catalytic properties of heparitinase I. If the penultimate glucosamine residue is *N*-acetylated, the deamination products from the non-reducing end should be tetrasaccharides or larger oligosaccharides with unsaturated hexuronic acid at the non-reducing terminal. If, however, the penultimate glucosamine residue is *N*-sulphated, the products should be disaccharide derivatives with unsaturated hexuronic acid. Although the chromatographic behaviours of such unsaturated disaccharides are not known, there is a possibility that some minor peaks in Fig. 5 represent such disaccharides. Molar ratios of the disaccharides (per mol of B16-H) estimated from the results of Table 4 and Fig. 5 are shown in Table 5. The characteristically high proportion of 2-*O*-sulphated iduronic acid-containing disaccharide units (4 mol/mol) suggests that clustering of these disaccharides may participate in the binding of bFGF.

Since heparitinase IV is only specific for glucosaminidic linkages containing 2-*O*-sulphated iduronic acid residues of heparan sulphate (K. Yoshida, H. Miyazono, A. Tawada, H. Kikuchi, K. Morikawa & K. Tokuyasu, unpublished work), digestion of heparan sulphate could yield oligosaccharides with unsaturated $HexUA(2SO_4)GlcNSO_3$ and/or $HexUA(2SO_4)GlcNSO_3(6SO_4)$ at the non-reducing terminal, but not those having $IdoA(2SO_4)GlcNSO_3$ and/or $IdoA(2SO_4)GlcNSO_3(6SO_4)$ units inside them. Our preliminary experiments showed that digestion of heparan sulphates with heparitinase IV resulted in a slight retardation of elution from the Sephadex G-50 column (about 20% was eluted in the area less than tetradecasaccharide) but in a complete loss of the binding to the bFGF affinity column (results not shown), consistent with the notion that clustering of $IdoA(2SO_4)GlcNSO_3$ and/or $IdoA(2SO_4)GlcNSO_3(6SO_4)$ units may form the binding site for bFGF.

Protective abilities of bFGF-bound and unbound heparan sulphates against proteolysis of ^{125}I-bFGF

It is known that heparin can protect FGFs against proteolysis. To examine whether or not heparan sulphate has such an activity and, if so, whether or not the ability is related to its binding to bFGF, ^{125}I-bFGF was incubated with 0.01% trypsin in the presence or absence of various GAGs, including bound HS and unbound HS. Aliquots of the digests were analysed by SDS/PAGE using 20% gels. Intact ^{125}I-bFGF moved as a single band of M_r 18000 on the gels. In the presence of 1 μg of heparin or 1 μg of bound HS, there was no significant decrease in the radioactivity of the bFGF band. In contrast, in the presence of chondroitin 6-sulphate or unbound HS the radioactive band became barely detectable (Fig. 6). To compare the protective abilities of various GAGs quantitatively, the remaining portions of the digests were precipitated with 10% cold trichloroacetic acid, and radioactivity in the supernatants was measured as described in the Experimental section. The relative protective abilities of GAGs against tryptic digestion are expressed as the concentrations of GAGs causing 50% inhibition of ^{125}I-bFGF degradation (Table 6). The unfractionated heparan sulphate was more than 100 times as active as chondroitin 6-sulphate. Further, bound HS was 50 times more active than unbound HS. None of the heparin and heparan sulphate preparations used inhibited the protease activity itself when Bz-DL-Arg-*p*-nitroanilide was used as a substrate. The results are consistent with the idea that bFGF-bound heparan sulphate may contain some specific structural units for binding to bFGF, thereby directly influencing the stability of bFGF.

Table 6. Relative protective ability of bound HS and unbound HS against proteolysis of ^{125}I-bFGF with trypsin

Relative protective ability was calculated from the concentration of GAG at which the degradation of ^{125}I-bFGF with trypsin was inhibited by 50%. The concentration of unfractionated heparan sulphate for 50% inhibition is taken as 1.

GAG	Relative protective ability
Unfractionated EHS tumour heparan sulphate	1.0
Bound HS	2.2
Unbound HS	0.04
Heparin	25
Chondroitin sulphate	$\leq$ 0.01

DISCUSSION

The present studies have shown that heparan sulphates are separated into two types with distinctive high or low affinity for bFGF; the ratio of the two varies with different preparations of heparan sulphate (Fig. 1). This suggests that expression of binding sites for bFGF on heparan sulphates may be regulated, depending upon differences in cell types, cell growth phase and cell transformation.

Hexadecasaccharides which were derived from pig aorta heparan sulphate by limited digestion with heparitinase I and which bound to bFGF gels characteristically comprised 3 mol of $IdoA(2SO_4)GlcNSO_3$ and 1 mol of $IdoA(2SO_4)GlcNSO_3(6SO_4)$ per molecule (Table 5). In addition, about 50% of the disaccharide units in the dodecasaccharides bound to the gels were $IdoA(2SO_4)$-containing units (3 mol per molecule). These results, and the fact that the octasaccharides were the smallest fragments for binding of bFGF and that their non-reducing ends were non-sulphated unsaturated hexuronic acid, suggest that clustering of three $IdoA(2SO_4)$-containing disaccharide units appears to form the site in heparan sulphate for the binding of bFGF. In relation to this structural requirement, it should be noted that the minimal structure of heparin required for acidic (a)FGF binding is $[IdoA(2SO_4)GlcNSO_3(6SO_4)]_3$ [26], which suggests the existence of common structural features for the binding of the FGF family growth factors.

Evidence for the clustering of $IdoA(2SO_4)$-containing disaccharide units were also obtained by taking advantage of the specificity of heparitinase IV. Since this enzyme can only cleave glycosaminidic linkages to 2-*O*-sulphated iduronic acid residues of heparan sulphates, unsaturated disaccharide products would be produced only from these clusters. In fact, digestion of pig aorta heparan sulphate with heparitinase IV released two-thirds of the total $IdoA(2SO_4)GlcNSO_3$ units and half of the total $IdoA(2SO_4)GlcNSO_3(6SO_4)$ units as unsaturated disaccharide products (results not shown). Furthermore, in view of the conformational versatility of IdoA or $IdoA(2SO_4)$ residues in heparan sulphates [27,28], it is tempting to assume that the

domains rich in these residues may be flexible enough to fit the binding site of bFGF. The clustering structure of two or three disaccharide units of $IdoA(2SO_4)GlcNSO_3$ and/or $IdoA(2SO_4)GlcNSO_3(6SO_4)$ has already been shown to occur in heparan sulphates from cultured skin fibroblasts, although their ability to bind to bFGF has not been tested [29].

Since we observed that the bound fraction of heparan sulphate from pig aorta had an M_r of 47000 and its $IdoA(2SO_4)GlcNSO_3$ plus $IdoA(2SO_4)GlcNSO_3(6SO_4)$ units comprised about 8% of the total disaccharide units present in the fraction, the bound heparan sulphate should contain 8 mol of $IdoA(2SO_4)$-$GlcNSO_3$ + $IdoA(2SO_4)GlcNSO_3(6SO_4)$ per mol of chain. In addition, as bound heparan sulphate from mouse EHS tumour had an M_r of 40000 and its $IdoA(2SO_4)GlcNSO_3$ + IdoA-$(2SO_4)GlcNSO_3(6SO_4)$ units comprised 3.5% of the total disaccharides, the bound tumour heparan sulphate should contain 3.3 mol of $IdoA(2SO_4)GlcNSO_3$ + $IdoA(2SO_4)GlcNSO_3(6SO_4)$ per mol of chain. These results suggest that there may be one or two bFGF-binding sites on each heparan sulphate chain.

A cluster of $IdoA(2SO_4)GalNAc(4SO_4)$ units has been shown to occur in skin dermatan sulphate as a binding site for heparin cofactor II, although the residues are relatively rare components in the dermatan sulphate preparation [30]. Therefore clustering of $IdoA(2SO_4)$-containing disaccharide units appears to be important for functions of both heparan sulphate and dermatan sulphate, and may be regulated by similar biosynthetic mechanisms in which hexuronosyl 5-epimerase and iduronosyl 2-*O*-sulphotransferase take a part [31–34].

Suggested biological roles of the interaction between bFGF and heparan sulphate are as follows. (1) The interaction enables bFGF to accumulate in the ECM [8,10] and protects bFGF from proteolysis (Fig. 6, Table 6; see [35–37]). (2) The binding of bFGF to heparan sulphate is necessary for the interaction of bFGF with its high affinity receptor [15,16]. A three-dimensional conformation change may occur in the receptor-binding domain of bFGF so as to make it accessible to the receptor [38–40]. The FGF bound to heparan sulphate proteoglycan in both the ECM and the cell surface could be released by attack with heparitinase [41] and/or proteases [36] as diffusible FGF–heparan sulphate oligosaccharide complexes [42]. For example, heparitinase from platelet cleaves a GlcA site of $GlcAGlcNSO_3$ [43] which may be localized outside the FGF-binding domain, and the bFGF–heparan sulphate oligosaccharide complex thus formed could diffuse freely to the FGF receptor.

It has been shown that bFGF is transported from the cell surface to the nucleus by endocytosis via endosomes and lysosomes [44]. Interestingly, heparin is also translocated into the nucleus [45]. Furthermore, heparan sulphate enriched in $GlcA(2SO_4)GlcNSO_3(6SO_4)$ has been found in the nuclei of rat hepatoma cells [2]. Therefore it is possible that bFGF–heparan sulphate oligosaccharide complexes are internalized by FGF-receptor-mediated endocytosis and transported into the nucleus under protection against proteolysis.

aFGF also binds to heparin and heparan sulphate [5,46], and exhibits 55% sequence identity with bFGF [47]. Both aFGF and bFGF have similar cell growth activity on a wide range of cultured cells, although their locations are different from each other in various tissues [5,6]. Further, the interaction between aFGF and heparin/heparan sulphate has also been shown to protect the mitogenic activity of aFGF against proteolysis [35,48]. In our preliminary work, the bound HS fraction also protected against the proteolytic inactivation of aFGF, but unbound HS did not. These data suggest that the aFGF-binding domain of heparan sulphate may be shared, at least in part, with the one for bFGF. Numerous studies have revealed that heparin binds various kinds of growth factors and ECM components (e.g. see [49–51]). It should be interesting to determine whether or not these proteins are able to interact with heparan sulphates as well and, if so, whether or not the specific binding structures in heparan sulphates are involved in such interactions. The biological significance of a structural microheterogeneity in heparan sulphate chains will be elucidated by such studies.

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